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## Inhibition of arenavirus infection by thiuram and aromatic disulfides

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### ABSTRACT

A selected group of aromatic disulfides, thiuram disulfides and thiosulfones, provided by the National Cancer Institute, were evaluated *in vitro* for their inhibitory activity against Junin virus (JUNV), the causative agent of Argentine hemorrhagic fever. The aromatic disulfides NSC4492 and NSC71033 and the thiuram disulfide NSC14560 were, respectively, the more potent virucidal and antiviral agents against JUNV, with inactivating concentration 50% (IC<sub>50</sub>) values of 0.2–0.5 μM for virucidal compounds and antiviral effective concentration 50% (EC<sub>50</sub>) of 8.5 μM for NSC14560. Both types of compounds exhibited inhibitory activity against three arenaviruses. Additionally, a comparable efficacy in the antiviral action of NSC14560 was observed in monkey, hamster or human cells with selectivity indices in the range 55.9–85.7. Time of addition experiments showed that the main antiviral activity of NSC14560 was situated before 5 h of infection, but a significant inhibition was still observed when the compound was added up 9 h *p.i.* This compound did not induce a refractory state to infection by cell pretreatment. Nor did it prevent viral entry, but the cytoplasmic and membrane expression of the main viral proteins was inhibited. The possible involvement of the RING finger motif of arenavirus Z protein as target for the thiuram disulfide is discussed.

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### 1. Introduction

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 glycoprotein precursor preGPC. Five arenaviruses, Lassa virus (LASV), Junín virus (JUNV), Machupo virus (MACV), Guanarito virus (GTOV) and Sabiá virus (SABV), are known to cause severe hemorrhagic fevers in humans (McCormick and Fisher-Hoch, 2002; Peters, 2002) and are listed as category A priority pathogens by the National Institute of Allergy and Infectious Diseases (NIAID) (Rotz et al., 2002). In particular, JUNV is the causative agent of the annual outbreaks of Argentine hemorrhagic fever (AHF), an endemo-epidemic disease recognized as a major public health problem in certain agricultural zones of Argentina (Damonte, 2002).

The danger of arenaviruses for human health and their increased emergence during recent years in North and South America has led to efforts to develop preventable vaccines or effective antiviral agents. 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 (Enría and Barrera Oro, 2002). Although vaccination is the major approach to control viral infections, vaccines probably may not be the complete answer for arenavirus control, due to the probability of ecological changes in the natural rodent reservoir (Cajimat et al., 2007) or the appearance of novel viral strains and related virus species, as frequently reported in recent years (reviewed in Charrel and de Lamballerie, 2010).

At present, no reliable drug therapy is available. The administration of standardized doses of convalescent plasma is the current therapy for AHF patients, however it is not effective when initiated after a week of illness and 10% of treated patients develop late neurological complications (Enría and Maiztegui, 1994; Enría et al., 2008). Administration of ribavirin, a broad spectrum antiviral agent, is the preferred method of treatment for Lassa fever (McCormick et al., 1986), but this compound did not reduce mortality in advanced cases of AHF and induced undesirable side effects (Enría et al., 2008; Fisher-Hoch et al., 1992). As occurs with other RNA viruses, the most explored approach of antiviral development has been the utilization of different kinds of nucleoside analogues to block viral RNA transcription and/or replication. However, all the nucleoside analogues tested against arenaviruses up to now have the serious drawback of a considerable level of toxicity (Andrei and De Clercq, 1990, 1993; Damonte and Coto, 2002).

Consequently, it is necessary to reinforce therapeutic capacity against JUNV and other pathogenic arenaviruses by analyzing alternative molecules. The presence in arenaviruses of the Z

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protein containing a highly conserved Zn-binding RING finger motif prompted us to initiate studies about this protein as a new antiviral strategy. We have assayed the anti-arenavirus activity of antiretroviral compounds with diverse chemical structures, provided by the National Cancer Institute (USA) and targeted to the Zn-finger motifs in the HIV nucleocapsid protein NCp7 (Rice et al., 1996, 1997). From the tested compounds, the disulfide NSC20625 was found to be a very potent and selective virucidal agent against arenaviruses (García et al., 2000, 2006, 2009). Based on these results and in an attempt to improve the effectiveness of arenavirus inhibition with virucidal as well as antiviral agents, we report here the studies about the *in vitro* inhibitory activity of a selected group of aromatic disulfides, thiuram disulfides and thiosulfones.

## 2. Materials and methods

### 2.1. Compounds

Twenty two compounds with the chemical structures and code numbers shown in Tables 1 and 2 were provided by the National Cancer Institute, Frederick, MD, USA. Ribavirin (Sigma–Aldrich) was used as reference anti-arenavirus substance. Stock solutions at a concentration of 100 mM were prepared in dimethylsulfoxide for all the compounds.

### 2.2. Cells and viruses

Vero (African green monkey kidney) cells were grown as monolayers in Eagle's minimum essential medium (MEM, GIBCO) containing 5% inactivated fetal bovine serum and 50 µg/ml gentamycin. The cell lines A549 (human lung carcinoma) and BHK-21 (baby hamster kidney) were cultivated in MEM supplemented with 10% fetal bovine serum and gentamycin. Maintenance medium (MM) consisted of MEM with 1.5% inactivated fetal bovine serum.

The attenuated strains IV4454 and XJCI3 of JUNV, the TRLV11573 strain of Tacaribe virus (TCRV), and the Armstrong (ARM) strain of lymphocytic choriomeningitis virus (LCMV) were used. Virus stocks were prepared in Vero cells and titrated by plaque assay on the same cells.

### 2.3. Cytotoxicity assay

Cytotoxicity was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich) method in Vero cells as previously described (García et al., 2000), and the cytotoxic concentration 50% (CC<sub>50</sub>), compound concentration required to reduce the MTT signal by 50% compared to controls, was calculated.

### 2.4. Virus yield inhibition assay

Cells grown in 24-well microplates were infected at a MOI of 0.1 PFU/cell. After 1 h adsorption at 37 °C, cells were washed and refed with MM containing serial 2-fold concentrations of the compounds. After 48 h of incubation at 37 °C, supernatant cultures were harvested and extracellular virus yields were determined by a plaque assay. The effective concentration 50% (EC<sub>50</sub>) was calculated as the concentration required to reduce virus yield by 50%, in the compound-treated cultures compared with untreated ones. All the determinations were performed thrice and each in duplicate.

### 2.5. Virucidal assay

Equal volumes of a virus suspension containing approximately  $1 \times 10^6$  PFU of virus 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. Then, 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 inactivating concentration 50% (IC<sub>50</sub>), the concentration required to inactivate virions by 50%, was calculated.

### 2.6. Effect of pretreatment of cells with compound prior to infection

Vero cell monolayers were pre-incubated with MM containing different compound concentrations during 2 h at 37 °C. Then, supernatants were removed; cells were thoroughly washed with PBS, and infected with JUNV at a MOI of 0.1 in the absence of compound. After 1 h incubation at 37 °C, inocula were discarded and MM was added. Virus yields were determined at 48 h p.i. by plaque formation in Vero cells.

### 2.7. Time of addition assay

Vero cells grown in 24-well microplates were infected with JUNV (MOI 0.1) and adsorption was allowed for 1 h at 4 °C. After removal of the inocula, the cells were washed with PBS, refed with MM and incubated at 37 °C. Duplicate wells were treated with 20 µM NS14560 at various times after infection. A control infected culture without drug treatment was performed simultaneously. In all the cases, extracellular virus yields were determined by plaque formation at 24 h post-infection.

### 2.8. Kinetics of virus adsorption

Vero cells were infected with JUNV at a MOI of 0.1 in the presence or in the absence of 20 µM NSC14560. After different times of adsorption at 4 °C, cells were washed with cold PBS to remove unadsorbed virus and disrupted by freezing and thawing. The amount of infectious bound virus was then measured by plaque formation.

### 2.9. Kinetics of virus internalization

Vero cells grown in 6-well microplates were infected with about 200 PFU of JUNV/well. After 1 h adsorption at 4 °C, unadsorbed virus was removed and cells were washed with PBS and incubated at 37 °C in the presence or in the absence of 20 µM NSC14560. At different times post-adsorption, cells were washed with PBS and treated with 0.1 ml of citrate buffer (citric acid 40 mM, KCl 10 mM, NaCl 135 mM, pH 3) for 1 min to inactivate adsorbed virus but not internalized virus. Cells were then washed with PBS and covered with MM containing 1% methylcellulose. Plaques were counted after 6 days of incubation at 37 °C.

### 2.10. Indirect immunofluorescence assay

Vero cells grown in coverslips were infected with JUNV at a MOI of 0.1 and MM containing or not 20 µM NSC14560 was added after virus adsorption. At 16 h p.i., cells were fixed in methanol for 15 min at –20 °C and cytoplasmic immunofluorescence staining was carried out by using the monoclonal antibody (mAb) SA02-BG12 for NP and the mAb QC03-BF11 reactive against GP1 and its precursor GPC (Sanchez et al., 1989), followed by fluorescein isothiocyanate (FITC)-goat anti-mouse IgG (Sigma–Aldrich Co.). After a final washing with PBS, cells were stained with Evans Blue and mounted in a glycerol solution containing 1,4-diazabicyclo[2,2,2]octane (DABCO). For membrane immunofluorescence, cells were fixed with 4% formaldehyde and probed with the mAb QC03-BF11 as described above. The percentage of fluorescent cells in each

**Table 1**  
Cytotoxicity, antiviral and virucidal activities of thiuram disulfides and thiosulfones.

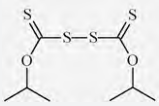
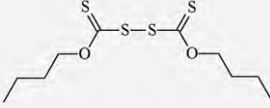
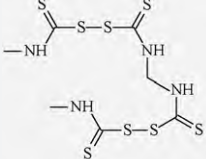
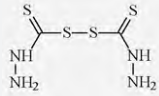
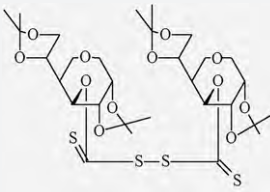
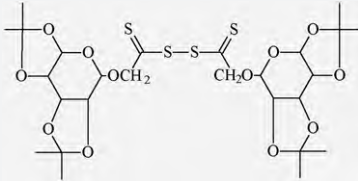
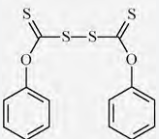
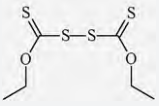
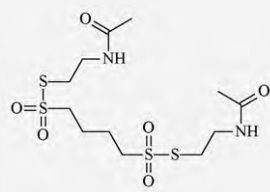
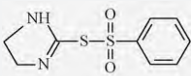
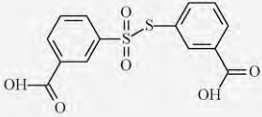
Compound	Structure	CC <sub>50</sub> [μM] <sup>a</sup>	EC <sub>50</sub> [μM] <sup>b</sup>	IC <sub>50</sub> [μM] <sup>c</sup>
Thiuram disulfides				
NSC 1339		>1000	>100	70.1 ± 1.4
NSC 5239		400.0 ± 0.2	18.5 ± 1.0	12.2 ± 0.4
NSC 14560		475.2 ± 0.2	8.5 ± 0.2	27.6 ± 0.8
NSC 27320		407.2 ± 0.3	>100	38.0 ± 0.9
NSC 93057		761.0 ± 0.4	>200	127.7 ± 2.4
NSC 93058		>1000	>200	121.1 ± 2.0
NSC 317926		528.4 ± 0.1	>200	101.9 ± 2.4
NSC 402561		989.2 ± 0.7	>100	17.9 ± 0.4
Thiosulfones				
NSC 76302		>1000	181.5 ± 4.6	>100
NSC 112801		297.0 ± 0.1	>200	>100

Table 1 (Continued)

Compound	Structure	CC <sub>50</sub> [ $\mu$ M] <sup>a</sup>	EC <sub>50</sub> [ $\mu$ M] <sup>b</sup>	IC <sub>50</sub> [ $\mu$ M] <sup>c</sup>
NSC 342015		>1000	>100	15.3 ± 0.5
Reference compound Ribavirin		>400	18.5 ± 1.7	>100

Values are the mean of three determinations ± standard deviation.

<sup>a</sup> Cytotoxic concentration 50%: compound concentration required to reduce cell viability by 50%, as determined by MTT assay.

<sup>b</sup> Effective concentration 50%: compound concentration required to reduce virus yield by 50%.

<sup>c</sup> Inactivating concentration 50%: compound concentration required to inactivate virion infectivity by 50%.

preparation was calculated from 20 randomly selected microscope fields.

### 3. Results

#### 3.1. Antiviral and virucidal activities of disulfides and thiosulfones against JUNV, strain IV4454

According to their chemical structures, the compounds assayed were grouped in three main categories: aromatic disulfides, thiuram disulfides and thiosulfones. Each category of compounds was first evaluated for cytotoxicity in Vero cells. As seen in Tables 1 and 2, most compounds exhibited low levels of cytotoxicity with CC<sub>50</sub> values higher than 100  $\mu$ M, with the only exception of two sulfonium-derivatized disulfides, NSC58950 and NSC83217. Then, the antiviral activity was assayed by a virus yield inhibition assay in the range of concentrations lower than the corresponding CC<sub>50</sub>, using JUNV strain IV4454 as model system. Finally, each compound was also tested against this virus strain for virucidal activity in a virion inactivation assay.

Most of the thiuram disulfide derivatives, six compounds from a total of eight, were inactive in antiviral assays up to 200  $\mu$ M and exhibited weak or moderate inactivating effect (IC<sub>50</sub> in the range 17.9–127.7  $\mu$ M) (Table 1). Only two thiuram compounds showed significant antiviral activity, NSC14560 and NSC5239 with EC<sub>50</sub> values of 8.5 and 18.5  $\mu$ M, respectively. These two compounds also had inactivating properties, but with different efficacy. For NSC5239, the values of EC<sub>50</sub> and IC<sub>50</sub> were in the same order, 18.5 and 12.2  $\mu$ M, respectively, suggesting that the antiviral action observed in Vero cells may be due to direct inactivation of the released virus. By contrast, the IC<sub>50</sub> for NSC14560 exceeded more than 3-fold the EC<sub>50</sub> (27.6  $\mu$ M vs 8.5  $\mu$ M, respectively), allowing to conclude that this disulfide can be considered an authentic antiviral agent with ability to interfere with any intracellular event in the multiplication cycle of JUNV in Vero cells. Furthermore, it is remarkable that the antiviral effectiveness and selectivity of NSC14560 against JUNV in Vero cells was higher than the reference compound ribavirin (Table 1). The selectivity index, calculated as the ratio between cytotoxicity (CC<sub>50</sub>) and antiviral activity (EC<sub>50</sub>) was 55.9 for NSC14560 whereas the index for ribavirin was 21.6. Even the effective concentration 90% (EC<sub>90</sub>, i.e. concentration required to reduce JUNV yield by 90%) was 23.02  $\mu$ M, a value 20-fold lower than the CC<sub>50</sub>, and also 4-fold lower than the EC<sub>90</sub> corresponding to ribavirin (96.7  $\mu$ M).

With regard to the thiosulfones, the derivatives NSC76302 and NSC112801 were inactive or showed negligible inhibitory activity in both antiviral and virucidal assays (Table 1). The aromatic thiosulfone NSC342015 also lacked antiviral activity but exhibited a moderate inactivating effect against JUNV virions, with an IC<sub>50</sub> of 15.3  $\mu$ M.

The group of aromatic disulfides included diverse substituted derivatives and represented the more effective inhibitors of JUNV. From a total of eleven disulfides, only three compounds (NSC38069, NSC238936 and NSC35825) were totally inactive lacking antiviral and inactivating properties (Table 2). Six compounds exhibited simultaneously moderate antiviral and virucidal activities with 0.5–7.5-fold differences between the EC<sub>50</sub> and IC<sub>50</sub> values. Finally, two compounds, the carboxamide-derivatized disulfide NSC4492 and the amino-nitro-derivative NSC71033 demonstrated moderate antiviral activity (EC<sub>50</sub> values in the range 27.7–32.4  $\mu$ M) but a very potent virucidal effect, with IC<sub>50</sub> values of 0.2 and 0.5  $\mu$ M for NSC4492 and NSC71033, respectively. Thus the ratio EC<sub>50</sub>/IC<sub>50</sub>, i.e. the relationship between antiviral and virucidal activity, for these two compounds is in the range 55.4–162, values exceptionally high that are indicative of the effectiveness of both substances to inactivate the infectivity of JUNV virions in comparison with their effect on intracellular virus multiplication. The inactivating property of both drugs against JUNV was not exerted through any cytotoxic effect, since the ratio CC<sub>50</sub>/IC<sub>50</sub> was 320 and 740 for NSC71033 and NSC4492, respectively.

#### 3.2. Spectrum of inhibitory activity

The thiuram disulfide NSC14560 and the aromatic disulfides NSC4492 and NSC71033 were chosen as the more potent antiviral and virucidal compounds, respectively, to further characterize their anti-arenavirus activity. To test their spectrum of virus activity, both types of agents were assayed against other strain of JUNV, the attenuated XJCl3 strain obtained by serial passage from the prototype XJ strain (Guerrero et al., 1969), against other New World arenavirus, TCRV, and against the Old World arenavirus and prototype of the family LCMV.

As seen in Fig. 1, the profile of susceptibility of the three viruses to these compounds was similar to that observed for the IV4454 strain of JUNV, with slight differences in the efficiency of inhibition against each arenavirus. The carboxamide-derivative NSC4492 exhibited the most effective inactivating properties against all the tested arenaviruses: the IC<sub>50</sub> values extrapolated from Fig. 1(A) were in the range 0.2–0.7  $\mu$ M. The IC<sub>50</sub> values for the nitro-derivative NSC71033 against arenaviruses calculated from Fig. 1(B) were also lower than 1  $\mu$ M, except for TCRV with an IC<sub>50</sub> of 2.5  $\mu$ M. The behavior of the thiuram disulfide NSC14560 was a little more variable among the different arenaviruses, but the EC<sub>50</sub>s extrapolated from Fig. 1(C) showed the susceptibility of all tested viruses with values of 11.9 ± 0.2, 15.8 ± 0.4 and 18.3 ± 0.7  $\mu$ M, for TCRV, LCMV and JUNV XJCl3, respectively.

For the thiuram disulfide NSC14560, the influence of the host cell on the inhibition of JUNV infection was also analyzed by determining its antiviral effectiveness in A549 and BHK-21 cell lines by virus yield inhibition assay. Both cell lines showed a similar

**Table 2**  
Cytotoxicity, antiviral and virucidal activities of aromatic disulfides.

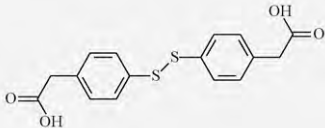
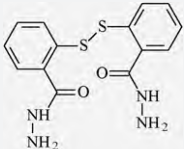
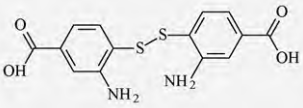
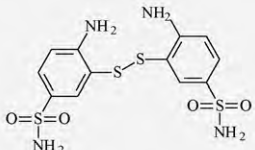
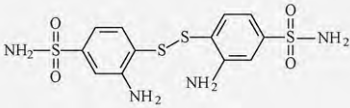
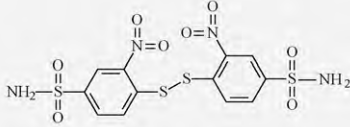
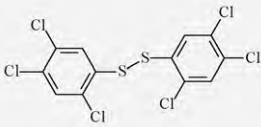
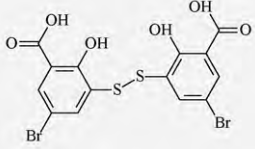
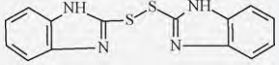
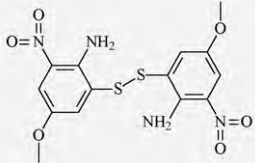
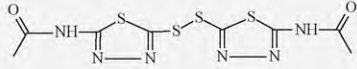
Compound	Structure	CC <sub>50</sub> [ $\mu$ M] <sup>a</sup>	EC <sub>50</sub> [ $\mu$ M] <sup>b</sup>	IC <sub>50</sub> [ $\mu$ M] <sup>c</sup>
Carboxyl/carboxamide-derivatized aromatic disulfides				
NSC 211		>1000	103.4 $\pm$ 3.8	22.7 $\pm$ 0.5
NSC 4492		148.0 $\pm$ 0.1	32.4 $\pm$ 0.9	0.2 $\pm$ 0.006
NSC 38069		>1000	>250	>100
Sulfonium-derivatized aromatic disulfides				
NSC 58950		<50	10.9 $\pm$ 0.3	21.0 $\pm$ 0.4
NSC 83217		51.2 $\pm$ 0.0	20.2 $\pm$ 0.5	16.2 $\pm$ 0.4
NSC 255089		>1000	133.4 $\pm$ 3.7	17.7 $\pm$ 0.3
Aromatic disulfides substituted with halogenated groups				
NSC 238936		387.6 $\pm$ 0.3	>200	>100
NSC 314654		400.0 $\pm$ 0.4	41.3 $\pm$ 1.2	9.4 $\pm$ 0.3
Miscellaneous aromatic disulfides				
NSC 35825		172.0 $\pm$ 0.2	>100	>100
NSC 71033		160.2 $\pm$ 0.2	27.7 $\pm$ 1.0	0.5 $\pm$ 0.02

Table 2 (Continued)

Compound	Structure	CC <sub>50</sub> [μM] <sup>a</sup>	EC <sub>50</sub> [μM] <sup>b</sup>	IC <sub>50</sub> [μM] <sup>c</sup>
NSC 327174		966.0 ± 0.1	89.7 ± 2.3	24.6 ± 0.6

Values are the mean of three determinations ± standard deviation.

<sup>a</sup> Cytotoxic concentration 50%: compound concentration required to reduce cell viability by 50%.

<sup>b</sup> Effective concentration 50%: compound concentration required to reduce virus yield by 50%.

<sup>c</sup> Inactivating concentration 50%: compound concentration required to inactivate virion infectivity by 50%.

level of sensitivity to the anti-arenavirus action of this compound (Fig. 1(D)). In the three cell systems tested, the EC<sub>50</sub> values of NSC14560 against JUNV IV4454 extrapolated from Fig. 1(D) were much lower than the levels of drug that caused toxicity to the host cells as determined by MTT method (Table 3). Altogether, these findings suggest that the studied disulfides possess an appropriate spectrum of inhibitory activity against different arenaviruses and host cell types.

### 3.3. Influence of time of treatment with compound NSC14560 on JUNV infectivity

To start the investigation of the mechanism of action of the most active compound with antiviral properties, a series of assays were carried out with NSC14560 to locate the stage at which it exerts the effect against JUNV.

To eliminate the possibility that a cellular binding factor was the target for the antiviral activity of NSC14560, a experiment was performed in which the cells were first incubated with compound, washed, and then infected with JUNV. Virus yields were determined

Table 3

Cytotoxicity and antiviral activity of NSC14560 in different cell types.

Host cell	CC <sub>50</sub> [μM] <sup>a</sup>	EC <sub>50</sub> [μM] <sup>b</sup>	SI <sup>c</sup>
Vero	475.2 ± 0.2	8.5 ± 0.2	55.9
A549	>500	6.8 ± 0.6	>72.9
BHK-21	485.3 ± 7.2	5.7 ± 0.7	85.7

Values are the mean of three determinations ± standard deviation.

<sup>a</sup> Cytotoxic concentration 50%: compound concentration required to reduce cell viability by 50%.

<sup>b</sup> Effective concentration 50%: compound concentration required to reduce JUNV IV4454 yield by 50%.

<sup>c</sup> Selectivity index: ratio CC<sub>50</sub>/EC<sub>50</sub>.

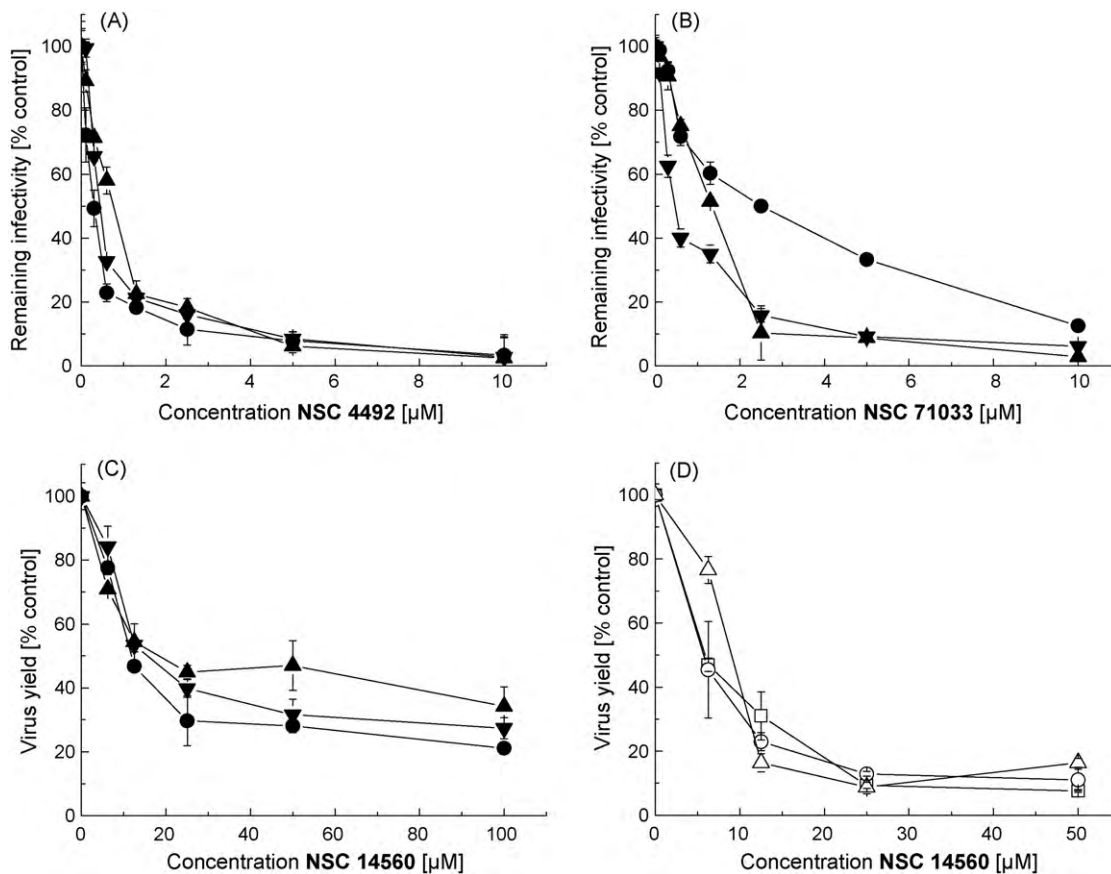
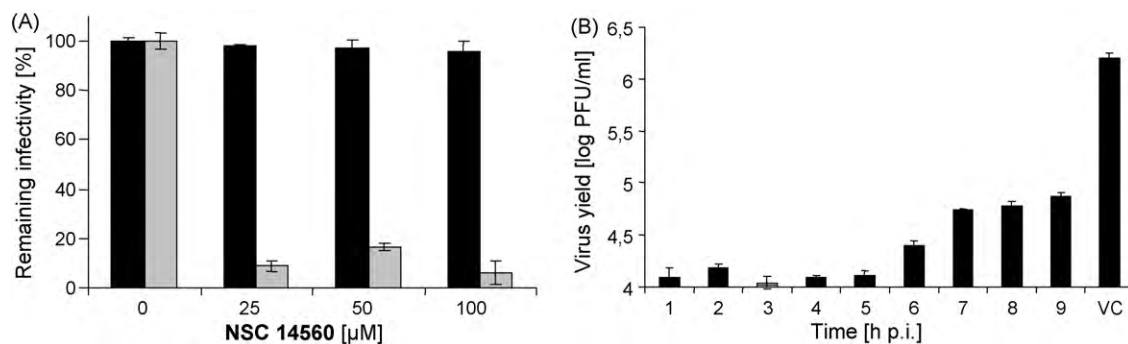


Fig. 1. Spectrum of inhibitory activity. (A and B) Suspensions of JUNV XJCl3 strain (▲), TCRV (●) and LCMV (▼) were incubated with different concentrations of NSC4492 (A) and NSC71033 (B) for 2 h at 37 °C and remaining infectivity was titrated. (C) Vero cells were infected with JUNV XJCl3 strain (▲), TCRV (●) and LCMV (▼) and MM containing different concentrations of NSC14560 was added. Virus yields were measured at 48 h p.i. (D) Vero (Δ), BHK-21 (□) and A549 (○) cells were infected with JUNV IV4454 strain and MM containing different concentrations of NSC14560 was added. Virus yields were measured at 48 h p.i. Values are the mean from three independent tests ± standard deviation.



**Fig. 2.** Influence of time of treatment with NSC14560 on anti-JUNV activity. (A) Pretreatment of cells (black bars): Vero cells were pre-incubated with MM containing different compound concentrations during 2 h at 37 °C; then, supernatants were removed and cells were infected with JUNV (MOI 0.1) in the absence of compound. Virus yields were determined at 48 h p.i. by plaque formation in Vero cells. Treatment during infection (grey bars): compound was added to Vero cells simultaneously with JUNV (MOI 0.1) and maintained during 48 h at 37 °C, when virus yields were determined. (B) Time of addition assay: Vero cells were infected with JUNV (MOI 0.1) and after 1 h adsorption at 4 °C, cells were washed, refed with MM (VC=viral control) and 20 µM NSC14560 was added at different times p.i. Extracellular virus yields were determined by plaque formation after 24 h of incubation at 37 °C. Values are the mean from three independent tests ± standard deviation.

after 48 h of infection. No inhibition was observed at any drug concentration tested (Fig. 2(A)). By contrast, the addition of compound to cells at the time of virus infection and during the whole incubation period, as in the screening assay shown in Table 1, produced a significantly high reduction of remaining infectious virus, supporting the conclusion that NSC14560 mainly targets an intracellular JUNV multiplication event.

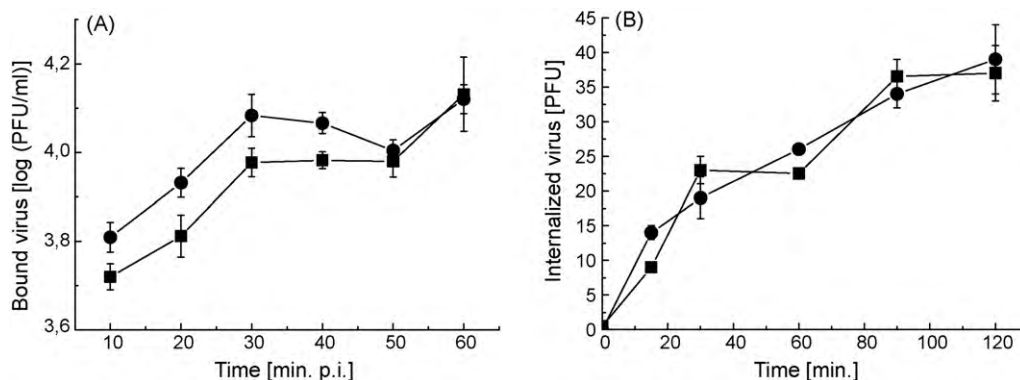
To determine the time dependence of the inhibitory action of NSC14560 on arenavirus replication, the effect of the time of addition of the compound on JUNV yield during infection was next examined. The replicative cycle of JUNV lasts approximately 12 h until infectious progeny is released from the cell (Mersich et al., 1981). However, virus production sufficient for an antiviral yield inhibition assay is only detected near 24 h p.i. Consequently, cells were infected with JUNV, adsorption proceeded during 1 h at 4 °C and then cultures were shifted to 37 °C to synchronize the start of virus cycle within the cell, and the drug was added at different times after adsorption. Extracellular virus yields from all infected cultures were titrated at 24 h p.i. As shown in Fig. 2(B), a similar level of inhibition, with reduction in virus yields higher than 99%, was achieved if the compound was added immediately after virus adsorption (time 1) or at 5 h p.i. A slightly lower but significant reduction in virus yield, about 95%, was still observed when compound was added at 5–9 h p.i. According to these results, the target for NSC14560 inhibition appears to be located at two probable sites of action, before and after 5 h of infection.

### 3.4. Effect on virus adsorption and internalization

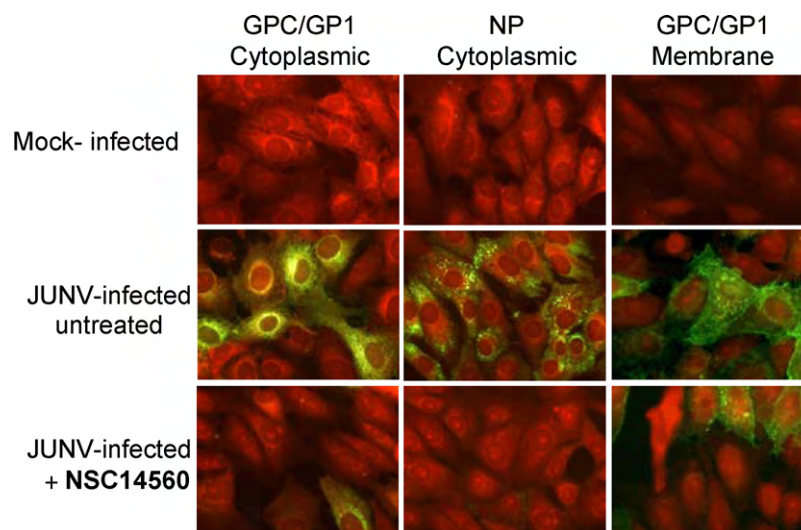
Given the extension of the time period for effective arenavirus inhibition evidenced in Fig. 2(B), it appears that the initial event of virus entry into the host cell is not affected by NSC14560. This hypothesis was further assessed by determining the effect of the drug during the virus adsorption and internalization processes. First, Vero cells were exposed to JUNV in the absence or in the presence of NSC14560 and the cell-bound infectivity after varying times of incubation at 4 °C was then determined. As seen in Fig. 3(A), virus adsorption to Vero cells was not inhibited by treatment with compound with respect to nontreated cells. Next, virus penetration was studied by inoculation of Vero cells with JUNV during 1 h at 4 °C followed by a variable time of incubation at 37 °C. Results obtained showed that virions were taken up by the cells with the same efficacy and kinetics independently of the presence or absence of the compound (Fig. 3(B)). Thus, NSC14560 does not prevent viral entry.

### 3.5. Expression and location of viral antigens

The effect of NSC14560 on the expression and cellular location of JUNV proteins was studied by indirect immunofluorescence. In control infected cells at 16 h p.i. the presence of the nucleocapsid protein NP, the earliest and more abundant viral protein, was revealed with mAb SA02-BG12 exhibiting a finely dotted regular distribution (Fig. 4). The amount of cells showing cytoplasmic flu-



**Fig. 3.** Effect of NSC14560 on virus adsorption and internalization. A. JUNV was adsorbed to Vero cells (MOI 1) at 4 °C in MM containing (■) or not (●) 20 µM NSC14560. At different times p.i., cell-bound infectious virus was determined by plaque assay. (B) Vero cells were infected with approximately 200 PFU of JUNV. After 1 h adsorption at 4 °C cells were incubated at 37 °C in the presence (■) or in the absence (●) of 20 µM NSC14560. At different times post-adsorption, cells were treated with citrate buffer, washed with PBS and covered with MM containing methylcellulose to quantify internalized virus. Each value represents the mean of three assays ± S.D.



**Fig. 4.** Effect of NSC14560 on JUNV antigen expression. Vero cells were mock-infected or infected with JUNV (MOI 0.1) and MM containing or not 20  $\mu$ M NSC14560 was added after adsorption. At 16 h p.i., cytoplasmic and membrane IF staining was performed using mAb SA02-BG12 for NP and mAb QC03-BF11 for GPC/GP1. Magnification: 400 $\times$ .

orescence for NP was drastically reduced in the presence of 20  $\mu$ M NSC14560. The inhibition in NP expression, quantified by counting the number of fluorescent cells in 20 randomly selected microscope fields, was 60%.

For JUNV glycoproteins, both cytoplasmic and membrane expression was monitored with mAb QC03-BF11 reactive against the precursor GPC and the mature envelope protein GP1 (Sanchez et al., 1989). As seen in Fig. 4 a similar decrease was observed in the pattern of JUNV glycoproteins dispersed throughout the cytoplasm as well as in cell surface after NSC14560 treatment.

#### 4. Discussion

There is currently no antiviral chemotherapy for treatment of arenavirus infections causing hemorrhagic fevers with an overall mortality of 15–30% in Africa and South America (McCormick and Fisher-Hoch, 2002; Peters, 2002). In the present study, we could find potent and selective inhibitors of the pathogenic arenavirus JUNV after the screening of diverse series of aromatic and aliphatic disulfides. This class of compounds was selected to evaluate anti-arenavirus activity based on our previous studies on diverse chemotypes, including disulfides, dithianes, azoic and hydrazide derivatives, with known reactivity against zinc-finger motifs. Among these compounds, several aromatic disulfides and dithianes were able to inactivate JUNV and LCMV virions through irreversible modifications in the matrix Z protein (García et al., 2006, 2009). Here, these studies were extended and the virucidal ability of aromatic disulfides against arenaviruses was confirmed. Two new compounds, the aromatic carboxamide-derivative NSC4492 and the amine-derivative NSC71033, have shown an improved efficacy in comparison to other disulfide-based compounds to inactivate JUNV in the range of submicromolar to low micromolar concentrations, turning these agents into very promising tools for prophylactic applications.

Furthermore, the screening of a variety of thiuram disulfides allowed to detect compounds with very effective inhibitory action exerted during the intracellular multiplication of arenaviruses. Thiuram disulfides represent an interesting class of compounds to be examined in extensor, since a member of this group, tetraethylthiuram disulfide (disulfiram or Antabuse), is a pharmacologically active drug approved for more than 50 years by Food and Drug Administration in USA for alcohol dependence therapy (Suh et al.,

2006). The thiuram disulfides have the common general structure  $R_1-C(S)-S-S-(S)C-R_2$  and differ by the nature of the R groups. Here, thiurams with diverse R substituents were assayed leading to the identification of the asymmetric aliphatic compound NSC14560 as a very effective inhibitor of arenavirus multiplication, without affecting cell viability.

An interesting feature of present findings is the spectrum of activity against New and Old World arenaviruses shown by both inactivating and antiviral compounds. The inhibitory effect against the three tested arenaviruses of the two virucidal agents NSC4492 and NSC71033 was dose-dependent up a concentration of 5  $\mu$ M, and at the highest concentration tested of 10  $\mu$ M the level of virus inactivation still increased reaching 96.6–100% inhibition, depending on the virus species, with the only exception of NSC71033 against TCRV, the least susceptible virus to inactivation among the arenaviruses tested (Fig. 1(B)). The antiviral compound NSC14560 was more effective against JUNV, strain IV4454, in comparison with other arenaviruses (Fig. 1(C)). Furthermore, a comparable efficacy in the antiviral action of NSC14560 against JUNV was observed using monkey, hamster or human cells, suggesting that the activity is not unique for a particular class of mammalian cell. The  $EC_{50}$  values in a variety of cell lines were lower than 10  $\mu$ M, with selectivity indices in the range 55.9–85.7. The  $EC_{90}$  values in Vero, BHK-21 and A549 fluctuated about 20–25  $\mu$ M, confirming the effectiveness of the inhibition exerted by NSC14560.

The molecular mechanism of antiviral activity of NSC14560 is cell type-independent but still remains unclear. Time of addition studies showed evidence that this compound appears to exert a dual effect during infection inhibiting two steps located before and after 5 h of infection in JUNV life cycle. Compound NSC14560 did not induce a refractory state to infection by cell pretreatment. Nor did it prevent viral entry (Figs. 2 and 3). The main level of inhibition was observed when NSC14560 was present during the first 5 hours after infection, but even when added at 9 h p.i., more than 90% inhibition in virus production was recorded. In accordance with this time-dependent activity of NSC14560, the expression of the main viral proteins, NP and GPC/GP1, was inhibited in the presence of the compound. In particular, for the viral envelope glycoprotein the percentage of inhibition in cytoplasmic fluorescence was similar to that obtained with membrane staining, indicating that the reduction in glycoprotein membrane insertion is a consequence of the reduction in viral protein expression in treated infected cells.



The reactivity of thiuram disulfides like NSC14560 with Zn-finger domains of the retroviral protein NCp7 has been demonstrated (Rice et al., 1996). Then, it can be hypothesized that, as shown for other disulfide-based compounds active against arenaviruses (García et al., 2006), the thiuram disulfide may interact with the zinc-binding motifs present in the arenavirus Z protein. This protein contains a central RING finger domain conformed by a highly conserved pattern of Cys and Hys residues, required for an adequate protein folding (Borden, 2000). The functionality of this matrix protein, connecting the viral lipid envelope with the internal nucleocapsid (Neuman et al., 2005), is not at present totally understood. However, there is no doubt that Z is critical for virus budding (Casabona et al., 2009; Perez et al., 2003; Strecker et al., 2003) and it also appears to be required for virion uncoating (García et al., 2009) as well as to regulate RNA transcription and replication (Cornu and de la Torre, 2002; López et al., 2001). It has been shown that disruption or mutation of the Z RING structure impaired some of these biological functions (Casabona et al., 2009; Jácomo et al., 2003). The multiple roles of Z in arenavirus cycle are in accordance with the early and late effects of NSC14560 observed during the virus multiplication cycle, but further investigation will be important to fully elucidate the target of this selective inhibitor compound in arenavirus infection.

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