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An antiviral disulfide compound blocks interaction between arenavirus Z protein and cellular promyelocytic leukemia protein

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ABSTRACT

The promyelocytic leukemia protein (PML) forms nuclear bodies (NB) that can be redistributed by virus infection. In particular, lymphocytic choriomeningitis virus (LCMV) influences disruption of PML NB through the interaction of PML with the arenaviral Z protein. In a previous report, we have shown that the disulfide compound NSC20625 has antiviral and virucidal properties against arenaviruses, inducing unfolding and oligomerization of Z without affecting cellular RING-containing proteins such as the PML. Here, we further studied the effect of the zinc-finger-reactive disulfide NSC20625 on PML–Z interaction. In HepG2 cells infected with LCMV or transiently transfected with Z protein constructs, treatment with NSC20625 restored PML distribution from a diffuse-cytoplasmic pattern to punctate, discrete NB which appeared identical to NB found in control, uninfected cells. Similar results were obtained in cells transfected with a construct expressing a Z mutant in zinc-binding site 2 of the RING domain, confirming that this Z–PML interaction requires the integrity of only one zinc-binding site. Altogether, these results show that the compound NSC20625 suppressed Z-mediated PML NB disruption and may be used as a tool for designing novel antiviral strategies against arenavirus infection.

Introduction

Lymphocytic choriomeningitis virus (LCMV) is an important etiologic agent of aseptic meningitis in humans. This prototype member of the *Arenaviridae* is carried as an inapparent chronic infection by rodents and occasionally transmitted to humans causing acute central nervous system disease and congenital malformations [1–3]. Human-to-human horizontal infection has not been documented, except for the unusual circumstances in which the virus was acquired through transplantation of infected tissues [4].

Arenaviruses encode five mature products: a nucleocapsid protein (NP), an RNA polymerase (L), the matrix protein (Z) and two envelope glycoproteins (GP1 and GP2). Despite the efforts of many groups, the precise role of Z is poorly understood. An early report suggested that Z was required for transcription of the arenavirus Tacaribe (TCRV) [5], but later studies have shown that Z inhibits transcription [6–8]. In addition to this regulatory role, Z has matrix functions, similar to other enveloped negative-stranded viruses and it is the main driving force for virion budding [9–12]. Z contains a conserved RING-finger domain flanked by an N-terminal hydrophobic domain with a myristoylation site and a phosphorylation site [13,14]. The C-terminal portion of the Z protein contains proline-rich motifs that were identified as late motifs in matrix proteins [15,16]. It was found that the integrity of the late motifs and the RING-finger domain is necessary for Z biological functions in the arenaviruses LCMV and Lassa [8,10,11] whereas budding of TCRV does not depend on late motifs [17].

Furthermore, Z has also been shown to interact with the promyelocytic leukemia protein (PML) [18] and other cellular proteins providing evidence for numerous virus-host interactions [10,19–21]. In particular, it has been observed that LCMV influences the disruption of PML nuclear bodies (NB) during infection, and transient transfection studies indicated that Z alone is sufficient to redistribute PML to the cytoplasm and that PML and Z colocalize [18].

Several zinc-finger-reactive antiretroviral compounds have shown antiviral and virucidal properties against arenaviruses in virus yield-inhibition and virion inactivation assays, respectively [22]. In particular, the disulfide NSC20625 (Fig. 1A) was able to

Abbreviations: PFU, plaque-forming units; MOI, multiplicity of infection; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; PML, promyelocytic leukemia protein; LCMV, lymphocytic choriomeningitis virus; NB, nuclear bodies; MM, maintenance media; DAPI, 4′,6′-diamidino-2-phenylindole, a DNA dye; pMLV, retroviral vector from murine leukemia virus; mAB, monoclonal antibody; cy5, cyanogen dye for microscopy; FITC, fluorescein isothyocyanate dye for microscopy.

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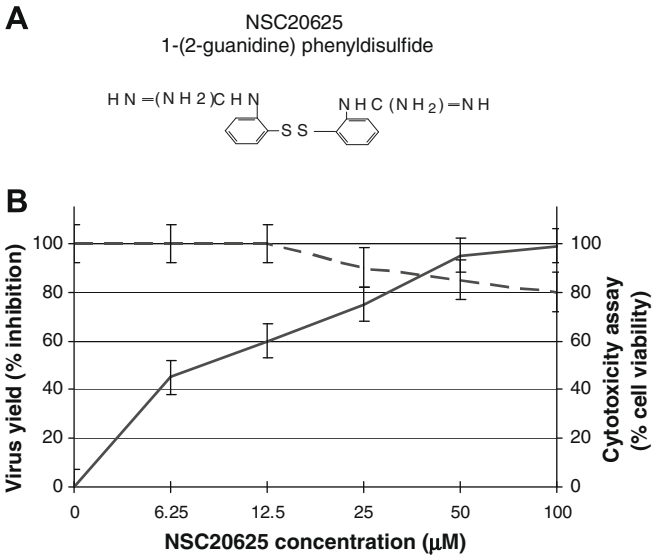

Fig. 1. (A) Chemical structure of NSC20625. (B) Concentration–response curves for cytotoxic and antiviral activity of NSC20625 against LCMV. HepG2 cells were incubated for 48 h in the presence of different concentrations of NSC20625 and then cell viability was determined by the MTT method (dash line). Another set of cultures was infected with LCMV (MOI 0.1) and after 48 h of infection in the presence of the compound, extracellular virus yields were determined (solid line). Each point is the mean value of duplicate independent determinations.

induce metal-ion ejection from purified Z protein, with the consequent loss of its native structure and stability. These results support the hypothesis that the Z protein, and in particular its RING-finger domain, is the most vulnerable target for this agent [23].

The studies reported here have extended the characterization of the zinc-finger-reactive compound NSC20625 to a human liver cell line frequently used in arenavirus studies. Similar to our results in primate fibroblasts, we have confirmed that this disulfide disrupts the interaction between the Z protein and the PML-associated NB and restores the nuclear prominence of the NB. Altogether, the experiments carried out in this study show that the compound can be used as a tool to study the interaction between Z and cellular proteins, and that it is a promising antiviral compound.

Materials and methods

Compound. The compound 1-(2-guanidine) phenyldisulfide (NSC20625) (Fig. 1A) was provided by the National Cancer Institute, Frederick, USA. Stock solutions at a concentration of 100 mM were prepared in dimethylsulphoxide.

Cells, viruses and plasmid constructs. HepG2 (ATCC HB-8065), Vero E6 (ATCC CRL-1587) and BHK-21 (ATCC CCL-10) cells were grown as monolayers in Eagle's minimum essential medium (MEM, GIBCO, USA) containing 10% inactivated fetal bovine serum (FBS) and 2 mM L-glutamine. Maintenance medium (MM) consisted of MEM supplemented with 5% calf serum. Stocks of LCMV strain WE were prepared in BHK-21 cell cultures (10^7 cells/T175) at a MOI of 0.1 PFU/cell. Supernatants were harvested at 48 and 72 h after infection and stored at 10^7–10^8 PFU/ml at −70 °C until needed. All virus stocks were titrated by plaque assay on Vero E6 cells.

The mammalian expression constructs containing Z (pcDNA3.1-Z, pMLV-Zwt, pMLV-Zmut) were prepared as described previously [18].

Cytotoxicity assay. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich) method in HepG2 cells as previously described [22]. Also calculated was the median cytotoxic concentration (CC_{50}), or compound concentration required to reduce the MTT signal by 50% compared to controls.

Virus yield-inhibition assay. HepG2 cells grown in 6-well microplates were infected at a MOI of 0.1 PFU/cell. After 1 h adsorption at 37 °C, cells were washed and re-fed with MM containing serial twofold dilutions of NSC20625. After 48 h of incubation at 37 °C, culture supernatants were harvested and extracellular virus yields were determined by a plaque assay in Vero E6 cells. The 50% effective concentration (EC_{50}) 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.

Transient transfection studies. HepG2 cells were transiently transfected using Lipofectamine 2000 Reagent with 1 μg of the appropriate mammalian expression construct (pcDNA3.1 or pMLV). At 24 h after transfection, cells were incubated in MM containing or not 40 μM NSC20625 for 4 or 24 h and then prepared for immunofluorescence.

Immunofluorescence and confocal microscopy. HepG2 cells grown on coverslips were infected with LCMV at a MOI of 1 or transfected with 1 μg of plasmid DNA, and incubated in MM containing or not 40 μM NSC20625. At the indicated time, cells were rinsed two times in PBS and fixed with methanol at −20 °C for 10 min. Then, cells were washed with PBS again and probed for 2 h at room temperature with mouse monoclonal anti-PML (mAB 5E10; 1:20) and rabbit polyclonal anti-Z serum, followed by incubation with a cy5-conjugated donkey anti-mouse and FITC-conjugated goat anti-rabbit antibody (Jackson Laboratory) for 1 h at room temperature. After incubation with the secondary antibody, cells were washed three times with PBS, dried, mounted in Vectashield mounting medium with DAPI (4′,6′-diamidino-2-phenylindole; Vector Laboratories) to visualize nuclei, and sealed. Confocal micrographs were detected using a magnification of 100× with an additional zoom factor of 2 on a Zeiss Meta 510 laser scanning confocal microscope. All channels were detected separately, and no cross-talk was observed between channels.

Results

Antiviral properties of NSC20625: effect on distribution pattern of PML protein in LCMV-infected cells

To study the effects of NSC20625 on the interaction of Z with the cellular PML protein, the human hepatic cell line HepG2 was the host used for infection or transfection. Since our previous studies have assayed the activity against arenaviruses of NSC20625 in Vero cells, we first evaluated the cytotoxicity and anti-LCMV inhibitory effect of this compound in HepG2 cells. The antiviral EC_{50} was 8.33 μM, whereas CC_{50} was >100 μM. After determining the effectiveness and selectivity of NSC20625 to inhibit LCMV infection in HepG2 cells, we studied the impact of the antiviral action of NSC20625 on Z–PML cellular localization. HepG2 cells were infected with LCMV, and after 72 h incubation, cells were treated with MM containing 40 μM of NSC20625 for different time periods (2 and 12 h); then, cells were fixed and indirect immunofluorescence was used to reveal the subcellular distribution of Z and PML. As controls, other cell cultures were mock-infected untreated (Fig. 2A) or treated with NSC20625 (Fig. 2B) during the 72 h period. Confocal images of infected cells have shown that, as reported before [18], PML underwent a dramatic rearrangement during the infection (Fig. 2C) becoming less distinct and disaggregated in comparison with uninfected cells (Fig. 2A). It appears that after infection many of the PML bodies become surrounded by increased diffuse fluorescence in the vicinity of the original structure, suggesting a marked efflux of PML protein. However, even after only 2 h of NSC20625 treatment (Fig. 2D), the PML protein started its accumulation again in PML NB. At longer treatments (12 h) (Fig. 2E) this diffuse fluorescence was less marked and the PML

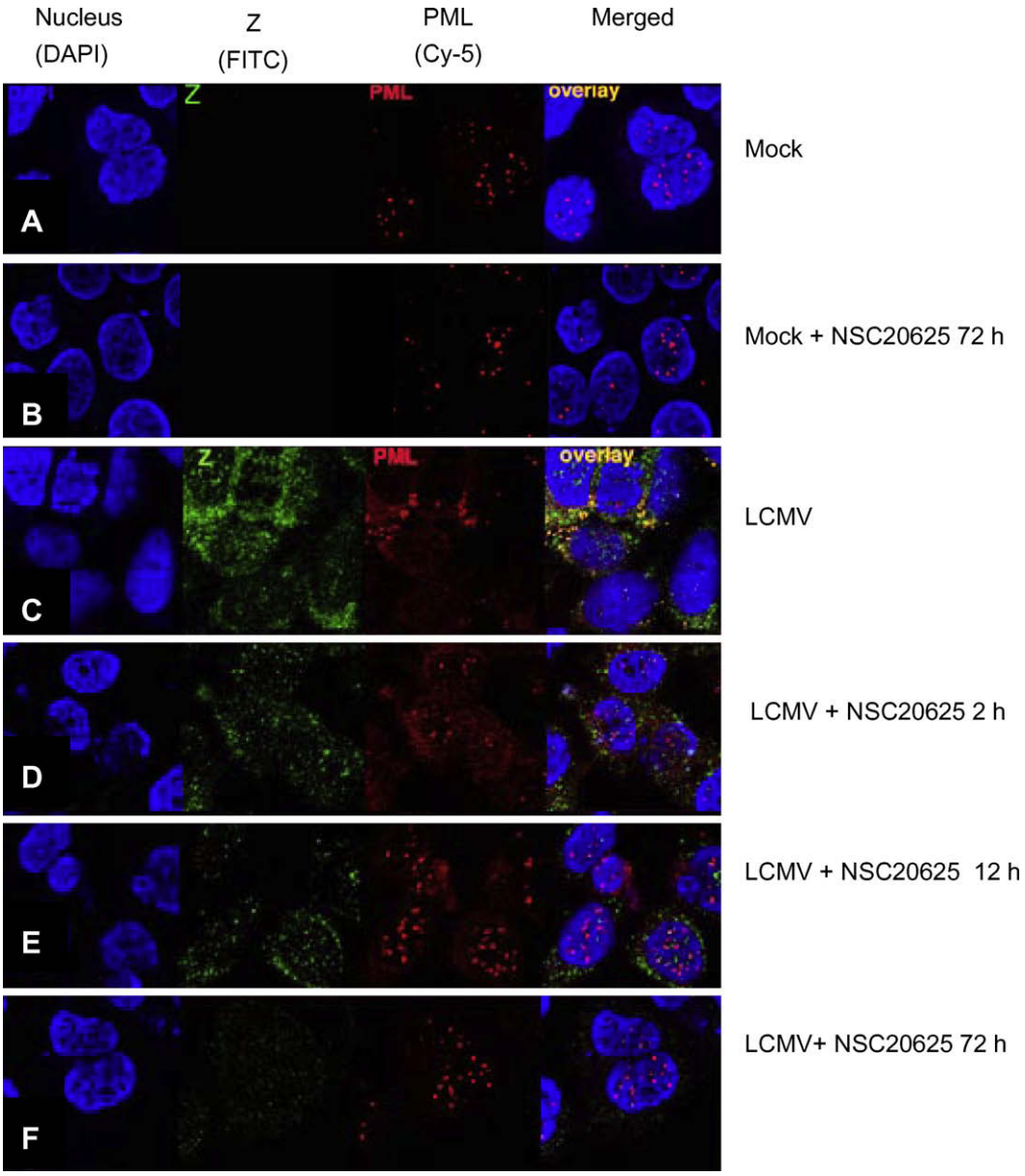

Fig. 2. Effect of NSC20625 on Z–PML subcellular distribution. HepG2 cells were infected with LCMV (MOI 1), and after 72 h incubation, cells were treated with maintenance medium containing 40 μM of NSC20625 for different time periods: 2 h (D) and 12 h (E); then, cells were probed for 2 h at room temperature with mouse monoclonal anti-PML and rabbit polyclonal anti-Z, followed by an incubation with a cy5-conjugated donkey anti-mouse and FITC- conjugated goat anti-rabbit. As controls, some uninfected cells were treated with NSC20625 (B) and some infected cells were not treated (C) or treated with NSC20625 (F) during the 72 h infection period. By confocal laser microscopy, blue (DAPI), green (Z) or red (PML) fluorescence was recorded independently (magnification, 400×). Images were overlaid in Photoshop. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

NB became distinct and well-defined, similar to their appearance before infection (Fig. 2A). It is worth mentioning that the reorganization of the PML NB began at an early stage of treatment, before extensive inhibition of Z was detectable by the image-capture conditions we used (Fig. 2D and E). The inhibition of Z expression was increasing with longer time treatments and was total after 72 h treatment (Fig. 2F). This evidence suggests but does not prove that PML NB structures have a role in arenavirus infection.

Direct effect of NSC20625 on cells expressing Z from a transfected plasmid

We knew from previous studies that transfection with the LCMV Z gene alone is sufficient to redistribute PML to the cytoplasm and to alter the appearance of PML from punctate nuclear to diffuse-cytoplasmic [18]. In order to verify the direct effect of NSC20625 on PML–Z interaction and distribution, we have performed transfection studies in HepG2 cells using the pcDNA3.1-Z construct, followed by short and long time treatments (4 and 24 h) with 40 μM of NSC20625. Confocal image (Fig 3A) shows the cellular distribution of overexpressed Z after 24 h of transfection. PML staining showed the same diffuse pattern at 24 h Z post-transfection as was seen in LCMV-infected cells. PML distribution in the presence of Z varies from mostly cytoplasmic to cytoplasmic and nuclear (Fig. 3A) and may reflect the fact that cells are at different stages in the cell cycle during transfection. Interestingly, the Z signal was diminished after 4 h NSC20625 treatment (Fig. 3B), and completely inhibited after 24 h treatment (Fig. 3C), and as expected, the PML pattern started

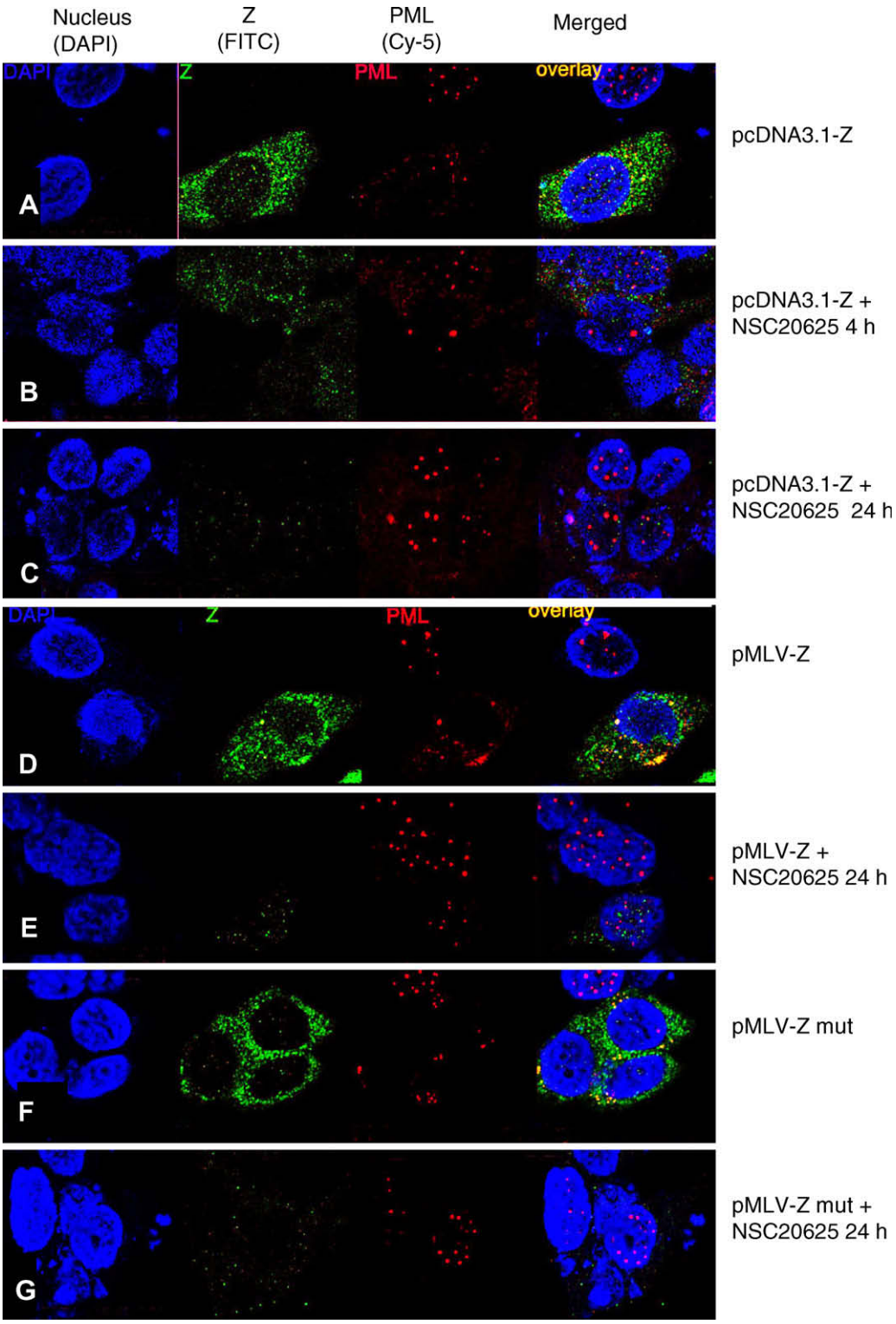

Fig. 3. Direct effect of NSC20625 on Z-expressing cells from a transfected plasmid. HepG2 cells were transfected with the pcDNA3.1-Z (1 μg), followed by non-treatment (A) or 4 h (B) and 24 h (C) time point treatments with 40 μM of NSC20625. In order to see the effect of NSC20625 on a Z mutant, HepG2 cells were transfected with 1 μg of pMLV-Z (D and E) or pMLV-Z mutant (F and G), respectively, followed by 24 h NSC20625 treatment (E and G), or non-treatment (D and F). Then, cells were probed with mouse monoclonal anti-PML and rabbit polyclonal anti-Z, followed by incubation with a cy5-conjugated donkey anti-mouse and FITC- conjugated goat anti-rabbit. By confocal laser microscopy, blue (DAPI), green (Z) or red (PML) fluorescence was recorded independently (magnification, 400×). Images were overlaid in Photoshop. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to return to discrete bodies after 4 h treatment (Fig. 3B), and was entirely recovered after 24 h NSC20625 treatment (Fig. 3C). As expected, cells transfected with the empty vector exhibited the normal PML distribution (data not shown).

Z mutant remains susceptible and interactive with NSC20625

The RING domain of Z protein contains a conserved cysteine-rich motif $CX_2CX_9CX_2HX_2CX_2CX_{10}CX_2C$ able to bind two zinc

atoms. Our previous studies showed that a single mutation at one zinc-binding site of the Z RING finger did not affect the ability of this mutant to colocalize with PML or to convert the punctate phenotype of PML to a diffuse one [24]. Here, we have used a construct expressing the Z protein mutated at the site 2 of the RING finger (Cys32Cys35ΔPhe32Gly35) in order to study the effect of NSC20625 on the remaining site-1 zinc ion of the RING-finger structure and its effect on PML distribution.

Transfection with the construct pMLV-Z containing wild-type Z showed the typical diffuse pattern of PML redistribution (Fig. 3D), and the treatment with NSC20625 restored discrete PML NB (Fig. 3E), as observed above in pcDNA3.1-Z transfected cells. Fig. 3F showed that a single mutation at the RING finger did not affect the normal cytoplasmic distribution of Z protein, but its nuclear localization was somewhat affected (Fig. 3F). Confocal images indicated that NSC20625 treatment impaired the detection of mutant Z and restored PML NB (Fig. 3G). It seems that the diminished staining pattern of Z mutant gets more punctate after the treatment, which could be indicative of aggregation. An effect of NSC20625 inducing misfolding and oligomerization of wild-type Z to high molecular mass aggregates was previously reported following treatment of a recombinant LCMV Z protein with the disulfide [23]. It appears likely that after treatment with NSC20625, unfolded and aggregated Z mutant loses its ability to bind PML leading to the reconstruction of PML NB. Altogether, these results indicate that Z–PML interaction depends on the integrity of at least one intact zinc-binding site within the Z RING-finger motif.

Discussion

Zinc-binding domains of proteins were first discovered as nucleic acid-binding domains, however the more globular RING structures discovered later also bound zinc, and were found to mediate protein:protein interactions [25]. The Z protein is the only arenavirus protein with a RING structure, but two other viral proteins bind zinc. A classical zinc-finger motif was found near the C-terminal of the NP [26], and a zinc-binding activity in the cytoplasmic domain of the arenavirus GP2 fusion protein was also reported [27]. The NP is responsible for maintaining the structure of the viral nucleocapsid, which is the key component of RNA transcription and replication. GP2 is mainly involved in the fusion step of virus entry. Although we cannot discount an interaction between the inhibitor and these viral proteins, at present, no alterations in NP and GP2 were detected after NSC20625 treatment of virions [23]. It must be noted that the Z protein (95 aa) might be more vulnerable to the action of this compound because its RING structure (60 aa) comprises the majority of its length and the integrity of this domain is important for LCMV Z biological functions.

Here we present new data about the effects of a zinc-binding antiviral on LCMV and its Z protein expression in a liver cell line. Liver cells are important targets for LCMV because their control of physiological functions such as energy storage, detoxification and activation of innate immunity is severely impacted by virulent viral infections [21,28,29].

It is remarkable that NSC20625 affected Z without any apparent effect on another RING protein, the cellular PML. This observation is in agreement with the selectivity exhibited by the compound in HepG2 cells, with inhibition of virus yield higher than 90% at concentrations minimally affecting cell viability (Fig. 1B). If the mode of action of this compound involves the oxidation of the cysteine thiolates in the Z RING finger, then any mild oxidizing agent could be considered a potential antiviral agent. Obviously, the number of such compounds is extremely large. However, in our experience, many of these agents exhibit severe toxicity in cell culture, few do not, and even fewer are selective for the viral target and effective as antivirals. The efficacy of these agents may be limited by their ability to resist intracellular metabolism. Further experiments are required to determine whether the effect of NSC20625 on arenaviruses is also associated with an interaction of the disulfide compound with the other viral and cellular zinc-containing proteins.

Viruses need to multiply in the infected host and have evolved different strategies to impair the cellular antiviral response, and to modulate apoptosis in infected cells [30,31]. PML is implicated in pro-apoptotic [32–34] and antiviral responses [35], which may explain why PML NB are frequent targets of viral infection. However, very little information is available about how RNA viruses affect PML NB. Since the RING domains of arenaviral Z proteins are highly conserved [36], it is likely that all arenaviruses will have similar effects on PML NB. In addition, PML is prominently expressed in reticuloendothelial cells [37], the most frequent targets of arenavirus infection [38]. The confocal images presented here clearly showed that PML is within discrete NB that are subject to dramatic reorganization during LCMV infection and that NSC20625 successfully inhibited these viral events. The interaction between PML and Z has been considered deleterious to virus replication because LCMV grows to higher titers in PML-negative cells [37]. Thus, disruption of PML–Z interactions with NSC20625 may have some positive impact on virus replication. Despite that possibility, there is an unmistakable depletion of LCMV and Z protein expression in cells treated with NSC20625 indicating that this compound is antiviral and probably damages aspects of the viral life cycle.

Finally, compounds that disrupt RING structures may help to elucidate the cross-talk between PML and the arenaviral Z protein during the infection cycle, and be used as tools for designing novel antiviral strategies against arenavirus infection. It is currently not entirely clear how viral effects on apoptosis, cell cycles, and NB distribution are connected to the systemic manifestations of disease caused by arenaviruses.

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