Antiviral Research 93 (2012) 23–29

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Identification of an antioxidant small-molecule with broad-spectrum antiviral activity

Rekha G. Panchal [a,*], St. Patrick Reid [a], Julie P. Tran [a], Alison A. Bergeron [a], Jay Wells [a], Krishna P. Kota [b], Javad Aman [c], Sina Bavari [a,*]

[a] United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011, USA
[b] PerkinElmer, Waltham, MA, USA
[c] Integrated Biotherapeutics, Germantown, MD, USA

ARTICLE INFO

Article history:
Received 8 July 2011
Revised 30 September 2011
Accepted 10 October 2011
Available online 18 October 2011

Keywords:
Broad-spectrum
Filoviruses
High-content
Antioxidant

ABSTRACT

The highly lethal filoviruses, Ebola and Marburg cause severe hemorrhagic fever in humans and non-human primates. To date there are no licensed vaccines or therapeutics to counter these infections. Identifying novel pathways and host targets that play an essential role during infection will provide potential targets to develop therapeutics. Small molecule chemical screening for Ebola virus inhibitors resulted in identification of a compound NSC 62914. The compound was found to exhibit anti-filovirus activity in cell-based assays and *in vivo* protected mice following challenge with Ebola or Marburg viruses. Additionally, the compound was found to inhibit Rift Valley fever virus, Lassa virus and Venezuelan equine encephalitis virus in cell-based assays. Investigation of the mechanism of action of the compound revealed that it had antioxidant properties. Specifically, compound NSC 62914 was found to act as a scavenger of reactive oxygen species, and to up-regulate oxidative stress-induced genes. However, four known antioxidant compounds failed to inhibit filovirus infection, thus suggesting that the mechanistic basis of the antiviral function of the antioxidant NSC 62914 may involve modulation of multiple signaling pathways/targets.

Published by Elsevier B.V.

1. Introduction

The filoviruses, Ebola viruses (EBOV) and Marburg viruses (MARV) are non-segmented, enveloped, negative strand RNA viruses that cause severe hemorrhagic fever in humans and non-human primates (Bray and Murphy, 2007; Ascenzi et al., 2008). Filoviruses cause periodic outbreaks with mortality rates as high as 90%. To date there are no FDA licensed vaccines or antiviral inhibitors to prevent or treat these infections, although vaccines and additional strategies that protect nonhuman primates from lethal filovirus challenge have been described (Falzarano et al., 2011; Feldmann and Geisbert, 2011). The development of efficacious therapeutics against filoviruses is hampered, in part by limited knowledge of the mechanisms underlying pathogenesis at the molecular level. Several mechanisms contributing to pathogenesis have been reported (Volchkov et al., 2001; Geisbert et al., 2003; Basler and Amarasinghe, 2009). In addition, recent advances in the study of viral assembly and budding have shed light on the critical role for the interaction of viral proteins with host factors (Liu and Harty, 2010). In particular, proteomic studies have identified several host proteins that are exploited during the filovirus life cycle and thus provide us with potential targets for developing host-based antiviral therapeutics (Spurgers et al., 2010).

Reactive oxygen species (ROS) are chemically reactive molecules produced as a by-product of oxygen metabolism. At relatively low concentrations ROS can act as cellular messengers playing a key role in antimicrobial and antitumor defense (Freeman and Crapo, 1982). However excessive production and accumulation of ROS can cause cellular damage and has been implicated in the pathogenesis of several disease states, including cancer and neurodegenerative diseases (Christen, 2000; Sawyer and Colucci, 2000; Valko et al., 2006). Under normal physiological conditions the damaging effects of ROS are kept in check by the cellular antioxidant defense system. In this system, antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidases can directly neutralize ROS (Jung and Kwak, 2010). The basal and inducible expression of these and a wide-array of additional antioxidant

Abbreviations: EBOV, Ebola virus; MARV, Marburg virus; ROS, reactive oxygen species; RVFV, Rift Valley fever virus.

* Corresponding authors. Address: Target Discovery & Cellular Microbiology Dept., 1425 Porter Street, USAMRIID, Frederick, MD 21702-5011, USA. Tel.: +1 301 619 4985 (R.G. Panchal), +1 301 619 4246 (S. Bavari).

E-mail addresses: rekha.panchal@amedd.army.mil (R.G. Panchal), sina.bavari@amedd.army.mil (S. Bavari).

0166-3542/$ - see front matter Published by Elsevier B.V.
doi:10.1016/j.antiviral.2011.10.011

genes are regulated by nuclear factor E2-related factor 2 (Nrf2) (Wakabayashi et al., 2010). Nrf2 is a basic leucine zipper redox sensitive transcription factor which binds to the antioxidant response element (ARE) present in the 5′ promoter region of many antioxidant genes (Nguyen et al., 2003).

ROS play an important role in viral infections and contribute to pathogenesis for a number of viruses (Schwarz, 1996; Peterhans, 1997; Akaike, 2001). For example, production of ROS has been demonstrated to play a role in pulmonary damage caused by influenza virus infection (Oda et al., 1989; Akaike, 2001). These data suggest that antioxidants may prove useful in combating viral pathogenesis. Indeed, antioxidants such as *N*-acetylcysteine (NAC) have been shown to have an inhibitory effect on influenza virus and HIV infection (Kalebic et al., 1991; Ghezzi and Ungheri, 2004; Garozzo et al., 2007). To date, the role of ROS in the pathogenesis of filovirus infection has yet to be determined, although increased levels of Cu/Zn superoxide dismutase (SOD), a marker for ROS buildup, has been found in the fluids of EBOV-infected adherent human monocytes/macrophages (Hensley et al., 2002).

Antioxidant therapy for prevention and treatment of various diseases including viral infections has not been very successful in clinical studies due to various reasons such as; oxidative stress is not the primary or the only cause of the disease, low bioavailability of the drug, poor target specificity, toxic side effects and inability of the drug to lower oxidative stress (Firuzi et al., in press; De Flora et al., 1997). Clinical evaluation of long term treatment with the antioxidant NAC in the therapy or prevention of influenza infection has revealed that NAC did not prevent influenza A/H1N1 virus infection but significantly reduced the incidence of clinically apparent disease by improving the patient's cell-mediated immunity (De Flora et al., 1997). To improve the success rate of antioxidant therapy it will be important to identify oxidative stress status biomarkers to measure the response to the antioxidant, select the right target, optimize time and duration of therapy, as excessive antioxidant can aggravate the disease condition, and probably consider antioxidant combination therapy.

In the present study we report the identification of a compound NSC 62914 (IUPAC name: 2-tert-butyl-6-[[3-[(3-tert-butyl-2-hydroxy-5-methylphenyl)methyl]-2-hydroxy-5-methylphenyl]methyl]-4methyl phenol), discovered by high-throughput screening for inhibitors of EBOV. Cheminformatic analysis predicted compound NSC 62914 to have antioxidant properties. Further experiments confirmed this antioxidant activity. NSC 62914 was demonstrated to be a potent ROS scavenger and was also able to induce expression of Nrf2-regulated antioxidant genes. We also demonstrated that *in vivo* compound NSC 62914 protected mice against lethal EBOV and MARV challenge. Taken together we have identified a novel antioxidant small-molecule with potent broad-spectrum antiviral activity.

2. Materials and methods

2.1. Chemical library

The small molecule diversity set library obtained from the National Cancer Institute is a collection of 1990 compounds chosen to cover a large and diverse range of molecular scaffolds and pharmacophoric features. A detailed description of the compounds in the diversity set library can be obtained from the web site: http://dtp.nci.nih.gov/dtpstandard/ChemData/index.jsp. The compounds from this library were screened in the EBOV-Zaire (ZEBOV) infection assay. Primary screening was done in duplicate at a single concentration of 20 μM and subsequent dose response studies were done to confirm the hits.

2.2. Screening for anti-viral inhibitors

Previously, we have described in detail the development of high-content imaging assays for EBOV and MARV infections and partial screening of the NCI diversity set library to identify anti-EBOV inhibitors (Panchal et al., 2010). In brief, Vero76 cells (4×10^4 cells/well) were seeded in 96-well BD high content imaging plates (BD Biosciences) and allowed to incubate overnight. The next day, cells were pretreated at 20 μM concentration of the small molecules for 2 h and then infected at a multiplicity of infection (MOI) of 5 with the mouse-adapted strain of ZEBOV that expresses the enhanced green fluorescent protein (GFP) (ZEBOV-eGFP) (Towner et al., 2005). After 48 h, cells were fixed in 10% formalin for 3 days, washed with PBS and stained with CellMask™ Deep Red cytoplasmic/nuclear stain (Invitrogen) and the nuclear Hoechst dye (Invitrogen).

For Lassa virus infections, Vero76 cells (3×10^4 cells/well) were seeded in 96-well BD high content imaging plates. The next day, cells were pretreated for 2 h with varying concentrations (0–20 μM) of NSC 62914 then infected at a MOI of 0.1 with Lassa virus. After 2 h, medium containing virus and compounds were removed, cells washed with PBS and then further incubated with medium containing appropriate concentrations of the compounds. After 48 h the cells were fixed in 10% formalin for 3 days, washed with PBS, blocked for 1 h at room temperature (RT) or overnight at 4 °C with 3% BSA prepared in PBS and then incubated with an anti-Lassa GP (L52-161-6) monoclonal antibody for 1 h at RT. After washing with PBS, the cells were stained for 1 h at RT with rabbit anti-mouse Dylight 488 secondary antibody, washed and then stained with CellMask™ Deep Red cytoplasmic/nuclear stain (Invitrogen) and the nuclear Hoechst dye (Invitrogen).

For Rift Valley fever virus (RVFV) the attenuated MP12 vaccine strain was used to infect A549, a human lung adenocarcinoma epithelial cell line. Briefly, A549 cells (3×10^4 cells/well) were seeded in a 96 well BD imaging plate. The next day the cells were pretreated for 2 h with varying concentrations of compound NSC 62914. The cells were infected at a MOI of 1 with the MP12 virus for 2 h, washed and further incubated in the presence of the compounds. After 24 h, cells were fixed, washed with PBS, blocked and stained with anti-RVF4D4 antibody for 1 h at RT. After washing with PBS, the cells were stained for 1 h at RT with rabbit anti-mouse Dylight 488 secondary antibody, washed and then stained with CellMask™ Deep Red cytoplasmic/nuclear stain (Invitrogen) and the nuclear Hoechst dye (Invitrogen).

For VEE TC 83 infections, Hela cells (10,000 cells/well) were seeded in 96-well BD imaging plates. The next day the cells were pretreated with different concentrations of NSC 62914 for 2 h and then infected at a MOI of 1. After 2 h virus was removed, cells washed and then further incubated with medium containing appropriate concentration of the compounds. After 20 h, cells were fixed, washed, blocked and then incubated for 1 h with anti-E2 (1A4A) monoclonal antibody. Subsequent staining was performed as described above. All assays were done in duplicate and repeated two independent times. Representative results are shown.

2.3. Antioxidant assay

To measure the antioxidant property of the compound NSC 62914, we employed the ABTS antioxidant assay (Cayman Chemical antioxidant assay kit). This assay relies on the ability of the antioxidant added to the sample to prevent oxidation of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate]) by a ferryl metmyoglobin radical, resulting in a decrease in the absorbance at 405 nm. The ability of the compound NSC 62914 to inhibit ABTS oxidation was compared with Trolox (a water soluble toco-pherol

analog), Pyrrolidine dithiocarbamate (PDTC), tert-butylhydroquinone (tBHQ) and *N*-acetyl cysteine (NAC).

For the cell-based antioxidant study the DCFH-DA assay was used (OxiSelect Intracellular ROS Assay Kit, Cell Biolabs, Inc.). Briefly, Hela cells (10,000 cells/well) seeded in a 96-well plate were loaded with a cell-permeable fluorogenic probe 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), diluted with serum free medium in the absence or presence of the indicated concentrations of Trolox, PDTC or compound NSC 62914. The cells were incubated for 1 h, washed twice with PBS. The DCFH-DA loaded cells were treated with hydrogen peroxide (H_2O_2), an oxidative stress inducer, and further incubated for one hour. The assay was terminated by adding cell lysis buffer, and fluorescence intensity was measured at 493 nm (ex)/523 nm (em) using a fluorescent plate reader. All assays were done in duplicates and repeated three independent times. Representative data is shown.

2.4. Quantitative real-time PCR

For quantitative real time PCR analysis, HepG2 cells were either left untreated or treated with 100 μM tBHQ or 10 μM of NSC 62914. At the indicated time points RNA was harvested using the Qiagen RNeasy mini isolation kit (Qiagen). Transcript levels were determined using inventoried Taqman primer/probes for NQO1 (Assay ID: Hs00168547_m1), GCLC (Hs00155249_m1) and Nrf2 (Hs00232352_m1) (Applied Biosystems), Taqman one-step RT-PCR kit (Applied Biosystems) and a 7900HT Fast real-time PCR instrument (Applied Biosystem). For each reaction 50 ng of input RNA was used.

2.5. In vivo efficacy studies

For EBOV studies, mouse-adapted ZEBOV (Bray et al., 1998) was used at a challenge dose of 1000 pfu (3000 LD_{50}). In the prophylaxis study, mice (C57BL/6, $n = 20$/group) were administered with the vehicle control (2% DMSO in 5% dextrose water) or compound NSC 62914 (2 mg/kg/injection) and after 1 hour, challenged with the mouse-adapted ZEBOV virus. Additional treatments with the vehicle control or compound were given on day 2 and day 5 post challenge. In a post-infection model, C57BL/6 mice ($n = 10$), were challenged with 1000 pfu of the mouse-adapted ZEBOV and treatment with the compound NSC 62914 (2 or 5 mg/kg/injection) was initiated day 1 post challenge. Two additional treatments were given on days 3 and 5 post challenge. All virus challenges and compound treatments were performed via the intraperitoneal (ip) route of injection.

For *in vivo* MARV studies, C57BL/6 mice ($n = 10$) were treated via the ip route with NSC 62914 (2 mg/kg/injection) for 1 h and then infected via the ip route with 1000 pfu of mouse-adapted MARV Ravn. Additional treatments were given at day 2 and day 5 post challenge.

All research was conducted under an IACUC approved protocol in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

3. Results

3.1. Screening of small molecule chemical library

Previously, we reported the screening of a partial set (580 compounds) of the NCI diversity library in an EBOV-eGFP infection assay (Panchal et al., 2010). Complete screening of this library (1990 compounds) resulted in the identification of 45 compounds that showed ⩾50% viral inhibition, at 20 μM final compound concentration. Based on the inhibition data, a single compound NSC 62914 was selected for further testing in a dose response study. The compound showed a dose-dependent inhibition of viral infection with an IC_{50} value of ∼5.2 μM (Fig. 1A). The chemical structure of the compound is shown in Fig. 1B and a representative image of viral inhibition by compound NSC 62914 is shown in Fig. 1C.

3.2. Structure-activity relationship (SAR)

The compound NSC 62914 is a triphenol compound that possesses antioxidative properties. The antioxidant property of NSC 62914 is predicted to be due to the presence of the 3 aryl-OH groups that function by scavenging ROS before they can interact with other molecules. Thus, by scavenging of radicals, NSC 62914 may prevent cellular damage to *in vivo* systems.

3.3. Antioxidant properties of compound NSC 62914

To confirm the antioxidant property of compound NSC 62914, we first investigated the scavenging potential of the compound in a cell-free ABTS assay. In this assay a reduction in absorbance results from an inhibition in the formation of the radical cation $ABTS^+$. At increasing concentrations compound NSC 62914 behaved similar to known antioxidant scavengers Trolox, tert-butylhydroquinone (tBHQ), *N*-acetyl cysteine (NAC) and pyrrolidinedithiocarbamate (PDTC) (Fig. 2A), confirming the scavenging function of the compound.

Next, we investigated the antioxidant potential of compound NSC 62914 in a cell-based assay. Hela cells were loaded with the cell-permeable DCFH-DA in the absence or presence of increasing concentration of Trolox, PDTC or NSC 62914. After one hour the cells were treated with H_2O_2 and further incubated for another hour. The assay relies on the compound DCFH-DA, a non-fluorescent probe that in cells is easily oxidized to the highly fluorescent compound dichlorofluorescein (DCF). A decrease in cellular fluorescence compared to the control cells, indicates the antioxidant capacity of the compounds added to the cells. In the presence of H_2O_2, DCFH-DA is oxidized to the fluorescent DCF. Although not as potent as the positive controls Trolox and PDTC, increasing concentrations of NSC 62914 reproducibly inhibited oxidation of DCFH-DA (Fig. 2B). This data further supports the antioxidant function of the compound.

In addition to scavenging activity many antioxidant small molecules can induce activation of the Nrf2-antioxidant defense system. To investigate whether compound NSC 62914 can activate this pathway, HepG2 cells were left untreated or treated with 10 μM of compound NSC62914 for various time points. As a positive control the well-established Nrf2 activator tBHQ was used. As expected, tBHQ induced activation of the Nrf2-regulated genes glutamate-cysteine ligase (GCLC) and NAD(P)H dehydrogenase quinine 1 (NQO1). Similarly, compound NSC 62914 induced expression at levels comparable to tBHQ (Fig. 2C). Interestingly, while tBHQ did not induce gene expression of Nrf2, compound NSC62914 was found to induce Nrf2 gene expression. Taken together, the data indicates that NSC 62914 acts as an antioxidant.

3.4. Anti-filovirus activity of known antioxidant scavengers

To test if the known antioxidants PDTC, Trolox, tBHQ and NAC exhibit anti-filovirus activity, Vero cells were pretreated for 1 h with two-fold dilution of the antioxidants and then infected with ZEBOV-eGFP for 48 h or Marburg Ravn for 72 h. Quantitative image analysis revealed that Trolox at very high concentrations

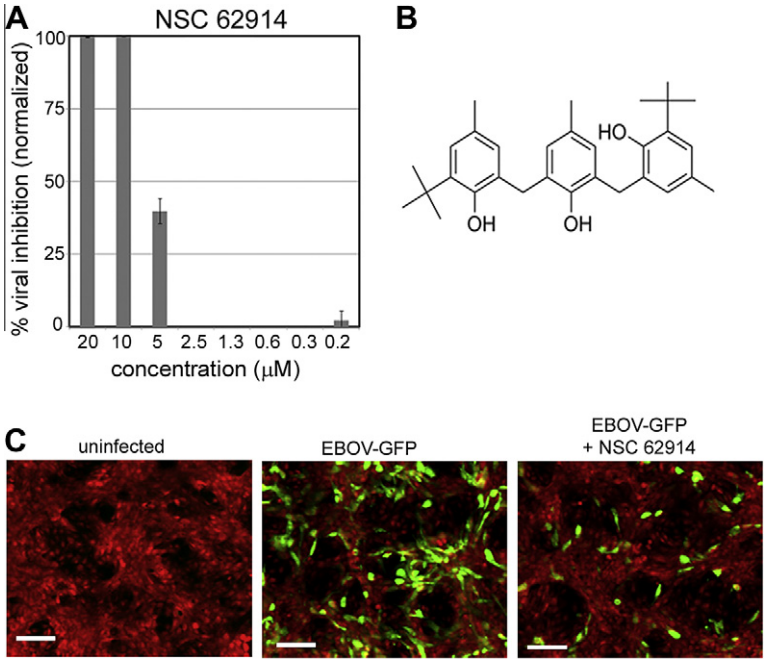

Fig. 1. NSC 62914 inhibits EBOV infection. (A) A dose response study showing the percent inhibition of ZEBOV-GFP infection at varying compound concentrations in Vero76 cells. (B) Chemical structure of NSC 62914. (C) Representative images acquired during high-content image analysis of cells that were either untreated (left panel), or treated with DMSO control and infected with EBOV-GFP virus (middle panel) or pre-treated with 10 μM of compound NSC 62914 and infected with EBOV-GFP virus. Green – EBOV-GFP and red – CellMask stain. Size marker = 10 μm.

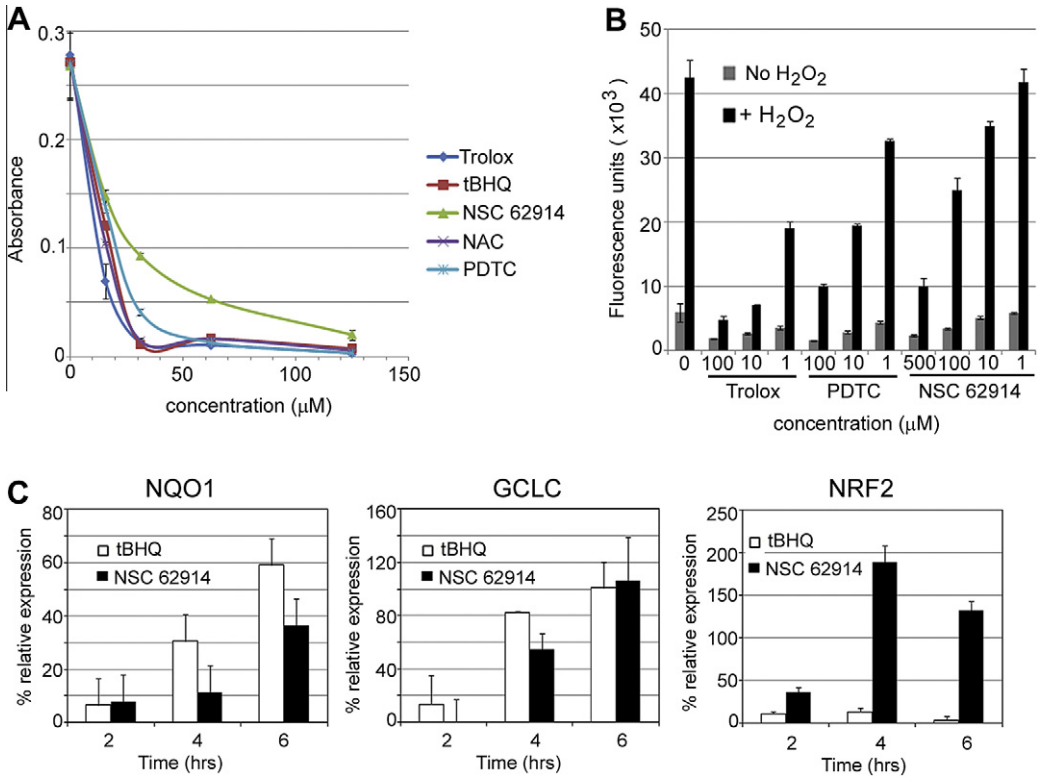

Fig. 2. Compound NSC 62914 exhibits antioxidant properties. (A) Cell-free ABTS assay. Varying concentrations of Trolox, tert-butylhydroquinone (tBHQ), Pyrrolidine dithiocarbamate (PDTC), *N*-acetyl cysteine (NAC) and NSC 62914 were tested for their ability to prevent ABTS oxidation, as measured by a decrease in the absorbance at 750 or 450 nm. (B) Cell-based DCFH-DA assay. Hela cells loaded with DCFH-DA were treated with various concentrations of the indicated compounds and then either left untreated or treated with H_2O_2 for 1 h. ROS accumulation was then determined by measuring fluorescence units. (C) qRT-PCR to measure expression of Nrf2-regulated genes. HepG2 cells were either left untreated or treated with tBHQ (100 μM) or NSC 62914 (10 μM) for the indicated time points and then subjected to qRT-PCR analysis to determine relative transcript levels. For each gene the expression level is plotted as a percentage of the transcript level in the untreated samples.

(⩾0.5 mM) showed a 40–70% inhibition of Ebola and Marburg virus infection (Fig. 3A) with minimal cytotoxicity (as measured by percent reduction in cell number, data not shown), while NAC and tBHQ at similar concentrations did not exhibit antiviral activity against these filoviruses (data not shown). The antioxidant PDTC showed a dose-dependent inhibition of Ebola and Marburg infection with a concomitant increase in cellular toxicity (Fig. 3B). Non-toxic concentrations of PDTC did not show any antiviral activity. These results suggest that the known antioxidants are not potent inhibitors of filovirus infection.

3.5. Broad spectrum anti-viral activity

Previous studies have shown that interference with the generation of ROS by use of antioxidants can drastically reduce the replication of seemingly unrelated viruses such as Sindbis, influenza A and Coxsackievirus B3 (Lin et al., 1995; Si et al., 2005; Geiler et al., 2010).The observed antioxidant activity of NSC 62914 prompted us to test the broad-spectrum antiviral activity of this compound against several different viral families, namely, *Bunyaviridae* (MP12, a vaccine strain of Rift Valley fever virus), *Arenaviridae* (Lassa virus), *Togaviridae* (Venezuelan Equine Encephalitis TC-83, a live attenuated vaccine derivative). In addition, we tested activity of the compound against the Ci67 strain of MARV. Similar to the initial EBOV screen, a dose-dependent inhibition of viral infection was observed across all virus families tested (Fig. 4). These results suggest that the compound NSC 62914 exhibits broad-spectrum antiviral activity.

3.6. In vivo efficacy studies

Based on the solubility and *in vitro* antiviral efficacy properties, compound NSC 62914 was further evaluated in the mouse model for EBOV infection (Bray et al., 1998). In this prophylaxis treatment model, where the compound (2 mg/kg/infection) was given 1 hour before challenge and at days 2 and 5 post-challenge, 80% of the compound-treated mice survived EBOV challenge (Fig. 5A). To determine if the compound is efficacious in a post exposure model, mice were challenged with EBOV and treatment with the compound was initiated day 1 post challenge. Two additional treatments were given on days 3 and 5 post challenge. Treatment dose of 2 mg/kg/injection conferred protection in 50% of the mice (Fig. 5B). Treatment with the higher dose of 5 mg/kg/injection did not improve survival of the mice which may be due totoxic side effects of the compound on the animals resulting in higher fatalities in a virus-independent manner. Alternatively the higher dose may have resulted in toxic effects in the animals, weakening their immune system and thus making them more susceptible to the subsequent virus infection. Together, these results suggest that the compound can provide a prophylactic effect and, to a lesser extent, a therapeutic effect on mice in a lethal EBOV challenge model.

To test the efficacy of the compound to protect mice in the MARV infection model, the mice were treated with NSC 62914(2 mg/kg/injection) for 1 h and then infected with 1000 pfu of mouse-adapted MARV Ravn virus. Additional treatments were given at day 2 and day 5. As shown in Fig. 5C, pretreatment of mice with the compound resulted in 90% survival rate. Taken together, these results suggest that that compound 62914 is effective in inhibiting filovirus infection.

4. Discussion

Small molecule chemical library screening for EBOV inhibitors lead to the identification of the compound NSC 62914, that has antiviral activity in cells and *in vivo* protects mice following EBOV and MARV challenge. The identified small molecule also exhibited

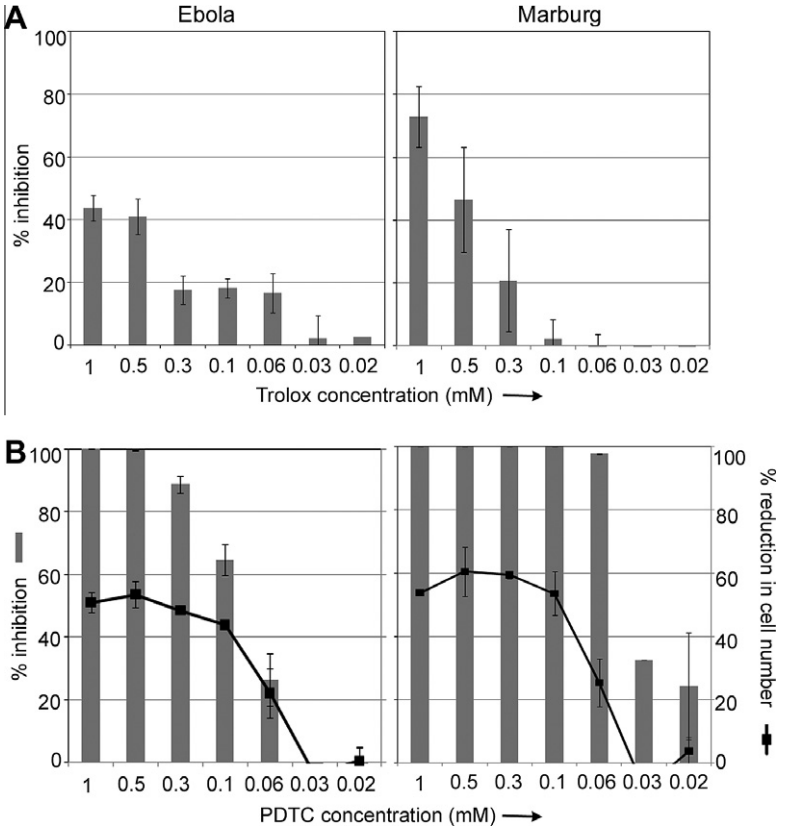

Fig. 3. Known antioxidants do not exhibit potent antiviral activity against filoviruses. A dose response study showing the percent inhibition of ZEBOV-GFP or Marburg Ravn infection at varying concentrations of Trolox (A) or PDTC (B). Cellular toxicity is shown by percent reduction in cell number with varying PDTC concentrations (B).

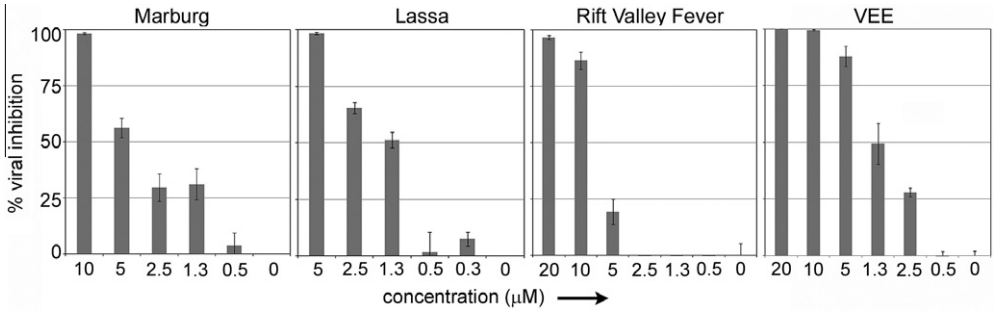

Fig. 4. Broad-spectrum antiviral activity of compound NSC 62914. A dose response study showing the percent inhibition of MARV (Ci67) and Lassa (Josiah) virus over varying concentrations of the compound in Vero76 cells. Similarly, dose response studies are shown for Rift valley fever virus (MP12) in A549 cells and for VEE TC 83 in Hela cells.

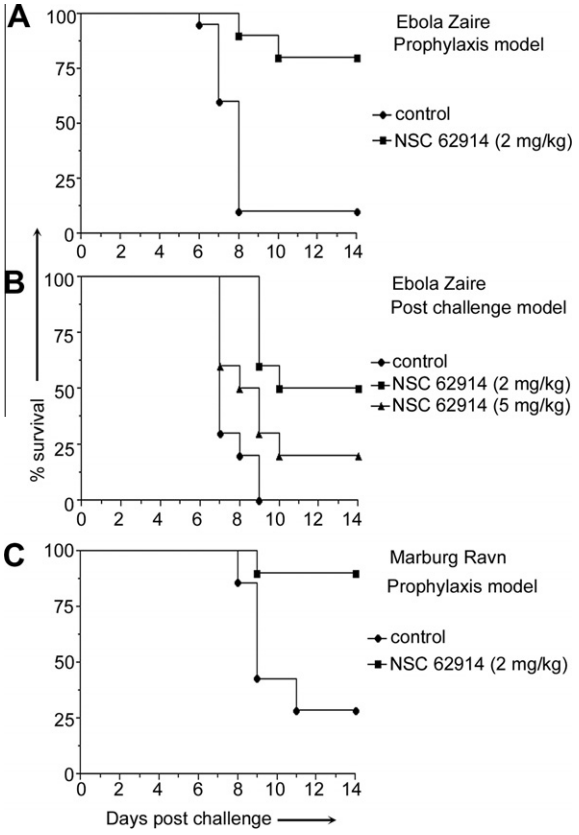

Fig. 5. Treatment with NSC 62914 protects mice from EBOV and MARV infection. (A) Testing compound NSC 62914 in a prophylactic model of EBOV infection. C57BL/6 mice were given the compound (2 mg/kg) 1 h prior to challenge with 1000 pfu (3000 LD_{50}) of mouse-adapted ZEBOV and then 2 and 5 days post challenge ($n = 20$ mice/group). (B) Testing compound NSC 62914 in a post challenge model of EBOV infection. Mice were infected with 1000 pfu of mouse-adapted ZEBOV and treatment with the compound (either 2 or 5 mg/kg) was initiated 1 day post challenge ($n = 10$ mice/group). Additional treatments with the compound were given days 3 and 5 post challenge. (C) Testing of the compound in prophylactic model of Marburg infection. Mice were treated with the compound (2 mg/kg) for 1 h prior to challenge with 1000 pfu of mouse-adapted MARV Ravn, and then 2 and 5 days post challenge. All virus challenges and compound treatments were done via intraperitoneal (ip) route.

broad-spectrum antiviral activity against several viruses, namely, Lassa, RVFV and VEE TC-83, thus suggesting that the compound targets a cellular pathway that is commonly exploited by multiple pathogens.

Based on structure activity studies, compound NSC 62914 was found to have the properties of an antioxidant. This property was confirmed in a series of studies. First, in a cell-free scavenging assay, the functional properties of NSC 62914 were similar to known antioxidant scavengers, Trolox, PDTC, tBHQ and NAC. Next, in a cell-based assay the compound inhibited H_2O_2 mediated production of the highly fluorescent DCF. The compound also displayed the ability to induce Nrf-2 regulated genes, indicating that it can activate the cellular antioxidant response system. However, in contrast to tBHQ, the compound induced Nrf2 gene expression, indicating that the compound likely mediates activation of additional signaling pathways. Proteomics-based approaches may help identify additional targets of the compound and thereby provide a better understanding of the protective effect of the compound.

Reactive oxygen species (ROS) contribute to the pathogenesis of a wide array of diseases including viral infections such as Japanese Encephalitis Virus, Respiratory syncytial virus (RSV) (Castro et al., 2006), Coxsackievirus B3 (Si et al., 2005) and H5N1 influenza A virus (Geiler et al., 2010). Filovirus pathogenesis is marked by dysregulation of multiple immune pathways and the severity of the disease pathology is a consequence of temporal host-induced disruption that occurs both at the cellular and molecular level. These viruses could potentially alter the host pro-antioxidant balance by increasing ROS production or by inhibiting antioxidant enzymes. However, to date there is no direct evidence of observed redox imbalance during filovirus infection and its influence on target tissues as well as on the whole organism.

Treatment with antioxidants like butylated hydroxyanisole (BHA) has been shown to ameliorate RSV-induced acute lung inflammation (Castro et al., 2006), while the antioxidant NAC inhibited replication of seasonal human influenza A viruses (Geiler et al., 2010). Progression of liver disease caused by hepatitis C virus is associated with oxidative stress due to mitochondrial respiratory chain dysfunction (Moriya et al., 2009). In recent studies Moriya et al. (2009) showed that Tacrolimus, a protector of mitochondrial respiratory function, could reverse the effect of the HCV core protein in the pathogenesis of HCV-associated liver disease. Thus there is precedence in the literature that treatment with antioxidants is beneficial by inhibiting viral infections. However, antioxidants can exhibit antiviral effects independent of their antioxidant activity. For example, the known antioxidant PDTC is reported to inhibit Coxsackievirus B3 replication through inhibition of the ubiquitin-proteasome pathway (Si et al., 2005) or inhibit influenza virus induced apoptosis by inhibiting viral macromolecular synthesis (Uchide et al., 2002). In our studies, known antioxidants PDTC, Trolox, tBHQ and NAC exhibited minimal to no anti-filovirus activity, thus suggesting that the antiviral effect of compound NSC 62914 may be independent of its antioxidant properties. Together our studies suggest that the compound NSC 62914 in addition to its antioxidant effects may potentially modulate additional signaling pathways, which in turn contributes to the observed broad-spectrum antiviral activity. Based on the antioxidant property of the identified small molecule, structure-activity relationship

(SAR) studies using NSC 62914 as a scaffold will help identify more potent antioxidants with broad spectrum antiviral activity.

Acknowledgements

We thank Xiaoli Chi, Julie Constantino and Cary Retterer for technical support, Dr. Robert (Dutch) Boltz for high-content imaging and image analysis. This project has been funded by Defense Threat Reduction Agency (to R.G. Panchal and S. Bavari). Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

References

Akaike, T., 2001. Role of free radicals in viral pathogenesis and mutation. Rev. Med. Virol. 11, 87–101.

Ascenzi, P., Bocedi, A., Heptonstall, J., Capobianchi, M.R., Di Caro, A., Mastrangelo, E., Bolognesi, M., Ippolito, G., 2008. Ebolavirus and Marburgvirus: insight the Filoviridae family. Mol. Aspects Med. 29, 151–185.

Basler, C.F., Amarasinghe, G.K., 2009. Evasion of interferon responses by Ebola and Marburg viruses. J. Interferon Cytokine Res. 29, 511–520.

Bray, M., Murphy, F.A., 2007. Filovirus research: knowledge expands to meet a growing threat. J. Infect. Dis. 196, S438–S443.

Bray, M., Davis, K., Geisbert, T., Schmaljohn, C., Huggins, J., 1998. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J. Infect. Dis. 178, 651–661.

Castro, S.M., Guerrero-Plata, A., Suarez-Real, G., Adegboyega, P.A., Colasurdo, G.N., Khan, A.M., Garofalo, R.P., Casola, A., 2006. Antioxidant treatment ameliorates respiratory syncytial virus-induced disease and lung inflammation. Am. J. Respir. Crit. Care Med. 174, 1361–1369.

Christen, Y., 2000. Oxidative stress and Alzheimer disease. Am. J. Clin. Nutr. 71, 621S–629S.

De Flora, S., Grassi, C., Carati, L., 1997. Attenuation of influenza-like symptomatology and improvement of cell-mediated immunity with long-term *N*-acetylcysteine treatment. Eur. Respir. J. 10, 1535–1541.

Falzarano, D., Geisbert, T.W., Feldmann, H., 2011. Progress in filovirusvaccineevelopment: evaluating the potential for clinical use. Expert Rev. Vaccines 10, 63–77.

Feldmann, H., Geisbert, T.W., 2011. Ebola haemorrhagic fever. Lancet 377, 849–862.

Firuzi, O., Miri, R., Tavakkoli, M., Saso, L., 2011. Antioxidant therapy: current status and future prospects. Curr. Med. Chem., in press.

Freeman, B.A., Crapo, J.D., 1982. Biology of disease: free radicals and tissue injury. Lab Invest. 47, 412–426.

Garozzo, A., Tempera, G., Ungheri, D., Timpanaro, R., Castro, A., 2007. *N*-acetylcysteine synergizes with oseltamivir in protecting mice from lethal influenza infection. Int. J. Immunopathol. Pharmacol. 20, 349–354.

Geiler, J., Michaelis, M., Naczk, P., Leutz, A., Langer, K., Doerr, H.W., Cinatl Jr., J., 2010. *N*-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. Biochem. Pharmacol. 79, 413–420.

Geisbert, T.W., Young, H.A., Jahrling, P.B., Davis, K.J., Kagan, E., Hensley, L.E., 2003. Mechanisms underlying coagulation abnormalities in ebola hemorrhagic fever: overexpression of tissue factor in primate monocytes/macrophages is a key event. J. Infect. Dis. 188, 1618–1629.

Ghezzi, P., Ungheri, D., 2004. Synergistic combination of *N*-acetylcysteine and ribavirin to protect from lethal influenza viral infection in a mouse model. Int. J. Immunopathol. Pharmacol. 17, 99–102.

Hensley, L.E., Young, H.A., Jahrling, P.B., Geisbert, T.W., 2002. Proinflammatory response during Ebola virus infection of primate models: possible involvement of the tumor necrosis factor receptor superfamily. Immunol. Lett. 80, 169–179.

Jung, K.A., Kwak, M.K., 2010. The Nrf2 system as a potential target for the development of indirect antioxidants. Molecules 15, 7266–7291.

Kalebic, T., Kinter, A., Poli, G., Anderson, M.E., Meister, A., Fauci, A.S., 1991. Suppression of human immunodeficiency virus expression in chronically infected monocytic cells by glutathione, glutathione ester, and *N*-acetylcysteine. Proc. Natl. Acad. Sci. USA 88, 986–990.

Lin, K.I., Lee, S.H., Narayanan, R., Baraban, J.M., Hardwick, J.M., Ratan, R.R., 1995. Thiol agents and Bcl-2 identify an alphavirus-induced apoptotic pathway that requires activation of the transcription factor NF-kappa B. J. Cell. Biol. 131, 1149–1161.

Liu, Y., Harty, R.N., 2010. Viral and host proteins that modulate filovirus budding. Future Virol. 5, 481–491.

Moriya, K., Miyoshi, H., Tsutsumi, T., Shinzawa, S., Fujie, H., Shintani, Y., Yotsuyanagi, H., Moriishi, K., Matsuura, Y., Suzuki, T., Miyamura, T., Koike, K., 2009. Tacrolimus ameliorates metabolic disturbance and oxidative stress caused by hepatitis C virus core protein: analysis using mouse model and cultured cells. Am. J. Pathol. 175, 1515–1524.

Nguyen, T., Sherratt, P.J., Pickett, C.B., 2003. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu. Rev. Pharmacol. Toxicol. 43, 233–260.

Oda, T., Akaike, T., Hamamoto, T., Suzuki, F., Hirano, T., Maeda, H., 1989. Oxygen radicals in influenza-induced pathogenesis and treatment with pyran polymer-conjugated SOD. Science 244, 974–976.

Panchal, R.G., Kota, K.P., Spurgers, K.B., Ruthel, G., Tran, J.P., Boltz, R.C., Bavari, S., 2010. Development of high-content imaging assays for lethal viral pathogens. J. Biomol. Screen. 15, 755–765.

Peterhans, E., 1997. Oxidants and antioxidants in viral diseases: disease mechanisms and metabolic regulation. J. Nutr. 127, 962S–965S.

Sawyer, D.B., Colucci, W.S., 2000. Mitochondrial oxidative stress in heart failure: ''oxygen wastage'' revisited. Circ. Res. 86, 119–120.

Schwarz, K.B., 1996. Oxidative stress during viral infection: a review. Free Radic. Biol. Med. 21, 641–649.

Si, X., McManus, B.M., Zhang, J., Yuan, J., Cheung, C., Esfandiarei, M., Suarez, A., Morgan, A., Luo, H., 2005. Pyrrolidine dithiocarbamate reduces coxsackievirus B3 replication through inhibition of the ubiquitin-proteasome pathway. J. Virol. 79, 8014–8023.

Spurgers, K.B., Alefantis, T., Peyser, B.D., Ruthel, G.T., Bergeron, A.A., Costantino, J.A., Enterlein, S., Kota, K.P., Boltz, R.C., Aman, M.J., Delvecchio, V.G., Bavari, S., 2010. Identification of essential filovirion-associated host factors by serial proteomic analysis and RNAi screen. Mol. Cell.Proteomics 9, 2690–2703.

Towner, J.S., Paragas, J., Dover, J.E., Gupta, M., Goldsmith, C.S., Huggins, J.W., Nichol, S.T., 2005. Generation of eGFP expressing recombinant Zaire ebolavirus for analysis of early pathogenesis events and high-throughput antiviral drug screening. Virology 332, 20–27.

Uchide, N., Ohyama, K., Bessho, T., Yuan, B., Yamakawa, T., 2002. Effect of antioxidants on apoptosis induced by influenza virus infection: inhibition of viral gene replication and transcription with pyrrolidine dithiocarbamate. Antiviral Res. 56, 207–217.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M., Mazur, M., 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact. 160, 1–40.

Volchkov, V.E., Volchkova, V.A., Muhlberger, E., Kolesnikova, L.V., Weik, M., Dolnik, O., Klenk, H.D., 2001. Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science 291, 1965–1969.

Wakabayashi, N., Slocum, S.L., Skoko, J.J., Shin, S., Kensler, T.W., 2010. When NRF2 talks, who's listening? Antioxid. Redox Signal 13, 1649–1663.