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## The cyanobacterial lectin scytovirin displays potent *in vitro* and *in vivo* activity against Zaire Ebola virus

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### Abstract

The cyanobacterial lectin scytovirin (SVN) binds with high affinity to mannose-rich oligosaccharides on the envelope glycoprotein (GP) of a number of viruses, blocking entry into target cells. In this study, we assessed the ability of SVN to bind to the envelope GP of Zaire Ebola virus (ZEBOV) and inhibit its replication. SVN interacted specifically with the protein’s mucin-rich domain. In cell culture, it inhibited ZEBOV replication with a 50% virus-inhibitory concentration (EC<sub>50</sub>) of 50 nM, and was also active against the Angola strain of the related Marburg virus (MARV), with a similar EC<sub>50</sub>. Injected subcutaneously in mice, SVN reached a peak plasma level of 100 nm in 45 minutes, but was cleared within 4 hours. When ZEBOV-infected mice were given 30 mg/kg/day of SVN by subcutaneous injection every 6 hours, beginning the day before virus challenge, 9 of 10 animals survived the infection, while all

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<sup>†</sup>This paper is dedicated to the memory of Dr. Barbara Giomarelli a valued colleague and friend (1971 – 2013)

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### Ethics statement

Animal research was conducted under an IACUC approved protocol at USAMRIID (USDA Registration Number 51-F-0021/728 & OLAW Assurance number A3473-01) in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

infected, untreated mice died. When treatment was begun one hour or one day after challenge, 70-90% of mice survived. Quantitation of infectious virus and viral RNA in samples of serum, liver and spleen collected on days 2 and 5 postinfection showed a trend toward lower titers in treated than control mice, with a significant decrease in liver titers on day 2. Our findings provide further evidence of the potential of natural lectins as therapeutic agents for viral infections.

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## Introduction

A number of cyanobacterial lectins bind with high affinity to high-mannose oligosaccharides on viral envelope glycoproteins, preventing virus attachment and entry into target cells (Boyd et al. 1997; Bokesch et al. 2003; Mori et al. 2005). These natural products might therefore be utilized to prevent or treat human viral diseases. One potential target for lectin therapy is the Zaire Ebola virus (ZEBOV), the causative agent of the current epidemic in West Africa. The surface of the EBOV virion bears multiple copies of a heavily glycosylated envelope glycoprotein (GP), which is cleaved by furin into two sub-units, GP1 and GP2, covalently linked by a disulfide bond. GP1 is a ~110 kDa protein that contains complex, hybrid and oligomannose oligosaccharides and a terminal mucin-rich region (Lee et al. 2008). It is responsible both for initiating viral attachment and entry and for evading immune response elements in the host (Takada et al. 1997; Lee, and Saphire 2009).

In an earlier proof-of-concept study, we found that the lectin cyanovirin (CVN), which is highly active against the human immunodeficiency virus (HIV), also inhibits the replication of ZEBOV (Boyd et al. 1997; Barrientos et al. 2003). Mice inoculated with ZEBOV and treated with repeated subcutaneous (s.c.) injections of CVN showed a significant prolongation of the course of illness, compared to untreated animals, but none survived the infection. Those findings suggested that additional lectins should be screened, to identify those with an enhanced ability to inhibit ZEBOV replication.

In the present study, we characterized the anti-ZEBOV activity of the lectin scytovirin (SVN), a 9.7 kDa monomeric protein isolated from the cyanobacterium *Scytonema varium* (Bokesch et al. 2003). SVN is known to bind with high affinity to the envelope GP of HIV and of the hepatitis C virus (HCV) (Bokesch et al. 2003; Takebe et al. 2013), but its activity against a highly pathogenic virus such as ZEBOV had not been assessed. We found that SVN was more active against ZEBOV than CVN, as it had a lower 50% virus-inhibitory concentration (EC<sub>50</sub>) in tissue culture and prevented the death of most ZEBOV-infected mice, when treatment was initiated the day before, the day of or the day after virus challenge, while all infected, untreated mice died. As discussed below, our findings suggest that researchers should continue to test other cyanobacterial lectins, to identify those with even greater activity against highly virulent pathogens.

## Materials and Methods

### SVN production

Scytovirin (SVN) was produced in *E. coli* and purified as previously described (Xiong et al. 2006). All protein samples were diluted to a final concentration of 1 mg/ml in PBS, as determined by amino acid analysis, and sterile-filtered before use.

### Virus and cells

Studies utilizing live ZEBOV and Marburg virus (MARV) were performed in Biosafety Level 4 (BSL-4) facilities at the U. S. Army Medical Research Institute of Infectious Diseases (USAMRIID); personnel wore positive-pressure protective suits fitted with HEPA filters and umbilical-fed air. USAMRIID is registered with the Centers for Disease Control and Prevention (CDC) Select Agent Program for the possession and use of biological select agents and toxins and has implemented a biological surety program in accordance with U. S. Army Regulation AR 50-1 “Biological Surety”. The construction of recombinant ZEBOV encoding green fluorescent protein (ZEBOV-eGFP) has been described (Towner et al. 2005). The virus was provided by John Towner at CDC and amplified in Vero E6 cells. Mouse-adapted ZEBOV has been described (Bray et al. 1998). The Angola strain of MARV was obtained from the USAMRIID collection. Vero E6 cells (ATCC: CRL-1586) were maintained in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum.

### In vitro assays for inhibition of ZEBOV-eGFP and MARV replication

For each assay, serial dilutions of SVN were added at a 4× concentration to 96-well plates of Vero E6 cells, which were then transferred to BSL-4 containment. ZEBOV-eGFP or MARV was added at a multiplicity of infection of 0.01. For ZEBOV, the plates were incubated for 48 hr and then read on a spectrofluorometer (Molecular Devices, Sunnyvale CA) at an excitation wavelength of 485 nm. For assays of MARV inhibition, the plates were incubated for 48 hours, then assayed for viral antigen by an immunofluorescence method using the 9G4 monoclonal antibody, as previously described (Radoshitzky et al. 2011). To confirm that decreases in fluorescence did not reflect cellular toxicity, uninfected cells were treated with the same concentrations of SVN and incubated for 48 hr, and their viability was assessed by an ATP-based method (Cell Titer-Glo, Promega, Madison, WI).

### ELISA of scytovirin binding to EBOV-infected cell lysates or recombinant GP1

Recombinant ZEBOV GP1 (rGP1) and the protein lacking the mucin region (rGP1 Muc) were provided by Dr. Erica Ollman Saphire, Scripps Research Institute. Wells of a 96-well protein binding plate (Nunc Immunosorp, Thermo Scientific, Waltham, MA) were inoculated with 100 ng of a EBOV-infected cell lysate, rGP1 or rGP1 Muc and allowed to incubate for 2 hr. The plate was then washed with PBS-0.5% Tween 20 and blocked overnight at 4° C with Superblock Buffer in PBS-Tween 20 (Pierce, Rockford, IL). The plate was washed three times and SVN was then added to triplicate wells at concentrations of 0.001-10 pmol/well and allowed to incubate for 2 hrs. After washing, the plate was incubated with a 1:1000 dilution of rabbit polyclonal anti-SVN IgG for 1hr, followed by

washing, incubation with a 1:5000 dilution of goat-anti-rabbit secondary antibodies ligated to horseradish peroxidase (HRP) (Immunopure, Thermo Scientific, Waltham, MA), washing and addition of HRP substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The reaction was stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and absorbance was measured at 450 nm. All tests were performed in triplicate.

### **Western blots of ZEBOV-infected cell lysates and rGP1**

Infected cell lysates and purified GP1 were electrophoresed by SDS-PAGE into a Tris-glycine 8 to 16% acrylamide gel (Invitrogen, Carlsbad, CA) under reducing conditions. Gels were stained with SimplyBlue SafeStain (Invitrogen, Carlsbad, CA) or blotted onto polyvinylidene difluoride (PVDF) membranes at a constant voltage of 25 volts for 2 hr. Membranes were blocked overnight at 4°C with Superblock Buffer in PBS containing 0.05% Tween 20. After washing, the membranes were incubated with buffered solutions of SVN (0.1 µg/ml) for 1 hr at room temperature, washed and incubated with polyclonal rabbit anti-SVN antibodies for 1 hr, washed, then incubated with goat anti-rabbit-HRP antibodies for 1 hr and washed again. Binding was detected with electrochemiluminescent reagents (Amersham, Buckinghamshire, UK).

### **Bioavailability of injected SVN in mice**

Four-8-week old BALB/c mice were obtained from the National Cancer Institute, Frederick, MD and housed under specific pathogen-free conditions. For bioavailability studies, 33 mice were injected s.c. with 10 mg/kg of SVN or with PBS; 3 mice were then sacrificed at each of 11 time points from 15 min to 48 hrs after injection, their blood was collected and the plasma separated. The concentration of SVN in the plasma of treated mice was determined using an adaptation of a method previously published for use with CVN (McFeeters et al. 2007). Briefly, wells of a 96-well plate were incubated with 100 ng/well of recombinant HIV-1 GP120 (Intracel, Columbia MD); after 2 hr, the wells were washed and then blocked overnight with 2% BSA in PBS. Subsequently, 50 µl of mouse plasma from varying time points was added to triplicate wells. After 2 hr incubation and repeat washing, binding of SVN was visualized by the antibody-bound HRP method described above. Samples with known concentrations of SVN in mouse plasma were tested on the same plate to provide a calibration curve for quantitation of the SVN in test samples.

### **Drug efficacy studies in EBOV-infected mice**

Virus challenge of BALB/c mice was performed by inoculating each animal intraperitoneally (i.p.) with 1000 plaque-forming units (pfu) of mouse-adapted ZEBOV (Bray et al. 1998). Treated mice were inoculated s.c. with 10, 20 or 30 mg/kg of SVN per day, divided into four equal doses given approximately every 6 hours; control mice received PBS in the same manner. One group was treated beginning 24 hours before virus challenge, with doses at -18, -12 and -6 hours, then one hour after infection and continuing every 6 hours. A second group was treated beginning 1 hr after virus challenge and a third beginning 24 hr after challenge. All groups were treated for a total of 10 days, and were monitored daily through day 28 for weight loss and survival. To assess adaptive immunity, the surviving mice and a naive control group were challenged with mouse-adapted EBOV at day 28 and monitored daily for additional 28 days.

## Quantitation of infectious virus by plaque assay and of viral RNA by qRT/PCR

Two experiments in mice were performed to determine titers of viral RNA and infectious virus in plasma and tissues. They were performed in the same manner as the efficacy studies just described, except that, instead of following the entire cohort for survival, some animals were sacrificed at various time points for plasma and tissue collection. As above, treated mice received 30mg/kg of SVN per day, divided into 4 s.c. doses, and control mice received PBS. In the first experiment, 3 mice were sacrificed on days 2, 5, 8 and 10; in the second, 5 mice were sacrificed on days 0, 0.5, 1, 2, 5, 6, 8, and 10. Titers of infectious virus in plasma and homogenized tissue were determined by the plaque assay method (Moe et al. 1981), in which 6-well plates of Vero E6 cells were infected with serial dilutions of samples in EMEM, followed by an agarose overlay and neutral red staining on day 7. Copy numbers of viral RNA in plasma and homogenized tissue were determined, based on detection of a viral nucleoprotein (NP) sequence by qRT-PCR. Samples were inactivated by the addition of Trizol LS (Invitrogen, Carlsbad, CA); extraction of viral RNA was obtained by mixing the inactivated sample with chloroform and incubating, then isolation of the aqueous phase through ethanol precipitation and centrifugation. This method was first described by Weidmann et al (2004).

## Histologic and immunohistologic studies

Liver, spleen, lungs and other tissues were collected at necropsy and preserved in 10% phosphate buffered formalin. Paraffin-embedded sections were stained by hematoxylin and eosin (H & E) and examined by light microscopy. Immunohistochemical staining using a polyclonal rabbit anti-EBOV antibody (R1645), kindly provided by Cindy Rossi at USAMRIID, was performed using standard methods (Raymond et al. 2011).

## Statistical analysis

For animal experiments involving the measurement of weight loss over time, we used two-way ANOVA to assess differences in weight loss between treated and control groups, with stepdown Bonferroni adjustment for pairwise comparisons.. We used Fisher's exact test to compare survival rates among groups, and a t-test to evaluate differences in geometric mean virus titers (GMT) in tissue and serum samples. Significance was set with a confidence interval of 95% and a *p* value < 0.05. All analyses were performed using GraphPad Prism® (GraphPad, La Jolla, CA).

## Results

### SVN inhibits ZEBOV and MARV replication in vitro

SVN inhibited the replication of EBOV-eGFP in Vero E6 cells with an EC<sub>50</sub> of 41 nM, which was lower than the level for CV-N tested simultaneously (EC<sub>50</sub> = 154 nM) (Figure 1). SVN also inhibited the replication of MARV, with an EC<sub>50</sub> similar to that for ZEBOV (Figure S1). Testing of cellular viability by an ATP-based assay failed to detect cytotoxicity at the more than 200-fold higher SVN concentration of 10.3 μM (Figure S2). Similarly, when other researchers tested SVN in Huh 7.5.1 cells, it showed no toxicity at

concentrations up to 2  $\mu\text{M}$  (Takebe et al, 2013). These results differed significantly from CV-N, which was cytotoxic at levels as low as 4  $\mu\text{M}$  (Barrientos et al. 2003).

### **SVN binds to ZEBOV GP1**

Western blotting showed that SVN bound specifically to a 120 kDA target in the ZEBOV-infected cell lysate, which migrated in parallel with rGP1 (Figure 2A). By ELISA, SVN showed increasing concentration-specific binding to rGP1 however, rGP1 lacking the mucin domain (rGP1 Muc) showed significantly reduced SVN binding indicating that the mucin domain is an important target for SVN (Figure 2B).

### **Injected SVN is bioavailable**

The serum concentration of SVN peaked approximately 45 min after s.c. injection at a level of 1.1  $\mu\text{g/ml}$  (113 nM) and decreased to baseline levels by 4 hr (Figure 3). The peak serum level was thus approximately twice the  $\text{EC}_{50}$  of SVN in cell culture.

### **Treatment with SVN protects mice against lethal ZEBOV challenge**

Treatment initiated on the day before virus inoculation was highly effective in preventing death from ZEBOV infection (Figure 4A). All mice given PBS succumbed to infection by day 6, while 90% of the mice that received 30 mg/kg/day of SVN beginning on day -1 survived. Those treated with 20 or 10 mg/kg/day showed 80% and 30% survival rates respectively. Infected, treated mice lost weight beginning on day 4-5, but started to regain weight on days 9-10 (Figure 4B). Uninfected control mice that received 30 mg/kg/day of SVN showed no weight loss or other signs of drug toxicity. Treated mice that survived infection were challenged a second time approximately 3 months later with ZEBOV and all survived; however, mice that were in a simultaneously infected control group (previously treated with 30 mg SVN but not challenged with ZEBOV) died (data not shown).

In a second experiment, SVN treatment was initiated 1 hr or 1 day after virus challenge, with the same regimens used in the first study. All control mice succumbed to infection by day 10, while 80% of the mice treated at day-1, 90% of those treated at +1 hr and 70% of those treated at day +1 survived infection (Figure 5).

### **SVN therapy reduces ZEBOV titers in serum and tissues**

Levels of infectious virus and viral RNA in serum, liver and spleen on days 2 and 5 post-infection were determined for mice treated with 30 mg/kg/day of SVN or with PBS, beginning the day before virus challenge (Figure 6). Titers of infectious virus were consistently lower in samples from treated than untreated animals, with decreases ranging from roughly 10-fold to more than 100-fold. As expected, copy numbers of viral RNA tended to be higher than titers of infectious virus in the same samples. Except for approximately equal titers in serum on day 5, SVN-treated mice had lower viral RNA levels than controls, but the extent of the decrease was more variable than for infectious virus. A repeat experiment gave similar results (data not shown).

## SVN therapy reduces tissue injury in ZEBOV-infected mice

The principal histologic abnormality in infected, untreated mice was extensive hepatic inflammation and necrosis, which corresponded with the presence of large amounts of viral antigen in hepatocytes and Kupffer cells (Supplemental Figure 3, A and B). Only minimal changes were seen in the livers of SVN-treated mice (C and D). Significant differences were also seen in the lungs, which showed mild inflammatory changes in treated mice (E), but moderate to severe pneumonia in control animals (F). Splenic lymphocytolysis and myocarditis were observed in less than half of untreated, infected mice, but were absent in SVN-treated animals (not shown).

## Discussion

The current epidemic of Ebola virus disease in West Africa has made it clear that safe and effective countermeasures are urgently needed against this devastating infection. Several approaches, including various small molecules and monoclonal antibodies, have proven beneficial for pre- and postexposure prophylaxis in ZEBOV-infected nonhuman primates, but none is yet approved for human use (Wong et al. 2014). Although cyanobacterial lectins are still in an early stage of development, we believe they hold promise as broad-spectrum antiviral agents, because their affinity for oligosaccharides commonly found on viral surface glycoproteins would allow them to inhibit many different pathogens.

In an earlier paper, we reported that treatment with the lectin CVN slowed the progression of illness in ZEBOV-infected mice, but did not prevent death (Barrientos et al. 2003). The present study has shown that SVN is a more potent inhibitor of ZEBOV replication than CVN. Its antiviral activity appears to result from specific binding to the mucin region of the enveloped GP, which is rich in N-linked high-mannose oligosaccharides (Lee and Saphire 2009) (Ritchie et al. 2010). A dose of 30 mg/kg/day of SVN produced no signs of toxicity, was protective in the majority of the mice when started the day before challenge, and prevented the death of nearly all mice when begun the day before or the day after challenge, suggesting that this lectin might be used in either a prophylactic or a therapeutic role for human ZEBOV infections. The fact that SVN was equally potent when begun the day before or the day after virus challenge indicates that its effects extend beyond the first round of infection. Since treated animals become ill and lose weight, it is apparent that SVN slows the progress of ZEBOV infection, rather than blocking it completely.

An interesting observation from our study is that only partial suppression of ZEBOV replication was needed to bring about survival. Under experimental conditions in which nearly all treated mice survived, but all control mice died, there was a general trend for titers of infectious virus in the serum, liver and spleen of treated mice on days 2 and 5 postinfection to be roughly 10- to 100-fold lower than in control animals (Figure 6). Levels of viral RNA, as measured by RT-PCR, appeared to be more variable, but were generally lower in treated mice at both time points; however, a statistically significant difference was only observed in liver tissue on day 2. The occurrence of considerable viral replication in mice that survived infection resembles that seen in some earlier evaluations of experimental ZEBOV therapy, such as an assessment of the small-molecule inhibitor FGI-103, which found that virus titers in the serum and tissues of treated mice differed by less than 100-fold

at days 4 and 6 post-infection from those of controls (Warren et al. 2010). It is also consistent with some studies in macaques, such as treatment with the anticoagulant rNAPc2, in which animals that survived infection developed peak serum virus titers approaching  $10^4$  pfu/mL, while mean titers were approximately 10-fold higher in treated macaques that showed a delay in death and 100-fold higher in fatally infected controls (Geisbert et al. 2003). However, more effective reduction in circulating virus in ZEBOV-infected macaques has recently been achieved by monoclonal antibody therapy, which resulted in complete suppression of viremia (Pettitt et al. 2013).

Although SVN was highly protective in ZEBOV-infected mice, its short serum persistence required dosing every 6 hours, a significant burden for researchers in Biosafety Level 4 containment and a disadvantage for any potential human therapy. A lectin that possesses SVN's strong antiviral activity, but a longer serum half-life, would therefore be highly desirable. We have recently shown that griffithsin, a red algal lectin that binds high-mannose oligosaccharides in a manner similar to CVN and SVN, has a remarkably long serum persistence in mice, and could still be detected two weeks after a single high-dose inoculation (Barton et al. 2014). Griffithsin is now being evaluated for its protective efficacy against EBOV. Given the inherent stability of lectins and their potential for large-scale production (O'Keefe et al. 2009), we believe that these proteins have promise as therapeutic agents against ZEBOV and other highly pathogenic filoviruses.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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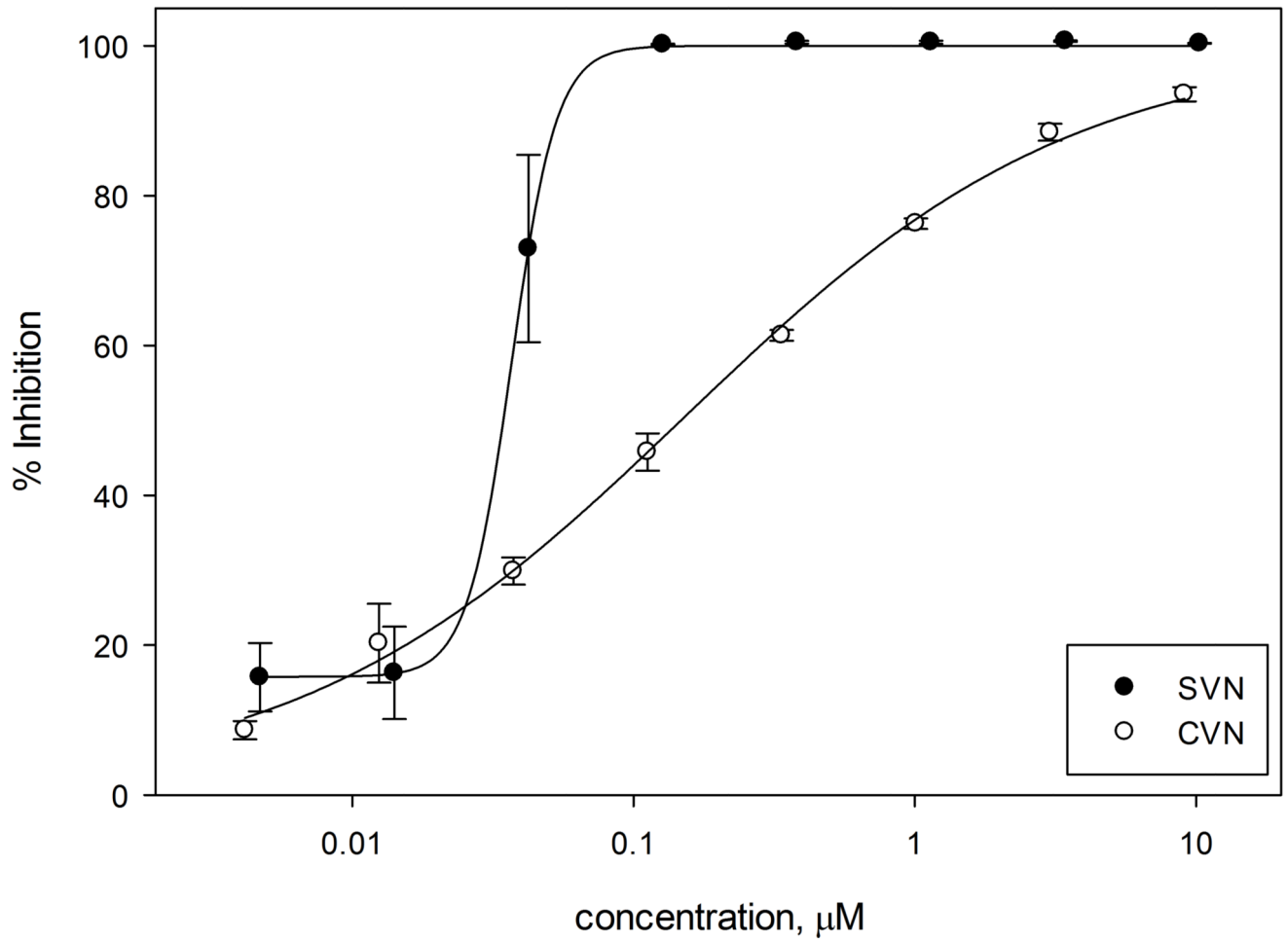
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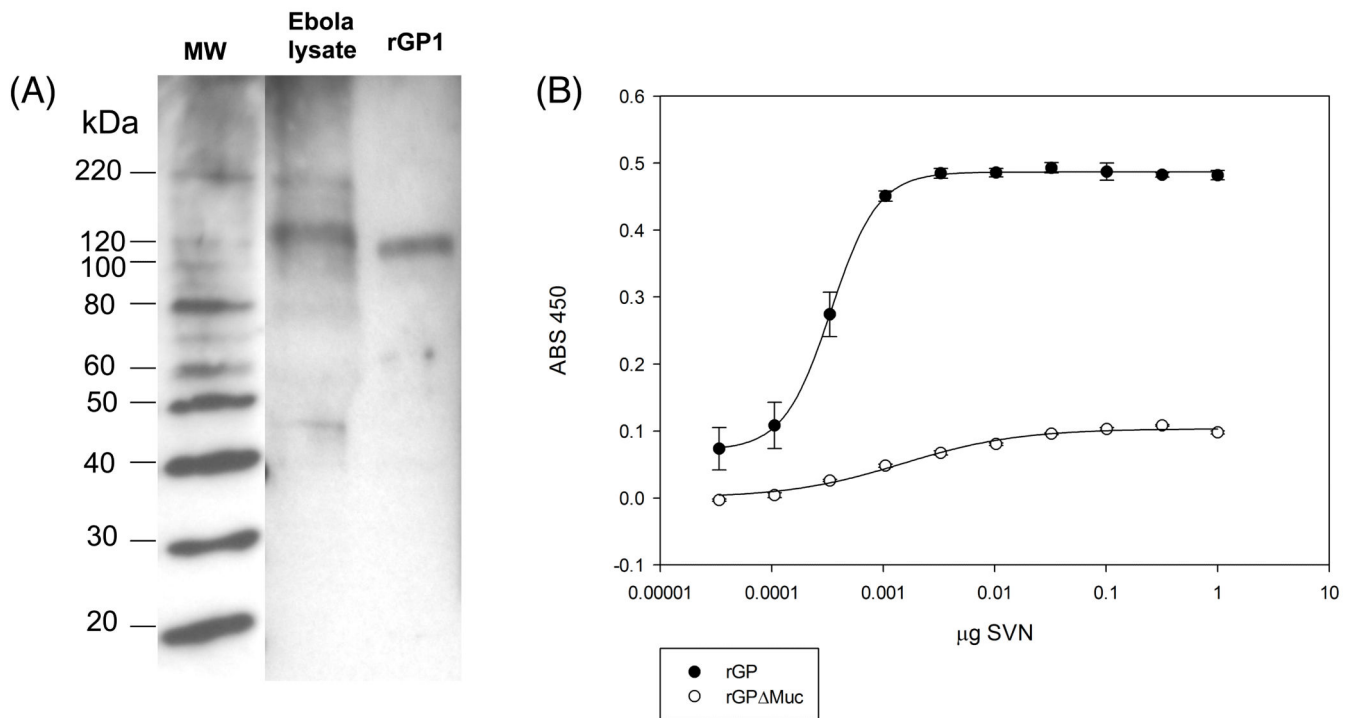
**Garrison et al. 2014 highlights revised**

- The cyanobacterial lectin scytovirin inhibits the replication of Zaire ebolavirus and marburgvirus *in vitro*.
- Scytovirin binds to the mucin domain of the ebolavirus glycoprotein.
- The compound caused no detectable toxicity in cell culture assays or in mice.
- Treatment of mice beginning the day before, the day of or the day after ZEBOV challenge resulted in 70-90% survival.
- The short persistence of scytovirin in the serum necessitated s.c. dosing every 6 hours.



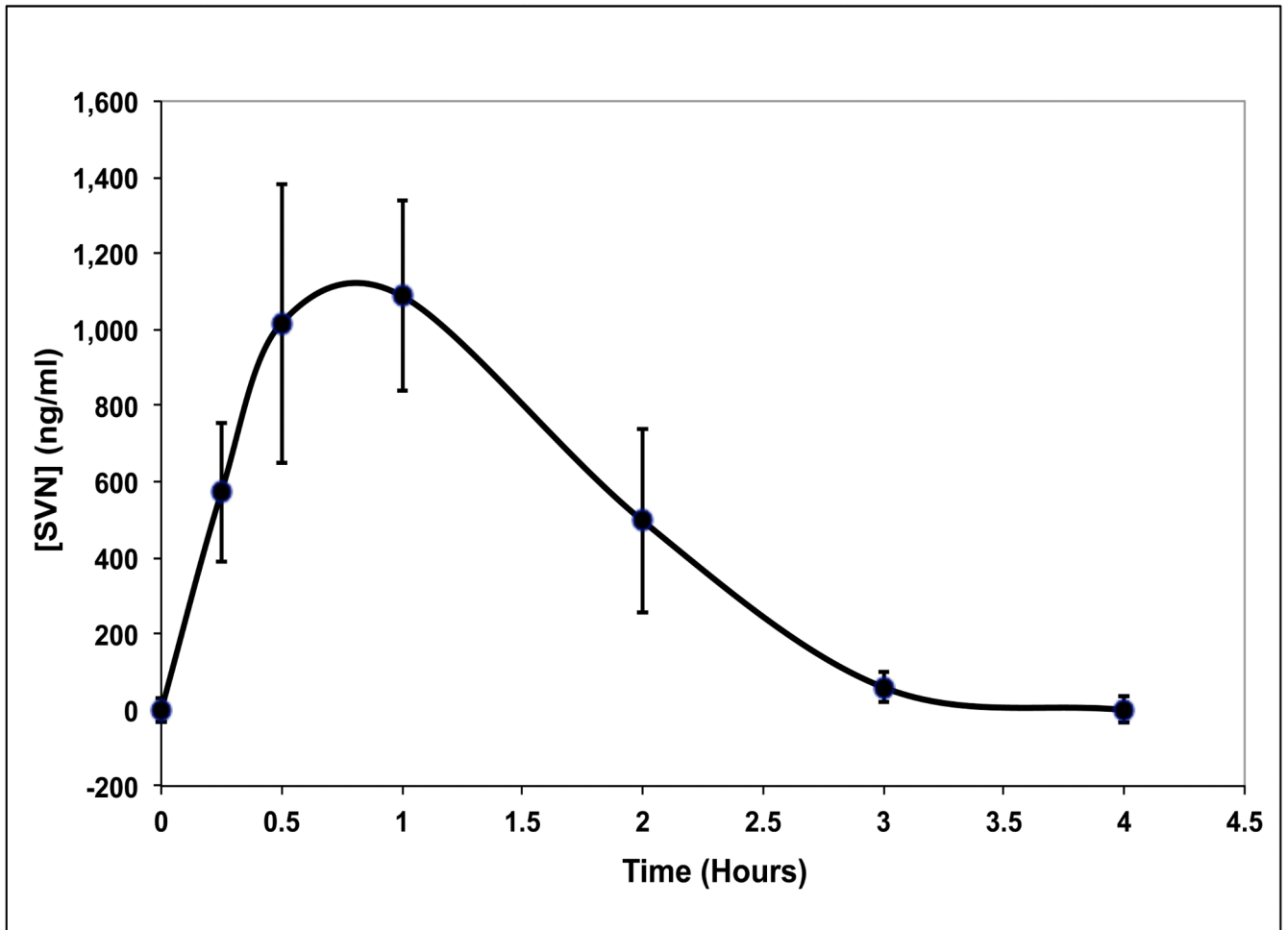
**Figure 1. SVN and CV-N inhibit ZEBOV replication *in vitro***

SVN (red circles) and CV-N (black squares) were tested for inhibitory activity against ZEBOV in Vero E6 cells, using a recombinant GFP-encoding virus. SVN exhibited an  $EC_{50}$  of 41 nM, while CV-N was found to have an  $EC_{50} = 154$  nM. The data shown are the average of triplicate experiments; error bars indicate standard deviations.



**Figure 2. SVN binds to the mucin-rich region of ZEBOV GP1**

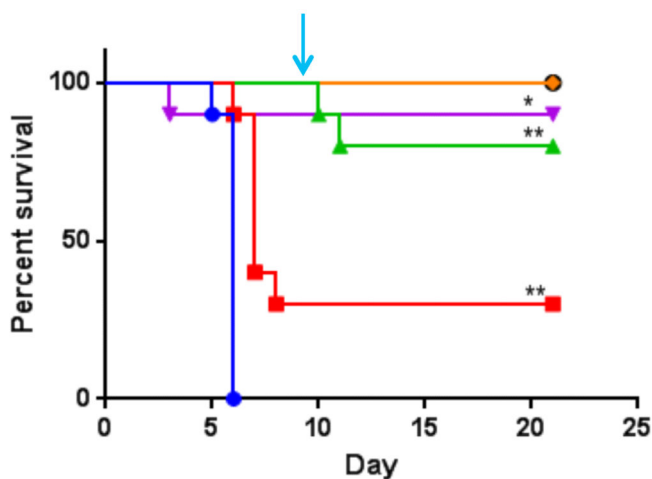
A. Western blot analysis showed that SVN bound predominantly to a single ~120 kDa protein in a EBOV-infected cell lysate, corresponding to GP1 (Lane 2), and to recombinant GP1 (Lane 3). Molecular weight markers (MW) are from a Coomassie stain of the corresponding SDS-PAGE gel. B. ELISA showing binding of SVN to rGP1 and rGP1  $\Delta$  Muc. The data shown are the average of triplicate experiments; error bars indicate standard deviations.



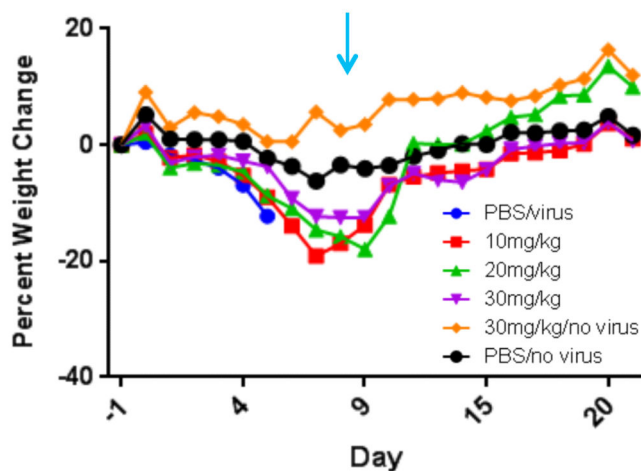
**Figure 3. SVN persists in the serum in mice for up to 4 hours**

Mice (n = 3 per time point, 33 total) were injected SC with 10 mg/kg SVN and 3 were euthanized at each indicated time point. The level of SVN in plasma was measured by HIV-1 gp120 capture ELISA. Each time point is the average of triplicate experiments; error bars indicate standard deviations.

## A. Percent Survival

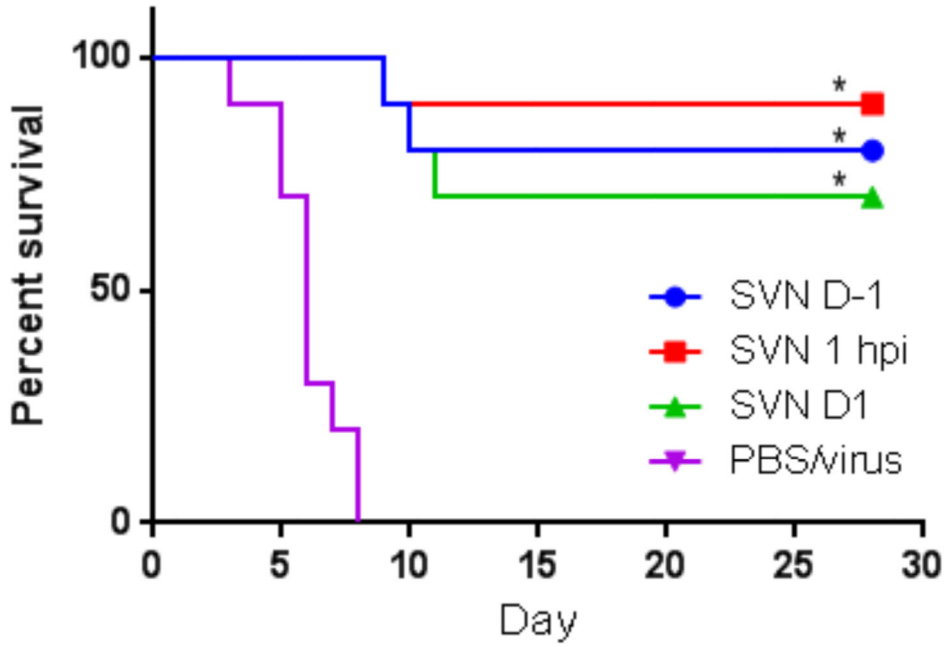


## B. Percent Weight Change

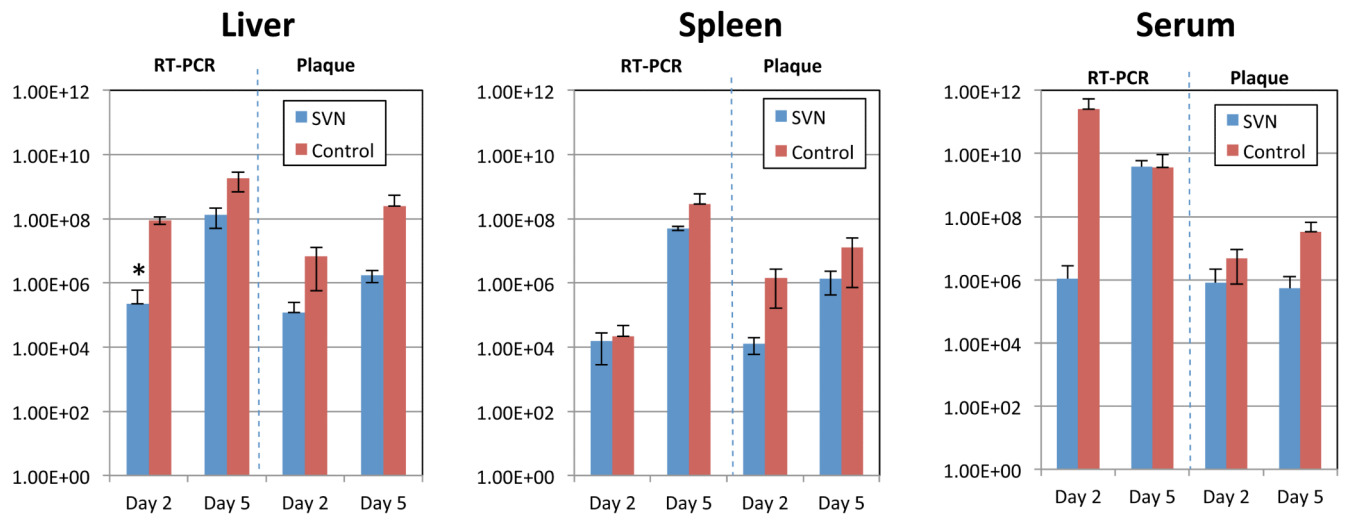


**Figure 4. SVN treatment protects mice when begun before virus challenge**

Groups of 10 mice were injected s.c. every 6 hours, beginning 24 hr before virus challenge, to give a total daily dose of 10 (red square), 20 (green triangle) or 30 (purple triangle) mg/kg. The initial doses were given at -24, -18, -12 and -6 hr, then 1 hr after i.p. virus challenge, and continuing every 6 hr through day 9 (arrow). Control groups were infected and treated with PBS (blue circle), or were left uninfected (black circle) and given a total daily dose of 30 mg/kg of SVN (orange diamond). A. Percent survival. A log-rank test was performed to compare survival of treated and untreated, infected mice; (\*) denotes statistical significance with a 95% CI and  $p = 0.0006$ , while (\*\*) denotes  $p < 0.0001$ . B. Percent weight change relative to mean group weight on day -1. A two-way ANOVA was performed and no significant difference was found in the weight changes between treatment groups and the uninfected, PBS-treated control group.



**Figure 5. SVN treatment protects mice when begun before or after virus challenge**  
Groups of 10 mice were injected every 6 hours with a total daily dose of 30 mg/kg, beginning 24 hr before (see description in previous figure) (blue circle), 1 hr after (red square) or 24 hours after (green triangle) ZEBOV challenge, continuing through day 9 (arrow). A control group (purple inverse triangle) received PBS beginning 1 hr after challenge. A log-rank test was performed, comparing treated and untreated groups; (\*) denotes statistical significance, with a 95% CI and  $p < 0.0001$ .



**Figure 6. SVN treatment reduces titers of infectious virus and viral RNA in serum and tissues** Mice ( $n = 3$ ) either untreated or treated beginning 1 hr after ZEBOV challenge with 30 mg/kg/day of SVN were sacrificed on days 2 and 5 postinfection. Samples of serum, spleen and liver were collected and assayed for infectious virus by plaque formation on Vero E6 cells and for levels of viral RNA by RT-PCR. SVN-treated samples are represented by blue bars and PBS controls by red bars. Error bars indicate standard deviation for three independent samples from three mice. There was a consistent trend toward lower mean viral titers in treated than untreated mice, but comparison by t-test found a statistically significant difference in only one sample set (\*), with a 95% CI and  $p = 0.0221$ .