Ceragenins: A Class of Antiviral Compounds to Treat Orthopox Infections

Michael D. Howell[1,2], Joanne E. Streib[1,2], Byung Eui Kim[1,2,3], Leighann J. Lesley[1,2], Annegret P. Dunlap[1,2], Dianliang Geng[4], Yanshu Feng[4], Paul B. Savage[4] and Donald Y.M. Leung[1,2]

Eczema vaccinatum is a potentially fatal, disseminated viral skin infection that develops in individuals with atopic dermatitis after exposure to the vaccinia virus (VV). Despite advances in modern medicine, there are few options for those suffering from disseminated VV infections. Ceragenins (CSAs) are synthetic antimicrobial compounds designed to mimic the structure and function of endogenous antimicrobial peptides (AMPs). We show that CSA-13 exhibits potent antiviral activity against VV by (1) direct antiviral effects against VV; and (2) stimulating the expression of endogenous AMPs with known antiviral activity against VV. In addition, we show that a topical application of CSA-13 penetrates the skin and reduces subsequent satellite lesion formation. This suggests that treatment with CSA-13 may be an intervention for individuals with a disseminated VV skin infection.

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INTRODUCTION

Orthopoxviruses are a family of enveloped DNA viruses that replicate their linear double-stranded DNA genome in the cytoplasm of infected cells (Marennikova *et al.*, 2005). Two main members of this family are vaccinia virus (VV) and variola major—the causative agent of smallpox. Owing to its ability to protect against smallpox infection, VV was routinely administered to individuals until the World Health Organization declared that smallpox had been eradicated in the late 1970s (World Health Organization, 1980). Despite its eradication in the 1970s, the potential use of smallpox as a biological weapon poses an imminent threat. It is estimated that 119 million residents of the United States have been born after the smallpox vaccination was discontinued. Such individuals are susceptible to smallpox infection, an epidemic of which could result in catastrophic numbers of death and disease (Bicknell, 2002). Although VV is highly effective at conferring protection, it also has the distinction of having a high rate of adverse events that lead to the discontinuation of its routine use in the general population. Adverse reactions caused by VV can range from mild discomfort or painless to life threatening.

Progressive vaccinia and eczema vaccinatum are potentially fatal adverse reactions that primarily develop in individuals with immunodeficiency disorders and eczema, respectively (Cono *et al.*, 2003). Although the use of Vaccinia Immune Globulin Intravenous (Human) has reduced the mortality of progressive vaccinia and eczema vaccinatum (Cono *et al.*, 2003), vaccinia immune globulin treatment is not effective for everyone. The seriousness of this was recently seen in a 28-month-old child who developed eczema vaccinatum after exposure to VV and who was unresponsive to treatment with vaccinia immune globulin alone. Additional treatment with Cidofovir, which had earlier been shown to be effective in animal models (Neyts *et al.*, 2004; Smee *et al.*, 2004), also failed to significantly reduce the viral load in the child (Vora *et al.*, 2008). Thus, new antiviral agents are needed for the treatment of disseminated VV infection.

Recent studies indicate that the increased propensity of atopic dermatitis patients toward eczema vaccinatum may be related to a deficiency of naturally occurring antimicrobial peptides (AMPs), such as cathelicidins or human beta defensins (HBD) (Howell *et al.*, 2006, 2007). Cathelicidins and HBD-3 have been shown to exhibit potent antiviral activity against VV (Howell *et al.*, 2004, 2007); however, their use as anti-VV agents is limited because of their rapid degradation by endogenous tissue proteases. Ceragenins (CSAs) are a new class of synthetic amphiphilic compounds

[1]*Division of Allergy and Immunology, Department of Pediatrics, National Jewish Health, Denver, Colorado, USA;* [2]*Department of Pediatrics, University of Colorado Denver, Denver, Colorado, USA;* [3]*Department of Pediatrics, Inje University, Seoul, Korea and* [4]*Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah, USA*

Correspondence: Dr Michael D. Howell, Division of Allergy and Immunology, Department of Pediatrics, National Jewish Health, 1400 Jackson Street, Room K1016a, Denver, Colorado 80206, USA.
E-mail: howellm@njc.org

Abbreviations: AMP, antimicrobial peptide; CSA, cationic steroid antimicrobial (ceragenin); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HBD, human beta defensin; KC, keratinocyte; NHK, primary human keratinocyte; PFU, plaque forming unit; SCID, severe combined immunodeficiency; VV, vaccinia virus

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designed with cationic and hydrophobic faces to mimic the structure of endogenous AMPs (Savage *et al.*, 2002; Ding *et al.*, 2004a). Their structures have been shown to disrupt bacterial membranes without damaging mammalian cell membranes (Ding *et al.*, 2004a); however, their activity against viruses is not known. On the basis of structural similarities with AMPs, we predict that CSAs may also be effective against orthopoxviruses. Owing to their synthetic nature, CSAs are not subject to human protease degradation and therefore have a longer tissue half-life. This makes them more attractive as antiviral agents than their endogenous AMP counterparts. This study was carried out to investigate the potential use of CSAs as antiviral agents against VV using *in vitro* and *in vivo* models.

RESULTS

Representative structures of the CSAs used in this study are shown in Figure 1. CSAs were originally optimized for antibacterial activity with variations made in the quantity and character of cationic groups, and in the length and nature of lipophilic chains (Savage *et al.*, 2002). Therefore, we evaluated the antiviral activity of CSA-13 and -31 using a standard viral plaque assay. On the basis of our earlier observations, LL-37 was included as a positive control (Howell *et al.*, 2006). CSA-13 exhibited potent antiviral activity against VV by inactivating 60.62 ± 4.94% of VV at 5 μM, 91.19 ± 1.37% at 10 μM, and 96.37 ± 0.52% at 25 μM (Figure 2a). Using nonlinear regression, we calculated IC90 (9.15 ± 1.09 μM) and IC95 (12.02 ± 1.13 μM) for CSA-13. In contrast, CSA-31 did not exhibit any activity against VV. Using electron microscopy, we further evaluated the mechanism by which CSA-13 kills VV. Similar to LL-37 (Howell *et al.*, 2004), we found that CSA-13 significantly disrupts the envelope and internal structure of VV (Figure 2b). In contrast, CSA-31 had no such effects.

Viral replication and pathogenesis require host involvement; therefore, additional experiments were carried out to determine whether earlier infected human keratinocytes (KC) could be rescued by CSA-13 treatment. KCs were infected with VV for 6 hours and then treated with CSAs for an additional 18 hours. In this experiment, KCs exposed to VV had significantly higher levels of VV gene expression than those cells exposed first to VV and then treated with CSA-13. Treating VV-infected KCs with CSA-13 dose responsively reduced the levels of VV gene expression to a significantly ($P<0.05$) lower expression after the addition of concentrations as low as 10 μM (0.76 ± 0.05 ng VV per ng GAPDH (glyceraldehyde-3-phosphate dehydrogenase)) as compared with VV alone (1.53 ± 0.10) (Figure 2c). In addition, CSA-13 protected the viability of KCs (Figure 2d). CSA-31, a structurally similar CSA with no antiviral activity, failed to reduce VV gene expression (Figure 2c) and did not protect KCs from further VV infection (Figure 2d).

The potential use of CSAs in antiviral applications may require selective targeting of viral envelopes over eukaryotic membranes. Earlier comparisons of the affinity for prokaryotic versus eukaryotic membranes used CSA-59, a fluorophore-labeled CSA. The fluorophore in CSA-59 is related to prodan (Ding *et al.*, 2004b), which is a small fluorophore that responds to the hydrophobicity of its environment through large changes in fluorescence emission wavelength and intensity (Weber and Farris, 1979). In water, fluorescence emissions of prodan and CSA-59 are centered at 550 nm, and in aprotic solvents, the emission intensity increases and is centered at 450 nm. Owing to the size of CSAs, addition of a fluorophore has the potential to modify the antibacterial/antiviral activities of the compounds. In our study, we found that CSA-59 exhibited significant antiviral activity at concentrations of 10 μM (33.70 ± 2.88% inactivation; $P<0.01$) and 25 μM (73.91 ± 3.26%; $P<0.01$) (Figure 3a). Although this activity is less than that observed for CSA-13, the structure and hydrophobicity of an appended fluorophore can be used to approximate the lipid chains found on CSA-13. The prodan fluorophore in CSA-59 contains 16 carbon atoms in substituted naphthalene, dimethylamine, and in the linker to the CSA. The carbonyl oxygen and amine offset the hydrophobicity of naphthalene and linker to a degree, and the fluorophore in CSA-59 may be considered to contribute hydrophobicity intermediate to that found in CSA-13.

The incorporation of CSA-59 into bacterial membranes correlates with an increased fluorescent intensity at 450 nm (Ding *et al.*, 2004b); therefore, we evaluated the association

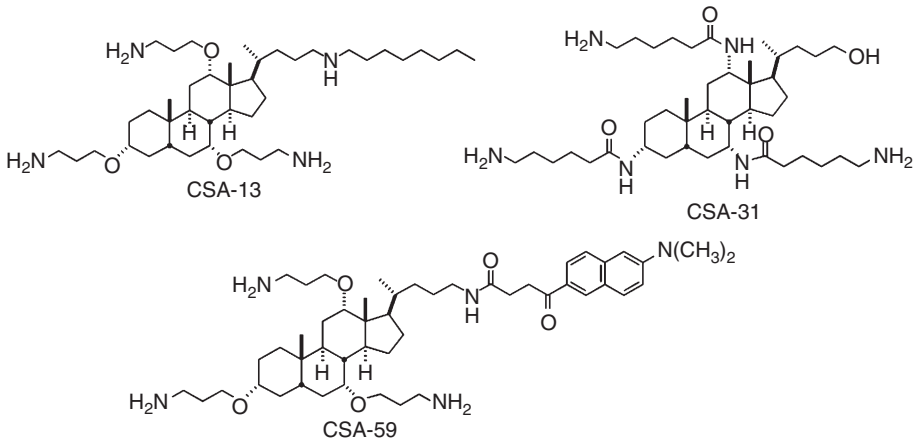

Figure 1. Chemical structures of ceragenins used in this study.

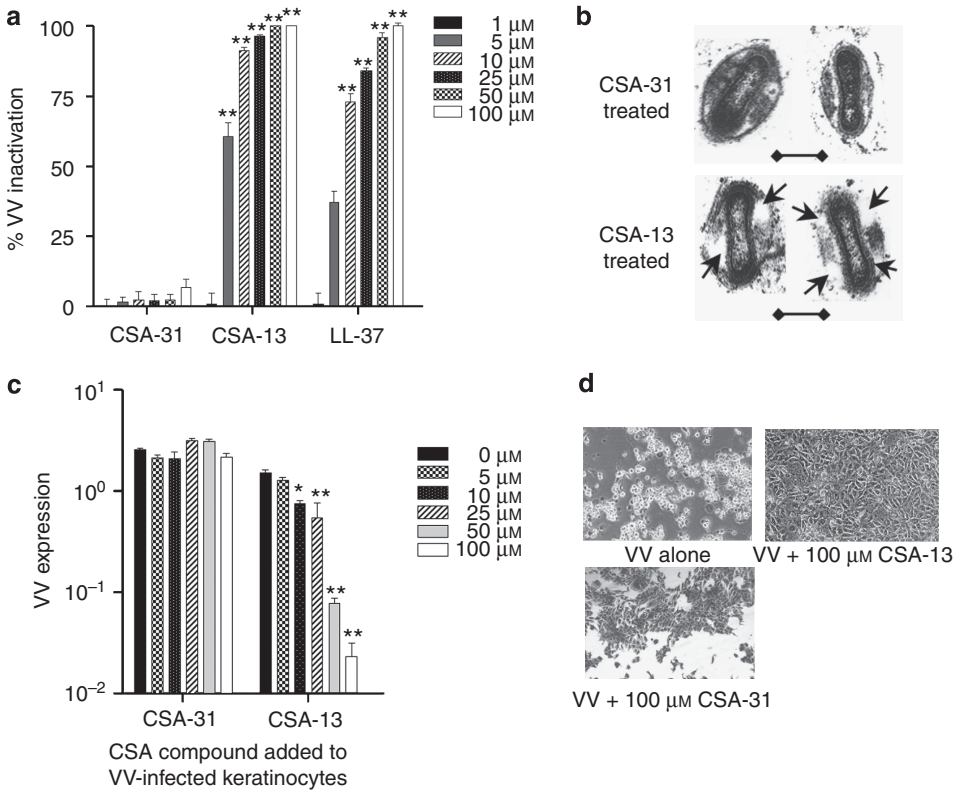

Figure 2. Ceragenins (CSAs) exhibit antiviral activity against vaccinia virus (VV). (**a**) Direct antiviral activity of LL-37, CSA-13, and -31 against VV (Wyeth), using a standard plaque assay. Data are represented as the percent VV inactivation. '**' indicates a significant difference of $P<0.01$ as compared with 0 μM. (**b**) Transmission electron micrographs (× 27,500 magnification) of vaccinia virions treated with 50 μM of CSA-13 or -31. Arrows indicate areas of disruption in the inner membrane and core wall. Bar indicates 0.1 μm. (**c**) CSA-13 rescues previously infected keratinocytes (KCs). Human KCs were infected with 0.05 PFU per cell of VV (Wyeth) for 6 hours and then treated with CSA-13 or -31 for an additional 18 hours. VV gene expression was evaluated by real-time reverse transcriptase PCR. '* and **' indicate significant differences of $P<0.05$ and $P<0.01$, respectively, as compared with 0 μM. (**d**) Phase contrast image comparing cell viability of KCs exposed to VV alone, VV plus 100 μM CSA-31, or VV plus 100 μM CSA-13. Images were collected at × 10 magnification.

of CSA-59 with VV and KCs on the basis of their surface area. The surface area of VV was based on approximate radii of 300 nm (Heuser, 2005), whereas that of KCs was approximated at 11 μm (Barrandon and Green, 1985). The relationships between surface area and fluorescence of CSA-59 at 450 nm are given in Figure 3b. In the presence of VV, fluorescence intensity of CSA-59 increases and the emission wavelength decreases; both results are consistent with the fluorophore on CSA-59 moving into a more hydrophobic environment. Earlier studies with CSA-59 correlated these fluorescence changes with the association of the compound with a lipid bilayer, and titrations of CSA-59 with prokaryotes or eukaryotic cells showed the lipid area necessary to sequester the antimicrobial compound (Ding *et al.*, 2004b). Titrations of CSA-59 with VV were carried out to determine whether the CSA would selectively incorporate into viral envelopes over eukaryotic membranes. In these titrations, CSA-59 incorporated into viral lipid bilayers in the presence of a much less lipid bilayer surface area than that required with KCs. This directly observed bilayer selectivity correlates well with the ability of CSAs to inactivate VV while remaining non-cytotoxic to KCs. The origins for this bilayer selectivity are less clear. They may be because of differences in bilayer composition, bilayer curvature (the viruses will experience much more membrane curvature than the larger KC), or a lack of membrane repair mechanisms.

We further tested the antiviral activity of CSA-13 using an *in vivo* murine model. For these experiments, CSA-13 was formulated as a cream that could be topically applied to test whether the topical application of CSA after VV infection would reduce viral pathogenesis. Severe combined immunodeficient (SCID) mice were infected with VV and then treated with CSA cream 2 hours after infection, and then daily thereafter. Satellite lesions, indicative of systemic viremia, began appearing by day 7 after infection and were monitored until day 10 (Figure 4a and b). SCID mice treated with CSA-13 ($n=35$) developed significantly less satellite lesions on days 8 (mean: 0.7 ± 0.2 satellite lesions; $P<0.05$) and 10 (mean: 4.5 ± 1.2 satellite lesions; $P<0.05$) as compared with SCID mice treated with control ointment ($n=41$; day 8: 2.5 ± 0.8 satellite lesions; day 10: 9.3 ± 1.6 satellite lesions). In addition to monitoring satellite lesion formation, the VV replication in primary lesions was determined by staining skin biopsies for A27L and measuring the mean fluorescent intensity (Figure 4c). A27L is a viral protein essential for virus-to-cell fusion (Rodriguez *et al.*, 1996). SCID mice inoculated with VV and treated with CSA-13 had significantly lower levels of VV (MFI (mean fluorescence intensity) of

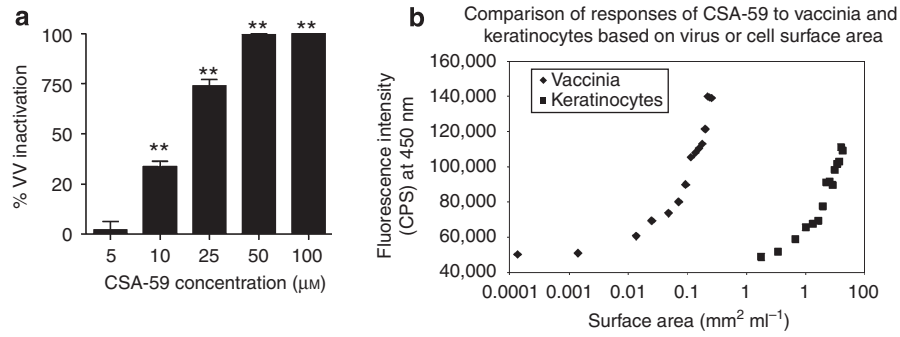

Figure 3. Ceragenins (CSAs) preferentially target viral envelopes. (**a**) Direct antiviral activity of CSA-59 against vaccinia virus (VV) (Wyeth) using a standard plaque assay. Data are represented as the percent VV inactivation. '**' indicates a significant difference of $P<0.01$ as compared with 0 μM. (**b**) CSAs selectively target viral envelopes than the HaCaT human keratinocyte (KC) cell line. The influence of VV and KCs on the fluorescence of CSA-59. Fluorescence changes are plotted *versus.* the surface area of the organisms and indicate that CSA-59 preferentially incorporates into viral lipid bilayers.

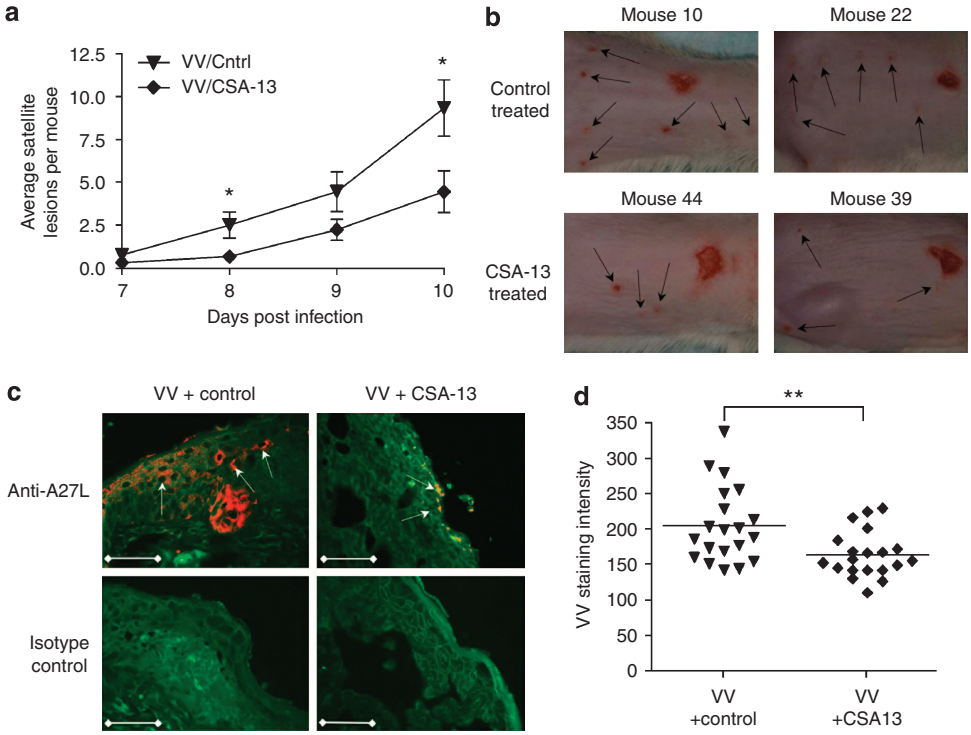

Figure 4. Ceragenin (CSA)-13 reduces vaccinia virus (VV) pathogenesis. (**a**) Satellite lesions on VV (WR1354)-infected severe combined immunodeficient mice were counted on days 7–10 after inoculation. '*' indicates a significant difference of $P<0.05$ between mice treated with control cream and mice treated with CSA-13. (**b**) Satellite lesion formation 10 days after VV infection. Arrows indicate the location of satellite lesions on the backs of mice treated with control or CSA-13. (**c**) A 6 mm biopsy was collected from the primary site of VV infection. VV replication was evaluated by staining for A27L, a viral protein essential for virus-to-cell fusion. Arrows indicate areas of intense VV staining in biopsies from mice treated with control cream and the lack of VV staining in biopsies from mice treated with CSA-13. (**d**) Mean fluorescence intensity was measured using confocal microscopy. '**' indicates a significant difference of $P<0.01$ between mice treated with control cream and mice treated with CSA-13. Bar indicates 50 μm.

163.9 ± 7.3; $P<0.01$) than did mice treated with control (MFI of 204.9 ± 11.9) (Figure 4d). Weight loss and mortality rates were similar in mice treated with control or CSA-13.

Severe combined immunodeficient mice are characterized by a lack of T and B cells, and therefore primarily rely on their innate immune response to prevent morbidity and mortality. The effect of CSAs on the innate immune response is currently unknown. Therefore, we further investigated the effect of CSA-13 on LL-37 and HBD-3, as they are known to have an anti-VV activity (Howell *et al.*, 2004, 2007). Primary human KCs (NHK) were stimulated with 0–50 μM of CSA-13 for 24 hours. In Figure 5, we show that stimulating NHK with CSA-13 induces LL-37 and HBD-3 in a dose-dependent manner. LL-37 gene expression was significantly higher in cells stimulated with 25 μM (1.25 ± 0.36 ng LL-37 per ng GAPDH; $P<0.05$) and 50 μM (6.19 ± 1.43; $P<0.01$) of

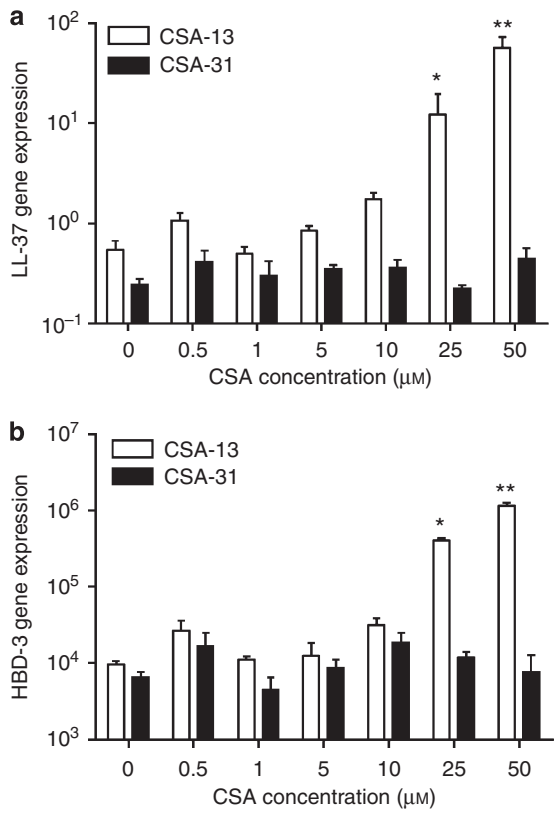

Figure 5. Ceragenin (CSA)-13 induces LL-37 and HBD-3 expressions in primary human keratinocytes. Cells were stimulated with 1–50 μM of CSA-13 or -31 for 24 hours. LL-37 (**a**) and HBD-3 (**b**) expressions were evaluated by real-time reverse transcriptase PCR. '* and **' indicate significant differences of $P<0.05$ and $P<0.01$, respectively, as compared with 0 μM.

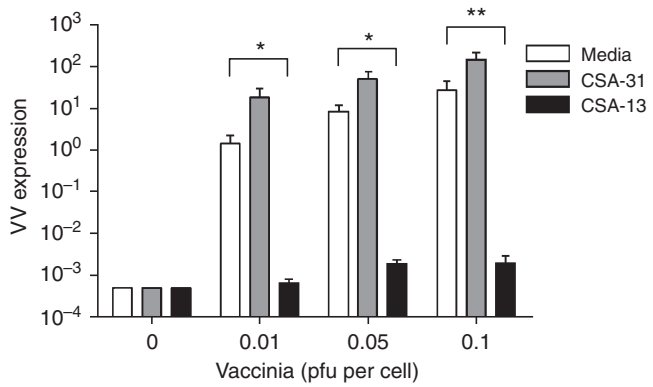

Figure 6. Pre-incubation with ceragenin (CSA)-13 inhibits vaccinia virus (VV) replication. Primary human keratinocytes were treated with 50 μM of CSA-13 or -31 for 6 hours and then infected with VV (Wyeth). Media were removed before infection to prevent extracellular VV killing by direct interaction with CSA. Cells were infected for 24 hours and then VV was evaluated using real-time reverse transcriptase PCR. '* and **' indicate significant differences of $P<0.05$ and $P<0.01$, respectively, between cells pretreated with media alone and cells pretreated with CSA.

CSA-13 than when stimulated with media alone (0.08 ± 0.02) (Figure 5a). Similarly, the HBD-3 expression was significantly induced after stimulation with 25 (4816 ± 677 ng HBD-3 per ng GAPDH; $P<0.05$) and 50 μM (10447 ± 2027; $P<0.01$) of CSA-13 than when stimulated with media alone (114 ± 43) (Figure 5b). On further testing, we found that stimulation with CSAs at concentrations >25 μM significantly modulates GAPDH expression. This suggests that CSAs exhibit a mild cytotoxic effect at concentrations >25 μM (data not shown).

On the basis of our observation that CSA-13 significantly induces AMPs with known antiviral activity, we further investigated whether a CSA-13-mediated induction of AMP is associated with reduced VV replication. NHK was stimulated with 50 μM of CSA-13 for 6 hours and then infected with VV for 24 hours. VV replicates in the cytoplasm of host cells; therefore, the cellular effluent containing CSA-13 was removed before infection to prevent the extracellular inactivation of VV. After 24 hours, VV replication was significantly decreased in NHK pretreated with CSA-13 as compared with that in NHK treated with media alone (Figure 6). Specifically, pretreatment with CSA-13 significantly reduced the VV expression in NHK infected with 0.01 PFU (plaque forming unit) per cell ($P<0.05$), 0.05 PFU per cell ($P<0.05$), and 0.1 PFU per cell ($P<0.01$), as compared with that in NHK treated with media alone.

DISCUSSION

Currently, there are limited treatment options for individuals suffering from orthopoxvirus infections. For decades, VV was used to protect individuals from infection with variola major (smallpox); however, potentially fatal VV infections can develop in individuals with immunodeficiency or skin disorders (Cono *et al.*, 2003). The recent emergence of monkeypox (Reynolds *et al.*, 2007) and cowpox (Kurth *et al.*, 2008) emphasizes the potential risk for human orthopox infection. It also highlights the need to both understand orthopoxviruses and develop antiviral agents to treat orthopox infections.

In our study, we evaluated the antiviral activity of a class of compounds. CSA-13 has earlier been shown to exhibit potent antibacterial activity, whereas CSA-31 did not (Rehman *et al.*, 1999; Savage *et al.*, 2002). Therefore, we used CSA-13 as the candidate compound for our study. Our first experiment evaluated the direct effect of CSA-13 on VV replication. Using the standard viral plaque assay as a functional readout, we show that CSA-13 exhibits potent antiviral activity against VV. Viruses use the host for survival; therefore, additional experiments were carried out using VV-infected KCs. In these experiments, we show that the addition of CSA-13 reduced VV replication and rescued the infected cells. It was further determined, using the flourophore-labeled CSA-59, that CSAs preferentially target viral envelopes to exhibit their activity.

As CSAs exhibit potent activity against VV, protect KCs *in vitro* against VV-mediated cell death, and preferentially target the negatively charged viral particles, the antiviral activity of CSA-13 was further tested using an *in vivo* murine model. We first established a murine model of disseminated vaccinia viral infection by infecting SCID mice with 5×10^6 PFU of VV. The infected mice developed satellite lesions (>2 mm from primary inoculation site) as early as day 7 after infection. Earlier studies with the antiviral agent, cidofovir,

showed that topical application provided better protection against VV infection than did parenteral administration (Smee *et al.*, 2004); therefore, CSA-13 was formulated as a cream and topically applied to the entire back of the mice after VV infection. To our knowledge, this is the first study to show that treatment with CSA-13 reduces the development of satellite lesions and inhibits VV replication in the epidermis of infected mice.

Severe combined immunodeficient mice primarily rely on their innate immune response for survival, as they do not have sufficient T and B cells. Earlier, we have shown that the innate immune response, namely, the AMPs, plays an important role in the regulation of VV pathogenesis. LL-37 and HBD-3 are AMPs produced by KCs in response to external stimulus and exhibit potent activity against VV (Howell *et al.*, 2004, 2007); therefore, we investigated whether stimulation of cells with CSA-13 modulated the expression of these AMPs. We show that stimulation with CSA-13 induces the expression of both LL-37 and HBD-3, suggesting that the antiviral effects of CSA-13 may be both direct and indirect. As the CSAs mimic the morphology of LL-37, it is likely that CSAs mimic the interaction of LL-37 with its receptors. These include $P2X_7$ (Gabel, 2007), formal peptide receptor-like 1 (Li *et al.*, 2008), and epidermal growth factor (Burgel and Nadel, 2008; Burgess, 2008). In addition, we showed that stimulating KCs with CSA-13 before infection reduced the ability of VV to replicate.

In our study, we show that CSA-13 exhibits a potent antiviral activity by preferentially targeting and inactivating VV directly and by inducing AMPs with a known activity against VV. In addition, we show that a topical administration of CSA-13 significantly reduces the development of satellite lesions. Taken together, our study suggests that CSAs may be effective as an antiviral agent against disseminated VV infections. The development of these synthetic agents for treatment of disseminated viral skin infections represents an exciting advance as we appreciate the important role that naturally occurring AMPs play in the human innate immune response.

MATERIALS AND METHODS

Virus preparation

These studies used the Wyeth (Center for Disease Control and Prevention, Atlanta, GA) and Western Reserve (VR1354; ATCC, Manassas, VA) strains of VV. The virus was propagated in HeLa S3 (ATCC#CCK-2.2) human adenocarcinoma cells as described earlier (Howell *et al.*, 2004).

CSAs preparation

Ceragenins were prepared as described in earlier studies (Li *et al.*, 1999; Ding *et al.*, 2002, 2004a). Representative CSA structures of those used in this study are shown in Figure 1.

Viral plaque assay

BS-C-1 (ATCC #CCL-26) African green monkey kidney cells were used for the viral plaque assay as described earlier (Howell *et al.*, 2004).

Ceragenin stocks were hydrated at 5 mg ml^{-1} in DMSO, mixed well, aliquotted, and stored at −80°C. CSAs were diluted to proper concentrations in 0.01 × tryptic soy broth containing 10 mM sodium phosphate buffer, pH = 7.4. Virus diluted in the same buffer was added to the compounds, and they were incubated for 24 hours at 37°C. A total of 20 µl of the peptide–virus mixture was added to the cells in 0.5 ml minimum essential medium containing 2.5% fetal calf serum and allowed to infect for 48 hours for plaque development. The medium was removed and the wells were overlaid with 0.5 ml 10% buffered formalin, and allowed to fix for 10 minutes at room temperature. The formalin was removed and 0.5 ml 0.1% crystal violet in phosphate-buffered saline was added to the wells for 5 minutes at room temperature. The wells were then aspirated and air-dried for observing plaques. We found the most accurate results, with the virus alone forming 50–80 plaques per well. Results are expressed as percent killing to allow comparisons between the compounds.

Electron microscopy

Concentrated VV stock (10^8 PFU) was treated with CSA-13 and -31 at a final concentration of 50 µM, and analyzed by transmission electron microscopy with a Phillips CM-10 as described earlier (Howell *et al.*, 2004).

KC cell culture

The HaCaT human KC cell line was grown in DMEM (Cellgro, Herndon, VA) as described earlier (Howell *et al.*, 2006). To investigate the antiviral activity of CSAs, the cells were seeded in 24-well plates at a concentration of 2×10^5 cells per well. The cells were infected with VV (0.05 PFU per cell) for 6 hours. After incubation, the virus was removed and the cells were washed with media to remove the remaining virus. CSAs (0–100 µM) were added to the infected KCs and allowed to incubate for an additional 18 hours. Total RNA was isolated from KCs using RNeasy Kits according to the manufacturer's guidelines (Qiagen, Valencia, CA) for analysis of VV gene expression by real-time reverse transcriptase PCR.

Primary human KC cell cultures were obtained from Cascade Biologics (Portland, OR) and maintained in serum-free EpiLife Medium (Cascade Biologics) supplemented with 1% human KCs growth supplement V2 (Cascade Biologics), 1% of penicillin and streptomycin, and 0.06 mM $CaCl_2$ as described earlier (Kim *et al.*, 2008). Before their use in experiments, NHK cells were differentiated in 1.3 mM $CaCl_2$ for 5 days. To understand the effects of CSA-13 on the innate immune response, differentiated NHK cells were stimulated with 1–50 µM CSA-13 for 24 hours. Total RNA was isolated from KCs using RNeasy Kits, according to the manufacturer's guidelines (Qiagen), for analysis by real-time reverse transcriptase PCR. Additional experiments were conducted to determine whether pretreating KCs for 6 hours with 50 µM of CSA-13 protects against VV infection.

Real time reverse transcriptase PCR

RNeasy Mini Kits (Qiagen) were used according to the manufacturer's protocol to further purify RNA from skin biopsies and to isolate RNA from cell cultures. RNA will be reverse transcribed into cDNA and analyzed by real-time reverse transcriptase PCR using an ABI Prism 7300 sequence detector (Applied Biosystems,

Foster City, CA), as described earlier (Howell *et al.*, 2006). Primers and probes for human GAPDH were purchased from Applied Biosystems. VV, LL-37, and HBD-3 primers and probes were prepared as described earlier (Ong *et al.*, 2002; Nomura *et al.*, 2003; Howell *et al.*, 2004). The primer and probe sequences for VV recognize a subunit of a DNA-directed RNA polymerase expressed within 2 hours of viral entry (Amegadzie *et al.*, 1991). To allow for comparisons between samples and group, quantities of all targets in test samples were normalized to the corresponding GAPDH or total cDNA levels as described earlier (Howell *et al.*, 2006).

Titration experiments

A fluorophore-labeled CSA (CSA-59) (Ding *et al.*, 2004b) was dissolved in phosphate-buffered saline at a concentration of 1.2 μg ml^{-1}. Purified VV (10^8 PFU ml^{-1}) was suspended in phosphate-buffered saline at concentrations of 10^4, 10^5, and 10^6 PFU ml^{-1} for these experiments. An aliquot of the CSA-59 solution was placed in a quartz cuvette in a FluoroMax-3 fluorometer (Horiba Jobin-Yvon, Edison, NJ). The temperature was held constant at 25°C. The VV was titrated into the CSA solution, and fluorescence emission was measured ($\lambda_{ex} = 340$ nm, $\lambda_{ex} = 450$ nm) in counts per second. Fluorescence intensities were corrected for changes in concentration during titration. Fluorescent experiments with KCs were carried out in a similar manner. Titration experiments were carried out twice or thrice with similar results.

Mice

CBySmn.CB17-*Prkdc*^{SCID}/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Crl:SKH1-*hr* (hairless) mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). All experiments using these animals were approved by the Institutional Animal Care and Use Committee at National Jewish Health. This institution has an animal welfare assurance number (A3026-1) on file with the Office of Protection and Research Risks.

Murine infection

The dorsal thoracic and lumbar regions of mice were clipped and treated with the depilatory agent, Nair, to remove all hair. At 72 hours after hair removal, the mice were anesthetized and inoculated with 5×10^6 PFU of Western Reserve VV by scarification. At 2 hours after inoculation, CSA-13 or control cream (petrolatum jelly) was applied to the dorsal thoracic and lumbar regions. Topical application was repeated daily until day 10 after inoculation. The mice were monitored for the formation of satellite lesions, weight loss, and mortality.

CSA penetration

To evaluate the ability of CSAs to penetrate the skin, a FITC-conjugated CSA-13 cream was applied to the dorsal region of hairless mice (Charles River Laboratories Inc.). Skin biopsies were collected 2, 4, 8, and 24 hours after application, immediately submerged in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA), and then cut into 5 μm sections. The slides were counterstained with wheat germ agglutinin conjugate, Alexa Fluor-633 (Molecular Probes, Eugene, OR), visualized with confocal microscopy (Leica, Wetzlar, Germany), and images were collected at ×40 with Slidebook 4.1 (Intelligent Imaging Innovations, Denver, CO).

VV immunofluorescent staining

Paraffin-embedded tissues were cut at 5 μm on frosted microscope slides. Using toluene and a series of ethanol washes, the slides were deparaffinized and then rehydrated. The slides were then immersed in an antigen-retrieval solution (0.01 mol l^{-1} citric acid, 0.05 mol l^{-1} NaOH, pH 6.0) and microwaved for 7 minutes to retrieve the masked antigens. Skin sections were then blocked with 5% BSA in Super Block (ScyTek Laboratories, Logan, UT) containing 10% non-immune donkey serum (Jackson Laboratories, West Grove, PA) for 60 minutes at 37°C. The slides were then stained with a mouse monoclonal anti-VV antibody (Santa Cruz Biotechnology, Santa Cruz, CA) directed against the 14 kDa protein of VV or control mouse IgG_3 (Santa Cruz Biotechnology) at 4°C overnight. The slides were washed with phosphate-buffered saline/Tween 0.05%, followed by incubation with a Cy3-conjugated donkey anti-mouse IgG (Jackson Laboratories). Immunohistochemical staining was visualized with confocal microscopy (Leica, Wetzlar, Germany). Images were collected at ×40, and levels of mean fluorescence intensity were measured with Slidebook 4.1 (Intelligent Imaging Innovations). The MFI was determined for each exposure group and was reported as mean MFI ± SE.

Statistical analyses

All statistical analyses were conducted using Graph Pad Prism, version 4.01 (San Diego, CA). Data were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA), followed by a Tukey–Kramer *post hoc* test (Tukey, 1977). Differences were considered significant at $P < 0.05$. IC90 and IC95 values were determined using nonlinear regression.

CONFLICT OF INTEREST

Dr Savage is a paid consultant to Ceragenix Pharmaceuticals, a company that has licensed the CSA technology.

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