



Nigericin is a potent inhibitor of the early stage of vaccinia virus replication

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ABSTRACT

Poxviruses remain a significant public health concern due to their potential use as bioterrorist agents and the spread of animal borne poxviruses, such as monkeypox virus, to humans. Thus, the identification of small molecule inhibitors of poxvirus replication is warranted. Vaccinia virus is the prototypic member of the *Orthopoxvirus* genus, which also includes variola and monkeypox virus. In this study, we demonstrate that the carboxylic ionophore nigericin is a potent inhibitor of vaccinia virus replication in several human cell lines. In HeLa cells, we found that the 50% inhibitory concentration of nigericin against vaccinia virus was 7.9 nM, with a selectivity index of 1038. We present data demonstrating that nigericin targets vaccinia virus replication at a post-entry stage. While nigericin moderately inhibits both early vaccinia gene transcription and translation, viral DNA replication and intermediate and late gene expression are severely compromised in the presence of nigericin. Our results demonstrate that nigericin has the potential to be further developed into an effective antiviral to treat poxvirus infections.

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1. Introduction

Vaccinia virus is the most widely studied member of the *Poxviridae*, a large group of complex DNA viruses which includes important human and animal pathogens (Moss, 2007). Vaccinia was used as a live vaccine for the eradication of smallpox caused by variola virus. Although variola no longer circulates amongst the human population, there remains concern about the deliberate release of variola virus as a bioterrorist agent. Furthermore, a small outbreak of monkeypox virus, which can cause fatal disease in humans, occurred in the USA in 2003 (Centers for Disease Control and Prevention, 2003). This raises the possibility that monkeypox, or other poxviruses of animals, could cross the species barrier and evolve into more efficient human pathogens. For these reasons, identification of therapeutic agents to treat poxvirus infection remains important.

Routine smallpox vaccination was abandoned in the early 1980s, and therefore a large proportion of the current worldwide population is susceptible to variola infection. Although stockpiles of smallpox vaccine exist, vaccination is accompanied by a relatively high incidence of adverse reactions and is contraindicated in the immuno-compromised and those with common skin conditions such as eczema (Aragon et al., 2003; Bristol, 2007; Centers for

Disease Control and Prevention, 2009). Moreover, vaccinia from the vaccination site can be transmitted to house-hold contacts and cause serious disease (Vora et al., 2008).

Currently there are no US Food and Drug Administration licensed pharmacological agents to treat poxvirus infection. However, cidofovir (CDV) may be used to treat severe smallpox vaccine complications under Investigational New Drug Protocols when vaccinia immune globulin treatment fails (Vora et al., 2008). CDV is a nucleoside analogue which inhibits the replication of numerous DNA viruses, including vaccinia (De Clercq et al., 1986). Although CDV is not orally bioactive, new derivatives have been developed to overcome this obstacle and also display greater potency (Kern et al., 2002). Another well characterized inhibitor of poxvirus replication in development is ST-246[®]. This compound targets the vaccinia F13 protein (Yang et al., 2005) which is required for efficient cell to cell spread of vaccinia (Blasco and Moss, 1991). ST-246 has demonstrated antiviral activity against vaccinia, cowpox virus, monkeypox virus and variola virus, both in vitro and in vivo (Berhanu et al., 2009; Duraffour et al., 2007; Smith et al., 2009; Huggins et al., 2009).

Given that there are no licensed antivirals to treat poxvirus infection, it remains important to identify additional candidate inhibitors for antiviral development. Furthermore, the Institute of Medicine has proposed that countries stockpile two distinct antivirals which inhibit different steps in poxvirus replication (LeDuc et al., 2002). To this end, we have identified nigericin as a potent inhibitor of vaccinia virus replication through a mechanism distinct from that of both CDV and ST-246. Nigericin (also known as antibiotic K-178, helexin C, azalomycin M, antibiotic X-464 and

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polyetherin A) is a carboxylic ionophore which readily incorporates into biological membranes and facilitates the exchange of mono-valent cations for protons (Pressman, 1976). Previously nigericin has been reported to inhibit Poliovirus and influenza virus replication (Irurzun et al., 1995; Alonso-Caplen and Compans, 1983). Nigericin has also been demonstrated to inhibit the replication of *Plasmodium falciparum* in vitro with the 50% inhibitory concentration (IC₅₀) reported as 0.8 pM (Adovelande and Schrevel, 1996). In vivo, nigericin suppresses *Plasmodium vinckei petteri* infection in mice, with the IC₅₀ achieved with a single daily subcutaneous dose of 1.8 mg kg⁻¹. Other potential applications of nigericin include its use to enhance the cytotoxic effect of chemotherapeutic agents which are more cytotoxic at low pH. For example, nigericin can potentiate the cytotoxic effect of mafosfamide (Jahde et al., 1991) and malphalan (Wood et al., 1995) on tumour cells.

We found that treatment of human HeLa, A549 and Huh7 cells with nigericin could almost completely abrogate vaccinia virus replication. The IC₅₀ of nigericin against vaccinia virus in HeLa cells was found to be approximately 7.9 nM, with a selectivity index (SI) of 1038. This antiviral effect of nigericin was not mediated by the inhibition of vaccinia entry into host cells. Instead, the mechanism by which nigericin exerts its antiviral effect appears to be through impairing early gene transcription and DNA replication. Correlated with this, intermediate and late vaccinia virus protein expression is compromised in the presence of nigericin. Furthermore, we have also characterized the antiviral activity of functionally related compounds which can alter proton concentrations within the cell, including monensin, and concanamycins A, B and C on vaccinia replication. Our results demonstrate that nigericin and other compounds mediating proton concentration have the potential to be further developed into effective antivirals to treat vaccinia virus and other poxvirus infections.

2. Materials and methods

2.1. Cell culture and viruses

HeLa, A549, Huh7, BHK21 and RK13 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin, at 37 °C, 5% CO₂. Recombinant vaccinia virus expressing green fluorescent protein (GFP) was constructed as previously described (Arsenio et al., 2008). Recombinant myxoma virus expressing GFP was a generous gift from Dr. Grant McFadden, University of Florida.

2.2. Reagents

Nigericin and monensin were purchased from Sigma and concanamycins A, B and C were purchased from Santa Cruz. Cidofovir was kindly provided by Gilead Sciences Inc., Foster City, CA. ST-246 was kindly provided by SIGA Technologies, Corvallis, OR. The vaccinia virus H3 antibody was a generous gift from Dr. Wen Chang, Institute of Biomedical Science, Taiwan. The vaccinia virus D12 and G8 peptide antibodies were made by Genscript. The actin antibody was purchased from Sigma.

2.3. Virus yield assays

For virus yield assays, confluent cells in 6-well plates were infected at an MOI of 0.1 with vaccinia virus (strain Copenhagen) or myxoma virus. Infections were done in the presence or absence of the indicated compound. After a 1 h incubation, the media containing the virus was removed, and the cells were washed three times with growth media. Fresh media with or without compound was then added to the cells. At 5 and 24 hpi for vaccinia virus, and 5 and 48 hpi for myxoma virus, cells were collected and the virus

was released by three cycles of freeze and thaw. Serial dilutions of supernatant were then added to confluent BHK21 cells for vaccinia virus titrations, or RK13 cells for myxoma virus titrations, in 12-well plates. All titrations were performed in triplicate and virus plaques were counted approximately 24 hpi.

2.4. Plaque reduction assays

The sensitivities of vaccinia virus to nigericin, monensin, and concanamycins A, B and C were determined by standard plaque reduction assays in HeLa cells. Sensitivities to cidofovir and ST-246 were also determined as reference compounds inhibiting vaccinia replication. Confluent HeLa cells in 12-well plates were infected with vaccinia virus to give approximately 50–60 plaques per well. After a 1 h incubation, the media containing the virus was removed. Two-fold dilution series of compounds were prepared as agarose overlays and added to the cells. All assays were done in duplicate and plaques were counted 24 hpi. The IC₅₀ was defined as the concentration of compound reducing the number of plaques by 50%. IC₅₀ values presented represent the mean of three independent assays ± one standard deviation.

2.5. Cell toxicity assays

HeLa cells were seeded in duplicate in 96-well plates at a density of $\sim 2 \times 10^4$ cells per well. After 24 h of growth, cells were approximately 90% confluent and were treated with serial dilutions of the indicated compounds in growth media. Following a 24 h incubation at 37 °C, 10 μ l of WST-1 reagent (Roche) was added to each well. The absorbance was measured at 450 nm in a SpectraMAX Plus luminometer (Molecular Devices). The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of compound reducing cell viability by 50%. Results represent the mean of three independent experiments ± one standard deviation.

2.6. Real time PCR

HeLa cells were pretreated with or without 2 μ M nigericin and then infected with vaccinia virus at an MOI of 1. RNA was extracted at 8 hpi using the RNeasy Mini Kit (Qiagen). RNA (1 μ g) was reverse transcribed using the Quantitect Reverse Transcription kit (Qiagen). Real time PCR was performed using Taqman Gene Expression Master Mix and primer/probe sets (Applied Biosystems) on an ABI 7500 FAST 96-well real time PCR machine (Applied Biosystems). Expression of actin was used as an internal standard. Errors bars represent the fold change or relative quantification (RQ) minimum and maximum. Primer sequences are listed below:

Primer	Sequence (5'–3')
Actin forward	CACACTGTGCCATCTACGA
Actin reverse	GCCAGCCAGGTCCAGAC
Actin probe	CCCATGCCATCCTGC
D12 forward	GATTAATAGCGTAAAGTATTATGGGAGGAGTAC
D12 reverse	CGCTGAGGTCGGCAACT
D12 probe	ACGGCGTCCGCTTTA
A1 forward	GGTGGTGAAGTAGGGTATTCTGTT
A1 reverse	TCTAAGAGCTACGTGAGACTTAACCA
A1 probe	AAGCCAACGAATCCCT
A17 forward	TCAAAAATAATGATGTTAGAACGTTACTCGGT
A17 reverse	GATAAATATCATCAATATAGAGATTAGAGAGGGCTA
A17 probe	CAAGACGAACAAAATC

2.7. Quantification of viral DNA replication

A real time PCR based approach was employed to analyze vaccinia virus DNA replication. Confluent HeLa cells were pretreated with 2 μ M nigericin for 1 h. Infected cells were also pretreated with or without 50 μ g/mL of cytosine arabinoside (araC) as a positive

control for inhibition of vaccinia DNA replication. Cells were then infected with vaccinia at an MOI of 1 in the presence or absence of compound. At 1 and 8 hpi, total DNA was isolated from the cells using the illustra triplePrep kit (GE Healthcare). Real time PCR was performed on 5 ng of DNA to detect the relative abundance of the vaccinia A1 gene as a measure of viral genome replication.

2.8. Western blotting

HeLa cells were pretreated with or without 2 μ M nigericin and then infected with vaccinia virus at a MOI of 1. Cells were collected at 2 and 8 hpi in 1 mL of PBS and pelleted by centrifugation at 6000 rpm for 2 min. Cell pellets were lysed in 200 μ L of 1 \times Laemmli buffer. Protein samples were separated on Criterion XT pre-cast gels (Bio-Rad) and transferred to Hybond-C nitrocellulose membranes (Amersham Bioscience). Membranes were developed with Western Lightning Chemiluminescent Reagent Plus (Perkin Elmer).

3. Results

3.1. Nigericin inhibits vaccinia virus replication

Nigericin has previously been demonstrated to inhibit the replication of RNA viruses including Poliovirus, influenza virus and Semliki Forest virus (Alonso-Caplen and Compans, 1983; Irurzun et al., 1995, 1997). We sought to determine whether nigericin could inhibit the replication of vaccinia virus, the prototypic member of the *Orthopoxvirus* genus of DNA viruses. HeLa cells were infected with vaccinia virus at a low MOI in the presence or absence of 0.2 or 2 μ M nigericin. Virus titres at 5 and 24 hpi were determined by plaque assays. We found that nigericin potently inhibited vaccinia virus replication in HeLa (Fig. 1A), A549 (Fig. 1B), and Huh7 cells (Fig. 1C). When used at 2 μ M, vaccinia replication was almost completely abrogated, representing a 3000, 380, and 1000-fold reduction in virus titres in HeLa, A549 and Huh7 cells, respectively. We observed ~10-fold decrease in viral titres using 0.2 μ M nigericin in each cell line tested. We also sought to investigate the potential broad anti-poxviral activity of nigericin. Given the high sequence similarity amongst members of the *Orthopoxvirus* genus, we reasoned that a poxvirus from a different poxvirus genus should be studied. To this end, we treated HeLa cells with 0.2 or 2 μ M nigericin and infected the cells with myxoma virus, a member of the *Leporipoxvirus* genus. Similar to vaccinia virus, myxoma virus replication was significantly inhibited in the presence of nigericin, with virus titres reduced 676-fold in 2 μ M nigericin treated HeLa cells (Fig. 1D).

3.2. Determination of the selectivity index of nigericin

We sought to determine the IC₅₀ of nigericin and compare it with a group of functionally related compounds, the concanamycins A, B and C, and monensin. Collectively, these compounds, and nigericin, can disrupt the electrochemical gradient across intracellular membranes (Pressman, 1976; Droese et al., 1993). Previously monensin (Payne and Kristensson, 1982) and concanamycin A (Townsend et al., 2006) have been demonstrated to inhibit vaccinia virus, but their specific antiviral activity in terms of IC₅₀ and SI were not characterized. We also compared the activity of nigericin to two well characterized inhibitors of vaccinia replication, cidofovir and ST-246. The antiviral activity of these compounds was determined by standard plaque reduction assays in HeLa cells (Table 1). We found the IC₅₀ of nigericin against vaccinia to be approximately 7.9 and 19.5 nM for the Copenhagen and Western Reserve strains, respectively. The IC₅₀ of monensin and concanamycins A, B and C against the Copenhagen strain of vaccinia was approximately 21.6, 1.7, 11 and 2.8 nM, respectively. The IC₅₀ for cidofovir was approximately

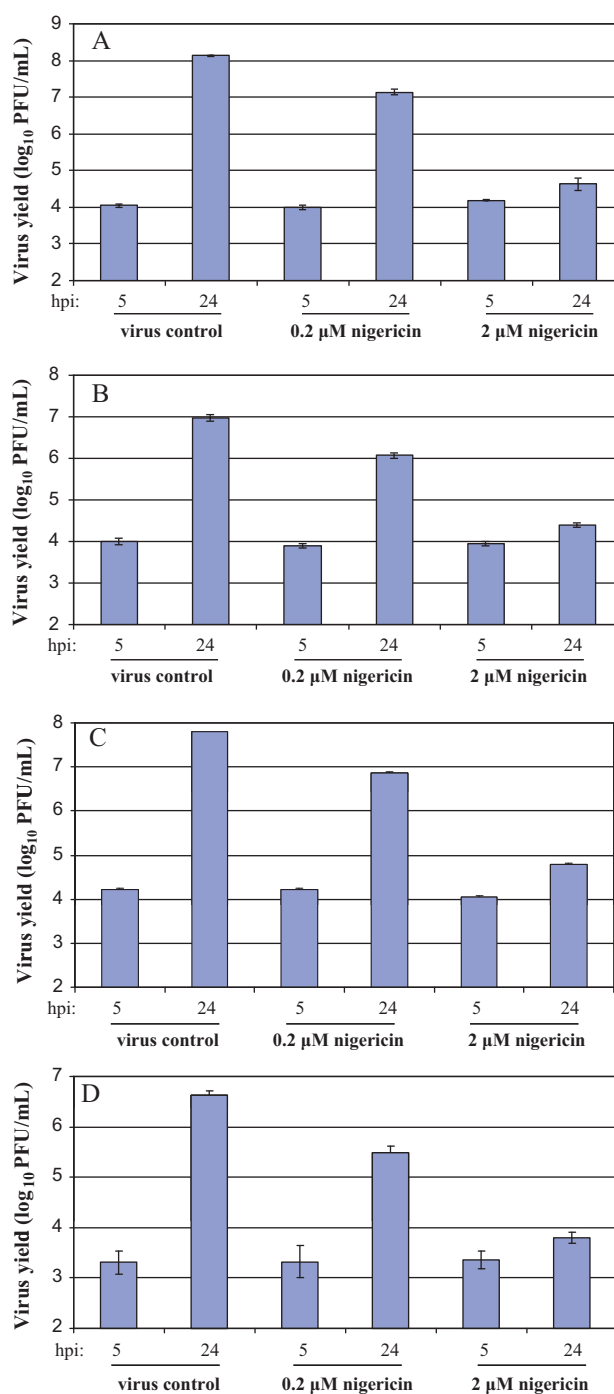


Fig. 1. Nigericin inhibits vaccinia virus replication in several human cell lines. Confluent HeLa (A), A549 (B) and Huh7 (C) cells in 6-well plates were treated with or without 0.2 or 2 μ M nigericin and infected with vaccinia virus (strain Copenhagen) at an MOI of 0.1. Virus titres were determined at 5 and 24 hpi by plaque assays on BHK21 cells. (D) HeLa cells in 6-well plates were treated with or without 0.2 or 2 μ M nigericin and infected with myxoma virus at an MOI of 0.1. Virus titres were determined at 5 and 24 hpi by plaque assays on RK13 cells. All results are representative of three independent experiments. Error bars represent the standard error of the mean.

110 μ M while the IC₅₀ for ST-246 was approximately 9.4 nM. Our results for ST-246 are similar to the previously reported IC₅₀ of 10 nM for ST-246 against vaccinia (Yang et al., 2005).

We also determined the level of cytotoxicity following treatment of cells with nigericin, monensin and the concanamycins (Table 1). HeLa cell monolayers were treated with serial dilutions of each compound and cell viability was measured after 24 h. The con-

Table 1

Inhibition of vaccinia virus replication by compounds in vitro.

Compound	IC ₅₀ ^a	CC ₅₀ ^b (μM)	Selectivity index ^c
Nigericin	7.9 ± 0.5 nM (Cop) 19.5 ± 5.7 nM (WR)	8.2 ± 1.8	1038 (Cop) 421 (WR)
Monensin	21.6 ± 4.4 nM	23.0 ± 2.9	1065
Concanamycin A	1.7 ± 0.2 nM	13.5 ± 5.7	7941
Concanamycin B	11.0 ± 1.9 nM	21.4 ± 0.6	1945
Concanamycin C	2.8 ± 0.8 nM	15.6 ± 3.2	5571
Cidofovir	110 ± 15 μM	>1000	–
ST-246	9.4 ± 2.4 nM	>1000	–

All results are the mean ± one standard deviation of three independent experiments.

^a IC₅₀ values were determined by plaque reduction assays in HeLa cells.^b CC₅₀ values were determined on cell monolayers using the WST-1 cell viability assay.^c The selectivity index was calculated as the CC₅₀ divided by the IC₅₀.

centration of nigericin at which cell viability was reduced by 50% (CC₅₀) was found to be approximately 8.2 μM. We were unable to obtain the CC₅₀ for cidofovir and ST-246, as no significant reduction in cell viability was observed with concentrations up to 1000 μM in our assay.

3.3. Inhibition of virus yield by nigericin, monensin and concanamycins A, B and C

We next sought to compare the inhibition of virus yield between the various compounds. HeLa cells were infected with vaccinia virus at an MOI of 0.1 in the presence or absence of each compound. For comparative purposes, compounds were used at a concentration of 2 μM, the concentration of nigericin which almost completely abrogates vaccinia replication in this assay. Virus titres were determined by plaque assays at 5 and 24 hpi (Fig. 2). Similar to the results in Fig. 1A, treatment of cells with 2 μM nigericin almost completely inhibited vaccinia virus replication. In contrast, CDV at 2 μM had no effect on vaccinia replication under these conditions as expected, given that our calculated IC₅₀ for CDV is 110 μM. ST-246 at 2 μM reduced viral titres approximately 7.4-fold compared to untreated controls. Monensin and concanamycins A, B and C displayed a similar potency in this assay, each reducing virus titres by approximately 20-fold.

3.4. Nigericin does not inhibit vaccinia virus entry and its effects on viral replication are reversible

Previously nigericin has been demonstrated to prevent entry of Semliki Forest virus into cells (Irruzun et al., 1997). We sought to determine whether nigericin also inhibits the entry of vac-

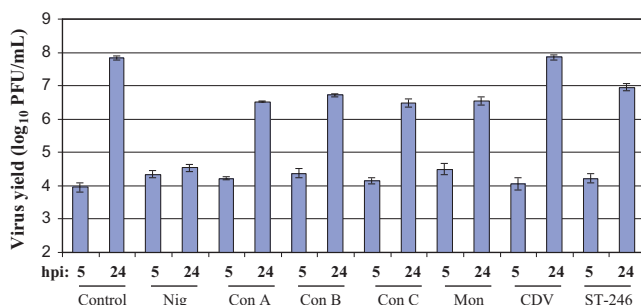


Fig. 2. Comparison of inhibition of virus yield between nigericin and functionally related compounds. Confluent HeLa cells in 6-well plates were left untreated or were treated with nigericin (Nig), concanamycin A (Con A), concanamycin B (Con B), concanamycin C (Con C), monensin (Mon), CDV or ST-246 at 2 μM and infected with vaccinia virus at an MOI of 0.1. Virus titres were determined at 5 and 24 hpi by plaque assays on BHK21 cells. Plaques were counted at approximately 24 hpi. Results are representative of three independent experiments. Error bars represent the standard error of the mean.

cinia virus into HeLa cells as its mechanism of action. To this end, we pretreated HeLa cells with nigericin (2 μM) for 1 h and then infected the cells at a low MOI with vaccinia virus in the presence of nigericin. At 1 hpi, the media containing the virus and nigericin was removed from the cells, and the cells were washed three times with growth media. Fresh growth media without nigericin was then added to the cells. Virus titres were determined at 5 and 24 hpi by plaque assays. When the inoculum containing nigericin was removed from the cells at 1 hpi, vaccinia replication approached the level of untreated controls (Fig. 3A). We also infected HeLa cells with vaccinia virus in the absence of nigericin and then treated cells with nigericin (2 μM) at 4 hpi. Cells were collected at 5 and 24 hpi and virus titres were determined by plaque assays. When nigericin was added 4 hpi to vaccinia infected cells, virus replication was reduced ~1600-fold compared to untreated controls (Fig. 3B).

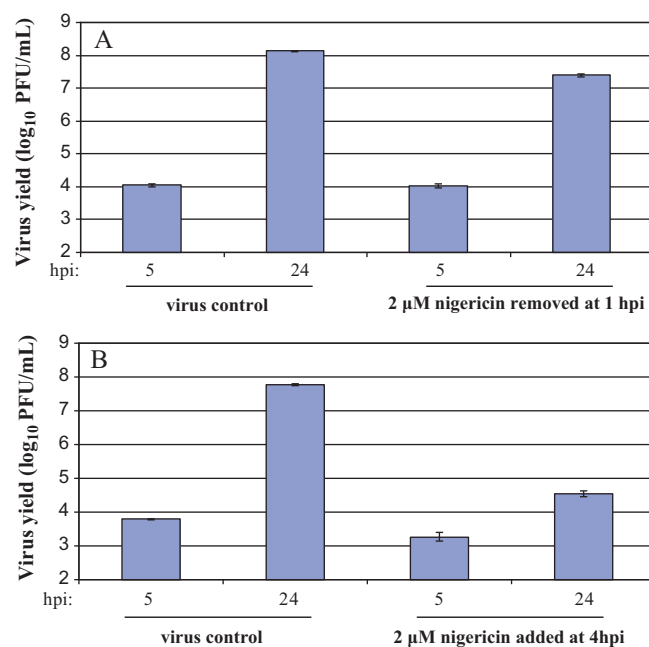


Fig. 3. Nigericin does not inhibit vaccinia virus entry. (A) HeLa cells in 6-well plates were pretreated with 2 μM nigericin for 1 h. Cells were then infected with vaccinia virus at an MOI of 0.1 in the presence of nigericin. At 1 hpi, the media containing the virus and nigericin was removed and the cells were washed three times with fresh growth media. Growth media without nigericin was then added to the cells and virus titres were determined at 5 and 24 hpi by plaque assays on BHK21 cells. (B) HeLa cells in 6-well plates were infected with vaccinia virus at an MOI of 0.1 in the absence of nigericin. At 4 hpi, the cells were left untreated or were treated with 2 μM nigericin. Virus titres were determined at 5 and 24 hpi by plaque assays on BHK21 cells. All results are representative of three independent experiments. Error bars represent the standard error of the mean.

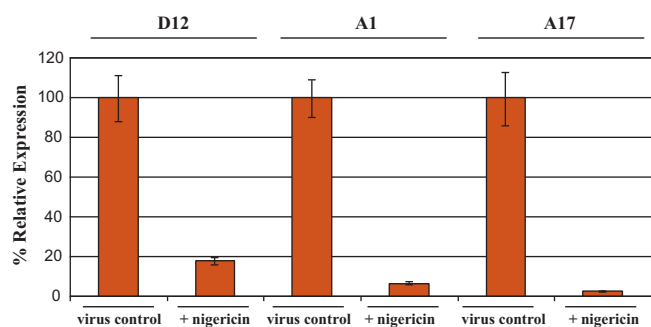


Fig. 4. Nigericin inhibits vaccinia virus transcription. HeLa cells in 6-well plates were left untreated or were pretreated for 1 h with 2 μ M nigericin. Cells were then infected with vaccinia virus at an MOI of 1 in the presence or absence of nigericin. The expression of vaccinia virus D12, A1 and A17 was quantified by real time PCR at 8 hpi. Expression of vaccinia virus genes was normalized to the expression level of actin within each sample to standardize RNA loading. The expression of each vaccinia gene in untreated, infected cells was assigned a value of 1. The fold change in expression of each gene in the presence of 2 μ M nigericin, as compared to untreated, infected cells was calculated. Results are shown as the percent relative gene expression in nigericin treated cells compared to untreated controls. All results are representative of three independent experiments. Error bars represent the fold change or relative quantification (RQ) minimum and RQ maximum values.

3.5. Nigericin inhibits vaccinia virus gene transcription

Next we investigated the mechanism by which nigericin exerts its antiviral effect against vaccinia. Vaccinia virus gene expression follows a cascade-like pattern, with early gene transcription proceeded by viral DNA replication (Broyles, 2003). Following DNA replication, intermediate and late genes are transcribed. We sought to determine at which stage of vaccinia virus replication nigericin acts. HeLa cells were infected with vaccinia virus at an MOI of 1 in the presence or absence of 2 μ M nigericin. Cells were collected at 8 hpi to examine viral gene expression. The expression of vaccinia early (D12), intermediate (A1) and late (A17) viral transcripts was quantified by real time PCR (Fig. 4). Compared to untreated controls, expression of D12, A1 and A17 was reduced in nigericin treated cells ~82, 94 and 98%, respectively.

3.6. Nigericin suppresses vaccinia DNA replication and intermediate/late protein translation

The potent inhibition of intermediate and late gene expression may be explained by inhibition of viral DNA replication by nigericin. To determine if nigericin blocks vaccinia DNA replication, we used real time PCR to quantify viral genome replication as measured by the relative abundance of the A1 gene. HeLa cells were infected with vaccinia virus at an MOI of 1 in the presence or absence of 2 μ M nigericin. Cells were also treated with cytosine arabinoside (araC), which blocks vaccinia DNA replication, as a positive control. Cells were collected at 1 and 8 hpi to examine the presence of the A1 gene. The fold change in A1 abundance of each sample was compared to the abundance of A1 at 1 hpi in infected, untreated cells. At 8 hpi, the A1 gene sequence was approximately 155-fold more abundant as compared to at 1 hpi (Fig. 5A). In contrast, there was no significant increase in the abundance of the A1 gene at 8 hpi in nigericin or araC treated cells. Similar results were obtained when quantifying the abundance of the vaccinia D12 gene (data not shown).

Potent inhibition of intermediate/late gene transcription and viral DNA replication should block intermediate and late viral translation. Thus, we also analyzed vaccinia virus protein translation. Identical samples to those described analyzing viral gene transcription were collected for Western blotting to determine the expression of D12 (early), G8 (intermediate) and H3 (late) (Fig. 5B).

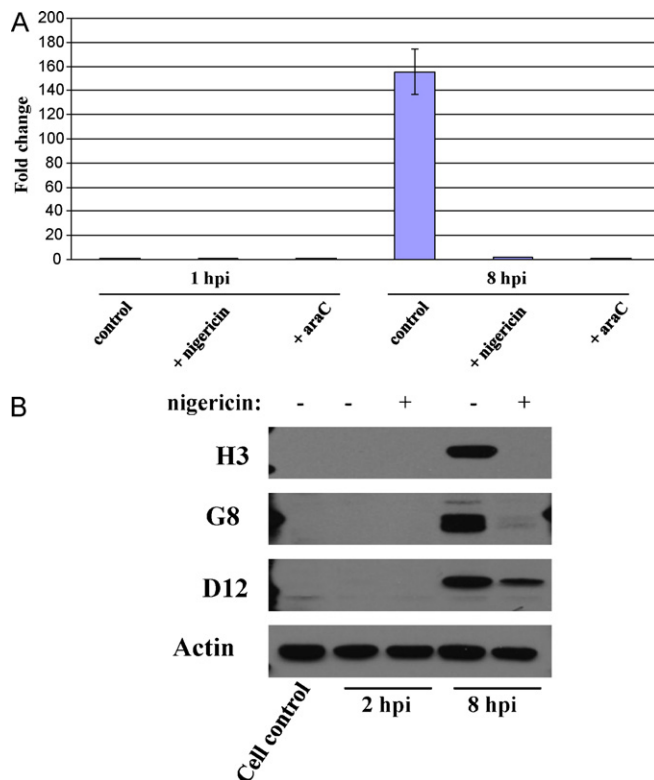


Fig. 5. Nigericin suppresses vaccinia DNA replication and intermediate/late translation. (A) HeLa cells in 6-well plates were left untreated or were pretreated for 1 h with 2 μ M nigericin. Cells were also pretreated with or without 50 μ g/mL of cytosine arabinoside (araC) as a positive control for inhibition of vaccinia DNA replication. Cells were then infected with vaccinia virus at an MOI of 1 in the presence or absence each of compound. Cells were collected at 1 and 8 hpi and total DNA was extracted. Real time PCR was performed to quantify the relative abundance of the vaccinia A1 gene. Results are representative of three independent experiments. Error bars represent RQ minimum and RQ maximum values. (B) Cells were treated with nigericin as in (A) and collected at 2 and 8 hpi and the expression of vaccinia virus D12, G8 and H3 was analyzed by Western blotting. Results are representative of three independent experiments.

The expression of actin was determined as a loading control. At 8 hpi, in the absence of nigericin, we were able to detect D12, G8 and H3 protein expression. Expression of D12 was partially inhibited in nigericin treated cells. The inhibitory effect of nigericin on intermediate protein translation was more pronounced, with very little G8 detected in nigericin treated cells compared to untreated controls. In contrast, we were unable to detect H3 protein in nigericin treated cells at 8 hpi.

4. Discussion

The threat of intentional release of poxviruses as bioterrorist weapons and the emergence of more human adapted strains of animal poxviruses underscores the need to develop therapeutic agents to treat human poxvirus infection. Furthermore, antivirals are also needed to treat severe reactions to poxvirus based vaccines. In this study, we have demonstrated that nigericin is a potent inhibitor of vaccinia virus replication in several human cell lines. Moreover, nigericin also inhibited myxoma virus, a distantly related poxvirus of the *Leporipoxvirus* genus. Given this and the high sequence similarity between vaccinia, variola virus and monkeypox virus, nigericin may have the potential to inhibit a broad array of poxviruses, including variola virus.

We have found that 2 μ M nigericin reduced vaccinia virus titres ~3000-fold in HeLa cells, representing a near complete block in virus replication (Fig. 1A). Although nigericin only reduced viral

titres 380-fold in A549 cells, the compound appears to be equally effective in inhibiting vaccinia replication in each cell line. The disparity between the reduction in titres between HeLa and A549 cells is a result of the increased replication capacity of vaccinia virus in HeLa cells as compared to A549 cells.

We used plaque reduction assays to compare the specific antiviral activity of nigericin to both cidofovir and ST-246, which represent well characterized inhibitors of poxvirus replication. We found that the IC_{50} of nigericin was 7.9 nM, with a corresponding SI of 1038 (Table 1). Nigericin was slightly less potent against the WR strain of vaccinia virus, which could be due to strain specific differences in post-entry events. In comparison, we found the IC_{50} of CDV and ST-246 to be 110 μ M and 9.4 nM, respectively. Previous studies have reported the IC_{50} of CDV against vaccinia virus to be 46.2 and 53 μ M (Kern et al., 2002; Andrei et al., 2006). The difference between our results and those previously reported for CDV may result from experimental variables such as the choice of cell line. Our calculated IC_{50} for ST-246 is similar to the previously reported IC_{50} of 10 nM (Yang et al., 2005). Thus, based on our results, nigericin displays a much greater potency than cidofovir and a similar potency to ST-246. We were unable to obtain the SI of cidofovir and ST-246 in our system, as significant toxicity was not observed when using up to 1000 μ M. Previous reports have also found CDV to be non-toxic at high micromolar concentrations (Jesus et al., 2009). In virus yield experiments in which the MOI is much higher than in plaque reduction assays, nigericin at 2 μ M was significantly more potent than either ST-246 or cidofovir at 2 μ M (Fig. 2). It is likely that nigericin is more potent than ST-246 in this assay because nigericin suppresses virus replication within the infected cell, while ST-246 only blocks cell to cell spread of the virus and does not affect intracellular replication.

We also compared the antiviral activity of nigericin with monensin and concanamycins A, B and C. Collectively, these compounds are functionally related to nigericin in several ways. Both nigericin and monensin are carboxylic ionophores with similar molecular structures. However, nigericin is preferentially selective for K^+ ions and monensin is preferentially selective for Na^+ ions (Pressman, 1976). By facilitating the exchange of K^+ ions for protons, nigericin can disrupt the electrochemical gradient across biological membranes. Similarly, the concanamycins inhibit endosomal function by blocking the activity of the vacuolar ATPases (Drose et al., 1993). Previously, both monensin (Payne and Kristensson, 1982) and concanamycin A (Townsend et al., 2006) were shown to inhibit vaccinia, but their antiviral activity in terms of IC_{50} and SI was not determined. We found that these compounds display low nanomolar IC_{50} values and correspondingly high SI values (Table 1). We found that even at much higher concentrations of 100–500 nM, these compounds were no more effective at inhibiting plaque formation than at the concentration listed in Table 1 as the IC_{50} . Therefore, the IC_{50} values and SI values for these compounds could vary widely. However, the lowest concentration of compound reducing the number of plaques by 50% was used, as this was deemed to be the most accurate measurement of antiviral activity. When comparing virus yield, which represents a higher MOI infection, monensin and concanamycins A, B and C were much less potent than nigericin (Fig. 2).

The high selectivity index of nigericin, combined with its superior potency in virus yield assays, suggests nigericin has the potential to be further developed into a therapeutic agent to treat poxvirus infection. Although nigericin is certainly more toxic than both cidofovir and ST-246, in vivo experiments demonstrate that nigericin can be used to provide positive clinical outcomes in mice. For example, nigericin treatment can protect mice from *Plasmodium vinckei petteri* infection at 1.8 mg kg⁻¹, while toxicity was observed with dosages above 10 mg kg⁻¹ (Adovelande and Schrevel, 1996). Furthermore, nigericin has been successfully used

in mice to potentiate the cytotoxic effects of melphalan on tumours when used at 2.5 mg kg⁻¹ (Wood et al., 1995). It should also be noted that nigericin has been reported to inhibit lymphocyte mitogenesis in cell culture (Daniele et al., 1978). This immunotoxic effect was not due to cytotoxicity and was reversible upon removal of nigericin. Again, the successful use of nigericin in vivo demonstrates that toxicity observed in cell culture may not manifest equally in vivo, or that the potency of nigericin may allow it to be used at sub-toxic doses.

We further characterized the mechanism of action of nigericin, since this is the first report to demonstrate that it can suppress vaccinia virus replication. To this end, we analyzed at which stage of vaccinia replication nigericin acts. Nigericin has been shown to prevent the entry of Semliki Forest virus into cells (Irurzun et al., 1997). It is unlikely that nigericin blocks vaccinia virus replication by impeding cell entry, since removal of the inoculum containing both the virus and nigericin at 1 hpi resulted in virus yields comparable to untreated controls (Fig. 3A). If nigericin blocked entry of vaccinia into the cell, then in this experiment, the virus would not enter the cell and would be removed during the media washes. In this scenario, the virus yield would be expected to be much lower than untreated controls. Thus, our results demonstrate that vaccinia virus enters the cells even in the presence of nigericin. Furthermore, treatment of cells with nigericin at 4 hpi also potentially inhibits vaccinia replication, demonstrating that nigericin can suppress post-entry events (Fig. 3B). Collectively, these results show that nigericin does not block vaccinia entry but rather targets vaccinia virus replication at a stage following entry of the virus. Therefore, the mechanisms by which nigericin inhibits Semliki Forest virus and vaccinia virus are distinct. Furthermore, that nigericin can suppress vaccinia infection when added both pre- and post-infection raises the possibility that nigericin could be used not only as a prophylactic but also for post-exposure antiviral treatment. This would be important in a setting in which prophylactic treatment would not likely have been given, such as accidental or deliberate release of a pathogenic poxvirus.

The earliest post-entry event that we found to be inhibited by nigericin was the suppression of early vaccinia gene expression (Fig. 4). These results are in agreement with previous reports that nigericin inhibits Poliovirus RNA synthesis (Irurzun et al., 1995). While early gene expression was significantly inhibited by nigericin, intermediate and late gene transcription was almost completely abrogated. Even moderate inhibition of early viral transcription would be expected to impair viral DNA replication to some extent. For this reason, we also analyzed vaccinia DNA replication in the presence of nigericin and found that the compound significantly blocked viral DNA replication. Correlated with this, intermediate/late gene transcription and intermediate/late translation were also inhibited. Thus, the combined impairment of early viral gene transcription and DNA replication likely accounts for the suppression of vaccinia virus replication by nigericin. However, the precise mechanism by which nigericin inhibits vaccinia virus gene transcription remains unknown. We have attempted to select for resistant viruses in the presence of nigericin, but were unsuccessful. Currently we do not know whether nigericin acts by targeting viral or host factors required by the virus.

An Institute of Medicine report has emphasized that two distinct antivirals targeting different stages of poxvirus replication should be stockpiled against a possible outbreak of variola virus (LeDuc et al., 2002). Two of the most well characterized inhibitors of poxvirus replication, ST-246 and CDV, suppress cell to cell spread or viral DNA synthesis, respectively (Blasco and Moss, 1991; Yang et al., 2005). In this report, we demonstrate that nigericin potentially inhibits vaccinia virus replication by suppressing early viral transcription leading to potent inhibition of viral DNA replication and subsequent intermediate/late gene expression. This mechanism is

therefore distinct from that described for ST-246 or cidofovir. Furthermore, we show that nigericin displays a very high potency against vaccinia replication and is effective when added to cells post-infection. Thus, additional work to investigate the potential of nigericin to be developed into an effective antiviral treatment for poxvirus infection should be explored. Synthesis of analogues of nigericin with higher potency and/or lower cell toxicity would be an important step in improving the activity of nigericin. Furthermore, experiments to study the potential of nigericin to protect mice from poxvirus challenge would be beneficial.

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References

- Adovelande, J., Schrevel, J., 1996. Carboxylic ionophores in malaria chemotherapy: the effects of monensin and nigericin on *Plasmodium falciparum* in vitro and *Plasmodium vinckei petteri* in vivo. *Life Sci.* 59, L309–L315.
- Alonso-Caplen, F.V., Compans, R.W., 1983. Modulation of glycosylation and transport of viral membrane glycoproteins by a sodium ionophore. *J. Cell Biol.* 97, 659–668.
- Andrei, G., Gammon, D., Fiten, P., De Clercq, E., Opdenakker, G., Snoeck, R., Evans, D., 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. *J. Virol.* 80, 9391–9401.
- Aragon, T.J., Ulrich, S., Fernyak, S., Rutherford, G.W., 2003. Risks of serious complications and death from smallpox vaccination: a systematic review of the United States experience, 1963–1968. *BMC Public Health* 3, 26.
- Arsenio, J., Deschambault, Y., Cao, J., 2008. Antagonizing activity of vaccinia virus E3L against human interferons in Huh7 cells. *Virology* 377, 124–132.
- Berhanu, A., King, D.S., Mosier, S., Jordan, R., Jones, K.F., Hruby, D.E., Grosenbach, D.W., 2009. ST-246 inhibits in vivo poxvirus dissemination, virus shedding, and systemic disease manifestation. *Antimicrob. Agents Chemother.* 53, 4999–5009.
- Blasco, R., Moss, B., 1991. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-Dalton outer envelope protein. *J. Virol.* 65, 5910–5920.
- Bristol, N., 2007. Adverse event related to smallpox vaccination. *Disaster Med. Public Health Prep.* 1, 11–12.
- Broyles, S.S., 2003. Vaccinia virus transcription. *J. Gen. Virol.* 84, 2293–2303.
- Centers for Disease Control and Prevention, 2003. Multistate outbreak of monkeypox—Illinois, Indiana, and Wisconsin, 2003. *MMWR Morb. Mortal. Wkly. Rep.* 52, 537–540.
- Centers for Disease Control and Prevention, 2009. Progressive vaccinia in a military smallpox vaccinee—United States, 2009. *MMWR Morb. Mortal. Wkly. Rep.* 58, 532–536.
- Daniele, R., Holian, S., Nowell, P., 1978. A potassium ionophore (Nigericin) inhibits stimulation of human lymphocytes by mitogens. *JEM* 147, 571–581.
- De Clercq, E., Holy, A., Rosenberg, I., Sakuma, T., Balzarini, J., Maudgal, P.C., 1986. A novel selective broad-spectrum anti-DNA virus agent. *Nature* 323, 464–467.
- Drose, S., Bindseil, K.U., Bowman, E.J., Siebers, A., Zeeck, A., Altendorf, K., 1993. Inhibitory effect of modified bafilomycins and concanamycins on P- and V-type adenosinetriphosphatases. *Biochemistry* 32, 3902–3906.
- Duraffour, S., Snoeck, R., de Vos, R., van Den Oord, J.J., Crance, J.M., Garin, D., Hruby, D.E., Jordan, R., De Clercq, E., Andrei, G., 2007. Activity of the anti-orthopoxvirus compound ST-246 against vaccinia, cowpox and camelpox viruses in cell monolayers and organotypic raft cultures. *Antivir. Ther.* 12, 1205–1216.
- Huggins, J., Goff, A., Hensley, L., Mucker, E., Shamblin, J., Wlazlowski, C., Johnson, W., Chapman, J., Larsen, T., Twenhafel, N., Karem, K., Damon, I.K., Byrd, C.M., Bolken, T.C., Jordan, R., Hruby, D., 2009. Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. *Antimicrob. Agents Chemother.* 53, 2620–2625.
- Irurzun, A., Sanchez-Palomino, S., Novoa, I., Carrasco, L., 1995. Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage. *J. Virol.* 69, 7453–7460.
- Irurzun, A., Nieva, J.L., Carrasco, L., 1997. Entry of Semliki forest virus into cells: effects of concanamycin A and nigericin on viral membrane fusion and infection. *Virology* 227, 488–492.
- Jahde, E., Glusenkamp, K.H., Rajewsky, M.F., 1991. Nigericin enhances mafosfamide cytotoxicity at low extracellular pH. *Cancer Chemother. Pharmacol.* 27, 440–444.
- Jesus, D.M., Moussatche, N., Damaso, C.R., 2009. In vitro activity of cidofovir against the emerging Cantagalo virus and the smallpox vaccine strain IOC. *Int. J. Antimicrob. Agents* 33, 75–79.
- Kern, E.R., Hartline, C., Harden, E., Keith, K., Rodriguez, N., Beadle, J.R., Hostetler, K.Y., 2002. Enhanced inhibition of orthopoxvirus replication in vitro by alkoxyalkyl esters of cidofovir and cyclic cidofovir. *Antimicrob. Agents Chemother.* 46, 991–995.
- LeDuc, J.W., Damon, I., Relman, D.A., Huggins, J., Jahrling, P.B., 2002. Smallpox research activities: U.S. interagency collaboration, 2001. *Emerg. Infect. Dis.* 8, 743–745.
- Moss, B., 2007. Poxviridae. In: Knipe, D., Howley, P. (Eds.), *Fields Virology*. Lippincott, Williams and Wilkins, Philadelphia, pp. 2905–2946.
- Payne, L.G., Kristensson, K., 1982. The effect of cytochalasin D and monensin on enveloped vaccinia virus release. *Arch. Virol.* 74, 11–20.
- Pressman, B.C., 1976. Biological applications of ionophores. *Annu. Rev. Biochem.* 45, 501–530.
- Smith, S.K., Olson, V.A., Karem, K.L., Jordan, R., Hruby, D.E., Damon, I.K., 2009. In vitro efficacy of ST246 against smallpox and monkeypox. *Antimicrob. Agents Chemother.* 53, 1007–1012.
- Townsend, A.C., Weisberg, A.S., Wagenaar, T.R., Moss, B., 2006. Vaccinia virus entry into cells via a low-pH-dependent endosomal pathway. *J. Virol.* 80, 8899–8908.
- Vora, S., Damon, I., Fulginiti, V., Weber, S.G., Kahana, M., Stein, S.L., Gerber, S.I., Garcia-Houchins, S., Lederman, E., Hruby, D., Collins, L., Scott, D., Thompson, K., Barson, J.V., Regnery, R., Hughes, C., Daum, R.S., Li, Y., Zhao, H., Smith, S., Braden, Z., Karem, K., Olson, V., Davidson, W., Trindade, G., Bolken, T., Jordan, R., Tien, D., Marcink, J., 2008. Severe eczema vaccinatum in a household contact of a smallpox vaccinee. *Clin. Infect. Dis.* 46, 1555–1561.
- Wood, P.J., Sansom, J.M., Newell, K., Tannock, I.F., Stratford, I.J., 1995. Reduction of tumour intracellular pH and enhancement of melphalan cytotoxicity by the ionophore Nigericin. *Int. J. Cancer* 60, 264–268.
- Yang, G., Pevear, D.C., Davies, M.H., Collett, M.S., Bailey, T., Rippen, S., Barone, L., Burns, C., Rhodes, G., Tohan, S., Huggins, J.W., Baker, R.O., Buller, R.L., Touchette, E., Waller, K., Schriewer, J., Neyts, J., DeClercq, E., Jones, K., Hruby, D., Jordan, R., 2005. An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus challenge. *J. Virol.* 79, 13139–13149.