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Nelfinavir inhibition of Kaposi's sarcoma-associated herpesvirus protein expression and capsid assembly

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Abstract

Background Antiviral therapies that target herpesviruses are clinically important. Nelfinavir is a protease inhibitor that targets the human immunodeficiency virus (HIV) aspartyl protease. Previous studies demonstrated that this drug could also inhibit Kaposi's sarcoma-associated herpesvirus (KSHV) production. Our laboratory demonstrated nelfinavir can effectively inhibit herpes simplex virus type 1 (HSV-1) replication. For HSV-1 we were able to determine that virus capsids were assembled and exited the nucleus but did not mature in the cytoplasm indicating the drug inhibited secondary envelopment of virions.

Methods For KSHV, we recently derived a tractable cell culture system that allowed us to analyze the virus replication cycle in greater detail. We used this system to further define the stage at which nelfinavir inhibits KSHV replication.

Results We discovered that nelfinavir inhibits KSHV extracellular virus production. This was seen when the drug was incubated with the cells for 3 days and when we pulsed the cells with the drug for 1–5 min. When KSHV infected cells exposed to the drug were examined using ultrastructural methods there was an absence of mature capsids in the nucleus indicating a defect in capsid assembly. Because nelfinavir influences the integrated stress response (ISR), we examined the expression of viral proteins in the presence of the drug. We observed that the expression of many were significantly changed in the presence of drug. The accumulation of the capsid triplex protein, ORF26, was markedly reduced. This is an essential protein required for herpesvirus capsid assembly.

Conclusions Our studies confirm that nelfinavir inhibits KSHV virion production by disrupting virus assembly and maturation. This is likely because of the effect of nelfinavir on the ISR and thus protein synthesis and accumulation of the essential triplex capsid protein, ORF26. Of interest is that inhibition requires only a short exposure to drug. The source of infectious virus in saliva has not been defined in detail but may well be lymphocytes or other cells in the oral mucosa. Thus, it might be that a "swish and spit" exposure rather than systemic administration would prevent virion production.

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Keywords Kaposi's sarcoma-associated herpesvirus, Nelfinavir, Virus inhibition, Integrated stress response, Virus assembly

Introduction

Kaposi sarcoma is a tumor associated with Kaposi's sarcoma-associated herpesvirus (KSHV also known as HHV-8). Transmission is believed to be salivary in most instances [1–6]. Agents that inhibit KSHV lytic replication have not been shown to be effective in the treatment of tumors [7]. However, agents that inhibit production of lytic virus might block salivary transmission [8]. Oral valganciclovir reduces KSHV shedding. Although well tolerated in the short term [8], valganciclovir is often associated with myelosuppression when used for long term treatment [9].

A topical therapy might inhibit lytic replication and viral transmission without associated systemic side effects. There is precedence for exploring topical agents to interfere with viral transmission. The Hendrix Lab has explored the use of rectal enemas with a variety of antiviral agents to block HIV transmission [10, 11].

Nelfinavir was developed as a protease inhibitor targeting the human immunodeficiency virus (HIV) aspartyl protease [12–14]. However, nelfinavir often leads to diarrhea [15]. With the advent of HIV protease inhibitors that are better tolerated, nelfinavir is rarely used in the treatment of HIV. A series of studies showed a variety of poorly understood off-target effects of nelfinavir [16]. Among them was a report showing antiviral activity against human herpesviruses [17]. This report led us to investigate the effects of nelfinavir on herpes simplex virus type 1 (HSV-1). We found that nelfinavir does not affect the activity of HSV-1 maturation protease; however, it alters glycoprotein maturation [18, 19]. We presented ultrastructural evidence that HSV type 1 infected cells treated with nelfinavir did not release virions into the intercellular space, but rather accumulated unenveloped virion particles in the cytoplasm [19]. The mechanism was unclear but other studies showed modulation of the unfolded protein response (UPR), cell cycle, apoptosis, autophagy, the proteasome pathway, oxidative stress and the integrated stress response (ISR) [20–23]. One of the hallmarks of the ISR is phosphorylation of the translation initiation factor eIF2 α to decrease overall translation initiation and increase the production of stress factors including the transcription factor ATF4 [24].

Gantt et al. reported that nelfinavir inhibited KSHV release by cells in tissue culture with an EC₅₀ of 7.4 μ M, which is 3.5 times more potent than ganciclovir [25]. Because nelfinavir has shown promising results

for KSHV inhibition, we theorized that it could be used to inhibit KSHV viral shedding. Since the site of shedding, the oral mucosa, is readily accessible, we hypothesize that a local antiviral formulation might inhibit shedding with minimal systemic exposure. Local antiviral drug delivery might involve a mouthwash or “swish and spit” therapy, that would reduce KSHV shedding in the saliva, and thus salivary transmission to uninfected partners. We sought to investigate further how nelfinavir may inhibit KSHV virus replication using a more tractable cell culture system and a recombinant KSHV virus developed by Jeffery Vieira [26]. This cell culture system could be used to examine all aspects of the KSHV replication pathway. In the investigations described in this report, we examine the activity of how nelfinavir impacts KSHV replication in cells and explore the effects of brief drug exposures on virus excretion such as might be achieved with a microbicidal mouthwash.

Materials and methods

Cells and viruses

All cell lines were grown in minimal essential medium (alpha medium– Gibco Invitrogen) supplemented with 10% fetal bovine serum (FBS– Gibco Invitrogen) and passaged as described previously [27]. The Vero cell line carrying the recombinant rKSHV.219 virus and the recombinant baculovirus Back50 were obtained from Jeffery Vieira [26]. These cells were used to subclone a Vero line that displayed almost 100% GFP positivity in the presence of puromycin (5 μ g/ml). The cell line iSLK developed by the Ganem Lab [28] was obtained from Jae Jung. 5r219 cells were maintained in 10 μ g/ml puromycin continuously.

Generation of an iSLK cell line harboring rKSHV.219

We created a KSHV positive iSLK cell line for these studies. To do this we derived virus from the Vero cell line harboring the KSHV recombinant virus rKSHV.219 [26] to infect the RTA-inducible iSLK cell line [28]. To get KSHV virus from the Vero cell line, we infected 1×10^6 cells with Back50 (baculovirus expressing ORF50-RTA) [26] for 3 h. This virus was removed and sodium butyrate added at a concentration of 0.5 mM in the growth medium. Four days following infection, the virus containing supernatant was used to infect 1×10^6 iSLK cells. The cells were monitored for the expression of GFP and four days following infection when more GFP positive cells were evident, the culture was trypsinized, diluted and

plated in media containing, initially high concentration (20 µg/mL) and then lower concentration (10 µg/mL) of puromycin. Individual GFP positive: puromycin resistant colonies were harvested using cloning cylinders and established lines were derived. One such clonal cell line designated 5r219 was used for all subsequent studies. For most experiments, 1×10^6 5r219 cells/well (12 well trays) were used. The monolayers were incubated with media containing 1 µg/mL doxycycline (DOX) for 1 h. This was then replaced with 1 ml of media containing the different concentrations of nelfinavir and 1 µg/ml of doxycycline or 1 µg/ml DOX and 1 mM sodium butyrate (DOX+NB). The supernatants containing virus were harvested at 72 h post-induction for analysis.

KSHV genome qPCR

Standards were created using KSHV DNA from the JSC-1 cell line [29] and all manipulations were done on ice. The PCR Master Mix was made using qPCR Taqman Universal Master Mix II (Thermo Fisher), 10 µM working stocks of the ORF73 Forward (5'-CCAGGAA GTCCACAGTGTTC-3') and Reverse (5'-GCCACCG GTAAAGTAGGACTAGAC-3') primers, 1 µM working stock of ORF73 Fluorescent Probe (5'/56-FAM/C ATCCGGGCTGCCAGCATTTG/36-TAMSp/3'), and dH₂O and vortexed for 10 s. The wells of a 96-well PCR plate were loaded with 40 µL of the PCR Master Mix and 10 µL of the standards, RNase/DNase-free H₂O as a negative control, or 10 µL of virus supernatant samples in multiple replicates. The plate was sealed with optical sealing tape and placed into the Bio-Rad CFX 96 qPCR Detection System. The thermal cycling parameters from the TaqMan Universal Master Mix II Protocol (Thermo Fisher) were used.

KSHV GFP titration

A GFP titration assay was generated by concentrating the virus present in the supernatant of a well (12 well size 1×10^6 cells) by centrifuging in a microfuge at 14000g for 60 min at +4°C. The supernatant was discarded and the virus resuspended in PBS (100 µL) and kept at +4°C overnight. The following day the virus suspension was vortexed for 20 s and the whole 100 µL volume added to Vero/HEK-293T cell monolayers in 12 well trays. The virus was absorbed to the cells for 2 h with intermittent shaking. Fresh media was added to the cells after the virus inoculum was removed. Cells were imaged in a Zoe (Bio-Rad) fluorescence microscope after 2–3 days.

Cell viability assays

5r219 cells (1×10^6) were incubated with the drug for the duration and concentration required. After

treatment, the cells were washed with PBS, trypsinized and resuspended in a final volume of 100 µL and transferred to 96 well tray. An equal volume of CellTiter-Glo reagent (Promega) was added to the cells, mixed on a shaker for 2 min and incubated at room temperature for 10 min before the luminescence signal was read in a GloMax plate reader (Promega).

Intracellular drug concentration

Nelfinavir was quantified in iSLK cells immediately after treatment and at 24, 48 and 72 h. Methanol (200 µL) was added to the pelleted iSLK cells (1×10^6) before extraction. The standard curve and quality control samples were prepared in methanol as a surrogate matrix for all matrices. Nelfinavir was extracted from 20 µL of sample with 80 µL of acetonitrile containing 125 ng/mL of the internal standard, ritonavir-d6. After centrifugation, the supernatant was then transferred into autosampler vials for LCMS/MS analysis. Separation was achieved with an UPLC BEH C18 (2.1 × 50 mm, 1.7 µm) column with a gradient elution using 0.1% formic acid in water (v/v; mobile phase A) and 0.1% formic acid in acetonitrile (v/v; mobile phase B). The flow rate was kept constant at 0.2 mL/min and the gradient started with 40% mobile phase B. Mobile phase B was held at 40% for 0.5 min before increasing to 100% mobile phase B over the span of 1.0 min. Next, mobile phase B was held at 100% for 1.0 min and then returned back down to 40% over 0.1 min. Finally, mobile phase B was allowed to equilibrate for 0.4 min for a total run time of 3.0 min. The column effluent was monitored using an AB Sciex triple quadrupole™ 4500 mass-spectrometric detector (Sciex, Foster City, CA, USA) using electrospray ionization operating in positive mode. The spectrometer was programmed to monitor the following MRM transitions: 569.1 → 330.0 for nelfinavir and 727.3 → 302.2 for ritonavir-d6. Calibration curves for nelfinavir were computed using the area ratio peak of the analysis to the internal standard by using a quadratic equation with a $1/x^2$ weighting function over the calibration ranges of 1.5 to 736.3 nM with dilutions up to 1:10 (v:v). Results were expressed in nmol/ 10^6 cells.

RT qPCR

RNA was extracted from cell pellets (1×10^6 cells) using the Qiagen RNeasy Kit according to manufacturer instructions. The resulting RNA samples were quantified using a NanoDrop 2000 (Thermo Fisher). cDNA was synthesized using the Bio-Rad iScript cDNA Synthesis Kit according to manufacturer instructions. Three PCR Master Mixes were created to detect DNA sequences associated with the GAPDH, CHOP, and Trib3 genes. Each of the master mixes contained the

Bio-Rad SsoFast EvaGreen supermix, RNase/DNase-free water, and forward and reverse primers [30, 31] associated with each gene. The wells of a 96-well PCR plate were loaded with 18 μ L of one of the PCR Master Mixes and 2 μ L of cDNA sample. Each sample had 2 or 3 technical replicates for each of the 3 genes being detected. The plate was sealed with optical sealing tape and placed into the Bio-Rad CFX 96 qPCR Detection System. The thermal cycling parameters from the SsoFast EvaGreen protocol were used.

Immunoblots

5r219 cells (1×10^6) were harvested 72 h post-induction. Cell pellets were lysed in 2X Laemmli buffer and 10% of this sample was resolved using NuPAGE 4–12% Bis-Tris gels (Thermo Fisher) and transferred to nitrocellulose membranes using the iBlot2 system (Thermo Fisher), as described by Luitweiler et al. [32]. Rabbit antibodies to KSHV proteins were used at a dilution of 1:500. Mouse antibody to ORF26 was purchased from Novus and Mouse monoclonal CHOP antibody from Cell Signaling Technology and used at similar dilutions. Rabbit polyclonal to GAPDH (Invitrogen) was used at 1:2500 dilution (Table S1). Blots were processed using the Clarity chemiluminescence kit (Bio-Rad) according the manufacturer's protocol and imaged using the iBright Imager (Invitrogen).

Transmission Electron Microscopy (TEM)

5r219 cells (1×10^6) cells were induced for lytic activation using 1 μ g/mL doxycycline for 60 min. The cells were then incubated with nelfinavir (15 and 20 μ M) for 72 h. No drug controls were also included. Samples were fixed in 2.5% glutaraldehyde, 3mM $MgCl_2$, in 0.1 M sodium cacodylate buffer, pH 7.2 for overnight at 4°C. After buffer rinse, samples were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (1 h) on ice in the dark. Following a DH_2O rinse and en bloc staining in 0.75% uranyl acetate for three hours, samples were dehydrated in a graded series of ethanol and embedded in Eponate resin overnight at 60°C. Thin sections, 60 to 90 nm, were cut with a diamond knife on a Leica UltracutE ultramicrotome and picked up with 2×1 mm formvar coated copper slot grids. Grids were stained with 2% uranyl acetate (aq.) and 0.4% lead citrate before imaging on a Hitachi 7600 TEM at 80 kV equipped with an AMT XR80 CCD.

Results

Generation of a KSHV virus producer 5r219 cell line

We wished to examine the antiviral activity of nelfinavir in greater detail using a more tractable cell culture system for KSHV. To this end we re-created an inducible SLK cell line harboring KSHV,

similar to iSLK.219 cl.10 that was originally described by Myoung and Ganem [28]. The KSHV recombinant rKSHV.219 was originally isolated by Jeff Vieira [26]. This virus expresses GFP constitutively (EF1a promoter) and upon lytic induction expresses RFP driven by the lytic KSHV PAN promoter. It also carries a puromycin resistance gene. Previously, we had obtained the Vero rKSHV-219 cell line from Jeff Vieira. This line requires infection with a recombinant baculovirus expressing ORF50 (RTA) and sodium butyrate treatment for lytic induction [26]. This was not conducive for our experiments. Therefore, we derived rKSHV.219 virus from the Vero cell line (after lytic induction) and infected iSLK cells. These cells express RTA under control of a doxycycline (DOX)-responsive promoter, the tetracycline-responsive element (TRE), for more efficient lytic induction [28]. Following infection of iSLK cells we derived cell lines that were GFP positive and puromycin resistant. Individual clones were selected for their lytic inducibility (RFP fluorescence) following the addition of doxycycline. One such clonal cell line designated 5r219 was used for all our experiments. Upon the addition of doxycycline (DOX) the cells began to express RFP indicating induction of lytic gene expression (Fig. 1A). We quantitated virus production from these cells following lytic induction (+ doxycycline) using a GFP plating assay on Vero and HEK-293T monolayers (Fig. 1B). The GFP fluorescence assay is a measure of biological infectivity in that GFP is only expressed after KSHV virus enters and begins to replicate in these cells. This assay demonstrated significant virus production from the 5r219 cells and because we observed similar results using either Vero or HEK-293T cells, we used Vero cells for subsequent plating assays. We chose to use a qPCR assay to quantitate viral genomes produced by 5r219 cells. The first experiments, shown here, were used to demonstrate the production of KSHV virus following induction with DOX (1 μ g/mL) alone or with DOX and 1 mM sodium butyrate (DOX/NB) (Fig. 1C). This was also visually observed when we infected Vero cell monolayers with virus harvested from 5r219 cultures at 72 h post-induction. The GFP signal indicative of KSHV infectious virus was high for both induced cultures (DOX or DOX/NB) (Fig. 1D). Because these observations showed that we could achieve significant virus yields at 3 days post-induction with either induction method, we thus eliminated sodium butyrate from the induction because of the toxicity of this compound. We also compared the qPCR and GFP titration methods using virus harvested (72 h) from 5r219 (DOX or DOX+NB) induced cells (Fig. S1). While the GFP assay is a visual reporter of infectious virus, it was not as easily quantifiable compared to the qPCR assay

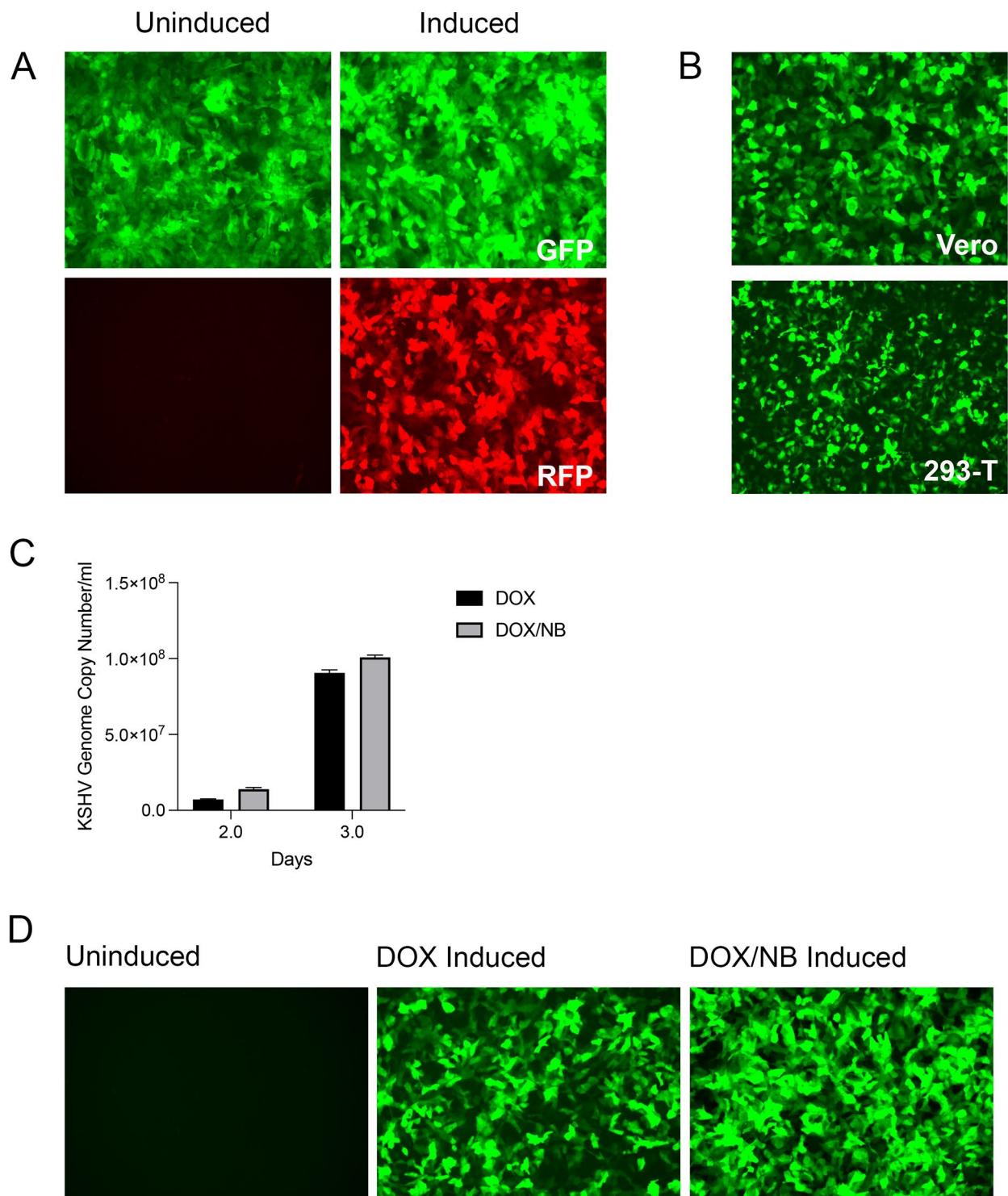


Fig. 1 Establishment of a tractable virus producer cell line for KSHV r219. The 5r219 cell line was generated following infection of iSLK cells with KSHV.r219 virus derived from Vero cells. Clonal isolates that displayed the highest level of lytic induction were analyzed further. The cells were first treated with doxycycline (1 $\mu\text{g/ml}$) and fluorescence visualized by light microscopy. Red fluorescence was only observed after lytic induction (**A**). The supernatants from these cultures were harvested at 72 h, concentrated by centrifugation and the virus was used to infect monolayers of Vero or HEK-293T cells. Numerous GFP positive cells were observed 48 h post-infection indicative of KSHV virus infection and replication (**B**). In order to more precisely quantitate virus yields and the optimal time of virus production, 5r219 cells were induced with doxycycline (DOX) or doxycycline plus 1 mM sodium butyrate (DOX/NB) and virus supernatants harvested, 2- and 3-days post-induction, and used in qPCR assays to determine KSHV genome copies (**C**). This was also visualized using GFP fluorescence following infection of Vero cell monolayers with extracellular concentrated virus (harvested after 72 h) from induced 5r219 cells (**D**)

because of the non-uniformity of the appearance of the GFP fluorescence.

Inhibitory activity of nelfinavir on KSHV replication

We first tested the effect of nelfinavir at different concentrations of drug. Using qPCR, we observed inhibition of genome copies at all concentrations used (Fig. 2A). There was an almost complete inhibition (97%) of virus genome copies at 20 μM . Even at 5 μM concentration of nelfinavir there was a 90% reduction of KSHV genomes. We analyzed the data in panel A using one-way ANOVA statistical test. As shown

in Fig. S2, we derived a $p < 0.0001$ for all comparisons, which is statistically significant. We also examined the inhibition of virus production using the GFP plating assay (Fig. 2B). In the absence of the drug, numerous GFP positive cells were evident, indicative of robust virus production. Adding nelfinavir significantly decreased the number of GFP positive cells. For concentrations of nelfinavir at or higher than 10 μM there was no GFP signal in the monolayer, although at 5 μM concentration of drug we observed some GFP positive cells, indicating production of some virus at this lower concentration. We also tested the toxicity

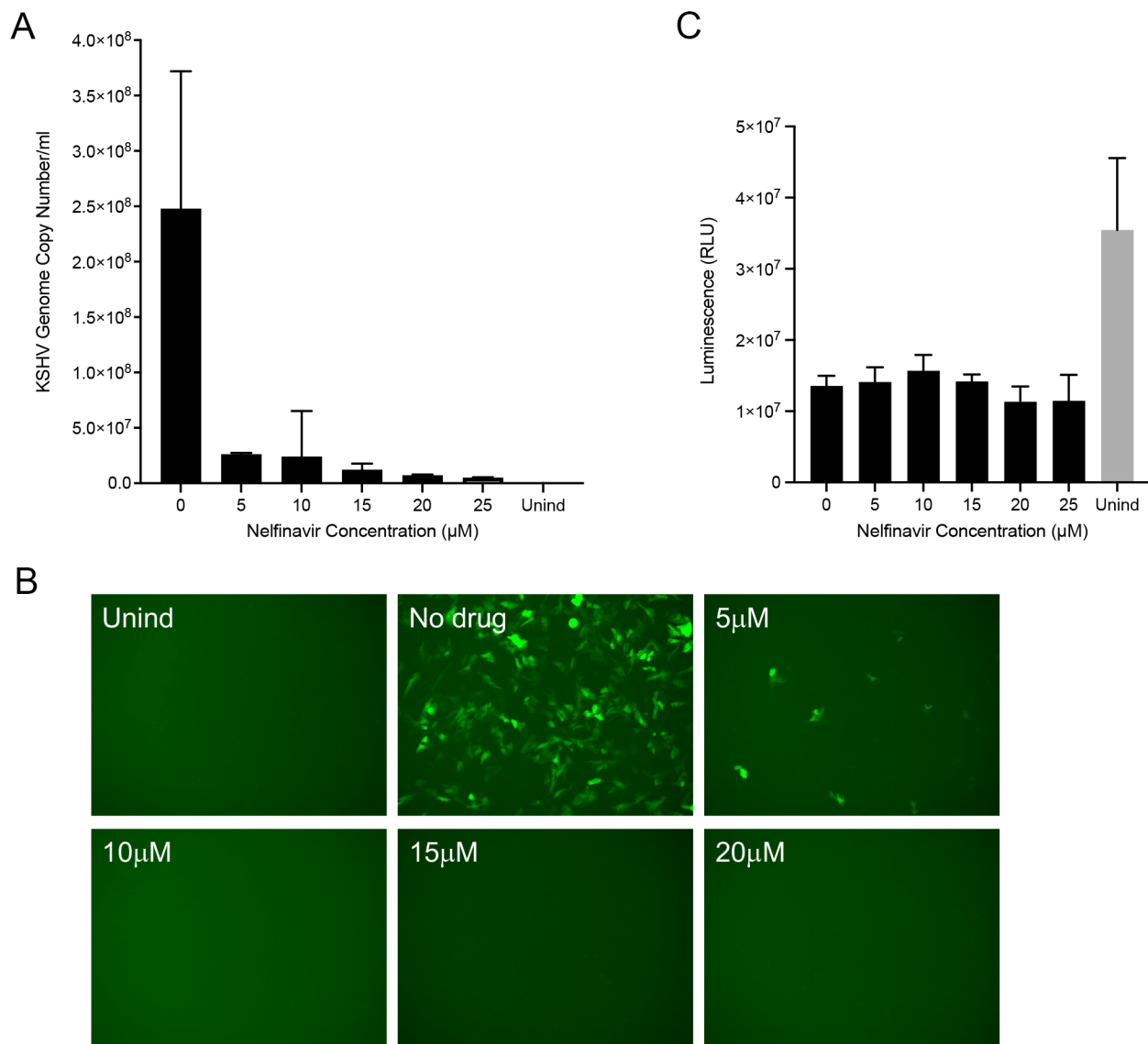


Fig. 2 Nelfinavir inhibits KSHV virus production. The effect of nelfinavir on KSHV virus production was examined using a standard dose-response assay. 5r219 cells were first incubated with media containing 1 $\mu\text{g}/\text{ml}$ doxycycline for 1 h. After that replicate cultures were incubated with varying concentrations of nelfinavir in the continued presence of doxycycline for 72 h. Uninduced (Unind) cultures were also examined in parallel. The culture supernatants were harvested, clarified and used in qPCR assays to enumerate viral genomes (A) as well as concentrated and the pelleted virus used to infect Vero cell monolayers (B). Similar 5r219 cell cultures were examined for cell viability using the CellTiter-Glo assay (C)

of the drug on replicate cultures, and again the data showed there was minimal toxicity even at the high concentrations of the drug (Fig. 2C). Thus, we chose a concentration of 20 μ M for many of the subsequent experiments. Because DOX induces RTA expression and thus the lytic gene expression program for KSHV in these cells, we do see an effect on the viability of the cells after 72 h compared to uninduced cells regardless of the presence of nelfinavir. The inhibitory activity of nelfinavir on KSHV as examined using 5r219 cells was similar to what we observed with HSV-1 infected Vero cells [19]. Gantt et al. also reported 80% inhibition of KSHV virus production using 10 μ M nelfinavir [17].

High-dose short duration exposure of KSHV infected cells to nelfinavir

In the above experiment the drug was incubated with the cells for 3 days at low micromolar concentrations. We then began to look at short duration exposure of high doses of nelfinavir and ganciclovir and their effect on KSHV production and cell toxicity. We initially started with 30 min exposure using 1 mM nelfinavir and 10 mM ganciclovir. The cells were exposed both after 1 h post-lytic induction (+DOX) and after 20 h post-lytic induction. We observed significant decrease in virus yield as judged by the GFP plating assay when the drug was incubated for only 30 min (data not shown). The inhibitory effect was more pronounced

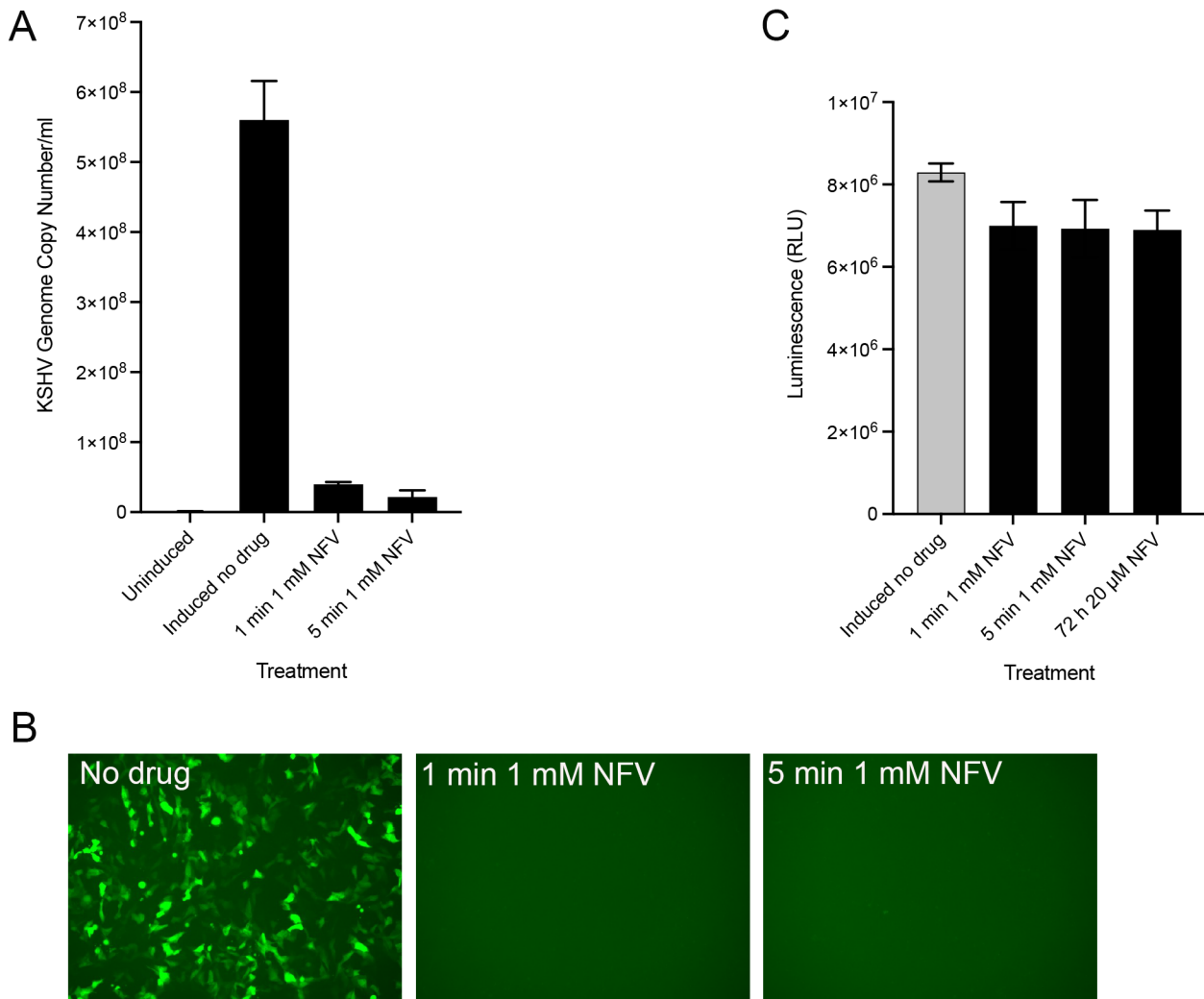


Fig. 3 High-dose short-duration exposure of nelfinavir and KSHV inhibition. 5r219 cells were lytically induced with doxycycline. After 1 h induction, the cells in replicates were treated with 1 mM nelfinavir mesylate (NFV) for 1 or 5 min. The drug was removed and the cells were incubated in medium containing only doxycycline. The cell culture supernatants were harvested at 72 h post-induction and viral genomes quantified using qPCR assays (**A**). Virus was also concentrated and the pelleted virus used to infect Vero cells. Fluorescence was examined after 48 h (**B**). Cell viability was examined for similarly treated cultures and compared to cells incubated with 20 μ M nelfinavir continuously for 72 h (**C**)

when the cells were treated after 1 h post-lytic induction (data not shown). This led us to try even shorter durations of drug exposure. We tested various short pulses of drug treatment. We observed that virus production was inhibited by very short pulses of nelfinavir, but for ganciclovir we had to treat the cells for longer times to achieve similar inhibition (data not shown). We settled on exposing the cells for 1 and 5 min with 1 mM nelfinavir only, as it was more potent. The cells were induced with DOX for 1 h and then the media removed and the cells pulsed (1 or 5 min) with 1 mM nelfinavir in a 100 μ L volume. The drug was removed and all the cells were incubated with DOX containing media for 72 h, except the uninduced controls. The uninduced cells were treated in a similar fashion to the other cells and replenished with media minus DOX. The intracellular drug concentrations remained

consistent over 72 h and were 4.0 ± 1.3 nmol/ 10^6 cells after 1 min of treatment and 6.7 ± 3.8 nmol/ 10^6 cells after 5 min of treatment. This treatment was as effective at inhibiting KSHV virus production as the continuous exposure of drug (Fig. 3A). Yields of virus were reduced by an average of 93% (1 min) and 96% (5 min). This was also visually observed using the GFP plating assay on Vero cells (Fig. 3B). There was complete absence of fluorescence indicative of absence of infectious virus. Even at the high doses of nelfinavir used here, the cells displayed minimal cell toxicity as judged by viability assays (Fig. 3C).

Nelfinavir induces the UPR in 5r219 cells

Because several studies have shown that nelfinavir is a potent inducer of the UPR [21], we wished to confirm that the same was true in the cell culture system

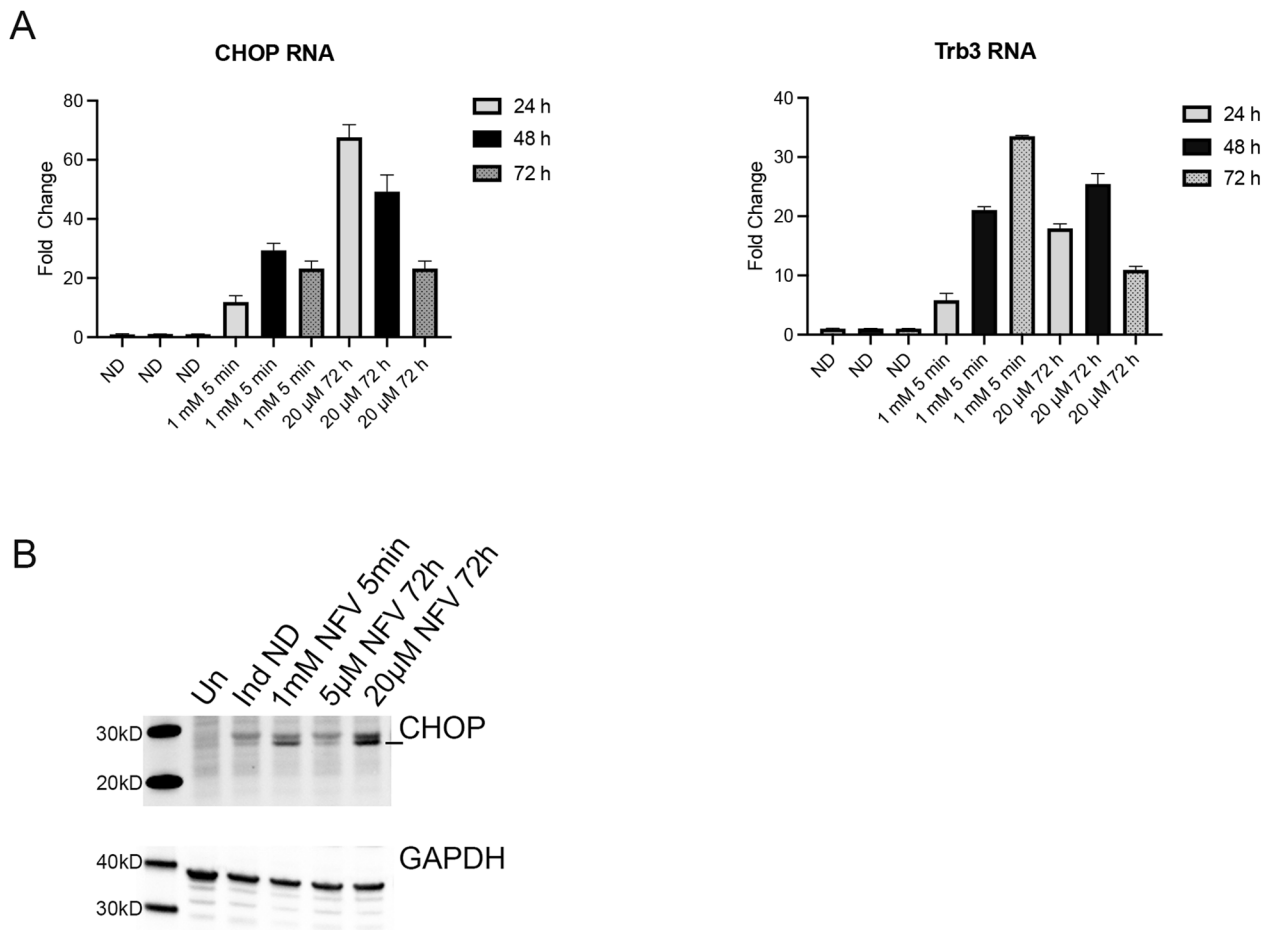


Fig. 4 Nelfinavir induces gene expression of CHOP and Trb3. 5r219 cell cultures were induced with doxycycline and then exposed to 1 mM of nelfinavir for 5 min or 20 μ M nelfinavir for 72 h or no drug (ND). At 24, 48 and 72 h after induction, RNAs were extracted from replicate cultures and were analyzed using RT qPCR methods (A). 5r219 cultures were induced with doxycycline and exposed to nelfinavir (1 mM for 5 min or 5 and 20 μ M of drug for 72 h). The cells were harvested at 72 h post-induction and the proteins were analyzed by western blot methods (B). Molecular weight standards (kD) are shown in the left lane. Un: uninduced, Ind ND: Induced with doxycycline no drug control. The induced CHOP polypeptide is indicated by a line on the right of the panel

that we have used in this study, both with continuous exposure and with the short duration drug treatment. We performed an RT qPCR assay to investigate this (Fig. 4A). Using RT qPCR assays we observed both CHOP and Trib3 RNA levels increase significantly following nelfinavir treatment for 5 min (1 mM) or nelfinavir treatment for 72 h (20 μ M). This was also confirmed using immunoblot methods using CHOP antibody (Fig. 4B). 5r219 cells were either pulsed with 1 mM nelfinavir (5 min) or incubated continuously with low (5 μ M) and high (20 μ M) concentrations of nelfinavir. CHOP polypeptide was observed in the cells treated with nelfinavir (either pulsed or 20 μ M).

Ultrastructural analysis of nelfinavir treated cells

We sought to determine where the block in KSHV virus production occurs in nelfinavir-treated cells. We treated 5r219 cells with nelfinavir and then processed and evaluated the cells by transmission electron microscopy

(TEM). Enveloped virions in the cytoplasm and nuclear capsids were evident in the no drug (control) cell cultures (Fig. 5A). These intranuclear angular capsids had an electron-dense (DNA) and scaffold (protein) cores. However, in nelfinavir-treated cells we did not see normal capsid assembly (Fig. 5B). There was no evidence of mature angular capsids in the nucleus of nelfinavir-treated cells. Instead, we observed nuclear aggregations with irregular densities, likely virus assembly compartments [33]. It is here that capsids would mature and become packaged with DNA. Thus, in the presence of drug the viral assembly compartments do not appear to facilitate the maturation of nuclear capsids. In addition, we did not observe any mature or enveloped virions in the cytoplasmic compartments of nelfinavir treated cells (Supplemental Fig. S3).

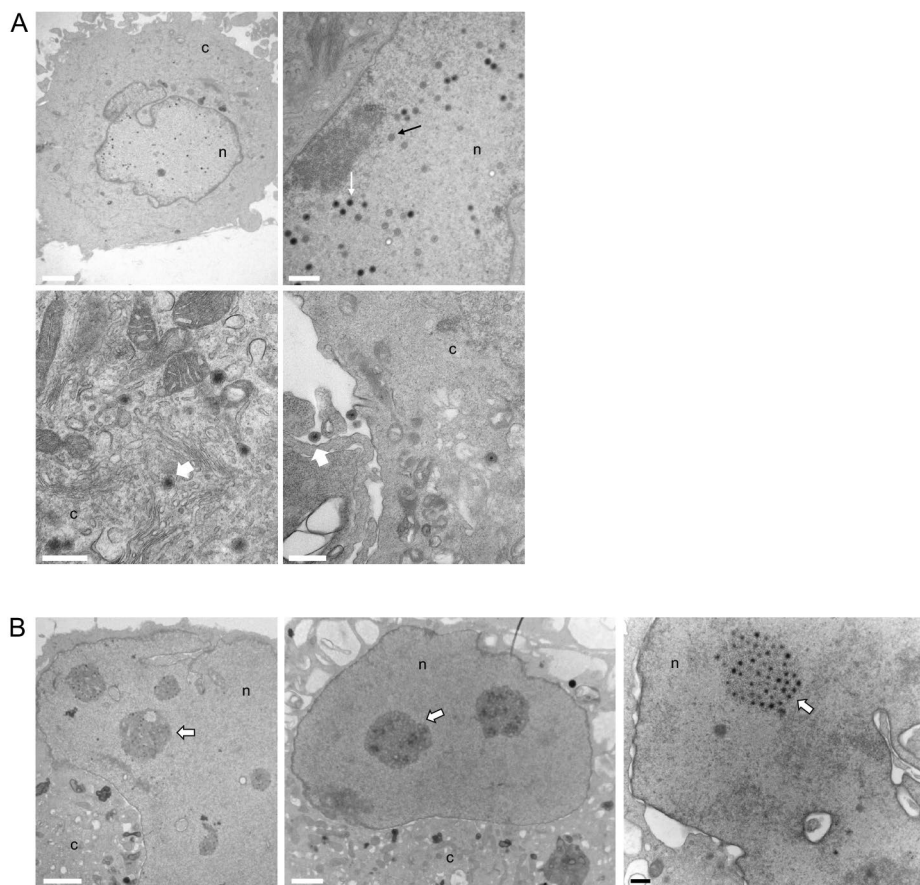


Fig. 5 Ultrastructural analyses of nelfinavir treated 5r219 cells. 5r219 cells were induced with doxycycline and then exposed to 20 μ M nelfinavir for 72 h. Cells were then processed for thin section and imaged by TEM. In the no drug controls (**A**), capsid structures were evident in the nucleus. Some contained an electron dense DNA core (white arrow) whereas others had an internal scaffold core (black arrow). In the cytoplasm and at the cell membrane, enveloped virions were evident (white arrowhead). In the nelfinavir treated cells (**B**), mature capsids were not observed however, within the nucleus, large dense aggregations were evident (white arrowheads). Scale bar = 1000 micron. c= cytoplasm, n= nucleus

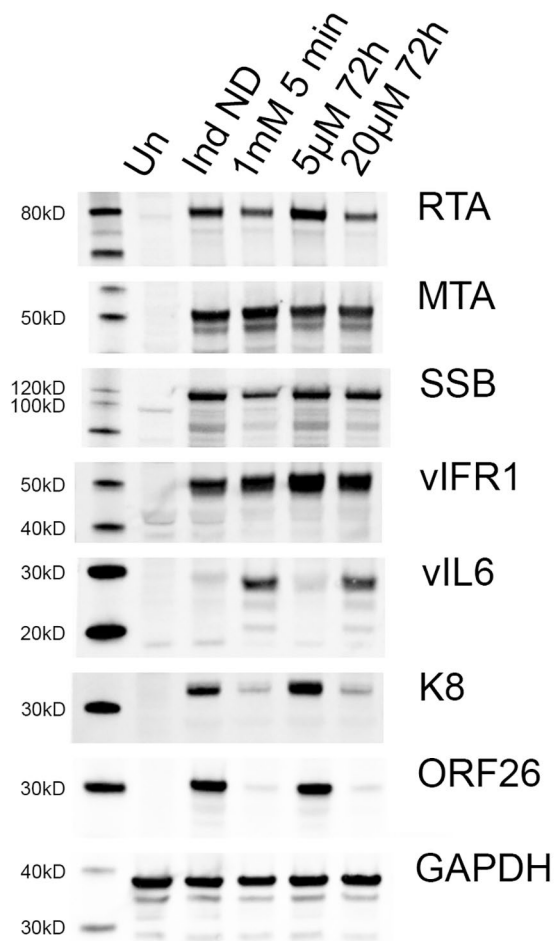


Fig. 6 Effects of nelfinavir on KSHV protein expression. 5r219 cultures were induced with doxycycline and exposed to nelfinavir (1 mM for 5 min or 5 and 20 μ M of drug for 72 h). The cells were harvested at 72 h post-induction and the proteins were analyzed by western blot methods. Molecular weight standards are shown in the left lane. Un: uninduced, Ind ND: Induced with doxycycline no drug control

KSHV virus protein expression in nelfinavir treated cells

Nelfinavir is a potent inducer of the ISR [20]. It has been shown that nelfinavir can decrease overall translation rates and facilitate transcriptional activity characteristic of the ISR [20]. We have also shown this recently with the antimicrobial drug, clofocetol and arsenic, in EBV positive cell lines [30, 31]. This may be one of the mechanisms by which nelfinavir inhibits virus capsid maturation: by altering the expression of an essential structural protein. We thus examined protein expression levels in 5r219 cells exposed to nelfinavir (both short pulse and continuous) using immunoblot methods. We chose several KSHV antigens to examine, some expressed early and others expressed late in the replication cycle (Table S1). The data are shown for uninduced cells as well as induced cells (+DOX), cells treated with a 5 min pulse of 1 mM

nelfinavir and cells treated continuously with 5 μ M and 20 μ M nelfinavir (Fig. 6). We chose these two latter concentrations of nelfinavir because we saw some virus production at the lower concentration (5 μ M) and almost none at the higher (20 μ M). For proteins such as MTA (ORF57) and vIRF1 there was minimal change in protein accumulation. For RTA and SSB (ORF6) there was a noticeable decrease in protein accumulation following treatment with nelfinavir, either a 1 mM 5 min pulse or 20 μ M of drug for 72 h. Interestingly, for vIL6 there was an increase in protein detected in the presence of nelfinavir (1 mM pulse or 20 μ M, 72 h). This correlates with studies by Hu et al. [34] that demonstrate the induction of vIL6 by XBP-1. Nelfinavir induces the expression of XBP-1 [35]. The promoter of vIL6 contains many potential XBP-1 response elements (XREs) that are functional and promote binding with XBP-1. The transport of vIL6 may also be disrupted because of the effect of nelfinavir on the ER [23]. The most significant effect is on K8 and the capsid triplex protein, ORF26. The levels of these proteins detected was significantly diminished in the presence of nelfinavir (1 mM pulse or 20 μ M– 72 h).

Discussion

In this study we used a more tractable culture system for KSHV to investigate how this drug prevents virion formation. Our data show that for KSHV, inhibition occurs at a stage earlier than capsid assembly. In the nuclei of cells treated with this drug, we observed large nuclear protein aggregates which are akin to virus assembly compartments seen in herpesvirus-infected cells [33]. These electron-dense bodies represent sites where virus proteins accumulate and begin the assembly of the capsid and subsequent DNA packaging of the assembled capsids. We did not observe any mature capsids in the nucleus. In cells not treated with nelfinavir we observed capsids with internal scaffold core and DNA cores. Thus, nelfinavir prevents capsid formation in this KSHV cell culture system. This was different to what was observed in HSV-1 infected cells: nelfinavir did not affect capsid assembly and DNA packaging, nor did it affect nuclear egress of mature capsids, but rather an essential step in the cytoplasm during secondary envelopment [19].

These different observations of how nelfinavir inhibits herpesvirus production could be related to the differences in how the two viruses replicate and the optimal cell culture system that each virus is grown in. We believe, for KSHV it is the effect of the drug on the ISR [24, 36] which then manifests as a block in virus capsid assembly. This was evident in the viral protein analysis which showed that the accumulation of some KSHV proteins was inhibited by nelfinavir.

The accumulation of RTA, the potent transactivator of KSHV, was reduced by the drug but not abolished, hence, its ability to turn on lytic genes was still active as demonstrated by the expression of lytic proteins. However, for some viral proteins, their accumulation was significantly inhibited by nelfinavir. This was observed for ORF26 which is an essential capsid protein. ORF26 is part of the triplex complex of the capsid shell. There are two copies of ORF26 in complex with the other triplex protein, ORF62, and this trimer has been shown to be essential for herpesvirus capsid assembly. If either is absent, capsids fail to assemble [37–43]. Hence, it seems likely that with the significant reduction in ORF26 protein accumulation, capsid assembly was abolished. This phenotype, perturbation of the ISR, was also observed when clofotol or arsenic, was used in EBV-infected cells [30, 31]. The expression of late structural proteins of EBV was also substantially decreased.

Previous studies in the lab have shown nelfinavir can induce the lytic replication cycle of KSHV in PEL cell lines and EBV in Burkitt lymphoma (BL) (data not shown). In those experiments, addition of 20 μ M nelfinavir resulted in elevated levels of ATF4, XBP-1 and CHOP-10 indicative of ER stress. Nelfinavir was also shown to induce the expression of the major transactivators of EBV (ZTA) and KSHV (RTA) due to the effect of the drug on ER stress and the UPR, similar to that observed with tunicamycin and thapsigargin [23, 44]. We similarly observed in the 5r219 cells that incubation with nelfinavir alone induced lytic gene expression as judged by expression of RFP from the Pan promoter. The mechanism of how the drug modulates the latency program to initiate lytic is unclear. Nelfinavir promotes phosphorylation of eIF2a which leads to increased expression of ATF4 and increased expression of known downstream targets genes [20]. The ISR leads to general decrease in mRNA translation and thus global reduction in protein translation [24, 36]. These events likely disrupt virus assembly and maturation because of their impact on the synthesis and accumulation of essential viral proteins.

In conclusion, our studies confirm the initial observations of Gantt et al. [17]. that nelfinavir inhibits KSHV virion production by disrupting virus assembly and maturation. A particularly interesting aspect of this inhibition is that it requires only a short exposure to drug. The source of infectious virus in saliva has not been defined in detail but may well be lymphocytes or other cells in the oral mucosa. Thus, it might be that a “swish and spit” exposure rather than systemic administration would prevent virion production. Much work remains to be done to better understand the mechanism of action of the antiviral effect and to characterize the source of infectious KSHV virions in saliva.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13027-024-00566-7>.

Supplementary Material 1. Fig. S1. Titration of KSHV virus produced from 5r219 cells. 5r219 cells (1×10^6) were induced with doxycycline (DOX) or doxycycline plus 1 mM sodium butyrate (DOX+NB) and virus supernatants harvested, 72 h post-induction. The virus in the supernatants were concentrated by centrifugation and resuspended in PBS overnight at +4°C. The virus titer was quantitated by qPCR assays (A) and infection of Vero cell monolayers (B). Undiluted (Undil) as well as serial ten-fold dilutions were assayed using both methods.

Supplementary Material 2. Fig. S2. Statistical analysis of nelfinavir inhibition of KSHV genome copy number. One way ANOVA was used to calculate *p* values for data in Fig. 2A. For all comparisons we derived a *p* value of **** $p < 0.0001$ which is statistically significant. All statistical analyses were performed with Prism V.9.0 Software (GraphPad Software).

Supplementary Material 3. Fig. S3. Ultrastructural analyses of nelfinavir treated 5r219 cells. 5r219 cells were incubated with 20 μ M nelfinavir for 72 h following induction. Whole cells were imaged by TEM. The panels show the nuclei (nuc) and cytoplasmic (cyto) regions of these cells. Electron dense aggregates were evident in the nuclei (white arrowheads). No mature or enveloped virions were observed in the cytoplasm of the nelfinavir treated cells. Scale bar = 2000 micron.

Supplementary Material 4. Table S1. Antibodies used in the immunoblot experiments presented here.

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Author contributions

ML, BJS, JL, JP, RW, NMA and PJD contributed to study design and data collection and interpretation. ML, BJS, JL, MAR, RFA and PJD contributed to study design, data interpretation and manuscript development. All authors reviewed the manuscript. We would like to dedicate this manuscript to the memory of Michelle A. Rudek who sadly passed away too early in life and who was a wonderful scientific colleague and human being.

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Data availability

All raw data used for this manuscript is available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors agree to publication.

Competing interests

The authors declare no conflict of interest.

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