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Anti-herpesviral effects of a novel broad range anti-microbial quaternary ammonium silane, K21

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ABSTRACT

We have created a novel quaternary ammonium silane, K21 through sol-gel chemistry, using an ethoxylated version of an organosilane quaternary ammonium compound and TetraEthyl Ortho Silicate (TEOS) as precursors. Previous studies using the precursor molecule quaternary ammonium compounds (QACs) and a methacryloxy version of K21, primarily designed for use in dental healthcare, have shown inhibited growth properties against several types of gram-positive and gram-negative bacteria including *Escherichia coli*, *Streptococcus mutans, Actinomyces naeslundii and Candida albicans etc*. Here we tested the effect of K21 on HSV-1, HHV-6A and HHV-7 in *in vitro* cell culture infection models. Our results show growth inhibitory effect of K21 on HSV-1, HHV-6A and HHV-7 infection.

1. Introduction

Quaternary ammonium compounds (QACs) have both surfactant properties and broad-spectrum antimicrobial activity and hence are widely used as anti-bacterial agents (McBain et al., 2004). Although the exact mechanism behind the antimicrobial properties of QACs is not fully understood, it is well accepted that QACs can solubilize phospholipid bilayers (Salton, 1968) leading to progressive cell lysis. Previously we have created a methacryloxy version of QAC, which was proven to be effective against the growth of varieties of pathogenic organisms including *Escherichia coli*, *Streptococcus mutans, Actinomyces naeslundii, Porphyromonas gingivalis and Candida albicans* (Gong et al., 2012a, 2014, 2012b). Recently, contact-killing antimicrobial activities of this compound were elucidated in a double-blind randomized clinical trial (Liu et al., 2016). In order to design a QAC with enhanced antimicrobial properties, we developed ethoxylated version of the QAC, called K21, which was tested effective against *Porphyromonas gingivalis* and *Enterococcus faecalis* (Meghil et al., 2015). With an intention of using K21 in dental materials, we tested efficacy of K21 against some of the human herpes viruses including HSV-1, HHV-6A, HHV-6B and HHV-7 that reside in the human oral cavities and are shed in the saliva to induce infection.

Here, we report antiviral nature of a novel compound K21, which acts as a prophylactic agent and reduces the infectivity of HSV-1, HHV-6A and HHV-7 at non-toxic concentrations. Our studies indicate reduced *in vitro* viral load in presence of K21 demonstrated at both DNA and protein levels.

2. Material and methods

2.1. Drug

The compound K21 was obtained from KHG fiteBac technologies, USA.

2.2. Cell lines and viruses

Primary human foreskin fibroblasts (HFFs) (ATCC SCRC-1041)

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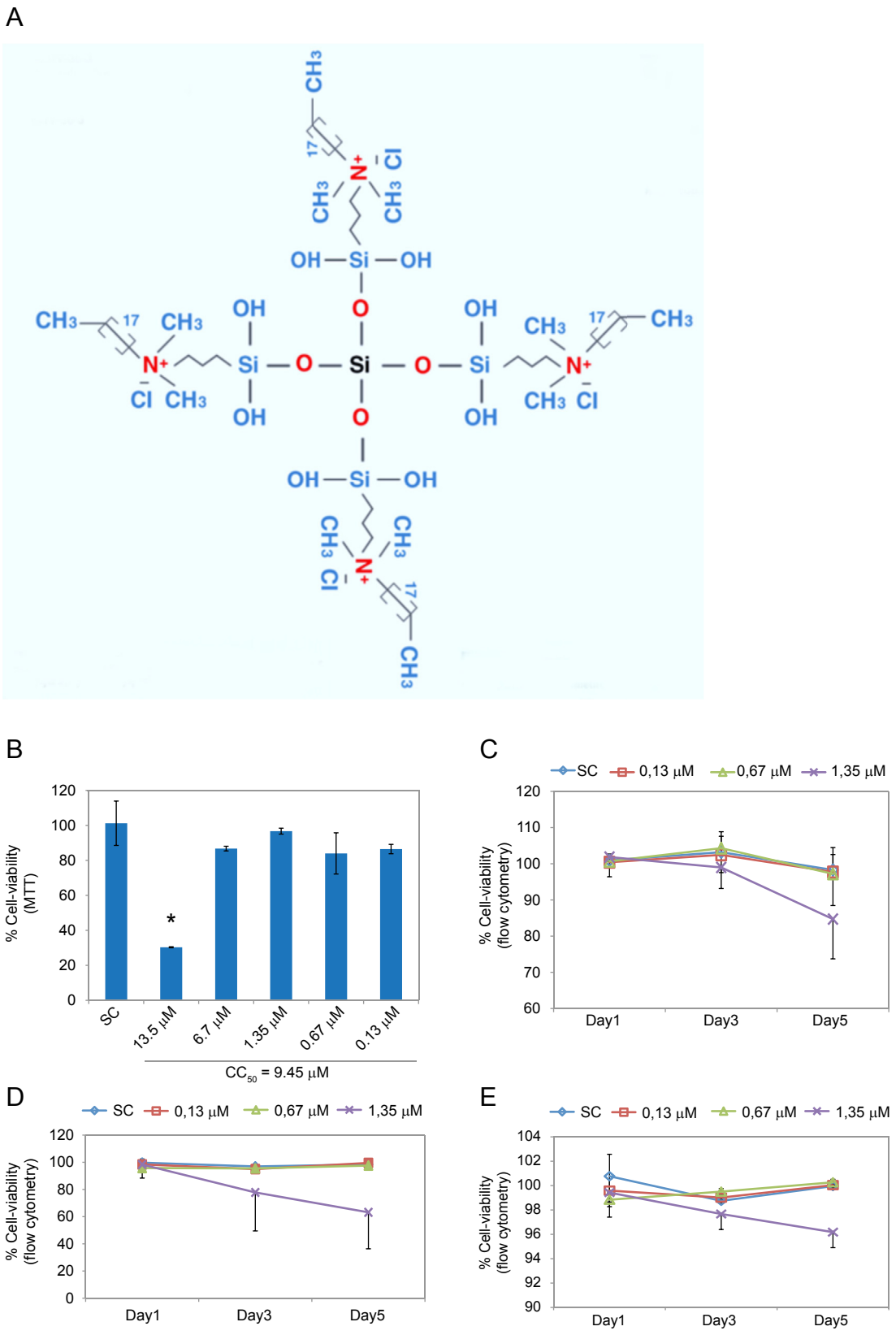

Fig. 1. Structure and cytotoxicity of K21. (**A**) Molecular structure of K21 molecule. (**B**) Cell viability (MTT) assay in primary human foreskin fibroblasts (HFFs) to determine cytotoxic dose (CC_{50}) of K21. HFFs were seeded into 96-well plates (10^3 cells/well). At 24 h in culture with varying amounts of K21, cells were analyzed for viability by MTT assay. Results show the mean % cell viability relative to solvent control treated cells (SC) from two repeated experiments performed in triplicate. $^*p < 0.05$. CC_{50} value was calculated to be 9.45 µM from the calculated slope equation. (**C**) Effect of K21 up on prolonged exposure to HFFs was studied by growing the cells in the presence or absence of different concentrations of K21 from 1 to 5 days and subsequently staining the cells with Annexin V and PI. Cells were counted and analyzed using flow cytometry. Data represents ±SEM of three independent experiments carried out on 3 different days. Similar cytotoxicity experiments were carried out in HSB-2 (**D**) and SupT-1 (**E**) cells.

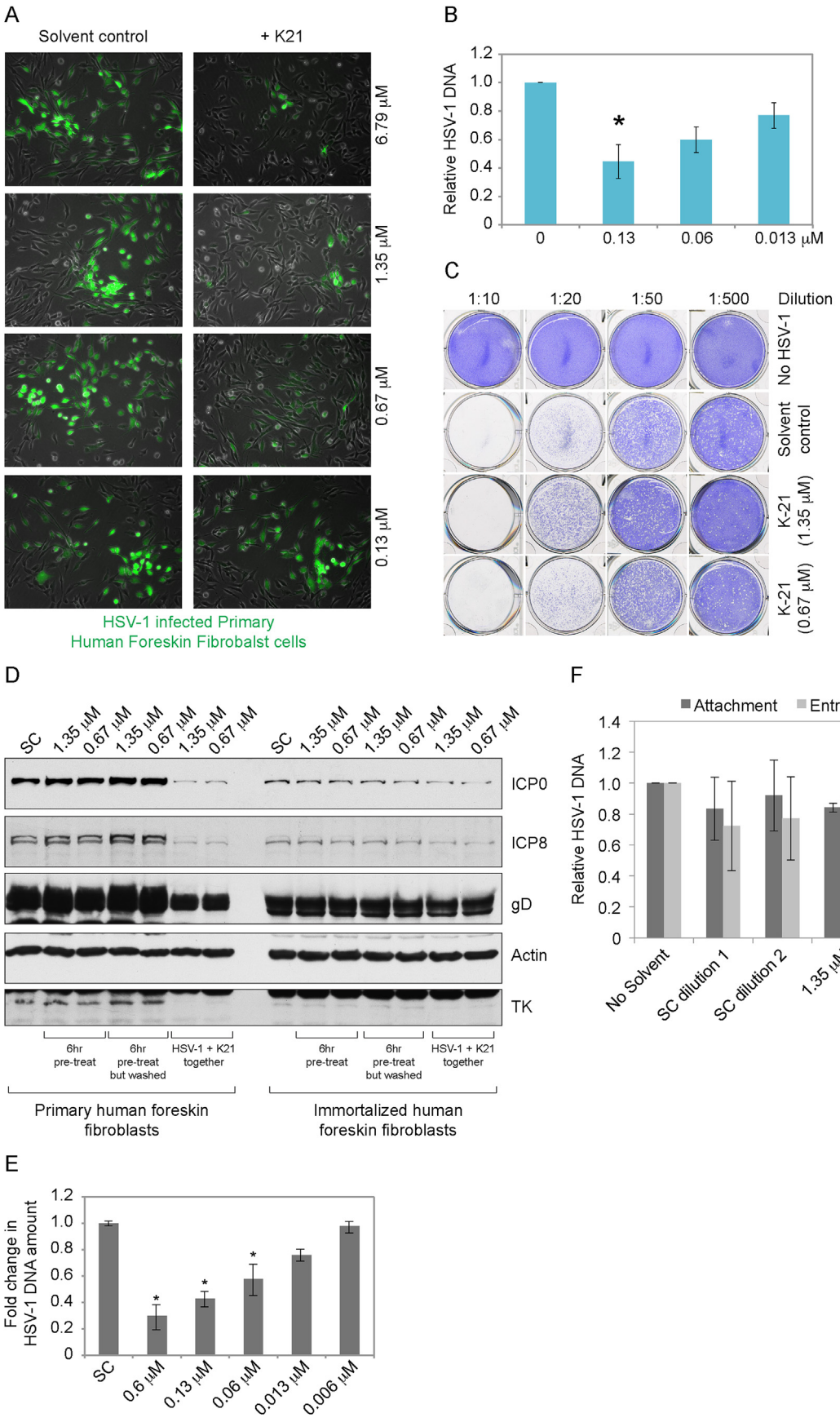

Fig. 2. K21 inhibits HSV-1 infection in primary human foreskin fibroblasts (HFFs). (**A**) HFFs were infected with HSV-1 that express eGFP in infected cells. Infected cells were treated with either solvent control or K21 at different concentrations. At 24 h post infection, cells were imaged using epifluorescence microscope. (**B**) Total genomic DNA was extracted from

were maintained in primary fibroblast basal medium (ATCC PCS-201-030) with recommended growth supplements and antibiotic (ATCC PCS-201-040) at 37 °C and 5% CO_2. HSB-2 and SupT-1 cells were maintained in RPMI 1640 with 10% FBS at 37 °C and 5% CO_2. The herpes simplex virus (HSV-1) strain (HSV1(17+)Lox-PMCMVGFP) used for infections have been described previously (Prusty et al., 2012). Human Herpes Virus 6A (HHV-6A) expressing GFP was kindly provided by Yasuko Mori, Japan. HSB-2 and SupT-1 cells were used for HHV-6A (Ablashi et al., 1988) and HHV-7 (JI) infection (Berneman et al., 1992a, 1992b) respectively. PBMCs were isolated and cultured as described earlier (Prusty et al., 2013b).

2.3. Cellular toxicity assay

MTT assay was performed to determine the CC_{50} value of the compound. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) salt was purchased from Sigma (M5655) and a stock solution of 5 mg/ml concentration was prepared. HFFs were seeded in different numbers and cells were grown for overnight. K21 was added in different concentrations and cells were allowed to grow for 24 h. Later, cells were washed once with 1× PBS and the MTT solution was added after diluting 10 times in the culture medium. The cells were incubated at 37 °C for about 4 h or until purple crystals were visible inside the cells. The plate was mixed thoroughly before measuring the optical density.

2.4. Flow cytometry

Cell viability and cytotoxicity of K21 was measured by Annexin V and Propidium Iodide (PI) staining using Dead Cell Apoptosis Kit (Life Technologies, Germany). Cells were incubated with K21 in 3 different concentrations and were stained after 1, 3 and 5 days post incubation according to manufacturer's instructions. Briefly, cells were pelleted at 500 g for 5 min and washed once with 1× PBS followed by staining with Annexin V Alexa Fluor 488 and PI in 100 μl of 1× Annexin binding buffer for 15 min at room temperature. Subsequently, additional 400 μl of 1× Annexin binding buffer was added to the reaction mix and mixed gently. Cells were immediately analyzed by flow cytometry. Cell count, around 6000–10,000, was kept constant for each cell type. For each day of treatment, cell count values were normalized against untreated cells. Data was acquired in BD Accuri C6 Cytometer (BD Biosciences, Germany) and BD Accuri C6 software. Data was analyzed using FCS Express 5 Plus (De Novo software).

2.5. DNA extraction and quantitative PCR

DNA was extracted with the DNAzol reagent (Life technologies) using the manufacturer's protocol. Quantitative PCR was performed using PerfeCTa qPCR supermix (Quanta Biosciences) on the StepOnePlus real time PCR platform (Applied Biosciences) using the manufacturer's protocol. The following primers were used for HHV-7 qPCR Forward 5′AGTGAGATGACCGCTATCCGC3′ and Reverse 5′GGGCCGCTAAGGAATCGCAT3′ and HSV-1 qPCR Forward 5′CCATACCGACCACACCGACGA3′ and Reverse 5′GGTAGTTGGTCGTTCGCGCTGAA3′. Primers for HHV-6A qPCR (U94), and control 5S primers are previously described (Prusty et al., 2013a, 2013b).

2.6. Immunoblotting

Infected cells were lysed in 2× Laemmli buffer containing 1 M Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 4% β-mercaptoethanol and 0.02% (w/v) bromophenol blue. The lysates were resolved by 12% sodium dodecyl sulfate (SDS) poly acrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were further blocked in 10% skimmed milk and subsequently probed with respective primary antibodies followed by HRP conjugated secondary antibodies. The proteins were visualized using the ECL system (Amersham). Antibodies against HHV-6 and HHV-7 were obtained from HHV-6 Foundation, USA. Antibodies against HSV-1 (sc-53330, sc-28038, sc-21719, sc-53070) were obtained from Santa Cruz Biotechnology, Inc.

2.7. Virus titer reduction assay

For viral titer reduction assay, primary human foreskin fibroblasts were infected with HSV-1 in the presence or absence of the drug and the supernatant containing virus was used subsequently to infect Vero cells in a 6 well plate.

2.8. Virus attachment and entry assays

For HSV-1, HFFs were grown in a monolayer in 6 well plates. HSV-1 particles were incubated with K21 or solvent control for 3 min and then were added to the HFF monolayer and the cells were incubated on ice for 30 min. For attachment assay, HFFs were washed extensively with 1× PBS and lysed for DNA extraction. For entry assay, cells were transferred to 37 °C for 1 h followed by washing with 1× PBS and mild trypsinization to dislodge the cells. Cells were pelleted and the pellet was washed with 1× PBS and used for DNA extraction.

For HHV-6A experiment, viral particles were incubated with K21 for 3 min and then added to HSB-2 cells in Eppendorf tubes and incubated on ice for 30 min. For attachment assay, cells were pelleted at 500 g for 5 min at 4 °C followed by washing the pellet several times with 1× PBS. The pellet was used for DNA extraction. For entry assay, cells were incubated in 37 °C for 30 min followed by pelleting the cells. Subsequently, cells were washed extensively with 1× PBS and the pellet was used for DNA extraction. DNA was extracted using DNAzol (Life technologies) and further used for quantitative PCR.

a parallel set of experiments and HSV-1 DNA amount in infected cells were quantified by qPCR. (**C**) In a similar set of experiment, infected cells were lysed after 24 h of infection and cell lysates were transferred to freshly seeded Vero cells at different dilutions. Plaque assay was carried out to check the infectious progeny. (**D**) Primary HFFs as well as transformed HFFs were infected with HSV-1 for 24 h in presence or absence of K21 (with solvent control (SC)). One set of cells was pre-treated with K21 for 6 h and HSV-1 was added to the cells after the pre-treatment. In another set of experiments, cells were treated with K21 for 6 h and then washed thoroughly with PBS and then infected with HSV-1. In third set of experiments, both HSV-1 and K21 was added to the cells at the same time. Total lysate from 24 h infected cells were used for immunoblotting. TK, Thymidine kinase. (**E**) Contact inhibition of K21 was studied against HSV-1 infection. HSV-1 viral particles were exposed to different concentrations of K21 and solvent control (SC) for 3 min at room temperature. Afterwards, drug treated viruses were allowed to infect HFFs for 24 h in the absence of further drug addition. Total DNA was extracted and used for viral DNA quantification. (**F**) In continuation to the previous contact inhibition experiment, HSV-1 entry and attachment was studied after exposure to different concentrations of K21 or solvent control. HSV-1 viral particles were exposed to 1.35 and 0.67 μM K21 for 3 min and were allowed to either attach to the surface of HFF or enter under different experimental conditions. Amount of viral DNA was quantified by qPCR.

3. Results

3.1. Structure of K21 and its cytotoxic evaluation

3-(trimethoxysilyl)propyldimethyl octadecyl ammonium chloride is a SiQAC by Dow Corning and possesses mild antiviral activity against influenza A virus (Tuladhar et al., 2012). We reacted ethoxylated SiQAC by Gelest with tetraethyl orthosilicate (TEOS), which permitted further derivatization of 3 additional molecules of 3-(triethoxysilyl)propyldimethyl octadecyl thus creating K21 (Fig. 1a). This molecule has four quaternary ammonium arms that impart four positive charges to the molecules, thereby conferring it its anti-microbial and anti-viral properties. Such QACs mainly disrupt the electrostatic interactions of the cytoplasm and bacterial cell wall thereby inhibiting bacterial survival (Melo et al., 2014). The three-silanol groups of each of the four arms of K21 are esterified with ethanol to prevent condensation polymerization. This makes K21 soluble in alcohols, but poorly soluble in water. Hence, we solubilized K21 in a mixture of 0.025% ethanol, 0.95% acetone, which is non-toxic to human cells (Chernos et al., 1972).

To test cytotoxicity of K21 and its effect on host cell viability, we first measured extent of cell viability and proliferation in presence of K21 in primary human fibroblasts (HFFs) by Microculture tetrazolium (MTT) assay. This colorimetric assay, that measures the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide into an insoluble, colored formazan product, represents metabolically active state of the cell, which is used as a read out for state of viability of the cell (Mosmann, 1983). The mean percent viability was higher than 50% in all the dilutions of K21 lower than 13.5 μM where it was around 30% (Fig. 1b). The cytotoxic dose (CC_{50}) value for K21 was calculated to be 9.45 μM in HFF cells. Similar CC_{50} values were also obtained in HSB-2 and SupT-1 cells. However, MTT assay has certain limitations and cannot compare the metabolic state of the cells in differentially growing cell types in particular when cells are grown for a prolonged time with the test compounds. Again, metabolic state of the cell may not represent the actual number of viable cells under different experimental conditions. Cell membrane integrity accurately represents the state of the cell, as most of the cells cannot survive with extensive membrane damage. As QACs disrupt the phospholipid bilayers, it also becomes essential to measure the extent of damage to cell membrane during exposure to K21. Therefore, we stained K21 treated and untreated cells with PI and Annexin V that penetrate necrotic and apoptotic cell membranes respectively depending upon differences in plasma membrane integrity and permeability (Vermes et al., 1995, 2000) and analyzed the cells using flow cytometry to count viable, apoptotic, or necrotic cells. We selected 3 concentrations of K21 (1.35 μM, 0.67 μM and 0.135 μM) below the CC_{50} value to evaluate cytotoxicity during prolonged culture for 5 days. Culture of HFFs for 5 days in the presence of 0.67 μM and 0.135 μM of K21 did not show any major effect on cell viability (Fig. 1C, S1). Up to 15% cell death (apoptotic and necrotic) was detected in presence of 1.35 μM K21 after 5 days of K21 exposure (Fig. 1C, S1). All the subsequent cell types were tested by flow cytometry till 5 days post K21 exposure. Thus, based on both the cell viability assays, we decided to use different concentrations of K21 below 1.35 μM for all subsequent experiments. In another set of experiments, 5 days of exposure of 1.35 μM K21 in HSB-2 cells caused up to 60% cell death (Fig. 3a and S2). However, we did not see any major effect of lower concentrations (0.67 μM and 0.135 μM) of K21 in these cells (Fig. 1c and S2). Also, we did not detect any major toxic effect of 1.35 μM K21 or lower concentrations on SupT-1 cell even after 5 days of exposure (Fig. 2d, S3). In addition, we tested effects of K21 on freshly isolated PBMCs and did not find any cytotoxic effects at 1.35 μM or lower concentrations (Fig. S4).

3.2. Antiviral activities of K21 against HSV-1

To evaluate inhibitory effects of K21 against HSV-1, we infected Primary HFFs with HSV-1 (strain 17+), carrying a GFP cassette under the regulation of a CMV promoter (Devadas et al., 2014), at multiplicity of infection (MOI) 1 in the presence or absence of different concentrations of the compound. 24 h post infection, infected cells were evaluated under a fluorescence microscope. We observed visible decrease in the GFP^+ HSV-1 infected cells at 0.67 μM, 1.3 μM and 6.7 μM concentrations as compared to the solvent control (Fig. 2a). Further analysis of viral DNA amount from the same cells revealed up to a 50% decrease in HSV-1 DNA in presence of 0.13 μM of K21 in comparison to solvent control ($EC_{50} = 0.13$ μM). Hence, we calculated the SI_{50} value of K21 to be 9.45 against HSV-1. Inhibition of viral infection was further verified by plaque assay, which showed a reduction in viral progeny in the presence of 0.67 μM of K21 in comparison to the solvent control (Fig. 2c). Subsequently, we analyzed expression dynamics of several different viral proteins in both primary and immortalized human foreskin fibroblasts by western blot analysis. A potential antiviral compound can act at several different stages of infection. To understand any direct effect of the drug on host cell membrane associated viral receptors that can prevent viral entry, infections were carried out under three different conditions. First, HFFs were pretreated with the compound at different concentrations for 6 h and then infected with the virus at MOI of 1 without removal of the drug. Second, HFFs were pretreated similarly but were washed thoroughly prior to virus infection. In a third approach, we infected HFFs with HSV-1 and added K21 simultaneously to the infected cells. At concentrations of 0.67 μM and 1.3 μM of K21, there was visible downregulation in the ICP0, ICP8, glycoprotein D (gD) and thymidine kinase (TK) proteins when the compound was added together to virus infected cells (Fig. 2d). Interestingly, K21 was more effective against HSV-1 in primary HFFs compared to their immortalized counterparts. These results indicate a direct inhibitory effect of K21 on HSV-1 viral particles, which might be independent of host cell receptor proteins. QACs exert their antibacterial action through solubilizing bacterial membrane and lysing the cells (Salton, 1968). Hence, we tested direct effect of K21 on viral envelope and its associated proteins through a contact inhibition experiment. Pre-treatment of HSV-1 viral particles with 0.13 μM K21 for 3 min reduced viral infection by 50% (Fig. 2e) indicating a direct inhibitory effect of K21 on viral particles possibly by damaging the viral envelope. Damage to the viral envelope could lead to reduced uptake of viral particles into host cell. To analyze if K21 exposure causes decreased viral attachment and entry, we further evaluated the ability of the viral particles to attach and enter into the host cell in presence of different concentrations of K21 (Fig. 2f). We did not observe any differences in viral attachment and entry at any of the tested K21 concentrations, suggesting decreased survival of viral particles inside the host cell as a possible mode of action of K21. Taken together, our data suggest that K21 can modulate HSV-1 viral particles within 3 min of direct exposure and in a manner that leads to subsequent decreased survival of HSV-1 within the host cell.

3.3. Antiviral activity of K21 against HHV-6A & HHV-7

We tested the effect of K21 on two beta-herpesviruses HHV-6A and HHV-7 in HSB-2 and SupT1 cells respectively. HHV-6A and HHV-7 are highly cell-associated viruses. Hence to evaluate the inhibitory effects of K21 on HHV-6A infection and its cell to cell

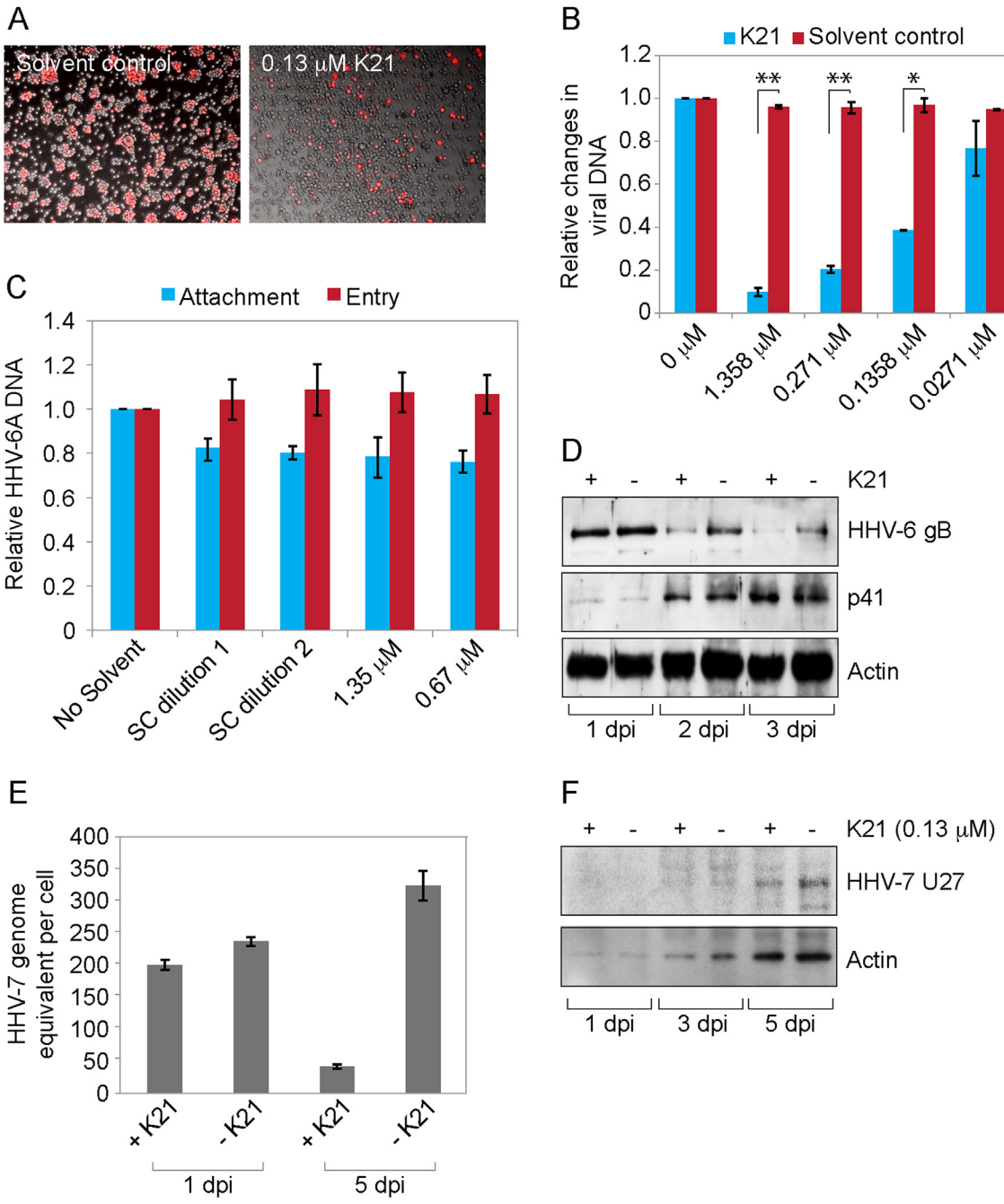

Fig. 3. K21 inhibits HHV-6 and HHV-7 infection. (**A**) Effect of K21 on HHV-6A infection was studied by fluorescence microscopy. HSB-2 cells were infected with HHV-6A that expresses mCherry protein in infected cells. Infected cells were treated with either solvent control or K21 (0.13 μM). At 72 h post infection, cells were imaged using epifluorescence microscope. (**B**) Total genomic DNA was extracted from a parallel set of experiment and HHV-6 DNA amount in infected cells were quantified by qPCR. (**C**) HHV-6A entry and attachment was studied up on exposure to different concentrations of K21 or solvent control. HHV-6A viral particles were exposed to 1.35 and 0.67 μM K21 for 3 min and were allowed to either attach to the surface of HSB-2 cells or enter under different experimental conditions. Amount of viral DNA was quantified by qPCR. SC, solvent control. (**D**) Effect of K21 (0.13 μM) on HHV-6 replication and growth was studied by immunoblotting. HHV-6A infected HSB-2 cells were mixed with uninfected HSB-2 cells at a ratio of 1:5 and were kept either in presence of K21 or solvent control for different time intervals. Total protein lysates were prepared and were analyzed for expression of HHV-6A early protein p41 (marker for viral replication) and late protein gB (marker for final viral particle formation). (**E**) Effect of K21 (0.13 μM) on HHV-7 infection was studied by qPCR. Total genomic DNA was extracted from SupT-1 cells infected with HHV-7 (JI) either in presence of K21 or solvent control. HHV-7 DNA amount in infected cells were quantified by qPCR. (**F**) Effect of K21 (0.13 μM) on HHV-7 growth was studied by immunoblotting. HHV-7 infected SupT-1 cells were mixed with uninfected SupT-1 cells at a ratio of 1:10 and were kept either in presence of K21 or solvent control for different time intervals. Total protein lysates were prepared and were analyzed for expression of HHV-7 late protein U27. Actin was used as loading control.

spread we mixed HHV-6A infected HSB-2 cells, carrying bacterial artificial chromosome (BAC) derived viral genomes encoding mCherry proteins (Tang et al., 2010), with uninfected HSB-2 cells in a ratio of 1:10, in the presence and absence of different concentrations of K21, and studied viral infection under an epifluorescence microscope after 72 h. We detected a decrease in viral growth and cell-to-cell spreading (Fig. 3a) in the presence of 0.13 μM of K21 (IC_{50} = 0.065 μM, SI_{50} = 145). Quantification of viral DNA from infected cells was consistent with visual analysis and showed up to a 63% decrease in HHV-6A DNA in presence of 0.13 μM of K21

(Fig. 3b). As with HSV-1, we did not find any effect of K21 on HHV-6A entry and attachment (Fig. 3c), corroborating our assumption on mode of action of K21 on the viral envelope and its subsequent survival inside the host cell. The effect of the compound on viral replication and growth was studied by Western blot analysis. HHV-6A infected HSB-2 cells were mixed with non-infected HSB-2 cells at a ratio of 1:10 in the presence or absence of K21. Total cell lysates were prepared after different time points to be analyzed by immunoblotting. Although we detected a decrease in the viral glycoprotein B (gB) after 2 dpi and 3 dpi, there was no visible reduction in the viral replication marker p41 (Fig. 3d) suggesting inhibition of viral infection independent of viral replication.

To test the effect of K21 on HHV-7 infection, SupT-1 cells were infected with HHV-7 (JI strain) and the infected cells were mixed with non-infected SupT-1 cells at a ratio of 1:10. Cells were grown in the presence of 0.13 μM K21 or absence of it (solvent control) for 5 days. Quantification of viral DNA revealed up to a 7-fold reduction in viral DNA after 5 days of K21 treatment (Fig. 3e). Total lysates from a similar set of infected cells were collected at different time points. Reduction in the expression of HHV-7 late protein U27 was observed in the K21 treated cells as compared to the solvent control treated cells (Fig. 3f). These results suggest a more robust inhibitory effect of K21 on HHV-6A and HHV-7 in comparison to HSV-1.

4. Discussion

We evaluated a novel compound, K21, for its potential to inhibit herpesviruses HSV-1, HHV-6A and HHV-7 infection *in vitro*. Our results suggest that K21 is a potent anti-herpesvirus compound and is strongly effective against HHV-6A and HHV-7. However, it is weakly effective against HSV-1. This might be due to differential nature of growth between alpha- and beta-herpesviruses. HSV-1 is a fast growing virus (Jenkins and Turner, 1996) and usually starts its replication within 15 h of infection. Whereas HHV-6A and HHV-7 are cell-associated (Klussmann et al., 1997), slow growing viruses that need at least 72 h to finish their life cycles. As K21 was most effective when directly exposed to the virus, we hypothesized that K21 might alter the infectious properties of the viral particles without significantly altering its attachment and entry. This type of inhibitory function is better reflected in the progeny of slow-growing pathogens than in fast growing ones. Immunoblot results with HSV-1 corroborated this hypothesis where we observed marked decrease in expression of early HSV-1 proteins (ICP0, ICP8, thymidine kinase etc.) whereas late proteins like gD was only marginally decreased. At the same time, different levels of regulation in primary and immortalized cells strongly suggest that key role(s) are played by host cell factors during viral infection. This assumption was further supported by results from HHV-6A testing. In the case of HHV-6A, the glycoprotein B (gB), which is expressed much later in the life cycle of the virus, was downregulated; however, the viral DNA replication-associated protein p41 remained more or less unmodulated (Fig. 3d). These results suggest that K21 may have 2 different modes of action. When directly exposed to the virus, it inhibits viral survival without altering viral attachment and entry possibly by altering the surface structure of the viral particles. K21 might also change cell physiology making it non-conducive for slow growing herpes viruses like HHV-6 and HHV-7. Hence, the ability of K21, to regulate infectious progeny formation and inhibit its spread to new cells may make it a highly effective antiviral compound.

Recent studies revealing potentiation by co-infection of pathogens (Prusty et al., 2013b) argue for the development of drugs that are effective against both bacteria and viruses. The effective broad range antimicrobial properties of K21 make it an ideal candidate for a multi-purpose anti-microbial. Further in-depth studies will be essential in order to understand exact mode of action of K21.

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Conflict of interests

None declared.

Author contributions

BKP, designed the study; NG and BKP, performed the experiments, wrote the manuscript; DA and GRK, analyzed the results and contributed to manuscript writting; KK and ADJ, developed and manufactured K21.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.05.004.

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