Differential Dependence on Host Cell Glycosaminoglycans for Infection of Epithelial Cells by High-Risk HPV Types

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Abstract

Human papillomavirus (HPV) infection is the leading cause of cervical cancer world-wide. Here, we show that native HPV particles produced in a differentiated epithelium have developed different strategies to infect the host. Using biochemical inhibition assays and glycosaminoglycan (GAG)-negative cells, we show that of the four most common cancer-causing HPV types, HPV18, HPV31, and HPV45 are largely dependent on GAGs to initiate infection. In contrast, HPV16 can bind and enter through a GAG-independent mechanism. Infections of primary human keratinocytes, natural host cells for HPV infections, support our conclusions. Further, this renders the different virus types differentially susceptible to carrageenan, a microbicide targeting virus entry. Our data demonstrates that ordered maturation of papillomavirus particles in a differentiating epithelium may alter the virus entry mechanism. This study should facilitate a better understanding of the attachment and infection by the main oncogenic HPV types, and development of inhibitors of HPV infection.

Introduction

Human papillomavirus (HPV) is the causative agent of cervical cancer and other anogenital cancers and oropharyngeal cancers [1,2]. The major capsid protein, L1, mediates primary attachment of viral particles to cells [3] and the extracellular matrix [4]. The minor capsid protein, L2, is essential for infection, having multiple roles in genome encapsidation, capsid stabilization, receptor-binding, endosomal escape, and escorting the viral genome to the nucleus [5–11]. Current papillomavirus vaccines target the major capsid protein L1 of the most common cancer-causing types, HPV16 and HPV18, which together account for 70% of cervical cancer cases, protecting against virus infection and development of neoplasias [12]. However, since current vaccines are type-specific, they do not offer protection against all cancer-causing HPV types. In addition, they are cost-prohibitive to most women around the world [13,14]. Thus, there is a need for the development of less expensive alternatives, such as universal microbicides in addition to the current vaccines.

HPV infects basal keratinocytes and the production of new particles is closely tied to the cellular differentiation pattern of epithelial cells. The complete HPV life cycle can be recapitulated in vitro in organotypic raft culture [15–18]. Thus far, infection and entry studies have mostly been done using pseudovirus (PsV), which are efficiently produced by over-expression and self-assembly of the capsid proteins in monolayers [19]. Using this system, most papillomaviruses have been observed to infect cells by first attaching to a form of glycosaminoglycan (GAG), heparin sulfate (HS), via L1 to the cell surface or extracellular matrix (ECM) [3,20,21]. Initial binding to laminin-332 (laminin 5) on the ECM has also been demonstrated [22,23]. HS attachment induces a conformational change allowing for the L2 N-terminus to be cleaved by a proprotein convertase (PC), furin and/or PC5/6 [24,25]. Following HS attachment and cleavage of L2 by furin and/or PC5/6, the virus is thought to be transferred to a secondary entry receptor [26,27]. Alpha6-integrin, growth factor receptors, and annexin A2 have been suggested as potential candidate receptors, however their role in infection is still unclear [11,28,29]. The conformational changes required for infectious entry of virus particles have been shown to be mediated by cyclophilin B for some, but not all, HPV PsV types tested [30]. Primary attachment to HS has been suggested to be a universal entry step for all papillomaviruses. However, noticeably, tissue-derived HPV31 native virus (NV) infection of human keratinocytes was shown not to require HS [31]. Furthermore, members of the closely related polyomavirus family have been shown to utilize different receptors [32–34]. Thus, a general hypothesis for HPV attachment and entry may not encompass all HPV types. In addition, while PsV has proven to be very important in the understanding of the process of HPV infection, it is not well understood what structural differences compared to authentic virions exist and how these structural alterations might affect the biology of the virus. In a study of the cross-neutralizing ability of neutralizing antibodies against L2 N-terminal epitopes, it was shown that there were differences in the neutralizing pattern of PsV as compared to NV particles, suggesting there may be overall structural differences between PsV and NV particles [35].
Consequently, structural differences between particles may impose functional differences on virus binding and infection.

To further the understanding of HPV entry, we set out to investigate whether diverse HPV types produced under physiologically relevant conditions of differentiating host tissue are dependent on GAG-mediated binding for infection. Here, we examined the requirement of the high-risk HPV types HPV16, HPV18, HPV31, and HPV45 for GAG binding during infection. Our results suggest that different HPV types show specific preferences for the type of GAG and the sulfation status of the GAGs for attachment and infection as seen with HPV18, HPV31, and HPV45. In contrast, HPV16 may infect their host cells independent of GAGs.

**Results**

**Infection and Neutralization for Analyses of High-risk HPVs**

The production and neutralization of the most common high-risk papillomaviruses HPV16, HPV18, and HPV45 in foreskin-derived organotypic raft culture has been previously shown [15–17]. Native HPV31 was produced from a cervical intraepithelial neoplasia type 1 biopsy-derived cell line CIN-612 9E [18]. To verify the specific particle-mediated infectious entry as well as the

![Figure 1. Particle-mediated infection.](image)

HPV16, HPV18, HPV31, and HPV45 were incubated with L1 type-specific antibodies H16.V5, H18.J4, H31.A6, and H45.N5, respectively, for 1 hour at 37°C prior to infection of HaCaT cells. Infections were analyzed by RT-qPCR measuring the relative amount of E1\^E4 transcript two days post-infection. doi:10.1371/journal.pone.0068379.g001

![Figure 2. Inhibitory effects of heparin against various HPV types.](image)

A) HPV16, B) HPV18, C) HPV31, and D) HPV45 were incubated with heparin at increasing concentrations (0 μg/ml, 1 μg/ml, 10 μg/ml, and 100 μg/ml) for 30 min’s at 37°C prior to infection and during infection of HaCaT cells. All infections were done at an MOI of 10. For HPV18 additional infections at a MOI of 100 and 1000 were performed. Infections were analyzed by RT-qPCR measuring the relative amount of E1\^E4 transcript two days post-infection. The data is plotted as relative infection at the different concentrations with infection at 0 μg/ml of heparin set equal to one. doi:10.1371/journal.pone.0068379.g002
Figure 3. Effect on absorption in the presence of heparin. A) HPV16, B) HPV18, C) HPV31, and D) HPV45 were mixed with heparin at increasing concentrations (0 μg/ml, 10 μg/ml, and 100 μg/ml) and added to HaCaT cells at an MOI of 25. The virus was allowed to attach to HaCaT cells for 2 hours at 4°C. E) HPV16, F) HPV18, G) HPV31, and H) HPV45 in the presence or absence of heparin (0 μg/ml and 100 μg/ml). After attachment at 4°C, the cells were shifted to 37°C for an additional 2 to 4 hours. Analysis of the number of particles was done by SYBG q-PCR with attachment in the absence of heparin set to 100%.

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specificity of the RT-qPCR assay for the analysis of HPV infection, we neutralized each virus with a type-specific monoclonal antibody targeting a major epitope in the L1 major capsid protein (figure 1).

Inhibitory Effects of the HS Mimetic Heparin

Heparin, a highly sulfated form of HS produced from mast cells, is thought to bind to HPV particles and prevent binding to the cells [3]. HPV16 infection of HaCaT cells was not blocked by heparin (figure 2A). In contrast, infection by HPV18 was efficiently blocked by heparin in a dose-dependent manner (figure 2B). HPV31, which is evolutionary related to and found in the same species (9) as HPV16, showed the same resistance to heparin as HPV16 (figure 2C). This confirms data from a previous study on infection by HPV31, where the presence of heparin had no effect on infection of HaCaT cells [31]. Infection by HPV45, which is related to and found in the same species (97) as HPV18, was also resistant to inhibition by heparin (figure 2D). Thus, functional studies rather than sequence conservation and relative relatedness between different HPV types are necessary for the prediction of responsiveness to a given blocking agent. At a multiplicity of infection (MOI) of 10 particles, HPV18 infection was unaffected by the presence of 1 μg/ml heparin, with 90% inhibition observed at 10 μg/ml heparin (figure 2B). When

Figure 4. Inhibitory Effects of Chondroitin A/C against various HPV types. HPV16, HPV18, HPV31, and HPV45 were incubated with or without chondroitin A/C (0 μg/ml, and 100 μg/ml) for 30 min's at 37 ºC prior to infection and during infection of HaCaT cells. All infections were done at an MOI of 10. Infections were analyzed by RT-qPCR measuring the relative amount of E1–E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 μg/ml of chondroitin A/C set equal to one. doi:10.1371/journal.pone.0068379.g004

Figure 5. Infection of sodium chlorate-treated HaCaT cells. HaCaT cells were treated with increasing concentrations of sodium chlorate (0 mM, 10 mM and 25 mM) 2 days prior to infection and during infection by A) HPV16, B) HPV18, C)HPV31, and D) HPV45 at an MOI of 10. Infections were analyzed by RT-qPCR measuring the relative amount of E1–E4 transcript two days post-infection. The data is plotted as relative infection at the different concentration with infection at 0 mM sodium chlorate set equal to one. doi:10.1371/journal.pone.0068379.g005
infections were done with an MOI of 1,000 particles the inhibitory ability of heparin decreased, with 50% inhibition observed at 10 μg/ml heparin. This is in contrast to a PsV inhibition assay at a similar inoculum where a 10-fold lower concentration is needed for 50% inhibition of infection [13], suggesting that HPV18 NV particles are less sensitive to inhibition by heparin than PsV. The observed capsid dose-dependence of inhibition further supports the direct binding of heparin to the HPV18 NV particles.

Heparin and Virus Attachment

The effect of heparin on total virus adsorption to HaCaT cells and the extracellular matrix was analyzed by measuring attachment at 4°C. No significant block was observed for attachment by HPV16 in the presence of heparin (figure 3A), suggesting HPV16 may attach to cells using a non-HS receptor. HPV18 attachment was completely blocked by heparin (figure 3B), supporting that the first step in the infectious pathway by HPV18 is to bind to HS.

Despite resistance to inhibition of HPV31 and HPV45 infection in the presence of heparin (figure 3C, D), a nearly complete block of attachment was observed for both virus types in the presence of heparin (figure 3C, D). After binding to cells, HPV virus particles have been shown to be surface bound for several hours [25,36–38] and conformational changes of surface-bound papillomavirus particles are well documented [8,25,39]. It has been suggested that conformational changes are responsible for the slow entry kinetics of the virus particles [36]. Thus, heparin, despite not being able to block infection may still bind to the particles and prevent efficient conformational changes thus preventing initial attachment to cells. We tested this by chasing attachment for 2 hours at 4°C with increased times at 37°C. HPV16 and HPV18 attachment in the presence of heparin did not change over time (figure 3E, F). For HPV31, switching the cells to 37°C allowed for an increased number of particles to adhere to the cells in the presence of heparin over time (figure 3G), suggesting that coating of the particles by heparin at 4°C may present a block to conformational changes that need to take place. HPV45 attachment in the presence of heparin remained blocked, even up to 8 hrs at 37°C (figure 3H and data not shown). Initial HPV45 receptor-binding may be of very low affinity and/or HPV45 transfer to a higher affinity entry receptor may require more time.

Effects of Chondroitin Sulfate

Two main categories of GAGs, glucosaminoglycans, including HS, and galactosaminoglycans, including chondroitin sulfate (CS), exist. To examine the possibility of HPV interactions with different GAG types, we investigated the role of CS for infection by adding chondroitin A/C exogenously during infection (figure 4). Only HPV18 was sensitive to the addition of CS, whereas HPV16, HPV31, and HPV45 were resistant. The sensitivity of HPV18 to both heparin and CS suggests that the determinant for attachment is a combination of the type of GAG and/or GAG sulfate modifications.

Infection in Absence of Sulfate Modifications

The sulfation patterns of GAGs have been shown to play a role in the ability to inhibit HPV PsV infection [40]. To examine infection of NV particles in cells deficient in cell surface GAG sulfate modifications, we treated HaCaT cells with increasing concentrations of sodium chlorate. HPV16 efficiently infected sodium chlorate-treated cells (figure 5A), suggesting HPV16 does not depend on GAG sulfate modifications for infection. In contrast, HPV18 was efficiently blocked (figure 5B). HPV31 and HPV45 were also inhibited by the absence of GAG sulfation in a dose-dependent manner (figure 5C, D). Given that HPV31 and

Figure 6. Attachment and infection in CHO parental cells and GAG-negative pgsA-745 cells. CHO Par and pgsA-745 cells were plated 2 days prior to infection by A) HPV16 and HPV18 at an MOI of 10 or attachment by B) HPV16 and HPV18, and D) HPV31 and HPV45. Infections were analyzed by RT-qPCR measuring the relative amount of E1*E4 transcript two days post-infection normalizing to infection by the CHO Par cells. Analysis of the number of particles was done by SYBR q-PCR after incubating for 2 hours at 4°C, normalizing attachment to the CHO Par cells.

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HPV45 infections are resistant to both heparin and chondroitin during infection, this supports a requirement for GAG sulfation by these virus types during infection that may display a different GAG specificity.

Attachment and Infection in the Absence of GAGs

To further analyze the specificity of NV HPV types for GAG binding, we took advantage of CHO cells either expressing or deficient for the expression of GAGs. We found that GAG-deficient pgsA-745 cells were infected by HPV16 at similar levels compared to CHO parental cells (figure 6A). Further, HPV16 binding to pgsA-745 cells was equivalent to that of CHO parental cells (figure 6B), supporting the ability of HPV16 to infect cells in a GAG-independent manner. In contrast, HPV18 infection was compromised in pgsA-745 cells (figure 6A). A decrease in HPV18 binding added to the evidence for a requirement of GAGs for primary attachment and infection (figure 6B).

Infection of CHO cells by HPV31 and HPV45 was too low to reproducibly detect in RT-qPCR infectivity assays. However, binding by HPV31 and HPV45, was consistently lower in the pgsA-745 cells compared to parental cells (figure 6C), suggesting that cell surface binding by HPV31 and HPV45 is dependent on cellular GAGs.

Differential Susceptibilities to Carrageenan in HaCaT cells

An in vitro screen of compounds that can effectively block infection by high-risk HPV PsV identified carrageenan, a highly sulfated polysaccharide derived from red algae, as a powerful inhibitor [13]. In contrast, for NV at an MOI of 10, carrageenan failed to inhibit infection by HPV16 at concentrations up to 100 μg/ml (figure 7A). For HPV18, when an MOI of 10 and 100 were used, significant levels of inhibition were observed at 1 μg/ml (figure 7B). When the titer was increased to an MOI of 1000, 50% inhibition increased to 10 μg/ml (figure 7B). In contrast, a 1000-fold lower IC50 in the ng/ml range was observed for various HPV PsV types [13], suggesting that the NV is more resistant to inhibition than PsV. Infection by HPV31 was also sensitive to inhibition by carrageenan (figure 7C), in contrast to the observed resistance to heparin and CS. This suggests a very selective requirement for a specific type of sulfated GAG for HPV31 infection. HPV45 did not show a dose-dependent decrease in infection in the presence of carrageenan (figure 7D), suggesting yet another preference for a different type of sulfated GAG.

We then analyzed virion attachment in the presence of increasing concentrations of carrageenan. HPV16 was not affected at the level of attachment (figure 8A). HPV18 attachment was completely blocked by carrageenan (figure 8B). HPV31 attachment was not as sensitive (figure 8C) as to account for the level of...
inhibition observed during infection (figure 7C). This suggests that carrageenan might have an additional post-attachment inhibitory effect on HPV31. HPV45 attachment in the presence of carrageenan was also not significantly reduced (figure 8D).

Antiviral Activities by Carrageenan in Primary Cells

We used primary cells in culture to verify key data generated using HaCaT keratinocytes. As PsV infection of primary cells in culture has been shown to be very inefficient [27], we first wanted to establish the ability to infect primary keratinocytes with NV. Infections by HPV16, HPV31, and HPV45 were comparable to, or better than, infection of HaCaT keratinocytes (figure 9A, C, D). In contrast, HPV18 infection is reproducibly weaker in primary keratinocytes (figure 9B).

We next investigated the efficiency of inhibition by carrageenan against infection by the different high-risk HPV types in primary keratinocytes. The lack of a dose-dependent inhibition of HPV16 infection of primary cells supports that HPV16 NV is insensitive to inhibition by carrageenan (figure 9E). An MOI of 100, instead of 10, was used for HPV18 infections, as this resulted in infection levels similar to that observed in HaCaT cells (data not shown). Infection by HPV18 was blocked by carrageenan (figure 9F), supporting the need for GAGs as primary attachment receptors for HPV18. HPV31 was not significantly blocked in the presence of carrageenan at 10 µg/ml, but was blocked only at the higher concentration of 100 µg/ml (figure 9G). Similarly to that seen in HaCaT cells, HPV45 infection of primary keratinocytes was not significantly blocked by carrageenan (figure 9H).

Discussion

As summarized in Table I, a complicated pattern of dependency on cell surface GAGs and/or GAG modifications, emerges from the comparison of infection by the four main cancer-causing HPV types, HPV16, HPV18, HPV31, and HPV45. We show here that raft-derived HPV18 requires HS binding for infection, similar to previously reported data using recombinant HPV particles. Noticeably, HPV18 infection is also sensitive to CS, whereas infections by HPV16 and HPV10 PsV, are not substantially inhibited by the presence of CS [23,41].

HPV31 and HPV45 display a different pattern of inhibition under our experimental conditions. The reduction in infectivity after sodium chlorate treatment of the cells, and the inability to attach to GAG-deficient cells, suggests a preference for a different type of sulfated GAG. Previous research has shown that the type and level of sulfation may play an important role for the interaction of PsV with polysaccharides [23,40]. Interestingly, despite not blocking infection, heparin effectively blocked attachment by HPV31 and HPV45. It is possible that despite not being able to block infection, heparin still attaches to the particles. HPV5 PsV has been shown to bind carrageenan beads even though no block was observed at the level of infection [13]. In a recent study attachment to the cell surface but not the ECM was blocked by heparin pre-incubated HPV16 PsV [23]. Further, it was demonstrated that bound heparin conferred a conformational change to the particles [23]. Thus, it is possible that residual levels of heparin-coated virus binding to the ECM are able to transfer directly to a secondary entry receptor on the cell surface.
Unexpectedly, native HPV16 does not follow the same mode of dependence on GAGs for attachment. Binding to GAGs is not required for attachment or infection by HPV16, as shown by incubation with soluble heparin, the ability of HPV16 to infect GAG-deficient cells, and by preventing sulfate modifications of GAGs by sodium chloride. In addition, PsV do not efficiently infect primary keratinocytes in culture [27], which was hypothesized to be due to altered structure of HS modifications during in vitro culturing of keratinocytes [42]. Interestingly, infection of primary keratinocytes by HPV18 NV is also inefficient, whereas primary keratinocytes are readily infected by HPV16, HPV31, and HPV45 NV. Together, these results suggest that different HPV types have distinct requirements for entry into their natural host cells. Further research is required to determine whether the GAG-dependent and independent HPV types post-attachment share the same receptor for internalization into their natural host cells. Interestingly, a recent publication demonstrated that HPV particles may be released from the cell surface in complex with HS, followed by re-attachment to the cell surface independent of cell surface GAGs, to initiate infection [29]. Additional studies are needed to determine the composition of native particles as they are released from the cells and whether that plays an important role in HPV NV infection.

The observed differences in GAG-dependency between different HPV types, as well as between PsV and NV particles, are unlikely to be explained simply due to the use of different cell lines and different requirements for in vivo and in vitro infections. Using the same HaCaT keratinocyte cell line, as used in previous PsV infection studies [13,41], we found that the sensitivity to polysaccharide compounds is greatly reduced or completely lacking for native HPV particles. It has been shown that the need for initial attachment to HS is tightly correlated with the need for cleavage by cellular furin during infection, where furin pre-cleaved PsV particles can bypass binding to HS for infection [27]. Bypassing HS-binding could allow for direct binding to a functional entry receptor. It is possible that HPV16, which does not need HS binding during infection, is pre-cleaved during virion morphogenesis. It is also conceivable that different HPV species utilize different molecules for initial attachment to the basement membrane and the host cell. Laminin-332 has been shown to be a preferential receptor for HPV11 and HPV16 PsV for initial attachment [22,23]. Studies using PsV show that different types of HPV may utilize different entry pathways [20], suggesting that binding to different receptor molecules on the cell surface may direct these viruses into different endocytic pathways. Moreover, although not required, it is still possible that HPV16 interacts with GAGs through conserved motifs on the virus surface [43]. In vivo, this may serve to concentrate the virus to the site of infection.

Importantly, neutralization by carrageenan was shown to be effective against HPV18 and HPV31 infections. However, HPV16 and HPV45 could not be blocked by carrageenan, suggesting carrageenan may not protect against all HPV types. Inhibition of dengue virus infection by carrageenan also showed differential efficacy depending on the serotype tested [44]. Different carrageenan compounds need to be considered, as a preparation of more than one type of carrageenan might be able to protect against more HPV types [13,44]. The efficient production of synthetic particles is invaluable for rapid analysis of many aspects of the papillomavirus life cycle. However, a complete comparison of the structure of PsV and NV has not been published and experimental results using PsV particles as a surrogate need to be verified with NV [45–47]. As described here, genital HPV's may use different strategies to attach to and infect their host. As a result, they may naturally exhibit differential susceptibility to various agents, an important factor to consider when developing new agents to block HPV transmission.

### Methods

#### Ethics Statement

The use of human cervical and foreskin keratinocyte tissues to develop cell lines as well as for infectivity assays for these studies was approved by the Institutional review Board at the Pennsylvania State University College of Medicine and by the Institutional review Board at Pinnacle Health Hospitals. Discarded, de-identified tissues were exempt from needing informed patient consent. Informed consent was waived by both Institutional Review Boards.

#### Cell Culture and Virus Production

HaCaT cells were maintained in DMEM supplemented with 10% FBS, 0.025 mg/ml Gentamicin, and 0.011 mg/ml sodium pyruvate. CHO cells were maintained in minimal essential α-medium supplemented with 10% FBS and antibiotics. Primary human keratinocytes from newborn foreskin circumcision and cervical biopsies were isolated as previously described [16]. Keratinocytes were maintained in 154 medium supplemented with Human Keratinocyte Growth Supplement Kit (Cascade

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### Table 1. GAG Dependence during Infection.

|       | Neutralized | Heparin | Chondroitin | Sod. Chl. | CHO ↔ GAGs | Carrageenan (HaCaT) | Carrageenan (HFkS) |
|-------|-------------|---------|-------------|-----------|------------|----------------------|-------------------|
| HPV16 | Yes         | No      | No          | No        | No         | Yes                  | Yes               |
| HPV18 | Yes         | Yes     | Yes         | Yes       | Yes        | Yes                  | Yes               |
| HPV31 | Yes         | No      | No          | Yes       | Nd         | Yes                  | Yes               |
| HPV45 | Yes         | No      | No          | Yes       | Nd         | No                   | No                |

Summary of the sensitivity of the different HPV types to neutralization by monoclonal antibodies, various polysaccharide compounds, sodium chloride treatment, and GAG-positive or negative cells. Nd = not done.

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Biologies, Inc., Portland, OR). Immortalized keratinocytes stably maintaining HPV episomes were cultured in E-medium with J2 3T3 feeder cells [18] and grown in raft culture to produce virus as previously described [18]. Mature virus particles were harvested from tissues after 20 days [17].

Rafts were harvested and virus was isolated by homogenization in phosphate buffer (0.005 M Na-phosphate, pH 8.2, 2 mM MgCl₂) as previously described [17]. All virus preps for titering and infectivity assays were treated with benzonase (375 U) at 37°C for 1 hour to remove any un-encapsidated viral genomes. Samples were adjusted to 1 M NaCl and centrifuged at 4°C for 10 minutes at 10,500 rpm to remove cellular debris.

Virus Titters

To release the viral genomes, 10 µl of a virus prep was re-suspended in a 200 µl HIRT DNA extraction buffer (400 mM NaCl/10 mM Tris-HCl, pH 7.4/10 mM EDTA, pH 8.0), with 2 µl 20 mg/ml Proteinase K, and 10 µl 10% SDS for 2 hours at 37°C. The DNA was purified by phenol-chloroform extraction followed by ethanol precipitation and re-suspended in 20 µl TE [17]. Titters were determined using a qPCR-based DNA encapsidation assay utilizing a Qiagen Quantitect SYBR Green PCR Kit. Amplification of the viral genome target was performed using previously described E2 primers against a standard curve of 10-fold serial dilutions from 10⁻⁶ to 10⁰ copies per µl [17,35].

Infections, Neutralizations, and Inhibition Assays

The poly saccharides heparin (H4784, high molecular weight) and chondroitin sulfate A/C (C4384), sodium chlorate (403016) and t-carrageenan (C4014) were purchased from Sigma-Aldrich. The anti-L1 antibodies were a gift from Neil D. Christensen. H16V5, H18J4, H31A6, and H45N5 were used to neutralize HPV16, HPV18, HPV31, and HPV45, respectively. HaCaT cells were seeded in 24-well plates, 50,000 cells per well 2 days prior to infection. CHO cells were seeded 30,000 per well and healthy low-passage primary cells were seeded 70,000 per well. Compounds were mixed with virus and media in a total volume of 500 µl prior to addition to cells. An MOI of 10 particles per cell was used unless otherwise noted. For sodium chlorate, cells were grown in the presence of the compound 2 days prior to and during the infection. Virus was incubated with the cells for 48 hrs at 37°C and mRNA was harvested using a Qiagen RNAeasy Kit. Infection was analyzed using a previously described RT-quantitative PCR-based infectivity assaying for E1/E4 transcript levels [17,35]. Results are representative of means and standard deviations for at least three independent infections from at least two different batches of virus preps for each virus type. Students t-test was performed with statistical significance calculated (p<0.05).

Attachment Assays

Cells were seeded the same way as in the infectivity assays. The virus was incubated with the cells at an MOI of 25 for 2 hours at 4°C with or without compound, and then shifted after the indicated number of hours to 37°C. Cells were washed 3x with PBS before lysing on the plate for 2 hours at 37°C using a HIRT lysis buffer, followed by phenol-chloroform extraction and ethanol precipitation. The number of particles attached to the cells was determined as the number of viral genomes by amplifying a target sequence in the E2 gene, as in the DNA encapsidation assay. Results are representative of means and standard deviations for at least three independent attachments assays from at least 2 different virus batches. Students t-test was performed with statistical significance calculated (p<0.05).

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Author Contributions

Conceived and designed the experiments: LC CM. Performed the experiments: LC. Analyzed the data: LC CM. Contributed reagents/materials/analysis tools: LC CM. Wrote the paper: LC CM.

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