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Prophylactic antiviral activity of sulfated glycomimetic oligomers and polymers.

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Antiviral research, virus, glycomimetics, GAG mimetics, precision glycooligomers, glycopolymers

ABSTRACT: In this work, we investigate the potential of highly sulfated synthetic glycomimetics to act as inhibitors of viral binding/infection. Our results indicate that both, long chain glycopolymers and short chain glycooligomers are capable of preventing viral infection. Notably, glycopolymers efficiently inhibit human papillomavirus (HPV16) infection *in vitro* and maintain their antiviral activity *in vivo*, while the glycooligomers exert their inhibitory function post attachment of viruses to cells. Moreover, when we tested the potential for broader activity against several other human pathogenic viruses, we observed broad-spectrum antiviral activity of these compounds beyond our initial assumptions. While the compounds tested displayed a range of antiviral efficacies, viruses with rather diverse glycan specificities such as Herpes Simplex Virus (HSV), Influenza A Virus (IAV) and Merkel Cell Polyomavirus (MCPyV) could be targeted. This opens up new opportunities to develop broadly active glycomimetic inhibitors of viral entry and infection.

INTRODUCTION

Viral diseases are a major health burden associated with significant socioeconomic loss.[1] Often, prevention and treatment of viral infections remains a major challenge. The most important and effective antiviral strategy is vaccination. However, vaccines are only available for a select number of viral infections. In addition, only a limited number of antiviral drugs exist, and those that do, typically target essential viral functions/proteins. As a consequence, there is a high risk of quick emergence of escape mutations in the virus, rendering viruses resistant to drug treatments.[2] Hence, the development of additional interventions with a broader range of targeted viruses is of highest interest.

A promising approach to fight viral infections is by targeting virus entry into host cells, i.e. the delivery of the viral genome to the intracellular replication site during initial infection. This could avoid primary infection and additionally limit spread within the organism and transmission between organisms. At best, a conserved and common mechanism for several viruses would be targeted. One such mechanism is the initial binding to cellular glycans for primary attachment. Many viruses have evolved to engage glycans that are presented on cell surface proteins or lipids to adhere to cells.[3,4] The glycans recognized by viruses are diverse, most often containing glycans such as sialic acids (Neu5Ac) and glycosaminoglycans (GAGs) like heparan sulfates (HS), which are composed of *N*-acetyl glucosamine (GlcNAc) and glucuronic acid (GlcA).[5]

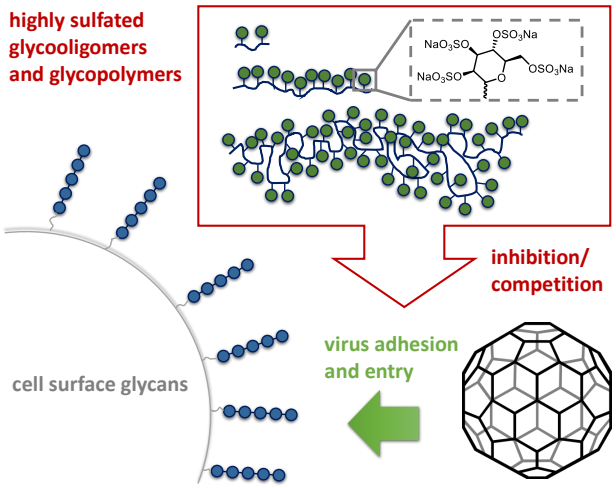
Inhibition of viral infections by negatively charged natural polysaccharides that compete with binding to cellular glycans such as HS is well documented.[6–8] Perhaps the most prominent example is carrageenan, a natural sulfated polysaccharide from red algae which interferes with infection of many viruses, for example human papillomaviruses (HPVs), herpes simplex virus type 1 (HSV-1) and influenza A virus (IAV).[7–9] The antiviral potential of carra-

geenan has also been realized in a clinical trial, where it reduced contraction of HPV16.[10] However, natural polysaccharides may present a number of challenges for safe clinical application. For one, these molecules are inherently heterogeneous. Preparations may consist of a variable mixture of heterogeneous glycans, and may contain a variety of impurities. Second, as natural glycans, they may also be inherently bioactive with the risk of biological side effects upon administration. In search of alternatives, synthetic glycan analogues or so-called glycomimetics have been chemically produced to provide more control over the structure of the compound. Such glycomimetics have been shown to retain or even exceed the activities of their corresponding natural polysaccharides while providing additional features such as improved stability, bioavailability and half-life.[11]

Glycomimetics of negatively charged natural polysaccharides can be differentiated into polyanionic systems and glycofunctionalized systems carrying either sialic acid motifs or sulfated glycan fragments.[12,13] Polyanionic systems and sialic acid functionalized glycomimetics have been widely studied for their antiviral activity.[14] For example, sialic acid carrying glycopolymers were introduced and studied by Roy and Whitesides and have been shown by several other groups to be potent inhibitors of influenza virus cell entry.[15,16] Haag, Azad and co-workers recently demonstrated that highly sulfated polyanionic polyglycerol dendrimers systems showed broad-spectrum antiviral activity against a number of viruses.[17] On the other hand, sulfated glycopolymers as glycomimetics of GAGs have been much less studied for their potential to inhibit viral infections[13], but rather as inhibitors of protein aggregation. For example, glycopolymer GAG mimetics as introduced by Hsieh-Wilson and co-workers were studied as anticoagulants showing activity comparable to commercial products such as Arixtra.[18] Miura and co-workers demonstrated an inhibitory effect of GAG mimetic glycopolymers on Alzheimer's b-secretase, playing an important role in the Alzheimer's disease.[19] A first study testing for the antiviral activity of sulfated glycopolymers was reported by Tengdelius and co-workers who introduced polymethacrylamides with pendant sulfated α-L-fucosides that exhibited inhibitory activity against HSV-1 similar to that of fucoidan, a natural anionic polysaccharide produced in algae.[20]

In this study, we synthesize and apply a series of sulfated glycomimetic oligomers and polymers to test for their antiviral potential. The compounds tested consist of a synthetic linear oligo(amidoamine) scaffold with carbohydrate side chains that are fully sulfated, thereby mimicking natural polysaccharides with high degrees of sulfation such as heparin. We are specifically interested in the role of the chain length or so-called degree of polymerization (dp), as it has been shown for natural GAGs[21] and GAG mimetics used as anticoagulants[18] that the chain length strongly affects bioactivity. Long chain glycopolymers are generated via controlled radical polymerization of glycomonomers. Short chain glycooligomers are generated by solid-phase synthesis, which allows for precise control of the degree of polymerization and thereby chain length and number of sulfated glycoside side chains.

As a model system to test inhibitory efficacy the sulfated glycomimetics, we initially used an HPV infection model. HPVs are small, non-enveloped DNA viruses with transforming potential that infect skin or mucosa. HPV virions consist of two structural proteins: the major capsid protein L1 that self-assembles into a total of 72 pentamers, and the minor capsid protein L2 situated beneath the pentamers.[22–24] High-risk HPV types (e.g. 16, 18 and 31) are responsible for a variety of anogenital and oropharyngeal cancers, causing most cervical cancers worldwide. The most prevalent HPV type is 16, which has been widely studied and serves as a prototype for studying HPV entry.[25] HPV16 attaches to basal cells or the basement membrane of epithelia cells via the glycan chains of heparan sulfate proteoglycans (HSPGs).[26–28] This interaction induces a conformational change in L1 that facilitates further structural processing of the capsid.[21] These crucial structural changes involve a proteolytic processing of L1 by secreted kallikrein-8, as well as cyclophilin-mediated externalization and subsequent furin-dependent cleavage of the N-terminus of L2.[29–31] These changes result in the loss of affinity for HSPGs and in the transfer of the virus to an elusive secondary receptor (complex), triggering viral internalization by a novel endocytic mechanism.[32–35]



Scheme 1. Model for the inhibition of viral entry by sulfated glycomimetic polymers by competing with cellular glycans.

In this work, sulfated glycomimetic oligomers and polymers were tested for their antiviral activity against HPV16 to gain insights into the potential differences in mode of action depending on their chain length (Scheme 1). Additionally, to test for their potential as viral inhibitors, first *in vivo* studies were performed and broad-spectrum activity was tested for other viruses such as Herpes Simplex

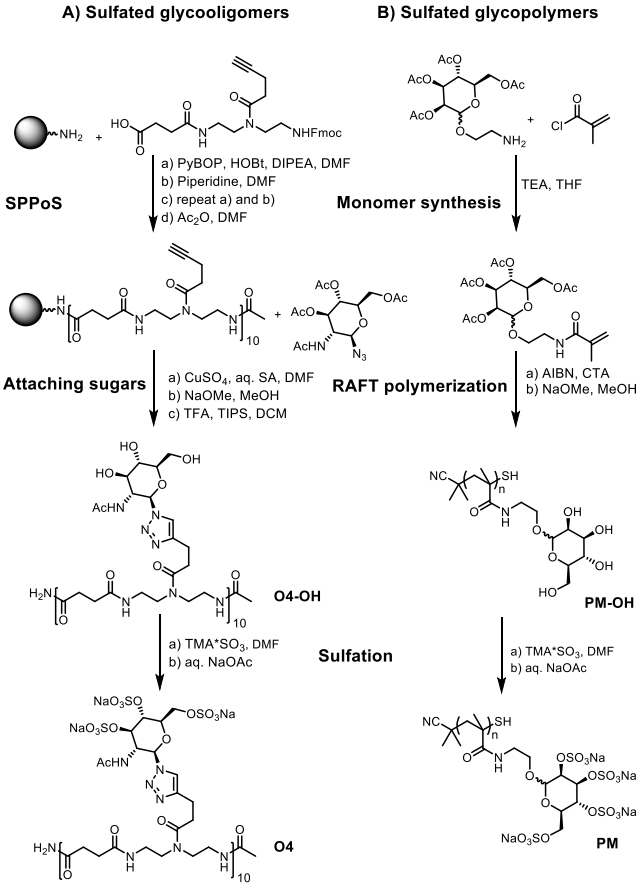
Virus (HSV), Influenza A Virus (IAV) and Merkel Cell Polyomavirus (MCPyV).

RESULTS

Synthesis of sulfated glycooligomers and glycopolymers

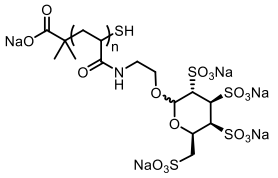
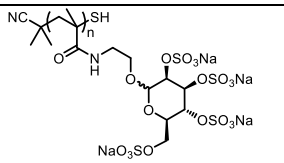
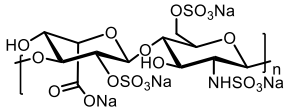
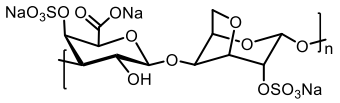
Glycopolymers were designed to resemble two major features of heparin and carrageenan - a polymeric display of saccharides and a high degree of negative charge through sulfation. Glycomonomers were first synthesized by attaching a methacrylamide unit to the anomeric position of the monosaccharide based on work by Yan et al.[36] and Wu et al.[37]. The corresponding monomers were then polymerized using controlled reverse addition-fragmentation chain-transfer (RAFT) polymerization using a cyano-2-propyl dodecyl trithiocarbonat chain transfer agent as described by Toyoshima et al.[38] (see SI for detailed description of synthesis) (Scheme 2). Two different glycopolymers carrying either Galactose (Gal) or Mannose (Man) side chains were generated. Gal is the constituting monomer of carrageenan, while Man is a commonly used monosaccharide in glycopolymer synthesis; the latter was used here as a control for comparison with Gal. For the Gal-presenting polymer, two batches were used: **PG1-OH** with an average dp of 40 and **PG2-OH** with dp 46, showing the good reproducibility of synthetic procedures (see SI). The Man-presenting polymer (**PM-OH**) was isolated with an average dp of 86 (see SI). As negative control, **PG-OH** with an average dp of 84, was used without further sulfation (see SI).

Glycooligomers were synthesized by applying solid phase polymer synthesis (SPPoS).[39–41] In contrast to glycopolymer synthesis, this allows for absolute control over the chain length and thereby the number and positioning of sugars within the glycooligomer. In short, tailor-made building blocks carrying a free carboxylic acid group and Fmoc-protected amine group were coupled in a stepwise fashion on solid support following standard Fmoc-peptide coupling protocols. When combining building blocks with alkyne side chain and hydrophilic main chain motifs, we could assemble monodisperse, sequence-defined oligo(amidoamine) scaffolds that allowed for site-selective introduction of azido functionalized carbohydrates via Cu-mediated azido-alkyne click reaction (Scheme 2). To more closely resemble the natural ligand of HPV16 in cells, GlcNAc one of the monomers forming HS, was used as carbohydrate residue. Here, for the first time, glycooligomers carrying 2 (**O1-OH**), 6 (**O2-OH**), 8 (**O3-OH**) or 10 (**O4-OH**) GlcNAc side chains were synthesized by SPPoS (see SI for detailed description of synthesis and analytical data) (Table 2).



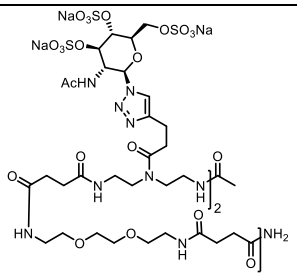
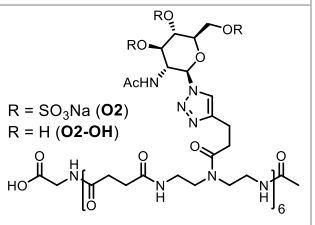
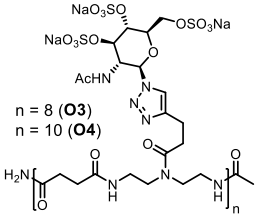
Scheme 2. General synthesis of sulfated glycooligomers (**A**) and glycopolymers (**B**).

Table 1. Structures of sulfated glycopolymers

#	N(sugars)	Degree of sulfation [%]	Dispersity[h]	Structure
PG1	40 [a]	n.d.[c]	2.0	
PG2	46 [a]	97.6[c,d]	1.08	see PG1
PM	86 [a]	n.d.[c]	1.25	
Heparin	30-34[b]	85%[e]/43%[f]	n.a.	
Carageenan	n.a.	25-34[g]	n.a.	

[a]Average number calculated from the M_n as determined by aqueous Gel Permeation Chromatography (GPC) for all polymer samples (see SI). [b]Of disaccharide repeating units as shown in the table, calculated from an average MW of 17-19 kDa as provided by the supplier. [c]According to NMR analysis, quantitative sulfation was achieved for glycopolymers. [d]For sample PG2, additional analysis via elemental analysis confirmed high degree of sulfation (see SI). [e]Corresponds to 2,4-2,6 sulfations per disaccharide[42], out of the 3 sulfations present in natural heparin (see structure). [f]As calculated for glycopolymers, counting all residues susceptible for sulfation [g]Literature values for food grade carrageenan (mainly iota type).[43] [h]Of the unsulfated precursor polymer as determined by aqueous GPC (see SI). n.d. = not determined, n.a. = information not available.

Table 2. Structures of sulfated glycooligomers

#	N(sugars)	Degree of sulfation [%][a]	Structure
O1	2	95-98.5	
O2	6	98.7	
O2-OH	6	0	
O3	8	89.9	
O4	10	85.2	

[a]Degree of sulfation as determined by ^{1}H-NMR (see SI).

Both, glycopolymers and glycooligomers, were then sulfated using sulfurtrioxide trimethylamine complex ($TMA*SO_3$) (Scheme 2). The degree of sulfation was determined via ^{1}H-NMR and additionally by elemental analysis for **PG2**, showing nearly full sulfation for all structures, sulfated glycooligomers (**O1**-**O4**) and glycopolymers (**PG1**, **PG2**, **PM**) (see SI for analytical data) (Table 1 and 2).

Sulfated glycopolymers are strong antagonists of HPV16

Natural sulfated polysaccharides such as heparin and carrageenan are good inhibitors of HPV16.[7,21,44] However, these naturally derived polysaccharides exhibit dispersity with respect to their carbohydrate content, length, and degree of sulfation. For example, heparin can occur in both high and low molecular weight forms with between 40-50% sulfation, while carrageenan exhibits between 25-35% sulfation (Table 1). This can make it difficult to determine precise structure/function relationships. In addition, biological sources of heparin can cause unwanted side effects within the host e.g. interfere with normal HS mediated cell processes.[45–47] Here, we aimed to analyze whether synthetic glycomimetic compounds may be alternatives to natural compounds.

To compare the antiviral efficacy of the synthesized glycomimetic compounds, glycopolymers were mixed in different concentrations with HPV16 capsids containing a reporter plasmid encoding for eGFP (pseudoviruses or PsV)[48] prior to infection. Despite HPV16 being a BSL2 pathogen, the use of PsV allows work under BSL1 conditions due to the incorporation of a pseudogenome instead of the viral genome. After binding to HeLa cells, the inoculum was removed, and the infection was scored 48h post infection (p.i.). Heparin and carrageenan were used as positive controls to evaluate glycopolymer binding. As expected, HPV16 infection was blocked by heparin and carrageenan in a dose-dependent fashion (Fig. 1A, [7]). Both heparin and carrageenan abrogated HPV16 infection at a concentration of 0.01mg/ml, although carrageenan was more efficient than heparin at lower concentrations. The half-maximal inhibitory concentration (IC_{50}) for heparin and carrageenan were 3·10-3 mg/ml and 4·10-5 mg/ml, respectively (Fig. 2A, B).

Next, the glycopolymers **PM** and **PG1** were tested for their inhibitory effect on HPV16 infection. Based on our unpublished work on the length of natural glycans, we expected that glycopolymers ≥dp40 would display similar efficacies in engaging the virus and preventing infection. Indeed, both abrogated infection at 0.01mg/ml, similarly to heparin and carrageenan (Fig. 1A). **PG1** and **PG2** provided highly similar results (data not shown), showing excellent reproducibility from batch to batch. However, no significant difference was observed for the inhibitory effects of **PM** and **PG1**. This confirmed that a dp of 40 is sufficient to yield maximal blocking efficacy. It may also provide a hint that the identity of the sugar, changing from galactose (**PG**) to mannose (**PM**), may not dramatically affect the inhibition potential. As mentioned above, we chose only one type of unsulfated control polymer (i.e. **PG-OH**), since it is already well established that binding of glycans to HPV16 requires a negative charge brought about by sulfation.[21,26] Similarly, mannose-based glycopolymers do not inhibit HPV16 infection.[49] Therefore, we omitted the addition of PM-OH as control. In line with the previous data, **PG-OH** did not significantly inhibit HPV16 infection (Fig. 1A). The IC_{50} of **PM** and **PG1** were 7·10-5 mg/ml and 2·10-4 mg/ml, respectively (Fig. 2C, D). Thus, the sulfated glycopolymers inhibited HPV16 infection as efficiently as carrageenan and 10-100 fold better than heparin, indicating that sulfated glycopolymers are effective inhibitors of HPV16 infection. Notably, incubation with the natural glycans or the glycopolymers displayed no cytotoxic effects in cell culture in the highest concentrations used (Fig. 1B).

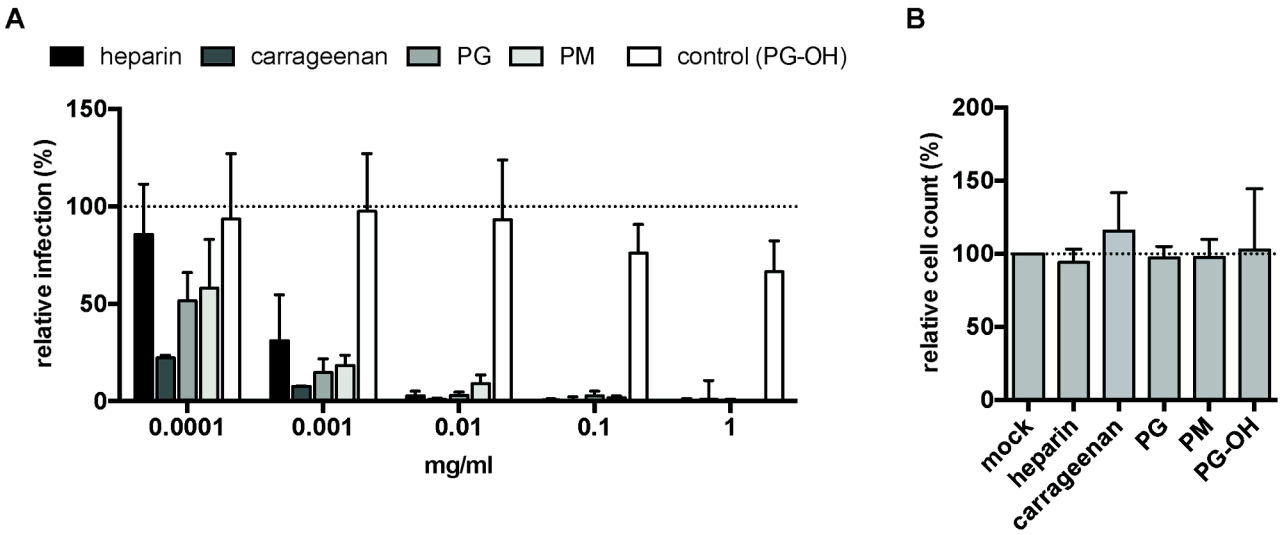


Figure 1. Natural polysaccharides and sulfated glycopolymers block infection of HPV16. (A) HPV16 was preincubated with the indicated amounts of heparin,carrageenan, glycopolymers **PM** and **PG1** and the unsulfated control **PG-OH** for 1h at RT. HeLa cells were infected and infection was scored 48h p.i. by microscopy (GFP signal). Results are shown in % relative to mock-incubated HPV16. All infection values are the mean ± SD of at least 3 independent experiments. (B) Relative cell count of cells treated as in A). The relative cell numbers after infection and treatment with glycopolymers or natural polysaccharides are shown relative (in %) to the untreated mock (uninfected) condition of each experiment and indicate the absence of toxicity by all treatments at 1mg/ml.

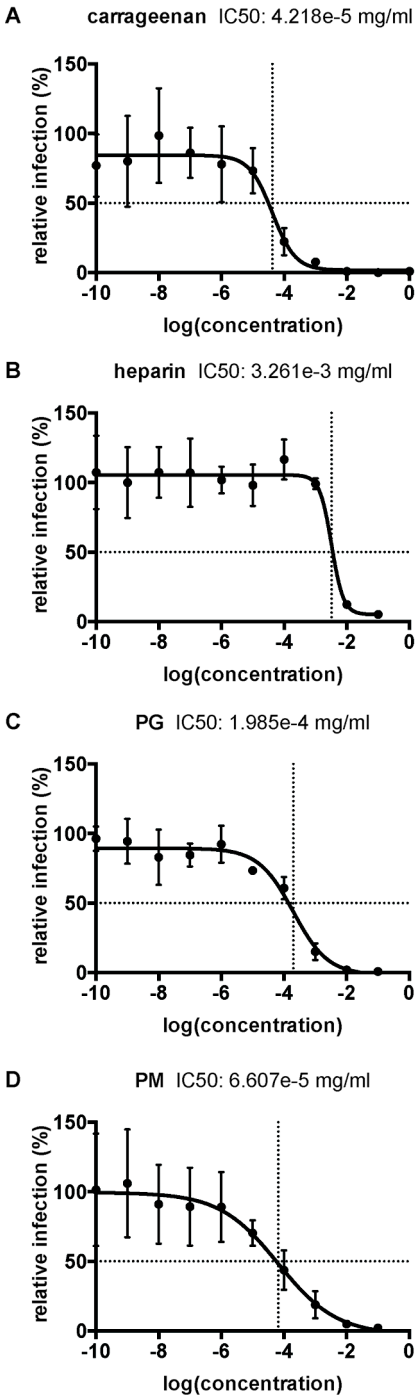


Figure 2. Inhibitory efficiency of glycopolymers **PM** and **PG1** and natural polysaccharides. HPV16 PsVs were preincubated with the indicated compounds for 1h at increasing log concentrations. The inoculums were added to HeLa cells for 2h and infection was scored 48h p.i. Inhibition curves and IC_{50} values for carrageenan (A), heparin (B), **PG1** (C) and **PM** (D) were calculated using GraphPad. Results are shown relative (in %) to mock-infected samples. All infection values are mean ± SD of at least 3 independent experiments.

HPV16 infection was reduced by sulfated glycooligomers *in vitro*

Our previous results indicated that the antiviral activity cannot be further increased above a critical dp of glycopolymers and we did not see a noticeable dependence on the type of sugar presented in the side chains. Therefore, we next looked at inhibition employing shorter glycooligomers of defined chain length going from 2 up to 10 sugars. Additionally, we decided to now include GlcNAc side chains in order to more closely mimic the natural HS fragments.[50,51]

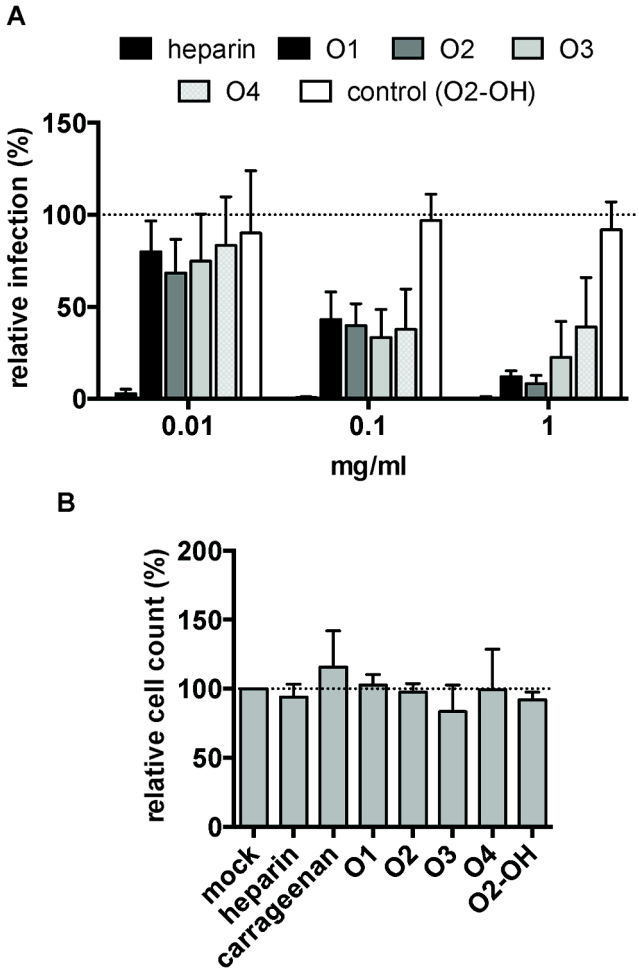


Figure 3. Antiviral activity by sulfated glycooligomers in HPV16 infection. **(A)** HPV16 infection assay was performed as described in Fig. 1 with glycooligomers with 2, 6, 8 or 10 GlcNAc residues (**O1**-**O4,** respectively) and unsulfated glycooligomer **O2-OH**. Results are shown relative (in %) to the mock-infected samples. All infection values are the mean ± SD of at least 3 independent experiments. (B) Relative cell count of cells treated as in A). The relative cell numbers after infection and treatment with glycooligomers or natural polysaccharides are shown relative (in %) to the untreated mock (uninfected) condition of each experiment and indicate the absence of toxicity by all treatments at 1mg/ml.

Since these glycooligomers are short in comparison to GAGs, we expected that they would exhibit lower inhibitory potential than the glycopolymers. HPV16 infection was also blocked by glycooligomers **O1**-**O4**, albeit at relatively high concentrations (Fig. 3A). We observed a reduction of 50% infection at a concentration of 0.1 mg/ml similar for all glycooligomers. Increasing the concentration of glycooligomers further reduced infection by about 90% for **O1** and **O2**, but surprisingly was less efficient for the longer glycooligomers, **O3** and **O4**. Thus, at higher concentrations the inhibitory effects of glycooligomers did not correlate with their length. This was unexpected, as longer sulfated polysaccharides display a higher number of binding moieties, which we reasoned would increase the affinity, avidity and therefore the probability of binding to the virus, thus inhibiting infection. This was not due to cytotoxic effects, since, similar to the glycopolymers, the glycooligomers did not exhibit any reduced cell numbers even upon high concentrations (Fig. 3B). Potentially, aggregation behavior of sulfated glycooligomers increases with increasing chain length, but further studies are required to investigate this in depth. Nevertheless, this first generation of glycooligomers were able to interfere with HPV16 infection, suggesting that they may be feasible antiviral candidates.

Two distinct mechanisms mediate inhibition of infection by glycooligomers and glycopolymers

Since the different glycomimetics displayed unexpected effects in inhibiting viral infection, we aimed to determine how they interfered with infection. Since we assumed that initial binding was blocked, we used fluorophore-labelled HPV16 particles that provided a visual and quantitative assessment of binding to cells while retaining their infectivity.[52] Glycopolymers, glycooligomers or natural polysaccharides were incubated with fluorophore-labelled HPV16, and subsequently added to cells to allow for binding to cell surface HS. Microscopic analysis showed that in untreated cells, bound virus particles are visible as bright dots (Fig. 4), whereas the natural glycans, heparin and carrageenan, blocked attachment of HPV16 to cells as expected (Fig. 4,[21]). Similarly, **PG1** and **PM** dramatically reduced virus binding to cells (Fig. 4). However, the short glycooligomers unexpectedly did not affect binding to a significant extent, even if infection was reduced by 90% at the same concentration (Fig. 4; compare Fig. 3). While the shorter **O1** and **O2** did not affect the distribution of virus signal, the longer **O3** and **O4** led to virus clustering (Fig. 4; brighter, bigger dots). None of the unsulfated controls (**PG-OH, O2-OH**) significantly affected HPV16 binding to cells (Fig. 4). In conclusion, while glycopolymers blocked binding as expected, glycooligomers allowed binding but not infection, indicating that infection occured at a post-binding step, thus representing a novel mode of interference with viral infection.

Glycopolymers display antiviral activity against HPV16 *in vivo*

As the *in vitro* experiments showed promising antiviral activity for both glycopolymers and glycooligomers, we tested whether they would also be effective in an *in vivo* infection scenario. For this, the HPV16 mouse vaginal challenge model was used.[53] Glycomymetics were incubated with HPV16-luciferase as for *in vitro* experiments and inoculums were applied in the mice vagina after mechanical disruption of the epithelia. Using non-invasive bioluminescence imaging, infection was scored by signal intensity after applying luciferin, the substrate of luciferase.

All mice in the untreated control group exhibited luciferase activity, indicating successful infection (2.0x105 ± 2.8x105 photons per second, p/s). As expected, mice inoculated with virus that were preincubated with carrageenan or heparin were not infected (Fig. 5; carrageenan: 1.5x104 ± 3.0x103 p/s, heparin: 2.1x104 ± 6.4x103 p/s; photon emission comparable to uninfected controls: $1.8x10^4$ ± $3.3x10^3$). The glycopolymer **PG2**, used as prototypical glycopolymer, also completely blocked infection in all mice (1.8x104 ± 4.5x103 p/s), supporting the *in vitro* results and further demonstrating the efficacy of this compound as an inhibitor of HPV16 infection. However, **O1** and **O4** showed little or no antiviral effect, respectively, in line with their reduced antiviral activity *in vitro* (SB1: 2.1x105 ± 3.8x105 p/s, SB4: 1.5x105 ± 9.8x104 p/s). In conclusion, the glycopolymer tested blocked HPV16 infection efficiently *in vitro* and *in vivo*, which is remarkable. To the best of our knowledge, this is the first study demonstrating *in vivo* efficacy of sulfated glycopolymers as antivirals.

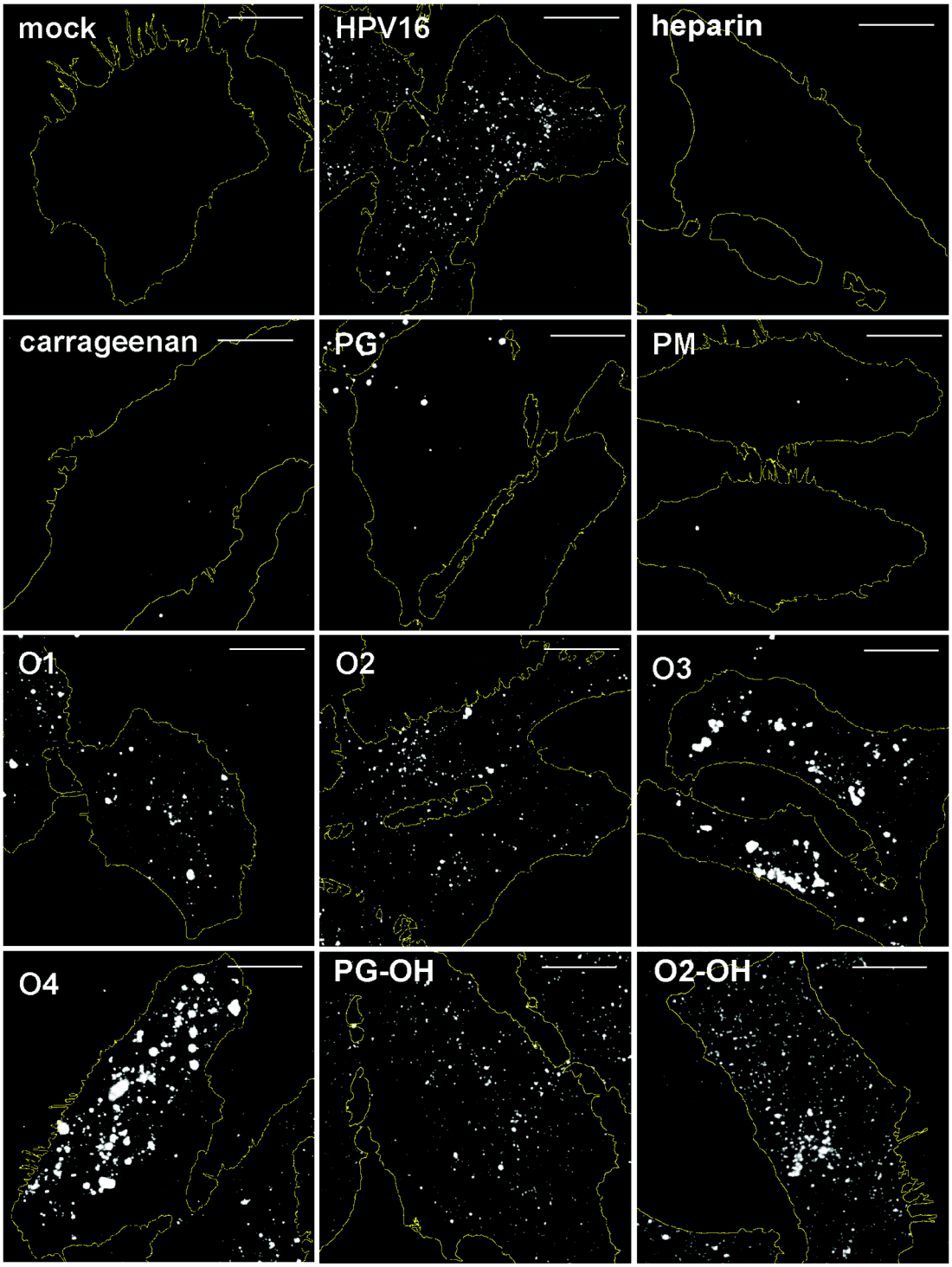


Figure 4. Different mechanisms of inhibiting HPV16 infection by sulfated glycopolymers and glycooligomers. Fluorophore-labeled HPV16 (white) was incubated with 1mg/ml of the indicated compounds for 1h. Preincubated PsV were allowed to bind to HeLa cells and fixed after 2h. Representative maximum intensity projections of stack images for each condition are shown. Cell outlines (yellow) were drawn in Fiji (ImageJ) based on phalloidin staining. Scale bars correspond to 20µm.

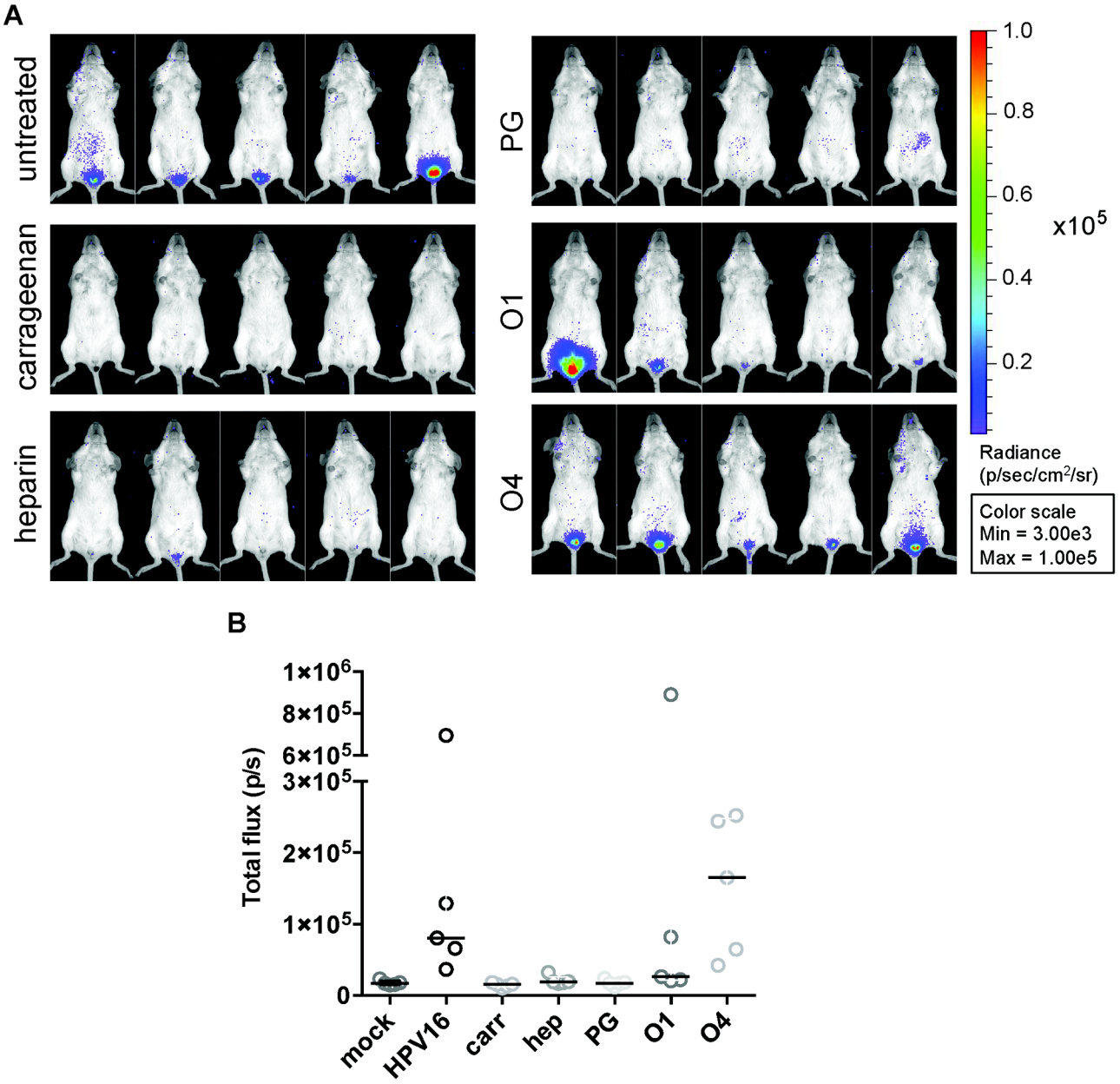


Figure 5. PG2 effectively inhibits HPV16-luciferase infection *in vivo*. (A) BalbC mice (5 per group), intravaginally inoculated with HPV16-luciferase preincubated with the respective compounds, were measured with an IVIS Spectrum to assess infection two days after virus inoculation. (B) The signal was quantified as photons per second (p/s). The data was analysed with Graphpad and infection level of each mouse is shown as a single data point. The line represents median of the values. **: $P < 0.01$ relative to uninhibited control.

Glycopolymers are potential broadband antiviral compounds

So far, our work has demonstrated that sulfated glycopolymers are good candidates as antiviral compounds against HPV16. Since the mechanisms by which these glycopolymers and glycooligomers block HPV16 are likely to be applicable to further HSPG-binding viruses, they potentially constitute broad-spectrum antivirals.

To test this notion, several viruses were subjected to infectivity assays similar to those described for HPV16. We selected viruses with different requirements for sulfated GAGs for successful viral entry: fully dependent (Herpes Simplex Virus-1 and Merkel Cell Polyomavirus), potentially dependent (Simian Virus 40 (SV40)) or independent (Influenza A Virus). HSV-1 is an enveloped virus that causes cold sores and in severe cases viral encephalitis with potential fatal outcome. HSV-1 requires HSPGs for attachment to cells.[54,55] MCPyV has been associated with the development of Merkel cell carcinoma, a severe and aggressive carcinoma of the skin.[56] This non-enveloped polyomavirus requires sequential engagement of sulfated

GAGs (such as HS) and sialylated glycans.[57,58] The animal polyomavirus SV40 requires binding to sialic-containing ganglioside GM1 but may also engage HSPGs.[59,60] As a potential negative control, IAV, a causative agent of severe respiratory infections, was used. IAV strictly binds sialylated receptors on the host cells to initiate viral entry.[61] MCPyV is a BSL2 organism, but since it was prepared as a PsV, work could be conducted under BSL1 conditions. All other viruses are BSL2 organisms, and work was conducted accordingly.

As indicated by reduction of infectivity, glycopolymers successfully blocked all HS-dependent viruses. HSV-1 was blocked with similar efficiency as HPV16 (Fig. 6A), whereas MCPyV required 100-fold higher doses of **PM** and **PG1** (Fig. 6B). Interestingly, SV40 was also susceptible to glycopolymer-mediated inhibition (Fig. 6C). While infection was reduced to 50% at a low glycopolymer concentration of 0.0001mg/ml, a basal level of infection of about 10% was resistant to inhibition. This may reflect direct binding to cells by low affinity interactions with sialic acids. In contrast to HPV16, the unsulfated **PG-OH** decreased infectivity of HSV-1 and SV40 to a minor extent, suggesting that the polymeric backbone or non-sulfated sugars may assist in engaging certain viruses.

As mentioned before, IAV strictly depends on sialylated glycans for binding and infection.[61] Sialylated glycans are structurally very different from HS: HS are long, unbranched and highly sulfated chains, whereas sialic acid is generally found as a terminal carbohydrate residue on short, branched and/or low or non-sulfated glycans. Hence, we reasoned that IAV should be less affected by the glycopolymers. Surprisingly, glycopolymers, despite resembling HS, inhibited IAV infection. Of note, while infectivity was reduced by 50% at a comparatively high concentration of 0.1mg/ml, it remained unaffected by heparin (Fig. 6D). This may suggest that the display of saccharides as side chains of a polymeric backbone rather than constituting the backbone itself is advantageous for a broad efficacy towards glycan binding viruses.

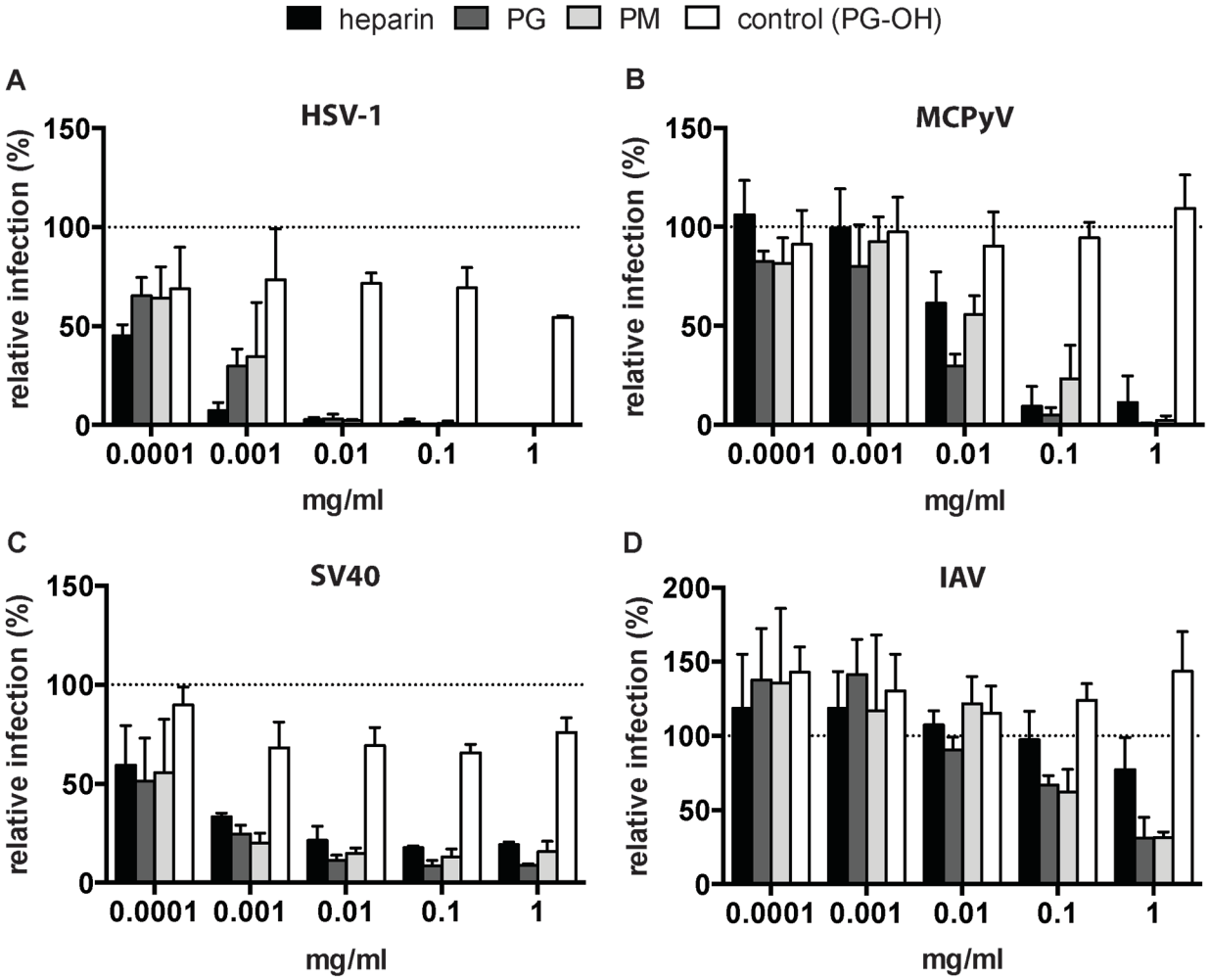


Figure 6. Broad-spectrum antiviral effects of sulfated glycopolymers. HSV-1 (A), MCPyV (B), SV40 (C) or IAV (D) were incubated with glycopolymers **PM**, **PG1** and **PG-OH** (as shown in Tab. 1) at the indicated concentrations. For more information on the infection assays of each virus, see Material and Methods (SI). Results are shown in relative infection (%) to an untreated condition. All infection values are the mean ± SD of at least 3 independent experiments.

As glycooligomers displayed some antiviral activity against HPV16, we also tested their potential broad-spectrum effect. HSV-1 was blocked by all glycooligomers at the highest concentration tested. At 0.1 mg/ml the intermediate-sized structure (**O2**) was the most efficient as compared to the unsulfated control (Fig. 7A) indicating that the dp does not influence the inhibitory potential in a straightforward manner.

Similar effects were observed for SV40. Infection was blocked by the short glycooligomer **O1** only at the highest concentration (Fig. 7C). **O2** was the most efficient compound and reduced infection by 50% at 0.1mg/ml, whereas longer glycooligomers **O3** and **O4** did not affect infection. Similar to the glycopolymers, a basal level of infection was resistant to inhibition.

As IAV was blocked by glycopolymers at high concentrations, it was not surprising that glycooligomers also exerted antiviral functions. Interestingly, for IAV the increase in glycooligomer chain length correlated with better neutralization of the virus (Fig. 7D).

In contrast, MCPyV infection was unaffected in the presence of glycooligomers suggesting that the oligomers displayed a low affinity to the virion (Fig. 7B).

In summary, sulfated glycopolymers displayed a good and broad anti-viral activity, whereas sulfated glycooligomers showed interesting potential. More importantly, the glycooligomers exhibited unexpected biological effects such as post-binding inhibition and non-linear size effects. The latter will need further in-depth characterization to allow for a more rational design of next generation glycooligomeric antivirals.

DISCUSSION

The strategy to interfere with viral attachment using sulfated polymeric glycans is not without precedent. Natural polysaccharides have long been used to interfere with viral attachment, mostly to characterize the glycan binding properties of a particular virus but also in light of their antiviral potential.

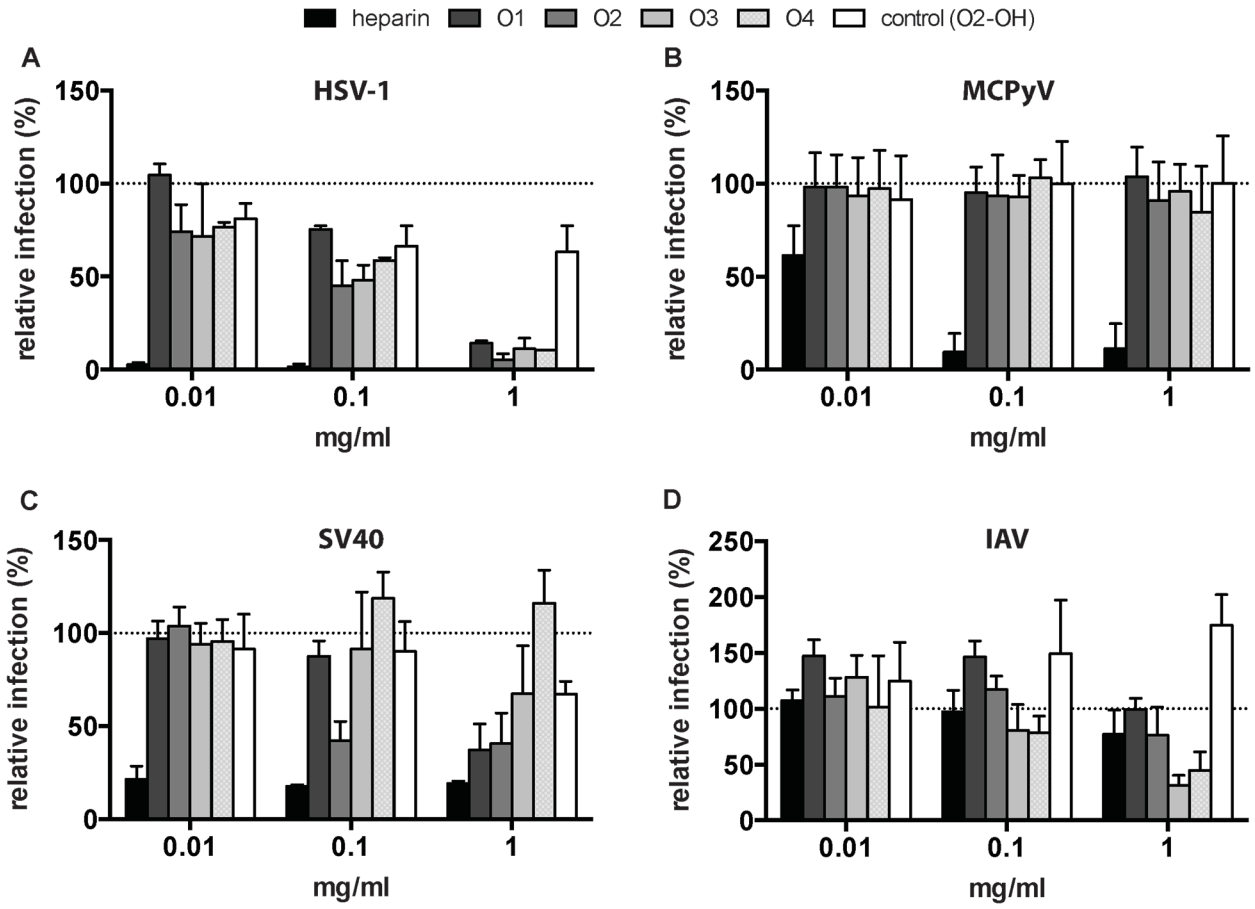


Figure 7. Sulfated glycooligomer inhibition profile of various viral infections. HSV-1 (A), MCPyV (B), SV40 (C) or IAV (D) were incubated with glycooligomers **O1**-**O4** and **O2-OH** (as shown in Tab. 2) at the indicated concentrations. For more information on the infection assays of each virus, see Material and Methods (SI). Results are shown in relative infection (%) to an untreated condition. All infection values are the mean ± SD of at least 3 independent experiments.

In this manuscript, we demonstrate for the first time, proof-of-principle that synthetic sulfated glycopolymers and glycooligomers can serve as powerful alternatives to naturally occurring polysaccharides, and that they provide a suitable platform to develop broad antivirals with the capacity to fine-tune the inhibitory mechanism. Our work revealed that sulfated glycopolymers efficiently blocked HPV16 and other viruses with a similar efficacy as carrageenan (Fig 1, 2, 5, 6). Moreover, glycopolymers and carrageenan inhibited viral infectivity more strongly than heparin, which serves as a mimic for the natural ligand (Fig. 1, 2). This provides an excellent foundation for future optimization of synthetic mimetics for antiviral therapies. In contrast to natural polysaccharides, synthetic glycomimetics are defined, homogeneous, replicable and tunable compounds that can be designed to vary in length, size and presentation. In addition, different sugars, linkers and spacings can also be explored.[62–64] Moreover, glycomimetics provide the advantage that they have, to the best of our knowledge, no further bioactive properties avoiding e.g. the anti-coagulating properties of heparin *in vivo*[65], which may cause inherent risks for certain applications. While the incorporation of different sugars into glycopolymers had only slight effects on their efficacy in this study, it has been previously noted that optimization of aspects such as length, composition and delivery may yield increased biological function beyond natural ligands.[18] Finally, glycomimetics offer very high batch to batch reproducibility (see SI) and also robust reproducibility of antiviral activities (data not shown).

The sulfated glycooligomers used in this study displayed a lower efficacy to block infectivity as compared to the glycopolymers (Fig. 3, 7). This is not unexpected, since the shorter oligomers display less sulfated sugars per molecule, which would result in lower avidity towards the multiple binding sites on virus particles. Nevertheless, they blocked the infectivity of several viruses at higher concentrations in vitro.

When analyzed for HPV16, glycopolymers blocked binding of the virus to cells (Fig. 4), indicating that glycopolymers likely engaged heparan sulfate binding sites on the virion as suggested for carrageenan and other natural sulfated polysaccharides.[7,21] Interestingly, glycooligomers blocked HPV16 infectivity at a post-binding step, as shown by the presence of cell-bound virus at concentrations in which infection was blocked (Fig. 3, 4). Hence, the glycooligomers did not effectively bind to sites in the virion that mediated initial binding to cells. HPV16 interaction with heparan sulfates involves several distinct sites on the virion[66], which are likely engaged in a stepwise fashion facilitating initial binding and conformational changes in the virion[44]. Post-attachment inhibition of infection thereby potentially involves binding to sites that are typically engaged in a second step of the HS-HPV16 interaction, blocking the conformational changes crucial for subsequent engagement of the internalization receptor.[29,32] Alternatively, one would have to propose engagement of sites independent of HS interaction, which seems less likely.

Another intriguing observation was that with increasing length of the glycooligomers, virions clustered on the cell surface (Fig. 4). Possibly, long glycomimetics bind to several virus particles thus linking them, whereas shorter oligomers may be unable to bridge virions. Alternatively, the longer oligomers may additionally engage other cellular proteins, which would lead to clustering on cells. We also observed some viral clustering with glycopolymers which absorbed to the coverslips during microscopy (Fig. 4, PG). This indicates that clustering also occurred with glycopolymers, but was not easily identifiable as virions were unable to bind cells.

Irrespective of the different modes of inhibition between glycopolymers and glycooligomers, our results demonstrate that additional work needs to be conducted to determine the rules of engagement in order to devise rational guidelines for the future design of sulfated glycomimetic antivirals. A variety of viruses engage cellular glycans for infectivity. Here, sulfated glycomimetic compounds interfered with infection of diverse viruses, enveloped vs. non-enveloped, HSPG-binding vs. non-HSPG binding viruses (Fig. 6, 7). Infection of HSV-1 and MCPyV, which also depend on heparan sulfate for infection of host cells, was blocked. Interestingly, viruses such as SV40 and IAV that engage sialic acids as main receptors were also blocked by the glycopolymers, although to a lesser extent, especially IAV. This is surprising, as the interaction between sialic acids and those viruses is not driven by the sulfation of the sugars, but rather structural constraints within the glycan and the virus. Nevertheless, SV40 seems to be susceptible to blocking by heparin (Fig. 6,[60]). Interestingly, this does not hold true for IAV. Heparin did not show inhibition of IAV infection, while the glycopolymers blocked infection (Fig. 6). Thus, it is likely that the flexibility of the linker between carbohydrate and polymeric backbone allows for an easier fit of the sugar residues into existing binding sites. The specific mechanism(s) by which the glycopolymers engage these viruses needs to be addressed in future work, but this finding further supports an advantageous role of glycomimetics over natural polysaccharides.

Importantly, the antiviral effect of glycooligomers does not allow predicting rules of engagement, since inhibitory effects did not correlate for all of the viruses we tested (Fig. 7). While for SV40 the antiviral activity correlated with length, the opposite was true for HPV16, whereas MCPyV was not blocked by any of the glycooligomers. Besides a more systematic analysis of all functional parameters of glycooligomers, it would be important to identify, how exactly the glycooligomers bind to different viruses.

Perhaps a rather straightforward approach to increase broad-spectrum antiviral potential would be to synthesize multifunctional compounds containing different biologically relevant glycans, for example combining GlcNAc (monomer present in HS) and sialic acid. To increase avidity, it may also be reasonable to present the glycooligomers on nanoparticles, as has previously been done for heparin[67] or even on micelles. A similar approach, using nanogold particles, improved the efficacy of glycomimetic-based vaccines[68] (and is also available for the multivalent presentation of glycooligomers as used in this study[69]).

Despite the fact that improvements would be necessary to generate sulfated glycomimetics with more efficient broad-spectrum antiviral activity, it is noteworthy that the glycopolymers were effective at preventing infection in a pre-clinical mouse model for vaginal infections of HPV16 (Fig. 5). Moreover, no cytotoxicity of sulfated glycopolymers and glycooligomers was observed during experimentation (Fig. 1B, 3B). Thus, it is already possible to start assessing essential properties for clinical applications. Some of these properties include how long protective effects last after initial application, how to best deliver the compounds, whether the glycomimetic compounds exhibit long-term adverse effects in various tissues/organisms, and the long term stability of these compounds. It is conceivable that the versatility of glycomimetics will allow for fine-tuning all these parameters and derive compounds with superior efficacy and versatility than natural polysaccharides. Despite their importance as proof-of-concept for the versatility of glycomimetics-mediated inhibition, delivery systems for the polyomaviruses MCPyV and SV40 may be challenging to develop, simply because the mode of transmission *in vivo* is still under discussion.[70] However, it is already easy to envision delivery systems for specific viruses tested in this paper: sulfated glycopolymers could be included in lubricants and vaginal creams (for HPV16), lip balms (for HSV-1), and in inhalations or nebulizers (for IAV).

CONCLUSION

In conclusion, our work is proof-of-principle that sulfated synthetic glycopolymers and glycooligomers have the potential to be used as broad-spectrum antivirals. We discovered different molecular mechanisms by which glycomimetics may exert antiviral activity, which will be advantageous to improve the next generation of sulfated glycomimetics. Glycooligomers derived by SPPoS as used in this study may also prove to be potential tools to study glycan function, structure and interaction in (viral) biology due to the versatility in generating precisely defined structures.

ASSOCIATED CONTENT

Supporting Information. Materials and Methods for synthesis of sulfated glycopolymers and glycooligomers. Analytical data for sulfated glycopolymers and glycooligomers and according precursors and intermediates. Additional information including Material and Methods and supplementary figures for biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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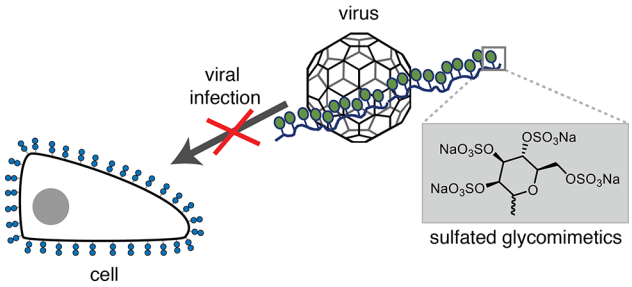
SYNOPSIS TOC.



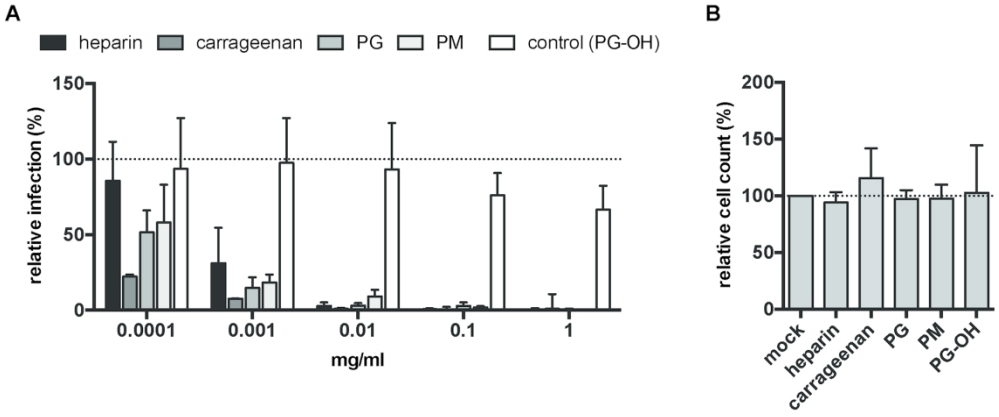


Figure 1. Natural polysaccharides and sulfated glycopolymers block infection of HPV16. (A) HPV16 was preincubated with the indicated amounts of heparin,carrageenan, glycopolymers PM and PG1 and the unsulfated control PG-OH for 1h at RT. HeLa cells were infected and infection was scored 48h p.i. by microscopy (GFP signal). Results are shown in % relative to mock-incubated HPV16. All infection values are the mean ± SD of at least 3 independent experiments. (B) Relative cell count of cells treated as in A). The relative cell numbers after infection and treatment with glycopolymers or natural polysaccharides are shown relative (in %) to the untreated mock (uninfected) condition of each experiment and indicate the absence of toxicity by all treatments at 1mg/ml.

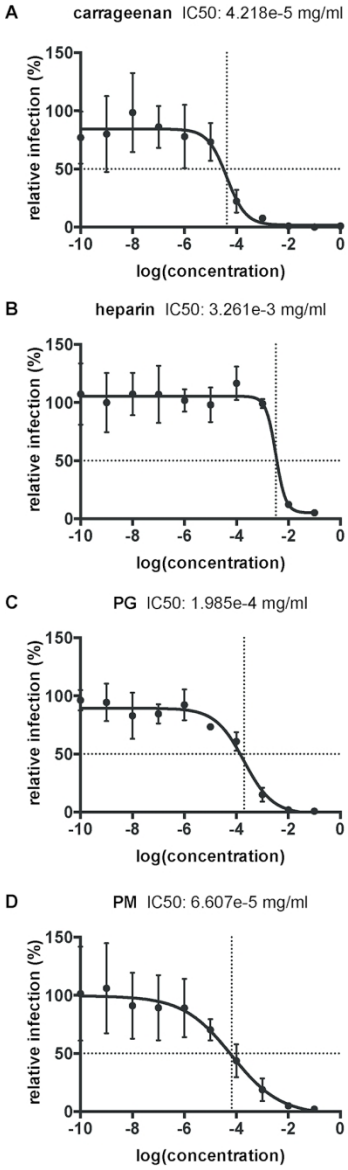


Figure 2. Inhibitory efficiency of glycopolymers PM and PG1 and natural polysaccharides. HPV16 PsVs were preincubated with the indicated compounds for 1h at increas-ing log concentrations. The inoculums were added to HeLa cells for 2h and infection was scored 48h p.i. Inhibition curves and IC50 values for carrageenan (A), heparin (B), PG1 (C) and PM (D) were calculated using GraphPad. Results are shown relative (in %) to mock-infected samples. All infection values are mean ± SD of at least 3 independent experiments.

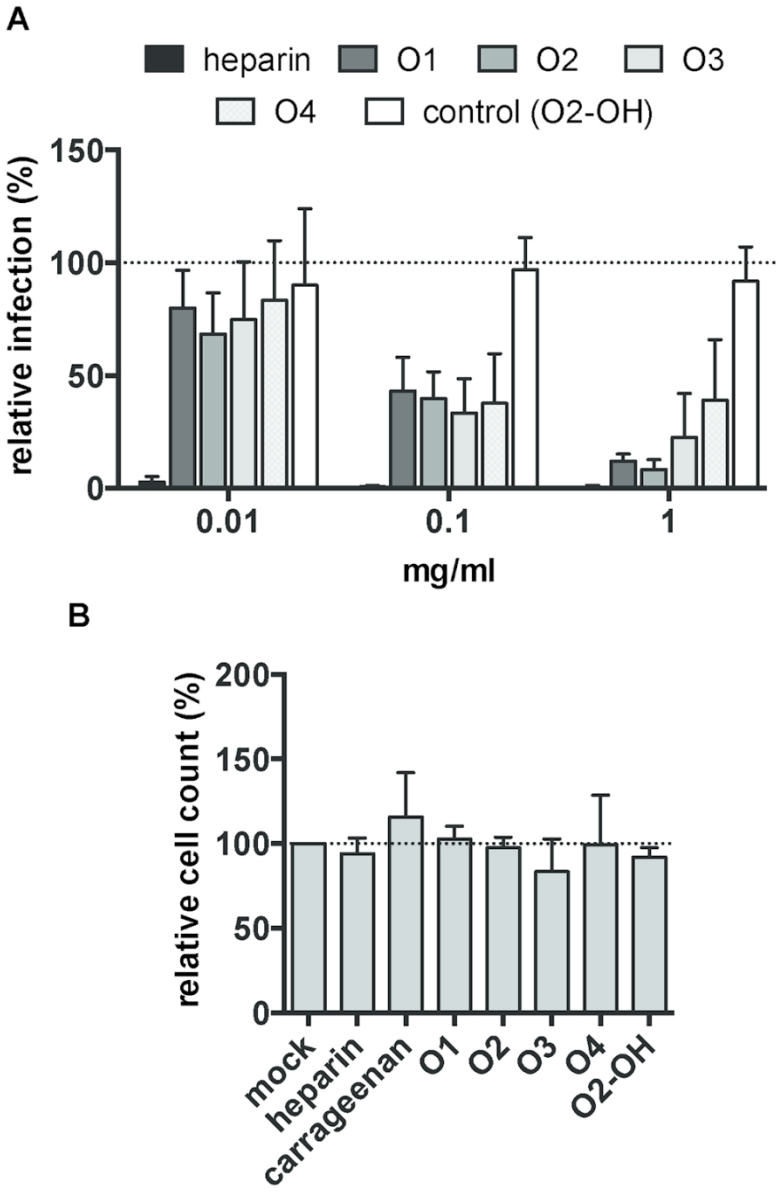


Figure 3. Antiviral activity by sulfated glycooligomers in HPV16 infection. (A) HPV16 infection assay was performed as described in Fig. 1 with glycooligomers with 2, 6, 8 or 10 GlcNAc residues (O1-O4, respectively) and un-sulfated glycooligomer O2-OH. Results are shown relative (in %) to the mock-infected samples. All infection values are the mean ± SD of at least 3 independent experiments. (B) Relative cell count of cells treated as in A). The rela-tive cell numbers after infection and treatment with glycooligomers or natural polysaccharides are shown relative (in %) to the untreated mock (uninfected) condition of each experiment and indicate the absence of toxicity by all treatments at 1mg/ml.

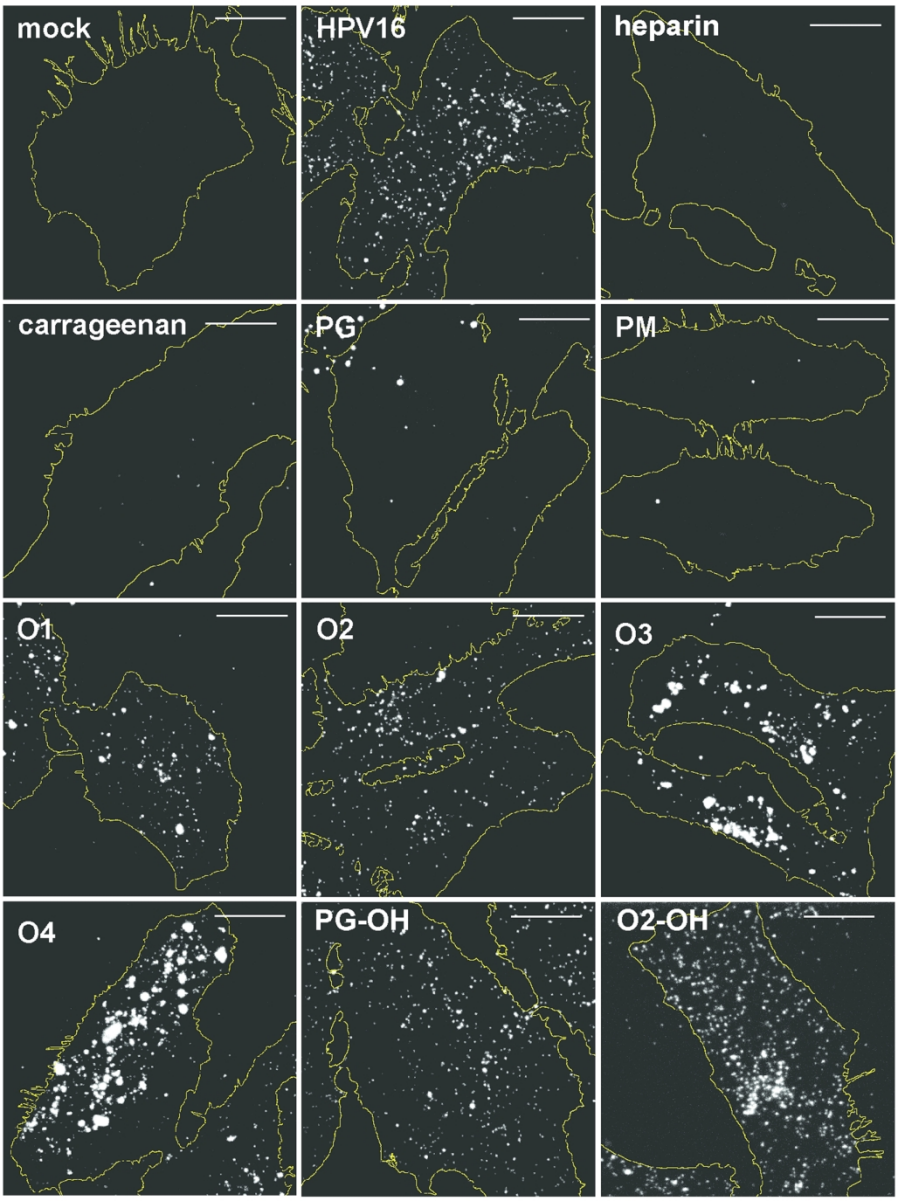


Figure 4. Different mechanisms of inhibiting HPV16 infection by sulfated glycopolymers and glycooligomers. Fluorophore-labeled HPV16 (white) was incubated with 1mg/ml of the indicated compounds for 1h. Preincubated PsV were allowed to bind to HeLa cells and fixed after 2h. Representative maximum intensity projections of stack images for each condition are shown. Cell outlines (yellow) were drawn in Fiji (ImageJ) based on phalloidin staining. Scale bars correspond to 20µm.

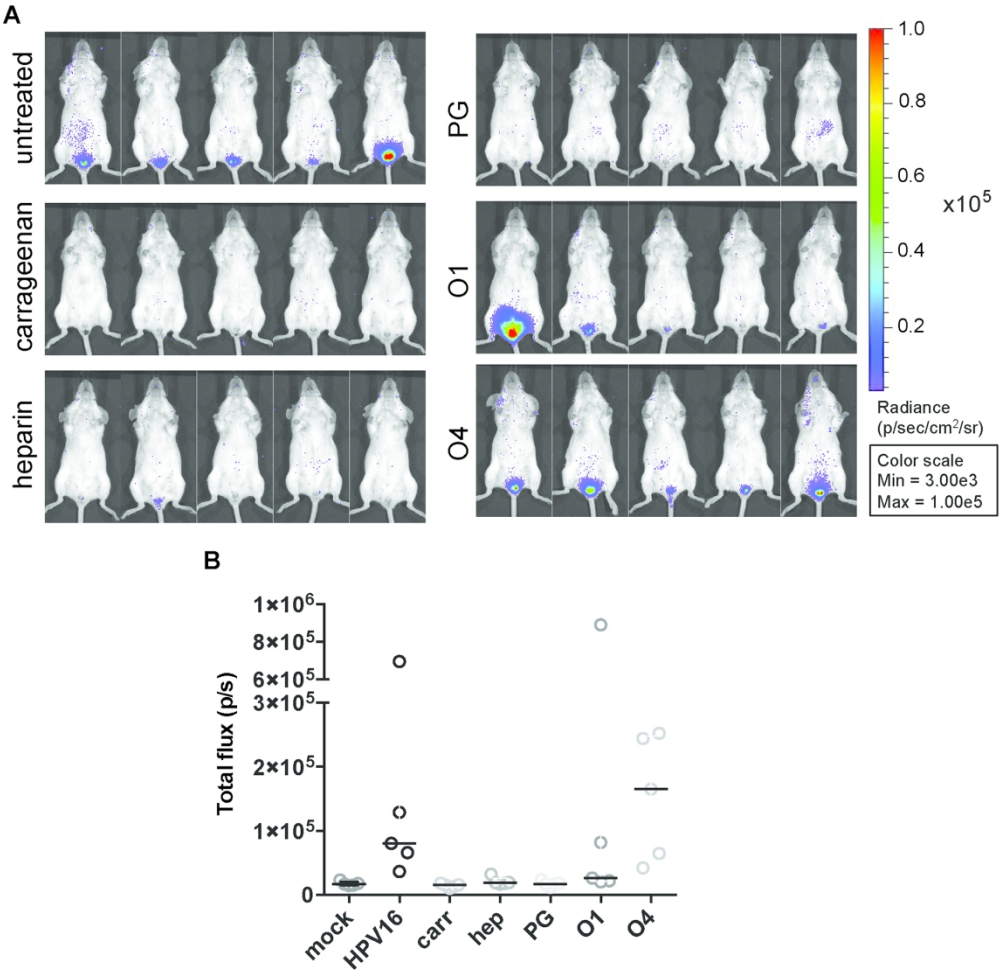


Figure 5. PG2 effectively inhibits HPV16-luciferase infection in vivo. (A) BalbC mice (5 per group), intravaginally inoculated with HPV16-luciferase preincubated with the respective compounds, were measured with an IVIS Spectrum to assess infection two days after virus inoculation. (B) The signal was quantified as photons per second (p/s). The data was analysed with Graphpad and infection level of each mouse is shown as a single data point. The line represents median of the values. **: $P < 0.01$ relative to uninhibited control.

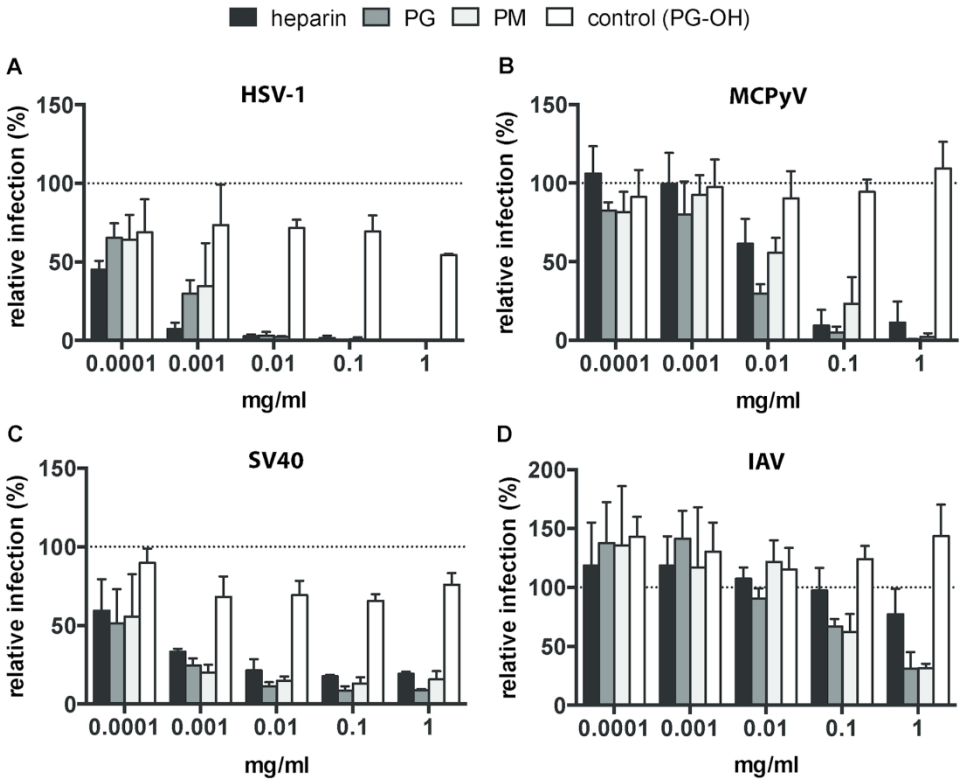


Figure 6. Broad-spectrum antiviral effects of sulfated glycopolymers. HSV-1 (A), MCPyV (B), SV40 (C) or IAV (D) were incubated with glycopolymers PM, PG1 and PG-OH (as shown in Tab. 1) at the indicated concentrations. For more information on the infection assays of each virus, see Material and Methods (SI). Results are shown in relative infection (%) to an untreated condition. All infection values are the mean ± SD of at least 3 independent experiments.

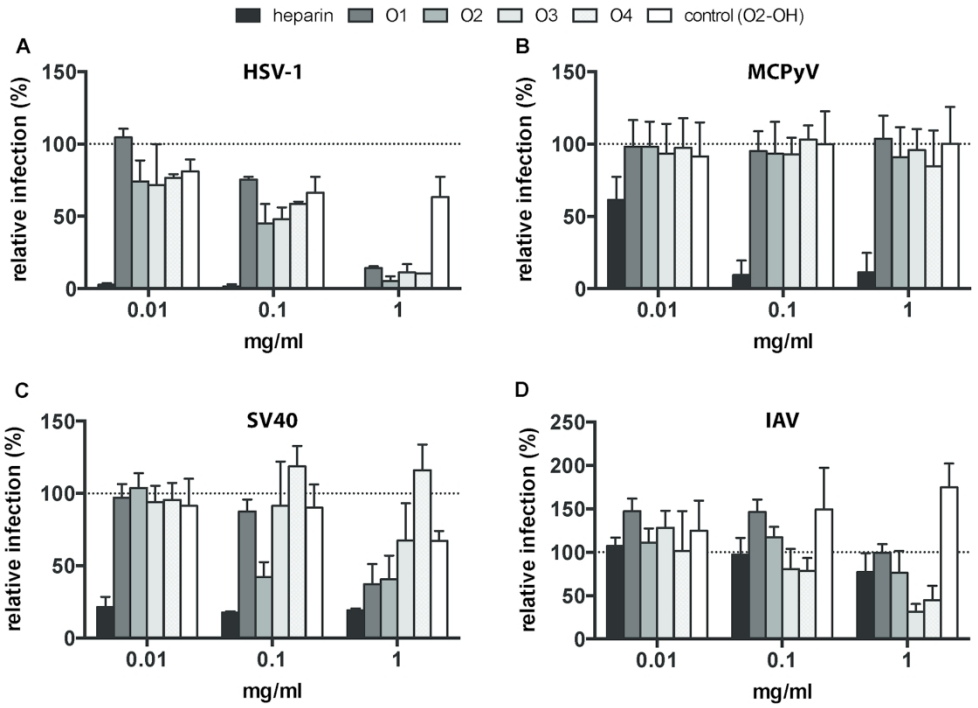


Figure 7. Sulfated glycooligomer inhibition profile of various viral infections. HSV-1 (A), MCPyV (B), SV40 (C) or IAV (D) were incubated with glycooligomers O1-O4 and O2-OH (as shown in Tab. 2) at the indicated concentrations. For more information on the infection assays of each virus, see Material and Methods (SI). Results are shown in relative infection (%) to an untreated condition. All infection values are the mean ± SD of at least 3 independent experiments.