Heparan sulfate as a receptor for poxvirus infections and as a target for antiviral agents

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Abstract
To establish the importance of virus–heparan sulfate (HS) interactions in virus infectivity, the poxvirus vaccinia virus (VACV) was used, as it binds HS and has both enveloped virus (EV) and non-enveloped mature virus (MV) forms. Initial studies showed that heparin inhibited plaque formation by both MV-rich WR and EV-rich IHD-J strains of VACV, with the EV-rich strain also losing trademark ‘comet’-shaped plaques. However, using GFP-tagged EV and MV forms of VACV, based on IC50 values, heparin was 16-fold more effective at inhibiting the infectivity of the EV form compared to the MV form. Furthermore, 6-O and N-sulfation of the glucosamine residues of heparin was essential for inhibition of the infectivity of both VACV forms. Several low-molecular-weight HS mimetics were also shown to have substantial antiviral activity, with glycosidic linkages, chain length and monosaccharide backbone being important contributors towards anti-VACV activity. In fact, the d-mannose-based sulfated oligosaccharide mixture, PI-88 (Muparfostat), was four-fold more active than heparin at inhibiting MV infections. Paradoxically, despite heparin and HS mimetics being potent inhibitors of VACV infections, removal of HS from cell surfaces by enzymatic or genetic means resulted in only a modest reduction in infectivity. It is unlikely that this paradox can be explained by steric hindrance, due to the low molecular weight of the HS mimetics (~1–2.5 kDa), with a more likely explanation being that binding of heparin/HS mimetics to free VACV initiates an abortive viral infection. Based on this explanation, HS mimetics have considerable potential as antivirals against HS-binding viruses.

INTRODUCTION
Viruses have been shown to exploit a wide variety of attachment molecules on cell surfaces and use them as receptors to invade cells and establish an infection [1]. Virus entry, spread and pathogenesis can be better understood by identifying the cell surface receptor(s) a particular virus targets [2]. Virus entry is mainly a two-step process, with the first step being attachment of the virus particle to a primary receptor, often ubiquitous cell surface-associated carbohydrate moieties, followed by interaction with a co-receptor that is usually a cell-specific transmembrane protein belonging to a well-defined receptor superfamily [3, 4]. Understanding the interaction between proteins on the surface of virus particles and the cell surface receptors exploited by the virus particles to enter cells is essential to understand viral tropism, which further helps in designing effective antiviral therapies.

Several published studies have suggested that a number of viruses use the heparan sulfate (HS) component of cell surface heparan sulfate proteoglycans (HSPG), which are ubiquitously expressed, as an initial receptor to attach to cells [1, 5–7]. HS is a glycosaminoglycan (GAG) composed of repeating disaccharides of glucosamine and hexuronic acid, joined in alternating sequences by 1,4-glycosidic linkages, which gives these carbohydrate chains the flexibility to bind to many different proteins [8–10]. The HS-binding
viruses are from widely different families and include herpes simplex virus (HSV) [11], human papillomavirus (HPV) [12], hepatitis B virus (HBV) [13], respiratory syncytial virus (RSV) [14], foot-and-mouth disease virus (FMDV) [15], human immunodeficiency virus type 1 (HIV-1) [16] and dengue virus [17]. Furthermore, alphaviruses like Sindbis virus (SINV) [18], Ross River virus (RRV) [19], Venezuelan equine encephalitis virus (VEEV) [20] and Semliki Forest virus (SFV) [21] have also been found to use cell surface HS as a co-receptor during the infection of cells. Poxviruses have also been shown to interact with cell surface HS [22–25]. Previous studies have shown that, in the case of vaccinia virus (VACV), there are at least three GAG-binding receptors on the surface of the mature virus, namely the envelope proteins H3L and A27L, which have been shown to bind HS, and D8L, which has been shown to interact with chondroitin sulfate (CS) [24, 26].

In order to better understand the initial interaction of viruses with cell surface HS, VACV was chosen as the prototype virus for this study. VACV has the advantage of having two forms of the virus, with possibly different HS binding properties. The two forms of VACV are the mature virus (MV) and the envelope virus (EV), with the EV form essentially being the MV form wrapped in at least one additional membrane (Fig. S1, available in the online Supplementary Material). The presence of one or more membranes around the EV makes this form of VACV structurally, chemically and antigenically different from the MV form of VACV [27]. The two strains of VACV used in this study were the Western Reserve (WR) and the International Health Department-J (IHD-J) strains. Both strains produce equal amounts of intracellular enveloped virus (IEV) and cell-associated enveloped virus (CEV) particles (Fig. S1), although a single codon mutation (Lys-151→Glu) located in the putative carbohydrate recognition domain of the A34R protein results in the IHD-J strain releasing much more of the EV form of VACV than the WR strain [28].

Our initial studies examined the dependence of cell entry on HS recognition by the MV and EV forms of VACV. Subsequent studies exploited a range of modified heparins and sulfated saccharides (HS mimetics) to determine the structural features of HS that the MV and EV forms of VACV interact with, a study which had the potential of identifying novel antiviral agents.

**RESULTS**

**Soluble heparin interferes with VACV plaque formation**

Previous studies have shown that VACV strains lacking the GAG binding proteins A27L, H3L or D8L have reduced plaque counts, as well as a reduced plaque size [24, 25, 29, 30]. Since it has been reported previously that MV surface proteins A27L and H3L interact with cell surface HS, the effect of soluble heparin, a highly sulfated form of HS, on plaque formation by the MV form of VACV was examined. In addition, the effect of soluble heparin on EV plaque formation was also investigated, particularly as some studies have suggested that heparin affects EV entry, but not MV entry, into cells [31]. Monolayers of 143B osteosarcoma cells were infected with WR (MV) or IHD-J (EV) strains of VACV either incubated with heparin (virus+heparin) or without any incubation (virus alone). Plaque assays indicated that heparin can induce a significant change in the plaque morphology of the IHD-J strain (Fig. 1a). The ‘comet’-shaped plaques, a hallmark of the IHD-J strain (EV rich), disappeared when soluble heparin was added to the culture medium. In fact, in the presence of heparin there was a >90% reduction (Fig. 1b) in the number of IHD-J strain plaques forming comets. Since comets result from the release of the EV form of VACV into the culture medium (Fig. S1), this finding suggests that EV particles interact with heparin more efficiently than MV particles. Furthermore, a detailed analysis of the plaque morphology revealed that the addition of heparin significantly increased the diameter of both WR-VACV and IHD-J-VACV plaques (Fig. 1c). This is a rather interesting finding, since any interference with virus infectivity usually causes a reduction in plaque size. Another important finding was that heparin also significantly reduced IHD-J-VACV plaque formation by 10-fold and WR-VACV plaque formation by 5-fold (Fig. 1d).

**Effects of heparin on infectivity of VACV**

In order to investigate the effects of soluble heparin and cell surface HS on the kinetics of VACV infections, GFP expressing recombinant WR and IHD-J strains of VACV were constructed (Fig. S2). The GFP expressing WR strain was used to purify MV particles (MV-GFP) and that expressing the IHD-J strain was used to purify EV particles (EV-GFP) (see the Supplementary Material for the purification protocols). The kinetics of infection by both MV-GFP and EV-GFP was readily detected by fluorescence-activated cell sorting 2–12 h post infection (Figs S3 and S4). The effect of soluble heparin on the kinetics of viral infectivity was then investigated (Fig. 2a). Heparin caused a significant inhibition of both EV-GFP and MV-GFP infection of 143B cell monolayers, with all three concentrations of heparin tested (1, 10 and 100 µg ml⁻¹) having an apparent impact on VACV infectivity. A direct comparison between EV-GFP and MV-GFP infectivity, however, indicated that heparin is more effective at inhibiting EV-GFP infections than MV-GFP infections. This difference was clearly evident at the 24 h time point, where as little as 1 µg ml⁻¹ of heparin could significantly inhibit EV-GFP infections, whereas the highest concentration of heparin (100 µg ml⁻¹) failed to significantly inhibit MV-GFP infectivity. However, as the MV-GFP and EV-GFP forms of VACV were purified from two different strains, it was essential to rule out the possibility that the differences seen in the interaction of the EV and MV forms of VACV with heparin were due to strain differences, rather than being an intrinsic property of the two virus forms. Thus, the MV and EV forms of VACV were isolated from cultures of GFP
expressing recombinant VACV WR and VACV IHD-J strains (see the Supplementary Material for the purification protocol), and used to infect adherent 143B cells in the presence of a wide concentration range of heparin (0.09 to 100 µg ml⁻¹). Overall, heparin inhibited the infectivity of the EV form of VACV much more effectively than the MV form, regardless of the VACV strain from which it was obtained (Fig. 2b). In fact, the heparin inhibition curves for the EV form of VACV obtained from both strains of VACV virtually overlap each other. Likewise, the heparin inhibition curves for the MV form of VACV isolated from the two different VACV strains also overlap each other. The IC₅₀ for heparin inhibition of the EV infections was 0.19 µg ml⁻¹, whereas the IC₅₀ for the MV infections was 4.7 µg ml⁻¹, which translates to a more than 16-fold difference in the IC₅₀ values for the two forms of VACV. These results indicate that the differential effects of heparin on EV and MV infectivity are due to the two different forms of VACV, and are not due to VACV strain differences. Therefore, for all subsequent assays, purified EV-GFP was obtained from the recombinant VACV IHD-J strain and purified MV-GFP from the recombinant VACV WR strain.

Effect of lack of cell surface HS on infectivity of VACV

A simple interpretation of the heparin inhibition findings is that cell surface HS represents a co-receptor for both EV and MV forms of VACV. To test this hypothesis directly, three different Flavobacterium heparinases (HPNSES) I, II, and III, and human platelet-derived heparanase (HPSE) were used to deplete cell surface HS from target cells (Fig. 2c, d). All four enzymes are capable of degrading heparin and HS, but with different substrate specificities. HPNSE 1 was the least effective of the three bacterial HPNSE enzymes, resulting in only a small (~30 %) but significant reduction in HS expression following treatment. The other two bacterial HPNSES were more effective at removing cell surface HS (~60 % depletion), although combining all three enzymes resulted in no improvement in HS removal (Fig. 2c, d). However, the most effective removal of HS was observed following mammalian HPSE treatment (~80 % depletion).

Treatment of the cell monolayers with all four enzymes significantly reduced both MV-GFP and EV-GFP infectivity,
although the effects were generally modest, being only 15–20% for MV-GFP and 15–50% for EV-GFP. As expected, among the enzymes tested, HPSE treatment of cell monolayers had the greatest effect on the infectivity of both MV-GFP and EV-GFP, with HPSE treatment of the cells reducing MV-GFP infectivity by about 20%, whereas the same treatment reduced EV-GFP infectivity by 50% (Fig. 2e).

VACV infection studies were also performed in pgsA-745 cells, a CHO-K1 cell-derived cell line that is genetically deficient in cell surface HS, to confirm the role of HS in VACV infectivity. This mutant CHO-K1 cell line has a defect in the xylosyltransferase that mediates the first sugar transfer reaction in GAG formation [32], with this mutation leading to the disruption of the synthesis of both cell surface HS and CS in pgsA-745 cells. It has been shown that the CHO-K1 host range (hr) gene is disrupted in the WR strain of VACV, making these cells non-permissive for VACV infections [33–35], however, VACV is able to infect CHO-K1 cells and initiate the virus replication cycle. The cycle is aborted at the intermediate gene expression stage, with expression of early VACV genes remaining unperturbed. Since an early/late promoter drives transcription of the GFP-Bsd cassette in both recombinant VACV WR and VACV IHD-J constructs, VACV cell entry assays can
A paradoxical aspect of this study is the fact that although enzymatic or genetic removal of HS from cell surfaces only had a modest effect on the ability of VACV to infect cells, soluble heparin was able to inhibit the infectivity of both the MV-GFP and EV-GFP forms of VACV almost completely. This paradox is considered in detail in the discussion.

**Effects of differently sulfated HS and chemically modified heparins on MV-GFP and EV-GFP infections**

So far, heparin, a highly sulfated form of HS present in mast cells, has been used to inhibit VACV infections. Since HS exists with different levels of sulfation, it was important to determine the ability of highly sulfated HS (HS\textsuperscript{hi}) and lowly sulfated HS (HS\textsuperscript{low}) to inhibit MV and EV infections. It was found that HS\textsuperscript{hi} was a much stronger inhibitor of MV-GFP infection than HS\textsuperscript{low}, with IC\textsubscript{50} values of 12.5 µg ml\textsuperscript{-1} and >100 µg ml\textsuperscript{-1}, respectively (Fig. 3a). EV infections were more strongly inhibited by HS\textsuperscript{hi} than by HS\textsuperscript{low}, i.e. IC\textsubscript{50} values of 0.39 µg ml\textsuperscript{-1} and 12.5 µg ml\textsuperscript{-1}, respectively, a 32-fold difference (Fig. 3b). Furthermore, compared with heparin, HS\textsuperscript{hi} was a two–three-fold less effective inhibitor of EV-GFP and MV-GFP infectivity based on IC\textsubscript{50} values. These studies with HS\textsuperscript{hi} and HS\textsuperscript{low} also confirmed that EV-GFP interacts with HS with much greater avidity than MV-GFP. Thus, highly sulfated GAGs, such as heparin and HS\textsuperscript{hi}, are much more potent inhibitors of EV infections than MV infections.

In order to understand the interaction of VACV with cell surface HS to be able to design antivirals, it was essential to first understand the effect of individual sulfate groups of heparin on the ability of heparin to inhibit VACV infectivity, similar to the HSV studies previously published [2, 36, 37]. To perform these assays, chemically desulfated heparin molecules lacking 2-O-sulfate (de2S), 6-O-sulfate (de6S) or N-sulfate (deNS) groups, or which had been completely desulfated (deS), were used to inhibit EV and MV infections (Fig. 4). Overall, the desulfated heparins tested had a greater inhibitory effect on the EV form of VACV than the MV form, indicating that EV interacts with the sulfate groups of heparin (or HS) with greater avidity than MV. Some sulfate groups, however, are not crucial for the inhibition of MV or EV infectivity, with the 2-O-sulfate of the iduronic/glucuronic acid residues being the least important (Fig. 4a). Thus, heparin molecules deficient in the 2-O-sulfate group were still quite effective inhibitors of VACV infections, exhibiting IC\textsubscript{50} values for EV and MV infections that were three-fold and five-fold higher than those for heparin, respectively (Table 1). In contrast, the 6-O-sulfate and N-sulfate groups were crucial for the inhibition of VACV infectivity; removing either of these two sulfate groups had a very significant impact on the ability of heparin to inhibit EV and MV infections. Thus, for EV infections, the IC\textsubscript{50} value for heparin lacking 6-O-sulfate groups on glucosamine residues was 65-fold higher than that of untreated heparin (Fig. 4b and Table 1). Similarly, heparin molecules lacking N-sulfate groups on their glucosamine residues were 98-fold less

Fig. 3. Effect of differently sulfated HS on MV-GFP and EV-GFP infections in vitro. 143B osteosarcoma cell monolayers were infected at an m.o.i. of 1 with (a) MV-GFP (obtained from VACV WR) or (b) EV-GFP (obtained from VACV IHD-J, in the presence of doubling dilutions of heparin, HS\textsuperscript{hi} or HS\textsuperscript{low}, starting from 100 µg ml\textsuperscript{-1}. Adherent cells were released by treatment with trypsin at 6 h post-infection, fixed and analysed using flow cytometry for % GFP-positive cells relative to control cells ±SEM (n=3). The data presented are representative of four independent experiments.

potential be performed in these CHO-K1 cell lines using MV-GFP and EV-GFP.

CHO-K1 and pgsA-745 cell monolayers were infected with a low [1] and a high [10] m.o.i. of both MV-GFP and EV-GFP, with a relatively low percentage of both cell types being infected by MV-GFP (18%) and EV-GFP (12%). However, the GFP expression by the MV-GFP infected CHO-K1 and pgsA-745 cell monolayers, measured as the mean fluorescence intensity (MFI), was almost identical at both VACV m.o.i.s used (Fig. 2f). This indicates that MV entry into cells is not heavily dependent on the presence of cell surface GAGs. In contrast, GFP expression by EV-GFP infected pgsA-745 cell monolayers, compared with CHO-K1 cell monolayers, was significantly reduced, by 60% at m.o.i. 1 and 35% at m.o.i. 10.
Fig. 4. Effect of different desulfated heparins on MV-GFP and EV-GFP infections in vitro. 143B osteosarcoma cells were infected with MV-GFP or EV-GFP viruses at an m.o.i. of 1 in the presence of doubling dilutions of different desulfated heparins, namely de-2-O-sulfate (de2S) (a), de-6-O-sulfate (de6S) (b), de-N-sulfate (deNS) (c) and totally desulfated (deS) (d) heparin. Adherent 143B cells were released with trypsin at 6 h post-infection, fixed and analysed by flow cytometry for GFP expression. A diagrammatic representation of different chemically modified heparins is shown next to the line plots for the respective modified heparins. Representative line plots show the mean (±SEM; n=3) percentage of GFP-positive cells relative to control cells infected with the viruses in the absence of inhibitors (100% positive control). The ability of unmodified heparin to inhibit MV-GFP and EV-GFP infections (dashed lines) is included in each graph for comparison. The data presented are representative of three independent experiments. IC\textsubscript{50} values for different desulfated heparins are presented in Table 1.
effective than heparin at inhibiting EV infections (Fig. 4c and Table 1). Furthermore, heparin lacking either 6-O-sulfate or N-sulfate groups was much less effective at inhibiting MV infections, although IC50 values could not be obtained, as they were not reached at the highest modified heparin concentration tested. As expected, removing all sulfate groups from heparin chains completely abolished the ability of heparin to have any inhibitory effect on VACV infectivity (Fig. 4d).

In contrast to desulfation, the removal of carboxyl groups from iduronic/glucuronic acid residues of heparin had a minimal effect on the ability of heparin to inhibit both MV and EV infections (Fig. 5a), with the IC50 values for EV and MV infections being, respectively, only 1.5-fold and 3-fold higher than for unmodified heparin. Although removal of the N-sulfate group from glucosamine residues dramatically reduced the ability of heparin to inhibit VACV infections (Fig. 4c), subsequent N-acetylation of glucosamine residues almost completely restored the ability of N-desulfated heparin to inhibit MV and EV infections (Fig. 5b), with the IC50 values for EV and MV infections being, respectively, two- and four-fold higher than those for untreated heparin (Table 1). These data suggest that the introduction of a positively charged amino group into glucosamine affects the inhibitory activity of heparin rather than the loss of N-sulfate. However, if the 2-O-sulfate group of N-desulfated/N-acetylated heparin is also deleted, the ability of heparin to inhibit MV and EV infections is further hampered (Fig. 5c). Finally, although chemical treatment of heparin to remove all three types of sulfate groups from the polysaccharide chain resulted in the total loss of inhibitory activity (Fig. 4d), re-N-sulfation of desulfated heparin only resulted in a slight restoration of inhibitory activity, based on IC50 values, with deS, reNS heparins being 200-fold less effective than heparin at inhibiting EV infections (Fig. 5d and Table 1). Furthermore, re-N-sulfation had no detectable effect on the inability of desulfated heparin to inhibit MV infections. Collectively, these data suggest that the 6-O-sulfate on glucosamine residues is the most important negatively charged group on heparin chains that interacts with VACV.

**Effects of different sulfated saccharides (HS mimetics) on MV-GFP and EV-GFP infections**

Heparin and HS not only interact with many viruses, but also bind to a wide range of functionally diverse proteins, such as growth factors, proteases, lipases, cell adhesion molecules and cytokines [38–43]. This plethora of interactions results in structurally diverse heparin having many undesirable side-effects in vivo. As a result, several small soluble polysulfated compounds (HS mimetics) have been developed that lack many of the undesirable interactions of heparin with HS binding proteins [44, 45]. In order to identify synthetic heparin/HS mimetics that could be used as antivirals for VACV infections, sulfated di- and/or oligo-saccharides of the D-glucose-based disaccharides maltose (Fig. S3), isomaltose (Fig. S6a–c), cellobiose (Fig. S6d–f) and laminaribiose (Fig. S7) were examined for their effects on VACV infections in vitro.

Of the four sulfated maltose-based saccharides tested, maltotetraose appeared to be the most potent inhibitor of EV infectivity with an IC50 value that was only four-fold higher than that of heparin (Table 2 and Fig. S5d). Maltotriose sulfated was not as potent inhibitor of EV infection as maltotetraose sulfated, but was more effective than maltose and maltotetraose sulfated, with maltotriose sulfated being 16-fold and maltose and maltotetraose sulfated being 50-fold less effective than heparin at inhibiting EV infections (Table 2 and Fig. S5). None of the sulfated maltose-based saccharides achieved IC50 values for MV infections, although the three sulfated malto-oligosaccharides were weakly inhibitory, with sulfated maltotetraose being the most active.

Of the three sulfated isomaltose-based oligosaccharides used as inhibitors of VACV infection, isomaltotetraose sulfated appeared to be the most effective inhibitor of EV and MV infections, with the IC50 values for EV and MV infections being, respectively, eight-fold and five-fold higher than those for heparin (Table 2 and Fig. S6c). Both of the other two sulfated isomaltose-based oligosaccharides, sulfated isomaltotetraose and isomaltohexaose, were much less effective inhibitors of EV infections (Table 2 and Fig. S6c–e), being 49-fold less active than heparin (Table 2). Similarly, isomaltotetraose and isomaltohexaose sulfated were weak inhibitors of MV infections, barely achieving IC50 values (Table 2).

Three cellobiose-based sulfated saccharides were tested, namely cellotetraose, cellopentaose and cellohexaose sulfated (Table 2 and Fig. S6d–f), and, overall, they appeared to be better inhibitors of VACV infections compared to the sulfated malto- and isomalto-oligosaccharides. In fact, all three oligosaccharides exhibited comparable antiviral activity against both the EV and MV infections, being ~8–16-fold

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**Table 1.** Comparison of IC50 values of various chemically modified heparins for EV and MV infections in vitro

<table>
<thead>
<tr>
<th>Heparin species*</th>
<th>IC50 (µg ml⁻¹) (fold change)†</th>
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<tbody>
<tr>
<td></td>
<td>EV</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.19</td>
</tr>
<tr>
<td>de2S</td>
<td>0.58</td>
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<tr>
<td>de6S</td>
<td>12.5</td>
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<td>deNS</td>
<td>18.75</td>
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<tr>
<td>deS</td>
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<tr>
<td>deCarboxyl</td>
<td>0.29</td>
</tr>
<tr>
<td>deNS, reNAc</td>
<td>0.39</td>
</tr>
<tr>
<td>de2S, deNS/reNAc</td>
<td>1.17</td>
</tr>
<tr>
<td>deS, reNS</td>
<td>37.5</td>
</tr>
</tbody>
</table>

*LMWH, low-molecular-weight heparin; de2S, de-2-O-sulfate; de6S, de-6-O-sulfate; deNS, de-N-sulfate; deS, de-sulfate; deCarboxyl, de-carboxylate; reNAc, re-N-acetyl; reNS, re-N-sulfate.

†Fold change (given in bold) represent the difference in IC50 values for the chemically modified heparins versus unmodified heparin.
Fig. 5. Effect of chemically modified heparins on MV-GFP and EV-GFP infections in vitro. 143B osteosarcoma cells were infected with MV-GFP or EV-GFP viruses at an m.o.i. of 1 in the presence of doubling dilutions of different chemically modified heparins, namely de-carboxylated (deCarboxyl) (a); de-N-sulfate, re-N-acetylated (deNS, reNAc) (b); de-2-O-sulfate, de-N-sulfate and re-N-acetylated (de2S, deNS/reNAc) (c); and de-sulfate, re-N-acetylated (deS, reNS) (d). Data were generated and presented as in Fig. 4. The diagrammatic representation of different chemically modified heparins is shown next to the line plots for respective modified heparins. IC\textsubscript{50} values for different modified heparins are shown in Table 1.
less effective than heparin (Table 2). Also, four sulfated laminarin-based saccharides, namely laminaribiase, laminaritriose, laminaritetraose and laminaripentaose, were tested for their ability to inhibit VACV infections in vitro (Fig. S7). All four had little or no effect on VACV infectivity, being 100–500-fold less effective than heparin against EV, and exhibiting no detectable inhibitory activity against MV (Table 2).

Finally, PI-88 (Muparfostat), a highly sulfated synthetic oligosaccharide derived from the extracellular phosphomannan produced by the yeast Pichia holstii [45, 46], was tested for anti-VACV activity. PI-88 is a multi-component mixture that is composed predominantly of phosphomannopentaose and phosphomannotetraose sulfates, which account for over 90% of the mixture (Fig. 6a) [47]. It has been developed as an anti-cancer drug that inhibits cancer metastasis and angiogenesis. PI-88 has the advantage that it has been used extensively in patients and, consequently, could be readily used clinically as an antiviral therapy. In fact, a previous report suggested that PI-88 could have potential antiviral effects in vivo [48].

PI-88-mediated inhibition of VACV infections gave some unexpected results (Fig. 6b), it being the only sulfated oligosaccharide tested in this study that could inhibit MV infections more efficiently than EV infections, with IC50 values for MV infections being 1.17 µg ml⁻¹ compared to 3.125 µg ml⁻¹ for EV infections. Moreover, PI-88 was a four-fold better inhibitor of MV infections than heparin. On the other hand, with EV infections, heparin was a much better inhibitor (i.e. 16-fold) than PI-88 (Fig. 6b and Table 2).

### Table 2. Comparison of IC50 values of various sulfated saccharides for EV and MV infections in vitro

<table>
<thead>
<tr>
<th>Sulfated saccharides</th>
<th>IC50 (µg ml⁻¹) (fold change)*</th>
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<tr>
<td>Heparin</td>
<td>0.19 (1)</td>
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<tr>
<td>Maltose</td>
<td>50 (263)</td>
<td>&gt;100 (&gt;21)</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>3.125 (16)</td>
<td>&gt;100 (&gt;21)</td>
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<tr>
<td>Maltotetraose</td>
<td>50 (263)</td>
<td>&gt;100 (&gt;21)</td>
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<tr>
<td>Maltopentaose</td>
<td>0.78 (4)</td>
<td>&gt;100 (&gt;21)</td>
</tr>
<tr>
<td>Isomaltotetraose</td>
<td>9.38 (49)</td>
<td>&gt;100 (&gt;21)</td>
</tr>
<tr>
<td>Isomaltopentaose</td>
<td>1.56 (8)</td>
<td>25 (5)</td>
</tr>
<tr>
<td>Isomaltohexaose</td>
<td>9.38 (49)</td>
<td>100 (21)</td>
</tr>
<tr>
<td>Celloctetraose</td>
<td>1.56 (8)</td>
<td>50 (11)</td>
</tr>
<tr>
<td>Cellobetaose</td>
<td>1.56 (8)</td>
<td>50 (11)</td>
</tr>
<tr>
<td>Cellobetaose</td>
<td>3.125 (16)</td>
<td>50 (11)</td>
</tr>
<tr>
<td>Laminaribiase</td>
<td>75 (395)</td>
<td>&gt;100 (&gt;21)</td>
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<td>Laminaritriose</td>
<td>100 (526)</td>
<td>&gt;100 (&gt;21)</td>
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<tr>
<td>Laminartetraose</td>
<td>25 (132)</td>
<td>&gt;100 (&gt;21)</td>
</tr>
<tr>
<td>Laminaripentaose</td>
<td>75 (395)</td>
<td>&gt;100 (&gt;21)</td>
</tr>
<tr>
<td>PI-88 (Muparfostat)</td>
<td>3.125 (16)</td>
<td>1.17 (0.25)</td>
</tr>
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</table>

*Fold change (given in bold) represents the difference in IC50 values for the sulfated saccharides versus unmodified heparin.

### DISCUSSION

The findings reported herein represent one of the few attempts at establishing the importance of heparin/HS recognition in a viral infection and as a target for antivirals, with VACV being chosen for the study due to the existence of two forms of the virus, MV and EV, with potentially different HS binding properties. This study had two main aims, first to determine the importance of cell surface HS in the infectivity of the MV and the EV forms of VACV, and second to investigate the structural features of heparin/HS required for inhibition of the infectivity of the two forms of VACV, with the long-term aim of developing antiviral agents.

Initial studies showed that soluble heparin reduced the plaque forming ability of both the MV-rich WR and EV-rich IHD-J strains of VACV, with the EV rich-IHD-J strain also exhibiting a dramatic loss of trademark comet-shaped...
plagues (Fig. 1). The study took a paradoxical turn when it was demonstrated that the enzymatic or genetic removal of HS from cell surfaces only had a modest effect on the ability of VACV to infect cells, whereas soluble heparin and some HS mimetics could almost completely inhibit the infectivity of both the MV-GFP and EV-GFP forms of VACV, with heparin inhibiting EV-GFP infectivity 16-fold more efficiently than MV-GFP, based on the IC₅₀ values (Fig. 2b). A possible explanation for this paradox is that the heparin and HS mimetics sterically hinder adjacent receptors on VACV that are essential for infecting cells. However, this explanation is unlikely as the HS mimetics are very small (1–2.5 kDa) with molecular weights 5–10-fold lower than heparin. In fact, one HS mimetic (PI-88) was a four-fold more effective inhibitor of MV infections than heparin (Fig. 6), a finding that is inconsistent with the steric hindrance hypothesis. A more likely explanation is that heparin binding to free VACV initiates an abortive infection in solution. Previous studies support this hypothesis, with it being shown that heparin binding to the EV form of VACV can result in the loss of the outer viral membrane [49]. Whatever the explanation for this paradox, it is clear that HS mimetics have considerable potential as antivirals against HS-binding viruses, with their antiviral properties involving much more than merely blocking HS-binding receptors on the surface of viruses.

With regard to the enzymatic and genetic removal of HS not markedly inhibiting infectivity, it is possible that enzymatic treatment to remove cell surface HS is incomplete, allowing virus particles to interact with ‘stubs’ left behind after the treatment and, therefore, initiate a slow but successful infection. Furthermore, VACV is also able to enter cells via a low-pH endosomal pathway or by direct fusion with the plasma membrane at pH 7 [50], a mechanism that would be independent of cell surface HS recognition and is likely to be the case in cells genetically deficient in cell surface HS.

Several studies have established that HS is the second largest group of carbohydrate-based cell surface receptors responsible for tissue specific tropism for viruses like influenza and HSV [51, 52]. Therefore, a detailed understanding of heparin/HS chain structure is crucial for the development of antivirals that are not only potent, but also safe to be administered. Initially, a simple evaluation of the effects of sulfation on the ability of HS to inhibit VACV revealed that highly sulfated HS (HS₄ₛ) molecules were more efficient at inhibiting EV infections than lowly sulfated (HS₂ₒ) molecules (Fig. 3), with inhibitory activity resembling that of heparin. Since heparin and HS₄ₛ share a very similar structure [8], although heparin is not present in the extracellular matrix like HS₂ₒ, it can be used as a readily available HS₄ₛ-like molecule for structural studies.

Furthermore, previous detailed studies investigating HSV attachment and infection of cells in vitro revealed that 2,3-O-sulfate and 6-O-sulfate groups are critical for heparin to interact with the gB and gC glycoproteins on the surface of HSV-1 (38, 39). Similarly, in the context of VACV, removal of N- and 6-O-sulfate groups from heparin chains was shown to have the most detrimental effect on heparin-dependent inhibition of VACV (Fig. 4). In contrast, removal of 2-O-sulfate groups or N-acetylation of all glucosamine residues along the heparin chain did not markedly affect the inhibitory ability of heparin for VACV infections, and nor did removal of the carboxyl groups of the iduronic/glucuronic acid residues of heparin (Fig. 5).

Furthermore, when several HS mimetics consisting of sulfated di- and oligo-saccharides of D-glucose were tested for their anti-VACV activity, the maltose-based saccharide series (Fig. S5), and sulfated isomaltopentaose from the isomaltose-based saccharide series (Fig. S6) were identified as the most efficient inhibitors of infection (activity approaching that of heparin). However, as with heparin, the HS mimetics were more effective inhibitors of the EV form of VACV than the MV form. These findings also suggest that an ideal carbohydrate-based inhibitor of VACV infectivity would be a sulfated pentasaccharide sequence, and that the glycosidic linkage of different D-glucose-based sulfated saccharides has a profound effect on the ability of the sulfated saccharides to inhibit VACV infections, with the order of potency being β(1→4)α(1→6)α(1→4)β(1→3) (Table S1). These findings support the hypothesis that the position of negatively charged sulfate groups in 3D space determines the antiviral activity of these molecules and, therefore, builds on the notion of a ‘sulfation code’ [53].

Interestingly, out of all the different modified heparins and HS mimetics tested, the heparanase inhibitor PI-88 (Mupafostat) was the only compound that was more effective at inhibiting MV compared to EV infectivity (Fig. 6). PI-88 is a sulfated oligosaccharide composed of α(1→3)- and α(1→2)-linked D-mannose residues [47], which differs in its monosaccharide constituents and glycosidic linkages from the D-glucose-based HS mimetics. Therefore, the remarkable effectiveness of PI-88 against MV infections, but not EV infections, could be attributed to the linkage of D-mannose residues, resulting in an orientation of sulfate groups that selectively blocks the interactions of MV particles with cell surface HS. Moreover, unlike other GAG-based molecules, PI-88 is highly heterogeneous, consisting of pentasaccharide and tetrasaccharide sulfates, along with small quantities of di-, tri- and hexa-phosphomannosulfates and a phosphotetrasaccharylamine sulfate [54]. Such a heterogeneous mixture may have the potential to target multiple HS binding sites on the surface of MV particles and, therefore, block the particles from interacting with cell surface HS. PI-88 has previously been demonstrated to have significant beneficial effects on disease outcomes in dengue virus and flaviviral encephalitis mouse models, such that the mortality rate in PI-88-treated mice was reduced to 39 % compared to 84 % in control untreated mice [48]. Furthermore, our results obtained with PI-88 inhibition of VACV infections support the hypothesis that cell surface HS receptors for MV and EV are structurally different. Collectively, our findings using MV and EV forms of VACV indicate that...
when designing HS-based antivirals, the amount of sulfation, the position of the sulfate groups, the glycosidic linkages and the saccharide chain length should be taken into consideration, as these structural features define the inhibitory activity of the HS mimetics and their effects on enveloped and non-enveloped viruses. HS mimetics, therefore, have considerable potential as antivirals against HS-binding viruses. Future studies should also address the nature of the HS-binding proteins on the surface of HS-binding viruses that are targeted by HS mimetics.

**METHODS**

**Cell culture**

Human osteosarcoma 143B cells (ATCC) were grown in MEM medium (GIBCO/Invitrogen, Grand Island, NY, USA) supplemented with 5 % heat-inactivated foetal bovine serum (FBS; GE Healthcare, Pittsburg, PA, USA), 50 µg ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin and 50 µg ml⁻¹ neomycin (all provided by Media Unit, JCSMR). Baby hamster kidney BHK-21 cells (ATCC), Chinese hamster ovary CHO-K1 cells and xylosyltransferase-I-deficient Chinese hamster ovary pgsA-745 cells (kindly provided by Dr Eva Lee, ANU), were all grown in RPMI-1640 (GIBCO/Invitrogen). Detached cells were subcultured at a cell concentration of 2 × 10⁵ cells ml⁻¹.

**Heparins, modified heparins and sulfated saccharides (HS mimetics)**

Heparin from porcine mucosa was supplied by Sigma-Aldrich, St Louis, MO, USA. Porcine mucosal HS⁺⁺ (HI-11098) and HS⁻⁻ (HO-10595) were obtained from Celsus Laboratories, Cincinnati, OH, USA. The chemically modified heparins and sulfated saccharides (HS mimetics) were a kind gift from Dr Craig Freeman (ANU). PI-88 (Muparfostat), a HS mimetic, was provided by Progen Pharmaceuticals (Brisbane, Australia).

**VACV strains**

The two strains of VACV used in this study were the International Health Department strain (IHD-J) (kindly provided by Dr David Tscharke, ANU) and the Western Reserve strain (WR). Green fluorescent protein (GFP) expressing recombinant IHD-J or WR strains of VACV were constructed as described in the Supplementary Material.

**Virus titration, plaque assays and plaque inhibition assays**

For the purpose of VACV titrations, 143B cells were used instead of BHK-21 cells, as VACV forms much larger plaques with these cells. Virus plaquing and titrations were performed as described previously [55]. The plaque diameters were measured using ImageJ particle analysis software with the following settings – pixel size, 0.05–5; circularity, 0.0–1.0 – and using an Olympus IX81 fluorescence microscope.

**Heparanase treatment of cells**

Cell surface HS on 143B cells was digested using either Flavobacterium heparinases (HPNSE) I, II and III (Sigma-Aldrich) or human platelet heparanase (HPSE) prepared as previously described [56]. Confluent 143B cell monolayers in 48-well flat bottom plates were washed with sterile PBS and then treated for 1 h at 37 °C with either bacterial HPNSES I, II and III (0.25 units ml⁻¹) individually or as a cocktail of all three enzymes, or with mammalian HPSE (4 µg ml⁻¹) in pH 5.5 serum-free MEM (100 µl well⁻¹). After incubation, the cell monolayers were washed with PBS and either detached and stained for cell surface HS expression or kept as monolayers and infected with VACV.

**Flow cytometry assays – HS staining**

HPNSE- or HPSE-treated 143B cell monolayers were detached by incubation with PBS containing 0.25 % trypsin-EDTA (GIBCO/Invitrogen) (100 µl/well) for 5 min at 37 °C. The cells were washed once with FACS buffer (PBS supplemented with 0.1 % BSA) and stained with the HS-specific mAb 10E4 (4 µg ml⁻¹) (SouthernBiotech, Birmingham, AL, USA) for 30 min at 4 °C. A mouse IgM κ-myeloma (20 µg ml⁻¹) (clone: C48-6; BD Pharmingen) was used as an isotype control with a secondary goat F(ab)₂ anti-mouse IgM-PE (2.5 µg ml⁻¹) (SouthernBiotech, Birmingham, AL, USA) being used to detect surface bound antibodies, and PE fluorescence being detected by a LSR benchtop flow cytometer and FlowJo software.

**Flow cytometry assays – infectivity of GFP-expressing VACV strains**

Purified MV-GFP and EV-GFP forms of VACV (see the Supplementary Material for the purification protocols) were used for FACS-based infection assays. Doubling dilutions of heparin, modified heparins or HS mimetics (0.09 µg ml⁻¹–100 µg ml⁻¹) in serum-free MEM were incubated with a virus m.o.i. of 1 for 1 h at 4 °C prior to addition (100 µl well⁻¹) to confluent 143B cell monolayers in 48-well plates and incubated for 1 h at 37 °C. Virus preparations without added inhibitors served as positive controls, whereas wells receiving no virus served as negative controls. In the case of heparanase-treated 143B osteosarcoma cell monolayers or HS-deficient CHO-K1 cells (pgsA-745), both MV-GFP and EV-GFP were used to infect the treated or deficient cells in 48-well plates, with untreated 143B cells or wild-type CHO-K1 cells being used as positive controls, respectively. Virus mixtures were then aspirated, the monolayers were washed with sterile PBS, fresh MEM (supplemented with 5 % FBS) was added (500 µl well⁻¹) and the cells were incubated for a further 2–24 h at 37 °C. All culture medium was then aspirated, 0.25 % trypsin-EDTA was added (100 µl/well) and the wells were incubated for 5 min at 37 °C, with detached cells being fixed in 0.5 % paraformaldehyde (100 µl well⁻¹) and run on a BD FACS Calibur, with 30 000 gated events.
being collected. Data collection and analysis were performed using FlowJo software.

**Statistical analysis**

The Mann–Whitney nonparametric t-test, one-way ANOVA (Dunnett’s multiple comparison) and two-way ANOVA (Bonferroni multiple comparisons) tests were performed using GraphPad Prism 5.0f software (GraphPad, Inc).

**Funding information**

C. R. P. was funded by a National Health and Medical Research Council (NHMRC) programme grant.

**Acknowledgements**

The authors would like to thank Dr Craig Freeman (ANU) for providing chemically modified heparins and heparan sulfate mimetics, Professor David Tscharke (ANU) for providing the IHD-J strain of Vaccinia virus and Dr Eva Lee (ANU) for providing CHO-K1 and pgsA-745 cells. PI-88 was provided by Progen Pharmaceuticals (Brisbane, Australia).

**Conflicts of interest**

We declare that there are no conflicts of interest.

**References**

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