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Herpes simplex virus type 1 shows multiple interactions with sulfonated compounds at binding, penetration, and cell-to-cell passage

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Abstract Herpes simplex virus type 1 (HSV-1) uses multicomponent mechanisms for binding, penetration, and cell-to-cell passage. These processes are affected by polysulfonated compounds. In this paper we have addressed the question of whether the same or different interactions of HSV-1 with polysulfonated compounds are involved in binding, penetration, and passage. For this, we have compared the inhibitory dose–response for a series of polysulfonated and cationic compounds known to block HSV-1 infections. These comparisons were done at the level of binding, penetration, and cell-to-cell passage. Variations in the parameters of the dose–response curves—IC_{50} and Hill coefficients (n_H)—are consistent with HSV-1 having multiple interactions with sulfonated cellular components in all these processes. Some of the interactions seem to be common to the three processes, while others are particular for each one.

Keywords HSV-1 · Infection · Polysulfonates · Binding · Penetration · Passage

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Introduction

Herpes simplex virus type 1 (HSV-1) may utilize multiple-component mechanisms for binding, penetration, and cell-to-cell passage involving different glycoproteins [1]. The initial attachment of HSV-1 to the cell is mediated by glycoprotein C (gC) interacting mainly with heparan sulfate—a glycosaminoglycan (GAG)—on the cell membrane; however, when gC is absent, gB can bind properly to the cells in vitro [2–4]. After binding, there is a fusion of the viral envelope with the cell membrane. This fusion is mediated by gB, gD, gK, and the complex gH–gL [3, 5–9]. Glycoprotein D exerts its action by interacting specifically with several cellular proteins that can serve as receptors for HSV-1 and other related viruses; the cellular proteins most frequently used as receptors belong to the nectin family [see 10–13 for reviews]. Cell-to-cell passage seems to be a process with overlapping but different mechanisms from virus entry, because it requires the same set of glycoproteins involved in virus entry plus the complex gE–gI that localizes at the cell–cell junctions facilitating virus transmission at these sites [14–16]. In addition, gG, gM, and U_L45 participate in cell-to-cell passage of syncytial strains, but have no role in viral entry [17–19]. The fact that some mutations in the same viral gene have different effects on cell entry and in cell fusion is further evidence that the mechanisms operating for the two processes are not completely congruent. [20].

GAGs are heteropolysaccharides with a characteristic repeat sequence, which are variably N- or O-sulfated and are involved in a diversity of cellular functions [21, 22]. Many virus, bacteria, fungi, parasites [23, 24], and, perhaps, prions [25] use GAGs as receptor and coreceptor molecules for cell attachment

and cell penetration. This is the case for HSV-1 infections in cells, as it has been known for many years that the initial binding of HSV-1 to cell membranes is mediated by heparan sulfate glycoproteins [26–28]. However, later evidence indicates that GAGs may also affect penetration and passage. Laquerre et al. [4] showed that virus with mutant gB, which does not bind to heparin (analog of heparan sulfate), had an slower rate of penetration, and formed smaller plaques, indicating that these types of interactions are important in penetration and in cell-to-cell passage. In agreement with this possibility, we showed that heparin and the polysulfonated compound suramin block productive infection after virus penetration and also reduce the size of the plaques [29]. Plaque reduction after penetration has been confirmed for other polysulfonates both in HSV-1 [30] and HSV-2 infections [31].

Since the processes of entry and cell-to-cell passage share some of their mechanisms, a relevant question is whether the same or different GAGs interactions—with either viral or cellular factors—are involved in binding penetration and cell-to-cell passage. To address this question we have compared the dose–response of the inhibition of productive infection by a series of sulfated polysaccharides and cationic compounds, known to inhibit virus binding [32–34]. Specifically, we have compared the IC_{50} (a measure of the strength of the interactions) and the Hill coefficients (n_H, an indicator of the complexity of the interactions). This comparison was done at the level of virus binding, penetration, and cell-to-cell passage. Our results are consistent with HSV-1 showing multiple interactions with sulfated GAGs in these three processes, with some of the interactions common and others particular for each individual process.

Materials and methods

Cells and viruses

We used rabbit skin fibrobrasts to grow the construct HSV-1 (dUTPase/LAT), in which the β-galactosidase gene is under the control of the dUTPase promoter (an early gene) at LAT site [35, 36]. Vero cells were used to analyze the effect of the different compounds. For this analysis, Vero cells cultures 90–100% confluent, in 24-well plates (~10^5 cells/well) were infected with 1000 PFU of this recombinant virus (multiplicity of infection of 0.01 PFU/cell). Rabbit skin fibroblast and Vero cells were cultured in Eagle's minimum essential medium (EMEM) containing 5% bovine serum and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin; HFF were cultured in EMEM containing 10% fetal calf serum and 100 μM G418. The cultures were maintained at 37°C under 5% CO_2.

Chemicals

Heparin, chondroitin, and suramin were purchased form Sigma, Dextran Sulfate from Pharmacia, De-N-acetylated Heparin, 2-O-desulfated Heparin, and 6-O-desulfated heparin were from Neoparin. All other reagents were of analytical grade.

Cytotoxicity

The cytotoxic effect of each compound was evaluated by the method of sulforhodamin B (SRB) [37]. Cells in 96-well plates were treated with different concentrations of each compound for either 8 or 24 h. After the treatment, the wells were washed three times with saline and fresh medium was added. Forty-eight hours after the initiation of the treatment with a compound, cells were fixed with 10% trichloroacetic acid (TCA) for 30 min at room temperature (RT). TCA was washed and the wells were dried overnight at RT. The cells were stained with 4% SRB in 1% acetic acid for 30 min at RT, washed with 1% acetic acid and dried overnight. SRB was extracted with 0.2 ml of 1 mM Tris Base and the densitometry read at 520 nm in an ELISA reader. Values were expressed as percent of controls from untreated wells in the same plate.

Binding

Binding of HSV-1 (dUTPase/LAT) to cells were carried out at 0°C in the presence and absence (control) of the compound to study (Fig. 1A). At this temperature the virus can bind, but cannot penetrate the cells [38]. After this incubation, the cells were washed three times with ice-cold saline solution to remove the unbound virus and compound. Then, warm overlay medium was added and the infection was allowed to proceed for 22–24 h at 37°C. In these conditions, the level of binding can be correlated with the level of productive infection [29]. The level of infection was determined by the expression of β-galactosidase reporter gene using the procedure previously described [29] utilizing 5 mM red-β-galactopyranoside (Boeringer Mannheim) as substrate. Using this procedure, we determined that for Vero cells maximum binding at 0°C is reached between 3 and 4 h. To determine the IC_{50} of each compound for blocking the binding of HSV-1, cells were incubated for 3 h with the virus, in the presence of different concentrations of the compound studied.

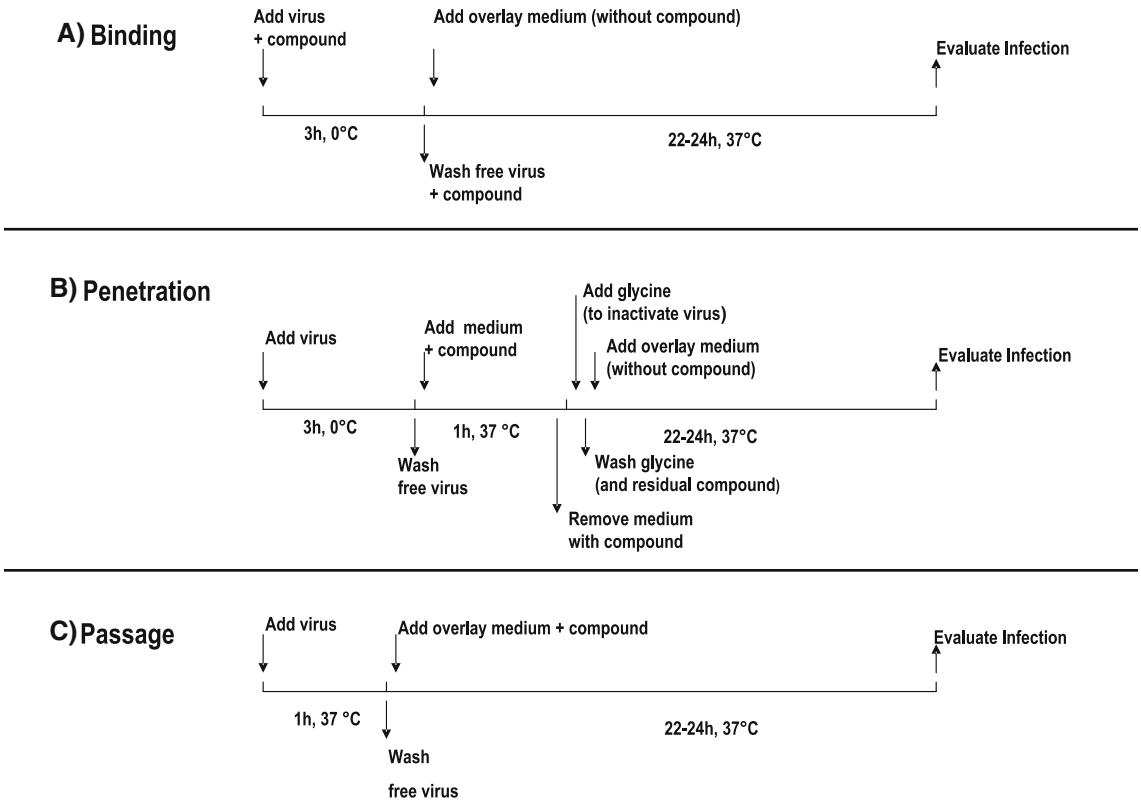

Fig. 1 Diagrams of the methods for: (**A**) Binding; (**B**) Penetration; (**C**) Passage

Penetration

For the study of penetration (Fig. 1B), HSV-1 (dUTPase/LAT) was bound to cells at 0°C for 3 h. After this incubation, the cells were washed three times with ice-cold saline solution to remove the unbound virus. After binding, penetration was initiated by adding overlay medium containing the compound to study at 37°C. After the maximum level of penetration was reached (see results), the overlay was removed and the cells washed once with PBS. The non-penetrated virus was inactivated by treating the infected cultures with 0.1 M glycine pH 3.0 for 1 min, and then the cells were washed twice with PBS to remove glycine and residual compound. Overlay media (without the compound under study) was replaced and incubation continued for 22–24 h at 37°C. The level of infection —that is correlated with the level of penetration—was determined by the β-galactosidase reporter gene activity as described previously.

Cell-to-cell passage

Cell-to-cell passage was determined by the same procedure described previously [29]. After virus penetration, the cells were washed with saline, overlay media containing the different compounds under study were added, and the level of infection determined by β-galactosidase reporter gene activity, as described above (Fig. 1C).

Data analysis

Data were analyzed using Graph Pad Prism 4 software. Curves were adjusted by a nonlinear regression analysis, and parameters were obtained using a sigmoidal dose–response model with variable slope. This model utilizes the equation: $I/I_0 = (1 + IC_{50}/[\text{compound}]^n)$, where I is the level of infection at a given compound concentration ([compound]), I_0 is the level of infection in the absence of compound (control), IC_{50} is the concentration of compound causing 50% inhibition and n is the Hill coefficient (n_H). Data for polysaccharides compounds were expressed in mg/ml instead of molar because they are a mixture of polymers.

Results

Cytotoxycity

The toxicity to cells of the different compounds utilized to block HSV-1 productive infection was deter-

mined at 8 and 24-h exposure. Heparin, chondroitin, and dextran sulfate at 5 mg/ml and suramin at 500 μM had no detectable toxic effect both after 8 and 24-h treatment. On the other hand, polylysine and neomycin show clear toxic effect at the higher concentrations used after 24-h treatment, but only a small effect after 8-h treatment (Fig. 2). This toxicity was not extensive enough to cause significant effect during the short time exposure in binding and penetration experiments, but interfered with after-penetration experiments.

Binding

It is known that at 0°C, HSV-1 can bind to cells but do not penetrate [38]. Therefore, to analyze the effect of the compounds on HSV-1 binding, cells were incubated with the virus at 0°C in the presence or absence (control) of the compound to be studied. After the appropriate length of incubation virus and compound were washed out, fresh medium was added and the infection continued for 22–24 h. At this time the level of infection was determined by the activity of a *β*-galactosidase reporter gene as we have previously described [29, 39].

We first determined the time course of this binding in order to establish an appropriate incubation period for the compounds studied. As shown in Fig. 3, a plateau is obtained after 3-h incubation at 0°C. Therefore, binding studies were carried out for 3 h at 0°C (Fig. 4, Table 1).

The inhibiton potencies (inverse value of IC_{50}) of the sulfated polysaccharides were in the rank Dextran > Heparin > De-N-acetylated heparin > chondroitin and for non-polysaccharides polylysine > suramin > neomycine. It is worth noting that all the

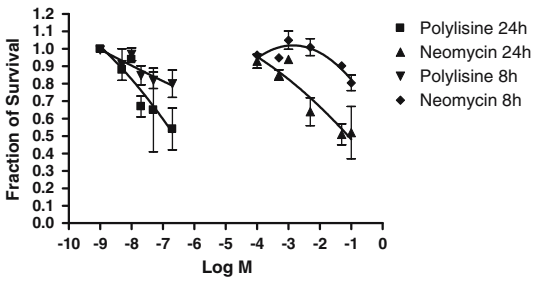

Fig. 2 Cytotoxic effect of polylysine and neomycin to Vero cells. The compound were added to the culture media at the indicated concentrations and left for either 8 or 24 h. After this time, the media were removed, and fresh media (without the compound) were added. Forty eight hours after the initiation of the treatment, survival cells were evaluated by sulforhodamine B method. Values are expressed as fraction of controls. Data are the average ±SD of at least three experiments by triplicate

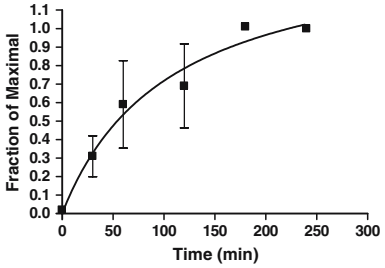

Fig. 3 Time-course of HSV-1 binding to Vero cell. Confluent Vero cells in 24-well plates were placed on ice for 5 min. The media were removed and the cells rinsed with ice-cold saline. HSV-1 (dUTPase/LAT) at MOI 0.01 in cold PBS-glucose was added and the incubation continued on ice for the indicated times. The cells were washed three times with ice-cold saline, warm ovelay medium was added and the incubation continued for 22–24 h at 37°C. Infection was evaluated by the level of *β*-galactosidase reporter gene. Points are the average ±SD of three separate determinations by duplicate

sulfated polycarbonates showed n_H smaller than 1 (Table 1). This is an indication of heterogeneous binding sites, or homogenous sites with negative cooperativity [40]; that is, occupation of one site difficult the occupation of another identical one. Other compounds, including the polysulfonate suramin, have n_H not dif-

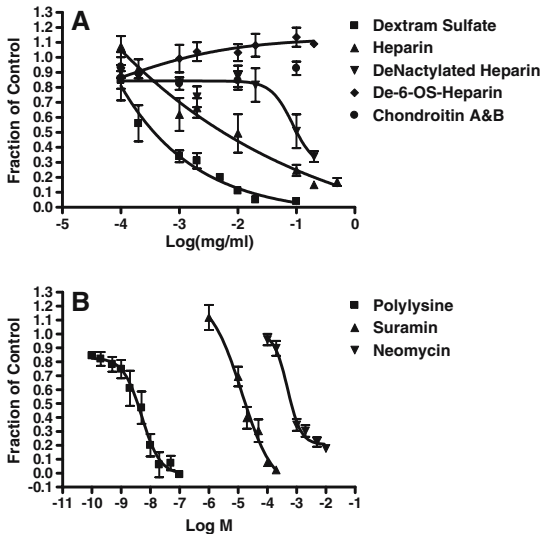

Fig. 4 Dose–response blockade of HSV-1 infection at the binding step. Confluent Vero cells in 24-well plates were placed on ice for 5 min. The media were removed and the cells rinsed with ice-cold saline. HSV-1(dUTPase/LAT) at MOI 0.01 was added in cold PBS-glucose containing the studied compound at the indicated concentrations. The incubations were continued on ice for 3 h. The cells were washed three times with ice-cold saline, warm ovelay medium was added and the incubation was continued for 22–24 h at 37°C. Infection was evaluated as the level of *β*-galactosidase reporter gene. Points are the average ±SD of at least 3 separate determinations by duplicate. (**A**) polysaccharide compounds, (**B**) non-polysaccharide compounds

Table 1 Dose–response parameters[a]

	Binding		Penetration		Passage	
	IC_{50}^{b}	n_H	IC_{50}^{b}	n_H	IC_{50}^{b}	n_H
Polysaccharides						
Dextran sulfate	$4.9 \pm 0.3 \times 10^{-4}$	0.69 ± 0.09	$2.4 \pm 0.6 \times 10^{-2}$	0.48 ± 0.05	$1.3 +0.3 \times 10^{-3}$	1.10 ± 0.33
Heparin	$8.7 \pm 0.6 \times 10^{-3}$	0.53 ± 0.08	$8.8 \pm 5.4 \times 10^{-1}$	0.41 ± 0.12	$3.0 \pm 0.6 \times 10^{-2}$	0.58 ± 0.05
De-N-acetylated Heparin	$1.1 \pm 1.1 \times 10^{-1}$	0.43 ± 0.12	>1.0		$3.5 \pm 1.3 \times 10^{-1}$	0.75 ± 0.21
Chondroitin	$9.7 \pm 2.0 \times 10^{-1}$	0.50 ± 0.17	>1.0		>1.0	
Non-Polysaccharides						
Suramin	$1.8 \pm 0.4 \times 10^{-5}$	1.30 ± 0.33	$1.6 \pm 0.5 \times 10^{-4}$	0.83 ± 0.23	$3.3 \pm 0.5 \times 10^{-5}$	3.1 ± 0.9
Polylysine	$2.8 \pm 0.5 \times 10^{-5}$	0.86 ± 0.12	$1.1 \pm 0.4 \times 10^{-7}$	0.73 ± 0.19		
Neomycin	$9.0 \pm 0.3 \times 10^{-4}$	1.0 ± 0.2	$2.2 \pm 0.2 \times 10^{-3}$	1.77 ± 0.3		

[a]Dose–response parameters were calculated as indicated in Materials and Methods, utilizing the equation: $I/I_0 = (1 + IC_{50}/[\text{compound}]^n)$, where I is the level of infection at a given compound concentration ([compound]), I_0 is the level of infection in the absence of compound (control), IC_{50} is the concentration of compound causing 50% inhibition and n is the Hill coefficient (n_H)

[b]IC_{50} is expressed in mg/ml for polysaccharide compounds and as molar for non-polysaccharide

ferent from 1, which is consistent with homogenous sites without cooperativity.

Penetration

For studying penetration, the virus was bound for 3 h at 0°C. After this period the virus was allowed to penetrate at 37°C for different lengths of time, before being inactivated with glycine. After this, cells were washed and culture media were added. Infection was allowed to proceed for 22–24 h and reporter gene activity was measured as an evaluation of original virus entry, and, thus, penetration. As shown in Fig. 5, a plateau was observed after 1-h incubation at 37°C. The time course of penetration was biphasic with near 60% of the penetration reached at 5 min incubation. This biphasic penetration has been observed by others [41].

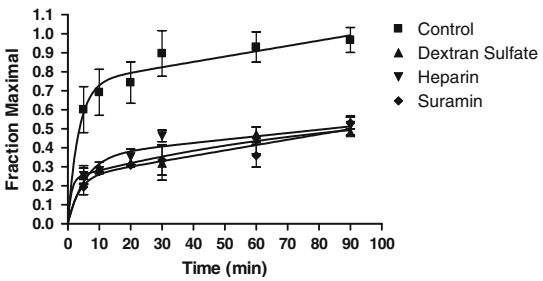

Fig. 5 Time-course of HSV-1 penetration. The binding of HSV-1 (dUTPase/LAT) at MOI 0.01 to Vero cells was carried out at 0°C for 3 h. After binding the media containing the virus, suspension was removed. Fresh overlay media warmed at 37°C containing either dextran sulfate (0.2 mg/ml), heparin (0.25 mg/ml), suramin (0.1 mM) or no compound (control) were added and the incubation continued at 37°C. At the indicated times, the unpenetrated virus was inactivated by treatment with 0.3 M glycine pH 3.0. The wells were rinsed three times with saline, overlay medium was added and the incubation continued at 37°C. The activity of β-galactosidase reporter gene was measured 22–24 h p.i. Data are the average ±SD of three duplicate determinations

Polysulfonated compounds clearly reduced the maximum level of HSV-1 penetration. As shown in Fig. 5, in the presence of dextran sulfate, heparin and suramin the plateau of penetration was reached after 1-h incubation at 37°C.

The inhibition potency rank for blocking penetration was the same as for blocking binding (Fig. 6, Table 1). However, all compounds tested showed lower potency in blocking penetration than binding. Heparin was the compound showing the largest decrease in potency, while neomycin showed the lower decrease. Neomycin also showed a n_H larger than 1 for the inhibition of penetration suggesting diversity of actions with positive cooperativity.

Cell-to-cell passage

We have shown that after penetration, the major action of a polysulfated compound is preventing cell-to-cell passage [29]. To analyze the effect of a compound on cell-to-cell passage, the compound was added after virus penetration and the infection continued for 22–24 h in the presence of the compound. The relative level of infection was determined by the reporter gene activity as before. We obtained dose–response curves for the inhibition of virus infection by suramin, dextran sulfate, heparin, and De-N-acetylated heparin after penetration (Fig. 7). It was not possible to obtain a complete dose–response curve for polylysine and neomycin because of the toxic effect of these compounds at higher concentrations during longer treatments. For other compounds, there was no blockade of infections at the higher concentrations used.

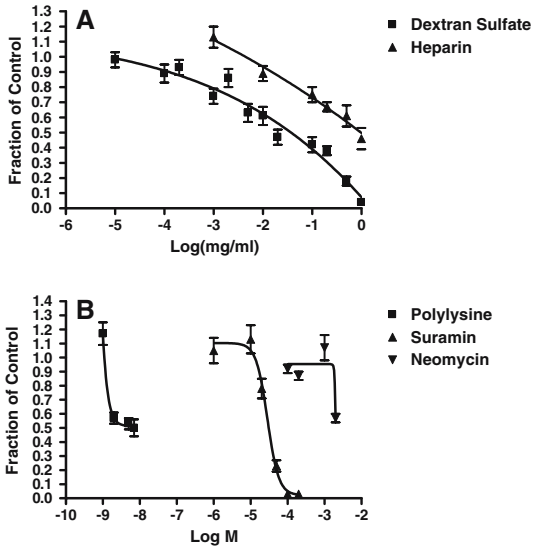

Fig. 6 Dose–response blockade of HSV-1 infection at penetration. The binding of HSV-1 (dUTPase/LAT) at MOI 0.01 in PBS-glucose to Vero cells was carried out at 0°C for 3 h. After binding the media containing the virus, suspension was removed and fresh media containing the indicated concentrations of compound was added. Ninety minutes later, the unpenetrated virus was inactivated by treatment with 0.3 M glycine pH 3.0. The wells were rinsed three times with saline, overlay medium was added and the incubation continued at 37°C. The activity of *β*-galactosidase reporter gene was measured 22–24 h p.i. Data are the average ±SD of three duplicate determinations. (**A**) polysaccharide compounds (polysaccharide compounds not shown were without clear effect at 1 mg/ml), (**B**) non-polysaccharide compounds

In every case, the IC_{50} after penetration was larger than the IC_{50} at the binding stage and smaller than the IC_{50} at the penetration stage (Table 1). The n_H for heparin was smaller than one suggesting a diversity of interactions of different affinity or negative cooperativity. On the other hand, the n_H for suramin was much greater than one, suggesting multiple interactions with positive cooperativity; that is, once an interaction takes place facilitates new interactions.

Combined action of anionic and cationic compounds

The proposed mechanism for the blockade of virus infection by polyanionic-sulfated compounds is that they interact with virus glycoproteins, preventing their binding to cellular sulfated GAGs [28]. On the other hand, cationic compounds seem to prevent this binding by interacting with cellular components [34]. It is possible that cationic compounds prevent virus interaction with sulfated cellular components. To test this mechanism we carried out experiments combining treatments. As shown in Fig. 8 in the presence of Polylysine (2×10^{-8} M) or Neomycin (0.01 M) the dose–response blockade of virus binding by heparin became bell-shaped. This is consistent with a mechanism in which the two compounds antagonize each other.

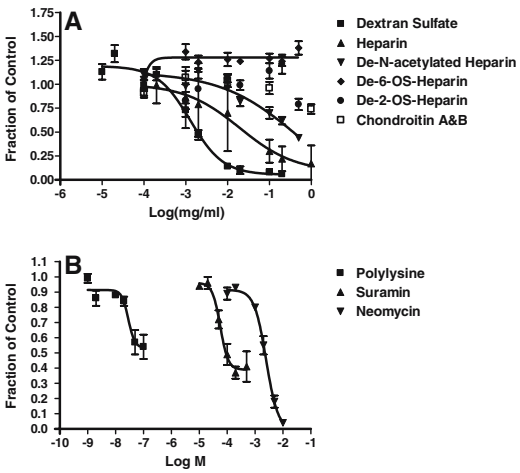

Fig. 7 Dose–response blockade of HSV-1 infection at cell-to-cell passage. Vero cells were incubated with HSV-1 (dUTPase/LAT) at MOI 0.01 in PBS-glucose at 37°C for 2 h. The virus suspension was removed and the wells rinsed with saline. Overlay medium containing the indicated concentrations of compound was added. The incubation was continued for 22–24 h at 37°C and the activity of *β*-galactosidase reporter gene determined. Data are average ±SD of at least three separate determinations by duplicate. (**A**) polysaccharide compound, (**B**) non-polysaccharide compounds

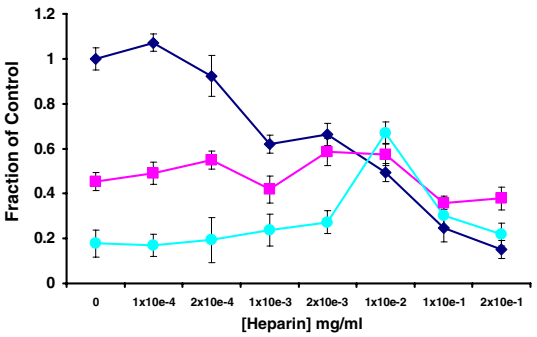

Fig. 8 Counteractive effect of heparin with polylysine and neomycine in blocking productive infections at the binding step. Confluent Vero cells in 24-well plates were placed on ice for 5 min. The media were removed and rinsed with ice-cold saline. HSV-1 (dUTPase/LAT) at MOI 0.01 was added in cold PBS-glucose containing polylisine (2×10^{-8} M) or neomycin (0.01 M) plus the indicated concentrations of heparin. The incubations were continued for 3 h on ice. The cells were washed three times with ice-cold saline, warm ovelay medium was added and the incubation continued for 22–24 h at 37°C. Infection was evaluated by the level of *β*-galactosidase reporter gene. Points are the average ±SD of at least three separate determinations by duplicate. Diamonds, heparine alone; squares, heparin plus neomycin; circles, heparin plus polylysine

The most parsimonious explanation of this antagonism is that cationic compounds interact with heparin negative charges instead of cellular sulfated GAGs preventing heparin binding to viral glycoproteins. However, there is no complete antagonization; that is, the value of the control (100%) is never reached in the presence of the two compounds. This is an indication that each compound interacts with some additional site(s) different from the other. We have observed similar results in the post-penetration phase but the data are erratic, possible due to the toxic effect that polylysine and neomycin show during longer treatments.

Discussion

Polysulfonated compounds are well-known for blocking, binding, and penetration of HSV-1 to the cells [24]. More recently we demonstrated their ability to also block cell-to-cell passage [29]. In this paper we have carried out a comparative analysis of the blockade of HSV-1 infection by polysufonated and polysulfonated binding compounds at the steps of binding, penetration, and passage. Our results are consistent with the virus showing multiple interactions with sulfated GAGs in each process, with some of these interactions being common and others unique for each process.

Multiple interactions of HSV-1 with sulfated GAGs seem to take place in binding, penetration, and cell passage. This is suggested by the n_H of the dose–response of blockade for several compounds (Table 1). At the binding step, all the polysaccharide compounds showed an n_H smaller than 1. This is consistent with multiple sites with different affinities or site with the same affinity with negative cooperativity. Given the diversity of sulfated GAGs in the cell membrane, it is likely that multiple binding sites with different affinities are involved in the binding step. A similar situation was observed in the blockade at the penetration step for heparin and dextran sulfate. Heparin also showed a n_H smaller than one for blocking at the cell passage step. Non-polysaccharide compounds did not show n_H smaller than one. However, these compounds are likely to compete also for the multiple interactions with membrane sulfated GAGs. It is possible that the differences in affinities are not large enough to result in a n_H smaller than one. An alternative is that there are multiple interactions (generating an $n_H < 1$) with positive cooperativity ($n_H > 1$) and the balance is an n_H close to 1. The possibility of positive cooperativity is indicated by the n_H larger than 1 for suramin blockade at the cell passage step (Table 1).

Another indication that there are multiple interactions involved is the fact that the blockade of infection by a polysulfated compound (heparin) is only partially neutralized by cationic compounds (polylysine and neomycin). A similar observation was obtained for neomycin and heparin in Hep-2 cells [32], and Jenssen et al. [42] have shown that the antiherpectic activity of lactorferrin analogs is only partially related to their ability to bind heparin. All these observations indicate that the cationic compounds only neutralize part of the interactions of heparin; therefore, there should be a diversity of sites.

Similar interactions are involved in each of the steps. This is suggested by the same potency rank for blocking at binding, penetration and passage. Thus, for polysaccharide compounds the potency rank is Dextran > heparin > De-N-Acetylated heparin > chondroitin for the blocking at the three steps. For the non-polysaccharide compounds the rank is Polylysine > suramin > neomycin, for binding and penetration. It was not possible to determine the IC_{50}s for polylysine and neomycin in blocking cell passage because of their toxic effect. However, when comparing IC_{10} (Fig. 7B) the potency rank is conserved.

However, viral interactions with membrane sulfated GAGs are not exactly the same in each step. This is inferred from the variations in the IC_{50} and the n_H for the same compound at each of the steps. The IC_{50} of each compound is clearly different for binding, penetration, and passage. Interestingly, for every compound tested the order of potency is always: binding > passage > penetration. Reduced access to the intercellular space—related to the size of the compound—has been proposed as a factor in reducing the potency for blocking intercellular passage, but the structure of the compound also counts [30]. Consistently, suramin—the smallest compound—showed the smaller variation in IC_{50} from binding to passage. Less accessibility to the sites involved in penetration than those in passage is unlikely. It seems that these compounds are more potent in preventing virus binding than penetration. During cell-to-cell passage the compounds may interact with sites involved both in binding and penetration and an intermediate potency is observed.

Variations in the values of n_H for each compound at the different steps is another indication of different interactions in the steps. The larger variation in n_H is shown by suramin. While its n_H is not different from 1 both at binding and penetration, it is much larger than 1 at intercellular passage. This is an indication of multiple interactions at this step with positive cooperativity. We observed a similar situation for the blockade of HSV-1 infection by DMSO [39]. Neomycin also

shows a variation in n_H with a value smaller than 1 for binding and larger than 1 for penetration. Finally, dextran sulfate shows an n_H smaller than 1 at binding and penetration, but not different from 1 at passage. With our data it is not possible to explain these variations; however, the take home lesson is that different sets of interactions are involved at each step.

In conclusion, our results show that similar but different interactions of HSV-1 and polylsulfonated are involved in binding, penetration, and passage. This is in consonance with the fact that different but overlapping sets of viral glycoproteins are involved in these steps. Further characterization of these interactions is important both for understanding the biology of the infection and for developing of new antiherpetic treatments.

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