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Antimicrob. Agents Chemother. **2014, 58(8):4782. DOI: 10.1128/AAC.02594-14.**
Published Ahead of Print 9 June 2014.

AAC Journals.ASM.org

Highly Sulfated K5 *Escherichia coli* Polysaccharide Derivatives Inhibit Respiratory Syncytial Virus Infectivity in Cell Lines and Human Tracheal-Bronchial Histocultures

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Respiratory syncytial virus (RSV) exploits cell surface heparan sulfate proteoglycans (HSPGs) as attachment receptors. The interaction between RSV and HSPGs thus presents an attractive target for the development of novel inhibitors of RSV infection. In this study, selective chemical modification of the *Escherichia coli* K5 capsular polysaccharide was used to generate a collection of sulfated K5 derivatives with a backbone structure that mimics the heparin/heparan sulfate biosynthetic precursor. The screening of a series of N-sulfated (K5-NS), O-sulfated (K5-OS), and N,O-sulfated (K5-N,OS) derivatives with different degrees of sulfation revealed the highly sulfated K5 derivatives K5-N,OS(H) and K5-OS(H) to be inhibitors of RSV. Their 50% inhibitory concentrations were between 1.07 nM and 3.81 nM in two different cell lines, and no evidence of cytotoxicity was observed. Inhibition of RSV infection was maintained in binding and attachment assays but not in preattachment assays. Moreover, antiviral activity was also evident when the K5 derivatives were added postinfection, both in cell-to-cell spread and viral yield reduction assays. Finally, both K5-N,OS(H) and K5-OS(H) prevented RSV infection in human-derived tracheal/bronchial epithelial cells cultured to form a pseudostratified, highly differentiated model of the epithelial tissue of the human respiratory tract. Together, these features put K5-N,OS(H) and K5-OS(H) forward as attractive candidates for further development as RSV inhibitors.

Human respiratory syncytial virus (RSV) is an enveloped single-stranded negative-sense RNA virus belonging to the genus *Pneumovirus* of the family *Paramyxoviridae* (1). It is the leading cause of bronchiolitis and pneumonia in infants and young children worldwide. More than half of all children are seropositive for RSV by 1 year of age, and almost all children have been infected by 2 years of age (2). Moreover, RSV is a pathogen of considerable importance in immunocompromised adults and the elderly, particularly in those with chronic obstructive pulmonary disease (3). In the United States alone, RSV is estimated to cause 120,000 hospitalizations each year and as many as 200 to 500 deaths in infants/young children, while around 160,000 fatalities occur annually worldwide (2, 4, 5). The economic burden related to RSV infection is approximately $500 million in the United States alone, without taking outpatient care into account (6, 7).

Currently, the treatment of RSV infections is mainly symptomatic (8), and the development of a preventive vaccine is hampered by difficulties in eliciting long-lasting protective immunity (9). Antiviral therapy is limited to ribavirin, a nonspecific antiviral drug that interferes with viral transcription; however, the nonnegligible side effects of ribavirin and the recent recommendation of the American Academy of Pediatrics not to routinely use this drug in children with bronchiolitis (10) call for the development of more selective and safe therapeutics for the treatment of RSV infection (11, 12). For immunoprophylaxis, a monoclonal humanized antibody, palivizumab, is available, but it is administered only to high risk premature newborns due to its high cost (13, 14). Another antibody, named motavizumab (an affinity-matured variant of palivizumab), was not provided with FDA approval due to safety concerns (15). Thus, in view of the continual rise worldwide in the morbidity and mortality of infants, the immunocompromised (in particular AIDS patients), and elderly individuals resulting from RSV infection (16, 17) and bearing in mind that no antiviral drug exists to combat this pathogen, RSV constitutes an important target for the development of new antiviral molecules.

The binding of RSV to cultured cells has been characterized at the molecular level: it involves an initial interaction between the positively charged basic amino acids present within the linear heparin-binding domain (HBD) (18) of the viral envelope proteins G and F (19, 20) and the negatively charged sulfated/carboxyl groups of the cell surface heparan sulfate proteoglycans (HSPGs). RSV attachment to HSPGs is followed by a second interaction with nucleolin, a cellular protein which is involved in attachment and entry of several viruses, including human parainfluenza virus type 3, Crimean-Congo hemorrhagic fever virus, Japanese encephalitis virus, and HIV (20, 21, 22, 23, 24, 25). Consequently, the interaction between the envelope glycoproteins of RSV and cellular HSPGs presents an attractive target for novel anti-RSV therapies.

HSPGs are associated with the cell surface; they consist of a protein core and glycosaminoglycan (GAG) side chains of unbranched sulfated polysaccharides, known as heparan sulfates (HS), which are structurally related to heparin. Heparin and HS consist of a sequence of glucuronic (GlcA) or iduronic acid (IdoA) residues that are α1→4 linked to a glucosamine (GlcN) molecule that can be N-sulfated or N-acetylated. The disaccharide sequence

Received 11 March 2014 Returned for modification 14 April 2014
Accepted 1 June 2014
Published ahead of print 9 June 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.02594-14.

doi:10.1128/AAC.02594-14

can also be O-sulfated in different positions: positions 3 and 6 on GlcN and position 2 on uronic acid. HS show high structural heterogeneity along their chains, with specific regions responsible for binding to different ligands.

In respect to HS, heparin is endowed with a high degree of sulfation and a more homogeneous disposition of sulfated groups along its saccharidic chain (26), and consequently, it usually binds to cognate ligands (both viral and eukaryotic) with a higher affinity than HS, resulting in the strongest HSPG-antagonist activity in competition experiments (27, 28, 29, 30). This identifies heparin as an ideal reference compound in studies aimed at the identification of polyanionic HSPG antagonist compounds.

Besides the case of RSV, HSPGs have also been demonstrated to act as attachment receptors for human immunodeficiency virus (HIV) (31), herpes simplex virus (HSV) (32), human papillomavirus (HPV) (33), human cytomegalovirus (HCMV) (34), dengue virus (35), and filoviruses (36); accordingly, several anti-HSPG strategies have been attempted for all of these viruses (29, 37, 38, 39, 40). Despite that fact that this huge amount of *in vitro* experimentation initially provided promising results, few polyanionic, heparin-like compounds progressed to clinical trial for different viral diseases (41, 42, 43). These compounds were safe and well tolerated in phase I and II studies but were devoid of any important clinical benefit in phase III study. This failure of clinical trials of earlier polyanionic antiviral compounds calls for the design of newer compounds endowed with more controlled structures and biological properties.

A peculiar class of compounds, namely, the sulfated derivatives of capsular K5 polysaccharide from *Escherichia coli*, has emerged as a promising biotechnological candidate agent for the development of novel antiviral drugs (44). In brief, the capsular K5 polysaccharide from *Escherichia coli* has the same structure as the heparin precursor *N*-acetyl heparosan. The chemical sulfation of K5 in various N and/or O positions along the polysaccharide results in the synthesis of K5 derivatives with different degrees of sulfation and charge distribution that are endowed with specific binding capacities and biological properties. These semisynthetic GAGs are devoid of the well-known anticoagulant activity that prevents the use of heparin and other sulfated polysaccharides as antivirals (26) and thus present a promising starting point for the development of new antiviral formulations.

In effect, K5 sulfated derivatives have been demonstrated to be endowed with inhibitory activity against different viruses, including herpes simplex virus (HSV) (38), human papilloma virus (HPV) (39), cytomegalovirus (CMV) (40), dengue virus (29), and HIV (37). Regarding this last virus, K5 polysaccharides have been demonstrated to classically act as antimicrobial agents, likely binding to the basic gp120 protein but also binding to and neutralizing other HIV proteins released by infected cells (i.e., Tat and p17) that contribute to HIV dissemination and to the onset of AIDS-associated infections (30, 45). Taken together, these data point to K5 sulfated derivatives as an interesting class of antiviral compounds endowed with a multitarget activity that can be explicated at different levels (i.e., against different viruses but also aimed at different proteins of a given virus) (44). Relevant to this point, a tight relationship exists between HIV, HSV, and HPV infection, suggesting the possibility of generating K5-based drugs with a multitarget mechanism of action that can control and/or prevent HIV, HPV, and HSV infection simultaneously (44). Interestingly, a positive correlation has also been described for RSV and HIV infections (46).

TABLE 1 Molecular weights and degrees of sulfation of the GAGs used in this work

Compound	Mol wt	SO_3^-/COO^- ratio
Unmodified heparin	13,700	2.14
Unsulfated K5	18,700	
K5-NS	15,600	1
K5-N,OS(L)	12,500	2.2
K5-N,OS(H)	14,700	3.68
K5-OS(L)	20,000	1.5
K5-OS(H)	18,800	2.7

The aim of the present work was to investigate whether the antiviral potential of K5 derivatives also extends to the respiratory virus RSV. To this purpose, a panel of N-sulfated (K5-NS), O-sulfated (K5-OS), and N,O-sulfated (K5-N,OS) derivatives was screened to identify compounds with RSV inhibitory activity; antiviral potency and the mode-of-action of the best-hit compounds were also investigated. Highly sulfated (H) K5 derivatives K5-N,OS(H) and K5-OS(H) emerged as nontoxic inhibitors of RSV infectivity in both cell culture and an *in vitro* tissue model of human tracheal/bronchial epithelium.

MATERIALS AND METHODS

Heparin and K5 polysaccharide derivatives. Unmodified unfractionated beef mucosal heparin was obtained from Laboratori Derivati Organici, Milan, Italy. K5 polysaccharide derivatives were obtained by N-deacetylation/N-sulfation and/or O-sulfation of a single batch of K5 polysaccharide as previously described (47). The N-deacetylation/N-sulfation of K5 polysaccharide is performed in one step and has been scaled to a 10-g amount. The average yield of compound of the N-deacetylation/N-sulfation is about 80%. The degree of N-sulfation is determined by ^{1}H nuclear magnetic resonance (^{1}H-NMR) at 500 MHz, and no signal of residual N-acetylation is detectable. The sulfate-to-carboxyl ratio of the final products is measured according to the method of Casu et al. (48). The antiviral results have been reproduced with two different batches of compounds. The main chemical features of these GAGs are shown in Table 1.

Cells and viruses. The epithelial cell lines HEp-2 (ATCC CCL-23) and A549 (ATCC CCL-185) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal calf serum (FCS) (Gibco/BRL). RSV strain A2 (ATCC VR-1540) was propagated in HEp-2 cells by infecting a freshly prepared confluent monolayer grown in MEM supplemented with 2% of FCS. When the cytopathic effect involved the whole monolayer, the infected cell suspension was collected and the viral supernatant was clarified. The virus stocks were aliquoted and stored at −80°C. The infectivity of virus stocks was determined on HEp-2 cell monolayers by standard plaque assay. The cell lines and the RSV were obtained from the American Type Culture Collection (Manassas, VA, USA).

Cell viability assay. Cells (A549 and HEp-2) were seeded at a density of 5×10^4/well in 96-well plates and treated the next day with serially diluted GAGs to generate dose-response curves. After 72 h of incubation, cell viability was determined using the CellTiter 96 proliferation assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Absorbances were measured using a microplate reader (model 680; Bio-Rad) at 490 nm. Fifty percent cytotoxic concentration (CC_{50}) values and 95% confidence intervals (CIs) were determined using Prism software (GraphPad Software, San Diego, CA).

Virus inactivation assay. Approximately 10^4 PFU of RSV and 3.6 μg/ml of each GAG (corresponding to 240 nM K5-N,OS(H), 191 nM K5-OS(H), and 263 nM heparin) were added to MEM and mixed in a total

volume of 100 μl. Virus-GAG mixtures were incubated for 2 h at 37°C or 4°C and serially diluted to the noninhibitory concentration of each test compound, and the residual viral infectivity was determined by the viral plaque assay.

Binding assay. Each GAG (10 μM) was added to an aliquot of RSV (5×10^4 PFU) and administered directly to HEp-2 or A549 cell monolayers in MEM supplemented with 2% FCS, incubated for 2 h at 4°C, and washed three times to remove unbound virus. Cells were then fixed with 4% paraformaldehyde, air dried, and blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline(PBS)-Tween. Bound virus was detected using RSV monoclonal antibody (Ab35958; Abcam, Cambridge, United Kingdom) (diluted 1:400), incubated for 1 h at room temperature, washed three times with PBS-Tween, and incubated for 2 h at 37°C with goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:1,000). At the end of incubation, plates were washed three times with PBS-Tween before adding the ABTS substrate [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] (Thermo Scientific, Rockford, IL) and reading the absorbance at 405 nm. Percent inhibition of virus binding was determined by comparing the absorbance measured in the presence of the compound to that measured in untreated cultures.

Viral plaque assay. To evaluate the capacity to inhibit RSV infection, GAGs were serially diluted to generate dose-response curves and added to RSV (multiplicity of infection [MOI], 0.01 PFU/cell). After 1 h of incubation at 4°C, the mixture was added to cells grown as monolayers in a 96-well plate at a density of 5×10^4/well. After 3 h of incubation at 37°C, monolayers were washed and overlaid with 1.2% methylcellulose medium. Three days postinfection, cells were fixed with cold methanol and acetone for 1 min and subjected to RSV-specific immunostaining using an RSV monoclonal antibody (Ab35958; Abcam, Cambridge, United Kingdom) and the UltraTech HRP streptavidin-biotin detection system (Beckman Coulter, Marseille, France). Immunostained plaques were counted, and the percent inhibition of virus infectivity was determined by comparing the number of plaques in compound-treated wells with the number in untreated control wells. Fifty percent inhibitory concentration (IC_{50}) values and 95% CIs were determined using Prism software. All data were generated from duplicate wells in at least three independent experiments.

To characterize the mechanism of the antiviral action of the K5 derivatives, the viral plaque assay was repeated, incorporating the following modifications.

Preattachment assay. HEp-2 and A549 cell monolayers in 96-well plates were incubated with increasing concentrations of the various GAGs for 2 h at 37°C. After removal of the compound and two gentle washes, cells were infected with RSV (MOI, 0.01 PFU/cell) in the absence of compounds for 3 h at 37°C. Cells were then overlaid with 1.2% methylcellulose medium, incubated for 72 h at 37°C, and successively fixed and immunostained as described above. Plaques were then counted.

Attachment assay. Serial dilutions of the various GAGs were preincubated with RSV (MOI, 0.05 PFU/cell) for 1 h at 4°C, added to cooled HEp-2 and A549 cells in 96-well plates, and incubated for 2 h at 4°C to ensure viral attachment but not entry. After two gentle washes, cells were overlaid with 1.2% methylcellulose medium, shifted to 37°C for 72 h, and successively fixed and immunostained as described above. Plaques were then counted.

Postattachment assay measuring viral yield. HEp-2 cell monolayers in 24-well plates were infected with RSV (MOI, 0.005 PFU/cell) in MEM supplemented with 2% FCS for 3 h at 37°C and then subjected to two gentle washes to remove unbound virus. Increasing concentrations of the various GAGs in MEM supplemented with 2% FCS were then added to cultures after washout of the viral inoculum or after 1, 2, 3, or 24 h. Incubation continued until the cytopathic effect involved the whole monolayer in the untreated wells. The infected cell suspensions were collected, and the supernatants were clarified. RSV infectivity was determined on A549 cell monolayers by standard plaque assay. Titrations were carried out at dilutions at which compounds were no longer active to exclude the possibility that a carryover of tested polysaccharides into the titration culture would block virus attachment.

Percent inhibition was determined by comparing the viral titer measured in the presence of the compounds to that measured in untreated wells.

Syncytium formation assay. The abilities of the various GAGs to block RSV cell-to-cell spread were evaluated using a previously described method (49) with minor modifications. Cell monolayers in 96-well plates were infected with RSV (MOI, 0.01 PFU/cell) in MEM supplemented with 2% FCS for 3 h at 37°C and then subjected to two gentle washes to remove unbound virus. Following inoculum washout, increasing concentrations of each GAG in 1.2% methylcellulose medium were then added to cultures. Incubation continued for 72 h postinfection at 37°C; cells were then fixed and immunostained. The immunostained syncytia were visualized using a Leica inverted microscope equipped with a Bresser MikroCam microscope camera and MikroCamLab software (Rhede, Germany). ImageJ software was used to quantify plaque sizes. Untreated RSV-infected monolayers were used as the control.

Rotavirus infectivity assay. Rotavirus infectivity assays were performed as previously described (50) with some modifications. Confluent MA104 cell monolayers in a 96-well plate were washed twice with MEM and then infected with human rotavirus strain Wa (ATCC VR-2018) at an MOI of 0.02 PFU/cell for 1 h at 37°C in the presence or absence of each test GAG. Virus was preactivated with 5 μg of porcine trypsin (Sigma)/ml for 30 min at 37°C. After the adsorption period, the virus inoculum was removed, cells were washed with MEM, and the cultures were maintained at 37°C for 16 h in medium with trypsin at 0.5 μg/ml. The infected cells were fixed and immunostained using an UltraTech HRP streptavidin-biotin detection system (Beckman Coulter).

EpiAirway tissues. EpiAirway tissues, cultured on collagen supports under air-liquid interface conditions, were obtained from MatTek Corp. (Ashland, MA, USA). These tissues consisted of normal human-derived tracheal/bronchial epithelial cells that are highly differentiated (i.e., contain cilia, tight junctions, sodium and chloride channels, etc.) and retain properties of normal respiratory tract epithelial tissue (i.e., actively secrete mucus, electrogenicity, etc.). Upon delivery, the EpiAirway tissue inserts were processed according to the supplier's protocol. Briefly, each tissue insert was transferred to a well in a 6-well plate prefilled with 900 μl prewarmed serum-free medium (Air-100-MM; MatTek Corp.) and incubated at 37°C in 5% CO_2 overnight (16 to 18 h). EpiAirway tissues were then used in the following three assays.

Cytotoxicity assay. The cytotoxicity of K5 derivatives on mucous membranes was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) ET_{50} tissue viability assay according to the manufacturer's instructions. K5 derivatives (10 μM) were applied to the cell culture insert on top of the EpiAirway tissue samples and incubated for 1, 4, or 18 h at 37°C in duplicate. At the end of the incubation, any liquid on top of the EpiAirway tissue was decanted, and inserts were gently rinsed with PBS to remove any residual material. Tissues were then processed according to the MTT kit protocol (MatTek Corporation) and read using an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 570 nm. Tissues incubated with assay medium were used as negative controls. The ET_{50} is the time required to reduce tissue viability to 50% and was determined using Prism software (GraphPad Software, San Diego, CA). According to the information provided by the supplier, ET_{50} values of $>$18 h indicate that a tested compound is not irritating.

Antiviral assay. To assess the antiviral activity of K5 derivatives on EpiAirway cultures, aliquots of 100 μl of medium containing 50,000 PFU of RSV with or without K5 derivatives (10 μM) were preincubated for 1 h at 4°C and then added to the apical surface of the tissues. After 3 h of incubation at 37°C, the medium was removed, and the cultures were washed apically with 100 μl of medium and then fed each day via the basolateral surface with 1 ml medium. To harvest the virus, 100 μl medium was added to the apical surface, and the tissues were allowed to

TABLE 2 Screening of K5 derivatives on A549 and HEp-2 cells[a]

Cell line	Compound	IC_{50}(nM)	95% CI	CC_{50} (nM)	SI
A549	K5	>600	NA	>24,000	>40
	K5- NS	257.40	164.5–402.8	>24,000	>93.24
	K5-N,OS(L)	36.81	23.3–58.2	>24,000	>651.99
	K5-N,OS(H)	2.56	2.07–3.18	>24,000	>9,375
	K5-OS(L)	25.42	15.2–42.5	>24,000	>944.14
	K5-OS(H)	1.07	0.704–1.624	>24,000	>22,429.91
	Heparin	3.52	2.08–5.96	>24,000	> 6,818.18
HEp-2	K5	>600	NA	>24,000	>40
	K5-NS	340	240–500	>24,000	>70.59
	K5-N,OS(L)	28.81	21.88–37.94	>24,000	>833.04
	K5-N,OS(H)	3.71	3.36–4.12	>24,000	>6,469
	K5-OS(L)	44.69	31.34–63.72	>24,000	>537.03
	K5-OS(H)	2.20	1.76–2.74	>24,000	>246,857
	Heparin	3.73	3.17–4.39	>24,000	>6,434.32

[a] IC_{50}, 50% inhibitory concentration; 95% CI, 95% confidence interval; CC_{50}, 50% cytotoxic concentration; NA, not assessable. Values are means and CIs from three separate determinations.

equilibrate for 30 min at 37°C. The suspension was then collected and stored at −80°C until viral titers were determined by plaque assay in A549 cell monolayers as described above. Collection of virus was performed sequentially from the same wells on each day postinfection.

Detection of RSV in EpiAirway tissue by immunohistochemistry. RSV was detected immunohistochemically using a specific mouse monoclonal antibody against RSV (Ab35958; Abcam, Cambridge, United Kingdom). Briefly, EpiAirway tissue cultures exposed to RSV in the absence or presence of K5 derivatives (10 μM) were fixed in buffered formalin and embedded (properly oriented) in paraffin together with adherent collagen membranes. Immunohistochemical sections were processed for antigen retrieval in citrate buffer using a dedicated pressure cooker (1 cycle for 5 min at 125°C, followed by 10 s at 90°C) in parallel with sections stained with conventional hematoxylin and eosin. Following incubation with the primary antibody (1:500 dilution), the reaction product was visualized using a biotin-free polymer-conjugated secondary antibody (Envision; Dako, Glostrup, Denmark). In the presence of a positive reaction, the antibody showed cytoplasmic and nuclear immunoreactivity, mostly recognizable in the cells of the superficial layers. Ten sections were analyzed for each experimental condition.

Statistical analysis. Inhibition of infectivity and formation of syncytia in the presence and absence of the putative antiviral compounds were compared by analysis of variance (ANOVA) followed by a Bonferroni posttest, if P values showed significant differences, using the GraphPad Prism 5.00 program (GraphPad Software). Results are expressed as means ± CI or standard errors of the means (SEM) or standard deviations (SD), as appropriate.

RESULTS

Screening of derivatives of *E. coli* K5 polysaccharide for RSV antiviral activity. Knowing that heparin is structurally related to HSPG and prevents RSV adsorption (19), we exploited the viral plaque assay to screen a panel of *E. coli* K5 polysaccharide derivatives, which have structures similar to those of heparin and HSPG (26). As reported in Table 2, all GAGs, except unmodified K5, showed a half-maximal inhibitory concentration (IC_{50}) in the nanomolar range. To exclude the possibility that the antiviral activity of K5 derivatives might be due to cytotoxicity, the GAGs were evaluated by MTT assays with uninfected HEp-2 and A549 cells. As reported in Table 2, none of the GAGs tested exhibited toxic effects in the range of the examined concentrations, hence the nondeterminable 50% cytotoxic concentrations (CC_{50}) and very favorable selectivity indexes (SI) for each active compound. K5-N,OS(H) and K5-OS(H) were endowed with the highest antiviral activities and were therefore selected for further investigation. Thus, the effect of K5-N,OS(H) and K5-OS(H) on cell viability was investigated with HEp-2 and A549 cells at higher doses than those reported in Table 2 (i.e., up to 200 μM) in order to determine the CC_{50} values. As shown in Fig. S1 in the supplemental material, K5-N,OS(H) and K5-OS(H) exerted a moderate dose-dependent reduction in cell viability only in HEp-2 cells at 50 μM, 100 μM, and 200 μM, which did not allow the calculation of CC_{50} values

K5 derivatives do not inactivate RSV particles. Since certain sulfated polysaccharides have been shown to exhibit direct virucidal activity (51), the K5 derivatives used in the present study were first subjected to a virus inactivation assay in our pursuit to understand their mechanism(s) of antiviral action. As shown in Fig. 1, the virus titers of samples treated with K5-N,OS(H), K5-OS(H), or heparin did not significantly differ from those determined in untreated samples ($P > 0.05$), indicating that the two K5 derivatives do not exert their antiviral activity via the direct inactivation of RSV particles.

K5 derivatives do not affect cell susceptibility to RSV infection. Some antiviral compounds are known to lower cell susceptibility to viral infection by downregulating or directly masking virus receptors. In particular, we recently demonstrated that the compound SB-105A10 exerts its anti-RSV activity by masking HSPGs on the cell surface (49). To investigate whether the K5 derivatives affect cell susceptibility to RSV infection, preattachment assays were performed as described above. To this end, HEp-2 and A549 cells were preincubated for 2 h with different concentrations of K5-N,OS(H) or K5-OS(H) or with heparin as a control. After incubation, cells were washed to remove unbound GAGs from the medium and infected with RSV. As shown in Fig. 2, under these experimental conditions, K5 derivatives and heparin do not exert any antiviral activity. This indicates that K5 derivatives do not affect cell susceptibility to RSV infection.

K5 derivatives block RSV binding to host cells. The antiviral activities of many sulfated polysaccharides correspond to their capacity to bind to and sequester the virus in the extracellular environment, thus hampering its attachment to the target cell (51). This possible mechanism was therefore investigated in relation to the K5 derivatives and RSV using the attachment assay described above, the conditions of which allow for the attachment of the virus to the cell surface but prevent its entry. As shown in Fig. 3, under these experimental conditions, K5 derivatives and heparin strongly inhibited RSV, with IC_{50}s that are comparable to those measured in the classical viral plaque assay (see Table 2), suggesting that the antiviral activities of these GAGs depend on their capacity to inhibit the attachment of the virus to the cell surface.

To substantiate this interpretation, binding assays in which we directly evaluated the amounts of virus particles bound to the cells in the presence or absence of the active GAGs were performed. Consistent with previous results, K5-N,OS(H), K5-OS(H), and heparin significantly reduced ($P < 0.05$) the amount of bound virus on HEp-2 and A549 cells (Fig. 3C and D, respectively), while unsulfated K5, which does not exhibit any antiviral activity, did not.

Taken together, these results indicate that the main mecha-

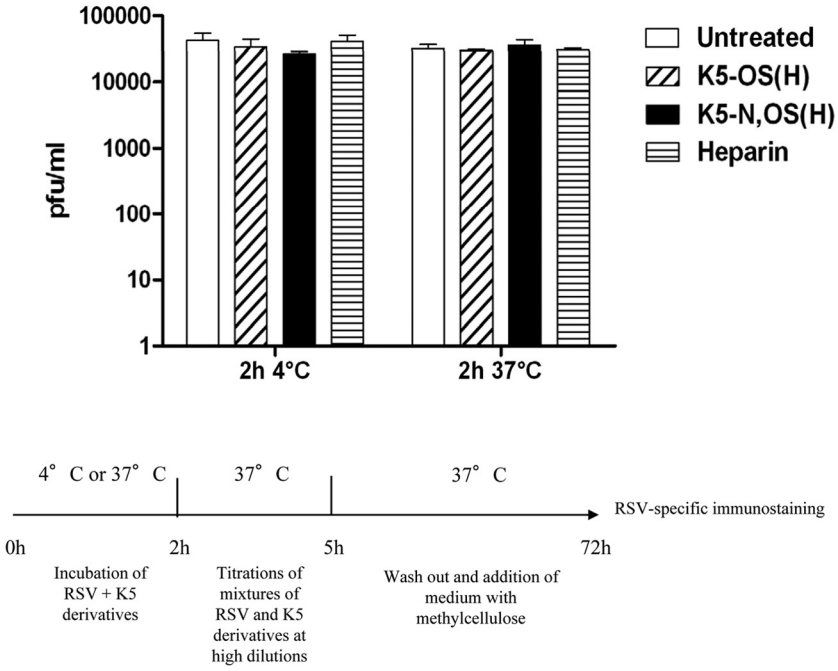


FIG 1 K5 derivatives are not active in a virus inactivation assay. RSV was incubated with 3.6 μg/ml of K5-N,OS(H) (240 nM), K5-OS(H) (191 nM), or heparin (263 nM) for 2 h at 4°C or 37°C. The mixtures were then titrated on A549 cells at high dilutions at which the concentrations of compounds were not active. The titers, expressed as PFU/ml, are means and SEM for triplicates.

nism of action of the K5 derivatives consists in their capacity to hamper the virus's interaction with an entry receptor(s) expressed on the surface of target cells.

K5 derivatives reduce viral yield for 24 h postinfection. To evaluate whether the reduction of RSV attachment and infection exerted by K5-N,OS(H) and K5-OS(H) is maintained in the long term, thus leading in a decrease in viral progeny production, post-attachment assays using virus yield were performed as described above; this stringent test allows multiple cycles of viral replication to occur before measuring the production of infectious viruses. In the first set of experiments, increasing concentrations of K5-N,OS(H), K5-OS(H), and heparin were added immediately after the removal of the viral inoculum in order to generate dose-response curves and to determine the IC_{50}s (Fig. 4A). Under these experimental conditions, the two K5 derivatives strongly reduced the RSV yield, with efficiencies that are similar to those measured in the classic viral plaque assay and in the attachment assay for K5-N,OS(H) and K5-OS(H), respectively. Interestingly, heparin exerted only modest inhibitory activity. Of note, K5-N,OS(H), K5-OS(H), and heparin were not cytotoxic even at the highest concentration tested (7 μM), as shown in Fig. S1 in the supplemental material.

In the second set of experiments, a single concentration of K5-N,OS(H), K5-OS(H), or heparin was added 1 h, 2 h, 3 h, and 24 h after the removal of the virus inoculum. The results shown in Fig. 4B demonstrate that reduction in viral yield is effective when the compounds are added up to 24 h postinfection. Once again, the inhibition profiles of K5-N,OS(H) and K5-OS(H) were better than that of heparin.

Taken together, these data indicate that the K5 derivatives but not heparin retain an antiviral activity for at least 24 h and are able to exert their inhibitory action over virions produced directly by the cell, thereby preventing further cell infection and viral yield production.

K5 derivatives inhibit cell-to-cell spread of RSV and syncytium formation. Massive viral production by infected cells triggers cell-to-cell spread of RSV that in turn triggers the formation of syncytia, the characteristic cytopathic effect of RSV; the formation of these large, multinucleated epithelial cells helps the infecting virus avoid antibodies present in nasal secretions (52, 53). We thus decided to investigate whether K5-N,OS(H), K5-OS(H), and heparin were able to block the cell-to-cell transmission of RSV. To this end, HEp-2 and A549 cells were infected with RSV in the absence of any GAG and then treated with different concentrations of K5-N,OS(H), K5-OS(H), or heparin after the removal of the virus inoculum. Three days postinfection, the cell-to-cell spread of RSV was evaluated by analyzing the size of the infection foci. As shown in Fig. 5A, all the compounds were able to reduce the transmission of RSV in a dose-dependent manner. A statistically significant reduction in syncytium dimension was observed in both A549 and HEp-2 cells treated with doses of K5-N,OS(H) ranging between 7 μM and 777.8 nM, and with doses of K5-OS(H) ranging between 7 μM and 259.3 nM. In contrast, a significant reduction in plaque size following treatment with heparin was observed in HEp-2 cells only at a dose of 7 μM ($P < 0.01$).

K5 derivatives do not exhibit antiviral activity against rotavirus. To date, a number of K5 derivatives that exhibit antiviral activity against a panel of HSPGs-dependent viruses, including HSV, HIV, and HPV (see the introduction), have been identified. Moreover, work from our own group has revealed the HSPG-binding dendrimer SB105A10 to be active against RSV infection (49), and the present study identifies additional K5 derivatives with capacities to block RSV infection. To provide further evi-

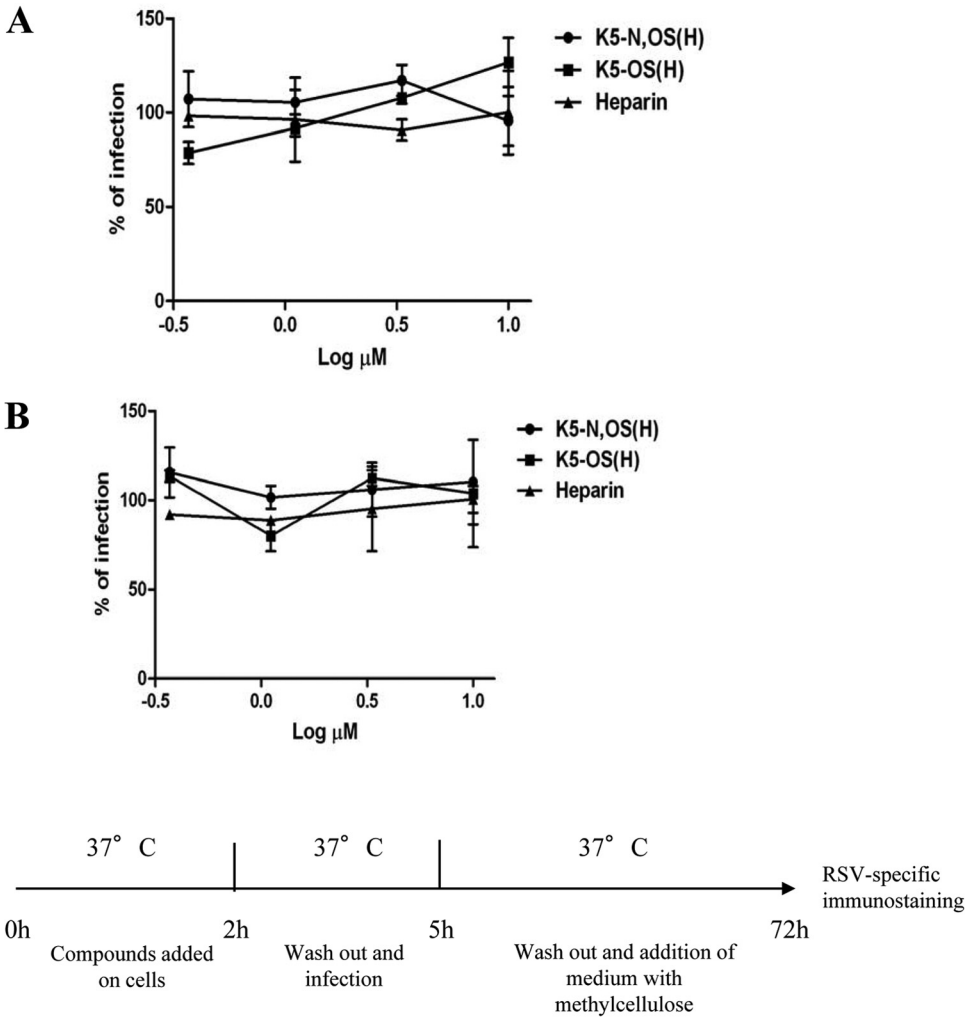


FIG 2 Preattachment assay with HEp-2 and A549 cells. HEp-2 (A) or A549 (B) cells were pretreated with increasing concentrations of K5 derivatives or heparin for 2 h at 37°C, washed, and infected. Three days postinfection, the cells were fixed and subjected to RSV-specific immunostaining, the plaques were counted, and the percent infection was calculated by comparing treated and untreated wells. The results are means and SEM for triplicates.

dence corroborating the hypothesis that the anti-RSV abilities of these K5 derivatives depend specifically on their capacity to prevent RSV from interacting with HSPGs on the target cell, the compounds were tested against human rotavirus, whose attachment and entry depend on interaction with integrins and heat shock proteins but not HSPG (54). Neither the K5 derivatives nor heparin could prevent rotavirus infection in MA104 cells when tested at doses up to 7 μM (Fig. 6), strongly indicating that these compounds are not active against viruses that do not bind to cell surface HSPGs.

Antiviral activities of K5 derivatives in EpiAirway tissue. The EpiAirway system consists of human derived tracheal/bronchial epithelial cells grown on a collagen-coated membrane to form a highly differentiated organotypic model with many of the features of respiratory mucosa, thus providing a useful *in vitro* means of assessing respiratory virus infections. We assessed the effect of K5-N,OS(H) and K5-OS(H) on RSV infection by measuring the titer of virus emerging from the apical surface of tissues infected with mixtures containing 50,000 PFU of RSV in the presence or absence of 10 μM K5-N,OS(H) or K5-OS(H) preincubated for 1 h at 4°C prior to virus application. At 72 h postinfection, the titer of virus in untreated control tissues was 1.45×10^3 PFU/ml. In tissues treated with K5-N,OS(H), the detected titer was 40 PFU/ml, whereas in the samples treated with K5-OS(H), the virus titer was undetectable (Fig. 7). Thus, the compounds inhibited the viral titer by 97.3% and 100%, respectively. The same tissues were fixed immediately after the virus harvest at 72 h postinfection and subjected to immunohistochemistry using an RSV-specific monoclonal antibody. All the sections derived from the infected tissue consistently showed the presence of cells expressing the RSV antigen in the upper cellular layer (Fig. 8B). No RSV-positive cells could be observed in sections from uninfected tissue (Fig. 8A), demonstrating the specificity of the signal. Furthermore, no RSV-positive cells could be identified in tissues treated with K5-N,OS(H) (Fig. 8C) or K5-OS(H) (Fig. 8D), corroborating the virus titer results. To verify that the antiviral action was not due to a cytotoxic effect, an MTT assay was performed with tissues treated with 10 μM (each) K5 derivative for 1, 4, or 18 h at 37°C. The results shown in Table 3

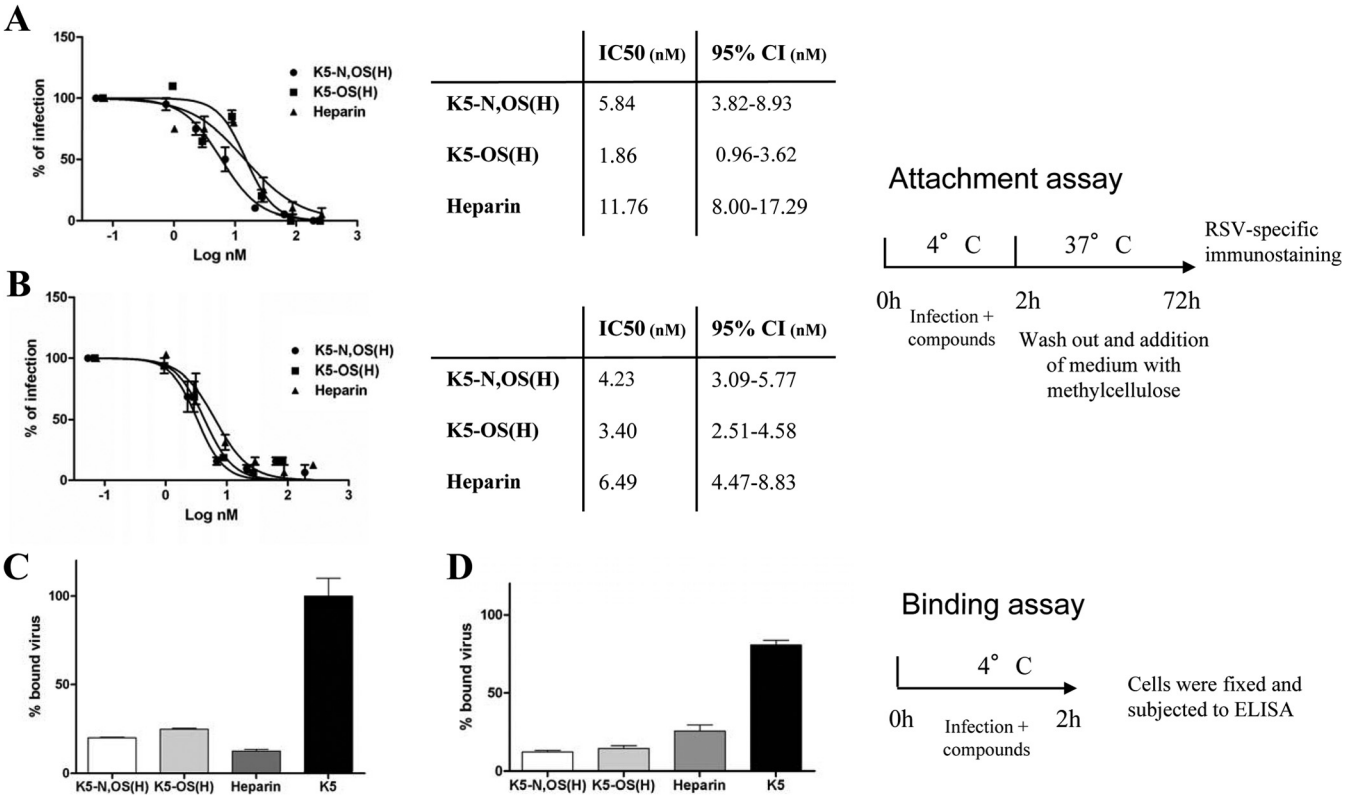


FIG 3 Investigation of inhibitory mechanisms of the hit compounds. In the attachment assay, RSV and compounds were added to HEp-2 (A) or A549 (B) cells for 2 h at 4°C. Cells were shifted to 37°C, and at 72 h postinfection, they were subjected to RSV-specific immunostaining, the plaques were counted, and the percentage of infection was calculated by comparing treated to untreated wells. In the binding assay, the virus bound to HEp-2 (C) or A549 (D) was detected by ELISA immediately after the removal of the virus inoculum. Each absorbance was mock subtracted, and the percentage of infection was calculated by comparing treated to untreated wells. The results are means and SEM from triplicates.

demonstrate that these K5 derivatives are not cytotoxic, and the time required to reduce tissue viability to 50% (ET_{50}) was greater than 18 h.

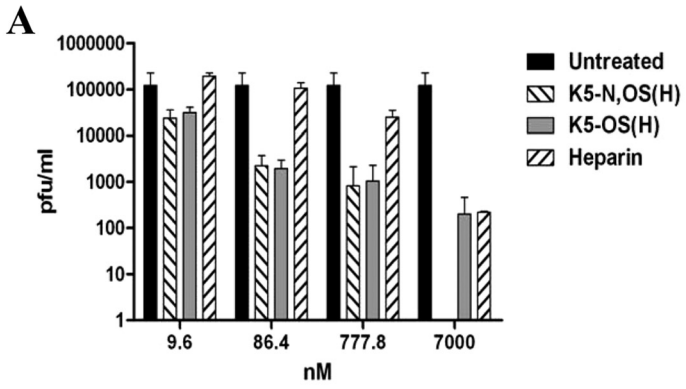
DISCUSSION

To infect target cells successfully, RSV needs to bind to HSPGs located on the cell membrane, and this interaction provides a target for the development of new anti-RSV compounds. Inhibition of the RSV/HSPG interaction can be achieved by two distinct approaches: the first involves receptor masking, usually achieved by means of polycationic compounds able to bind to the negatively charged sulfate groups present on the GAG side chains of HSPGs, and the second involves the use of polyanionic compounds that bind to and antagonize the virus. We recently confirmed the feasibility of the first approach by demonstrating that a highly positively charged dendrimer effectively binds to HSPGs, inhibiting RSV infection (49). Accordingly, positively charged peptides derived from the HBD of the RSV G protein also block virus infection (18). The feasibility of the second approach, on the other hand, has been supported by the demonstration that heparin (19, 55) and other negatively charged polysaccharides, such as chondroitin sulfate (56) and dextran sulfate (57, 58), are able to bind RSV, preventing its cell attachment and infection.

Due to their structural heterogeneity, heparin, heparan sulfate, and other GAGs are able to bind to a wide range of molecules and exert a number of biological activities that can interfere with one another, leading to the risk of toxicity and undesired side effects. The solution therefore lies in the production of tailor-made heparin-like compounds endowed with specific antiviral activities; however, this requires detailed knowledge of the molecular basis of the heparin/HSPG interaction with viral envelope proteins. In the case of RSV, we know that the glycoproteins G and F are responsible for the heparin/HSPG interaction, and specific basic amino acid sequences acting as HBDs have even been identified in the each of these proteins (18, 19, 20). Nevertheless, little has been done to date to characterize the structural features of heparin/HSPGs that mediate their binding to RSV protein, although it is very likely that the negatively charged sulfated groups of the GAG chains are those involved in the interaction, as demonstrated for almost all the other viral heparin-binding proteins (59).

The capsular K5 polysaccharide from *Escherichia coli* can be chemically sulfated in selected positions, resulting in the synthesis of completely N-sulfated compounds with different amounts of O-sulfation in different positions or completely N-acetylated molecules differing only in the position and degree of O-sulfation (30). Due to these features, sulfated K5 derivatives have been useful in the study of the structure-activity relationship of the interactions of several viral proteins with heparin and used in the design of specific antiviral polysaccharides.

Here, we found that selected K5 sulfated derivatives exert a strong anti-RSV effect. Experiments aimed at elucidating their mechanism(s) of anti-RSV action indicate that the inhibitory ef-



	IC50 (nM)	95% CI (nM)
K5-N,OS(H)	2.79	1.17-6.86
K5-OS(H)	4.32	3.38-5.64
Heparin	175.5	121.6-253.4

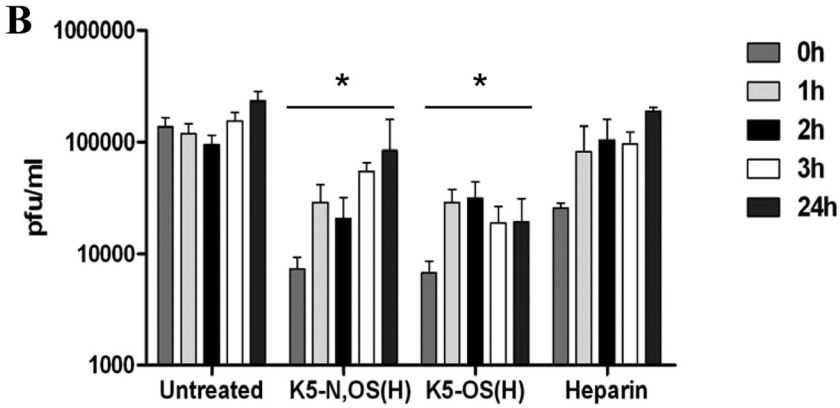


FIG 4 Reduction of viral yield. (A) HEp-2 cells were infected and subsequently treated with different concentrations of compounds. When the cytopathic effect involved the whole monolayer of untreated wells, supernatant were harvested and titrated. The results are means and SEM from triplicates. The table in panel A shows the IC_{50} and 95% CI values for each compound tested. (B) The same procedure was followed, with a fixed dose of 10 μg/ml added to infected cells at different times postinfection, ranging from 0 h to 24 h. The results are means and SEM for triplicates. *, $P < 0.05$ in a 2-way ANOVA.

fect is due mainly to the capacity of K5-N,OS(H) and K5-OS(H) to interact with the virus particles, rather than with cell components, and thereby prevent virus attachment to the cell surface. Several lines of evidence support this. First, cells pretreated with K5 derivatives remained susceptible to RSV infection, thus excluding the possibility that these compounds form stable interactions with one or more cellular components, preventing their interaction with viral glycoproteins. Second, the results of the binding and attachment assays demonstrate that K5-N,OS(H) and K5-OS(H) block the adsorption of RSV virions to the cell surface with a potency similar to that of heparin, which has been shown to prevent RSV infection by competing with cellular HSPGs for binding to virion components (60, 61) Third, preincubation of RSV virions with the active sulfated K5 derivatives did not result in a loss of infectivity, suggesting that the antiviral activity of the compounds does not rely on inactivation of a virion component(s). A similar mechanism of action was previously observed for heparin when it was tested against HSV-1 and RSV (49, 50) and when K5 derivatives were tested against HCMV (40).

Unsulfated K5 polysaccharide, unlike K5-N,OS(H) and K5-OS(H), did not show any significant RSV antagonist activity, indicating that the sulfate groups, rather than the backbone structure, mediate the interaction with RSV. Moreover, a good correlation exists between the degree of sulfation of the GAGs tested and their capacities to inhibit RSV infection (Fig. 9). However, this correlation is lost in the highly sulfated GAGs (see the left part of Fig. 9); thus, in addition to the degree of sulfation, the position of the sulfate groups along the polysaccharide chain is also important. Furthermore, complete sulfation of the N positions confers very limited RSV antagonist activity to K5-NS, while

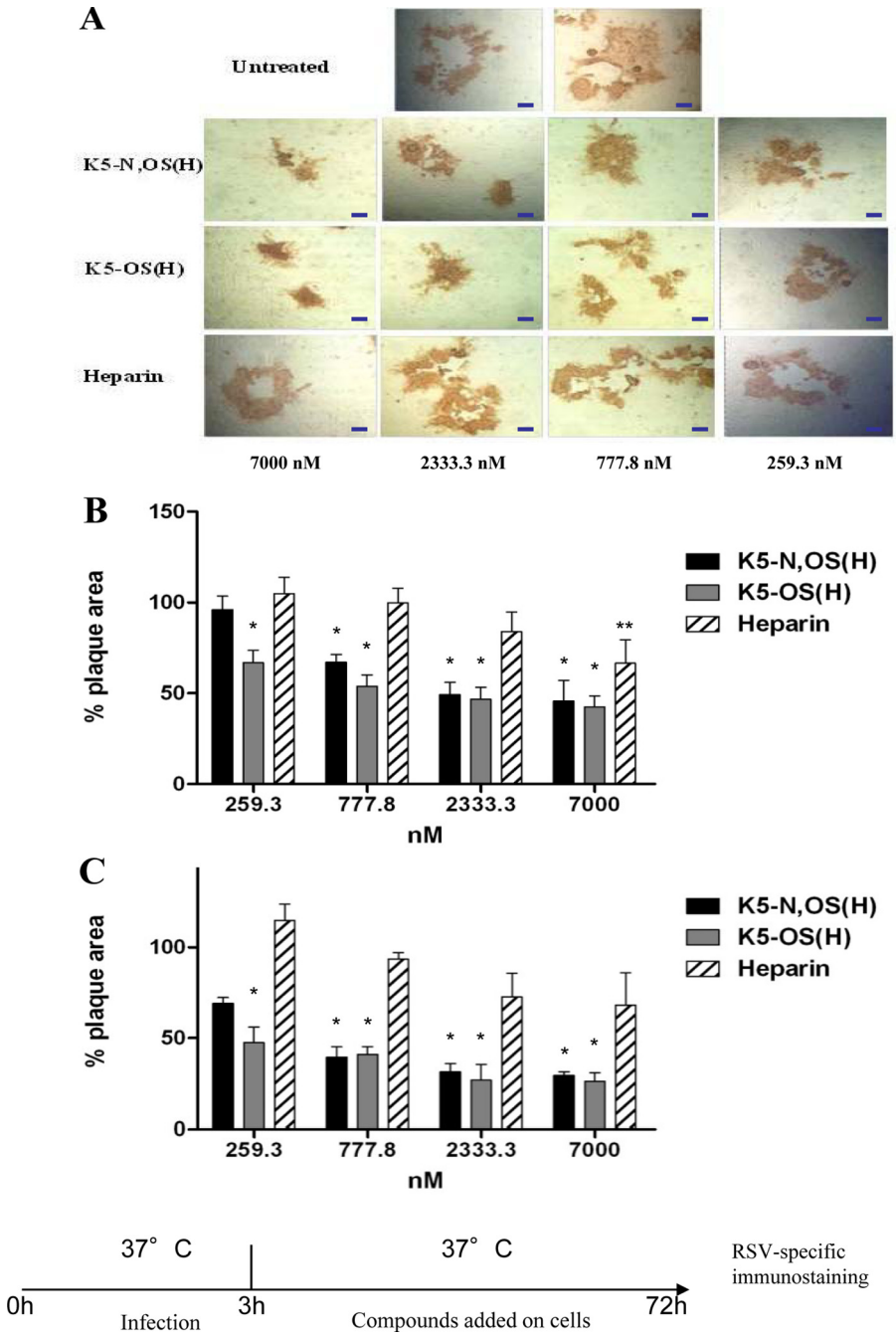


FIG 5 Inhibition of RSV-induced syncytium formation by K5 derivatives and heparin. The images in panel A show representative syncytia in HEp-2 cells, with horizontal bars corresponding to 20 μm. HEp-2 cells (B) or A549 cells (C) were infected with RSV in the absence of compounds. The inoculum was removed at 3 h postinfection, and cells were left untreated or incubated in the presence of the following concentrations of substances in 1.2% methylcellulose medium: 7,000 nM, 2,333.3 nM, 777.8 nM, and 259.3 nM. Formation of syncytia was assessed 72 h after infection, by immunostaining. The histograms show the percentage of plaque area of treated wells compared to that of untreated wells as a function of compound concentration. The pictures and histograms shown are representative of many analyzed plaques, ranging from 15 to 25 per condition. *, $P < 0.001$; **, $P < 0.01$.

sulfation of the O position confers an inhibitory capacity to K5-OS(L) that is almost 10 times higher than that of K5-NS (Table 2) despite a similar $SO_3^-/COOH^-$ ratio (1 and 1.5, respectively) (Table 1). Similarly, K5-OS(H) is endowed with an inhibitory capacity that is 30 times higher than that of K5-N,OS(L) (Table 2) despite their similar $SO_3^-/COOH^-$ ratios (2.7 and 2.2, respectively) (Table 1). Finally, the greater $SO_3^-/COOH^-$ ratio of K5-OS(H) than that of K5-NOS(H) (from 2.7 to 3.68) (Table 1) does not confer any additional anti-RSV potency.

Taken together, these data suggest that O- rather than N-sulfated groups mediate the binding of RSV to K5 polysaccharides. In apparent contrast with our findings, Hallak and coworkers demonstrated that N-sulfation of heparin is necessary for RSV infection (55). In this regard, it must be pointed out that heparin (but

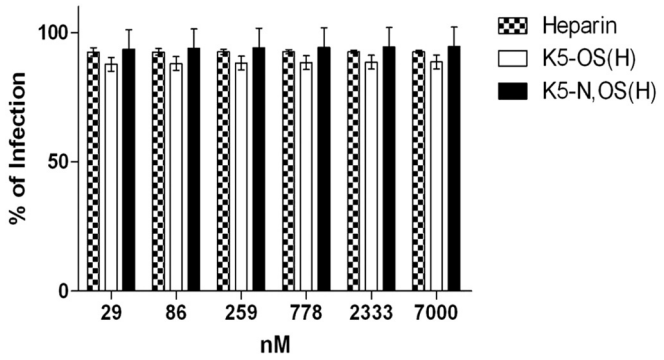


FIG 6 Antiviral assay with MA104 cells infected with human rotavirus. MA104 cells were infected in the presence of K5 derivatives or heparin. Sixteen hours postinfection, the cells were fixed and subjected to rotavirus-specific immunostaining. The infected cells were counted, and the percent infection was calculated by comparing treated and untreated wells. The results are means and SEM from triplicates.

not K5 derivatives) is epimerized and that the presence of iduronic acid instead of glucuronic acid residues confers on heparin greater flexibility (62) that in turn may allow a better presentation of the N-sulfated groups to the RSV envelope proteins G and F. In accordance with this hypothesis, when the RSV antagonist capacities of the K5 derivatives are compared to that of heparin, it is evident that their activities are enhanced by the presence of IdoA, since K5 N,OS(L) is much less active despite a similar sulfate-to-carboxyl ratio (2.2 and 2.4). Thus, the epimerization of K5 derivatives represents a promising approach for the design of even more active and specific anti-RSV compounds.

K5-OS(H) and K5-N,OS(H) are also revealed as exhibiting more potent anti-RSV activity than heparin in viral yield reduction assays (Fig. 4) and in limiting RSV cell-to-cell spread (Fig. 5). However, in the classic viral plaque assay and in the attachment assays, K5-OS(H) and K5-N,OS(H) show IC_{50}s that are comparable to or only 2 to 5 times higher than that of heparin. It should be mentioned, however, that these two assays somehow "favor" the HSPG antagonist action of GAGs that are allowed to bind to the virus before its administration to cells. Indeed, although these assays are useful in their own right and are widely used for screening purposes, they do not resemble the *in vivo* situation, which is characterized by the continuous release by infected cells of virions that promptly interact with neighboring cells, often resulting in direct cell-to-cell spread and syncytium formation.

Interestingly, we found that when K5-OS(H) and K5-N,OS(H)

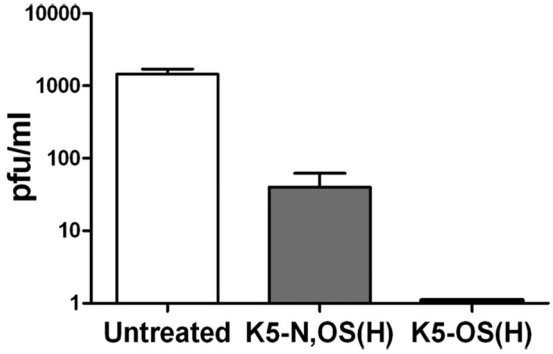


FIG 7 Reduction of viral yield on EpiAirway tissue. Fifty thousand PFU and 10 μM K5-N,OS(H) or K5-OS(H) were preincubated for 1 h at 4°C and subsequently added to the apical surface of the EpiAirway tissues. After 3 h of incubation at 37°C, the medium was removed and the cultures were washed apically with 100 μl of medium. At 72 h postinfection, 100 μl of medium was added to the apical surface, and the tissues were allowed to equilibrate for 30 min at 37°C. The suspension was then collected and titrated on A549 cells. The results are means and SEM from triplicates.

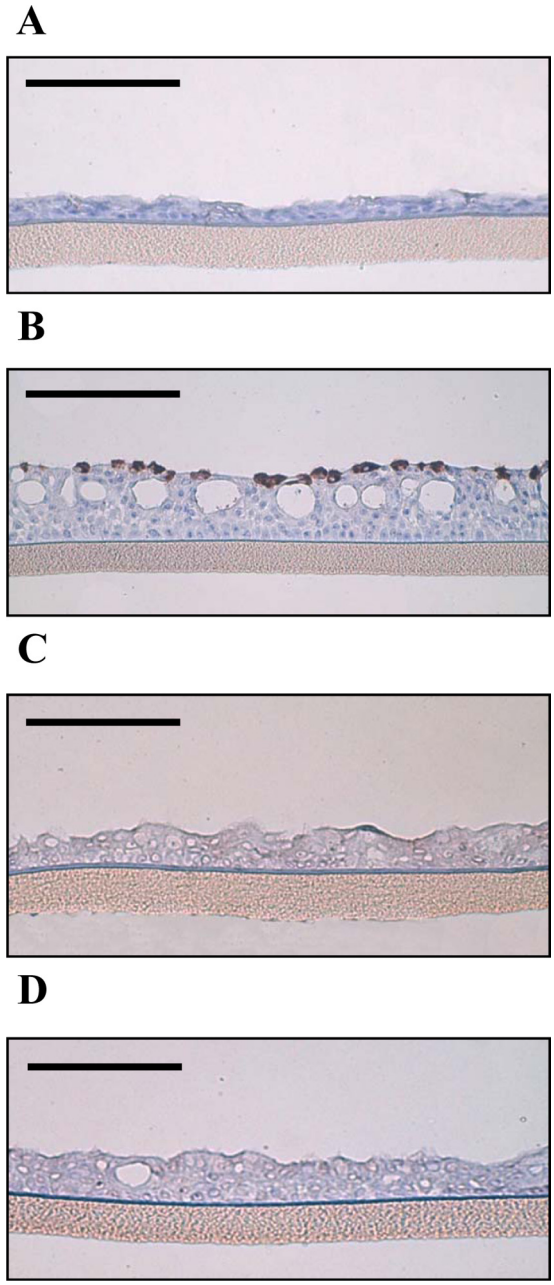


FIG 8 Reduction of RSV-infected cells in EpiAirway tissue by K5-N,OS(H) and K5-OS(H). (A) Immunohistochemistry of control tissue; (B)RSV-infected tissue (50,000 PFU); (C) RSV-infected tissue treated with 10 μM K5-N,OS(H); (D) RSV-infected tissue treated with 10 μM K5-OS(H). Three days postinfection, RSV-infected cells were identified using a RSV-specific monoclonal antibody (brown signal). The pictures shown are representative of many analyzed sections, ranging from 5 to 12 per condition. Horizontal bars correspond to 100 μm.

TABLE 3 Viability on EpiAirway tissue

Conditions	% of viability[a]
Untreated [1 h]	100
K5-N,OS(H) [1 h]	129 ± 9.8
K5-OS(H) [1 h]	127.2 ± 11.2
Untreated [4 h]	100
K5-N,OS(H) [4 h]	89.2 ± 7.9
K5-OS(H) [4 h]	90.8 ± 8.5
Untreated [18 h]	100
K5-N,OS(H) [18 h]	80.8 ± 10.2
K5-OS(H) [18 h]	81.3 ± 6.8

[a] The results presented are means and SD from triplicate tissues.

were assayed in the more stringent postinfection assay using HEp-2 cells, they retained a long-lasting RSV-inhibitory capacity comparable to that measured in the viral plaque assay, while heparin was indicated to be less effective, remaining active for only for short periods of time at concentrations that are 40 to 60 times lower than those of the two K5 derivatives (Fig. 4). Accordingly, K5-OS(H) and K5-N,OS(H) also presented significantly better inhibitory profiles than heparin when assayed for their capacity to inhibit RSV-induced syncytium formation (Fig. 5). K5-OS(H) and K5-N,OS(H) have a backbone structure more similar to that of HS than heparin, since they contain only GlcA, the presence of which, along with their high sulfate contents, might make these molecules more efficient than heparin in preventing electrostatic interactions between the RSV glycoproteins G and F and HSPGs at the cell surface. Alternatively, the peculiar structure of K5 derivatives may render these molecules more stable than heparin, thus contributing to their persistent RSV-inhibitory activity.

In conclusion, not only are the active K5 derivatives able to interfere with the virus adsorption process, but they also limit the cell-to-cell spread of RSV in a dose-dependent manner at nontoxic concentrations. These antiviral properties may be useful in the clinical setting, where K5-OS(H) and K5-N,OS(H) might be able to block both cell-to-cell spread and cell-free virus within the extracellular space—the two predominant routes of dissemination for RSV *in vivo* (63, 64, 65, 66).

As mentioned above, heparin and heparan sulfates cannot be used as anti-RSV drugs due to their anticoagulant activity and/or aspecific activities. K5-OS(H) and K5-N,OS(H), on the other hand, are endowed with a significantly lower anticoagulant activity (67). Moreover, since their structure is very similar to those of natural heparan sulfates, they can be metabolically recognized and easily catabolized without inducing toxicity, and they are expected to be tolerated by the immune system. Accordingly, recent results have shown that proinflammatory cytokines are not mobilized in the presence of K5 derivatives (67) but rather can even exert an anti-inflammatory effect (68).

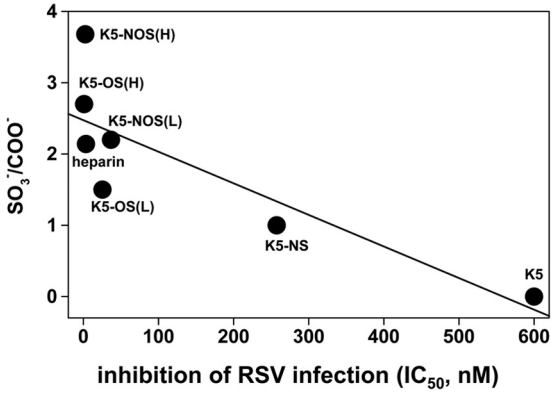


FIG 9 Correlation between the IC_{50}s of K5 derivatives and heparin with their degree of sulfation (SO_3^-/COO^-). Correlation coefficient, −0.83829; $P < 0.01848$.

Besides viral proteins (44), K5 derivatives are known to bind a wide array of eukaryotic proteins (26), implying possible adverse effects associated with their therapeutic administration. Relevant to this point, this class of molecules can be suitably tailored to produce countless compounds endowed with peculiar structural features (degree of sulfation, disposition of sulfated groups, length of GAG chain, and epimerization) (26) whose modulation impacts their binding capacity (see the discussion above), thus suggesting the possibility of producing selected K5 sulfated derivatives with specific binding capacities and biological effects.

With regard to a potential administration of K5 derivatives for the prevention or treatment of RSV infections, we assessed their antiviral activities in human tracheal/bronchial histocultures (EpiAirway). This model system avoids species extrapolation and the use of animal models at the early preclinical phase of drug development and provides a better simulation of the human respiratory tract than the cell monolayers used in standard antiviral assays. It carries the same cell type composition and polarity, mucus-secreting function, and mucociliary movements as the airway epithelium *in vivo*. Moreover, the HSPG composition and expression level *in vivo* are expected to be well duplicated in the EpiAirway tissue. In agreement with previous literature, we observed that RSV infects the lumenal ciliated columnar airway epithelial cells via the apical surfaces of the cultures, as shown in Fig. 8B (69). Both the virus yield assays and the immunohistochemical analysis of histological cross sections showed that K5-OS(H) and K5-N,OS(H) exhibit clear antiviral activity in the EpiAirway tissue at a dose of 10 μM with no signs of cytotoxic effect, indicating that this inhibitory strategy may well be effective *in vivo*. Studies to assess the clinical potential of these inhibitors against RSV infections are ongoing in animal models.

ACKNOWLEDGMENT

This work was supported by a grant from Ricerca Finanziata dall'Università degli Studi di Torino (ex 60%) 2012 to D.L.

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