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

UNIVERSITÀ DEGLI STUDI DI TORINO

Highly sulfated K5 *Escherichia coli* polysaccharide derivatives inhibit respiratory syncytial virus infectivity in cell lines and human tracheal-bronchial histocultures

Valeria Cagno[1], Manuela Donalisio[1], Andrea Civra[1], Marco Volante[2], Elena Veccelli[3], Pasqua Oreste[3], Marco Rusnati[4], David Lembo[1*]

[1] *Department of Clinical and Biological Sciences, University of Torino, 10043 Orbassano, Torino, Italy;* [2] *Department of Oncology, University of Torino 10043 Torino, Italy,* [3] *Glycores 2000 Srl, 20155 Milano, Italy;* [4]*Department of Biomedical Sciences and Biotechnology, University of Brescia, 25123 Brescia, Italy*

Running title: "Anti-RSV activity of K5 derivatives"

* Corresponding author: Prof. David Lembo
Department of Clinical and Biological Sciences
University of Torino, S. Luigi Gonzaga Hospital
Regione Gonzole, 10
10043, Orbassano, Torino, Italy
Phone: +39 011 6705484
Fax: +39 011 2365484
E-mail: david.lembo@unito.it

ABSTRACT

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 pre-attachment assays**.** Moreover, antiviral activity was also evident when the K5 derivatives were added post-infection, 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.

INTRODUCTION

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. Over 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 200-500 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 US 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 non specific antiviral drug that interferes with viral transcription; however, the non-negligible side effects of ribavirin and the recent recommendation of American Academy of Pediatrics not to use routinely 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 only administered 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 to 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 α 1–4 linked to a glucosamine (GlcN) molecule that can be N-sulfated or N-acetylated. The disaccharide sequence 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 highly 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 a strongest HSPG-antagonist activity in competition experiments (27, 28, 29, 30). This identify heparin as an ideal reference compound in studies aimed at the identification of polyanionic HSPG-antagonist compounds.

Besides 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 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 resulted to be safe and well tolerated in phase I and II studies but devoid of any important clinical benefit in phase III study. This failure of clinical trials on 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 of 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 semi-synthetic 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), CMV (40) dengue virus (29) and Human Immunodeficiency Virus (HIV) (37). Regarding this latter virus, K5 polysaccharides has been demonstrated to classically act as antimicrobial agent, likely binding to the basic gp120 protein but also to bind and neutralize 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 to generate 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 been described also for RSV and HIV infections (46).

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. K5-N,OS(H) and K5-OS(H) emerged as non toxic inhibitors of RSV infectivity both in 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 10 gr 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-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 Casu et al. (48). The antiviral results have been reproduced on 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 x 10^4/well in 96-well plates and treated the next day with serially diluted GAGs to generate dose-response curves. After 72 hours of incubation, cell viability was determined using the CellTiter 96 Proliferation Assay Kit

158 (Promega, Madison, WI, USA), according to the manufacturer's instructions. Absorbances were
159 measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. 50% cytotoxic
160 concentration (CC_{50}) values and 95% confidence intervals (CIs) were determined using Prism
161 software (GraphPad Software, San Diego, CA).
162
163 **Virus inactivation assay.** Approximately 10^4 PFU of RSV and 3.6 μg/ml of each GAG
164 (corresponding to 240 nM K5-N,OS(H), 191 nM K5-OS(H), and 263 nM heparin) were added to
165 MEM and mixed in a total volume of 100 μl. Virus-GAG mixtures were incubated for 2 hours at 37
166 °C or 4°C, serially diluted to the non-inhibitory concentration of each test compound, and the
167 residual viral infectivity determined by the viral plaque assay.
168
169 **Binding assay.** Each GAG (10 μM) was added to an aliquot of RSV ($5x10^4$ PFU) and administered
170 directly to Hep-2 or A549 cell monolayers in MEM supplemented with 2% FCS, incubated for 2
171 hours at 4°C, and washed three times to remove unbound virus. Cells were then fixed with 4%
172 paraformaldehyde, air dried and blocked with 5% BSA in PBS-Tween. Bound virus was detected
173 using the RSV monoclonal antibody RSV monoclonal antibody (Ab35958, Abcam, Cambridge,
174 UK) (diluted 1:400) incubated for 1 hour at room temperature, washed three times with PBS-Tween
175 and incubated for 2 hours at 37°C with goat anti-mouse IgG conjugated to horseradish peroxidase
176 (1:1000). At the end of incubation, plates were washed three times with PBS-Tween before adding
177 ABTS substrate (Thermo scientific, Rockford) and reading the absorbance at 405 nm. Percent
178 inhibition of virus binding was determined by comparing the absorbance measured in the presence
179 of the compound to that measured in untreated cultures.
180
181 **Viral plaque assay.** To evaluate the capacity to inhibit RSV infection, GAGs were serially diluted
182 to generate dose-response curves and added to RSV (MOI 0.01 PFU/cell). After 1 hour of
183 incubation at 4°C the mixture was added to cells grown as monolayers in a 96-well plate at a

density of 5 x 10^4/well. After 3 hours of incubation at 37°C, monolayers were washed and overlaid with 1.2% methylcellulose medium. Three days post-infection, cells were fixed with cold methanol and acetone for 1 minute and subjected to RSV-specific immunostaining using an RSV monoclonal antibody (Ab35958, Abcam, Cambridge, UK) and the UltraTech HRP streptavidin-biotin detection system (Beckman Coulter, Marseille, France). Immunostained plaques were counted and the percent inhibition of virus infectivity determined by comparing the number of plaques in compound treated wells with the number in untreated control wells. 50% inhibitory concentration (IC50) 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:

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

ii) Attachment assay. Serial dilutions of the various GAGs were pre-incubated with RSV (MOI 0.05 PFU/cell) for 1 hour at 4°C, added to cooled Hep-2 and A549 cells in 96-well plates, and incubated for 2 hours 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 hours and successively fixed and immunostained as described above. Plaques were then counted.

iii) Post-attachment 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 hours at 37°C, 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 hours. Incubation continued until the cytopathic effect involved the whole monolayer in the untreated wells. The infected cell suspensions were collected and the supernatants clarified. RSV infectivity was determined on A549 cell monolayers by standard plaque assay. Titrations were carried out at dilutions at which compounds were no more 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 viral titer measured in the presence of the compounds to that measured in untreated wells.

Syncytium formation assay. The ability of the various GAGs to block RSV cell-to-cell spread was 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 hours at 37°C, 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 hours post-infection 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 MOI 0.02 PFU/cell for 1 h at 37°C in the presence or absence of each test GAG. Virus was pre-activated 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 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 pre-filled with 900 μl pre-warmed serum free media (AIR-100-MM, MatTek Corp.) and incubated at 37°C in 5% CO_2 overnight (16-18 h). EpiAirway™ tissues were then used in the following three assays:

i) Cytotoxicity assay. The cytotoxicity of K5 derivatives on mucous membranes was assessed using the 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 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 ELISA plate reader at a wavelength of 570 nm. Tissues incubated with assay medium were used as negative controls. The ET50 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, ET50 values > 18h indicate that a tested compound is not irritating.

ii) 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 1h 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, 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 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. Viral collection was performed sequentially from the same wells on each day post-infection.

iii) Detection of RSV in EpiAirway tissue by immunohistochemistry. RSV was detected immunohistochemically using a specific mouse monoclonal antibody against RSV (Ab35958, Abcam, Cambridge, UK). 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 post-test, if *P* values showed significant differences, using GraphPad Prism 5.00 (GraphPad Software). Results are expressed as means ± CI or SEM or SD, as noted in the figure legends.

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 structure similar to 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 on 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 non-determinable 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 activity and were therefore selected for further investigation. Thus, the effect of K5-N,OS(H), K5-OS(H) on cell viability was investigated on 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, K5-N,OS(H) and K5-OS(H) exerted a moderate dose-dependent reduction of cell viability only in Hep-2 cells at 50 μM, 100 μM and 200μM that 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 to 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 down-regulating 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, pre-attachment assays were performed as described above. To this end, Hep-2 and A549 cells were pre-incubated for 2 h with different concentrations of K5-N,OS(H) or K5-OS(H), or 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 activity of many sulfated polysaccharides corresponds 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} values that are comparable to those measured in the classical viral plaque assay (see Tab. 2), suggesting that the antiviral activity of these GAGs depends on their capacity to inhibit the attachment of the virus to the cell surface.

To substantiate this interpretation, binding assays were performed in which we directly evaluated the amounts of virus particles bound to the cells in the presence or absence of the active GAGs. 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 (Figures 3C and 3D, respectively) while unsulfated K5, which does not exhibit any antiviral activity, did not.

Taken together, these results indicate that the main mechanism-of-action of the K5 derivatives consists in their capacity to hamper the virus's interaction with entry receptor(s) expressed on the surface of target cells.

K5 derivatives reduce viral yield for 24 h post-infection. To evaluate whether the reduction of RSV attachment and infection exerted by K5-N,OS(H) and K5-OS(H) is maintained on 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} values (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 only exerted a modest inhibitory activity. Of note, K5-N,OS(H), K5-OS(H) and heparin were not cytotoxic even at the highest concentration tested (7µM) as shown in Fig. S1.
In the second set of experiments a single concentration of K5-N,OS(H), K5-OS(H), or heparin was added 1h, 2h, 3h, and 24h after the removal of the virus inoculum. The results shown in Fig. 4B and C demonstrate that viral yield reduction is effective when the compounds are added up to 24 h post infection. Once again, the inhibition profile of K5-N,OS(H) and K5-OS(H) was 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 syncytia formation. A 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 help 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 post-infection, 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 syncytia 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 to 259.3 nM. In contrast, a significant reduction in plaque size following treatment with heparin was only observed in Hep-2 cells 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 have been identified that exhibit antiviral activity against a panel of HSPGs-dependent viruses, including HSV, HIV, and HPV (see introduction). 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 evidence corroborating the hypothesis that the anti-RSV ability of these K5 derivatives depends 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 depends 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 activity 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 of K5-N,OS(H) or K5-OS(H) preincubated for 1h at 4°C prior virus application. At 72 h post-infection, the virus titer in untreated control tissues was $1.45x10^3$ 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 post-infection 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 in tissues treated with 10 μM of each K5 derivative for 1, 4 or 18 h at 37°C. The results shown in Table 3 demonstrate that these K5 derivatives are not cytotoxic, and the time required to reduce tissue viability to 50% (ET50) 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 RSV G protein also block virus infection (18). The feasibility of the second approach, on the other hand, has been supported from the demonstration that heparin (19; 55), as well as 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).

438 The capsular K5 polysaccharide from *Escherichia coli* can be chemically sulfated in selected
439 positions, resulting in the synthesis of completely N-sulfated compounds with different amounts of
440 O-sulfation in different positions or completely N-Acetylated molecules differing only in the
441 position and degree of O-sulfation. (30). Due to these features, sulfated K5 derivatives have been
442 useful in the study of the structure-activity relationship of the interactions of several viral proteins
443 with heparin, and used in the design of specific antiviral polysaccharides.
444 Here, we found that selected K5 sulfated derivatives exert a strong anti-RSV effect. Experiments
445 aimed at elucidating their mechanism(s) of anti-RSV action indicate that the inhibitory effect is
446 mainly due to the capacity of K5-N,OS(H) and K5-OS(H) to interact with the virus particles, rather
447 than with cell components, and thereby preventing virus attachment to the cell surface. Several lines
448 of evidence support this. First, cells pre-treated with K5 derivatives remained susceptible to RSV
449 infection, thus excluding the possibility that these compounds form stable interactions with one or
450 more cellular components, preventing their interaction with viral glycoproteins. Second, the results
451 of the binding and attachment assays demonstrate that K5-N,OS(H) and K5-OS(H) block the
452 adsorption of RSV virions to the cell surface with a potency similar to that of heparin, which has
453 been shown to prevent RSV infection by competing with cellular HSPGs for binding to virion
454 components (60, 61) Third, pre-incubation of RSV virions with the active sulfated K5 derivatives
455 did not result in loss of infectivity, suggesting that the antiviral activity of the compounds does not
456 rely on inactivation of virion component(s). A similar mechanism-of-action was previously
457 observed for heparin when tested against HSV-1 and RSV (50, 49) and when K5 derivatives were
458 tested against HCMV (40).
459 Unsulfated K5 polysaccharide, unlike K5-N,OS(H) and K5-OS(H), did not show any significant
460 RSV-antagonist activity, indicating that the sulfate groups, rather than the backbone structure,
461 mediate the interaction with RSV. Moreover, a good correlation exists between the degree of
462 sulfation of the GAGs tested and their capacities to inhibit RSV infection (Fig. 9). However, this
463 correlation is lost in the highly sulfated GAGs (see 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 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 a 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) compared to K5-NOS(H) (from 2.7 to 3.68, Tab.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 co-workers demonstrated that N-sulfation of heparin is necessary for RSV infection (55). To this regard, it must be pointed out that heparin (but not K5 derivatives) is epimerized and that the presence of iduronic acid instead of glucuronic acid residues confers heparin greater flexibility (62) that, in turn, may allow a better presentation of the N-sulfated groups to 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} that are comparable or only 2-5 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 widely used for screening purposes, they do not resemble the *in*

vivo situation, which is characterized by the continuous release of virion by infected cells that promptly interact with neighboring cells, often resulting in direct cell-to-cell spread and syncytia formation.

Interestingly, we found that when K5-OS(H) and K5-N,OS(H) were assayed in the more stringent post-infection assay using Hep-2 cells, they retained a long-lasting RSV-inhibitory capacity comparable to that measured in the viral plaque assay, while heparin resulted as being less effective, remaining active for only for short periods of time at concentrations that are 40-60 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 syncytia 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 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 non-toxic 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 an anti-RSV drug due to its anticoagulant activity and/or aspecific activities. K5-OS(H) and K5-N,OS(H), on the other hand, are endowed with a significantly lower anti-coagulant activity (67). Moreover, since their structure is very similar to 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).

Beside viral proteins (44), K5 derivatives are known to bind a wide array of eukaryotic proteins (26), inferring 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, epimerization) (26) whose modulation impacts their binding capacity (see discussion above), thus suggesting the possibility to produce selected K5 sulfated derivatives with specific binding capacity and biological effects.

With regard to a potential administration of K5 derivatives for the prevention or treatment of RSV infections, we assessed their antiviral activity in human tracheal/bronchial histocultures (EpiAirway^{TM}). 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^{TM} 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^{TM} 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 are ongoing in animal models to assess the clinical potential of these inhibitors against RSV infections.

ACKNOWLEDGEMENTS

This work was supported by a grant from “Ricerca finanziata dall'Università degli Studi di Torino (ex 60%) 2012” to DL.

REFERENCES

1. **Collins PL, Crowe Jr, JE.** 2007. Respiratory syncytial virus and Metapneumovirus. p 1601–1646. *In* Knipe DM, HowleyPM(ed) Fields virology, 5th ed. Lippincott Williams and Wilkins, Philadelphia, PA.

2. **Shay DK, Holman RC, Roosevelt GE, Clarke MJ, Anderson LJ.** 2001. Bronchiolitis-associated mortality and estimates of respiratory syncytial virus-associated deaths among US children, 1979–1997. J. Infect. Dis. **183**:16 –22.

3. **Falsey AR, Formica MA, Hennessey PA, Criddle MM, Sullender WM, Walsh EE.** 2006. Detection of respiratory syncytial virus in adults with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **173**:639–643.

4. **Leader S, Kohlhase K.** 2002. Respiratory syncytial virus-coded pediatric hospitalizations, 1997 to 1999. Pediatr. Infect. Dis. J. **21**:629–632.

5. **World Health Organization.** 2009. Initiative for vaccine research (IVR). Acute Respiratory Infections. Geneva, Switzerland. http://www.who.int/vaccine_research/diseases/ari/en/.

6. **Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P.** 2009. The burden of respiratory syncytial virus infection in young children. N. Engl. J. Med. **360**:588 –598.

7. **Pelletier AJ, Mansbach JM, Camargo Jr, CA.** 2006. Direct medical costs of bronchiolitis hospitalizations in the United States. Pediatrics **118**:2418– 2423

8. **Corsello G.** 2007. Bronchiolitis: the new American Academy of Pediatrics guidelines. J. Chemother. **19**:12–14.

9. **Castilow EM, Varga SM.** 2008. Overcoming T cell-mediated immunopathology to achieve safe RSV vaccination. Future Virol. **3**:445– 454.

10. **American Academy of Pediatrics Subcommittee on Diagnosis and Management of Bronchiolitis.** 2006. Diagnosis and management of bronchiolitis. Pediatrics. **118**(4):1774-1793.

11. **Leyssen P, De Clercq E, Neyts J**. 2008. Molecular strategies to inhibit the replication of RNA viruses. Antiviral Res. **78**:9-25.

12. **Sidwell RW, Barnard DL**. 2006. Respiratory syncytial virus infections: recent prospects for control. Antiviral Res. **71**(2-3):379-90.

13. **Johnson S, Oliver C, Prince GA, Hemming VG, Pfarr DS, Wang SC, Dormitzer M, O'Grady J, Koenig S, Tamura JK, Woods R, Bansal G, Couchenour D, Tsao E,Hall WC, Young JF.** 1997. Development of a humanized monoclonal antibody (MEDI-493) with potent in vitro and in vivo activity against respiratory syncytial virus. J. Infect. Dis. **176**:1215–1224.

14. **Wu H, Pfarr DS, Losonsky GA, Kiener PA.** 2008. Immunoprophylaxis of RSV infection: advancing from RSV-IGIV to palivizumab and motavizumab. Curr. Top. Microbiol. Immunol. **317**:103–123.

15. **Welliver RC.** 2010. Pharmacotherapy of respiratory syncytial virus infection. Curr. Opin. Pharmacol. **10**:289 –293.

16. **van Drunen Littel-van den Hurk S, Watkiss ER.** 2012. Pathogenesis of respiratory syncytial virus. Curr Opin Virol. **2**:300 – 305

17. **King JC Jr.** 1997. Community respiratory viruses in individuals with human immunodeficiency virus infection. Am. J. Med. **102**:19-24

18. **Crim RL, Audet SA, Feldman SA, Mostowski HS, Beeler JA.** 2007. Identification of linear heparin-binding peptides derived from human respiratory syncytial virus fusion glycoprotein that inhibit infectivity. J. Virol. **81:**261-271.

19. **Feldman SA, Audet S, Beeler JA**. 2000. The fusion glycoprotein of human respiratory syncytial virus facilitates virus attachment and infectivity via an interaction with cellular heparan sulfate. J Virol. **74**:6442-6457.

20. **Feldman SA, Hendry RM, Beeler JA.** 1999. Identification of a linear heparin binding domain for human respiratory syncytial virus attachment glycoprotein G. J. Virol. **73:**6610–6617.

622 **21. Tayyari F, Marchant D, Moraes TJ, Duan W, Mastrangelo P, Hegele RG.** 2011.
623 Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. Nat.
624 Med. **17**:1132–1135.
625
626 **22. Bose S, Basu M, Banerjee AK.** 2004. Role of nucleolin in human parainfluenza virus type
627 3 infection of human lung epithelial cells. J. Virol. **78:** 8146–8158.
628
629 **23. Xiao X, Feng Y, Zhu Z, Dimitrov DS.** 2011. Identification of a putative Crimean-Congo
630 hemorrhagic fever virus entry factor. Biochem. Biophys. Res. Commun. **411:**253–258.
631
632 **24. Thongtan T, Wikan N, Wintachai P, Rattanarungsan C, Srisomsap C, Cheepsunthorn**
633 **P, Smith DR.** 2012. Characterization of putative Japanese encephalitis virus receptor
634 molecules on microglial cells. J. Med. Virol. **84:**615–623.
635
636 **25. Nisole S, Krust B, Callebaut C, Guichard G, Muller S, Briand JP, Hovanessian AG.**
637 1999. The anti-HIV pseudopeptide HB-19 forms a complex with the cell-surface-expressed
638 nucleolin independent of heparin sulfate proteoglycans. J. Biol. Chem. **274:**27875–27884.
639
640 **26. Rusnati M, Oreste P, Zoppetti G, Presta M.** 2005. Biotechnological engineering of
641 heparin/heparan sulphate: a novel area of multi-target drug discovery. Curr Pharm
642 Des. **11**:2489-2509
643
644 **27. Matos PM, Andreu D, Santos NC, Gutiérrez-Gallego R.** 2013. Structural requirements of
645 glycosaminoglycans for their interaction with HIV-1 envelope glycoprotein gp120. Arch
646 Virol. In press.
647

28. **Rusnati M, Coltrini D, Oreste P, Zoppetti G, Albini A, Noonan D, D'Adda di Fagagna F, Giacca M, Presta M.** 1997. Interaction of HIV-1 Tat protein with heparin. Role of the backbone structure, sulfation, and size. J Biol Chem. **272**(17):11313-20.

29. **Vervaeke P, Alen M, Noppen S, Schols D, Oreste P, Liekens S.** 2013. Sulfated Escherichia coli K5 Polysaccharide Derivatives Inhibit Dengue Virus Infection of Human Microvascular Endothelial Cells by Interacting with the Viral Envelope Protein E Domain III. PLoS One. **8**:e74035.

30. **Bugatti A, Giagulli C, Urbinati C, Caccuri F, Chiodelli P, Oreste P, Fiorentini S, Orro A, Milanesi L, D'Ursi P, Caruso A, Rusnati M.** 2013. Biochemical characterization of HIV-1 matrix protein p17 interaction with heparin. J. Biol. Chem. **288**:1150-1161.

31. **Patel M, Yanagishita M, Roderiquez G, Bou-Habib DC, Oravecz T, Hascall VC, Norcross MA.** 1993. Cell-surface heparan sulfate proteoglycan mediates HIV-1 infection of Tcell lines. AIDS Res Hum Retroviruses **9**:167–174.

32. **Shieh MT, Dunn DW, Montgomery RI, Esko JD, Spear PG**.1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol **116**:1273–1281.

33. **Giroglou T, Florin L, Schafer F, Streeck RE, Sapp M.** 2001. Human papillomavirus infection requires cell surface heparan sulfate. J Virol **75**:1565–1570.

34. **Compton, T.** 2004. Receptors and immune sensors: the complex entry path of human cytomegalovirus. Trends Cell Biol **14:**5-8.

35. Chen Y, Maguire T, Hileman RE, Fromm JR, Esko J, Linhardt RJ, Marks RM. 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med **3**:866–871.

36. Salvador B, Sexton NR, Carrion R Jr, Nunneley J, Patterson JL, Steffen I, Lu K, Muench MO, Lembo D, Simmons G. 2013. Filoviruses utilize glycosaminoglycans for their attachment to target cells. J Virol **87**:3295-3304.

37. Vicenzi E, Gatti A, Ghezzi S, Oreste P, Zoppetti G, Poli G. 2003. Broad spectrum inhibition of HIV-1 infection by sulfated K5 Escherichia coli polysaccharide derivatives. AIDS **17**:177−181.

38. Pinna D, Oreste P, Coradin T, Kajaste-Rudnitski A, Ghezzi S, Zoppetti G, Rotola A, Argnani R, Poli G, Manservigi R, Vicenzi E. 2008. Inhibition of herpes simplex virus types 1 and 2 in vitro infection by sulfated derivatives of Escherichia coli K5 polysaccharide. Antimicrob Agents Chemother. **52**: 3078-84

39. Lembo D, Donalisio M, Rusnati M, Bugatti A, Cornaglia M, Cappello P, Giovarelli M, Oreste P, Landolfo S. 2008. Sulfated K5 Escherichia coli polysaccharide derivatives as wide-range inhibitors of genital types of human papillomavirus. Antimicrob Agents Chemother. **52**:1374-1381.

40. Mercorelli B, Oreste P, Sinigalia E, Muratore G, Lembo D, Palù G, Loregian A. Sulfated derivatives of Escherichia coli K5 capsular polysaccharide are potent inhibitors of human cytomegalovirus. Antimicrob Agents Chemother. **54**:4561-4567.

700 **41. Ludwig M, Enzenhofer E, Schneider S, Rauch M, Bodenteich A, Neumann K, Prieschl-**
701 **Grassauer E, Grassauer A, Lion T, Mueller CA.** 2013. Efficacy of a carrageenan nasal
702 spray in patients with common cold: a randomized controlled trial. Respir Res. **14**:124.
703
704 **42. Marais D, Gawarecki D, Allan B, Ahmed K, Altini L, Cassim N, Gopolang F, Hoffman**
705 **M, Ramjee G, Williamson AL.** 2011. The effectiveness of Carraguard, a vaginal
706 microbicide, in protecting women against high-risk human papillomavirus infection. Antivir
707 Ther. **16**(8):1219-26.
708
709 **43. Pirrone V, Wigdahl B, Krebs FC.** 2011. The rise and fall of polyanionic inhibitors of the
710 human immunodeficiency virus type 1. Antiviral Res. **90**(3):168-82.
711
712 **44. Rusnati M, Vicenzi E, Donalisio M, Oreste P, Landolfo S, Lembo D.** 2009. Sulfated K5
713 Escherichia coli polysaccharide derivatives: A novel class of candidate antiviral
714 microbicides. Pharmacol Ther. **123**: 310-322.
715
716 **45. Urbinati C, Bugatti A, Oreste P, Zoppetti G, Waltenberger J, Mitola S, Ribatti**
717 **D, Presta M, Rusnati M.** 2004. Chemically sulfated Escherichia coli K5 polysaccharide
718 derivatives as extracellular HIV-1 Tat protein antagonists. FEBS Lett. **568**(1-3):171-177.
719
720 **46. Moyes J, Cohen C, Pretorius M, Groome M, von Gottberg A, Wolter N, Walaza**
721 **S, Haffejee S, Chhagan M, Naby F, Cohen AL, Tempia S, Kahn K, Dawood H,Venter**
722 **M, Madhi SA; South African Severe Acute Respiratory Illness Surveillance Group.**
723 2013. Epidemiology of respiratory syncytial virus-associated acute lower respiratory tract

infection hospitalizations among HIV-infected and HIV-uninfected South African children, 2010-2011. J Infect Dis. **208**(3):S217-26.

47. Leali D, Belleri M, Urbinati C, Coltrini D, Oreste P, Zoppetti G, Ribatti D, Rusnati M, Presta M. 2001. Fibroblast growth factor-2 antagonist activity and angiostatic capacity of sulfated *Escherichia coli* K5 polysaccharide derivatives. J. Biol. Chem. **276:**37900–37908

48. Casu B, Gennaro U. 1975. A conductimetric method for the determination of sulphate and carboxyl groups in heparin and other mucopolysaccharides. Carbohydr Res. **39**(1):168-76.

49. Donalisio M, Rusnati M, Cagno V, Civra A, Bugatti A, Giuliani A, Pirri G, Volante M, Papotti M, Landolfo S, Lembo D. 2012. Inhibition of human respiratory syncytial virus infectivity by a dendrimeric heparan sulfate-binding peptide. Antimicrob Agents Chemother. **56**:5278-5288.

50. Graham KL, Zeng W, Takada Y, Jackson DC, Coulson BS. 2004. Effects on rotavirus cell binding and infection of monomeric and polymeric peptides containing alpha2beta1 and alphaxbeta2 integrin ligand sequences. J Virol. **78**:11786-11797.

51. Ghosh T, Chattopadhyay K, Marschall M, Karmakar P, Mandal P, Ray B. 2009. Focus on antivirally active sulfated polysaccharides: from structure-activity analysis to clinical evaluation. Glycobiology. **19**:2-15.

52. Morton CJ, Cameron R, Lawrence LJ, Lin B, Lowe M, Luttick A, Mason A, McKimm-Breschkin J, Parker MW, Ryan J, Smout M, Sullivan J, Tucker SP, Young PR. 2003**.** Structural characterization of respiratory syncytial virus fusion inhibitor escape

mutants: homology model of the F protein and a syncytium formation assay. Virology. **311**:275-288.

53. Black CP. 2003. Systematic Review of the Biology and Medical Management of Respiratory Syncytial Virus Infection. Respir Care **48**:209-231

54. Lopez S, Arias CF. 2004. Multistep entry of rotavirus into cells: a Versaillesque dance. Trends Microbiol. **12:**271–278.

55. Hallak LK, Spillmann D, Collins PL, Peeples ME. 2000. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. J. Virol. **74**:10508 –10513.

56. Hallak LK, Collins PL, Knudson W, Peeples ME. 2000. Iduronic acid containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. Virology **271**:264 –275.

57. Kimura K, Ishioka K, Hashimoto K, Mori S, Suzutani T, Bowlin TL, Shigeta S. 2004. Isolation and characterization of NMSO3-resistant mutants of respiratory syncytial virus. Antiviral Res. **61**:165–171.

58. Hosoya M, Balzarini J, Shigeta S, De Clercq E. 1991, Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. Antimicrob Agents Chemother. **35**(12):2515-2520.

775 **59. Rusnati M, Urbinati C.** 2009. Polysulfated/sulfonated compounds for the development of
776 drugs at the crossroad of viral infection and oncogenesis. Curr. Pharm. Des. **15**:2946-2957.
777
778 **60. Krusat T, Streckert HJ.** 1997. Heparin-dependent attachment of respiratory syncytial virus
779 (RSV) to host cells. Arch Virol. **142**:1247-1254.
780
781 **61. Bourgeois C, Bour JB, Lidholt K, Gauthray C, Pothier P.** 1998. Heparin-like structures
782 on respiratory syncytial virus are involved in its infectivity in vitro. J Virol. **72**:7221-7227
783
784 **62. Mulloy B, Forster MJ.** 2000. Conformation and dynamics of heparin and heparan sulfate.
785 Glycobiology **10**(11):1147-56.
786
787 **63. Delage G, Brochu P, Robillard L, Jasmin G, Joncas JH, Lapointe N.** 1984. Giant cell
788 pneumonia due to respiratory syncytial virus. Occurrence in severe combined
789 immunodeficiency syndrome. Arch. Pathol. Lab. Med. **108**:623-625.
790
791 ***64.*** **Neilson KA, Yunis EJ.** 1990. Demonstration of respiratory syncytial virus in an autopsy
792 series. Pediatr. Pathol. **10**:491-502.
793
794 **65. Collins PL, Graham BS.** 2008. Viral and host factors in human respiratory syncytial virus
795 pathogenesis. J Virol. **82**:2040-2055.
796
797 **66. Richardson LS, Belshe RB, Sly DL, London WT, Prevar DA, Camargo E, Chanock**
798 **RM.** 1978. Experimental respiratory syncytial virus pneumonia in cebus monkeys. J Med
799 Virol. **2**:45-59.
800

67. Oreste P, Zoppetti G. 2012. Semi-synthetic heparinoids. Handb Exp Pharmacol **(207):**403-422.

68. Collino M, Castiglia S, Manoni M, Salsini L, Chini J, Masini E, Fantozzi R. 2009. Effects of a semi-synthetic N-,O-sulfated glycosaminoglycan K5 polysaccharide derivative in a rat model of cerebral ischaemia/reperfusion injury. Thromb Haemost. **102**(5):837-45.

69. Zhang L, Peeples ME, Boucher RC, Collins PL, Pickles RJ. 2002. Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology. J Virol. **76**:5654-5666.

812 **FIGURE LEGENDS**

813 **FIG 1.** K5 derivatives are not active in a virus inactivation assay.

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

818

819 **FIG 2.** Pre-attachment assay in Hep-2 and A549 cells.

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

824

825 **FIG 3.** Investigation of the hit compounds inhibitory mechanism.

826 In the attachment assay RSV and compounds were added to Hep-2 cells (A) or A549 (B) for 2 h at
827 4°C. Cells were shifted to 37°C, and at 72 h postinfection they were subjected to RSV-specific
828 immunostaining, the plaques were counted, and the percent of infection was calculated by
829 comparing treated to untreated wells. In the binding assay, the virus bound to Hep-2 (C) or A549
830 (D) was detected by ELISA immediately after the removal of the virus inoculum. Each absorbance
831 was mock subtracted and the percent of infection was calculated by comparing treated to untreated
832 wells. The results are mean and SEM for triplicates.

833

834 **FIG 4.** Reduction of viral yield.

835 (A) Hep-2 cells were infected and subsequently treated with different concentrations of compounds.
836 When the cytophatic effect involved the whole monolayer of untreated wells, supernatant were

harvested and titrated. The results are mean and SEM for triplicates. The table in panel A shows the IC_{50} and the 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 post-infection ranging from 0 h to 24 h. The results are mean and SEM for triplicates. * P<0.05 in a 2 way ANOVA .

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) and 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:7000 nM, 2333.3 nM, 777.8 nM and 259.3 nM. Formation of syncytia was assessed 72 h after infection, by immunostaining. The histograms show the % of plaque area of treated wells compared to untreated wells as a function of compounds 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

FIG 6. Antiviral assay on MA104 infected with Human Rotavirus.

MA104 cells were infected in presence of K5 derivatives or heparin. 16 hours post-infection, 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 for triplicates.

FIG 7. Reduction of viral yield on EpiAirway tissue.

50,000 pfu and 10 μM of K5-N,OS(H) or K5-OS(H) were pre-incubated for 1h at 4°C and subsequently added on the apical surface of the EpiAirway tissues. After 3 h of incubation at 37°C,

863 the medium was removed, the cultures were washed apically with 100 ml of medium. At 72 hours
864 post-infection, 100 μl of medium was added to on the apical surface and the tissues were allowed
865 to equilibrate for 30 minutes at 37°C. The suspension was then collected and titrated on A549 cells.
866 The results are mean and SEM for triplicates.
867
868 **FIG 8.** Reduction of RSV-infected cells in EpiAirway tissue by K5-N,OS(H) and K5-OS(H).
869 (A) Immunohistochemistry of control tissue; (B)RSV-infected tissue (50,000 PFU); (C) RSV-
870 infected tissue treated with 10 μM of K5-N,OS(H); (D) RSV-infected tissue treated with 10 μM of
871 K5-OS(H). Three days postinfection RSV-infected cells were detected using a RSV-specific
872 monoclonal antibody (brown signal). The pictures shown are representative of many analyzed
873 sections, ranging from 5 to 12 per condition. Horizontal bars correspond to 100 μm.
874
875 **FIG 9.** Correlation between the IC_{50} values of K5 derivatives and heparin with their degree of
876 sulfation (SO3-/COO-). Correlation coefficent: -0.83829 $p<0.01848$.

TABLE 1. Molecular weight and sulfation degree of the GAGs used in this work

	MW	**SO3-/COO-**
Unmodified Heparin	13700	2.14
Unsulfated K5	18700	-
K5-NS	15600	1
K5-N,OS(L)	12500	2.2
K5-N,OS(H)	14700	3.68
K5-OS(L)	20000	1.5
K5-OS(H)	18800	2.7

TABLE 2. Screening of K5 derivatives on A549 and Hep-2 cells.

	A549			
Compound	**IC_{50} * (nM)**	**95% CI****	**CC_{50}*** (nM)**	**SI**
K5	>600	NA	> 24000	> 40
K5- NS	257.40	164.5 – 402.8	> 24000	> 93.24
K5-N,OS(L)	36.81	23.3 – 58.2	> 24000	> 651.99
K5-N,OS(H)	2.56	2.07 – 3.18	> 24000	> 9375
K5-OS(L)	25.42	15.2 – 42.5	> 24000	> 944.14
K5-OS(H)	1.07	0.704 – 1.624	> 24000	> 22429.91
Heparin	3.52	2.08 – 5.96	> 24000	> 6818.18

	Hep-2			
Compound	**IC_{50} * (nM)**	**95% CI****	**CC_{50}*** (nM)**	**SI**
K5	> 600	NA	> 24000	> 40
K5-NS	340	240 – 500	> 24000	> 70.59
K5-N,OS(L)	28.81	21.88 – 37.94	> 24000	> 833.04
K5-N,OS(H)	3.71	3.36 – 4.12	> 24000	> 6469
K5-OS(L)	44.69	31.34 – 63.72	> 24000	> 537.03
K5-OS(H)	2.20	1.76 – 2.74	> 24000	> 246857
Heparin	3.73	3.17 – 4.39	> 24000	> 6434.32

* IC_{50}: 50% inhibitory concentration

** 95% CI: 95% confidence interval

***CC_{50}: 50% cytotoxic concentration

Values are means and CIs for three separate determinations.

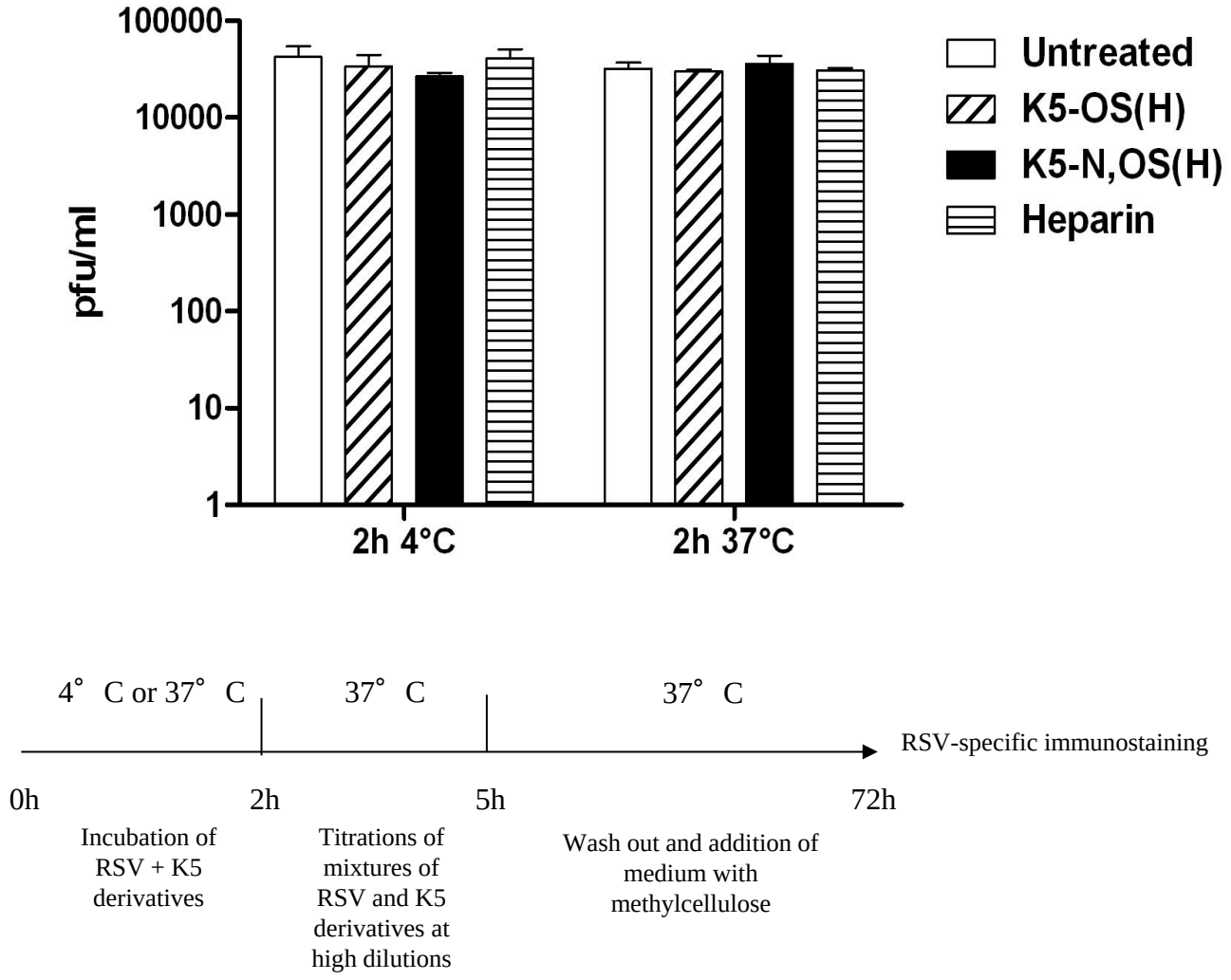
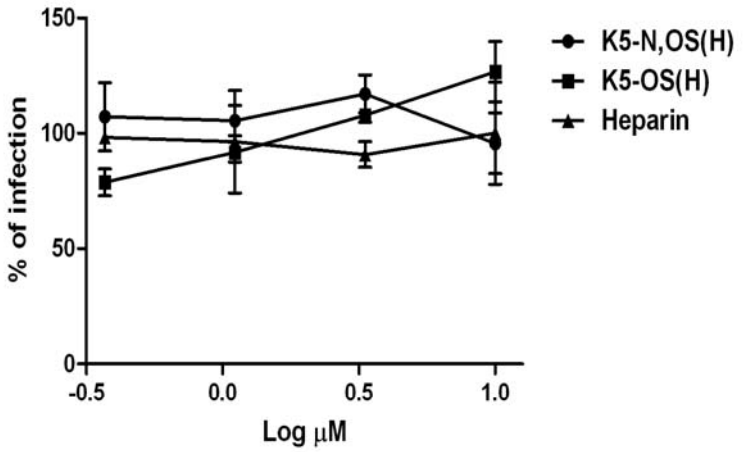
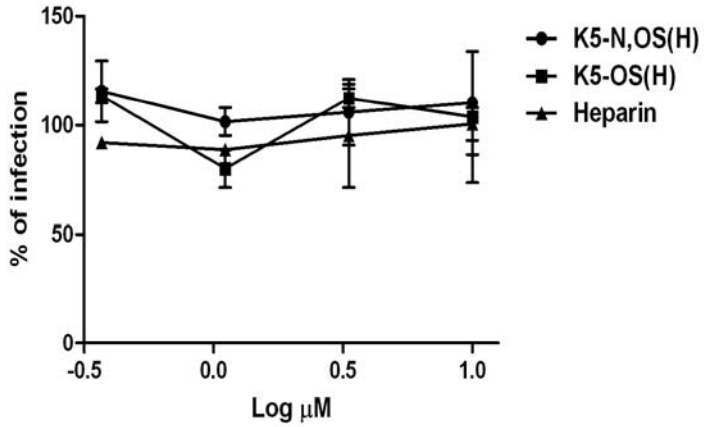

100000
10000
1000
100
10
1
pfu/ml
2h 4°C
2h 37°C
Untreated
K5-OS(H)
K5-N,OS(H)
Heparin
4° C or 37° C
37° C
37° C
RSV-specific immunostaining
0h
2h
5h
72h
Incubation of RSV + K5 derivatives
Titrations of mixtures of RSV and K5 derivatives at high dilutions
Wash out and addition of medium with methylcellulose

Figure 1

A


150
100
50
0
% of infection
-0.5
0.0
0.5
1.0
Log μM
K5-N,OS(H)
K5-OS(H)
Heparin

B


150
100
50
0
% of infection
-0.5
0.0
0.5
1.0
Log μM
K5-N,OS(H)
K5-OS(H)
Heparin

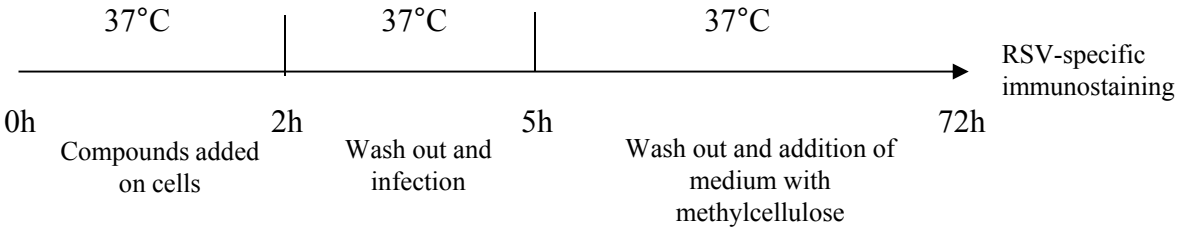
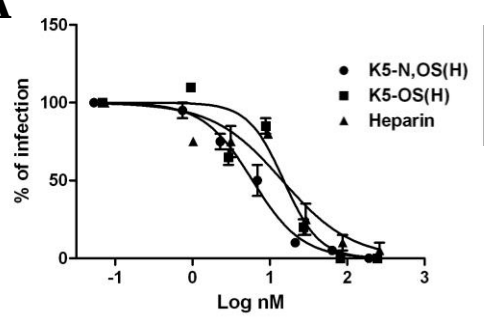

37°C
37°C
37°C
RSV-specific immunostaining
0h
2h
5h
72h
Compounds added on cells
Wash out and infection
Wash out and addition of medium with methylcellulose

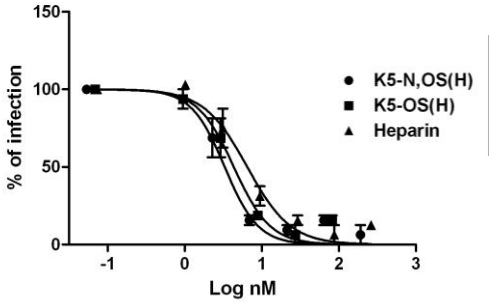
Figure 2

A



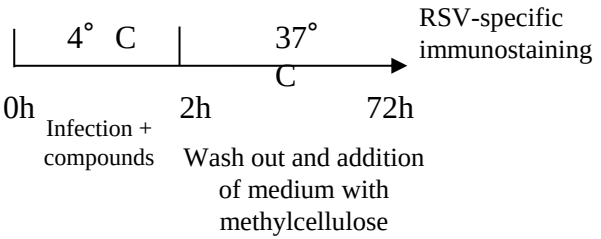
	IC50 (nM)	95% CI (nM)
K5-N,OS(H)	5.84	3.82-8.93
K5-OS(H)	1.86	0.96-3.62
Heparin	11.76	8.00-17.29

B

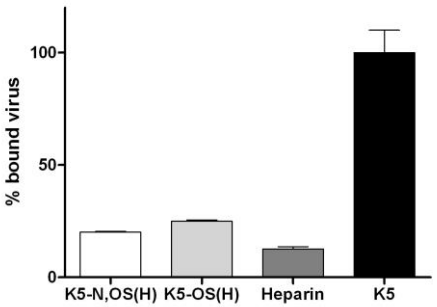


	IC50 (nM)	95% CI (nM)
K5-N,OS(H)	4.23	3.09-5.77
K5-OS(H)	3.40	2.51-4.58
Heparin	6.49	4.47-8.83

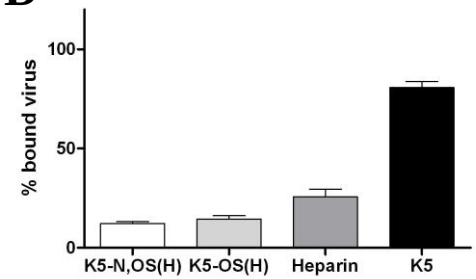
Attachment assay



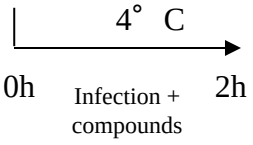
C



D



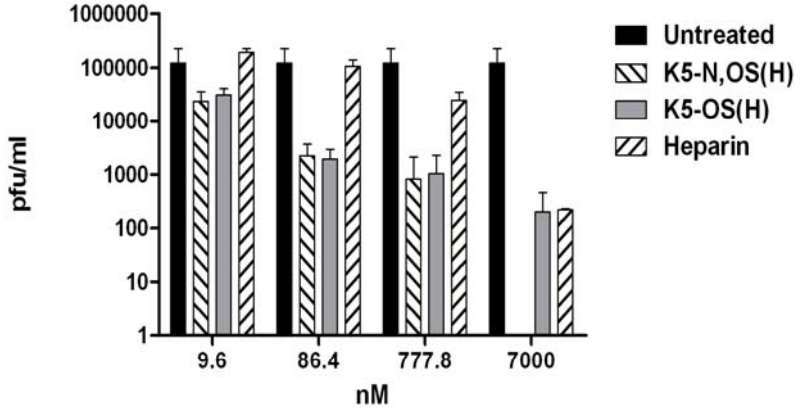
Binding assay



Cells were fixed and subjected to ELISA

Figure 3

A



	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

B

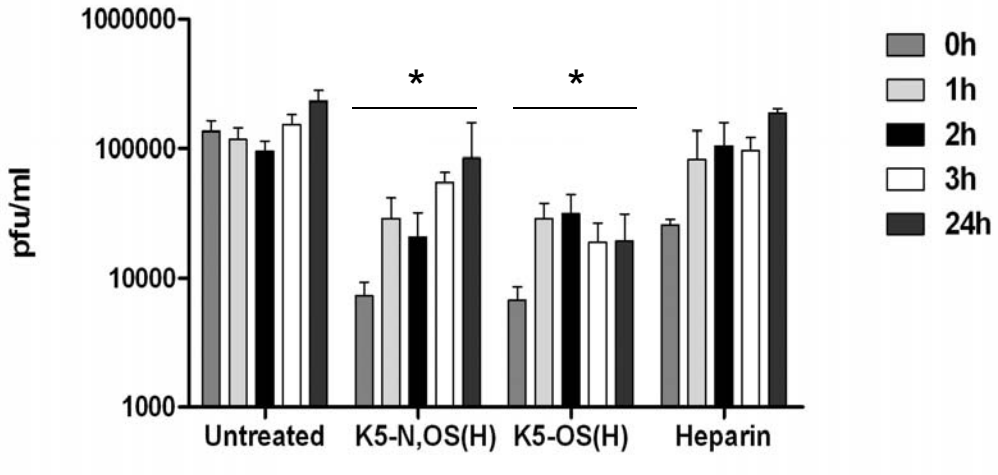
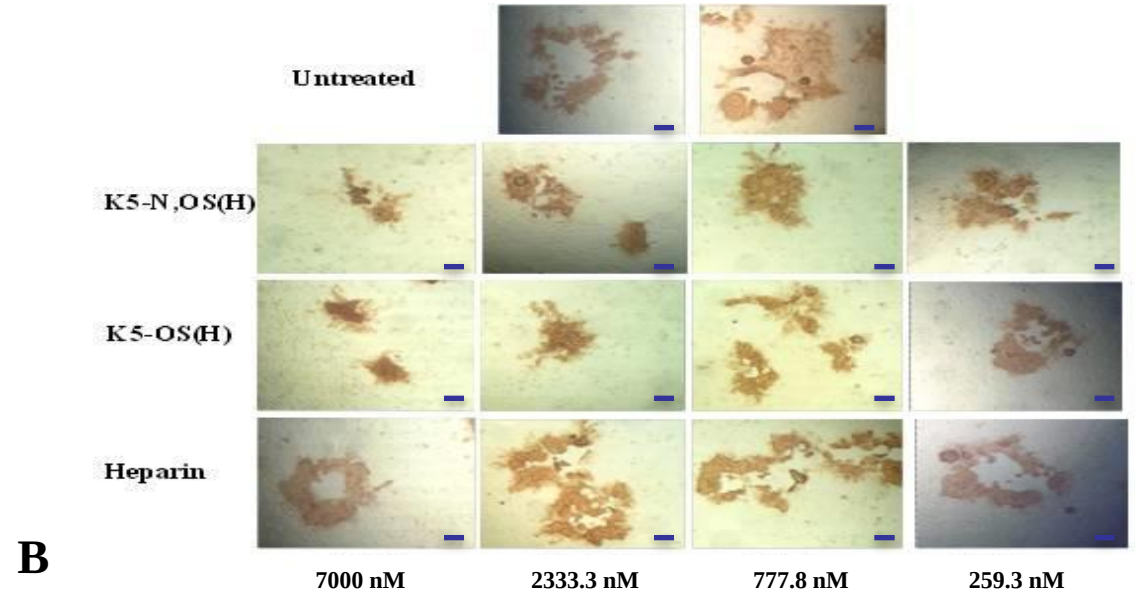
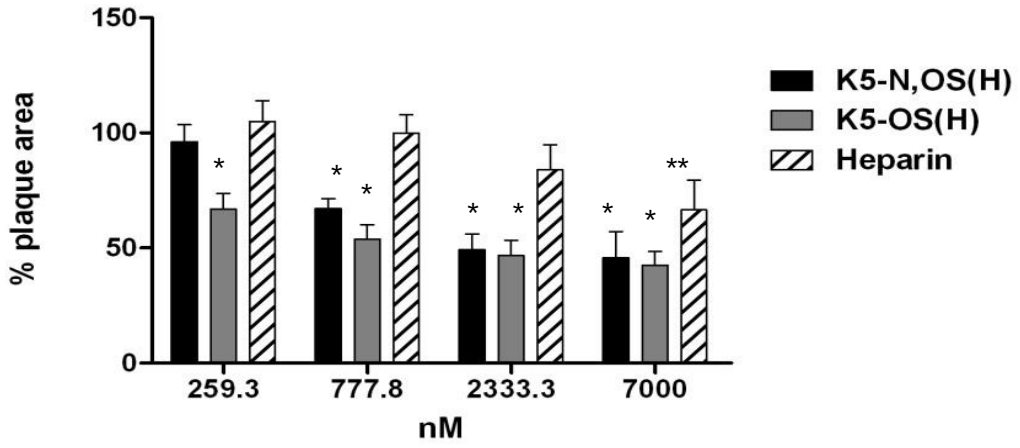


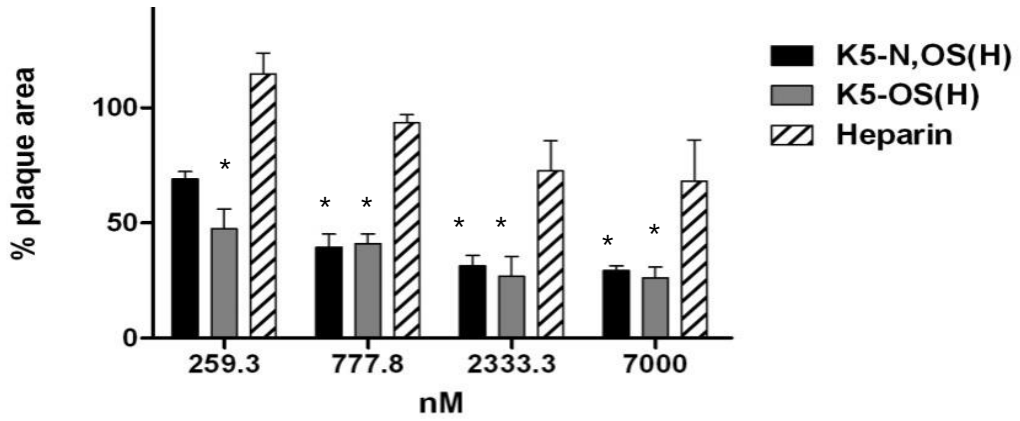
Figure 4


Untreated
K5-N,OS(H)
K5-OS(H)
Heparin
7000 nM
2333.3 nM
777.8 nM
259.3 nM

B


% plaque area
150
100
50
0
K5-N,OS(H)
K5-OS(H)
Heparin
259.3
777.8
2333.3
7000
nM

C


% plaque area
100
50
0
K5-N,OS(H)
K5-OS(H)
Heparin
259.3
777.8
2333.3
7000
nM

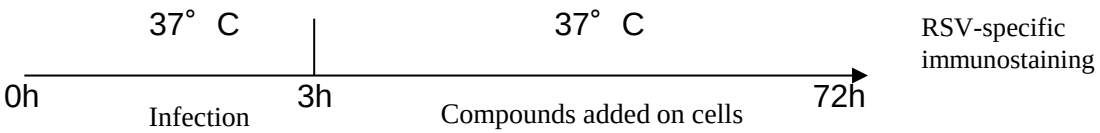

37° C
37° C
RSV-specific
immunostaining
0h
3h
72h
Infection
Compounds added on cells

Figure 5

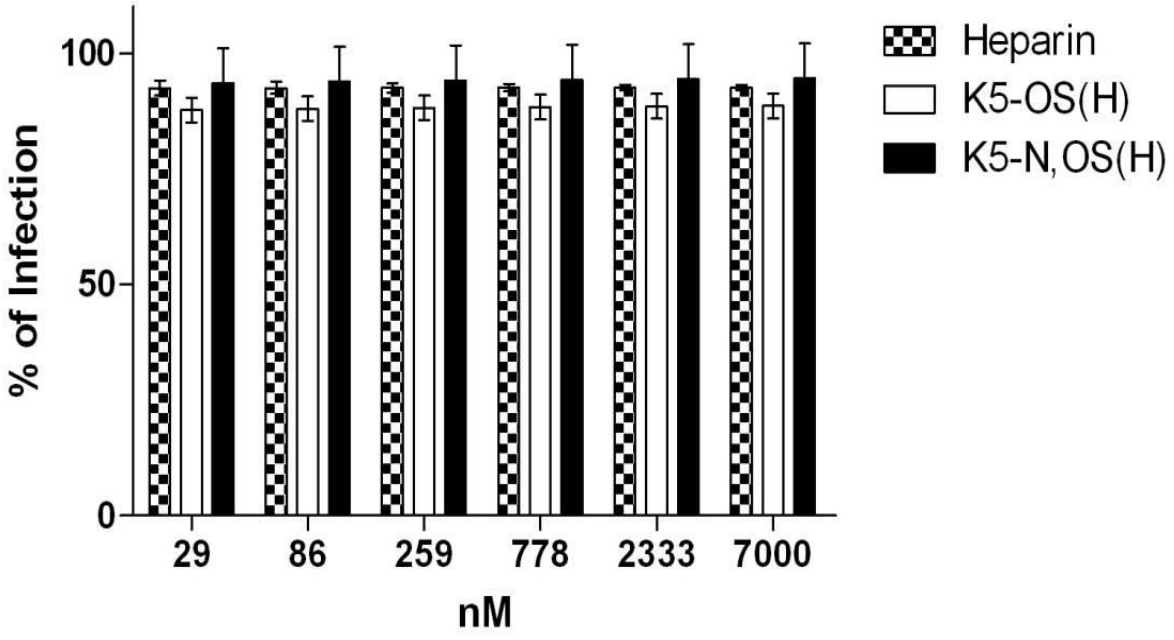

100
50
0
% of Infection
29
86
259
778
2333
7000
nM
Heparin
K5-OS(H)
K5-N,OS(H)

Figure 6

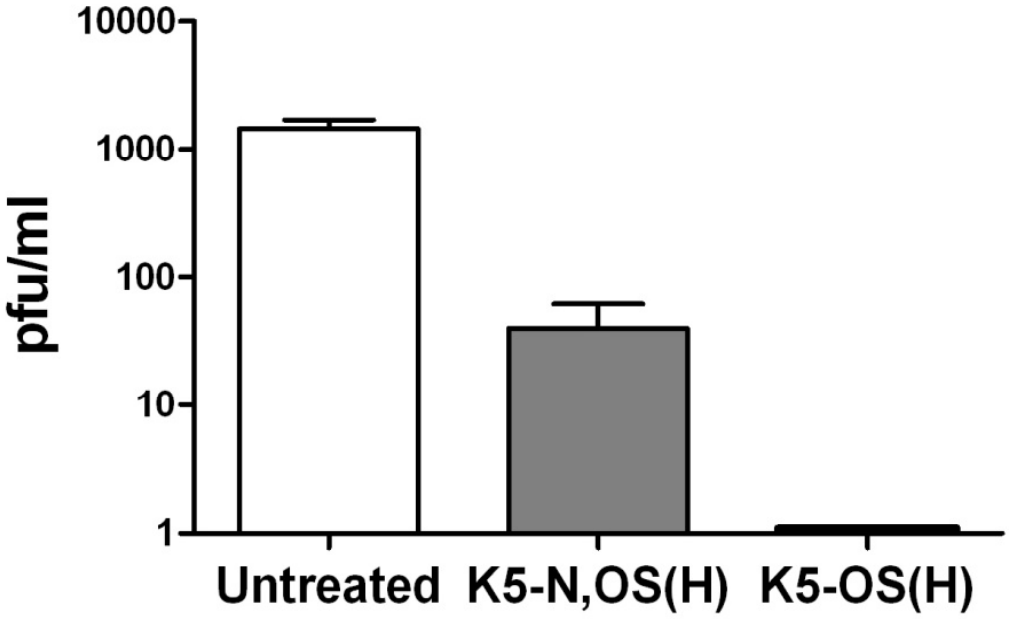
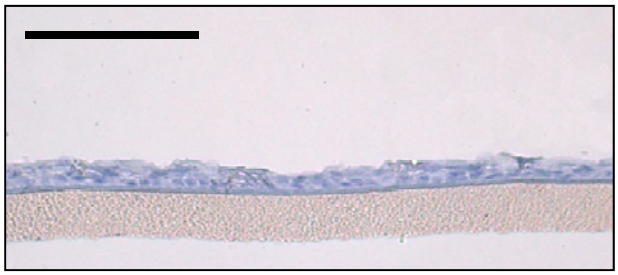
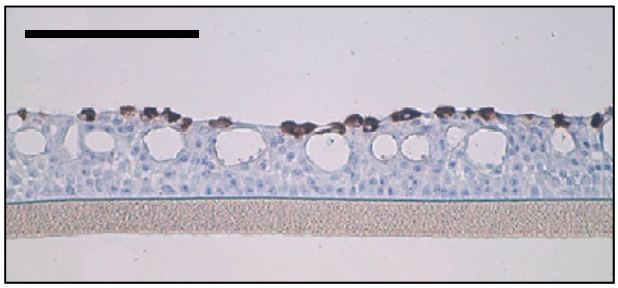


Figure 7

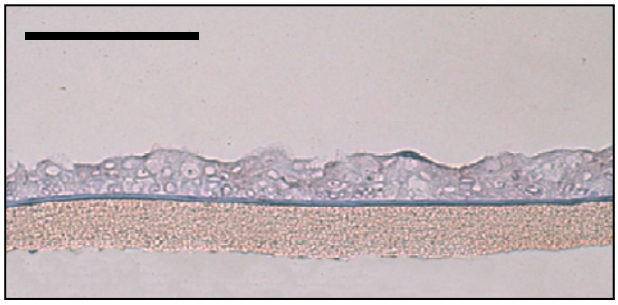
A



B



C



D

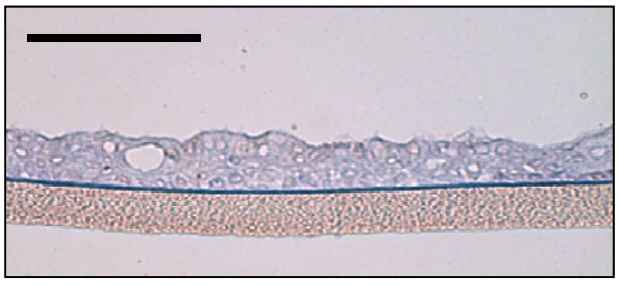


Figure 8

TABLE 3. Viability on EpiAirway tissue.

Conditions	**% of viability**
Untreated [1h]	100
K5 N,OS (H) [1h]	129 ± 9.8
K5OS (H) [1h]	127.2 ± 11.2
Untreated [4h]	100
K5 N,OS (H) [4h]	89.2 ± 7.9
K5OS (H) [4h]	90.8 ± 8.5
Untreated [18h]	100
K5 N,OS (H) [18h]	80.8 ± 10.2
K5OS (H) [18h]	81.3 ± 6.8

The results presented are mean and SD for triplicate tissues

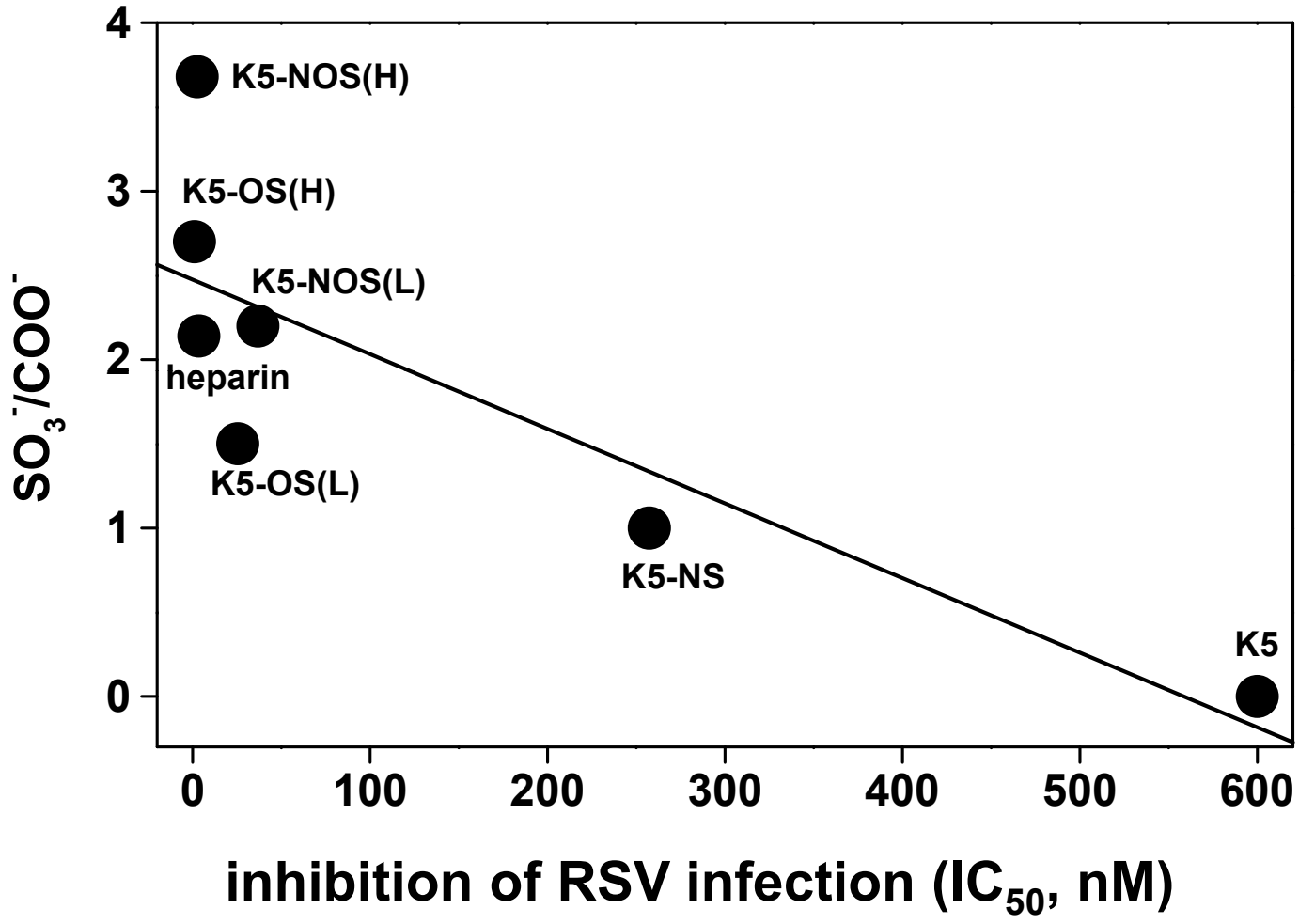

4
3
2
1
0
K5-NOS(H)
K5-OS(H)
K5-NOS(L)
heparin
K5-OS(L)
K5-NS
K5
0
100
200
300
400
500
600
SO3-/COO-
inhibition of RSV infection (IC50, nM)

Figure 9