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Inhibition of Human Respiratory Syncytial Virus Infectivity by a dendrimeric heparan sulphate-binding peptide

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Abstract

Respiratory syncytial virus (RSV) interacts with cell surface heparan sulphate proteoglycans (HSPGs) to initiate infection. The interaction of RSV with HSPGs thus presents an attractive target for the development of novel inhibitors of RSV infection. In the present study, a minilibrary of linear, dimeric and dendrimeric peptides containing clusters of basic amino acids was screened with the aim of identifying peptides able to bind HSPGs and thus block RSV attachment and infectivity. Of the hit compounds identified, the dendrimer SB105-A10 was the most potent inhibitor of RSV infectivity, with IC50 values equal to 0.35 μM and 0.25 μM measured in Hep-2 and A549 cells, respectively. SB105-A10 was found to bind to both cell types via HSPGs, suggesting that its antiviral activity was indeed exerted by competing with RSV for binding to cell-surface HSPGs. SB105-A10 prevented RSV infection when added before the viral inoculum, in line with its proposed HSPG-binding mechanism of action; moreover, antiviral activity was also exhibited when added post-infection, being able to reduce the cell-to-cell spread of the virus. The antiviral potential of SB105-A10 was further assessed using human-derived tracheal/bronchial epithelial cells cultured to form a pseudo-stratified, highly differentiated model of the epithelial tissue of the human respiratory tract. SB105-A10 strongly reduced RSV infectivity in this model and presented no signs of cytotoxicity or pro-inflammatory effects. Together, these features render SB105-A10 an attractive candidate for further development as a RSV inhibitor to be administered by aerosol delivery.

Introduction.

Human respiratory syncytial virus (RSV) is an enveloped RNA virus which belongs to the *Pneumovirus* genus of the *Paramyxoviridae* family (6). It is the leading cause of lower respiratory tract infections, such as bronchiolitis and pneumonia, in infants and young children worldwide. The risk factors for severe RSV disease include premature birth, low birth weight, bronchopulmonary displasia, congenital heart disease, immunodeficiency and the timing of birth in relation to the winter season (32, 50). As RSV infection does not produce any long lasting immunity, recurrent infections may occur throughout life, although they are milder in healthy children and adults. RSV causes severe morbidity and mortality in the elderly, particularly in those with chronic obstructive pulmonary disease (14). In the United States alone, as many as 120,000 hospitalisations and 200-500 deaths occur annually as a result of RSV infections in infants and young children; and as many as 160,000 fatalities occur worldwide (27, 39, 49). The hospitalisation of RSV patients in the United States produces an annual economic burden of approximately $500 million, and considerable further costs should be added to this figure in relation to outpatient care (18, 33). The management of RSV infection is primarily symptomatic (8) and any antiviral treatment is limited to the use of ribavirin, a drug with controversial activity and associated with significant side effects (11, 42, 47). Palivizumab, a humanised monoclonal antibody, has been approved for the immunoprophylaxis of RSV infection in just one narrowly defined patient group: high-risk prematurely born infants (23, 51). However, a major problem with palivizumab is its high costs, which may lead to the progressive restriction of its use (8, 41). Motavizumab, an affinity-matured variant of palivizumab, was expected to replace palivizumab for the prevention of RSV infection in infants, however it was recently denied approval by the FDA on the basis of concerns about safety and allergic reactions.

No vaccine for RSV is currently available. Previous vaccine attempts failed to elicit a long-lasting protective immune response (4) and the approval of a new RSV vaccine is not expected before 2020. This scenario makes RSV an important target for antiviral research and development (42).

Recently developed drugs under investigation as therapeutic agents against RSV were recently reviewed (48).

While ample evidence demonstrates the binding of RSV to cultured cells to involve an interaction between the viral envelope glycoproteins G and F and cell surface heparan sulphate proteoglycans (HSPGs), as well as other sulphated proteoglycans (3, 13, 15, 16, 19, 21, 24, 26, 31, 44), compelling evidence recently identified the cellular protein nucleolin as a specific receptor for RSV (43). The authors propose that RSV binds to cell surface proteoglycans to allow the RSV fusion protein to interact with nucleolin. The interactions between RSV and cell surface HSPGs required for RSV attachment and entry into host cells therefore represent a valid target for the inhibition of RSV infectivity.

HSPGs consist of a core protein bearing glycosaminoglycan (GAGs) chains composed of unbranched heparan sulphate (HS) chains, which are structurally related to heparin (1). On the molecular level, the negatively charged sulphated/carboxyl groups of HSPGs/heparin (20) interact with a cluster of positively charged basic amino acids present within the linear heparin binding domain (HBD) of RSV glycoprotein G (16). Interestingly, a similar putative HBD was also identified in RSV glycoprotein F (15), suggesting that the HSPG-HBD interaction is a common theme in the RSV proteins that mediate infection, making it a preferential target for the development of antiviral compounds against RSV.

Sulphated polysaccharides are a large, heterogeneous group of charged polymers that are able to mimic HS chains and thereby interact with viral glycoproteins and block virus attachment to HSPGs by competitive inhibition (36, 37). Sulphated polysaccharides have been extensively studied as broad range antiviral compounds and some of them [e.g. heparin (20), chondroitin sulphate (19) and dextran sulphate (25)] have proved to be active against RSV.

In principle, a second type of inhibitors could be designed that act through the opposite mechanism: i.e. molecules that inhibit virus attachment by masking the host cell RSV binding sites. For example, polycationic molecules able to bind to HSPGs would mask these receptors from the virus,

thereby preventing its attachment to the cell surface. The feasibility of this alternative anti-viral strategy is sustained by two observation: a) basic fibroblast growth factor (bFGF) and protamine, two prototypic heparin-binding molecules, reduce RSV infection by binding to HSPGs (19); and b) linear heparin-binding peptides derived from RSV F glycoprotein inhibit RSV infectivity (9). Little research attention has been placed onto the development of antivirals that act via this latter mechanism in comparison with that placed on the use of sulphated polysaccharides. The reason is most likely due to the fact that cell surface HSPG molecules are expressed at a concentration of 10^5-10^6 molecule/cell, and each molecule bears several saccharidic chains endowed with multiple binding sites. Thus, the amount of HSPG-binding required to inhibit virus attachment is much higher than that required when the binding sites on the virus itself are targeted.

Dendrimers have recently come to the limelight as attractive potential new therapeutics due to their multivalency and consequent capacity to interact with biological recognition processes (5). Accordingly, dendrimers have been studied as antiviral drugs endowed with multiple binding sites that can efficiently prevent/disrupt the molecular interactions mediating cell attachment and hence virus infection (2). Indeed, HSPG-binding dendrimers have successfully been found to inhibit the infection of human papillomaviruses (HPV), human cytomegalovirus (HCMV) and herpes simplex virus (HSV) (10, 28, 29). Antiviral dendrimeric peptides consist of a lysine peptidyl branching core and four covalently attached surface peptide functional units, containing clusters of basic amino acids that bind to the negatively charged sulphate and carboxyl groups of HS.

The aim of the present work was to screen a minilibrary of linear, dimeric and dendrimeric peptides for their RSV-antagonist activity and to investigate the antiviral potency, the mode of action and the biocompatibility of the best hit compound. The HSPG-binding peptide dendrimer SB105-A10 emerged as a potent inhibitor of RSV infectivity both in cell culture and in a model of human-derived tracheal/bronchial epithelium.

MATERIALS AND METHODS

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% foetal 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 clarified viral supernatant then adjusted to 100 mM $MgSO_4$ and 50 mM Tris-HCl (pH 7.5) to stabilise the viruses as previously reported (17). The virus stocks were aliquoted and stored at -80°C. The infectivity of virus stocks was determined on Hep-2 cell monolayers and calculated using the Reed-Muench method; titres are reported as 50% tissue culture infectious doses ($TCID_{50}$) (22).

The cell lines and the RSV were obtained from the American Type Culture Collection (Manassas, VA, USA).

Reagents. Heparin (13.6 kDa) was obtained from Laboratori Derivati Organici Spa (Milan, Italy). Heparinase III, a glycosidase that digests the glycosaminoglycan moiety of HSPGs (12), was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Antiviral compound minilibrary. The potential antiviral compounds were synthesised as peptides in tetrameric, dimeric or linear forms, as described previously (10).

Cell viability assay. Cells were seeded at a density of 5 x 10^4/well in 24-well plates and treated the next day with serially diluted peptidic compounds to generate dose-response curves. After 48 or 72 hours 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, BIORAD) at 490 nm. 50% cytotoxic concentration (CC_{50}) values and 95% confidence intervals (CIs) were determined using Prism software (GraphPad Software, San Diego, CA).

Infectivity inhibition assay. To assay the antiviral activity of the linear dimeric, and dendrimeric peptides, the compounds were diluted to a concentration of 33 μg/ml (for minilibrary screening) or serially diluted (for further characterisation) and added to cells grown as monolayers in a 96-well plate. After 45 minutes of incubation at 4°C, virus (diluted to yield 20 to 40 plaques/well in 100 μl) was added to linear or dendrimeric peptide-treated and untreated control wells and allowed to adsorb for 3 hours at room temperature; the monolayers were then 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 linear or dendrimeric peptide-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.

Syncytium formation assay. The ability of the dendrimeric peptide SB105A10 to block RSV cell-to-cell spread was evaluated using a previously described method (30) with minor modifications. Cell monolayers in 96-well plates were infected with RSV in MEM supplemented with 2% FCS for 3 hours at room temperature, then subjected to two gentle washes to remove unbound virus. Increasing concentrations of the dendrimeric peptide SB105A10 in 1.2% methylcellulose medium were then added to cultures after washout of the inoculum. Incubation continued for 72 hours post-infection at 37°C; cells were then fixed and immunostained. The immunostained syncytia were visualised 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.

Cell pretreatment assay. Hep-2 cell monolayers in 96-well plates were incubated with increasing concentrations of the dendrimeric peptide SB105-A10 for one hour at 4°C. After removal of the

compound and two gentle washes, cells were infected with RSV according to the protocol described above for the infectivity inhibition assays (except for the absence of compounds during the infection). Cells were then overlaid with 1.2% methylcellulose medium, incubated for 3 days at 37°C and successively fixed and immunostained as described above. Plaques were then counted.

Attachment assay. Various concentrations of the dendrimeric peptide SB105-A10 were preincubated with RSV for 1 hour at 4°C. The mixture was added to cooled Hep-2 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.

Binding assay. The dendrimeric peptide SB105-A10 or heparin (100 μg/ml) was added to the aliquot of RSV (10^4 PFU), incubated for 1 hour at 4°C, added to Hep-2 cell monolayers in DMEM supplemented with 2% FCS, further incubated for 2 hours at 4°C and finally washed two times to remove unbound virus. Cells were fixed with 4% paraformaldehyde, air dried and blocked with 5% BSA in PBS-Tween. Bound virus was detected using the RSV monoclonal antibody (diluted 1:200) incubated for 1 hour at room temperature, washed three times with PBS-Tween and incubated for 2 hours at 37°C with goat anti-mouse IgG conjugated to horseradish peroxidise (1:1000). At the end of incubation, plates were washed three times with PBS-Tween before adding ABTS substrate (Thermo scientific, Rockford) and reading the absorbance at 655 nm. Percent inhibition of virus binding was determined by comparing the absorbance measured in the presence of the dendrimeric peptide SB105-A10 to that measured in untreated wells.

Cell-binding assay for SB105-A10-PEG-biotin peptide: Monolayers of A549 and Hep2 cells in 96-well plates were incubated for 2 hours at 4° C in phosphate buffered saline (PBS) containing 0.1 mg/ml $CaCl_2$, 0.1 mg/ml $MgCl_2$ and 0.1 % gelatin and: i) increasing concentrations of SB105-A10-PEG-biotin peptide; or ii) a fixed amount (220 nM) of SB105-A10-PEG-biotin peptide in the absence or presence of heparin (100 μg/ml); or iii) SB105-A10-PEG-biotin peptide (220 ng/ml) alone, then washed with PBS containing 2 M NaCl – a treatment known to remove cationic

polypeptides from cell surface HSPGs (35); or iv) pre-incubated for 48 h at 37°C with DMEM containing 40 mM sodium chlorate to abrogate GAG sulphation (38); or v) pre-incubated for 1 h at 37°C with heparinase III (15 mU/ml) before proceeding with the binding assay.

At the end of incubations, cells were washed with PBS and the amount of cell associated SB105-A10-PEG-biotin peptide determined with horseradish peroxidase-labelled streptavidin (1/5,000) and the chromogen substrate ABTS (Kierkegaard & Perry Laboratories, Gathersburg, MA).

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.). EpiAirway™ tissues have an average trans epithelial electric resistance (TEER) of 391.2 ± 50.2 Ω/cm^2. Upon delivery, the 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 ml pre-warmed serum free media (AIR-100-MM, MatTek Corp.) and incubated at 37 °C in 5% CO_2 overnight (16-18 h).

Evaluation of SB105A10 cellular toxicity in EpiAirway™ tissues. The cytotoxicity of the dendrimeric peptide SB105A10 on mucous membranes was performed using the MatTek EpiAirway™ System and the MTT ET-50 Tissue Viability Assay followed by the analysis of lactate dehydrogenase (LDH) levels, according to the manufacturer's instructions.

SB105A10 (100 μg/ml) was added to the cell culture insert on top of the EpiAirway™ tissue samples and incubated for 0.5, 1, 4, or 18 hours 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. Then, tissues were 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 ultrapure water were used as the negative control. 1.0% Triton X-100 was used as the positive control. The ET-50 value refers to the time required to reduce tissue viability to 50% and was

determined using Prism software (GraphPad Software, San Diego, CA, USA). To analyse the release of lactate dehydrogenase from the treated EpiAirway™ tissues into the culture medium, the LDH Cytotoxicity Detection Kit (TAKARA bio inc, Japan) was used, following the manufacturer's protocol.

Analysis of inflammatory response. To evaluate the inflammatory response, EpiAirway™ tissues were treated with the dendrimeric peptide SB105A10 (100 μg/ml) for different exposure times of 0.5, 1, 4, and 18 hours, as reported previously. After incubation, the concentration of interleukin 8 (IL-8) in culture medium was measured according to the instructions provided by the manufacturer using an IL-8 ELISA assay kit from ImmunoTools (Friesoythe, Germany). The concentration of IL-8 was calculated by interpolation of a standard calibration curve.

Antiviral assay in EpiAirway™ Tissue. EpiAirway™ cultures were pre-incubated with SB105A10 by applying 100 μl of medium containing 100 μg/ml of the compound to the apical surface and incubating for 2 hours at 37°C. The medium was then removed and the cultures infected with 66,000 pfu of RSV for two hours at 37°C in presence of SB105A10. Cultures were then washed apically with 100 μl of medium, placed at 37°C and fed each day via the basolateral surface with 0.9 ml medium. To harvest the virus, 100 μl medium was added to the apical surface and cells then allowed to equilibrate for 30 minutes at 37°C. The suspension was then collected and stored at -80°C until viral titres were determined by plaque assay in Hep-2 cell monolayers as described above. The viral collection was performed sequentially on the same wells of cells on each day post-infection.

Detection of RSV in EpiAirway™ Tissue by immunohistochemistry. RSV in EpiAirway™ cultures was detected immunohistochemically using a specific mouse monoclonal antibody against RSV subtypes A and B (AbCam, Cambridge, UK). Briefly, EpiAirway™ tissue cultures were fixed in buffered formalin and, properly oriented, embedded 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 minutes at 125°C, followed by 10 seconds at 90°C)

in parallel with sections stained with conventional haematoxylin and eosin. Following incubation with the primary antibody (1:500 dilution), the reaction was visualised 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 recognisable in the cells of the superficial layers. Several sections were analysed 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 using Student's *t* tests. Results are expressed as means ± 1 standard deviation, as noted in the figure legends. P values ≤ 0.05 were considered as significant.

RESULTS

Screening of a minilibrary of linear, dimeric and dendrimeric peptides for RSV-antiviral activity. In the search for RSV inhibitors, we screened a peptide minilibrary that was previously generated to identify HSPGs antagonists. The minilibrary was composed of nine tetrameric, dimeric or linear peptide derivatives containing clusters of basic amino acids that hold the potential to bind to the negatively charged sulphate and carboxyl groups of the glycosaminoglycan chains of HS. The minilibrary was evaluated for *in vitro* activity against RSV using an infectivity inhibition assay on Hep-2 cells. As reported in Figure 1, one dimeric peptide, SB056-dim5, and three tetrameric compounds, SB007-pyrE, SB105 and SB105-A10, exerted statistical significant inhibitory activity (80–100%) at a concentration of 33 μg/ml ($P < 0.05$).

Activity of hit compounds. The ability of the four hit compounds to inhibit RSV infection was investigated further by generating dose-response curves for two cell lines commonly used in RSV studies: Hep-2 and A549. As shown in Table 1, the half maximal inhibitory concentrations (IC_{50}) for peptides SB056-dim5, SB007-pyrE and SB105 were determined to be between 1.1 and 4.4 μM, whereas SB105-A10 was found to potently block RSV infection in A549 and Hep-2 cells with IC_{50} values equal to 0.25 and 0.35 μM, respectively. Moreover, RSV-specific immunostaining revealed the absence of any RSV antigen-positive cell in the cultures treated with the highest concentrations of SB105-A10, suggesting that the dendrimer inhibits RSV at an early step of the replication cycle (data not shown). The CC_{50} were above 20 μM for all compounds tested, indicating that the inhibitory activities observed were not due to cytotoxicity. Since SB105-A10 was the most active of the peptides tested, it was subjected to further investigations.

Investigation of the SB105-A10 inhibitory mechanism. To determine the step in which SB105-A10 interferes with the infection process of RSV, two types of assays were performed on Hep-2 cells (see Materials and methods). In the "attachment assay", increasing concentrations of SB105-A10 were preincubated with RSV at 4°C and the mixture then added to cooled cells for a further 2

hours of incubation at 4°C. It is important to note that this procedure allowed viral attachment to occur, but until the cells were washed twice (to remove unbound virus) and the temperature raised to 37°C, viral entry and infection could not proceed. Under these experimental conditions, SB105-A10 strongly inhibited the infectivity of bound virus to the cell surface ($IC_{50,}$ = 1.74 μM, Figure 2A). Surprisingly, when the amount of virus bound to cells in these experimental conditions was quantified by enzyme-linked immunosorbent assay (ELISA; see "binding assay" in materials and methods), we found that preincubation of the virus with SB105-A10 increased the amount of RSV bound to the surface of Hep-2 (Figure 2B); the bound virus was unable, however, to proceed with the infectious process, as previously demonstrated in the attachment assay. Under the same experimental conditions, heparin (a polyanionic molecule that binds to RSV preventing its interaction with the cell surface; 40) effectively reduced the amount of RSV bound to Hep-2 cells to almost background levels.

To investigate further the mechanism of action of SB105-A10, a "pre-attachment assay" was performed where cell monolayers were incubated with increasing concentrations of SB105-A10 for 1 hour at 4°C to allow its interaction with the cell surface. After removal of unbound compound, cells were further incubated with RSV for 3 hours at room temperature, washed and shifted to 37 °C for 70 hours. Under these experimental conditions, SB105-A10 retained its capacity to inhibit RSV infection with an IC_{50} (0.37 μM); a value that is even higher than that achieved in the attachment assay (Figure 2C). These results suggest that SB105-A10 exerts its inhibitory activity mainly by binding to the virus binding sites present on the cell surface (likely HSPGs), making them unavailable for productive binding to the virus.

SB105-A10-PEG-biotin peptide binds HSPGs on the surface of epithelial A549 and Hep2 cells.

On the basis of the results and considerations reported above, we investigated the direct binding of SB105-A10 to cell surfaces. To this purpose, in a first set of experiments we evaluated the capacity of SB105-A10-PEG-biotin to bind to the surface of epithelial A549 and Hep2 cells and the possible involvement of HSPGs in such an interaction. As shown in Fig. 3A, SB105-A10-PEG-biotin

peptide binds to A549 and Hep2 cells in a dose-dependent manner with EC_{50} values between 80-220 nM and saturation binding values of about 1100 nM of the dendrimer in both cell lines. To evaluate the possible involvement of cell surface HSPGs in the cells' interaction with SB105-A10-PEG-biotin, different approaches were used. First, as shown in Fig. 3B, heparin, a structural analogue of the saccharidic chains of HSPGs, was found to compete with SB105-A10-PEG-biotin for binding to the cell surface. Moreover, washing with 2.0 M NaCl, a treatment known to interfere with the binding of basic polypeptides to heparin/HSPGs (35), removed SB105-A10-PEG-biotin peptide from the cell surface. Finally, inhibiting the sulphation of HSPG saccharidic chains by treatment with sodium chlorate (38), as well as removing the saccharidic chains themselves by heparinase III, significantly reduced the capacity of both A549 and Hep2 cells to bind SB105-A10-PEG-biotin peptide.

SB105-A10 inhibits syncytium formation. Syncytia formation is a well known mechanism of cell-to-cell infection that contributes significantly to virus spread *in vivo*. To determine whether SB105-A10 prevents cell-to-cell spread of virus after infection, a syncytium formation assay was performed on infected Hep-2 cells. Increasing concentration**s** of the dendrimer were added to cells immediately after RSV adsorption, and monolayers were immunostained and examined for syncytia formation, as described in Materials and Methods. As shown in Fig. 4A, large syncytia were visible by RSV-specific immunostaining in untreated Hep-2 cells 72 hours post-infection. In contrast, the area of syncytia decreased in a dose-dependent manner in SB105-A10-treated cells and, at a concentration of 100 μg/ml, singly infected cells were mainly seen. When used at high concentrations, SB105-A10 not only decreases the area of the syncytia, but it also reduces the number of syncytia present and even the number of infected cells. Similar results were obtained using A549 cells (Fig. 4B).

Antiviral activity of SB105A10 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 same features of respiratory mucosa; thus

providing a useful *in vitro* means to assess respiratory infections, such as RSV infection. We assessed the effect of SB105A10 on RSV infection in EpiAirway™ cultures by measuring the titre of virus emerging from the apical surface. At 72 hours post-infection, the virus titre of untreated control tissue was 1.05×10^5 pfu/ml whereas the titre of the tissue treated with 10 μg/ml of SB105-A10 was 1.19×10^4 pfu/ml. Thus the compound inhibited the viral titer by 88.7%.

To reinforce this finding, the same tissues were fixed immediately after the virus harvest at 72 hours 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. 5B). The signal was considered as specific since no RSV positive cells could be observed in sections from uninfected tissue (Fig.5A). The sections from infected tissues that had been treated with SB105-A10 showed either no signal or single RSV positive cells (Fig. 5C).

Since EpiAirway™ Tissue is ideally suited for toxicology studies, we also exploited it to evaluate the irritation and inflammatory potential of SB105A10. Briefly, SB105A10 (100 μg/ml) was applied to the apical surface at the air-tissue interface of tissues for 0.5, 1, 4 and 18 hours at 37°C and tissues were then analysed for: i) the reduction of tetrazolium salt (MTT), to study the metabolic activity of the living cells; ii) lactate dehydrogenase (LDH) release, to measure the accumulation of dead cells; and iii) the release of IL-8, to evaluate inflammatory activation of cells (see Materials and Methods for further details). As reported in Table 2, SB105A10 was not cytotoxic and the time required to reduce tissue viability to 50% (ET_{50}) was greater than 18 hours. No significant difference in the release of the cytoplasmic enzyme LDH was observed between SB105-A10-treated and untreated tissues. Finally, our results indicate that human-derived tracheal/bronchial epithelial cells exposed to SB105A10 do not exhibit significant differences in levels of the pro-inflammatory cytokine IL-8 (Table 2) compared to untreated samples.

DISCUSSION

Many viruses exploit HSPGs as co-receptors, or even entry-receptors, to infect target cells. On the molecular level, the sulphated groups on HSPGs interact with clusters of basic amino acids (also defined as 'heparin binding domains', HBDs) present within proteins on the virus's surface that act as determinants of infectivity (37). The dependency of infection upon HSPGs is common among viruses, making it an attractive target for the development of novel antiviral drugs. So far, this approach has mainly been tackled using polyanionic/HSPG-antagonist heparin-like compounds that bind to viruses and thus sequester the infectious agents within the extracellular environment (37, 46). Conversely, polycationic/HBD-mimicking compounds able to bind to mask HSPGs, thus hampering virus attachment, have been largely neglected.

In the present study, we screened a minilibrary of linear, dimeric and tetrameric peptides containing clusters of basic amino acids in order to identify novel inhibitors of RSV infection. Four hit compounds were identified, and the dendrimer SB105-A10 emerged as being the most potent inhibitor with IC50 values equal to 0.35 μM and 0.25 μM when measured in Hep-2 and A549 cells, respectively. Of note, SB105-A10 prevented RSV infection when added before the viral inoculum (see the results of the pre-attachment assay, Fig.2C), but it was also able to reduce the cell-to-cell spread of the virus when added post-infection (Fig. 4).

To date, SB105-A10 has been found to be active against three DNA viruses, namely HSV, HCMV and HPV (10, 28, 29). RSV (an enveloped RNA virus) shares with these viruses the capacity to bind heparin and the requirement to interact with HSPGs on the target cell surface for infection. The binding of the aforementioned viruses to heparin/HSPGs depends on the presence of HBDs in their surface proteins (37). The peptide that functionalises SB105-A10 contains the following stretch of basic amino acids ASLRVRIKK, which mimics the HBD of RSV envelope glycoproteins G and F. Accordingly, it has been demonstrated that SB105-A10 binds to heparin immobilised to a BIAcore sensor chip – a model which allows protein interactions with cell surface HSPGs to be studied in a "cell-free" system (10). Moreover, the results here reported demonstrate that the binding of SB105-

A10 to two different epithelial cell types mainly depends on HSPGs. These findings suggest that SB105-A10 exerts its antiviral activity by competing with glycoprotein G or F for binding to cell surface HSPGs. This hypothesis is further sustained by the observations that SB105-A10 effectively blocks the binding of other HSPG-dependent viruses (including HPV, HSV and HCMV), but does not exert any antiviral activity against HSPG-independent viruses (including rotavirus and adenovirus) (10, 28, 29).

Despite the features of SB105-A10 described above and its effective capacity to inhibit RSV infection in the attachment assay (see Fig. 2A), our binding assay revealed that SB105-A10 actually increases the number of virus particles that bind to the cell surface (see Fig. 2B). We can hypothesise that, due to its dendrimeric structure and multivalent binding nature, SB105-A10 sets up cross-binding interactions between cell surface HSPGs and RSV, generating the "abortive" binding of large macromolecule complexes to the cell surface that hamper the capacity of HSPGs to mediate infection.

As mentioned above, the interaction of viral HBD with negatively charged sulfated/carboxyl groups of HSPGs is one of the major targets of antiviral research. As a consequence, HSPG-antagonist sulphated polysaccharides (i.e. polyanionics) have been largely investigated as antivirals for decades (37). In contrast, polycationic/HBD-mimicking compounds that bind HSPGs (like SB105-A10 studied here) have received the little research attention to date. Nevertheless, several important features render this latter class of compounds (i.e. HSPG-binding/HBD-mimicking compounds), and in particular SB1015-A10, highly attractive molecules for further development: first, while polyanionic/HSPG-antagonists are directed at the virus, which hold the capacity to develop resistance to the drug, compounds like SB105-A10 target a cellular component, making the emergence of resistant viruses less likely. Secondly, polyanionic/HSPG-antagonists have a narrow therapeutic window, acting only in the presence of viruses. Moreover, as they exert their action within body fluids, they can be rapidly cleared. In contrast, SB1015-A10 acts by binding to cell surface HSPGs, an interaction that is known to be long-lasting (35) and that thereby protects bound molecules from inactivation processes (7), suggesting that, once administered, SB1015-A10

maintains its antiviral activity for prolonged periods of time. Indeed, the present study found that even if SB1015-A10 was administered to cells prior to the RSV challenge, its antiviral effect was effectively maintained.

On the other hand, both polyanionic/HSPG-antagonists and polycationic/HBD-mimicking compounds share common drawbacks, such as the potential to interfere with physiological cellular signalling cascades and their consequent cellular responses. Indeed, HSPGs interact with a variety of physiological extracellular ligands, participating in an array of cellular events during cell adhesion, migration, proliferation and differentiation (1, 34, 45). This issue should be carefully addressed in preclinical studies before candidating SB105-A10 as an antiviral for the prevention and/or control of RSV infections.

With regard to the potential administration of SB105-A10 as an aerosolized formulation, we assessed its biocompatibility on human-derived tracheal/bronchial epithelial cells cultured to form a pseudo-stratified, highly differentiated model (EpiAirway^{TM}) which closely resembles the epithelial tissue of the respiratory tract. This model system avoids species extrapolation and the use of animal models at the early, preclinical phase of development. The results demonstrate that SB105-A10 is well tolerated by the tissue and we observed no signs of cytotoxic or pro-inflammatory effects with a 100 μg/ml dosage. Besides biocompatibility, we also assessed the antiviral activity of SB105-A10 on RSV-infected EpiAirway^{TM} tissues. This model system 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 HSGP composition and expression level *in vivo* are expected to be well duplicated in the EpiAirway^{TM} tissue. Both the virus yield assays and the immunohistochemistry analysis of histological cross-sections showed a clear antiviral activity of SB105-A10 in the EpiAirway^{TM} tissue at a dose of 10 μg/ml, indicating that this inhibitory strategy may well be effective *in vivo*.

In conclusion, the biocompatibility and confirmed antiviral activity of SB105-A10 in an *in vitro* model that closely resembles the epithelial tissue of the human respiratory tract indicate the

compound as a good candidate antiviral treatment for aerosol delivery. Studies are ongoing in animal models to assess the clinical potential of this inhibitor.

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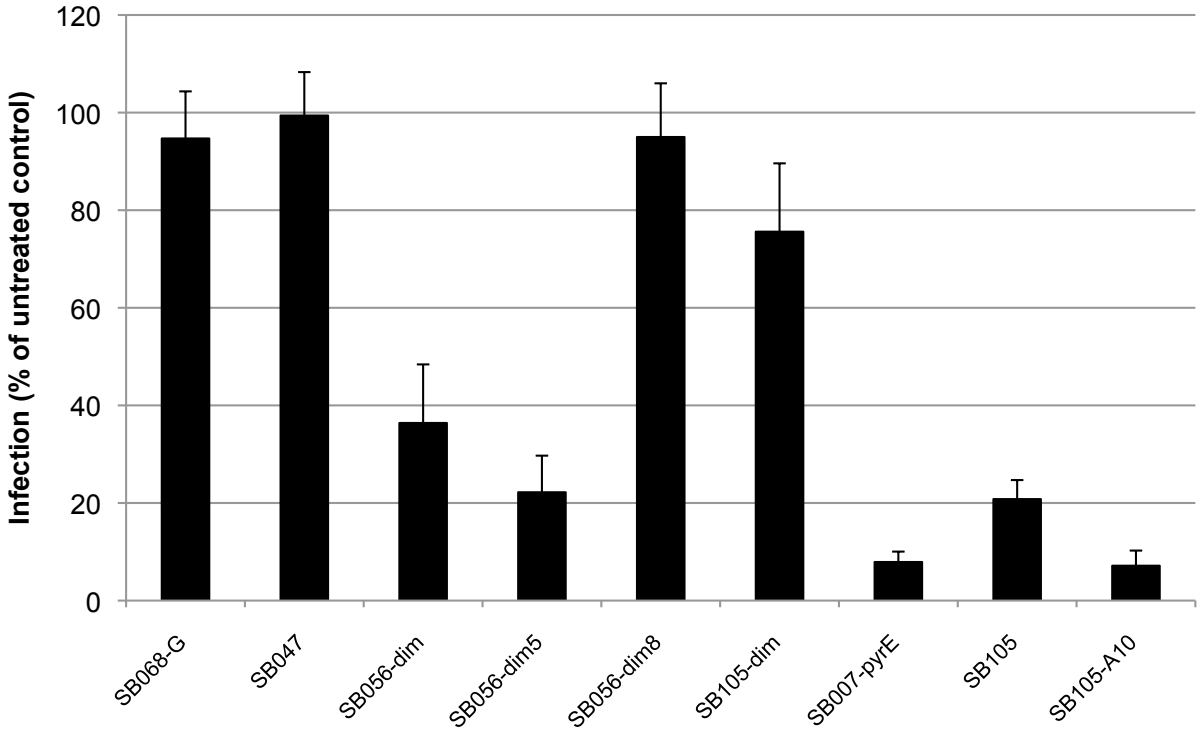
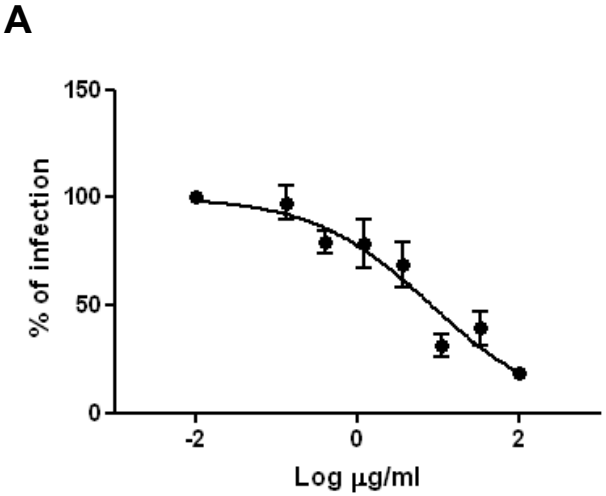
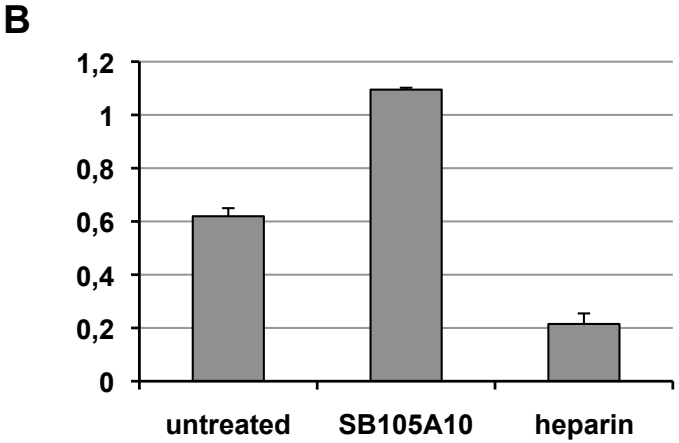


Figure 1. Screening of a peptide minilibrary against RSV. Hep-2 cells were preincubated with compounds (33 μg/ml) for 45 minutes at 4°C before the addition of virus for 3 hours at room temperature. Cells were then washed to remove unbound virus and overlaid with 1.2% methylcellulose medium. Three days post-infection, cells were fixed and subjected to RSV-specific immunostaining. Plaques were counted and the percent inhibition of virus infectivity determined with respect to untreated control wells. The data represent the mean of at least three independent experiments. * $P < 0.05$.

TABLE 1. Inhibition of RSV infection of Hep-2 and A549 cells by hit compounds.

Compound	**Hep-2 cells**		**A549 cells**	
	IC_{50} (μM)	**CC_{50} (μM)**	**IC_{50} (μM)**	**CC_{50} (μM)**
SB056-dim5	4.4 ± 2.2	>20	3.2 ± 2.0	>20
SB007-pyrE	1.19 ± 1.8	>20	2.4 ±1.2	>20
SB105	2.8 ± 1.2	>20	3.0 ± 0.9	>20
SB105-A10	0.35 ± 0.1	>20	0.25 ± 0.1	>20





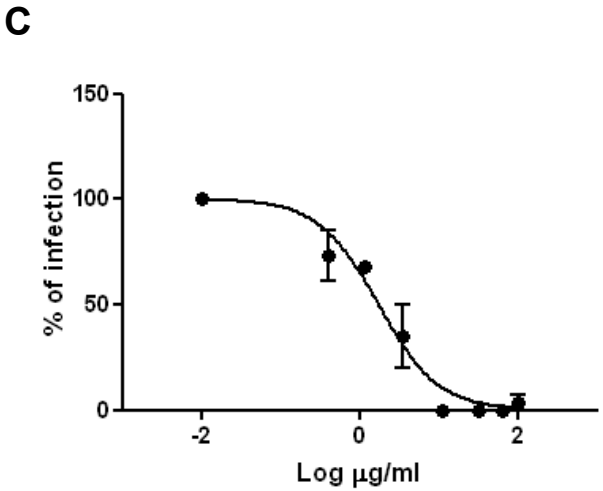


Figure 2. Investigation of SB105-A10 antiviral mechanism in Hep-2 cells. (A) Pre-attachment assay. Hep-2 cells were pretreated with increasing concentrations of SB105-A10 for 1 hour at 4°C, washed and then infected. Three days post-infection the cells were fixed, subjected to RSV-specific immunostaining and the plaques counted. (B and C) Quantification of virus binding to cells and infectivity in the presence of SB105-A10. RSV was added to cells for 2 hours at 4°C for preincubation in the absence or presence of SB105-A10. In the "attachment assay" (B), cells were shifted to 37°C and 72 hours post-infection subjected to RSV-specific immunostaining and the plaques counted. In the "binding assay" (C), the virus bound to cells was detected by enzyme-linked immunosorbent assay (ELISA) immediately after the removal of the virus inoculum. The results are given as the mean ± standard deviation of triplicates.

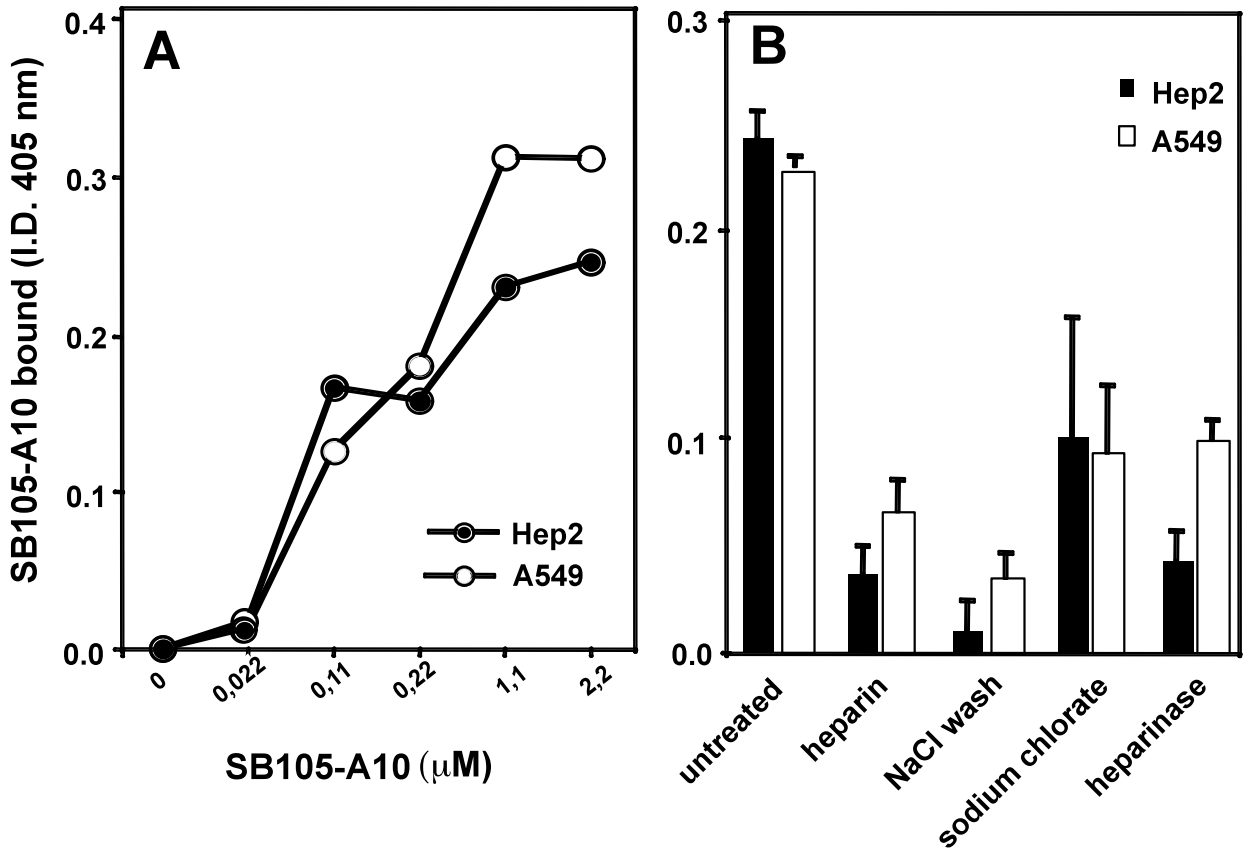
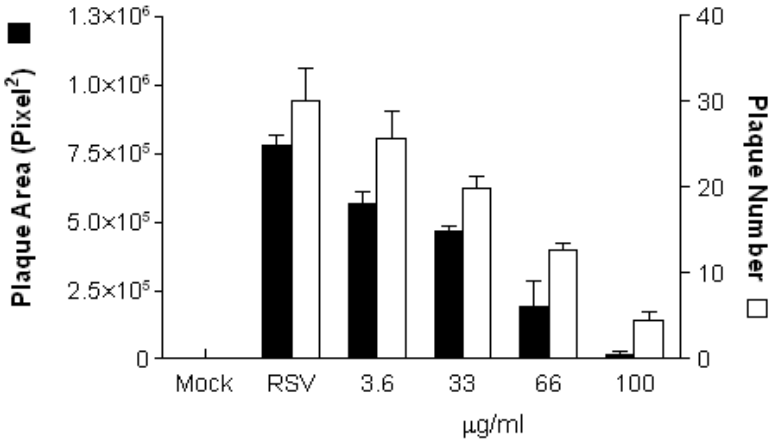


Figure 3. Binding of SB105-A10-PEG-biotin to HSPGs expressed at the surface of A549 and Hep2 epithelial cells. Monolayers of A549 and Hep2 epithelial cells were incubated with increasing concentrations (A) or 220 nM (B) of SB105-A10-PEG-biotin peptide alone or in the presence of heparin (100 µg/ml) and washed with PBS containing 2 M NaCl. Alternatively, cell monolayers were pretreated with sodium chlorate (40 mM) or heparinase III (15 mU/ml) before incubation with SB105-A10-PEG-biotin. The amount of biotinylated SB105-A10 bound to cells was subsequently measured. Each point is the mean ± SEM of 3-4 determinations performed in duplicate.

A


Plaque Area (Pixel²)
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1.0×10⁶
7.5×10⁵
5.0×10⁵
2.5×10⁵
0
Plaque Number
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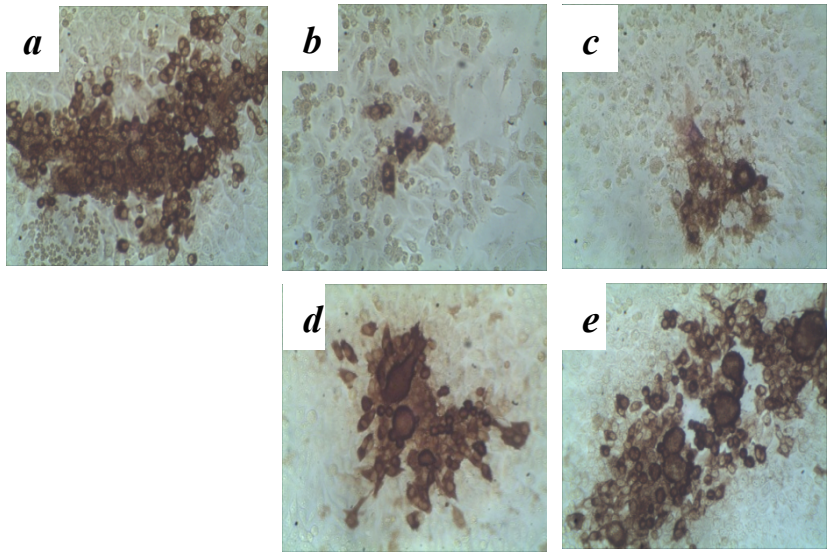
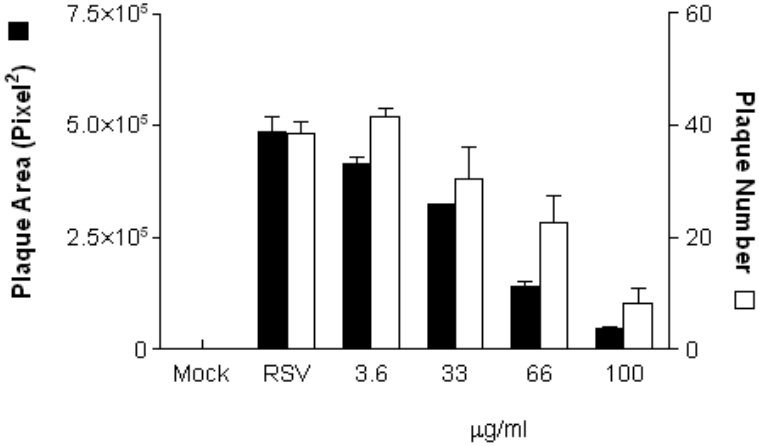
30
20
10
0
Mock
RSV
3.6
33
66
100
μg/ml


a
b
c
d
e

B


Plaque Area (Pixel²)
7.5×10⁵
5.0×10⁵
2.5×10⁵
0
Plaque Number
60
40
20
0
Mock
RSV
3.6
33
66
100
μg/ml

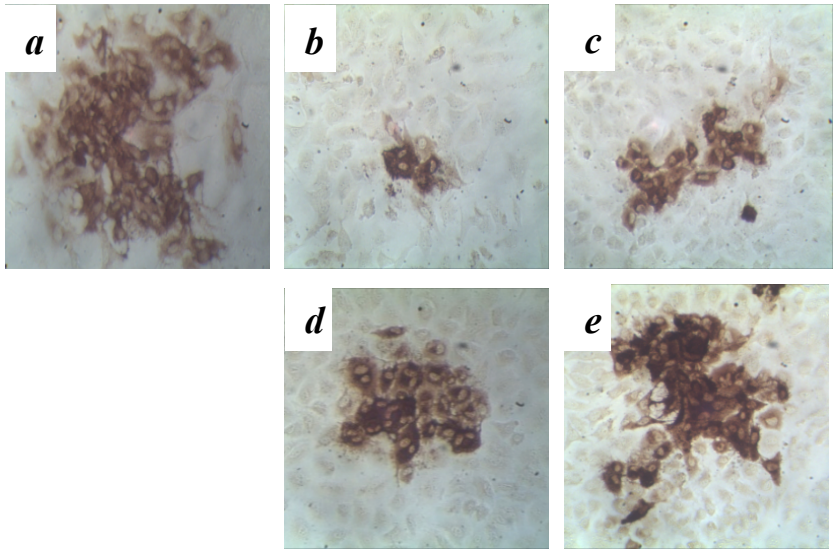

a
b
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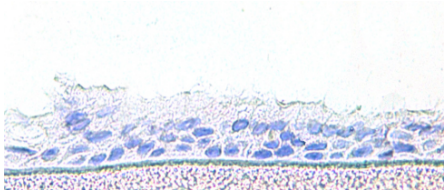
Figure 4. Inhibition of RSV-induced syncytia formation by SB105-A10. (A) Hep-2 cells and (B) A549 cells were infected with RSV in the absence of SB105-A10. The inoculum was removed at 3 hours post-infection and cells incubated in the presence of the following concentrations of SB105A10 in 1.2% methylcellulose medium: (*b*) 100 μg/ml, (*c*) 66 μg/ml, (*d*) 33 μg/ml, (*e*) 3.6 μg/ml. Formation of syncytia was assessed 72 hours after infection, as described in Materials and Methods. The histograms show the plaque area and the plaque count as a function of SB105-A10 concentration. The images in panels (C) and (D) show representative syncytia in Hep-2 and A549 cells, respectively. The results presented in this figure are representative of three independent experiments. * $P < 0.05$; ** $P < 0.001$.

A



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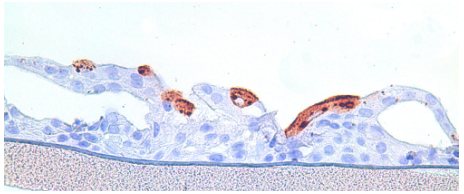

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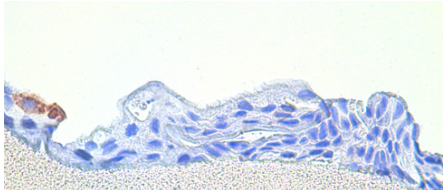

Figure 5. **Reduction of RSV infected cells in EpiAirway tissue by SB105-A10.** Immunohistochemistry of control tissue (A), RSV-infected tissue (66,000 PFU) (B) and RSV-infected tissue treated with SB105-A10 (10 μg/ml) at 3 days post-infection (C), using a monoclonal antibody to RSV (brown signal). The pictures shown are representative of many analysed sections.

TABLE 2. Evaluation of the irritation potential of SB105-A10 (100 μg/ml) on the EpiAirway™ Tissue Model**.**

	% of viability	**LDH Release (Abs)**	**IL-8 Release (pg/ml)**
Untreated (30 min.)	100	0.25 ± 0.02	481.0 ± 19.0
SB105-A10 (30 min.)	98.2 ± 12.6	0.25 ± 0.08	489.5 ± 12.0
Untreated (1 hour)	100	0.23 ± 0.02	496.2 ± 11.0
SB105-A10 (1 hour)	84.9 ± 6.7	0.23 ± 0.01	491.5 ± 1.9
Untreated (4 hours)	100	0.22 ± 0.21	441.0 ± 39.3
SB105-A10 (4 hours)	95.3 ± 3.0	0.20 ± 0.02	477.6 ± 38.3
Untreated (18 hours)	100	0.17 ± 0.06	484.8 ± 25.1
SB105-A10 (18 hours)	94.7 ± 0.05	0.17 ± 0.03	476.0 ± 1.2