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Potent anti-respiratory syncytial virus activity of a cholestanol-sulfated tetrasaccharide conjugate

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ABSTRACT

A number of different viruses including respiratory syncytial virus (RSV) initiate infection of cells by binding to cell surface glycosaminoglycans and sulfated oligo- and polysaccharide mimetics of these receptors exhibit potent antiviral activity in cultured cells. We investigated whether the introduction of different lipophilic groups to the reducing end of sulfated oligosaccharides would modulate their anti-RSV activity. Our results demonstrate that the cholestanol-conjugated tetrasaccharide (PG545) exhibited ~5- to 16-fold enhanced anti-RSV activity in cultured cells compared with unmodified sulfated oligosaccharides. Furthermore, PG545 displayed virus-inactivating (virucidal) activity, a feature absent in sulfated oligosaccharides. To inhibit RSV infectivity PG545 had to be present during the initial steps of viral infection of cells. The anti-RSV activity of PG545 was due to both partial inhibition of the virus attachment to cells and a more profound interference with some post-attachment steps as PG545 efficiently neutralized infectivity of the cell-adsorbed virus. The anti-RSV activity of PG545 was reduced when tested in the presence of human nasal secretions. Serial passages of RSV in the presence of increasing concentrations of PG545 selected for weakly resistant viral variants that comprised the F168S and the P180S amino acid substitutions in the viral G protein. Altogether we identified a novel and potent inhibitor of RSV, which unlike sulfated oligo- and polysaccharide compounds, could irreversibly inactivate RSV infectivity.

1. Introduction

To initiate infection of susceptible cells, viruses frequently bind to cell surface carbohydrate residues such as sialic acid or sulfated glycosaminoglycan (GAG) chains, which represent attractive targets for antiviral intervention. In fact, specific mimetics of sialic acid are already approved for treatment of influenza virus infections (Von Itzstein et al., 1993). In contrast, mimetics of GAG chains such as sulfated polysaccharides or other polysulfonated compounds potently inhibit infection of cultured cells by many different GAG-binding viruses including human immunodeficiency virus (HIV), herpes simplex virus (HSV), and respiratory syncytial virus (RSV) (for reviews, see Vaheri, 1964; Witvrouw and De Clercq, 1997; McCarthy et al., 2005). However, in the case of HIV infection, these compounds failed to show protective effects in humans (Abrams et al., 1989; Van de Wijgert and Shattock, 2007; Cohen, 2008). While the reason of this failure is unclear, it should be emphasized that GAGs and their mimetics are composed of long chains bearing anionic residues that bind to viral attachment proteins via multiple electrostatic interactions and consequently this binding is relatively weak and reversible (non-virucidal) (Neyts and De Clercq, 1995). In the case of HSV it was found that this kind of interaction requires the permanent presence of a GAG mimetic during the virus attachment to cells, and a simple dilution of the virus-inhibitor complexes was shown to liberate infectious virus (Vaheri, 1964). These data emphasize the need for structural modifications of GAG-mimetics in order to confer irreversible binding to the viral attachment protein and thereby cause permanent inactivation of viral infectivity.

Human RSV targets ciliated cells of the bronchial epithelium and type 1 pneumocytes in the alveoli (Zhang et al., 2002; Johnson et al., 2007; Welliver et al., 2007) causing acute bronchiolitis and pneumonia in infants, the elderly, and immunocompromised individuals (for review, see Collins and Graham (2008)). Experiments in cultured cells revealed that an initial step of the RSV infectious cycle is the binding of the virus attachment protein G (Levine

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et al., 1987) to cell surface sulfated GAGs (Krusat and Streckert, 1997), mainly to iduronic acid-containing GAGs such as heparan sulfate or chondroitin sulfate B (Hallak et al., 2000). It is uncertain whether RSV uses GAGs to infect humans since heparan or chondroitin sulfate chains are poorly or not at all expressed at the surface of airway epithelium (Zhang et al., 2005; Monzon et al., 2006). However another type of GAG chain, i.e., keratan sulfate, is abundantly expressed on the apical surface of ciliated cells of well differentiated cultures of bronchial epithelium (Zhang et al., 2002). This suggests that GAGs or GAG-like receptors may promote RSV infection of humans, and that compounds that mimic GAG chains may protect humans from RSV.

The anti-cancer drug candidate muparfostat (formerly known as PI-88) (Parish et al., 1999) is a mixture of highly sulfated mannose-containing di- to hexasaccharides with penta- and tetrasaccharides as predominant components. In addition to anti-cancer activities (for review, see Kudchadkar et al., 2008), it also exhibits anti-HIV (Said et al., 2010), anti-HSV (Nyberg et al., 2004), anti-dengue and -encephalitic flavivirus (Lee et al., 2006), and anti-malarial (Adams et al., 2006) activities. In an attempt to improve antiviral activity of muparfostat we paid attention to an observation that certain polysulfonated compounds such as PRO2000, composed of chains of aromatic/lipophilic moieties instead of relatively hydrophilic sugar residues, exhibited virucidal activity (Cheshenko et al., 2004) and provided some protection of women against HIV (Cohen, 2009). It has also been reported that the peptide-based inhibitors of cell entry of HIV (Ingallinella et al., 2009) or some paramyxoviruses (Porotto et al., 2010) exhibited greatly enhanced antiviral activity when conjugated with cholesterol. In the present work we conjugated specific lipophilic groups to the reducing end of sulfated tetra- and pentasaccharides and tested whether this modification would affect anti-RSV activity. Our study demonstrated that the cholestanyl-conjugated tetrasaccharide glycosides exhibited improved anti-RSV potency including virucidal activity, a feature absent in native sulfated oligosaccharides.

2. Materials and methods

2.1. Cells and viruses

Human laryngeal epidermoid carcinoma (HEp-2) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS), 1% L-glutamine, 60 μg/ml of penicillin, and 100 μg/ml of streptomycin. In most of experiments, 1-day-old cultures of cells at ~70% confluence were used. Madin–Darby canine kidney (MDCK) cells were propagated in Eagle's medium supplemented with 5% FCS, 1% tricine and antibiotics. Laboratory RSV strain A2 (Lewis et al., 1961) was used throughout the experiments, and its stock was prepared as described by Hallak et al. (2000) with some modifications (Lundin et al., 2010). In some experiments the tissue culture adapted strain A/PR/8/34 of influenza A virus (IAV) and the Indiana strain of vesicular stomatitis virus (VSV) were used.

2.2. Compounds

Polysulfated tetra- and pentasaccharide glycosides composed of α(1 → 3)/α(1 → 2)-linked mannose residues with specific lipophilic groups attached to the reducing end (Table 1) were all prepared and characterized by ^{1}H NMR, ^{13}C NMR, mass spectrometric, and microanalytical techniques as described previously (Johnstone et al., 2010). PG545, the cholestanyl β-glycoside of polysulfated maltotetraose was prepared in a similar fashion (Ferro et al., 2008). Muparfostat was prepared as described previously (Cochran et al., 2003). All test compounds were solubilized in de-ionized water to a final concentration of 10 mg/ml and stored at −20 °C. All test compounds maintained good solubility upon their dilution in the cell culture media.

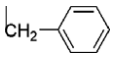
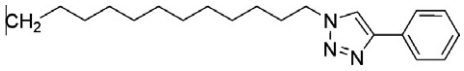
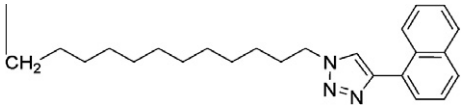
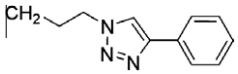
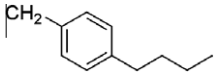
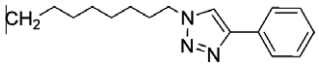
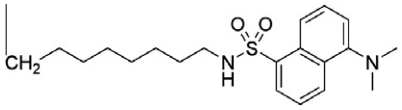
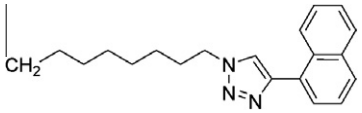
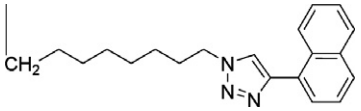
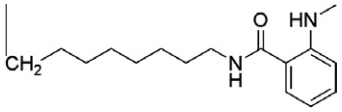
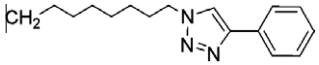
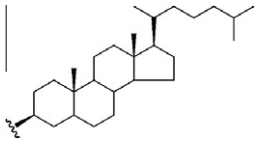
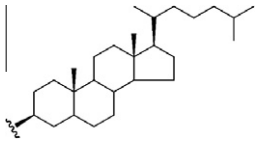
2.3. Viral plaque assays

The plaque number-reduction assay was performed as described by Lundin et al. (2010). Briefly, test compounds were serially 5-fold diluted in either DMEM supplemented with 1% L-glutamine, antibiotics, and 2% heat-inactivated FCS (DMEM-S) or the same medium without addition of serum (DMEM-NS). Subsequently ~200 PFU of RSV A2 strain in 50 μl of respective medium was added to test compounds and incubated for 10 min at room temperature. HEp-2 cells, seeded in 12-well plates to achieve confluence of ~70% after one day of culture, were washed once and 0.5 ml of the virus-compound mixture was added. After co-incubation of the virus-compound mixture with cells for 2–3 h at 37 °C in a humidified 5% CO_2 atmosphere, the medium was collected and 1.5 ml of 0.75% methylcellulose solution in DMEM-S was added. To visualize the viral plaques the cells were stained with 1% solution of crystal violet after 3 days of incubation at 37 °C. The effect of test compounds on VSV infectivity in HEp-2 cells was tested in the same manner as for RSV using the DMEM-S medium. The effect of test compounds on IAV was tested in MDCK cells using the viral cytopathic effect (CPE) reduction method. Briefly, 5-fold dilutions of test compounds in Eagle's medium supplemented with 0.25% bovine serum albumin (BSA), 10 mM HEPES, 0.8 μg/ml of TLCK trypsin, and antibiotics were mixed with ~1000 $TCID_{50}$ of the virus and incubated for 10 min at room temperature. MDCK cells, seeded in 96 well plates, were washed twice with the above described medium, and the virus-compound mixtures were transferred to these cells and incubated for 2 h at 37 °C. Subsequently the cells were washed once, and incubated in the same medium for 2 days at 37 °C. For the RSV plaque size-reduction assay, the cells were inoculated with ~100 PFU of the virus for 4 h at 37 °C. After washing of cells, specific concentrations of test compound in 0.4% methylcellulose solution were added and incubated with infected cells for 6 days at 37 °C. The cells were stained with 1% solution of crystal violet, and the area of captured images of viral plaques measured by using IM500 image software (Leica) as described previously (Ekblad et al., 2010). Statistical analysis was performed using Student *t*-test.

2.4. Purification of radiolabeled virus and virus binding assay

Virus radiolabeling with the expre^{35}S protein labelling mix (Perkin Elmer, Upplands Vasby, Sweden) followed by two rounds of virus purification using a 25–55% sucrose linear gradient was performed as described by Techaarpornkul et al. (2001). For the virus binding assay, the virus was adjusted with Eagle's medium supplemented with 1% BSA to contain 6×10^5 cpm/ml. Serial 5-fold dilutions of test compounds in Eagle's medium (0.16–100 μg/ml) were mixed with the virus (~2×10^4 cpm/well) and incubated for 10 min at 4 °C. HEp-2 cells, seeded in 24 well plates to reach a confluence of ~90–95% after 2 days of culture, were precooled at 4 °C for 45 min and subsequently washed twice with Eagle's medium. The virus-compound mixtures were added to these cells and incubated for 90 min at 4 °C under moderate agitation. The cells were then washed thrice with cold Eagle's medium, lysed with 200 μl of 5% SDS solution in PBS, and transferred to scintillation vials for quantification of radioactivity. To study the effect of test compounds on the post attachment step(s), the cells were inoculated with ~200 PFU of non-labeled RSV in DMEM-S for 2 h at 4 °C, then washed twice with cold medium, and incubated with specific

Table 1
Anti-RSV activity of oligosaccharide glycosides found in a screening assay.

Compound	Structure of glycoside		Residual infectivity (%)[a]
	Oligosaccharide component Manα(1 → 3)/Manα(1 → 2) (No. of residues)	Aglycone component	
1	Tetrasaccharide		59.0 ± 9.3
2	Tetrasaccharide		26.6 ± 18.2
3	Pentasaccharide		0.9 ± 1.5[c]
4	Pentasaccharide		2.3 ± 0.6[c]
5	Pentasaccharide		0.0 [c]
6	Pentasaccharide		8.9 ± 2.3
7	Tetrasaccharide		13.6 ± 8.1
8	Tetrasaccharide		20.7 ± 14.2
9	Tetrasaccharide		4.8 ± 3.0
10	Pentasaccharide		3.8 ± 5.4
11	Tetrasaccharide		4.0 ± 5.2
12	Tetrasaccharide		10.3 ± 7.6
13	Pentasaccharide		7.8 ± 6.8
14	Tetrasaccharide		0.0[c]
PG545	Tetrasaccharide[b]		0.0[c]
Muparfostat (PI-88)	Mainly penta- and tetrasaccharide	None	13.5 ± 5.1

[a] Percentage of a number of viral plaques found with drug treated virus (100 μg/ml) relative to mock treated controls. Three screening assays were performed.
[b] Composed of α(1 → 4)-linked glucose residues (maltotetraose).
[c] Statistically significant inhibitory effect as related to muparfostat at P value of <0.05 (n = 3).

concentrations of test compounds in the same medium for 2 h at 37 °C. The rest of the procedure was as described in Section 2.3.

2.5. Time-of-addition assay

HEp-2 cells were incubated with test compound (20 μg/ml) in DMEM-S for periods of 2 h at 37 °C which occurred either prior to, during, or after inoculation of cells with ~200 PFU of RSV A2 strain for 2 h at 37 °C. Following each treatment and infection stage, the compound and/or the virus were removed, and the cells were washed twice and overlaid with a 0.75% methylcellulose solution in DMEM-S.

2.6. Virus inactivation assay

Approximately 10^5 PFU of RSV A2 strain and the test compound at concentrations 1, 10 and 100 μg/ml were added to DMEM-S or DMEM-NS and mixed in a total volume of 500 μl. The virus-

compound mixture was incubated for 15 min in a 37 °C water bath, then diluted serially to the non-inhibitory concentration of test compound, and the residual viral infectivity determined by the viral plaque assay.

2.7. Cytotoxicity assay

HEp-2 cells were seeded in 96 well plates to reach a confluence of ~60% after 1 day of culture. The cells were washed with either DMEM-S or DMEM-NS and 100 μl of the same medium containing serial 5-fold dilutions of test compound at a concentration range 0.032–500 μg/ml were added in duplicate. The cells and the test compounds were co-incubated for 72 h at 37 °C, and 20 μl of the CellTiter 96® Aqueous One Solution reagent (Promega, Madison, USA) was added to each well. Following further incubation for 1–2 h at 37 °C, the absorbance at 490 nm against a background of 650 nm was recorded.

2.8. Modulation of PG545 antiviral activity by nasal secretions

Human nasal secretions were obtained from three healthy volunteers. To collect a sample, a cotton swab was inserted into the posterior area of the nasal cavity and left for ~10 s to adsorb secretions. Swabs were immediately immersed into 1 ml of PBS in 10 ml tubes, then left at room temperature for 15 min, and extensively vortexed. Next, the cotton swabs were transferred to empty, sterile syringes inserted into 12 ml tubes and centrifuged for 10 min at 3000*g* to collect fluid remaining in the swab. This fluid was pooled with the rest of the sample and stored at −80 °C.

Modulation of PG545 activity by nasal secretions was tested as follows. PG545 at 10-fold increasing concentration (1–1000 μg/ml) in 25 μl of distilled water was mixed with 200 μl of pooled nasal secretions and 25 μl of DMEM-NS medium comprising ~10^5 PFU of the virus. The mixtures were incubated for 15 min at 37 °C water bath, and the residual virus infectivity tested by the plaque assay.

2.9. Preparation and analysis of the drug resistant RSV variants

Plaque purified RSV A2 strain was subjected to 6 or 10 consecutive passages in HEp-2 cells in the presence of muparfostat (50 μg/ml) or to 13 passages in the presence of increasing concentrations (1–4.5 μg/ml) of PG545 in DMEM comprising 1% heat-inactivated FCS. The same virus was also passaged in the absence of test compound to serve as control material. Any resistance to these compounds was investigated by using the viral plaque number-reduction assay. Viral variants that survived the selective pressure of these compounds were plaque purified twice and subjected to nucleotide sequencing analysis of genes coding for the viral G and F proteins as described previously (Lundin et al., 2010).

3. Results

3.1. Anti-RSV activity of lipophile-conjugated sulfated oligosaccharides

Although sulfated oligo- and polysaccharides inhibit RSV infectivity potently, their interaction with viral particles is weak, reversible, and non-virucidal (Neyts and De Clercq, 1995), and complete virus blockade is difficult to achieve even at relatively high concentrations of these compounds (e.g. Hallak et al., 2000, 2007). To search for GAG mimetics with improved anti-RSV activity polysulfated tetra- and pentasaccharides were chemically modified by introduction of different aromatic/lipophilic groups to the reducing end of the oligosaccharide chain (Table 1). These glycosides were then screened at 100 μg/ml for anti-RSV activity in cultures of HEp-2 cells. Because sulfated oligo and polysaccharides including muparfostat are mixtures of carbohydrate chains of different molecular weight the test compounds were compared based on μg/ml rather than on molar values. In contrast to the unmodified sulfated oligosaccharides of muparfostat, compounds possessing dodecyl (**3**), 12-(4-naphthalen-1-yl-[1,2,3]triazol-1-yl)dodecyl (**5**) or cholestanyl (**14** and PG545) as the aglycone component demonstrated complete or near-complete inhibition of RSV infectivity (Table 1). Moreover, these four glycosides exhibited more favorable IC_{50} values than muparfostat, and showed virucidal activity, a functional feature absent in muparfostat oligosaccharides (Table 2). Since PG545 exhibited the most pronounced virucidal activity, this glycoside was selected for detailed evaluation of anti-RSV potency. Note that although both PG545 and **14** are composed of a lipophilic cholestanyl group conjugated to a sulfated tetrasaccharide, PG545 contains maltotetraose while **14** possesses a mannose $\alpha(1\rightarrow3)/(1\rightarrow2)$-linked tetrasaccharide, as found in muparfostat, as the oligosaccharide component.

The dose response effects of PG545 on the viability of HEp-2 cells and on infection of these cells by RSV are shown in Fig. 1A. The anti-RSV activity of the cholestanol-sulfated oligosaccharide conjugate (PG545) was ~5 times greater than that of unmodified sulfated oligosaccharide of muparfostat. PG545 completely blocked RSV infectivity at concentrations of $\geqslant$20 μg/ml while unmodified sulfated oligosaccharides of muparfostat did not demonstrate complete inhibition even at 500 μg/ml. At a concentration range of 0.16–500 μg/ml muparfostat demonstrated no cytotoxicity while PG545 reduced viability of HEp-2 cells with CC_{50} value of 230 μg/ml. Given the presence in PG545 of cholestanol, a sterol that could interact with many different lipophiles such as serum apolipoproteins, we tested the cytotoxicity and anti-RSV activity of PG545 using serum-free media. Under these conditions, the anti-RSV activity of PG545 was ~16 times greater than that of muparfostat. Note that the absence of serum in the culture medium enhanced both the anti-RSV activity and cytotoxicity of PG545 by ~5-fold (Fig. 1B) as opposed to data obtained in the presence of serum (Fig. 1A).

We also tested the effect of PG545 on infectivity of IAV or VSV. The former virus uses sialic acid for initial interaction with cells. While the cellular receptor for VSV is not known (Coil and Miller,

Table 2
Antiviral activity and cytotoxicity of lipophile-conjugated sulfated oligosaccharide glycosides.

Compound	Cytotoxicity (CC_{50}; μg/ml)	Anti-RSV potency (IC_{50}; μg/ml)	Virucidal activity[a] (% of residual infectivity)
Muparfostat	>400	9.9 (>40)[b]	94.0
3	>400	4.6 (>87)	47.3
5	NT	5.7	57.3
14	113	1.7 (66)	13.3
PG545	230	2.2 (105)	9.9

[a] Residual RSV infectivity after incubation for 15 min at 37 °C of ~10^5 PFU of the virus with 100 μg/ml of a test compound.
[b] In parentheses are the values for selectivity index.

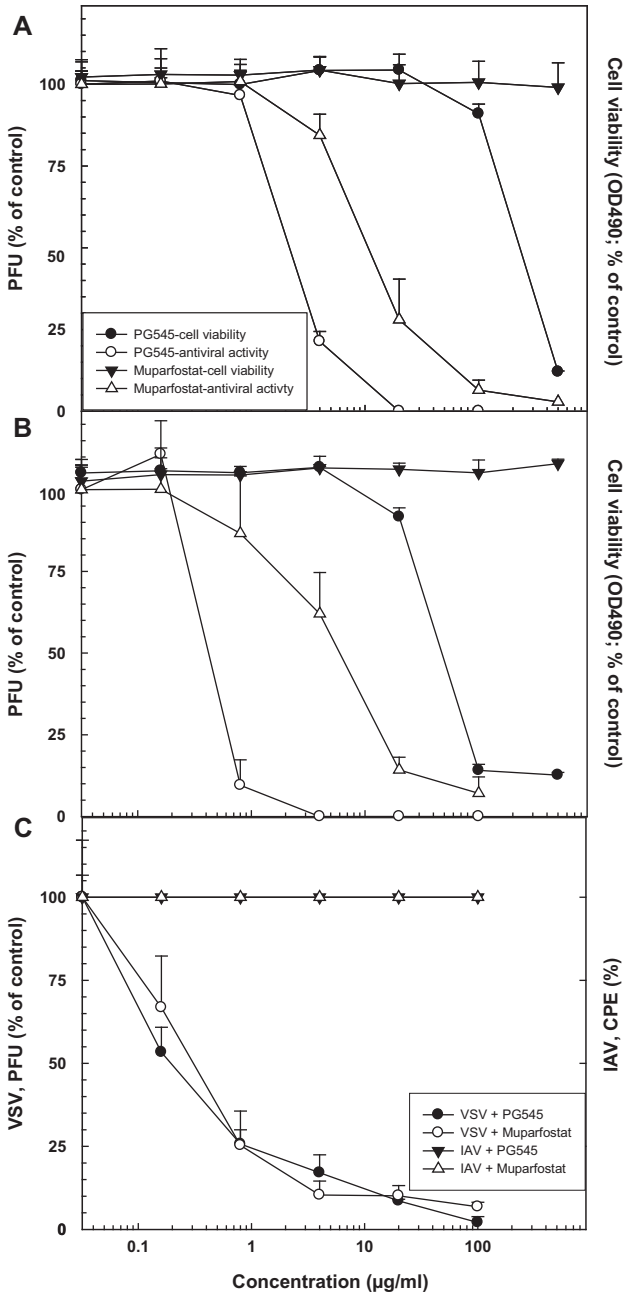


Fig. 1. Effect of PG545 or muparfostat on RSV infectivity and HEp-2 cell viability. The test compounds at specific concentrations in medium comprising 2% heat-inactivated fetal calf serum (A) or in serum-free medium (B) were either added to cells and incubated for 3 days at 37 °C or incubated with ~200 plaque-forming units (PFU) of RSV A2 strain for 10 min prior to and during 2–3 h period of virus infection of cells. The results are expressed as a percentage of the number of viral PFU found with drug-treated RSV (infectivity assay) or the number of OD_{490} units found with drug-treated cells (cell viability assay) relative to mock-treated controls. Values shown are means of four determinations from two separate experiments. (C) The effect of PG545 or muparfostat on vesicular stomatitis virus (VSV) infectivity in HEp-2 cells was tested in the same manner as for RSV using the medium supplemented with fetal calf serum. In case of influenza A virus (IAV), the test compounds at indicated concentrations in Eagle's medium supplemented with 0.25% bovine serum albumin (BSA), 10 mM HEPES, 0.8 μg/ml of TLCK trypsin, and antibiotics were mixed with ~1000 $TCID_{50}$ of the virus and incubated for 10 min at room temperature. The virus-compound mixtures were transferred to MDCK cells and incubated for 2 h at 37 °C. Subsequently the cells were washed once and incubated in the same medium for 3 days at 37 °C. Two separate experiments were carried out in quadruplicate wells and the results are expressed as a cumulative percentage of wells in which cytopathic effect was detected. Calculations were performed according to Reed–Muench method for computation of 50% endpoints.

2004) this virus is highly sensitive to GAG mimetics (Baba et al., 1988). PG545 and muparfostat efficiently inhibited infectivity of VSV while showing no effect on IAV infectivity (Fig. 1C).

3.2. Mode of anti-RSV action of PG545

To identify which step of the infectious cell cycle of RSV is affected by PG545, the compound was added to HEp-2 cells at different time points relative to the virus inoculation. The presence of compound during the 2 h period of virus attachment to and entry into the cells resulted in near complete blockade of RSV infectivity (Fig. 2) indicating that one of the initial steps of RSV infection of cells is the major target of PG545 activity. In contrast, incubation of PG545 with cells for a 2 h period occurring just prior to the RSV inoculation of cells reduced the virus infectivity by ~20% suggesting that PG545 could target, although to lesser extent, the non-infected cells. Likewise, incubation of PG545 with cells for a 2 h period occurring just after 2 h period of inoculation of cells with RSV resulted in ~60% reduction of RSV infectivity suggesting that PG545 could affect some steps occurring after virus penetration into the cells or target the virus particles remaining on the cell surface since the cell entry rate of RSV is known to be relatively slow (Techaarpornkul et al., 2001).

To clarify which event of the early RSV-cell interaction is targeted by PG545, the effect of this compound on the virus attachment to cells was tested. PG545, at a concentration range of 0.8–100 μg/ml, reduced the binding to cells of purified and radiolabeled RSV particles by ~50% (Fig. 3A). In contrast muparfostat at 4–100 μg/ml prevented ~75% of RSV virions from their binding to cells. Due to partial reduction of RSV binding to cells by PG545, we sought to investigate whether this compound could interfere with the events of RSV cycle occurring after the virus attachment to cells. To this end, the virus was adsorbed to cells for 2 h at 4 °C prior to the addition of PG545 in warm medium to trigger the entry of preadsorbed virus into the cells. Under these conditions PG545, and to lesser degree muparfostat,

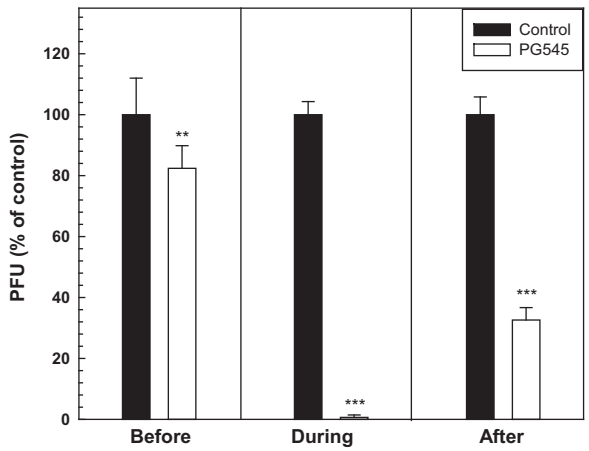


Fig. 2. Anti-RSV activity of PG545 added to cells at different time-points relative to RSV inoculation. One day old cultures of HEp-2 cells, seeded in 12 well plates, were washed twice with 1 ml of DMEM-S medium, and PG545 (20 μg/ml) in the same medium was added and incubated for 2 h period which occurred either prior to, during, or after inoculation of the cells with ~200 plaque-forming units (PFU) of RSV A2 strain for 2 h at 37 °C. The cells were washed twice with 1 ml of DMEM-S medium after each period of their incubation with the drug and/or the virus and overlaid with 1.5 ml of 0.75% methylcellulose solution. After 3 days of incubation at 37 °C, the cells were stained with 1% solution of crystal violet and the viral plaques counted. Three separate experiments were carried out in duplicate, and the results are expressed as a percentage of the number of viral PFU found with compound treated virus and/or cells relative to mock-treated controls. Statistically significant differences as related to controls at P values of <0.005 (***) or <0.01 (**).

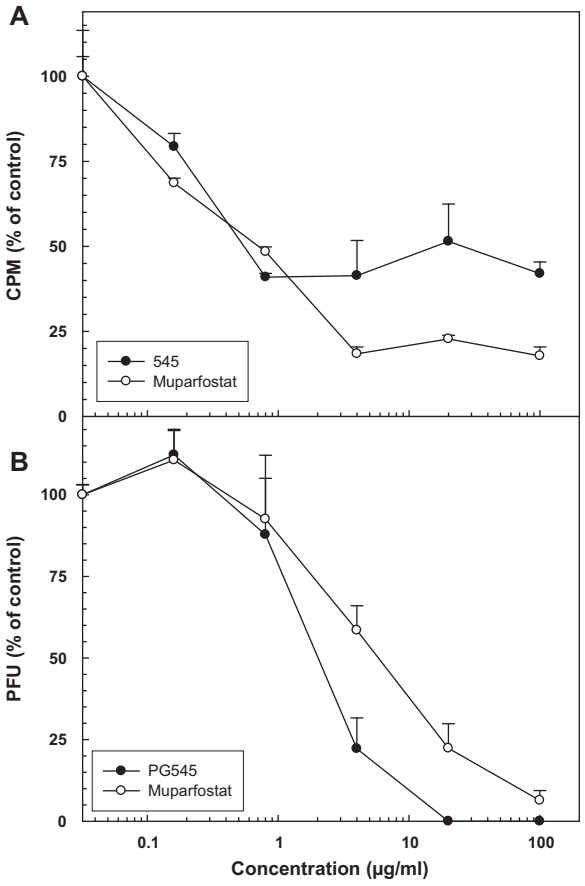


Fig. 3. Effect of PG545 on the binding of RSV to cells and on infection of cells by preadsorbed virus. The test compounds at indicated concentrations were mixed with purified [^{35}S]-labeled RSV virions (~20,000 cpm) and incubated at 4 °C for 10 min prior to and during 90 min period of virus attachment to HEp-2 cells under moderate agitation. The cells were washed 3 times with cold Eagle's medium, lysed with 5% SDS, and transferred to scintillation vials for quantification of radioactivity (A). The cells were inoculated with ~200 plaque-forming units (PFU) of RSV A2 strain in DMEM-S medium for 2 h at 4 °C, then washed with cold medium, and incubated with specific concentrations of test compounds for 2 h at 37 °C (B). Two separate experiments were carried out in duplicate, and the results are expressed as a percentage of attached viral cpm (A) or a percentage of a number of PFU (B) found with compound-treated virions relative to mock-treated controls.

inhibited infection of cells by the pre-adsorbed virus (Fig. 3B) indicating that this compound could either displace the cell-attached virus or block the virus entry into the cells. Altogether, these data suggest that PG545 acts, at least in part, through inhibition of RSV attachment to and entry into the cells. Furthermore, presence of PG545 in culture medium throughout the development of viral plaques reduced their size by ~42% ($P < 0.005$). In particular, the mean area of viral plaques ($n = 28$) developed 6 days after inoculation in mock-treated cells and in the presence of PG545 (4 μg/ml) was 0.31 ± 0.13 and 0.18 ± 0.06 mm^2, respectively (data not shown).

To identify which component of RSV particles is targeted by PG545, we attempted to select viral variants resistant to this compound. For comparative purposes, we also attempted to select viral variants resistant to muparfostat. To this end, plaque purified RSV A2 strain was subjected to 10 passages in HEp-2 cells in the presence of muparfostat (50 μg/ml) or to 13 passages in the presence of increasing concentrations (1–4.5 μg/ml) of PG545. The virus was also mock-passaged in the absence of the test compounds to serve as controls. However, the PG545 resistance of RSV generated in this way was not apparent. In particular, this virus could resist a maximum 4.5 μg/ml of the compound. Furthermore, it required 2–3 days longer to produce complete CPE in the presence of PG545 than in its absence, and its resistance to PG545 assessed by the dose-dependent viral plaque assay, was only ~3- to 4-fold higher than that of original virus (Table 4). Nonetheless, sequencing of the PG545 resistant virus and its comparison with the original and mock-passaged RSV revealed presence of the F168S and the P180S amino acid substitutions in the G protein in all three virus variants examined, and the V516I amino acid alteration in the F protein in variant A (Table 4). Because alteration in the F protein was not found in all variants tested and the resistance of this variant was not substantially different from variants lacking this alteration, this mutation in contrast to alterations in the G protein is likely to be irrelevant for the resistant phenotype.

RSV variants generated by selective pressure from muparfostat in 10 passages in HEp-2 cells were readily selected and appeared to be ~7–9 times more resistant to this compound than original virus. All three plaque variants of resistant virus comprised the N191T amino acid change in the viral attachment protein G (Table 4). In addition to this mutation, variant A also contained the D126E amino acid substitution and the t642c (silent) nucleotide alteration in the G component. Because the drug resistance of variant A was similar to variants B and C, the N191T amino acid change in the G protein seemed to confer RSV resistance to muparfostat. In repetition of this experiment, the RSV was subjected to 6 passages in HEp-2 cells in the presence of muparfostat and two viral variants were plaque purified and analyzed. Both variants were resistant to muparfostat and in contrast to initial or mock-passaged virus comprised the N191T amino acid substitution in the G protein (data not shown). One of these plaques also contained the K197T alteration in the G protein. These data confirm that the N191T alteration in the G protein is responsible for resistance of RSV to muparfostat.

3.3. Virucidal activity of PG545

Data presented in Table 2 indicate that, unlike the sulfated oligosaccharides of muparfostat, inhibition of RSV infectivity by PG545 is associated with virucidal activity of this compound. The term "virucidal activity" is usually applied to agents that are capable of neutralizing, inactivating or destroying a virus permanently. We tested the virucidal potency of PG545 in a dose dependent manner. To this end, PG545 at the indicated concentrations and ~10^5 PFU of RSV A2 strain were mixed in medium comprising 2% heat-inactivated FCS or in serum-free medium and incubated for 15 min at 37 °C. Subsequently, the virus-compound mixture was serially diluted and the residual virus infectivity determined at the non-inhibitory concentrations of PG545. In contrast to muparfostat, PG545 exhibited virucidal activity (Table 3). This activity of PG545 was most pronounced in the serum-free medium where 10 μg/ml of compound completely inactivated infectivity of ~10^5 PFU of RSV. These results indicate that some components of FCS decreased anti-RSV activity of PG545.

Since RSV infecting cells of human airway epithelium represents an obvious target for PG545, we sought to determine whether nasal secretions would modify anti-RSV activity of this compound. To this end pooled nasal secretions, collected and prepared as described in Section 2.8, were mixed with different concentrations of PG545 and ~10^5 PFU of RSV, and incubated for 15 min at 37 °C. Comparative analysis of infectious titers of survived virus (Table 5) revealed that human nasal secretions decreased RSV infectivity by ~4.4-fold. Moreover, human nasal secretions reduced anti-RSV activity of PG545. This effect was clearly seen at a concentration of 10 μg/ml of PG545 that completely inhibited (⩾99.98%) RSV infectivity in the absence of nasal

Table 3
The virus-inactivating activities of muparfostat and PG545.[a]

Compound concentration (μg/ml)	Muparfostat		PG545	
	Serum	No serum	Serum	No Serum
100	122.4 ± 8.5 [b]	151.1 ± 10.7	6.1 ± 5.4	0
10	97.9 ± 3.0	113.7 ± 21.7	62.8 ± 2.4	0
1	101.5 ± 3.6	116.7 ± 16.8	88.8 ± 2.8	35.5 ± 26.5

[a] Test compounds at specific concentrations in the medium supplemented with 2% fetal calf serum or in the serum-free medium were mixed with $\sim 10^5$ plaque-forming units of RSV A2 strain and incubated for 15 min at 37 °C water bath. The mixture was then serially diluted in the same medium and the residual virus infectivity determined.
[b] Mean percentage of residual virus infectivity as related to mock-treated virus found in two separate experiments.

Table 4
Alterations detected in the PG545 or muparfostat resistant variants of RSV.

	Virus variant	Alteration[a]		Sensitivity IC_{50} (μg/ml)
		Nucleotide	Amino acid (protein)	
Muparfostat	Initial[b]	None	None	11
	A	t486 g	D162E (G)	>100 (>9)[c]
		a572c	N191T (G)	
		t642c	Silent	
	B	a572c	N191T (G)	100 (9)
	C	a572c	N191T (G)	80 (7)
PG545	Initial	None	None	1.4
	A	t503c	F168S (G)	6.2 (4)
		c538t	P180S (G)	
		g1546a	V516I (F)	
	B	t503c	F168S (G)	4.8 (3)
		c538t	P180S (G)	
	C	t503c	F168S (G)	4.6 (3)
		c538t	P180S (G)	

[a] Detected by comparison of the nucleotide sequences of RSV subjected to serial passages in the presence of PG545 or muparfostat with these of initial virus or mock-passaged virus in the absence of test compounds.
[b] Plaque purified RSV A2 strain that served as starting material for the virus passages.
[c] Fold resistance to test compounds as related to initial virus is shown in parentheses.

Table 5
Modulation of anti-RSV activity of PG545 by human nasal secretions[a].

PG545 (μg/ml)	Residual RSV infectivity (PFU/ml) with	
	Nasal secretions (+)	Nasal secretions (−)
0	2.5×10^4	1.1×10^5
1	3.1×10^4	4.0×10^4
10	9.9×10^3	$<2 \times 10^1$
100	$<2 \times 10^1$	$<2 \times 10^1$
1000	$<2 \times 10^1$	NT

[a] PG545 at indicated concentration in 25 μl of distilled water was mixed with 200 μl of pooled nasal secretions and 25 μl of DMEM-NS medium comprising $\sim 10^5$ PFU of the virus. The mixtures were incubated for 15 min at 37 °C water bath, and the residual virus infectivity tested by the plaque assay. Results shown are means of two determinations from two separate experiments.

secretions but reduced the RSV titer by 60.4% in the presence of this body fluid. The inhibitory effect of nasal secretions on anti-RSV activity of PG545 was not detected at concentrations $\geqslant$100 μg/ml. The IC_{50} values for PG545, calculated based on data shown in Table 5, were 7 and 0.6 μg/ml when tested in the presence and absence of nasal secretions, respectively. This suggests that under experimental conditions described above ~11 times more of PG545 would be required to overcome inhibitory effect of nasal secretions.

4. Discussion

We found that the anti-RSV activity of polysulfated oligosaccharides was greatly improved following their conjugation with cholestanol, a derivative of cholesterol, a molecule that is a frequent component of antimicrobial lipids of airway secretions (Do et al., 2008). In addition to improved IC_{50} values, this modification endowed oligosaccharides with virucidal activity, a feature that seems to be of importance in possible clinical application of GAG mimetics. This possibility is supported by observation that polysulfonated compound PRO2000, a linear polymer of relatively hydrophobic naphthalene 2-sulfonate, exhibited virucidal activity when tested with HSV (Cheshenko et al., 2004) and provided some protection of women against HIV (Cohen, 2009). In contrast, sulfated oligo- and polysaccharides such as cellulose sulfate or carrageenan that exhibited little or no virucidal activity (Carlucci et al., 1999; Cheshenko et al., 2004) failed in large clinical trials to protect women against HIV infection (Van de Wijgert and Shattock, 2007; Cohen, 2008) in spite of their potent antiviral activity in cultured cells.

The most active glycoside PG545, an anticancer drug candidate currently in Phase I clinical trials (Dredge et al., 2011), composed of cholestanol conjugated to polysulfated maltotetraose, inhibited RSV infection of HEp-2 cells with an IC_{50} value of 2.2 μg/ml while the 50% cytotoxic dose of this compound was 230 μg/ml. The structural design of PG545 is to some extent similar to that of NMSO3, a glycoside known for its potent anti-RSV activity (Kimura et al., 2000). This glycoside is composed of polysulfated mono-sialic acid conjugated to two alkyl chains of $C_{22}H_{45}$ as the lipophilic aglycone component, and its IC_{50} value for RSV Long strain ranged from 0.3 (Kimura et al., 2000) to 6 μg/ml (Wyde et al., 2004) while 50% cytotoxic concentration ranged from 750 μg/ml (Wyde et al., 2004) to >1 mg/ml (Kimura et al., 2000). Modification of sulfated oligosaccharides with a relatively short alkyl chain (dodecyl) was employed in glycoside **3** (Table 1) which exhibited a favorable IC_{50} value and no cytotoxicity (Table 2), however, due to modest

virucidal activity this compound was not extensively studied. More pronounced virucidal activity was observed in PG545 and it is difficult to compare it with NMSO3 since no data on the virucidal activity of this compound was reported. We found that the virucidal activity of PG545 was decreased in the presence of FCS in culture medium, and this observation is not surprising since sterols can interact with several serum proteins including apolipoproteins.

More importantly, because PG545 would need to target RSV infecting cells of the airway, we tested whether the antiviral activity of this compound is modulated in the presence of human nasal secretions. We found that pooled preparations of nasal secretions can inhibit RSV infectivity. The anti-RSV activity of nasal secretions could be exerted by some components of this body fluid such as surfactant proteins (Ghildyal et al., 1999), antimicrobial peptides (Laube et al., 2006; Kota et al., 2008), mucins (Rubin, 2002), or cholesteryl esters (Do et al., 2008). Moreover, we found that human nasal secretions reduced anti-RSV activity of PG545, however, this inhibitory effect could be overcome by using higher concentrations of PG545. Further studies employing a model of RSV infection of well-differentiated cultures of human airway epithelium (Zhang et al., 2002) are needed to assess modulation of anti-RSV activity of PG545 by airway mucus.

The capability of PG545 and related glycosides to interact with serum proteins did not seem to limit their in vivo application. In fact, the presence of the lipophilic moiety in PG545 and related glycosides helped to overcome two major disadvantages associated with in vivo usage of sulfated oligo- and polysaccharides, i.e., it greatly attenuated their anticoagulant activity and prolonged the half life of these compounds in the body (Johnstone et al., 2010; Dredge et al., 2011). Due to the presence of sulfate groups in PG545 and related glycosides, these compounds can inhibit the interaction between a plethora of different proteins and sulfated GAGs. Thus, interference of PG545 with the activity of vascular endothelial and fibroblast growth factors inhibited angiogenesis, a key process in tumor development, while binding to heparanase, an enzyme abundantly expressed on neoplastic cells, limited their metastasis (Dredge et al., 2010, 2011; Johnstone et al., 2010). Both these functional features confer potent anti-cancer activities on PG545 (Dredge et al., 2011).

How exactly does the presence of a lipophilic moiety alter the interaction of sulfated oligosaccharides with RSV? We found that PG545 preferably interacted with RSV and to lesser extent with cells, and blocked initial events of RSV invasion of cells. Sulfated oligo- and polysaccharides (Krusat and Streckert, 1997; Kwilas et al., 2009) including muparfostat (this report) target mainly the RSV attachment protein G. Indeed, analysis of the viral variants resistant to muparfostat revealed a G protein mutation, N191T, occurring in the heparin-binding domain (Feldman et al., 1999) responsible for interaction of this protein with GAGs. Interestingly, in HSV muparfostat targeted proteins that, like RSV G protein, contain the mucin-like region, and the resistant variants of HSV-1 expressed attachment protein gC with the entire mucin-like segment deleted (Ekblad et al., 2007) while HSV-2 produced no envelope glycoprotein gG (Adamiak et al., 2007). In contrast to muparfostat, RSV variants resistant to PG545 exhibited only a weak resistance to this drug. Nonetheless, these weakly resistant variants comprised two amino acid substitutions F168S and P180S in the central region of the G protein that includes the cysteine noose. Thus, analysis of RSV variants resistant to muparfostat and PG545 indicates that both these compounds target the G protein. However, in comparison with muparfostat, PG545 reduced the virus attachment to cells less extensively while demonstrating a more pronounced inhibitory effect on infection of cells by virus that was adsorbed to cells at 4 °C prior to the addition of PG545. Collectively, poor resistance of RSV to PG545 and moderate reduction of the virus binding to cells by this compound suggest that in addition to the G protein PG545 may target other components of the viral envelope. Indeed, an expected affinity of cholestanol component of PG545 for lipid membranes suggests that this compound could be inserted into the viral lipid envelope thus creating a coat of artificial sulfo-glycolipids/sterols, a structure that could prevent fusion of viral and cellular membranes and thereby neutralize the virus. Lack of PG545 activity against influenza A virus, a pathogen that does not require GAGs for initial binding to cells, suggests that the sulfated oligosaccharide component of PG545 can be responsible for specific affinity of this compound for the GAG-binding viruses, an event followed by hydrophobic interaction of cholestanol with viral lipids. Thus, it is likely that PG545 may target more than one viral component to exhibit anti-RSV activity. Mutations detected by us in the G protein were not found in the published sequences of clinical isolates of RSV. It is noteworthy that another cholestanol- tetrasaccharide conjugate **14** failed to generate resistance in HSV-2 (Ekblad et al., 2010). Kimura et al. (2004) generated NMSO3 variants of RSV Long strain which, following 15 and 33 passages in HEp-2 cells, achieved 4.8- and 9.3-fold resistance to this drug. The NMSO3 resistant phenotype was due to numerous mutations in the G protein, however a single amino acid substitution in the F protein was also detected (Kimura et al., 2004). One of the mutations detected by these authors in the G protein after 33 NMSO3 passages (i.e., F168S) was also detected by us in the PG545 resistant RSV.

In conclusion, PG545 is a novel inhibitor of RSV that exhibits virucidal activity, and the development of RSV resistance to this compound is slow. Given the fact that PG545 and related glycosides also exhibited potent virucidal activities against HSV (Ekblad et al., 2010) and HIV (Said et al., 2010), the cholestanyl-conjugated oligosaccharides are promising lead compounds for treatment and/or prevention of infections caused by viruses that use GAGs as initial receptors.

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