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Lipophile-conjugated sulfated oligosaccharides as novel microbicides against HIV-1

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ABSTRACT

With the aim of providing compounds suitable for further development as microbicides active against human immunodeficiency virus 1 (HIV-1) a library containing 37 lipophile-conjugated sulfated oligosaccharides was screened for antiviral and virucidal activity against this virus. Four highly active compounds had low drug inhibition concentrations (IC_{50}) for HIV-1 and inactivated viral particles, suggestive of virucidal properties. Two of these compounds comprising a sulfated tetrasaccharide linked to a cholestanol group by a glycosidic bond, showed low toxicity and high selectivity indices. The two compounds were active both against CCR5 and dual-tropic CCR5/CXCR4 clinical HIV-1 isolates. Since herpes simplex virus type 2 (HSV-2) may be a cofactor for HIV-1 infection, the virucidal effect of the compounds was demonstrated against both viruses when mixed and incubated together on permissive cells. Incubation of compounds with serum, and to a lesser degree, cervical secretions, reduced the HIV-1 inactivating capacity, which suggests the need for molecular modification to reduce host protein binding. Considering the virucidal effect and low toxicity, these sulfated oligosaccharides with lipophilic tails may offer new possibilities of microbicide development.

1. Introduction

The global prevalence of the human immunodeficiency virus (HIV) in 2008 was estimated to be 33.4 million people, of which 15.4 million were women (UNAIDS, 2009). Prophylaxis against HIV transmission to women is essential to curb the pandemic and a prerequisite to stop infection of the newborn. The male condom is to date the only protection available against sexually transmitted infections (STIs) such as HIV type 1 (HIV-1) and herpes simplex virus type 2 (HSV-2) (Holmes et al., 2004). Women, especially in sub-Saharan Africa, often have difficulties to negotiate male condom use because of socio-cultural issues (Balmer et al., 1995; Ulin, 1992; Varga, 1997). A safe and effective vaginal microbicide which, when applied topically, can inhibit HIV-1 and HSV-2 infection could be an alternative and female-controlled prevention method. In several reports, HSV-2 (as well as other ulcerating STIs) was shown to play an important role for the spread of HIV-1 by enhancing the transmission of the virus between individuals (Holmberg et al., 1988; Keet et al., 1990; Stamm et al., 1988). Some studies have also indicated that HSV-2-infected individuals without recurrent herpetic lesions are at increased risk of being HIV-1 infected (Kapiga et al., 2007; Quinn, 1987). Taken together, these results clearly indicate the necessity of the development of microbicides fully active against both HIV-1 and HSV-2.

HIV-1 initially infects T-cells and macrophages expressing the CD4 receptor on their cell surface (Dalgleish et al., 1984). For the virus to successfully fuse and enter into the cell, either of the two chemokine receptors CXCR4 or CCR5 (or both) is needed (Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996). It has been suggested that CCR5 is the major co-receptor involved in sexual transmission of HIV-1. In contrast, many of the cell lines utilized in preclinical research are infected via the CXCR4 receptor. Most of the microbicides tested to date can indeed inhibit infection by CXCR4-binding HIV-1 but fail to inhibit CCR5-binding HIV-1 to the same extent (Neurath et al., 2002). For a drug to prevent HIV-1 transmission, it is most crucial for it to target primarily the CCR5-tropic viruses, but preferably both types. A common denominator to inhibit transmission via both co-receptors might be the blocking of HIV-1 binding to another group of receptors in the form of sulfated carbohydrates such as glycosaminoglycans (GAGs) present on the cell surface.

In recent reports, cell surface heparan sulfate (HS) was suggested to enhance HIV-1 infection through binding to an

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arginine-rich domain of envelope glycoprotein gp120 (Crublet et al., 2008; Vivès et al., 2005). Sulfated and sulfonated polymers and polysaccharides, such as dextrin 2-sulfate (Javan et al., 1997), cellulose sulfate (Anderson et al., 2002), and PRO-2000 (naphthalene sulfonate polymer) (Fletcher and Shattock, 2008) are some of the antimicrobial agents that have been taken to clinical trials (El-Sadr et al., 2006; Keller et al., 2006; Kilmarx et al., 2006; Malonza et al., 2005; Piret et al., 2000; Rusconi et al., 1996; Schaeffer and Krylov, 2000) and that have shown efficient inhibition of HIV infection *in vitro*. Because of their overall negative charge, these oligosaccharide/polymeric compounds may hinder viral attachment and/or entry into the target cells, presumably through blocking of the above-mentioned HS-binding site of gp120. As a further development of such compounds, here we have screened a library containing 37 lipophile-conjugated sulfated oligosaccharides for virucidal activity against HIV-1 and HSV-2. One group of highly active molecules was derived from hydrophobic extension of the oversulfated oligosaccharide such as those found in muparfostat (i.e. PI-88), a mixture of sulfated di- to hexasaccharides previously shown to efficiently hinder entry and cell-to-cell spread of HSV-2 (Nyberg et al., 2004). Of these molecules, the cholestanol conjugates P3, P4, P5 and P6 were shown to have low 50% drug inhibitory concentrations (IC_{50}) for both HIV-1 and HSV-2. Furthermore, these compounds blocked infectivity of free viral particles, a feature suggestive of virucidal properties. We suggest that these substances may be promising as candidate compounds for further development of microbicides against HIV-1 and HSV-2.

2. Materials and methods

2.1. Compounds

The polysulfated phosphomannan muparfostat (formerly known as PI-88), prepared by hydrolysis and sulfonation of yeast phosphomannan (Ferro et al., 2001; Yu et al., 2002) was obtained from Progen Pharmaceuticals Ltd. (Australia) along with a library of 37 fully sulfated oligosaccharides with reducing end lipophilic modifications (Fig. 1). This library included α-glycosides of the Manα(1→3)/(1→2)-linked tetra- or pentasaccharide sequence found in muparfostat, e.g., P1, P2 and P3, as well as β-linked conjugates derived from maltotriose and maltotetraose, such as P4, P5 and P6. The lipophilic modifications included straight chain alkyl groups of various lengths, with or without terminal aromatic substituents, and steroids such as cholestanol, with or without a spacer (e.g. triazole). The test compounds were synthesized in a similar fashion to that described previously for related compounds (Johnstone et al., 2010; Karoli et al., 2005). Full details of the synthesis and characterization of some compounds (e.g. P1 and P2) have recently been disclosed (Johnstone et al., 2010) and the details for the remaining compounds will be published in due course. Four of the analogues, the cholestanol conjugates P3, P4, P5 and P6, showed potential of virucidal properties and were selected for detailed studies.

2.2. Cells and viruses

H9-cells were cultured in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 20% heat-inactivated fetal calf serum (FCS), 0.1% penicillin and streptomycin (PEST) and 0.01% polybrene (2.24 μg/mL). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors and purified using the Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. The cells were then stimulated with 2.5 μg/mL phytohemagglutinin (PHA, Becton Dickinson Microbiology systems, Sparks, MD) in RPMI supplemented with 10% FCS and 0.1% PEST for 3–5 days. The cells were cultured in RPMI medium containing 10% FCS, interleukin-2 (200 IU/mL proleukin, Chiron, Amsterdam, The Netherlands), hydrocortisone (5 μg/mL, Sigma, St. Louis, MO), polybrene (2.24 μg/mL, Sigma) and 0.1% PEST. African green monkey kidney (GMK AH1) epithelial cells (Gunalp, 1965) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 2% FCS, 0.05% Primaton RL substance (hydrolysate of lactoalbumin, Kraft Inc., Norwich, CT) and 1% PEST. Three HIV-1 strains, HIV-1^{IIIB}, the dual-tropic CXCR4/CCR5 HI92-206 isolate (X4/R5), and the CCR5-tropic HI05-91 isolate (R5) were used. HIV-1 isolations were performed using blood collected to EDTA solution. PBMCs purified with Ficoll-Hypaque were co-cultured with PBMCs from healthy blood donors. Stocks of HIV-1 isolates were prepared after one passage by collecting supernatants from infected cultures. The HIV-1^{IIIB} laboratory strain was kindly provided by Dr. R.C. Gallo and Dr. M. Popovic (at that time at the National Cancer Institute, NIH, Bethesda, MD). The HSV-2 strain used was HSV-2 333 (Duff and Rapp, 1971).

2.3. Screening for compounds with antiviral activity against HIV-1

Screening of the compound collection for anti-HIV-1 activity was performed as follows. Serial fivefold dilutions of each test compound were added to fresh medium in 48-well plates to receive final concentrations ranging from 0 to 100 μg/mL. Subsequently, 100 $CCID_{50}$ (100 × 50% cell culture infective dose) of laboratory strain HIV-1^{IIIB} or clinical isolate X4/R5 or R5 was added and incubated at room-temperature for 5–10 min before addition of H9 (5×10^5 cells/mL) cells or PBMCs (6×10^5 cells/mL). The mixtures were incubated at 37 °C for 6 days, after which half of the medium volume was exchanged to fresh supplemented medium without drug. On day 13, the degree of viral replication was established by observation of the cytopathic effect (CPE), in this case syncytium formation (visible in H9 cells only). Pentosan polysulfate served as a positive control substance and was added at 100 μg/mL, since it has been described as a potent inhibitor of HIV-1 infection *in vitro* (Baba et al., 1988). Cells infected with HIV-1 in the absence of an inhibitor and mock-infected cells served as additional controls. Samples showing no visible syncytium formation were further examined for p24 antigen contents, using an *in house* antigen enzyme-linked immunosorbent assay (ELISA) as previously described (Horal et al., 1991). The p24 amount was calculated by using a recombinant p24 standard (Protein Sciences Corporation, Meriden, CT) with fixed concentrations and a detection limit of approximately 500 pg/mL. The IC_{50} value was determined by plotting the p24 antigen contents against the drug concentration. To control for any inhibitory effect (i.e. altered IC_{50} values) by polybrene, a control experiment using compound P4 together and without this substance was performed.

2.4. Cell proliferation assay

Fifty microlitres of supplemented medium containing H9 cells (5×10^5 cells/mL) or the PHA-stimulated PBMCs (6×10^5 cells/mL) were added to a 96-well plate. Fifty microlitres of serial two fold dilutions of each drug at a concentration range of 1.5–800 μg/mL were then added to the cells, and incubated at 37 °C for 24 h. Thereafter, 20 μL of the CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI) was added to each well and the plate was further incubated for 4 h at 37 °C. Controls consisted of cells incubated without test compounds. The absorbance was measured at 490 nm with 650 nm background subtractions using an ELISA plate

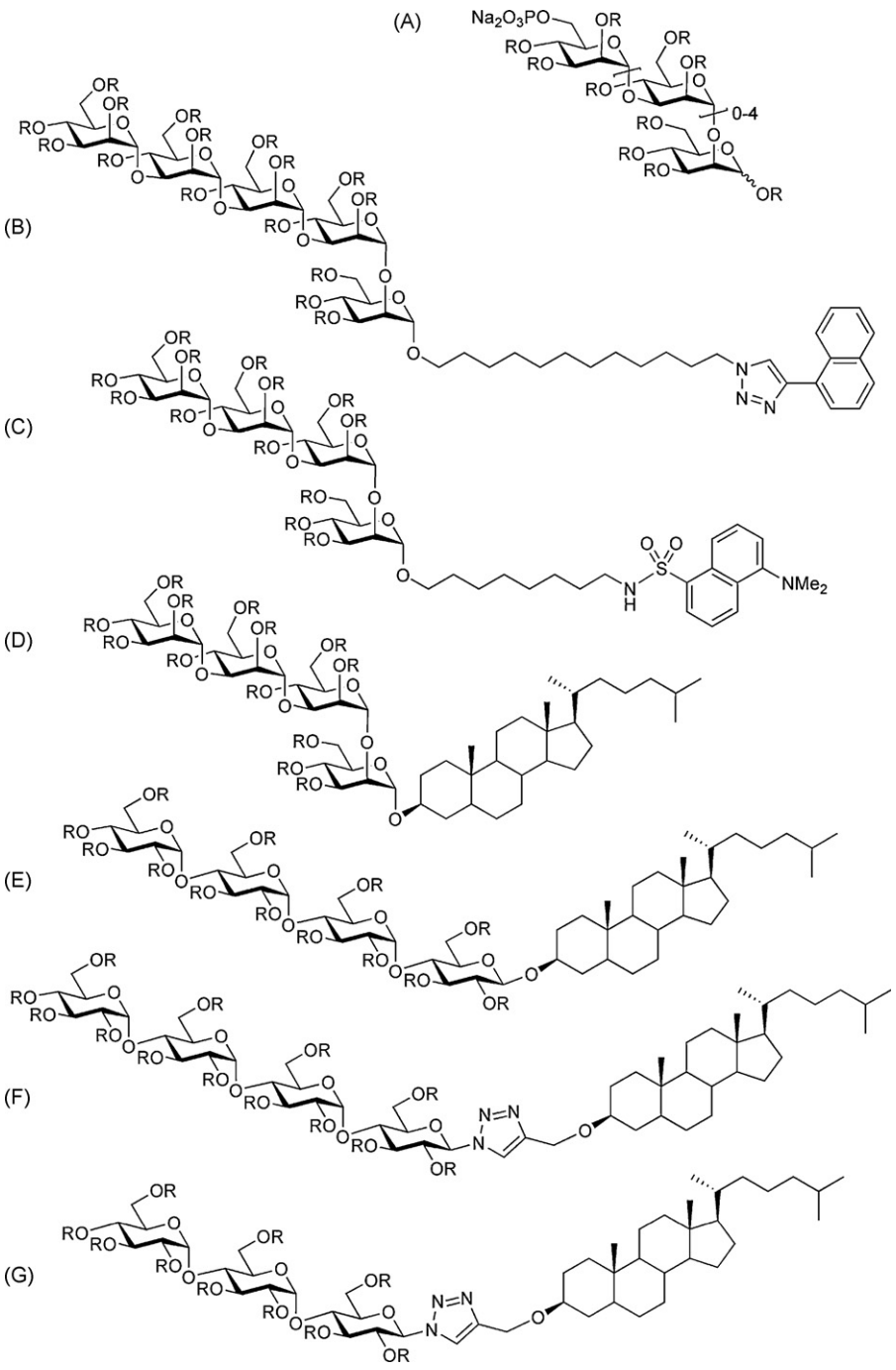

Fig. 1. Structures of muparfostat (A) and some lipophilic sulfated oligosaccharide conjugates described in this study, where R = SO_3Na. Compounds P1 (B), P2 (C) and P3 (D) are α-glycosides of the Manα(1→3)/(1→2)-linked penta- or tetrasaccharide sequence found in muparfostat. Compounds P4 (E), P5 (F) and P6 (G) comprise a cholestanyl group, with or without a triazole spacer, β-linked to a sulfated maltotetraose or maltotriose.

reader. The cell proliferation activity was then calculated as % of the cell control for each drug concentration.

2.5. Time-of-addition assay

For all four lines of experiments (see below), all samples were plated on 24-well plates with 5×10^5 H9 cells/mL and infected with 100 $CCID_{50}$ of HIV-1^{IIIB} followed by an incubation at 37 °C for 13 days, including change of medium on day 6 and collection of supernatants on days 8 and 13. The p24 antigen was quantified by ELISA and compared with a negative control prepared in the same manner but without addition of test compound.

2.5.1. Pre-treatment of cells with compound

H9 cells were treated with 100 μg/mL of compound P3 and incubated in a test tube at 37 °C for 2 h. The mixture was then centrifuged at 1200 rpm for 7 min at 20 °C and washed once with fresh RPMI. Finally, 100 $CCID_{50}$ of HIV-1^{IIIB} was added together with fresh medium.

2.5.2. Pre-treatment of virus with compound

HIV-1^{IIIB} was mixed with compound P3 and incubated at room temperature for 5–10 min in a test tube. Thereafter, the virus/compound mixture was added to another test tube containing

H9 cells, and incubated on ice for 2 h. The cells were then washed with RPMI and fresh medium was added.

2.5.3. Compound treatment of cells with pre-attached virus

Pre-cooled H9 cells were added to a test tube together with HIV-1^{IIIB}. The mixture was incubated on ice for 2 h and then washed with RPMI. Fresh medium was added together with compound P3 at room temperature and the mixture was then incubated at 37 °C for 2 h. The mixture was again washed with RPMI and fresh medium was added.

2.5.4. Treatment of cells with compound after the virus infection

H9 cell suspension was mixed with HIV-1^{IIIB} strain in a test tube and incubated at 37 °C for 2 h. The cells were washed once with RPMI and fresh medium was added. The compound P3 was finally added to the cell/virus mixture.

2.6. Determination of virucidal effect

The compound (100 μg/mL) was incubated together with 1000 $CCID_{50}$ of HIV-1^{IIIB} or with 100–1000 $CCID_{50}$ of either of the clinical isolates R5 or X4/R5 at 37 °C for 60 min. The mixture was then diluted 10-fold and 100-fold (in duplicate) in 24-well plates and incubated together with H9 cells (5×10^5 cells/mL) and PBMCs (6×10^5 cells/mL), respectively, at 37 °C for 13 days. A sample without the compound served as a control. The level of p24 antigen for each dilution was measured by ELISA in supernatant fluids collected at day 6, 8, and 13.

2.7. HIV-1 and HSV-2 co-infection in H9 cells

This assay was performed in the same manner as the above-mentioned inactivation experiment including 200 plaque forming units (pfu) of the HSV-2 333 virus, which was added to the test tube together with 1000 $CCID_{50}$ of HIV-1^{IIIB} and 100 μg/mL of test compounds P4, P5 or P6. Supernatants were collected and analyzed by ELISA for the presence of p24 antigen.

2.8. HIV-1 and HSV-2 co-infection in GMK cells–plaque reduction assay

The inactivation experiment (as above) of virus infectivity was performed at 37 °C for 60 min. The mixture contained 200 pfu of the HSV-2 333 strain, 1000 $CCID_{50}$ of the HIV-1^{IIIB} strain and 100 μg/mL of test compounds P4, P5 and P6 in EMEM. The virus/drug mixture was then diluted 10-fold and 100-fold in EMEM supplemented with 2% FCS and 1% PEST and added to the confluent monolayer of GMK AH1 cells growing in 12-well cluster plates. The cells were incubated at 37 °C for 2 h, whereupon the wells were washed with fresh EMEM, and 1 mL volume of 1% methylcellulose solution in supplemented EMEM was added to each well. After 2–3 days of incubation the methylcellulose was removed and the cells were stained with 1% solution of crystal violet. The viral plaques were counted under a microscope. Included in the assay were three controls containing (i) HSV-2 and HIV-1 but no compound, (ii) HSV-2 and compound but not HIV-1 and (iii) HSV-2 without both the drug and HIV-1.

2.9. HIV-1 inactivation in the presence of cervical secretions and fetal calf serum

Cervical secretions (CS) and RPMI containing 10% FCS were included in an inactivation assay each in order to find out whether any of these components would affect the activity of the test compound. CS were obtained by swabbing the uterine cervix. The swabs were immersed in isotonic NaCl and pre-diluted 10-fold in the same medium. 100 μL of the CS dilution were used in the inactivation assay (constituting 50% of the total volume). Three control samples were also prepared for each assay, one without the drug, another with drug but without CS or FCS and the third without both drug and CS or FCS. Supernatants from day 13 were analyzed by ELISA.

2.10. Transmission electron microscopy

HIV-1^{IIIB} ($100 \times CCID_{50}$) was treated with 100 μg/mL of compound P4 before being incubated with H9 cells for 7 days. A negative control with cells and virus only was prepared in the same manner. The cells were collected, re-suspended and fixed in a mixture of 2.5% glutaraldehyde with 2% paraformaldehyde in 0.05 M Na cacodylate buffer for 3 h; and thereafter they were stored in a 0.15 M Na cacodylate buffer, at 4 °C. The cells were rinsed in 0.15 M Na cacodylate buffer and fixed for 2 h at 4 °C in a 1% OsO_4 and 1% $K_4Fe(CN)_6$ fixative, rinsed three times in distilled water and treated with a 0.5% uranylacetate solution for 1 h in the dark at room temperature. The samples were dehydrated stepwise in ethanol (70%: 5 min; 85%: 5 min; 95%: 5 min; 99.5%: 4×5 min), acetone (3×5 min), acetone/Agar 100 resin (1:1 ratio: 5 min) and acetone/Agar 100 resin (1:1 ratio: 2 h). Specimens were embedded in Beem capsules (Balzers Union BU 011027-T) in Agar 100 resin and polymerized for 15 h at 40 °C followed by 48 h at 60 °C. Ultra thin sections of 60 nm for transmission electron microscopy were obtained with a diamond knife on a Reichert-Jung Ultracut E microtome (Leica Microsystems, Vienna, Austria) and collected on copper grids, which were then contrasted with uranyl acetate (5% in 25% ethanol) and lead citrate. Sections were studied with a LEO 912AB Omega transmission electron microscope (Oberkochen, Germany). Digital image files were captured with a Mega View III camera (Soft Imaging Systems, Münster, Germany) and processed in Adobe Photoshop CS2.

3. Results

3.1. Lipophile-conjugated sulfated oligosaccharides inhibit HIV-1 infection of cultured cells

The aim of the screening assay was to find compounds inhibiting infection of HIV-1^{IIIB} *in vitro*. The compounds ($n = 37$) were incubated with cells and virus at 37 °C for 13 days, including a medium change on day 6. Ten compounds, together with the control substance pentosan polysulfate, inhibited syncytium-forming activity of HIV-1. Furthermore, their anti-HIV activities were confirmed by ELISA, which showed lack of p24 antigen production. One of these compounds, i.e. the cholestanol conjugate P3 (designated as 14 in the referred paper) was recently shown to exhibit virucidal effects *in vitro* on HSV-2 (Ekblad et al., 2010). Thus, some compounds fulfilled the criteria of inhibiting both HIV-1 and HSV-2, and were selected for further experiments.

3.2. Anti-HIV potential of lipophile-conjugated sulfated oligosaccharides

The dose-response tests, in which the compounds P3, P4, P5, and P6 were diluted 5-fold (resulting in final concentrations of 100 μg/mL, 20 μg/mL, 4 μg/mL, 0.8 μg/mL and 0.16 μg/mL) and including a negative control, were performed on H9 cells with the laboratory strain HIV-1^{IIIB} and on PBMCs with the R5 tropic and the X4/R5 tropic clinical isolates. Supernatants were collected at 6, 8 and 13 days post infection and analyzed by ELISA for p24 antigen. IC_{50} values for each compound were calculated by plotting the p24 level against the compound concentration. The lowest IC_{50} value for the HIV-1^{IIIB} strain, i.e. 7 μg/mL, was established for both compound P3 and P6 (Table 1). For the clinical isolates, the IC_{50}

Table 1
Anti-HIV-1 activities of selected compounds in H9 cells and PBMCs.

Compound	Virus[a]	Cells	IC_{50} (μg/mL)[b], $M \pm$ S.D.[c]	CC_{50} (μg/mL)[d], $M \pm$ S.D.	SI[e]
Muparfostat	HIV-1^{IIIB}	H9	53 ± 2	n.d.[f]	
P3	HIV-1^{IIIB}	H9	7.0 ± 4	>800	>110
	R5 virus	PBMCs	52 ± 33	>800	>15
	X4/R5 virus	PBMCs	24 ± 20	>800	>33
P4	HIV-1^{IIIB}	H9	14 ± 4	550 ± 40	39
	R5 virus	PBMCs	4.0 ± 3	650 ± 75	160
	X4/R5 virus	PBMCs	5.0 ± 3	650 ± 75	130
P5	HIV-1^{IIIB}	H9	12 ± 3	290 ± 4	24
	R5 virus	PBMCs	6.0 ± 5	360 ± 115	60
	X4/R5 virus	PBMCs	4.0 ± 2	360 ± 115	90
P6	HIV-1^{IIIB}	H9	7.0 ± 3	190 ± 29	27
	R5 virus	PBMCs	2.0 ± 2	300 ± 90	150
	X4/R5 virus	PBMCs	8.0 ± 5	300 ± 90	38

[a] R5 represents the CCR5-tropic clinical isolate and X4/R5 the dual-tropic clinical isolate, preferably using the CXCR4 receptor but can also infect cells by the CCR5 receptor.
[b] The compound concentration that inhibits the virus replication by 50%.
[c] *M* represents the mean and ± the standard deviations (S.D.) for three independent experiments.
[d] The compound concentration that reduces the cell proliferation by 50%.
[e] The selectivity index (SI) defined as the ratio of 50% cytotoxic concentration (CC_{50}) to 50% virus inhibitory concentration (IC_{50}).
[f] n.d.: not done.

values were in most cases comparable to or even lower than those found for HIV-1^{IIIB}. Note, however, that in the case of the R5 tropic strain, compound P3 was found to have the highest IC_{50} value of 52 μg/mL while compound P6 showed the lowest IC_{50} for this virus of 2.0 μg/mL. Compounds P4 and P5 showed the lowest IC_{50} for the X4/R5 virus of 4.0–5.0 μg/mL as compared with 8.0 μg/mL for compound P6. A control experiment of eventual altering effect of polybrene on compound P4 showed similar IC_{50} values with and without this substance for all three HIV-1 viruses tested (data not shown).

The concentrations of test compounds that reduced the cell proliferation by 50% (CC_{50}) were calculated for each compound based on the results of the cell proliferation assay. As compared with compounds P5 and P6, the CC_{50} for compounds P3 and P4 were higher for both cell types (H9 and the PBMCs) and ranged between 550 μg/mL and >800 μg/mL. The CC_{50} values for compounds P5 and P6 were at a range of 190–360 μg/mL. It is likely that the structural basis for these differences is the additional triazole ring (spacer) present between the oligosaccharide chain and the cholestanol group (Fig. 1) in compounds P5 and P6. The compound with the highest selectivity index (SI; CC_{50}/IC_{50}) was P4, which exhibited SI values of 160 for the R5 virus and 130 for the X4/R5 virus. Compound P3 had the lowest SI of more than 15 for the R5 virus, and the highest SI of more than 110 for strain HIV-1^{IIIB}.

3.3. Mode of anti-HIV activity of compound P3

One of the four active compounds, P3, was synthesized in higher quantity and therefore available for analysis of mode of action by four sets of experiments. In the first assay we wanted to investigate whether pre-treatment of cells would block virus infection indicative of cells rather than the virus being a target for compound P3. H9 cells were pre-incubated for 2 h in the presence of 100 μg/mL of compound P3 prior to the addition of HIV-1^{IIIB}. Although a difference in p24 quantity was observed between the drug-treated and non-drug treated cells at 8 days after HIV infection, no difference was found at day 13 (Fig. 2A). These results argue against interpretation that most of the anti-HIV effect of compound P3 was due to blocking of susceptible cells. However, pre-treatment of HIV-1 with compound P3 followed by incubation with cells for 2 h on ice resulted in inhibition of infection on day 13 (Fig. 2B). The low incubation temperature enabled the virus to attach to its receptor without conformational changes of gp120 and gp41 that normally occur prior to viral entry (Demaria and Bushkin, 1996). However, the interaction of the compound with the virus could occur, as well as the attachment of the virus to the host cells. These results suggest that compound P3 blocked viral attachment/entry, and/or acted as a virucidal compound. Furthermore, when compound P3 was added to cells after a 2 h period of virus attachment on ice, and the temperature was raised to 37 °C, no virus infection was seen on day 8, but a substantial amount of p24 was detected on day 13 (Fig. 2C). This suggests that the compound may somewhat decrease the infectivity of cell-attached virions; however, the presence of compound P3 at 2 h after infection of cells at 37 °C, as well as presence in the culture medium, resulted in no detectable amounts of p24 at day 13 (Fig. 2D). This indicates that, in addition to blocking the virus binding/entry steps, compound P3 may exhibit some other effects on virion or on cells, as suggested by the delay in HIV replication in P3 pre-treated cells (Fig. 2A). Since these experiments were performed at low multiplicity of infection, the effects of P3 observed in Fig. 2D may be attributed to inhibition of HIV-1 egress and/or cell-to-cell spread of progeny virions.

3.4. Virucidal properties of lipophile-conjugated sulfated oligosaccharides

Since the anti-HIV-1 effects of test compounds could be due to virucidal properties, an inactivation assay was performed with compounds P1, P2, P3, P4, P5 and P6. These compounds were chosen because of their potency to inhibit HIV-1^{IIIB} in the screening procedure, as well as the ability of P3 to inhibit HSV-2 infection (Ekblad et al., 2010). The HIV-1^{IIIB} strain was first incubated with each compound for 1 h at 37 °C whereafter the mixture was diluted 10-fold and then added to H9 cells. Supernatants were collected on day 6, 8 (not shown) and 13 and p24 quantities were determined by ELISA. No p24 was detected in samples where the virus was treated prior to the addition to cells with compounds P4, P5, P6 (not shown) and P3 (Table 2). In contrast compounds P1 and P2 did not inactivate the virus when the virus/compound mixture was diluted 100-fold prior to the testing for residual infectivity (Table 2).

3.5. Inactivation of clinical HIV-1 isolates by lipophile-conjugated sulfated oligosaccharides

Two different clinical isolates, the R5 tropic and the X4/R5 tropic variants were also tested for their inactivation by the compounds P4, P5 and P6. All three compounds inactivated the R5 viral variant

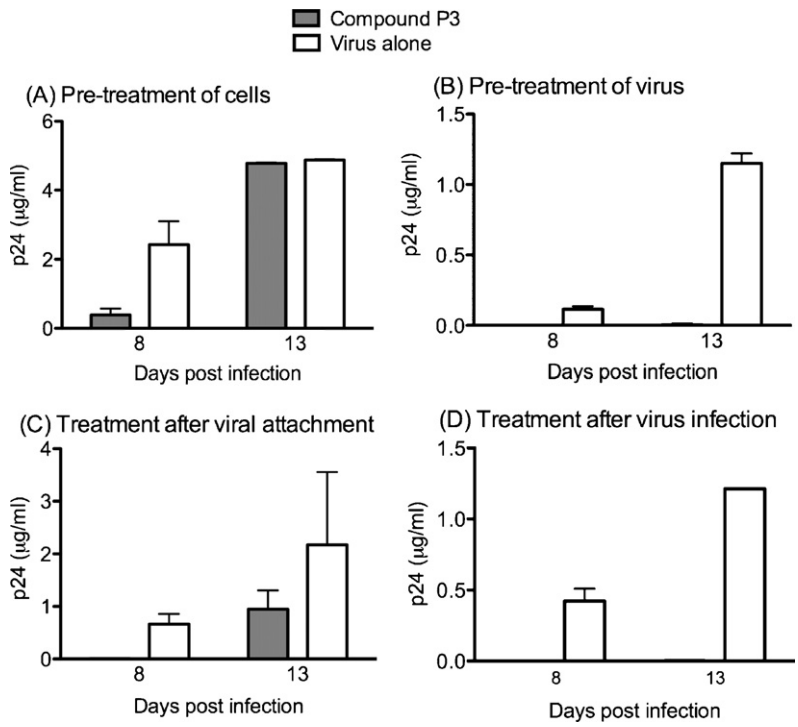

Fig. 2. Time-of-addition and anti-HIV activity of compound P3. Antiviral activity was measured 8 and 13 days post infection by ELISA. (A) H9 cells were incubated with compound (100 μg/mL) for 2 h at 37 °C whereafter the medium was exchanged for fresh medium (without compound) containing 100 $CCID_{50}$ of HIV1-1^{IIIB}. (B) Virus and compound were mixed and incubated at room temperature for 5–10 min prior to addition of the mixture to cells and incubation for 2 h on ice. The medium was then exchanged for fresh medium without new compound added. (C) Cells were incubated with the virus for 2 h at 4 °C after which the medium was exchanged and compound added. The mixture was incubated at 37 °C for 1 h and the medium was again exchanged for fresh medium and antiviral activity was measured with ELISA day 8 and 13. (D) Virus was incubated with cells for 2 h at 37 °C, the medium was then exchanged for fresh medium containing the compound and the antiviral activity was assayed by ELISA for quantification of the p24 antigen.

Table 2
The HIV-inactivating activity of selected test compounds.

Compound (100 μg/μL)	Virus[a]	Final $CCID_{50}$[b]	Residual infectivity (%)[c], $M \pm$ S.D.[d]
P1	HIV-1^{IIIB}	100	0
		10	32 ± 1
P2	HIV-1^{IIIB}	100	0
		10	17 ± 3
P3	HIV-1^{IIIB}	100	0
		10	0
	X4/R5	100	0
		10	0
	R5	100	1 ± 1
		10	63 ± 30
P4	X4/R5	100	1 ± 1
		10	87 ± 34
	R5	100	0
		10	0
P5	X4/R5	100	0
		10	84 ± 33
	R5	100	0
		10	0
P6	X4/R5	100	1 ± 1
		10	89 ± 34
	R5	100	0
		10	0

[a] The stock solution titers were 1×10^4 $CCID_{50}$ for HIV-1^{IIIB}, 1×10^3 $CCID_{50}$ for the CCR5 tropic isolate and 1×10^2 $CCID_{50}$ for the CXCR4/CCR5 tropic isolate.
[b] Denotes the $CCID_{50}$ value in wells after dilutions of the virus/compound mixture.
[c] Results are expressed as a percentage of residual virus infectivity as related to the virus proliferation in the absence of drug.
[d] *M* represents the mean and ± the standard deviations (S.D.) for three independent experiments.

(Table 2). Note that the X4/R5 variant was inactivated by compounds P4, P5 and P6 in the 10-fold dilution, while the hundred-fold dilution of the same compounds inhibited infection of this virus by only 13% (87% residual infectivity), 16% (84% residual infectivity) and 11% (89% residual infectivity), respectively.

3.6. The virus-inactivating potential of lipophile-conjugated sulfated oligosaccharides in the presence of serum and cervical secretions

Since protein-rich environments might alter the activity of molecules such as charged oligosaccharides, we decided to study the HIV-1^{IIIB} inactivating capability of compounds P4, P5 and P6 in the presence of cervical secretions (CS) and of different concentrations of heat-inactivated FCS. All three compounds inactivated HIV^{IIIB} in the absence of FCS, but showed decreased activity in the presence of 10% FCS followed by dilution of the drug–virus mixture 100-fold (Table 3). At 10-fold dilution of the drug–virus mixture, compound P5 blocked infection while P4- and P6-treated virus at the same dilution retained 9% and 6% original infectivity, respectively. Cervical secretions decreased the virus inactivating activities of the compounds at dilution of 1:20 less efficiently than the effect observed with FCS. Compounds P4 and P6 were not at all or very little affected by the presence of CS whereas P5 blocked infection only to 30% (residual infectivity 70%) when the drug–virus mixture was diluted 1:100.

3.7. Inactivation of HIV-1^{IIIB} and HSV-2 during simultaneous infection

Inactivation assays were performed with compounds P4, P5 and P6 each at a concentration of 100 μg/mL and dual infection

Table 3
The HIV-inactivating activity of selected test compounds in the presence of cervical secretions or fetal calf serum.

Compound (100 μg/μL)	Virus	Final $CCID_{50}$[a]	FCS[b] concentration during inactivation (%)	CS[c] dilution during inactivation	Residual infectivity (%), $M \pm$ S.D.[d]
P4	HIV-1^{IIIB}	100	0	0	0
		10	0	0	0
		100	10	0	9 ± 2
		10	10	0	100
		100	0	1:20[e]	0
		10	0	1:20	3 ± 2
P5	HIV-1^{IIIB}	100	0	0	0
		10	0	0	0
		100	10	0	0
		10	10	0	100
		100	0	1:20	0
		10	0	1:20	70 ± 35
P6	HIV-1^{IIIB}	100	0	0	0
		10	0	0	0
		100	10	0	6 ± 3
		10	10	0	100
		100	0	1:20	0
		10	0	1:20	0

[a] The $CCID_{50}$ value in the well after dilutions of the virus/compound mixture. Prior to the dilutions the virus titre was corresponding to 1×10^3 $CCID_{50}$.
[b] Fetal calf serum.
[c] Human cervical secretions.
[d] *M* represents the mean and ± the standard deviations (S.D.) for at least two independent experiments.
[e] CS was first diluted 10-fold in deionized water and in the inactivation assay mixed at a 1:2 ratio.

of H9 cells by HIV-1^{IIIB} and the HSV-2 333 strain. No p24 was detected in the supernatant 13 days post infection in any of the dilutions suggesting that the virus was fully inactivated in spite of the presence of HSV-2 333 (Table 4). Similarly, to address the question whether the compounds in the presence of HIV-1^{IIIB} would inactivate the HSV-2 333 strain, a plaque reduction assay was performed. When the cells were analyzed after 2 or 3 days, no HSV-2 plaques were formed in any of the dilutions (Table 4). These results indicate that during HSV-2 and HIV co-infection, these two viruses did not seem to interfere with the inactivating potency of any of the three compounds towards the co-infecting virus.

Table 4
Anti-HIV-1 and HSV-2 activity of selected test compounds in co-infected GMK and H9 cells.

Compound (100 μg/μL)	Virus	Cells	Final $CCID_{50}$ of HIV-1^{IIIB}[a]	Number of HSV-2 plaques, $M \pm$ S.D.[b]	Residual HIV-1 infectivity (%), $M \pm$ S.D.
P4	HIV-1^{IIIB} + HSV-2 333	GMK	100	0	
			10	0	
	HIV-1^{IIIB} + HSV-2 333	H9	100		0
			10		0
P5	HIV-1^{IIIB} + HSV-2 333	GMK	100	0	
			10	0	
	HIV-1^{IIIB} + HSV-2 333	H9	100		0
			10		0
P6	HIV-1^{IIIB} + HSV-2 333	GMK	100	0	
			10	0	
	HIV-1^{IIIB} + HSV-2 333	H9	100		0
			10		0
No compound	HIV-1^{IIIB} + HSV-2 333	GMK	100	115 ± 24	
			10	14 ± 2	
No compound	HIV-1^{IIIB} + HSV-2 333	H9	100		100
			10		9 ± 1
No compound	HSV-2 333	GMK	100	99 ± 13	
			10	7 ± 5	
No compound	HIV-1^{IIIB}	H9	100		100
			10		8 ± 12
P4	HSV-2 333	GMK	100	0	
			10	0	
P5	HSV-2 333	GMK	100	0	
			10	0	
P6	HSV-2 333	GMK	100	0	
			10	0	

[a] The $CCID_{50}$ value in the well after dilutions of the virus/compound mixture. Prior to the dilutions the HIV-1^{IIIB} virus titer was corresponding to 1×10^3 $CCID_{50}$ and the stock solution of HSV-2 333 was 200 pfu/ml.
[b] *M* represents the mean and ± the standard deviations (S.D.) for three independent experiments.

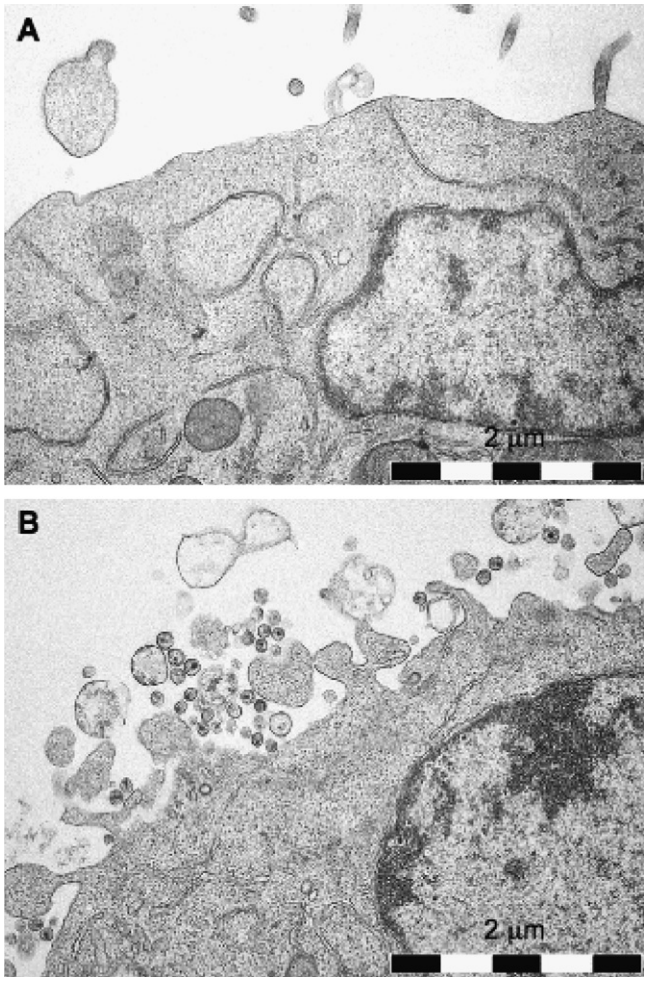

Fig. 3. Transmission electron microscopy (TEM) images of sections of HIV-1^{IIIB}-infected H9 cells. (A) Drug treated (compound P4, 100 µg/mL), HIV-1^{IIIB} infected H9 cell with visible outgrowths projecting from the cell surface. (B) Infected H9 cell untreated with compound P4. Virions are visible as clusters at the cell surface.

3.8. Transmission electron microscopy (TEM)

Analysis of TEM specimen sections revealed that there were no detectable virus particles of HIV-1^{IIIB} in the P4-treated samples (Fig. 3). In contrast, in the non-treated control sections, large numbers of viral particles were observed (Fig. 3). These results further support the HIV-inactivating capacity of the compound.

4. Discussion

To prevent sexual transmission of HIV-1, much effort has been directed towards development of effective microbicides for prophylactic use by vaginal application. Polyanions, often sulfated, have in several studies shown effect as inhibitors of HIV-1 infection in vitro and have therefore been suggested as potential microbicides (Pearce-Pratt and Phillips, 1996; Tao et al., 2007; Vzorov et al., 2007). However, the human clinical trials involving topical gel formulations of sulfated polysaccharides have been disappointing. In particular, a recent phase III trial of cellulose sulfate was prematurely terminated because of indications of increasing the risk of HIV infection (Tao et al., 2008). Furthermore, the sulfated polysaccharide carrageenan (Cohen, 2008) and polysulfonated compound PRO-2000 (Microbicides Development Program, 2009) have shown weak protection against transmission of HIV. In fact, some sulfated polymers such as dextran sulfate appear to alter the vaginal innate immune response and/or ulcerate mucous surface linings (Trifonova et al., 2009). Another possible explanation as to why sulfated polysaccharides have failed in clinical trials is that naturally occurring polyanions, such as HS are known to facilitate HIV entry in certain cell types at low concentrations (Moulard et al., 2000; Zhang et al., 2002). Thus, due to the difficulties of reaching a truly antiviral concentration in the vaginal tract of any microbicide formulation based on polyanions, the risk of transmission from a few remaining HIV-1 particles must be considered.

Here, in search of an effective microbicide, we have selected compounds that not only block HIV-1 entry but also inactivate the virus. We found that such an inactivating capacity could be achieved by linkage of short, sulfated carbohydrates to hydrophobic tails, the latter with potency of interacting with the lipid envelope of HIV. This virus inactivation was also seen against R5-tropic strains, which might be more representative for sexual transmission than the X4/R5 tropic strains often used in evaluation of microbicide potency. In addition to HIV-1, the lipophile-conjugated oligosaccharides outlined in this report showed inactivating activity on HSV-2, another enveloped virus that has a profound enhancing effect on sexual transmission of HIV-1 (Ekblad et al., 2010; Kapiga et al., 2007).

Of the four most promising conjugates, compound P3 and P4 had a lesser effect on the cell proliferation (CC_{50}) values than compounds P5 and P6. Because the latter two compounds differ from P3 and P4 by the presence of a triazole ring connecting the oligosaccharide chain with the cholestanyl group this structural feature might be responsible for the increased toxicity. Overall, substance P4 showed the highest SI values making it the most promising candidate for microbicide development.

As a possible mechanism of action, we suggest that the herein described HS mimetics P3, P4, P5 and P6, act by blocking the binding of HIV gp120 to cell surface GAGs such as HS. After the binding to CD4-molecules, gp120 interacts with cell surface HS through four domains on the V3 loop (Crublet et al., 2008; Mondor et al., 1998; Roderiquez et al., 1995; Vivès et al., 2005). Since the test compounds all have a much higher degree of sulfation than all cell surface GAG molecules including HS, these compounds could block infection through a higher binding affinity for the positively charged HS binding site of gp120. Earlier studies from us and others on entry blockers of HS-binding viruses have shown that the higher degree of sulfation of an oligosaccharide, the higher affinity for positively charged entities on the viral surface. Non-sulfated saccharides, such as the muparfostat precursor component molecule PM_5, are devoid of antiviral activity (Nyberg et al., 2004; Witvrouw and De Clercq, 1997). However, direct binding assays of the here presented compounds to gp120 is an essential requirement in order to clarify their mechanism of action, and such experiments are under way.

The herein suggested mechanism of action as an entry blocker was supported by kinetic experiments where we wanted to define whether the compounds were active against attachment/entry of the virus, or after entry, or if it affected host cells. Here, we selected compound P3 as a model, being the first molecule in the library to be demonstrated to possess virucidal properties against the laboratory strain HIV-1^{IIIB}. Pre-treatment of HIV-1^{IIIB} with compound P3 inhibited viral entry, which supported the idea that the molecule blocked viral attachment to the cell surface through binding to the virus. Further support of this was given when cells were treated after viral attachment resulting in only partial blocking of infection. Most interestingly, if the compound was added after viral entry and was present throughout the experiment, no viral replication could be found, suggesting that budding virions were blocked from infecting new cells in a second replication cycle in the presence of the drug.

With respect to the virus-inactivating (virucidal) activity, compound P3 was previously found in our laboratory to inactivate

HSV-2. This stems from the observation that when the mixture of compound P3 and HSV-2 was diluted to non-inhibitory compound concentrations and thereafter added to GMK AH1 cells, no residual infectivity remained (Ekblad et al., 2010). In the present study, inactivation of both HSV-2 in the presence of HIV-1^{IIIB} in GMK AH1 cells and HIV-1^{IIIB} in the presence of HSV-2 333 in H9 cells was shown to occur. Thus, the compound P3 seemed to have the ability to inactivate both these viruses, suggesting that P3 could target, in addition to the HS-binding site of gp120, the virus lipid envelope most likely through its lipophilic cholestanol tail. Although the mechanisms responsible for this inactivating activity remain to be elucidated, including eventual effects also on cellular membranes, it is interesting to note that we failed to detect any structured HIV particles by TEM after the incubation of HIV-1^{IIIB} with P4.

Protein-rich additives in the cell growth medium, such as FCS, substantially decreased the virucidal capability of compounds P4, P5 and P6 when present at high concentration. When two clinical HIV-1 isolates, R5 and X4/R5 were assayed for inactivation with compounds P4, P5 and P6, the former virus was more efficiently inactivated than the latter at the 100-fold dilution of virus stock. One possible explanation is that the stock of X4/R5 virus, which had a 10-fold lower infectious titer and therefore 10-fold higher concentration of FCS than the R5 virus was somehow better protected against inactivation by the compounds tested. However, attempts to diminish this negative effect of host proteins should be performed through molecular modifications of compounds such as P4.

It has been reported that the net charge of the HIV-1 gp120 V3 loop of R5-binding viruses increases throughout the course of infection by selection of cationic amino acid substitutions in gp120 which leads to a higher positive viral charge and seems to drive the co-receptor switch from CCR5 to CXCR4 (Pollakis et al., 2001). In addition, another study indicated that in a host where co-receptor switching does not seem to occur and where CCR5-using viruses dominate through the end-stage of the disease (Borggren et al., 2008), mutations in proximity to the V2 and V4 loops leading to an increased positive net charge of gp120 also emerged (Repits et al., 2008). These end-stage viruses were associated with reduced sensitivity to entry inhibitors, such as T-20 and TAK-779 (Repits et al., 2005). In contrast, we suggest that the use of strong anionic compounds in the present study might be especially efficient for such viruses with increased positive charge of gp120, and ongoing experiments are addressing this issue.

Herein, we describe a novel set of compounds of amphipathic character, combining a highly sulfated oligosaccharide with a lipophilic tail, which show potency as virucidals against both HIV-1 and HSV-2 and that are promising candidate molecules as microbicides for HIV prevention with a broad activity against both CCR5- and CXCR4-tropic strains. A decrease of inactivation in the presence of serum proteins needs to be abrogated by adjustments in the molecular structure, although this inhibition was less emphasized when using cervical secretion as additive. The herein reported simultaneous inactivation of both HIV and HSV-2 virions by single compounds warrants further development of this group of molecules including elucidation of their mechanism of action.

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