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A unique class of lignin derivatives displays broad anti-HIV activity by interacting with the viral envelope

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ABSTRACT

In Gordts et al. (2015), we have shown that lignosulfonic acid, a commercially available lignin derivative, possesses broad antiviral activity against human immunodeficiency virus (HIV) and Herpes simplex virus (HSV) by preventing viral entry into susceptible target cells. Because of the interesting safety profile as potential microbicide, we now determined the antiviral activity of a series of lignosulfonates in order to understand better which molecular features can contribute to their antiviral activity. Here, 24 structurally different lignosulfonates were evaluated for their capacity to inhibit HIV and HSV transmission and replication in various cellular assays. These derivatives differ in origin (hardwood or softwood), counter-ion used during sulphite processing (Na^+, Ca^{2+}, or NH_4^+), sulphur content, carboxylic acid percentage, and molecular weight fraction, which allowed to determine structure-activity relationships. We demonstrate that the broad antiviral activity of lignosulfonates is mainly dependent on their molecular weight and that their mechanism of action is based on interactions with the viral envelope glycoproteins. This makes the lignosulfonates a potential low-cost microbicide that protects women from sexual HIV and HSV transmission and thus prevents life-long infection.

1. Introduction

More than 30 years after its discovery, human immunodeficiency virus (HIV) is still a major health problem. According to UNAIDS data, an estimated 36.9 million people (including 1.8 million children) were living with HIV in 2017. Although incidence has decreased in the last decade, almost 1 million people died of AIDS-related illnesses in 2017. Moreover, for women of reproductive age (15–49), it is one of the main causes of death globally (WHO data). The vast majority of people living with HIV are from low- and middle- income countries,19.6 million people are living in eastern and southern Africa, where 800 000 new HIV infections occurred in 2017 (UNAIDS). Women in sub-Saharan Africa have a higher risk of becoming HIV-infected, because of biological, structural and social reasons (Antimisiaris and Mourtas, 2015; Dellar et al., 2015). Examples are increased genital inflammation due to the more vulnerable female genital tract, intergenerational relationships, man-controlled communities, polygamy, and lack of schooling. Furthermore, current preventive measures like condom use and male circumcision are mainly under control of men. This underscores that efforts are needed to increase the number of measures that can be controlled by women. As long as an adequate HIV vaccine does not exist, research should still focus on the development of other preventive mechanisms.

Microbicides are self-administered topical drugs that, when applied vaginally or in the rectum, prevent transmission of HIV and possibly other sexually transmitted infections (STI). Implemented in pre-exposure prophylaxis (PrEP) they have great potential to protect women. Microbicides have several requirements: next to efficacy on the long term they also have to be safe, affordable to at-risk populations, and easy to apply (Shattock and Rosenberg, 2012). Drugs that have been considered as microbicides cover a wide array of origins: surfactants, acid-buffering gels, polyanionic compounds, and antiretroviral drugs (Antimisiaris and Mourtas, 2015). Currently, microbicide research is also focused on compounds such as polyanionic dendrimers (Sepulveda-Crespo et al., 2016) and on development of user-friendly application methods (Machado et al., 2016; Malcolm et al., 2016). Several antiretroviral microbicides are under clinical investigation (Benítez-Gutiérrez et al., 2018; Hoesley et al., 2018; McBride et al., 2019).

Naturally occurring compounds are an attractive source for the development of novel antivirals (Grice and Mariottini, 2018; Linnakoski

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et al., 2018). They possess several advantages such as reduced side effects, increased cost-efficiency, higher bioavailability, and various modes of action. Furthermore, their high diversity and chemical variability could contribute to overcome problems with drug-resistant viral strains (Hassan et al., 2015). Lignin is the second most abundant natural polymer, after cellulose Boerjan et al., 2003). It is a class of cross-linked phenolic polymers that confers structural integrity to plants. Until now, lignin is highly underutilized. 98% of the lignin isolated in the pulping industry is burned as a low-value fuel (Upton and Kasko, 2016). Because of their abundance, low cost and sustainability, lignins are attractive sources of novel applications in times of environmental problems. Recent studies indicate this application potential, such as conversion into renewable chemicals as biofuel, carbon fiber and activated carbon (Bugg and Rahmanpour, 2015; Chatterjee and Saito, 2015; Upton and Kasko, 2016) and thermoplastics (Wang et al., 2016).

Lignin derivatives are generated during the processing of lignocelluloses in the paper industry or bioethanol production. One of the employed methods is sulphite pulping. In the course of this process lignin is extracted from the wood using salts of sulfurous acid. This process leads to the formation of lignosulfonic acid (LA) – a lignosulfonate – as byproduct (Vishtal and Kraslawski, 2011) (Fig. 1). Due to their dispersing, binding, complexing, and emulsifying properties, lignosulfonates have been utilized for several decades. At the end of the 19th century, it was used as a tanning agent (May, 1879). Furthermore, it has several other applications such as protection agent of protein degradation (Mansfield and Stern, 1994; Windschitl and Stern, 1988), pellet binder in animal food (Acar et al., 1990), binding agent in phenolic resins (Alonso et al., 2005), plasticizer in cement (Grierson et al., 2005), and use in detergents, glues and surfactants (Vishtal and Kraslawski, 2011).

In Gordts et al. (2015), we have shown that a commercially available lignosulfonate (LA^{low}) possesses broad antiviral activity against HIV and Herpes simplex virus (HSV) by preventing viral entry into susceptible target cells. Because of these interesting properties, we determined the antiviral activity profiling of a series of lignosulfonates in order to deduce which molecular features contribute to the antiviral activity and to discover compounds with potential higher antiviral potency. Here, we evaluated 24 structurally different lignosulfonates and defined their capacity to inhibit viral (HIV and HSV) transmission and replication in several cell-based assays. These lignin derivatives differ in origin (hardwood or softwood), counter-ion used during sulphite processing (Na^+, Ca^{2+}, or NH_4^+), sulphur content, carboxylic acid percentage, and molecular weight fraction, which allows to determine structure-activity relationships. We demonstrate that the antiviral activity of lignosulfonates is dependent on their molecular weight, and that their mechanism of action is based on interactions with viral envelope glycoproteins.

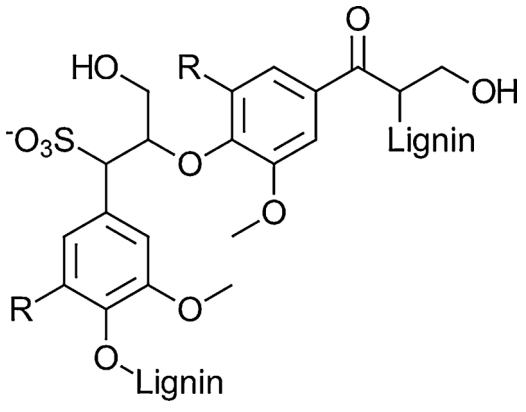

Fig. 1. Schematic structure of a lignosulfonate dimer. For softwood R = H, for hardwood R = H or CH_3 in approximately 1:1 ratio.

2. Materials and methods

2.1. Cell lines, plasmids and virus strains

Human CD4$^+$ T-lymphocytic SupT1 cells, C8166 cells, HuT-78 cells, HEp-2 cells, HeLa cells, Vero cells and human embryonic lung (HEL) fibroblasts were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). HIV-1 IIIB and HIV-1 NL4.3 persistently infected HuT-78 cells were obtained by infecting HuT-78 cells (HuT-78/IIIB and HuT-78/NL4.3, respectively). Human CD4$^+$ T-lymphocytic MT-4 cells were a kind gift from Dr. L. Montagnier (formerly at the Pasteur Institute, Paris, France). The human B-lymphoma cell line Raji transfected with DC-SIGN (Raji$^{DC\text{-}SIGN}$) was obtained from Dr. L. Burleigh (Pasteur Institute). These cells were cultured in RPMI-1640 medium (Thermo Fisher, Gent, Belgium) containing 10% fetal calf serum (FCS; Thermo Fisher) and 2 mM L-glutamine (Thermo Fisher). TZM-bl cells were a kind gift from Dr. G. Vanham (ITG, Antwerp, Belgium). This cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher) supplemented with 10% FCS and 0.01 M HEPES buffer (Thermo Fisher). All cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. They were subcultivated every 2 to 3 days. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors (Red Cross, Leuven), by density gradient centrifugation. They were cultured in RPMI-1640 medium supplemented with 10% FCS and 2 mM L-glutamine and stimulated with 2 μg/ml of the mitogenic phytohemagglutinin (PHA; Sigma-Aldrich) for 3 days at 37 °C before their further use in antiviral assays. Plasmids pQCXIP-AcGFP, pCG-gagpol, and pCF-VSVG were kindly provided by Dr. L. Naesens. The HIV-1 strains NL4.3 (X4), IIIB (X4) and BaL (R5) were originally obtained from the National Institute of Allergy and Infectious Disease AIDS Reagent Program (NIAID). The dual-tropic HIV-1 strain HE (R5/X4) was originally isolated in the University Hospitals and Rega Institute (Leuven, Belgium) from an AIDS patient and later on propagated in various CD4$^+$ T cell lines. The HIV-2 strain ROD (R3/R5/X4) was obtained from the Medical Research Council (MRC; London, UK). The HSV-1 strain KOS and the HSV-2 strain G were used as reference laboratory HSV strains. Vesicular stomatitis virus (VSV) and Zika virus (ZIKV) strain MR766 were obtained from ATCC (Manassas, VA, USA). Laboratory-adapted dengue virus serotype 2 (New Guinea C, NGC, DENV-2) was kindly provided by Dr. V. Deubel (Institut Pasteur, Paris, France). All viruses were obtained and used as approved according to the rules of Belgian equivalent of IRB (Departement Leefmilieu, Natuur en Energie, protocol SBB 219 2011/0011n and the Biosafety Committee at the KU Leuven).

2.2. Test compounds

The polyanionic compounds LA (sodium salt) (MW: ca. 8 000 g/mol and ca. 52 000 g/mol, further referred to as LA^{low} and LA^{high}, respectively) were obtained from Sigma-Aldrich (Diegem, Belgium). Twenty-four lignosulfonates (LA01-LA24) were provided by Borregaard LignoTech (Sarpsborg, Norway). The polyanionic compounds dextran sulfate (DS 5000 and DS 10,000; MW: ca. 5000 g/mol and 10,000 g/mol, respectively) were purchased from Sigma-Aldrich, and PRO 2000 (MW: ca. 5000 g/mol) was kindly provided by Dr. A.T. Profy (formerly at Indevus Pharmaceuticals Inc., Lexington, MA, USA). Maraviroc (MW: 514 g/mol) and AMD3100 (MW: 830 g/ml) were donated by Dr. G. Bridger (formerly at AnorMed, Langley, Canada). Acyclovir (MW: 225 g/mol) was obtained from GlaxoSmithKline (Brentford, UK). Enfuvirtide (T20; MW: 4.5 kDa) was kindly provided by Dr. E. Van Wijngaerden (University hospitals, Leuven, Belgium). The anti-gp120 mAb 2G12 was purchased from Polymun Scientific (Vienna, Austria). Tenofovir (MW: 287 g/mol) was obtained from Gilead Sciences (Foster City, CA, USA). AZT was purchased from Tocris (Abingdon, UK).

2.3. Anti-viral cellular assays

Three different anti-viral cellular screening assays were performed in our laboratory.

First, a colorimetric antiviral assay was performed, which was originally described by Pauwels et al. (1988). We adapted the protocol as described in detail in Van Hout et al. (2017). Briefly, $CD4^+$ $CXCR4^+$ MT-4 cells (1×10^6 cells/ml; 50 µl) or HEL cells were seeded in cell culture medium in a 96-well plate (Falcon, BD Biosciences, Erembodegem, Belgium) and pre-incubated with 5-fold dilutions of test compound (100 µl) at 37 °C for 30 min, in duplicate. Virus (50 µl; HIV strains NL4.3, IIIB, HE, or ROD, HSV-1 strain KOS and HSV-2 strain G) was added according to cell culture infectious dose ($CCID_{50}$) which was determined by titration of the stock. After an incubation period of 5 days, virus-induced cytopathic effect (CPE) was scored with light microscopy and IC_{50} was calculated using the spectrophotometric MTS/PES viability staining assay (Cell-Titer96 Aqueous One Solution Proliferation Assay kit; Promega, Leiden, The Netherlands). Absorbance was measured using the Versamax microplate reader and SoftMax Pro software (Molecular Devices, Sunnyvale, Ca, USA). To determine the compounds potential cellular cytotoxicity the assays were performed without the addition of virus. The reference compounds AMD3100, maraviroc or acyclovir were included as controls in all assays.

Second, a luminescence-based antiviral assay was carried out, as described in detail in Montefiori (2009). $CD4^+$ $CXCR4^+$ $CCR5^+$ TZM-bl cells are transfected to contain the reporter gene for firefly luciferase under control of the HIV promotor. Cells (3.4×10^5 cells/ml) were pre-incubated with serial dilutions of test compound in duplicate, as described above. Cell culture medium was supplemented with 15 µg/ml diethylaminoethyl-dextran (DEAE-Dextran; Sigma-Aldrich) to facilitate infection. Next, virus (HIV-1 NL4.3 or BaL) was added according to the $CCID_{50}$ of the stock. After an incubation period of 2 days, viral replication was measured by luminescence. Steadylite Plus reagent (Perkin Elmer, Zaventem, Belgium) and lyophilized substrate were mixed according to the manufacturer's guidelines. Supernatant was removed from the plates and 75 µl of the mix solution was added. After 10 min incubation in the dark in a plate shaker, 100 µl was transferred to white 96-well plates (Greiner Bio-One, Frickenhausen, Germany). To determine viral replication, relative luminescence units were measured using the SpectraMax L microplate reader (Molecular Devices) and SoftMax Pro software with an integration time of 0.6 s. and dark adaptation of 5 min.

Finally, an HIV-1 p24 Ag ELISA-based antiviral assay was performed in PBMCs, as described previously (Schols et al., 1997). PHA-stimulated PBMCs (5×10^5 cells/ml; 200 µl) were seeded in 48-well plates (Costar, Elscolab NC, Kruibeke, Belgium) and pre-incubated for 30 min with various concentrations of test-compound (250 µl) in the presence of 2 ng/ml interleukin 2 (IL-2) (Roche Applied Science, Vilvoorde, Belgium). Next, virus (HIV-1 NL4.3 and BaL) was added and 2 ng/ml IL-2 was supplemented again at day 3 and 6 after infection. After 10 days of infection, supernatant was collected and stored. Viral replication was determined by HIV-1 p24 Ag ELISA (Perkin Elmer), according to the manufacturer's instructions.

2.4. Time-of-drug addition assay

To determine at what time point the test compounds intervene in the viral life cycle, we performed a time-of-drug addition assay as described previously (Daelemans et al., 2011). Briefly, MT-4 cells (5×10^5 cells/ml) were infected with HIV-1 NL4.3 (to reach an MOI of 0.5) and seeded in a 96-well culture plate and incubated at 37 °C. Test compounds (LA02, LA13, LA14, LA20, LA^{low}, and the reference anti-HIV compounds with a specific mode of action i.e. PRO 2000, tenofovir and AZT) were diluted to 1000-fold their IC_{50} in cell culture medium. At defined time points after infection, the compounds were added to the cell cultures. Thirty-one hours after infection, cell cultures were examined light microscopically for CPE and supernatant was collected. We performed HIV-1 p24 Ag ELISA to determine the level of HIV-1 infection and replication.

2.5. HIV-1 gp120 cellular binding assay

This gp120 binding assay was described in detail by Gordts et al. (2015). $CD4^+$ SupT1 cells (2×10^5) were seeded in a 48-well plate and incubated with dilutions of test compound and HIV-1 NL4.3 stock (2×10^5 pg of HIV-1 p24 Ag). After an incubation time of 2 h at 37 °C, cells were transferred to 15 ml conical polypropylene tubes (Falcon, BD) and thoroughly washed 2 times with 12 ml PBS/FCS 2% solution. Then the cells were transferred to 5 ml polypropylene tubes and virus binding was detected using mouse monoclonal anti-gp120 Ab NEA-9205 (diluted 1/50; NEN, Boston, MA, USA). After 30 min incubation time, cells were thoroughly washed and PE-conjugated Goat-anti-Mouse mAb was added (GaM-PE; diluted 1/100; Biolegend, ImTec Diagnostics NV, Antwerp, Belgium). After an additional incubation time of 30 min, cells were washed and fixed with a 1% paraformaldehyde solution. Aspecific binding on the T cells was determined using GaM-PE only. Inhibition of gp120 binding by the test compounds was analyzed by flow cytometry (Accuri C6, BD) and FlowJo software (Tree Star, San Carlos, CA, USA).

2.6. HSV-2 gD antibody cellular binding assay

MT-4 cells, which express the HSV receptors nectin-1 and nectin-2, were infected with HSV-2 strain G. Four days after infection, cells (3×10^6) were transferred to 5 ml polypropylene tubes and incubated with dilutions of test compound for 1 h at 37 °C. Virus binding was detected using mouse monoclonal anti-HSV-1 + HSV-2 gD Ab (clone 2C10, diluted 1/50; Abcam, Cambridge, UK). After 30 min incubation time, cells were thoroughly washed and Alexa Fluor 488-conjugated Goat-anti-Mouse mAb was added (GaM-AF488; diluted 1/200; Abcam). After an additional incubation time of 30 min, cells were washed and fixed with a 1% paraformaldehyde solution. Aspecific binding on the T cells was determined using GaM-AF488 only. Inhibition of HSV gD mAb binding by the test compounds was analyzed by flow cytometry and FlowJo software.

2.7. HIV giant cell co-culture assay

This assay was performed as described by Férir et al. (2012). Briefly, $CD4^+$ $CD8^+$ SupT1 cells are very susceptible to virus-induced giant cell formation when co-cultured with persistently HIV-infected HuT-78 T cells. Test compounds (5-fold dilutions) were added to a 96-well culture plate and incubated with an equal mix of SupT1 (1×10^5 cells) cells and persistently infected $HLA\text{-}DR^+$ HuT-78 cells (HuT-78/IIIB or HuT-78/NL4.3) at 37 °C for 24 h. Afterwards, giant cell formation was scored light microscopically and cells were stained with specific mAbs to accurately calculate IC_{50} values. Samples were incubated with FITC-conjugated anti-CD8 (clone SK1, diluted 1/50, BD) and PerCP-Cy5.5-conjugated HLA-DR (clone L243, diluted 1/50, BD) mouse monoclonal antibodies to distinguish between both cell populations ($HLA\text{-}DR^+$ HuT-78 cells and $CD8^+$ SupT-1 cells). After 30 min incubation, cells were washed with PBS and fixed with 1% paraformaldehyde solution. Samples were analyzed by flow cytometry and IC_{50} values were determined using FlowJo software.

2.8. DC-SIGN capture/transmission assay

Cells expressing DC-SIGN are described to capture and transmit HIV to $CD4^+$ target cells. This can be experimentally simulated in capture/transmission assays, as published previously (Balzarini et al., 2007). In the capture assay, test compound was diluted in cell culture medium in 15 ml conical polypropylene tubes and incubated with HIV-1 HE stock

(100,000 pg) at 37 °C for 30 min. Raji$^{DC\text{-}SIGN}$ cells (5×10^5 cells) were added and incubated at 37 °C for 1.5 h. Afterwards, cells were washed thoroughly in cell culture medium and then resuspended in a triton X-100 solution and viral presence was quantified by HIV-1 p24 Ag ELISA.

In the transmission assay, Raji$^{DC\text{-}SIGN}$ cells (5×10^5 cells) were incubated with HIV-1 HE stock (100 000 p of p24 HIV-1 Ag) at 37 °C for 1.5 h. Afterwards, cells were washed thoroughly and resuspended in cell culture medium (2×10^6 cells/ml). Test compounds were diluted in a 96-well plate and incubated for 30 min with CD4$^+$ CXCR4$^+$ CD25$^+$ C8166 cells (2×10^6 cells/ml). Then, the virus-exposed Raji$^{DC\text{-}SIGN}$ cells were added. After an incubation period of 48 h, all wells were scored microscopically for syncytium formation and samples were washed and stained with PE-conjugated anti-CD25 (clone 2A3, diluted 1/50, BD) and FITC-conjugated anti-DC-SIGN (clone DCN46, diluted 1/50, BD) mAbs to distinguish the cell populations. Aspecific binding was determined using SimulTest control (IgGγ1-FITC/IgGγ2a-PE; BD). After fixation in 1% paraformaldehyde solution, samples were analyzed by flow cytometry to determine the IC_{50} values.

2.9. Antiviral assays with several resistant HIV strains

LA^{low} and LA^{high} resistant NL4.3 HIV-1 were obtained by passaging the virus in increasing concentrations of the respective compound in MT-4 cells. Every 5 days, virus-induced CPE was scored light microscopically and when full CPE was observed, cell supernatant was used to infect the next passage of cells. After 85 and 100 passages respectively, the virus was able to replicate in the presence of 90 μg/ml LA^{high} (1.7 μM) and LA^{low} (11.3 μM). This virus was collected by centrifugation, RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and the *env* gene was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit to determine relevant mutations compared to wild-type virus. Reactions were run on an ABI3100 Genetic Analyzer (Applera, Nieuwerkerk a/d Ijssel, The Netherlands). The sequences were analyzed using Sequence Analysis version 3.7 and SeqScape version 2.0 (Applera). Antiviral assays with resistant viruses were performed in MT-4 cells as described above. Additionally, we performed antiviral assays with HIV-1 strains that were generated resistant to several well-defined entry inhibitors such as AMD3100, PRO 2000, DS 5000, 2G12 mAb and T20. These resistant virus strains were characterized in earlier publications (de Vreese et al., 1996; Esté et al., 1997; Huskens et al., 2007).

2.10. Surface plasmon resonance (SPR) analysis

SPR technology was used to determine the binding of gp120 and gp41 with lignosulfonates. Recombinant gp120 HIV-1 strain IIIB (ImmunoDiagnostics Inc., Woburn, MA), recombinant gp41 HIV-1 strain HxB2 (Acris Antibodies GmbH, Herford, Germany) and human serum albumin (Sigma) were covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip in 10 mM sodium acetate, pH 4.5 (gp120) or pH 5 (gp41 and HSA), using standard amine coupling chemistry. A reference flow cell was used as a control for non-specific binding and refractive index changes. Interaction studies were performed at 25 °C on a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden) in HBS-P + (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20; pH 7.4). Lignosulfonates were serially diluted, covering a concentration range between 10,000 and 1.5 nM by using 3-fold dilution steps. Samples were injected for 2 min at a flow rate of 30 μl/min and the dissociation was followed for 4 min. The sensor chip surface was regenerated with 50 mM NaOH and stabilized for another 2 min. Several buffer blanks were used for double referencing. Binding affinities (K_D) were derived after fitting the experimental data to the 1:1 binding model in the Biacore T200 Evaluation Software 2.0. Because of a large deviation from the 1:1 binding model at higher LA concentrations, only the lower concentrations were used in the fitting procedure and the apparent K_D values are reported.

2.11. Antiviral assay with VSVG-HIV-1 pseudovirus

Pseudovirus was produced using plasmids encoding GFP (pQCXIP-AcGFP), VSV envelope (pCF-VSVG) and HIV vector (pCG-gagpol). They were co-transfected into HEp-2 cells using FuGENE HD transfection reagent (Promega) according to the manufacturer's protocol. After 24 h incubation, medium was replaced by fresh transfection medium containing 1 mM sodium butyrate (Sigma-Aldrich). 48 h post-transfection, supernatant containing VSVG-HIV-1 pseudovirus was collected, centrifuged and used for transduction. HeLa cells (1.5×10^4 cells/well) were seeded in a 96 well plate and after overnight incubation, cells were incubated for 15 min with various concentrations of lignosulfonates. Cells were then transduced with VSVG-HIV-1 pseudovirus. Viral infection was quantified microscopically, 48 h after transduction. As a control, antiviral activity of lignosulfonates was also determined against replication competent VSV.

3. Results

3.1. Lignosulfonates with broad anti-HIV and anti-HSV activity

Twenty-four lignosulfonates with varying molecular properties were evaluated (Table 1). Inhibition of viral replication by these compounds was tested in two well-described cellular assay systems (MT-4 cells and TZM-bl cells) against two HIV-1 strains and one HSV-2 strain (Table 2). The antiviral activity between the compounds varied by 100- to even 1000-fold. Overall, compounds LA06, LA07, LA08, LA09, LA13, LA14, and LA20 showed the most prominent antiviral activity in these assays (IC_{50}: 10–300 nM), while LA04 showed the weakest inhibitory effect (IC_{50}: 30.0–236.6 μM). The compounds had a more prominent activity towards HIV replication inhibition when compared to HSV, but the best-rated compounds still have anti-HSV IC_{50} values in the low nanomolar range (70–300 nM).

Fig. 2 shows that the antiviral activity of the lignosulfonates in the different screening assays correlate with each other (Fig. 2A,B), and with their molecular weight (Fig. 2C). We also observed a weak but statistically significant correlation between the antiviral activity and

Table 1
Chemical characteristics of 26 lignin-derived compounds used in the study.

Compound	MW (g/mol) [a]	Counter ion [a]	Raw material [a]	Organic sulfur (%) [a]	COOH (%) [a]
LA01	6300	Sodium	Softwood	1.8	15.1
LA02	6300	Sodium	Softwood	1.8	0
LA03	8000	Sodium	Softwood	1.6	ND
LA04	900	Sodium	Softwood	2.0	ND
LA05	40,000	Sodium	Softwood	5.7	9.0
LA06	58,100	Sodium	Softwood	5.6	6.0
LA07	58,100	Sodium	Softwood	5.6	0
LA08	60,000	Sodium	Softwood	5.8	8.8
LA09	54,900	Sodium	Softwood	7.1	6.9
LA10	6700	Sodium	Hardwood	4.6	10.0
LA11	6600	Calcium	Hardwood	4.3	13.9
LA12	41,000	Calcium	Softwood	5.0	8.2
LA13	58,000	Calcium	Softwood	5.4	6.5
LA14	82,500	Calcium	Softwood	5.5	6.5
LA15	12,500	Calcium	Hardwood	5.6	10.6
LA16	10,400	Calcium	Hardwood	4.8	8.6
LA17	6500	Calcium	Hardwood	4.3	14.3
LA18	40,000	Ammonium	Softwood	5.0	ND
LA19	28,000	Ammonium	Softwood	5.0	ND
LA20	58,000	Ammonium	Softwood	5.5	6.3
LA21	26,000	Sodium	Softwood	9.4	9.6
LA22	16,100	Sodium	Softwood	10.5	9.6
LA23	11,000	Calcium	Hardwood	4.7	ND
LA24	16,500	Calcium	Hardwood	3.3	ND
LA^{high}	52,000	Sodium	Softwood	6.3	6.2
LA^{low}	8000	Sodium	Softwood	5.6	6.5

[a] Characterized by Borregaard LignoTech; ND: not determined.

Table 2
Antiviral activity of the evaluated lignosulfonates.

	IC_{50} (μM) [a]				CC_{50} (μM) [b]	
	MT-4		TZM-bl		MT-4	TZM-bl
	HIV-1 NL4.3	HSV-2	HIV-1 NL4.3	HIV-1 BaL		
LA01	0.2 ± 0.03	3.9 ± 0.7	0.4 ± 0.1	0.6 ± 0.02	> 40.0	> 40.0
LA02	0.8 ± 0.08	6.4 ± 1.2	0.5 ± 0.06	0.7 ± 0.06	24.0 ± 1.2	25.0 ± 2.4
LA03	0.1 ± 0.02	2.1 ± 0.3	0.2 ± 0.05	0.4 ± 0.04	26.5 ± 2.6	> 31.0
LA04	30.0 ± 0.9	236.6 ± 26.2	73.2 ± 0.01	104.8 ± 0.01	> 278.0	> 278.0
LA05	0.05 ± 0.02	0.6 ± 0.04	0.09 ± 0.02	0.1 ± 0.03	> 6.0	> 6.0
LA06	0.02 ± 0.001	0.2 ± 0.06	0.05 ± 0.01	0.07 ± 0.02	> 4.5	> 4.5
LA07	0.02 ± 0.002	0.3 ± 0.02	0.04 ± 0.02	0.09 ± 0.02	> 4.5	> 4.5
LA08	0.02 ± 0.002	0.3 ± 0.03	0.06 ± 0.01	0.07 ± 0.01	> 4.0	> 4.0
LA09	0.02 ± 0.001	0.2 ± 0.07	0.06 ± 0.003	0.09 ± 0.001	> 4.5	> 4.5
LA10	0.7 ± 0.07	10.7 ± 1.7	1.7 ± 0.3	3.2 ± 0.3	> 38.0	> 38.0
LA11	0.9 ± 0.04	9.6 ± 3.8	0.8 ± 0.2	3.0 ± 0.4	> 38.0	> 38.0
LA12	0.05 ± 0.01	0.5 ± 0.02	0.1 ± 0.02	0.3 ± 0.08	> 6.0	> 6.0
LA13	0.02 ± 0.004	0.1 ± 0.02	0.05 ± 0.003	0.07 ± 0.004	> 4.5	> 4.5
LA14	0.01 ± 0.007	0.07 ± 0.004	0.04 ± 0.004	0.05 ± 0.002	> 3.0	> 3.0
LA15	0.5 ± 0.05	9.3 ± 0.8	0.8 ± 0.2	2.3 ± 0.2	> 20.0	> 20.0
LA16	0.6 ± 0.08	8.2 ± 0.8	1.1 ± 0.2	2.3 ± 0.08	> 24.0	> 24.0
LA17	0.7 ± 0.04	11.6 ± 4.6	1.2 ± 0.1	3.2 ± 0.2	> 38.5	> 38.5
LA18	0.04 ± 0.01	0.5 ± 0.01	0.1 ± 0.02	0.2 ± 0.01	> 6.0	> 6.0
LA19	0.08 ± 0.04	0.8 ± 0.03	0.2 ± 0.06	0.4 ± 0.1	> 9.0	> 9.0
LA20	0.02 ± 0.003	0.2 ± 0.04	0.05 ± 0.02	0.08 ± 0.005	> 4.5	> 4.5
LA21	0.02 ± 0.007	0.6 ± 0.02	0.2 ± 0.02	0.3 ± 0.04	> 9.5	> 9.5
LA22	0.07 ± 0.01	1.3 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	> 15.5	> 15.5
LA23	0.2 ± 0.03	1.2 ± 0.5	0.2 ± 0.04	0.6 ± 0.01	> 22.5	> 22.5
LA24	0.09 ± 0.02	1.3 ± 0.06	0.4 ± 0.02	1.2 ± 0.1	> 15.0	> 15.0
LA^{low}	0.1 ± 0.04	1.1 ± 0.5	0.4 ± 0.03	0.5 ± 0.09	> 31.3	> 31.3
LA^{high}	0.03 ± 0.01	0.09 ± 0.02	0.06 ± 0.01	0.1 ± 0.02	> 4.8	> 4.8

[a] Compound concentration required to inhibit viral replication by 50%. Antiviral activity was determined in MT-4 cells against HIV-1 NL4.3 (X4) and HSV-2 G and in TZM-bl cells against HIV-1 NL4.3 and HIV-1 BaL (R5) replication. Mean ± SEM of three independent experiments is shown.
[b] Compound concentration required to decrease cell viability with 50%. Cellular cytotoxicity was determined in MT-4 cells and in TZM-bl cells.

organic sulfur content (Fig. 2D). A significant correlation between carboxylic acid content and antiviral activity was not detected.

The cellular cytotoxicity of the lignosulfonates was also evaluated in $CD4^+$ MT-4 and TZM-bl cells. Most compounds did not show any cellular cytotoxicity, except for LA02 and LA03 (Table 2). However, their selectivity index in MT-4 cells against HIV-1 NL4.3 replication was 30 and 189, respectively. When we included a washing step after incubation of the cells with the compound, and thus removed the

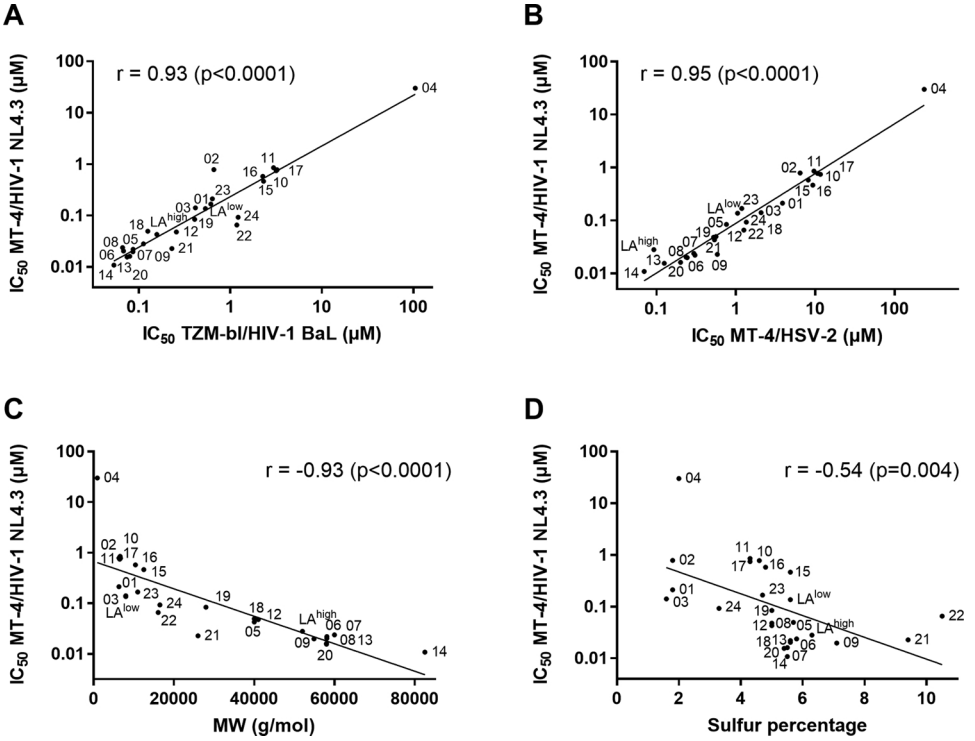

Fig. 2. IC_{50}s of lignosulfonates correlate with each other and with molecular weight and sulfur content. Correlation of the IC_{50} value of the compounds for inhibition of HIV-1 NL4.3 replication in MT-4 cells with (A): their IC_{50} for inhibition of HIV-1 BaL in TZM-bl cells ($p < 0.0001$), (B): their IC_{50} for inhibition of HSV-2 in MT-4 cells ($p < 0.0001$), (C): their molecular weight ($p < 0.0001$), (D): their sulfur content ($p = 0.004$). Correlations were calculated in Graphpad using the Spearman correlation test, except for (D) where Pearson correlation test was used (p-values < 0.05 were considered as statistically significant).

Table 3
Antiviral activity profile of selected lignosulfonates.

	IC_{50} (μM) [a]				
	MT-4			PHA-blasts	
	HIV-1 IIIB	HIV-1 HE	HIV-2 ROD	HIV-1 NL4.3	HIV-1 BaL
LA02	1.2 ± 0.3	1.2 ± 0.3	2.5 ± 0.09	0.8 ± 0.2	4.5 ± 1.5
LA04	58.8 ± 9.2	38.9 ± 5.6	28.1 ± 6.8	43.6 ± 24.1	46.0 ± 8.5
LA13	0.02 ± 0.007	0.03 ± 0.003	0.04 ± 0.004	0.02 ± 0.009	0.06 ± 0.02
LA14	0.02 ± 0.005	0.02 ± 0.004	0.03 ± 0.002	0.02 ± 0.004	0.05 ± 0.02
LA20	0.04 ± 0.009	0.04 ± 0.004	0.05 ± 0.005	0.01 ± 0.006	0.06 ± 0.02
LA^{low}	0.2 ± 0.03	0.2 ± 0.002	0.3 ± 0.01	0.1 ± 0.05	0.4 ± 0.1
LA^{high}	0.04 ± 0.004	0.03 ± 0.009	0.06 ± 0.002	ND	ND

[a] Compound concentration required to inhibit viral replication by 50%. The antiviral activity of 7 selected lignosulfonates was determined in MT-4 cells against HIV-1 IIIB (X4), HIV-1 HE (R5/X4), HIV-2 ROD (R3/R5/X4). In PHA-blasts antiviral activity was determined against HIV-1 NL4.3 (X4) and HIV-1 BaL (R5) replication. Mean ± SEM of three to four independent experiments is shown.

compound during the viral infection period, no anti-viral activity whatsoever was observed. This is in contrast with data obtained with compounds that bind to the target cells, such as the CXCR4 antagonist AMD3100 (data not shown). Based on the results, we decided to perform most subsequent experiments with three lignosulfonates with high viral inhibiting properties (i.e. LA13, LA14, and LA20) and two derivatives with lower inhibiting properties (i.e. LA02 and LA04). We also observed that the antiviral activity of these lignosulfonates was irrespective of viral tropism or co-receptor use, since they inhibited equally well CXCR4-using or X4 (NL4.3, IIIB), CCR5-using or R5 (BaL), dual–using R5/X4 (HE) HIV-1 strains. They also inhibited with equal potency the multi-tropic (R3/R5/X4) HIV-2 (ROD) strain (Table 3). Furthermore, also in PBMCs expressing CD4 and various chemokine receptors (defined as PHA-blasts) comparable antiviral activity of the compounds was observed (Table 3). These PHA-blasts simulate the physiological target cells and can be seen as a model for the blood barrier that has to be crossed by the virus after vaginal/rectal intercourses (García-Gallego et al., 2015).

Finally, the test compounds also inhibited HSV-1 and HSV-2 replication when evaluated in HEL cells and, importantly, dual HIV-1 and HSV-2 infections (when both viruses were added simultaneously at the start of the assay) in susceptible MT-4 cells (Table 4). This is not observed with the well-described compounds AMD3100 and acyclovir, which respectively inhibit HIV and HSV replication. These compounds have no activity in inhibiting virus-induced CPE when dual viral infections were performed in MT-4 cells (data not shown). To determine if there was a broad antiviral activity against enveloped viruses, we also determined the activity of LA^{low} and LA^{high} against the flaviviruses ZIKV and DENV-2, but no activity was observed at the highest concentration tested (100 μg/ml) (Table 4).

3.2. Lignosulfonates interfere with viral entry

In the time-of-drug addition experiments, antiviral activity of the lignosulfonates started to decline when they were added more than 30 min after infection. When added after 2 h, no inhibition of viral replication was observed (Fig. 3). This coincides with the HIV entry process, which is described to be completed in less than 1 h in MT-4 cells (Daelemans et al., 2011). A comparable pattern was observed for the reference entry inhibitor PRO 2000. Two different reverse transcriptase inhibitors, tenofovir and AZT, still exerted their full antiviral activity when they were added up to 5 h after infection, which concurs with the reverse transcription process (that starts 4–6 h after infection (Daelemans et al., 2011)). We thus conclude that lignosulfonates exert their antiviral activity at an early time point of infection by interfering with the viral entry process, which includes binding and adsorption to the surface of target cells, interaction with (co)receptors, and subsequent fusion.

This interaction with cellular (co)receptors is a complex process that is mediated through interaction between the viral glycoprotein gp120 and the CD4 receptor expressed on the target cell (Wilen et al., 2012). To determine if lignosulfonates exert their antiviral activity by interfering with this process, we evaluated HIV binding to the surface of $CD4^+$ T cells with a specific anti-envelope gp120 antibody. As shown in Fig. 4A, all lignosulfonates dose-dependently inhibited this binding. LA14 (IC_{50}: 0.15 ± 0.03 μM), LA13 (IC_{50}: 0.24 ± 0.03 μM) and LA20 (IC_{50}: 0.25 ± 0.01 μM) were the most potent inhibitors, followed by LA02 (IC_{50}: 2.6 ± 0.2 μM) and LA^{low} (IC_{50}: 5.2 ± 0.7 μM). LA04 was much less active in anti-HIV replication assays and in this assay the compound showed no inhibition (IC_{50}: > 111 μM). The polyanionic virus-binding inhibitor PRO 2000 also inhibited this process (IC_{50}:

Table 4
Antiviral activity of lignosulfonates against HSV, dual infection with HIV-1 and HSV-2, VSV, ZIKV and DENV-2.

	IC_{50} (μM) [a]					
	HEL		MT-4	HEp-2	Vero	
	HSV-1	HSV-2	HIV-1 + HSV-2	VSV	ZIKV	DENV-2
LA02	5.8 ± 0.5	1.2 ± 0.03	4.6 ± 1.0	> 15.9	ND	ND
LA04	> 111.1	41.2 ± 2.8	101.9 ± 1.3	> 111.1	ND	ND
LA13	0.1 ± 0.01	0.02 ± 0.008	0.1 ± 0.04	0.7 ± 0.2	ND	ND
LA14	0.09 ± 0.009	0.02 ± 0.004	0.07 ± 0.03	0.4 ± 0.2	ND	ND
LA20	0.1 ± 0.002	0.09 ± 0.06	0.1 ± 0.06	0.9 ± 0.1	ND	ND
LA^{low}	1.0 ± 0.006	0.2 ± 0.03	1.0 ± 0.5	4.5 ± 1.0	> 25.0	> 25.0
LA^{high}	0.2 ± 0.02	0.09 ± 0.06	0.2 ± 0.09	0.8 ± 0.1	> 3.8	> 3.8

[a] Compound concentration required to inhibit viral replication by 50%. The antiviral activity of selected lignosulfonates was determined in HEL cells against HSV-1 KOS and HSV-2 G, in MT-4 cells against dual virus infection with HIV-1 NL4.3 and HSV-2 G, in HEp-2 cells against VSV and in Vero cells against ZIKV strain MR766 and DENV-2. Mean ± SEM of three independent experiments is shown.

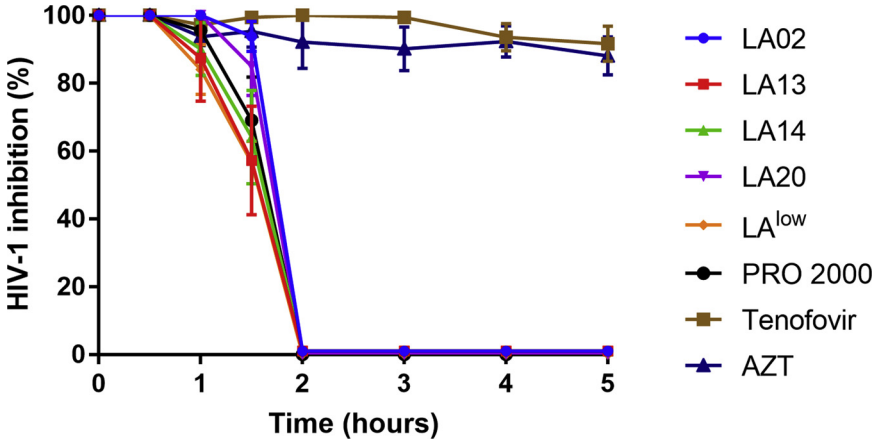

Fig. 3. Lignosulfonates inhibit viral replication at an early time point in the viral life cycle. MT-4 cells were infected with HIV-1 NL4.3 and 100 µg/ml lignosulfonates (16 µM LA02, 1.7 µM LA13, 1.2 µM LA14, 1.7 µM LA20, 13 µM LA^{low}), 20 µM PRO 2000, 1 µM AZT, and 100 µM tenofovir were added at different time points post-infection (0–5 h). After 31 h, viral replication was determined in the supernatant by specific HIV-1 p24 Ag ELISA. Data were plotted relative to the positive control to show the percentage inhibition of viral replication over time. Mean ± SEM of three independent experiments is shown.

0.43 ± 0.1 µM). These results indicate that lignosulfonates perturb the interaction between the viral glycoprotein gp120 and the cellular receptor CD4. To determine if lignosulfonates also interact with HSV glycoproteins, we evaluated if they interfere with anti-gD envelope mAb binding to HSV-2-infected MT-4 cells. Fig. 4B shows that they dose-dependently inhibit the gD mAb binding. Again, LA14 (IC_{50}: 0.94 ± 0.1 µM), LA13 (IC_{50}: 1.2 ± 0.1 µM), and LA20 (IC_{50}: 1.5 ± 0.1 µM) were the most potent inhibitors. LA^{low} (IC_{50}: 10.0 ± 0.7 µM) and LA02 (IC_{50}: 13.1 ± 2.3 µM) were less active, and LA04 showed no activity at all (IC_{50}: > 111 µM). These IC_{50} are higher than those of HIV binding inhibition (Fig. 4A), which corresponds to the lower activity of lignosulfonates against HSV replication compared to HIV in MT-4 cells (Table 2). These results suggest that lignosulfonates interact with the HSV gD glycoprotein.

3.3. Lignosulfonates interact with HIV glycoproteins

SPR experiments further supported this hypothesis of compounds interacting with HIV envelope proteins since we showed that they interacted with the gp120 glycoprotein. The affinity of the lignosulfonates for gp120 – as presented by their equilibrium dissociation constant k_D – reflected their antiviral potency. LA14 (k_D: 0.38 ± 0.05 nM), LA20 (k_D: 0.58 ± 0.29 nM), and LA13 (k_D: 0.79 ± 0.26 nM) had the highest gp120 affinities, followed by LA^{high} (k_D: 0.98 ± 0.25 nM), LA^{low} (k_D: 4.62 ± 1.26 nM), and LA04 (k_D: 118.0 ± 28.3 nM). The gp120 affinity k_D of the polyanionic control compound PRO 2000 was 2.6 ± 0.70 nM, which also reflects its antiviral activity (HIV-1 NL4.3 EC_{50}: 0.2 ± 0.07 µM). We also determined binding affinities between LA^{high} /LA^{low} and HIV gp41 and human serum albumin (HSA). Interestingly, there was also a potent interaction with gp41 (LA^{high} k_D: 1.65 ± 0.81 nM, LA^{low} k_D: 3.59 ± 0.14 nM). HSA was included to determine aspecific binding. For this interaction, the k_Ds were very low varying from 0.36 ± 2.97 µM for LA^{high} and 0.31 ± 0.17 µM for LA^{low} (Fig. 5). Finally, we evaluated if these compounds also inhibit VSVG-HIV-1 pseudovirus production and we observed that this production was inhibited in the presence of these compounds (SFig. 1). This suggests a mechanism of action based on electrostatic interactions between positively charged envelope proteins and the polyanionic character of the compounds. Replication competent VSV was dose-dependently inhibited as well, albeit with lower potency than against HIV or HSV (Table 4).

3.4. Lignosulfonates inhibit transmission and subsequent infection by cell-associated HIV

HIV is capable of inducing syncytia ('giant cells') formation between HIV-infected and uninfected $CD4^+$ T cells. We simulated this process in a co-cultivation assay by mixing persistently HIV-infected and uninfected $CD4^+$ T cells. Three lignosulfonates (LA13, LA14, and LA20) strongly and dose-dependently inhibited giant cell formation by two HIV-1 strains (IC_{50}: 0.1-0.3 µM) (Table 5, SFig. 2). They were approximately ten times more active than LA^{low} (IC_{50}: 1.4–2.5 µM). LA04

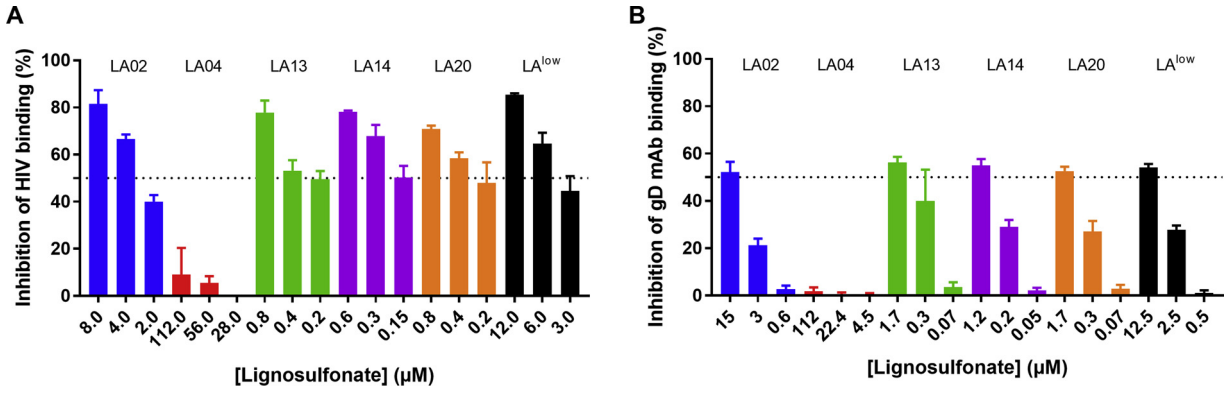

Fig. 4. Lignosulfonates interfere with the viral glycoproteins that are involved in entry. (A) SupT1 cells were incubated with HIV-1 NL4.3 in the presence or absence of different concentrations lignosulfonates (8.0 – 4.0 – 2.0 µM LA02, 112.0 – 56.0 – 28.0 µM LA04, 0.8 – 0.4 – 0.2 µM LA13, 0.6 – 0.3 – 0.15 µM LA14, 0.8 – 0.4 – 0.2 µM LA20, 12.0 – 6.0 – 3.0 µM LA^{low}). After 2 h, binding of HIV-1 gp120 to CD4 was measured using anti-gp120 NEA-9205 mAb. The graph demonstrates the percentage inhibition of HIV-1 gp120 binding to $CD4^+$ SupT1 cells. Mean ± SEM of three independent experiments is shown. (B) MT-4 cells were infected with HSV-2 G. After four days, they were incubated with various concentrations of lignosulfonates (15.0 – 3 – 0.6 µM LA02, 112.0 – 22.4 – 4.5 µM LA04, 1.7 – 0.3 – 0.07 µM LA13, 1.2 – 0.2 – 0.05 µM LA14, 1.7 – 0.3 – 0.07 µM LA20, 12.5 – 2.5 – 0.5 LA^{low}). After 1 h, binding of HSV-2 gD envelope antibody to the infected cells was determined. The graph shows the percentage inhibition of gD mAb binding to HSV-2-infected MT-4 cells. Mean ± SEM of three independent experiments is shown.

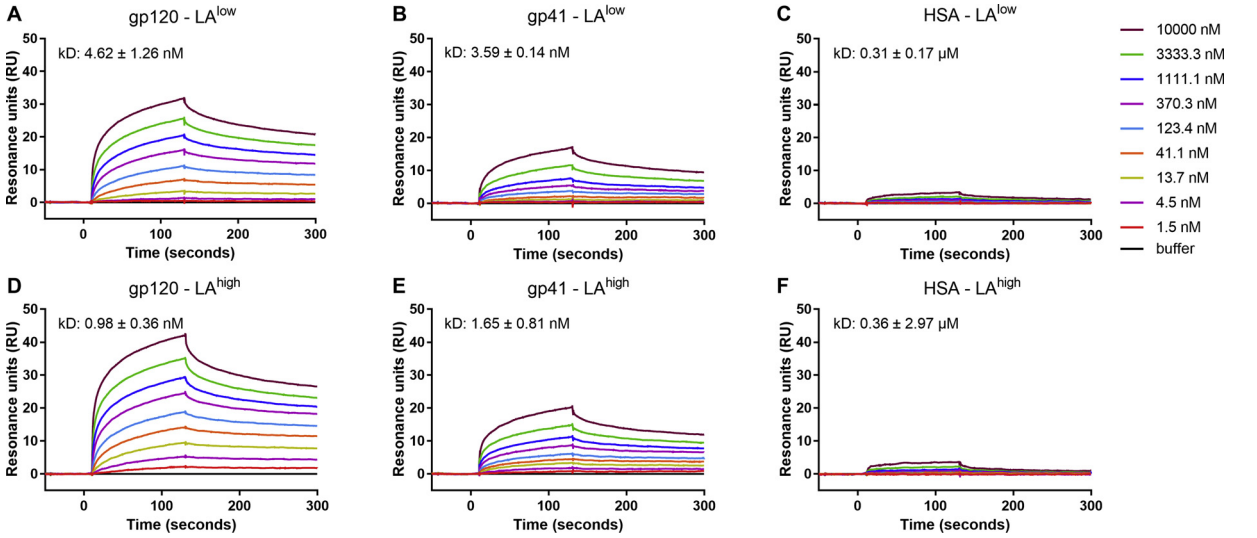

Fig. 5. Lignosulfonates interfere with the interaction of viral gp120 and cellular CD4 by binding to gp120 and to a lesser extent to gp41. SPR sensorgrams of the interaction between gp120 (left panels A,D), gp41 (middle panels B,E), and HSA (right panels C,F) and various concentrations of LA^{low} (upper panels A–C) and LA^{high} (lower panels D–E). The binding curves of 0–120 s show the association, whereas those of 120–300 s show the dissociation phase. The equilibrium dissociation constant k_D is shown. One representative experiment out of three is shown.

Table 5
Lignosulfonates inhibit the HIV-induced formation of giant cells.

	IC_{50} (µM) [a]	
	HIV-1 NL4.3	HIV-1 IIIB
LA02	5.6 ± 0.4	3.9 ± 1.1
LA04	> 111.1	> 111.1
LA13	0.3 ± 0.01	0.2 ± 0.02
LA14	0.2 ± 0.01	0.1 ± 0.01
LA20	0.3 ± 0.02	0.2 ± 0.02
LA^{low}	2.5 ± 0.2	1.4 ± 0.1

[a] Compound concentration needed to reduce virus-induced giant cell formation with 50%. Giant cell formation of HIV-1 NL4.3 and IIIB was evaluated. Mean ± SEM of three independent experiments is shown.

was not able to inhibit this giant cell formation process (IC_{50}: > 111 µM) and LA02 was weakly active (IC_{50}: 3.9–5.6 µM). The observed IC_{50}s were somewhat higher against HIV-1 NL4.3 than against HIV-1 IIIB.

Cells that express DC-SIGN, e.g. immature dendritic cells, bind pathogens and present them to T cells to evoke an appropriate immune response. Viruses like HIV exploit this receptor to facilitate access to and infection of target T cells. We simulated this capture by DC-$SIGN^+$ cells and the subsequent transmission to $CD4^+$ T cells in two separate assays. In the virus capture assay, only LA13 and LA14 inhibited DC-SIGN-mediated HIV capture, as indicated in Fig. 6. This inhibition was dose-dependent although not that potent, since their respective IC_{50} values were 2.0 and 2.4 µM. However, this observation is important, as LA^{low} is not able to inhibit this process (IC_{50}: > 31.0 µM). Table 6 and SFig. 3 show that all lignosulfonates inhibited the transmission of HIV-1 presented by DC-$SIGN^+$ cells to $CD4^+$ T cells. LA13, LA14, and LA20 had the highest inhibitory activity, which was ˜10-fold more potent compared to LA^{low}. LA02 and LA04 had the lowest HIV-1 transmission inhibition potential, which is in agreement with the other results, as presented above.

3.5. Cross-resistance of lignosulfonates against HIV entry inhibitor resistant strains

HIV-1 NL4.3 was cultured in MT-4 cells in the presence of increasing concentrations of lignosulfonates to obtain resistant strains. The commercially available LA^{low} (MW 8000 g/mol) and LA^{high} (MW 51,900 g/mol) were used. The IC_{50} of LA^{low} against LA^{low} resistant NL4.3 HIV-1 was 9.2 ± 0.7 µM, compared to 0.1 ± 0.04 µM against the wild type virus. The IC_{50} of LA^{high} against LA^{high} resistant NL4.3 HIV-1 increased

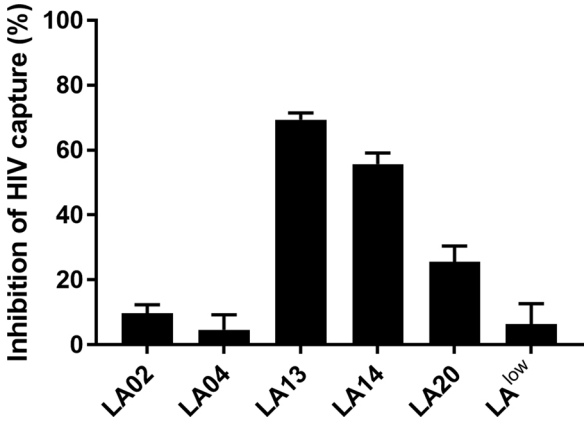

Fig. 6. Lignosulfonates inhibit DC-SIGN-mediated capture of HIV-1 to $CD4^+$ target cells. DC-$SIGN^+$ Raji cells were infected with HIV-1 HE for 2 h in the presence of lignosulfonates. All lignosulfonates were tested at 250 µg/ml (LA02: 39.7 µM, LA04: 277.8 µM, LA13: 4.3 µM, LA14: 3.0 µM, LA20: 4.3 µM, LA^{low}: 31.3 µM). Viral binding percentage was determined using HIV-1 p24 Ag ELISA. Mean ± SEM of two independent experiments is shown.

Table 6
Lignosulfonates inhibit DC-SIGN-mediated capture and transmission of HIV-1 to $CD4^+$ target cells.

	IC_{50} (µM) [a]	
	Capture	Transmission
LA02	> 40.0	1.6 ± 0.7
LA04	> 278.0	35.8 ± 18.1
LA13	2.01 ± 1.2	0.04 ± 0.006
LA14	2.4 ± 1.7	0.02 ± 0.002
LA20	> 4.3	0.03 ± 0.007
LA^{low}	> 31.0	0.3 ± 0.06

[a] Compound concentration needed to reduce DC-SIGN-mediated capture or transmission of HIV-1 by 50%. Mean ± SEM of two to three independent experiments is shown.

Table 7
Antiviral activity of lignosulfonates against LA-resistant HIV-1 NL4.3.

	IC_{50} (μM)[a]	
	LA^{low} res	LA^{high} res
LA02	> 31.8	> 31.8
LA04	> 222.2	> 222.2
LA13	1.2 ± 0.2	1.3 ± 0.1
LA14	0.8 ± 0.2	0.9 ± 0.1
LA20	1.3 ± 0.2	1.2 ± 0.2
LA^{low}	9.2 ± 0.7	9.0 ± 1.8
LA^{high}	1.5 ± 0.1	1.7 ± 0.1
PRO 2000	3.7 ± 0.3	2.9 ± 0.8

[a] Compound concentration required to inhibit HIV-1 replication by 50%. This assay was performed in MT-4 cells. Mean ± SEM of three independent experiments is shown.

from 0.03 ± 0.01 μM to 1.7 ± 0.1 μM. This respective 90- and 60-fold increase in IC_{50} is a clear indication of the presence of compound resistance. Subsequently, the activity of five selected lignosulfonates against these resistant viruses was evaluated. All compounds showed decreased antiviral activity, varying between 50- and 90-fold. LA02 and LA04 were completely inactive (Table 7, Fig. 7A). The polyanionic entry inhibitor PRO 2000 also exerted a ˜20-fold decreased antiviral activity against these resistant strains (from 0.2 ± 0.07 (wildtype NL4.3 EC_{50}) to 3.7 ± 0.3 μM (LA^{low} resistant NL4.3 EC_{50}) and 2.9 ± 0.8 μM (LA^{high} resistant NL4.3 EC_{50})), indicating a comparable mechanism of action of this compound. The CXCR4 inhibitor AMD3100 showed no cross-resistance (< 4-fold increase in IC_{50}), which was expected since its mechanism of action works through an interaction with the CXCR4 receptor expressed on the cell surface instead of the virus itself. The sequencing of these lignosulfonate-resistant strains shows eleven mutations that occurred in both LA^{low}- and LA^{high} -resistant NL4.3, of which seven mutations occurred in gp120 and four mutations in gp41 (Fig. 8). Eight of these residues were also mutated in the PRO 2000-resistant HIV-1 NL4.3 which explains the observed cross-resistance. In gp120, two mutations (R116 K, S160 N) occurred in the V1V2 loop, three (V170 N, R389 T, F393I) in or adjacent to the CD4-induced epitopes, and two (N271E, Q280 H) in the V3 loop (Fig. 8A). These gp120 regions are involved in CD4 and coreceptor binding, so the mutations in these regions further support the presumed mechanism of action of lignosulfonates, i.e. inhibiting the binding of gp120 to the cellular HIV receptors. In gp41, all four mutations (N42D, K77Q, N126 K, H132Y) occurred in or adjacent to the two heptad repeat regions (Fig. 8B). These are involved in the formation of a hairpin structure that precedes fusion of viral and cellular membranes during the entry process. The occurrence of mutations in gp41 suggests perhaps an additional mode of action of the lignosulfonates, which was also suggested by the results obtained in the SPR experiments.

We evaluated the presence of cross-resistance against several other entry inhibitor resistant HIV-1 NL4.3 or HIV-1 IIIB strains that were obtained previously (de Vreese et al., 1996; Esté et al., 1997; Huskens et al., 2007) (Table 8, Fig. 7B). When lignosulfonates were tested against 2G12 mAb- or T20-resistant strains, the IC_{50} changed between 0.5–2.4 fold. However, the 2G12 mAb IC_{50} increased > 100-fold, and the T20 IC_{50} > 17-fold, against the respective resistant viruses. 2G12 mAb is a unique mAb that binds to several specific mannose residues on gp120, while T20 is a biomimetic gp41 peptide interfering with the HIV fusion process. These results indicate that lignosulfonates have an alternative mechanism of action. The lignosulfonates' IC_{50} increased between 2.1 and 5.8 fold when evaluated against the AMD3100-resistant strain. AMD3100 itself had an inhibitory activity that was > 40-fold higher compared to the wild type virus (IC_{50} increased from 20.3 ± 3.1 nM to 911.0 ± 128.0 nM). Two HIV-1 strains that were resistant against the polyanionic entry inhibitors DS 5000 and PRO 2000 were also evaluated. The lignosulfonates' IC_{50} against DS 5000-resistant HIV-1 increased between 2.1 and 4.4-fold, while the DS 5000 IC_{50} increased 200-fold (from 0.05 ± 0.02 μM to 10.0 ± 5.3 μM). However, IC_{50} values increased between 11-and 24-fold when antiviral activity was tested against the PRO 2000-resistant strain. The PRO 2000 IC_{50} increased 20-fold (from 0.08 ± 0.01 μM to 1.7 ± 0.1 μM). Cross-resistance against PRO 2000-resistant HIV-1 could be attributed to a more comparable structure of PRO 2000 and lignosulfonates, being both sulfonated phenolic polymers, while DS 5000 is a sulfonated polysaccharide (Pirrone et al., 2011).

4. Discussion

In this study, we identified the antiviral activity against HIV and HSV of 24 sulfonated lignin derivatives with varying properties (Table 1). All lignosulfonates inhibited replication of various strains of HIV-1, HIV-2, HSV-1, and HSV-2 (Tables 2–4). The compounds also inhibit dual HIV-1/HSV-2 co-infection (Table 4). This is an important observation since it is described that an infection with HSV-2 increases the risk of acquiring HIV, and conversely (Suazo et al., 2015). Because HSV infections often remain unnoticed and both viruses seem to co-operate and facilitate their infections, it is perhaps essential that a microbicide has dual antiviral activity, which is associated with health benefits and decreased costs (Thurman and Doncel, 2012).

We selected five lignosulfonates with low IC_{50} values (LA13, LA14, LA20, LA^{low}, and LA^{high}) and two with high IC_{50}s (LA02 and LA04) to assess in more detail their antiviral profile, and to determine which properties contribute to this activity. Time-of-drug addition experiments showed that lignosulfonates interfere with the viral entry process

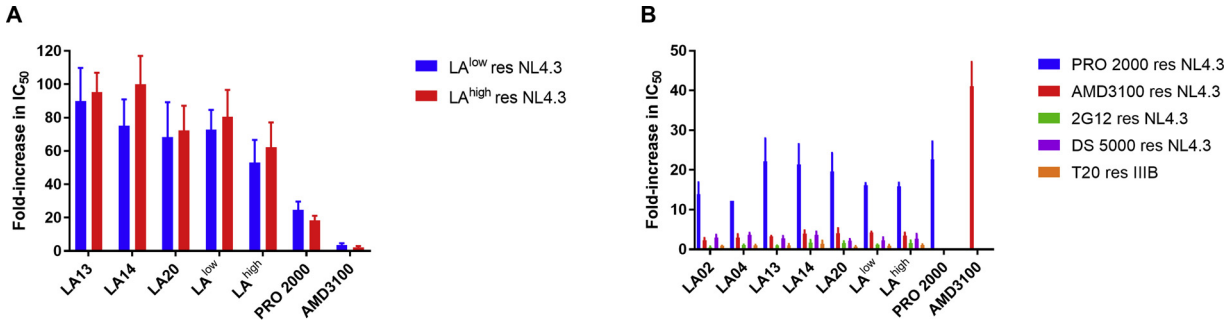

Fig. 7. Lignosulfonates show cross resistance against HIV-1 strains that are resistant against the commercially available LA and PRO 2000. (A) Antiviral activity (IC_{50}) of lignosulfonates and control compounds PRO 2000 and AMD100 against LA-resistant HIV-1 strains was compared to their IC_{50} against the wild type HIV-1 strain. The ratio of LA02 and LA04 could not be determined as these compounds completely lost their antiviral activity. (B) Antiviral activity (IC_{50}) of lignosulfonates against HIV-1 strains resistant to various entry inhibitors was compared to IC_{50} against the wild type HIV-1 strain. The ratios of control compounds 2G12, DS 5000 and T20 is not shown since they completely lost antiviral activity against their respective resistant viral strains. Mean ± SEM of three independent experiments is shown.

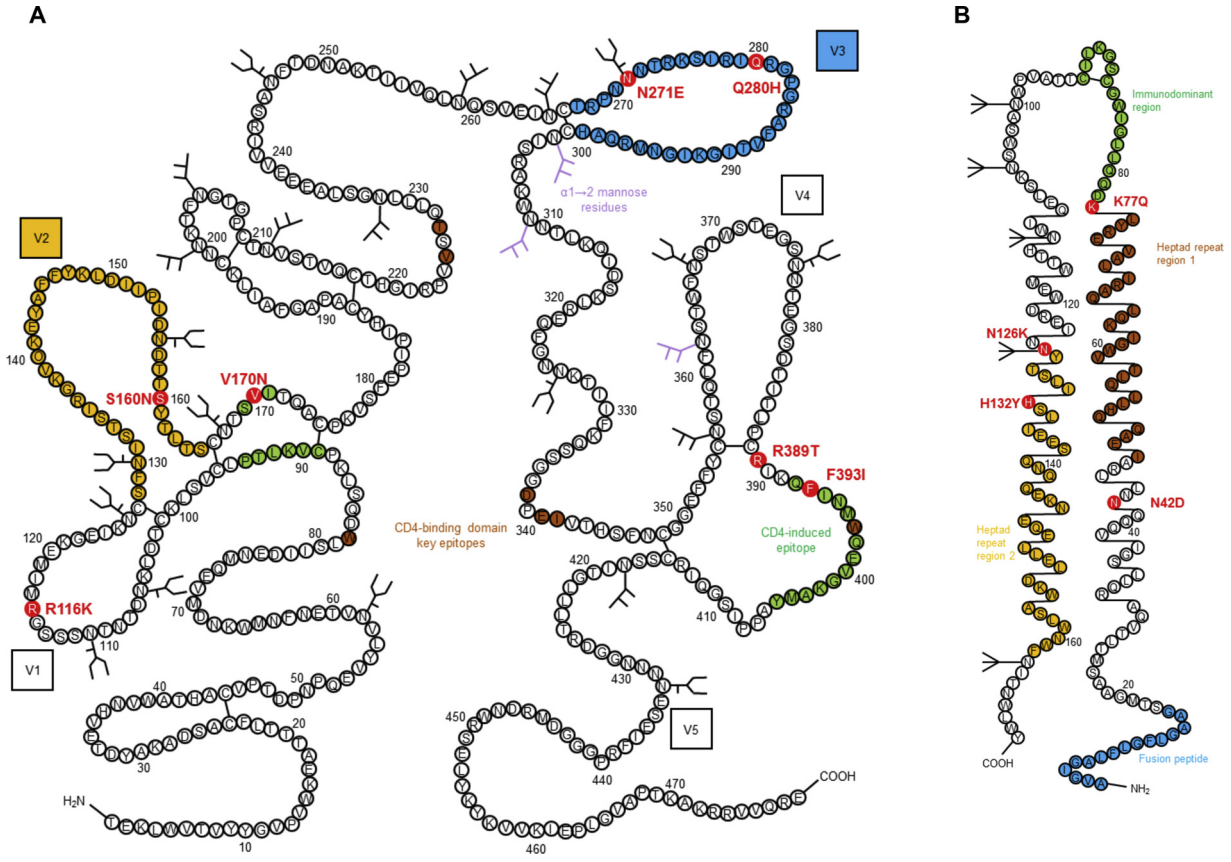

Fig. 8. Schematics of HIV envelope glycoproteins gp120 and gp41, depicting the mutations observed in LA^{low} and LA^{high} resistant HIV-1 NL4.3. (A) HIV-1 gp120 molecule showing the mutations in the resistant strains in red. The glycosylation sites containing high mannose-type and/or hybrid-type oligosaccharide structures are indicated by the branched structures, and glycosylation sites containing complex-type oligosaccharide structures are indicated by the U-shaped branches. The variable regions (V1-V5) are marked in boxes. Epitopes that induce neutralizing antibodies are marked in color: the CD4-binding domain key epitopes (brown), the CD4-induced epitope (green), an epitope composed of α1→2 mannose residues (purple), the V2 loop (orange) and the V3 loop (blue). Fig. 8A is adapted from Leonard et al. (1990) and Zolla-Pazner (2004). (B) HIV-1 gp41 molecule depicting the mutations in the resistant strains in red. Forked structures show the glycosylation positions. Important gp41 regions are marked in color: N-terminal heptad repeat region 1 (brown), C-terminal heptad repeat region 2 (orange), immunodominant region (green), fusion peptide (blue). Fig. 8B is adapted from Gallaher et al. (1989) and Sanders et al. (2002).

Table 8
Antiviral activity of selected lignosulfonates against various classes of viral entry inhibitor resistant HIV-1 strains.

IC_{50} (μM) [a]	AMD3100-res	PRO 2000-res	2G12-res	DS 5000-res	T20-res
LA02	1.6 ± 0.2	8.9 ± 2.3	0.4 ± 0.1	1.8 ± 0.3	1.1 ± 0.4
LA04	65.0 ± 2.8	> 111.1	28.8 ± 10.7	64.0 ± 7.8	59.7 ± 8.9
LA13	0.07 ± 0.02	0.4 ± 0.06	0.03 ± 0.004	0.07 ± 0.02	0.03 ± 0.01
LA14	0.05 ± 0.006	0.2 ± 0.05	0.02 ± 0.003	0.03 ± 0.002	0.03 ± 0.02
LA20	0.09 ± 0.02	0.4 ± 0.05	0.04 ± 0.002	0.07 ± 0.02	0.03 ± 0.01
LA^{low}	0.8 ± 0.05	3.0 ± 0.5	0.2 ± 0.002	0.5 ± 0.2	0.2 ± 0.06
LA^{high}	0.1 ± 0.03	0.7 ± 0.04	0.07 ± 0.02	0.1 ± 0.05	0.05 ± 0.009

[a] Compound concentration required to inhibit HIV-1 replication by 50%. All tests were performed in MT-4 cells. All resistant viruses were derived from culturing HIV-1 NL4.3 virus in MT-4 cells with increasing concentrations of the products AMD3100, PRO 2000, 2G12 mAb and DS 5000, except virus generated resistant to T20 was derived from the HIV-1 IIIB. Mean ± SEM of three independent experiments is shown.

as was expected since these are polyanionic molecules (Gordts et al., 2015; Pirrone et al., 2011) (Fig. 3). Entry inhibitors are still attractive microbicide candidates, because inhibiting this early step in the viral life cycle also prevents the establishment of a latent phase infection in the host cells. The lignosulfonates also inhibit transmission of cell-associated virus (Tables 5 and 6, SFigs. 2 and 3). This is beneficial since it prevents further spread of infection in the case viral transmission has already occurred. To conclude, a topical application of these compounds lies in the prevention of viral transmission and of further spread to neighboring cells after an established infection.

The mechanism of action of these polyanionic compounds is inhibition of glycoprotein adsorption to the susceptible target cells (Fig. 4), through electrostatic interactions between this glycoprotein and negatively loaded lignosulfonates. This mechanism of action is supported by the finding that lignosulfonates show cross-resistance against the HIV virus strain resistant to PRO 2000, another polyanionic compound (Table 8 and Fig. 7B). In SPR experiments, binding between HIV gp120 and the lignosulfonates was shown (Fig. 5A and D), further supporting the proposed mechanism of action. The lignosulfonates with the highest antiviral activity (LA14, LA20 and LA13) have the highest

affinity for gp120 binding. Importantly, SPR experiments and viral resistance experiments also suggest an important interaction between the lignosulfonates and the HIV glycoprotein gp41 (Figs. 5B and E and 8). Lignosulfonates are known to interact with proteins through hydrogen bonding, dipole-dipole, charge-charge, and hydrophobic interactions, further supporting the mechanism of action (Huang et al., 2003; Windschitl and Stern, 1988). Lignosulfonates also inhibit HIV-1 pseudovirus with a VSV envelope, which is positively charged as well, showing that these interactions are not limited to HIV glycoproteins alone (Carneiro et al., 2002). This decrease in VSVG HIV-1 pseudovirus production by sulfated polymers has been observed previously (Prokofjeva et al., 2013; Sepúlveda-crespo et al., 2018).

The compounds with the highest antiviral activity in the screening assays in MT-4 and TZM-bl cells were associated with a higher molecular weight (Fig. 2C). This is in line with previous studies on sulfated polyanionic macromolecules and is attributed to multivalent interactions between larger molecules and multiple HIV gp120 molecules or attachment proteins of other enveloped viruses (Ghosh et al., 2009). This is supported in the SPR experiments as the lignosulfonates with higher MW bind with a higher affinity to gp120. The absence of activity of LA04 (900 g/mol) suggests that a certain 'treshold' molecular weight is necessary to obtain significant antiviral activity. There was also a weak association between organic sulfur content and antiviral activity (Fig. 2D). Sulfur has been shown before to be an important determinant of antiviral activity and a higher sulfation degree is associated with better antiviral activity (Ciejka et al., 2016; Hwu et al., 2011; Witvrouw and De Clercq, 1997). LA21 (sulfur content 9.4%) is 4-fold more active than LA19 (sulfur content 5%) which has a comparable molecular weight. Another observation was that some hardwood lignosulfonates (e.g. LA10, LA11, LA15, LA16 and LA17) possess less antiviral activity compared to softwood lignosulfonates (LA01, LA02, LA22 and LA^{low}) of comparable molecular weight. Hardwood lignosulfonates possess extra $-OCH_3$ groups on the aromatic rings (Fig. 1), which prevents this carbon position from further chemical reactions during both the lignification and pulping process. This makes them in general more linear and thus less branched polymers, which could be an explanation for the decreased antiviral activity (Monteil-Rivera, 2016). LA23 and LA24 are more active than expected from their known properties (low molecular weight and low sulfur content). This can be related to other chemical properties that are unknown. We did not find correlations between antiviral activity of lignosulfonates and other characteristics such as counter-ion or carboxylic acid content. In solution, the counter-ions are probably dissociated from the acidic lignosulfonate, preventing them to contribute to the antiviral properties. Taken together, our results suggest that the molecular weight of lignosulfonates has the most decisive influence on antiviral activity, but it would be interesting to study high molecular weight lignosulfonates with higher sulfur contents and from hardwood origin. This would allow us to more accurately determine the contribution of chemical properties to their antiviral activity.

Apart from having identified compounds with higher antiviral activity, our results are in line with those obtained previously for the lignosulfonate LA^{low}, that was included as a reference compound in this study (Gordts et al., 2015; Qiu et al., 2012). In Gordts et al. (2015), we additionally showed that LA^{low} does not harm the vaginal microbiota. Together with its low cost, non-toxicity, and inhibition of dual HIV/HSV infection these are all good properties for the application of lignosulfonates as microbicide.

Currently, three sulfated polyanionic compounds have been evaluated as topical microbicides in phase III clinical trials. The first one, carrageenan, was considered as safe and it suggested efficacy, but it failed the trial because of absence of statistical significance (Altini et al., 2010; Skoler-Karpoff et al., 2008). The second one, cellulose sulfate, was also safe, but the trial was aborted prematurely because the first results suggested even an increased risk of HIV transmission. However, these results turned out to be non-significant (Van Damme et al., 2008). Because of this early closure, a parallel phase III trial was stopped as well, although in this study results suggested efficacy, albeit non-significant (Halpern, 2008). Finally, a 0.5% and 2% PRO 2000 (naphthalene sulfonate) gel was evaluated in phase III clinical trials. Despite safe and accepted use, efficacy as microbicide could not be demonstrated (McCormack et al., 2010). It turns out that in all three cases there is a clear discrepancy between promising results in preclinical and early clinical testing, and absence of efficacy in phase III clinical trials. Several reasons for these negative results were proposed: influence of vaginal pH by the product, decrease of activity in the presence of seminal fluid, product loss due to dilution or leakage, or disruption of vaginal epithelial integrity (Pirrone et al., 2011). These potential problems should be investigated during preclinical testing. The most important issue was the low drug adherence during the trial period which profoundly influenced the reported outcome (Skoler-Karpoff et al., 2008). Special efforts need to be made to increase drug adherence, for example by making application methods more user-friendly, e.g. through microbicide-containing intravaginal rings (Ugaonkar et al., 2015). Also, the way that adherence can be measured and reported should be improved, since self-reporting of drug adherence has been shown to be incongruent with actual adherence (Ramjee et al., 2010; Skoler-Karpoff et al., 2008).

Although none of these polyanionic compounds has made it to the market, it has never been demonstrated that they are unsafe or even could increase the risk of HIV transmission. Their antiviral activity profile and potential applications remain interesting. The findings in this study aid to develop specific nanotechnology-based polyanionic microbicide candidates, such as polyanionic dendrimers. Recently, more attention is going to this class of compounds. They are highly branched, star-shaped, and nano-sized molecules with a central core, several layers, and functional groups at the outer surface. Lignosulfonates resemble dendrimers somewhat, both structurally (being branched polymers) and behaviorally (exposing their sulfonate groups when in solution) (Myrvold, 2008). The implementation of several end-groups in dendrimers allows multivalent interactions with the virus envelope proteins. Polyanionic dendrimers possess several benefits such as structural uniformity and monodispersity, stability, and a high targeting-efficiency (Ceña-Diez et al., 2016; Sepulveda-Crespo et al., 2016).

This study reveals that the conversion of wood pulp into paper produces a variation of lignin-derived byproducts with broad antiviral activity by interfering with the viral entry process, that is dependent on their molecular weight. The results obtained here suggest that lignosulfonates might be useful as potential microbicides against HIV and HSV sexual transmission. However, thorough preclinical development needs to be done, e.g. by studying the interaction with biological entities (epithelia, semen, …) and especially the application method. Currently, drug-releasing vaginal rings are at the forefront of ongoing efforts to develop microbicide-based strategies for prevention of heterosexual HIV transmission (McBride et al., 2019).

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197760.

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