

Inhibition of HIV-1 and HSV-2 infection by glycosaminoglycan mimetics

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To my family

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HIV-1 is a sexually transmitted pandemic pathogen that causes progressive defects in cell-mediated immunity for which curative treatment and prophylaxis are lacking. In addition to CD4 and chemokine receptors of protein nature, the virus may utilize glycosaminoglycan chains of heparan sulfate (HS) for attachment to cells. HS mimetics such as sulfated oligosaccharides act as inhibitors of HIV-1 infection *in vitro* by interfering with the interaction between positively charged domains of the viral envelope gp120 and negatively charged HS chains at the cell surface. Also HSV-2, which causes recurrent genital lesions and increases the risk of HIV-1 acquisition, uses HS for attachment. Attempts to develop microbicides as prophylactic agents against genital transmission of HIV-1 have hitherto failed, since no compound was found to be effective and safe in clinical trials.

In pursuit of a topical microbicidal compound that can be used vaginally for prevention of HIV-1 and HSV-2 infection, we screened a library of analogues of sulfated oligosaccharide muparfostat. We here present a novel set of cholestanol-coupled sulfated oligosaccharides as potential microbicides. Several compounds displayed potent antiviral activity of which P4/PG545 was chosen for further studies. The compound exhibited virucidal properties against strain HIV-1IIIB and various HIV-1 clinical isolates *in vitro*, including both CCR5-using and dual-tropic CXCR4/CCR5 viral variants. A closely related compound, P3, was used to elucidate the mode of antiviral activity. A “time-of-addition” experiment showed that the compound interfered with the attachment of HIV-1 to cellular receptors and/or by hindered the egress of newly produced virions from cells. To further clarify a mechanism of action of these compounds, we generated muparfostat-resistant HIV-1 mutants by passaging the virus in cell culture in the presence of increasing amounts of compound. By sequencing of genes coding for viral envelope gp120 and gp41, escape mutations selected for by antiviral pressure of muparfostat were identified. Mildly resistant virus variants, with ~3-4 times decreased sensitivity to muparfostat, displayed several unique mutations including amino acid (a.a.) substitutions in the V2 and V3 loops, and a deletion of five a.a. in the V4 region of gp120. In addition, a mutation in the transmembrane region of gp41 was identified. Selection of these variants by muparfostat suggested that the compound interfered with viral binding to cell surface HS.

In a murine model of vaginal HSV-2 infection, PG545 was found to efficiently inactivate the virus, and abrogate clinical disease and death after preincubation of HSV-2 with high doses of PG545. Low-dose inoculation prevented HSV-2 infection of the second order of sensory neurons in the spinal cord, which might have had bearings to a favorable outcome. Furthermore, PG545 was found to reduce mortality and clinical disease when instilled vaginally shortly before or after infection. In conclusion, glycoconjugate PG545 showed virucidal activity against HIV-1 infection *in vitro*, and against HSV-2 in an animal model. These results pave the way for further studies of a microbicide with a potential use as a prophylactic compound against genital transmission of HIV-1.

Keywords: HIV-1, HSV-2, Glycosaminoglycan, Heparan sulfate, Microbicide, Muparfostat, Virucidal activity, Escape mutant, gp120, gp41

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SAMMANFATTNING PÅ SVENSKA

Humant immunbristvirus typ 1, HIV-1, sprids sexuellt mellan män och kvinnor eller mellan män. Botande behandling eller vaccin saknas. Även om prevalensen har minskat de senaste åren, bland annat i afrikanska länder söder om Sahara, ökar antalet infekterade individer i andra delar av världen. Kondom utgör en god barriär mot sexuellt överförbara sjukdomar som HIV-1 och herpes simplex virus typ 2 (HSV-2), som orsakar återkommande genital herpes och som ökar smittoöverföring av HIV-1. Det kan dock vara problematiskt, inte minst för gifta kvinnor, att förhandla med sin partner om användning av kondom. Ett kompletterande skydd i form av ett virusdödande medel applicerat som vaginal-gel eller vagitorier, en så kallad mikrobicid, skulle kunna användas före, under eller efter samlag för att minska risken för att bli smittad av HIV-1 och HSV-2.

Syftet med den här avhandlingen var att utveckla en sådan mikrobicid genom att: (i) screening-undersöka bibliotek av kolhydratbaserade substanser som liknar virusreceptorn heparansulfat (HS) på cellmembraners yta, (ii) bestämma antivirala egenskaper samt toxicitet i cellkultur hos utvalda substanser, (iii) selektera fram resistent virusmutanter mot den HS-liknande kolhydraten muparfostat, i syfte att klarlägga dess verkningsmekanism samt (iv) utvärdera den virusdödande substansen PG545 i en musmodell av vaginal HSV-2-infektion.

Biblioteket som undersöktes utgjordes av korta, högsulfaterade kolhydrater, baserade på en grundmolekyl kallad muparfostat. Derivatet kopplades till olika sidogrupper med förmåga att binda till lipidskikt i virusmembranet. En av substanserna, PG545, uppvisade antiviral potens och låg toxicitet. Förutom att substansen effektivt hämmade infektion av laboriestammen HIV-1^{III_B} och olika kliniska isolat, avdödade PG545 dessa virus i cellkultur. Verkningsmekanismen för PG545 och dess snarlika substans P3 studerades kinetiskt genom att tillsätta substansen vid olika tidpunkter i förhållande till virusinfektion i cellkultur. Den antivirala effekten var mest uttalad när substansen kunde blockera virus inbindning till cellen, eller dess utträde.

Vidare kartlades de exakta positionerna på det virala ytproteinet som påverkades vid närvaro av muparfostat genom att selektera fram muparfostat-resistent HIV-1-mutanter. För att få fram sådana mutanter odlades och passerades virus upprepade gånger i cellkultur med ökande koncentration av muparfostat, varefter resistent virusvarianter gensekvenserades. HIV-1-varianter som uppvisade 3-4 gånger lägre känslighet mot muparfostat

innehöll en förändrad gensekvens som resulterade i flera aminosyreförändringar i virusets höljeprotein, både i den yttre glykoproteindelen gp120 och den membranbundna regionen gp41. Vidare eliminerades fem aminosyror inom den variabla V4-regionen hos gp120, en förändring som tidigare har beskrivits för HIV-1 virus-resistenta mot andra sulfaterade kolhydrater. Vid passage vid högre koncentration av muparfostat fann vi dessutom ett aminosyreskifte inom V3-regionen som gav ökad negativ laddning till regionen, vilket sannolikt försämrade HS-bindningen hos gp120. Resultatet styrker att muparfostat, och därmed troligen även PG545, inhiberar interaktionen mellan HIV-1 och HS-molekyler på cellytan.

För att undersöka om PG545 hämmade virusinfektion även *in vivo*, användes en djurmodell i vilken möss infekterades vaginalt med HSV-2. Möss som behandlats med PG545 kort tid innan infektion uppvisade hög grad av överlevnad i ett kinetiskt försök, följt av möss där substans tillfördes 2 h efter virusinfektion. Resultaten speglade ovan beskrivna data från cellkultur. I ett annat försök blandades istället HSV-2 med olika koncentrationer av PG545, och virus och drog inokulerades vaginalt. Vid högre koncentrationer (500 och 100 µg/mL) klarade sig möss undan både lokal inflammation och CNS-påverkan. Möss som behandlades med en lägre dos (20 µg/mL) genomgick ett mildare förlopp av vaginal infektion jämfört med obehandlade kontrollmöss, och de tillfrisknade snabbt utan några skador på nervsystemet. Mängden viral arvs massa i form av RNA, mätt med kvantitativ PCR, såväl som mängden infektiöst virus visade liknande nivåer som hos kontrollmössen. Med hjälp av immunhistokemi, som påvisar viralt antigen, verkade infektionen dock ha begränsats till nervceller som sträcker sig in till ryggmärgen, medan de obehandlade kontrollmössen var infekterade även i ryggmärgens nervceller.

Sammantaget har avhandlingen visat att PG545, ett konjugat bestående av en kort, högsulfaterad kolhydrat i form av muparfostat kopplad till en molekyl som band till lipidmembraner, hämmade både HIV-1- i cellkultur och HSV-2-infektion i djurmodell. Baserat på kinetiska försök samt på gensekvensering av resistenta HIV-1-varianter identifierades en trolig verkningsmekanism: PG545 hämmade virus inbindning till samt utträde ur cellen. Substansen gav fullständig blockering av vaginal HSV-2-infektion i en musmodell, samt skyddade ryggmärgen mot virusinfektion vid lägre dos. PG545 kan därför betecknas som en lovande läkemedelskandidat för utveckling av en mikrobicid substans som kan appliceras vaginalt för att skydda mot sexuellt överförd HIV-1-smitta.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

- I. **Said, J**; Trybala, E.; Andersson, E.; Johnstone, K; Liu, L; Wimmer, N; Ferro, V; Bergström, T. *Lipophile-conjugated sulfated oligosaccharides as novel microbicides against HIV-1*.
Antiviral Research 2010; 86: 286-295.
- II. **Said, J**; Andersson, E; Trybala, E; Bergström, T. *HIV-1 variants with reduced sensitivity to sulfated oligosaccharide muparfostat contain mutations in the envelope glycoproteins gp120 and gp41*.
J Antivir Antiretrovir 2013; 5: 050-056.
doi: 10.4172/jaa.1000063
- III. **Said, J**; Trybala, E; Görander, S; Ekblad, M; Liljeqvist, J-Å; Jennische, E; Lange, S.; Bergström, T. *Anti-HSV-2 activity of the glycoconjugate PG545 in a mouse model of genital herpes infection*.
In manuscript

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
C1-C5	Constant regions 1 to 5 of gp120
cART	Combination antiretroviral therapy
CCR	CC chemokine receptor
CD4	Cluster of differentiation
CPE	Cytopathic effect
CRF	Circulating recombinant forms
CS	Chondroitin sulfate
CSPG	Chondroitin sulfate proteoglycan
CXCR	CXC chemokine receptor
DRG	Dorsal root ganglia
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope gene
Gag	Group antigen gene
GAG	Glycosaminoglycan
GALT	Gut-associated lymphoid tissue
gB, gC, gD, gG, gH/gL	Envelope glycoproteins B, C, D, G, gH/gL of HSV-2
gp	Glycoprotein
HAART	Highly antiretroviral therapy
HCMV	Human cytomegalovirus
HIV-1	Human immunodeficiency virus type 1
HS	Heparan sulfate

HSPG	Heparan sulfate proteoglycan
HSV-2	Herpes simplex virus type 2
HVEM	Herpes virus entry mediator
IC ₅₀	50% inhibitory concentration
Nef	Negative factor
NNRTI	Non-nucleoside/nucleotide reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
PCR	Polymerase chain reaction
Pol	Polymerase gene
R5 HIV-1	CCR5-using HIV-1
RER	Rough endoplasmic reticulum
Rev	Regulator of virion expression
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
STI	Sexually transmitted infection
Tat	Transactivator of transcription
TCID ₅₀	50% tissue culture infective dose
V1-V5	Variable regions 1 to 5 of gp120
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VZV	Varicella-zoster virus
X4 HIV-1	CXCR4-using HIV-1
X4/R5 HIV-1	CXCR4 and CCR5-using HIV-1

1 INTRODUCTION

Human immunodeficiency virus (HIV) targets essential cells of the immune system causing a progressive defect in cell-mediated immunity, which is manifested clinically as an increased vulnerability to seemingly harmless pathogens. This pathogenic process is the cause of the high morbidity and mortality in conjunction with HIV infections around the globe. According to the UNAIDS report (2012) an estimated 34 million [31.4 million-35.9 million] individuals were living with HIV in 2011. The virus is predominantly spread by sexual intercourse and is found in all communities and populations regardless of ethnicity, gender and sexuality. However, the prevalence in the poorer parts of the world is significantly higher than in the richer countries, where antiretroviral therapy is readily available and prophylactic measures are implemented more strongly. Being infected with HIV is still a stigmatising condition in many parts of the world, as was the case with tuberculosis during the first part of the former century. Despite the fact that the number of patients newly infected with HIV worldwide is declining, as documented by a decrement of approximately 20% in the yearly HIV incidence since the beginning of the pandemic (UNAIDS), the virus continues to spread. The majority of infections are transmitted through heterosexual coitus from males to females. Herpes simplex virus type 2 (HSV-2), the cause of recurrent genital herpes, strongly contributes to the transmission of HIV-1 since it has been documented in several studies that the risk of acquiring the virus increases significantly in HSV-2 infected individuals [1-3]. Despite their molecular and phenotypical differences, the two viruses have several common denominators.

- Both are sexually transmitted viruses
- No vaccines are available
- Both infections are treatable (but not curable) with antivirals
- Both viruses cause life-long infections by establishing latent or persistent infections
- Both viruses carry envelope glycoproteins that interact with cell surface glycosaminoglycans during cell entry

A microbicide that can be applied topically prior to, during or after sexual intercourse as a new preventive strategy against HIV and other sexually transmitted infections with similar ways of transmission, such as HSV-2, is therefore highly desirable. The efforts to develop such microbicides have

hitherto failed [4], which prompted our current attempts to utilize novel strategies aimed at this goal.

1.1 Origin of HIV-1 and HSV-2

1.1.1 HIV-1

HIV constitutes a group of retroviruses belonging to the family of Retroviridae and the genus [5] Lentivirinae [6]. HIV is further divided into two main species; HIV type 1 (HIV-1) and HIV type 2 (HIV-2). Both viruses have evolved from zoonotic transfers to man. HIV-1 is responsible for the current global pandemic, while HIV-2 is only endemic in West Africa with limited documented spread to southern Asia, North America and southern Europe [7]. HIV-2 is associated with lower infectivity rate and slower disease progression. The virus probably originated from SIVsm, which has sooty mangabey monkeys as its natural host. The virus is genetically divided into 7 sub-species, groups A-H [8, 9]. Since the current work has studied intervention of HIV-1 but not HIV-2 infections, we now focus on the evolutionary aspects of the former virus.

There are strong data supporting that HIV-1 originated from the non-human primate simian immunodeficiency virus (SIV), which has chimpanzee as its natural host. Most likely, zoonotic transmissions from chimpanzee to man have occurred several (at least 4) times, based on phylogenetic analyses (Figure 1). During the first decades of the 20th century, concurrent with emergence of larger cities in Central Africa such as Kinshasa, HIV-1 was firmly established in humans and expanded thereafter (for a review, see [9]). HIV-1 is genetically divided into genogroups M, N, O and the recently discovered group P. Group M, which constitutes the dominant part of the global epidemic, is in turn divided into 9 genetic subtypes and additionally 37 circulating recombinant forms (CRF) of these subtypes [5, 10]. Group N (which to date has been found only in a few individuals in Cameroon) is related to group M, but both were probably zoonotically transmitted independently of each other from the chimpanzee *Pan troglodytes troglodytes* to humans in Central Africa [11] (Figure 1). Group O, mainly found in West-Central African countries, and P, so far identified in only two individuals in Cameroon, may have originated from SIV found in Western Lowland gorillas *Gorilla gorilla gorilla* (SIVgor) in Cameroon [12, 13]. Interestingly, an alternative hypothesis is that also SIVgor resulted from a

zoonotic transmission from chimpanzee to gorilla. In this case, both these genogroups also emerged from the chimpanzee.

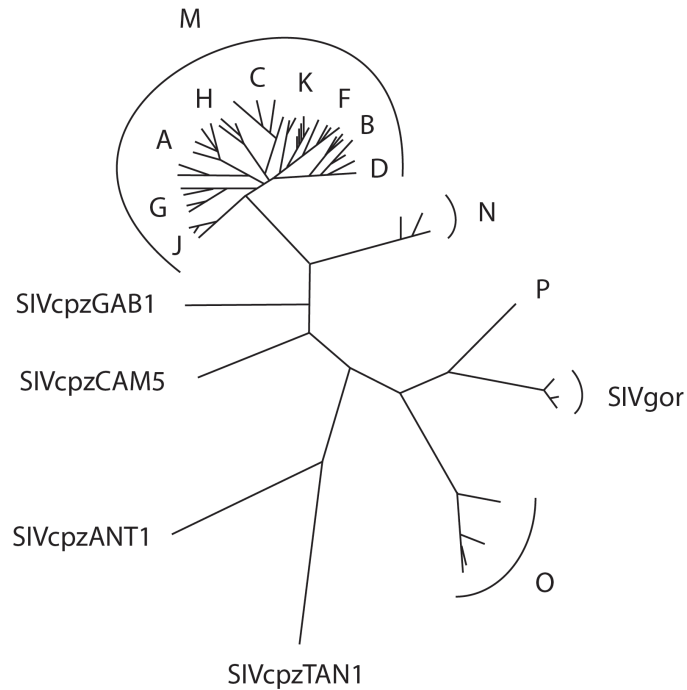


Figure 1. Phylogenetic tree of the origin of HIV-1. HIV-1 group M, including subtypes A-D, F-H, J and K and groups N, O and P are all based on reference sequences of polymerase gene region of SIV and HIV-1. SIV sequences from chimpanzees, *SIV_{cpz}CAM5* and *SIV_{cpz}GAB1* derived from *Pan troglodytes troglodytes* whereas *SIV_{cpz}ANT1* and *SIV_{cpz}TAN1* derived from *schweinfurthii*. Adapted with permission from Hemelaar, J. Trends Mol Med 2011.

The profound genetic diversity of circulating HIV is mainly due to three conditions: (i) the viral reverse transcriptase (RT) is extremely error-prone producing ~ 0.2 errors per genome during each replication cycle [14], and further errors introduced by the host cell RNA pol II during transcription of integrated DNA to RNA are also frequent, (ii) a rapid replication rate of $\sim 10^{10}$ - 10^{12} viral particles per day [15] in one individual results in a large population of genetic variants, and, (iii) recombination and natural selection of viral variants is frequent.

1.1.2 HSV-2

In contrast to HIV-1, members of the herpesviridae family are DNA viruses that seldom are zoonotic but have evolved together with their respective hosts for at least 400 million years. It is likely that the subfamily of α -herpesviruses, including Herpes simplex virus type 1 (HSV-1), HSV-2 and varicella-zoster virus (VZV), diverged from other herpesviruses 180-210 million years ago [16]. The proofreading mechanism of HSV viruses is considerably more efficient, resulting in only 3×10^{-8} substitutions per site per year [17], compared to HIV viruses with $\sim 10^{-3}$ substitutions per site per year [18, 19]. Herpesviruses have been detected in mammals, reptiles, birds, fishes and even molluscs, and are almost always host species-specific [20]. However, zoonotic infections do sometimes occur and although mutations are relatively rare, new viral species appear, mostly through genetic recombination [21]. Intra-host recombination may also occur if the host is co-infected with two different viral strains that both manage to enter the same cell. Recombination may be advantageous for the virus if beneficial parts of two genomes are combined and this might drive the evolutionary process as reviewed by [22].

1.2 HIV-1 genome and structure

1.2.1 HIV-1 genome

Ever since the HIV pathogen was identified as the causative agent of AIDS in the early 1980s [23, 24] intense efforts have been made to define its genome organization and functions of its particular components. Although the HIV-1 genome is relatively small (~ 10 kb) its structural organization is more complex than that of many other retroviruses (Figure 2). The three major parts of the genome (gag, pol, and env) are translated as polyproteins, which are then cleaved by viral or cellular proteases into the mature

structural proteins of the HIV virion. Thus, the Gag polyprotein is cleaved into the matrix (p17), capsid (p24), nucleocapsid (p7), and p6 proteins. The Pol precursor protein is processed into the reverse transcriptase (RT), integrase (In), and protease (Pr) proteins while the Env precursor gp160 is cleaved by a cellular protease into the viral membrane associated components gp120 and gp41. The *vif*, *vpr*, *vpu*, *rev*, *tat*, and *nef* are auxiliary proteins that play different roles in the virus life cycle [6].

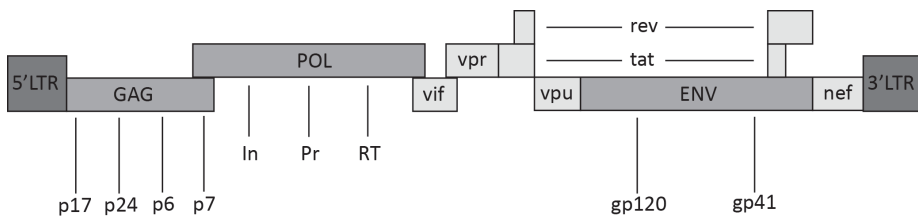


Figure 2. Genomic organization of HIV-1.

Table 1. HIV-1 proteins and their functions [6, 25]

Gene	Protein	Function
<i>Structural</i>		
Gag	p17	Matrix protein, interacts with gp41
	p24	Capsid protein
	p6	Core protein that binds Vpr
	p7	Nucleocapsid protein/Binds viral RNA
Pol	In (p32)	Integrates proviral DNA into host cell DNA
	Pr (p10)	Cleaves precursor proteins Gag and Pol
	RT (p66/p51)	Catalyzes reverse transcription from viral RNA to DNA
Env	gp120	Viral envelope protein, interacts with cell receptors
	gp41	Viral transmembrane protein, involved in fusion with host cells
<i>Regulatory</i>		
Rev (regulator of expression of virion protein)	p19	Shuttles viral unspliced and singly spliced mRNA from nucleus
Tat (trans-activator of transcription)	p14	Regulates LTR-driven transcription, immune suppression
<i>Auxiliary</i>		
Vif (virion infectivity protein)	p23	Prevents antiviral activity by APOBEC, efficient cell-free transmission
Vpr (viral protein R)	p18	Arrests cell division, shuttles DNA to nucleus, enhances viral replication
Vpu (Viral protein U)	p16	Enhances viral budding, involved in CD4 degradation on host cell membranes
Nef (negative regulatory factor)	p27	Modulates cell replication, down-regulates expression of host cell CD4

The HIV-1 virion harbors two single RNA strands tightly bound to the nucleocapsid protein p7, enzymes RT, and protease and auxiliary proteins Vif, Vpr and Nef (Figure 3). These viral components are all encapsulated by the capsid protein p24. The viral core is protected by an envelope consisting of the matrix protein p17 located within the host-derived membrane, which includes host-derived proteins and adhesion molecules acquired by the virus upon budding from the host cell [6]. The only strictly viral component of the envelope protruding to the outside of the viral particle is the glycoprotein region gp120 and parts of the transmembrane part gp41. The trimeric gp120 spikes are relatively few on the spherical virion (especially compared to the large number of envelope proteins on HSV-2 virions) ranging from four to 35 [26, 27]. An increasing number of viral envelope glycoproteins were associated with higher infectivity [28]. In addition, large parts of the envelope glycoproteins are glycosylated, making it possible for the virus to escape the host immune system.

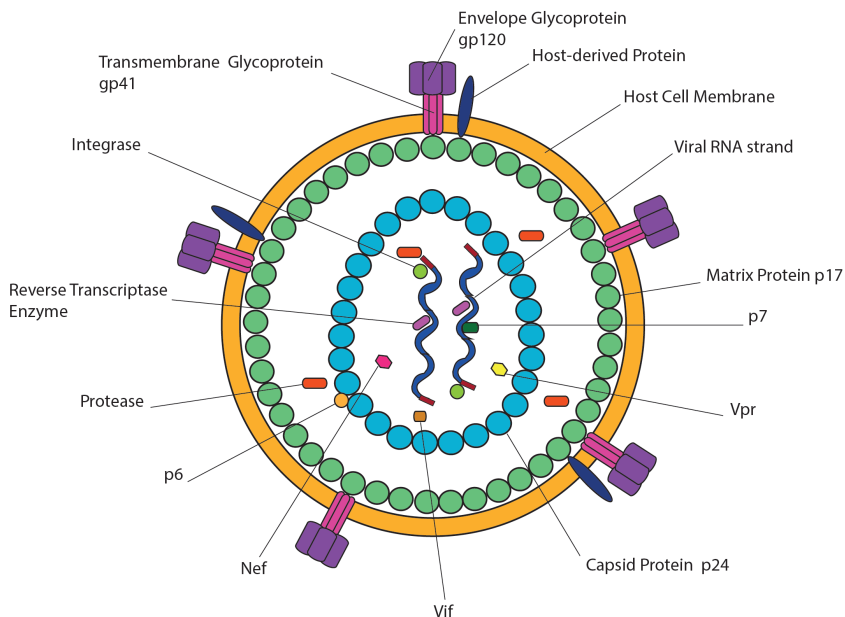


Figure 3. Structure of the HIV-1 virion.

1.2.2 HIV-1 envelope structure and glycosylation

The HIV-1 envelope gene constitutes approximately one fourth of the entire genome and consists of two subunits, the ectodomain part referred to as gp120 and the transmembrane domain gp41, which protrudes into the virion particle. The gp120 complex is composed of three non-covalently bound heterodimers which interact with cellular receptors in the viral attachment process. The gp41 region mediates the following step during cell entry, which is the fusion between viral and cellular membranes [29].

The first crystal structure of gp120 was that of a monomer bound to a 2-domain soluble CD4 molecule and a neutralizing monoclonal antibody [30]. Although only ~60% of the entire structure was covered by the crystal, some essential elements could be identified, which paved the way for further investigations of the gp120 architecture. However, the variable loops are notably cumbersome to crystallize since some of these regions are not involved in antibody- or receptor binding. Furthermore, some regions of gp120 may structurally differ when crystals are generated from complexes with varying antibodies (as reviewed by [31]). This phenomenon indicates that the molecule is very flexible and dynamic depending on the target it interacts with.

The domains of HIV-1 gp120 can be divided into five variable regions, V1-V5 [32], which may vary extensively in amino acid (a.a.) sequence between strains, and into five more conserved regions, C1-C5 [33] (Figure 4 A). Mutations in variable loops commonly occur without abolishing vital properties such as recognition of and binding to receptors. However, mutations in V3 loop, the major site for attachment/binding to chemokine receptors [34] and heparan sulfate [35], can alter the overall loop charge and change viral tropism for its co-receptors. For instance, viruses using the CXCR4 (X4) chemokine receptor carry a higher number of positively charged a.a. in the V3 region than viruses using the CCR5 (R5) chemokine receptor [36, 37]. In addition, R5 viruses tend to increase their net charge during end stage disease when the selection pressure from the immune response is decreasing [38]. The V1/V2 region also tends to vary significantly in the a.a. sequence, and it has been reported that co-receptor usage may be determined by charge alterations in this region as well [39].

The transmembrane protein gp41 is linked to gp120 and mediates fusion between the viral surface and the host cell membrane. The protein may be divided into three major segments; the N-terminal ectodomain, the transmembrane domain and the intraviral C-terminal segment, which

interacts with the matrix protein p17 [29] (Figure 4 B). The external domain of the protein comprises the fusion peptide involved in the crucial fusion process, the N-terminal heptad repeat region, the immunodominant region, which appears to be recognized by neutralizing antibodies [40, 41] and the C-terminal heptad repeat region. The transmembrane domain penetrates the viral membrane while the intraviral segment with the cytoplasmic tail protrudes to the inward of the virion and has been described to play a role in replication and infectivity [42].

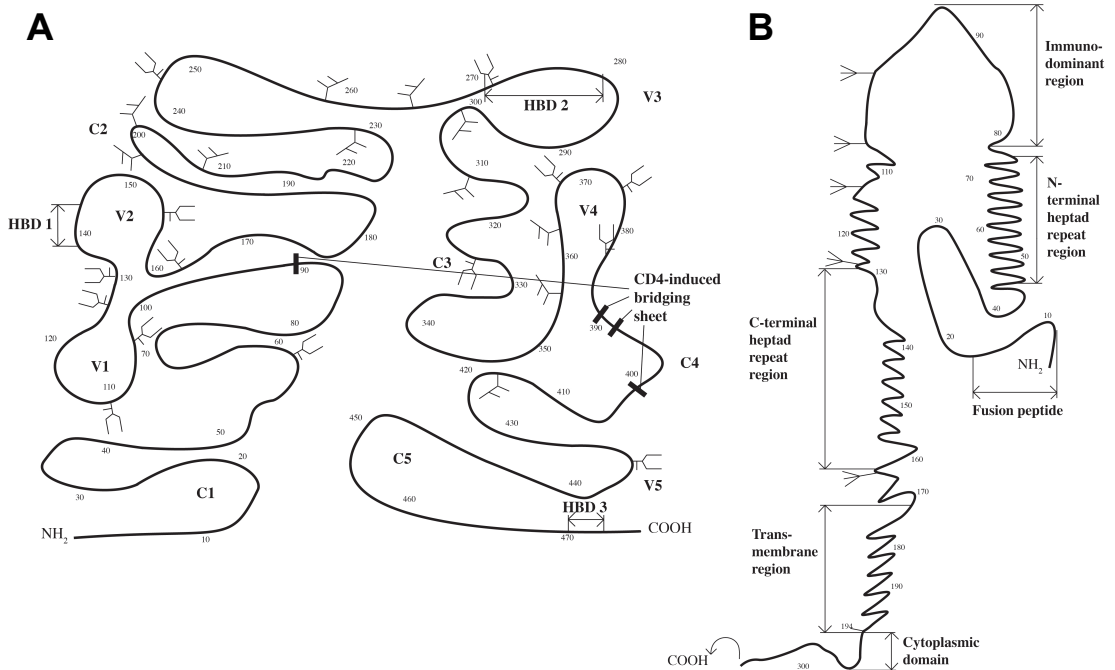


Figure 4. Schematic view of the HIV-1 envelope. Functional domains and structures of (A) the envelope glycoprotein gp120 and (B) the transmembrane region gp41.

Synthesis of the HIV-1 envelope protein precursor gp160 takes place in polyribosomes associated with the rough endoplasmic reticulum (RER) of the host cell. During this step the protein undergoes glycosylation with high mannose glycans [43-45], followed by an oligomerization most frequently to a trimer. This form of gp160 is then transported to Golgi where it is subjected to other posttranslational modifications including the proteolytic

cleavage of gp160 by cellular furin-like proteases into the two biologically active subunits gp120 and membrane-associated gp41, an essential step for HIV-1 infectivity [46-48]. During transport from the RER to Golgi some of the high mannose glycans are trimmed by host mannosidase, and subsequently elongated into complex type glycans by host cell-specified Golgi-associated glycosyltransferases, and in the fully processed gp120, the glycans are located in the external envelope part gp120 [49]. A gp120 monomer contains approximately 20-30 potential N-glycosylation sites, most of which are occupied, while the transmembrane subunit gp41 possesses 3-5 such sites. Thus, roughly 50% of the molecular mass of gp120 is constituted by N-linked oligosaccharides [43]. The carbohydrate molecules “shield” the virus from recognition by the host immune response, not least by neutralizing antibodies [50, 51]. The glycans present on the HIV-1 envelope glycoprotein are mainly of N-linked type, i.e., attached to asparagine (N) residue within a motif sequence Asn-X-Thr/Ser (where X is any amino acid except proline). Also from an antiviral point of view it is important to note that these glycans can occur as high-mannose or complex type. The high-mannose oligosaccharides, which are more frequent in the HIV envelope than the complex type glycans [52-54], represent a known target of anti-HIV intervention. High-mannose include several mannose residues while complex type instead possesses sialic acid, galactose and/or fucose.

1.2.3 HIV-1 cell attachment and entry

The major target cells for HIV-1 are the CD4⁺ T-lymphocytes, and this molecule constitutes the principal receptor for the virus. However, the CD4 receptor alone is not sufficient for successful attachment and entry into the cell. A co-receptor is needed, and, during transmission, the R5 receptor is preferentially utilized. Once the primary infection is established, the R5-tropic viruses are often the most predominant variants [55]. However, in some individuals, viruses are selected that utilize an additional, or entirely unique, co-receptor in form of the X4 receptor. These viral X4 variants are associated with a more virulent form of infection and a faster disease progression. Viruses able to use both of the co-receptors are referred to as dual-tropic (X4/R5).

HIV-1 infects target cells, such as macrophages and T-lymphocytes, by a sequence of events commencing with recognition and binding of envelope gp120 to CD4. This episode induces several major conformational changes including those of the heparin binding domains of the variable loops V1/V2

and V3 of gp120 [56], leading to a subsequent exposure of the binding site for the R5 or X4 co-receptors [45] (Figure 5). Further, the gp120 and co-receptor interaction results in the repositioning of the gp41 fusion peptide, which enables its insertion into the host cell plasma membrane followed by an association of separate heptad repeat domains located at the amino- and carboxy-terminal regions of the gp41 extodomain and formation of a six-helix bundle in a hairpin structure [57-59]. A consequence of the forceful association of the heptad domains of gp41 is that the cellular and viral membranes are brought into close proximity to each other, which permits fusion and allows the viral capsid to enter into the cytoplasm.

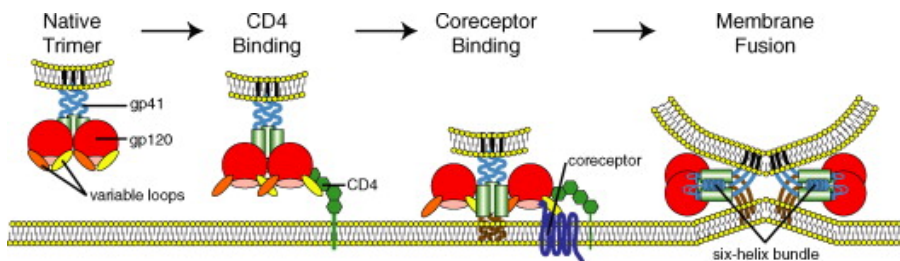


Figure 5. Receptor-attachment, co-receptor binding and fusion of HIV-1 envelope glycoproteins and cellular membrane. Adapted with permission from Tilton, J.C. and Doms, R. W. *Antivir Res* 2010.

It should be noted that CD4 is not the only surface molecule of importance for successful HIV-1 entry. Glycoprotein gp120 can interact with and bind to cells with a low or no CD4 expression through heparan sulfate proteoglycans (HSPG) [60]. Since heparan sulfate (HS) is present on most cell surfaces, tissues such as stratified layers of epithelial cells in the vagina are able to bind virions, translocate them across the epithelium, and present them to their principal target cells [61, 62]. In macrophages, the presence of HS may compensate for the low CD4 expression by capturing and concentrating a number of HIV-1 particles on the cell surface thus enabling their efficient infection [63]. Furthermore, the presence of HS of syndecan type proteoglycans on epithelial CD4-deficient cells may enhance infection not by replacing the CD4 receptor but through absorption of large quantities of virus onto the cell surface, protecting virions from neutralization by different components of cell environment, and/or by virion transmission to CD4+ T lymphocytes (in *trans* mechanism) [64, 65].

In addition, microvascular endothelial cells of the blood brain barrier harbor HS molecules that can bind infectious virions and assist them in the spread to brain tissue [66].

The binding of negatively charged cell surface HS chains to the basic residues of the V3 loop of gp120 is probably mediated by electrostatic interactions [56, 67]. However, it has been shown that upon interaction with CD4, gp120 binds more avidly to HS chains than native gp120 suggesting that the CD4-induced chemokine co-receptor binding site is also involved in the interaction of gp120 with HS chains [67]. Further studies confirmed the presence of four heparin binding domains in gp120 located in the V3 region and the co-receptor binding site, in the distal part of the V1/V2 loop, and a domain close to the C-terminal part of the protein [56]. These heparin-binding domains may contribute to the interaction of gp120 with HS chains [56]. In summary, HIV-1 gp120 binding to HS seems to occur in tandem with the CD4 binding thus increasing the virus attachment to and infection of cells. Moreover, it has been reported that HS chains can interact with the fusion peptide domain of gp41, an overall hydrophobic region, which is then activated leading to initiation of fusion between viral and cellular membranes [68]. Finally, HS chains may promote cell “surfing” of virus particles on spermatozoa and dendritic cells, a phenomenon that can be accounted for as a strategy for the virus to relocate to the receptor binding sites or to cells of preference [69, 70].

1.2.4 HIV-1 replication

After the fusion step, the viral capsid is released into the cell, and the viral RNA strands together with the RT, protease and integrase proteins initiate the replication process. In the early replicative phase, the two RNA strands are transcribed into double-stranded DNA catalyzed by RT, whereafter the proviral DNA can be transported to the nucleus and integrated into the host cellular DNA by the enzymatic activity of the integrase. This step of the replication cycle creates intra-host reservoirs of viral DNA, which enables life-long infection. From integrated DNA, new viral mRNA can be transcribed in the nucleus at any given time before being transported into the cytoplasm where it is spliced into smaller parts. Spliced viral mRNA is translated into proteins Tat and Rev and the latter binds the spliced mRNA or aids unspliced mRNA to exit the nucleus. At this time point other viral proteins are being translated, such as Env and Gag. When full-length mRNA constituting the viral genome is bound to Gag encoded proteins and the production of new virions can take place. In the last phase viral particles are

being assembled near the host cell plasma membrane. The polyprotein precursor, gp160, is cleaved into the envelope components gp120 and gp41 in the Golgi and is then transported to the plasma membrane where all viral parts, including the Gag and Gag-Pol polyproteins, are assembled to form new virions. The virus particles are then released from the cell surface by budding, using the host cell membrane as its own outer envelope. With this strategy the virus can at least partly circumvent the host immune response. A virion that has recently budded from a cell surface is immature and non-infectious until the Gag polyprotein is cleaved into its proper components by the protease. After maturation the virion can progress to infect other cells and the replication cycle is completed.

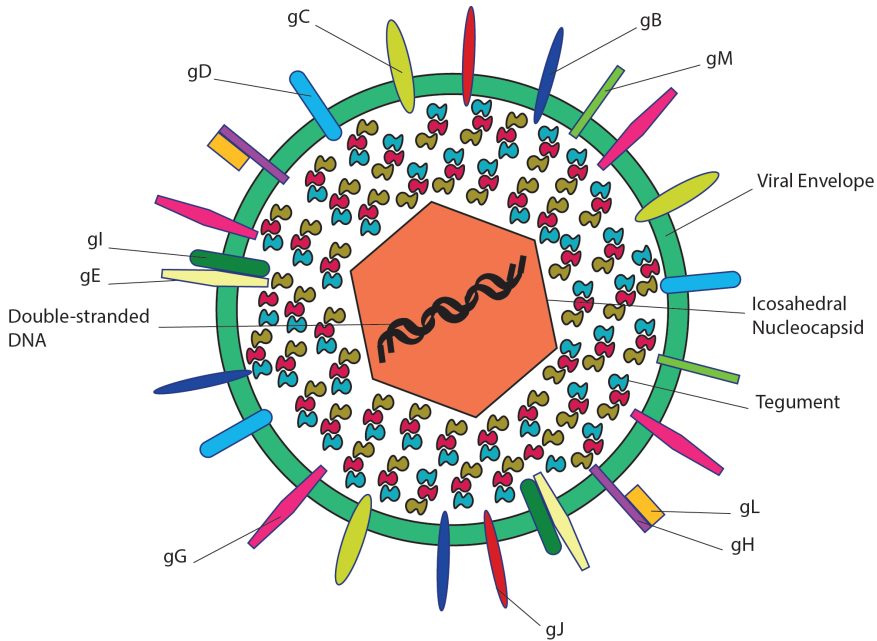
1.3 HSV-2 structure and viral entry

1.3.1 HSV-2 structure and genome

Herpes simplex viruses (HSV) are DNA viruses belonging to the order *Herpesvirales* [71] and the family *Herpesviridae*, which is further divided into three sub-families; alpha-, beta- and gammaherpesvirinae. HSV-1, HSV-2 and Varicella-Zoster virus (VZV) all belong to the human alphaherpesvirus sub-family. Human cytomegalovirus (HCMV) is a member of the betaherpesvirus sub-family while Epstein-Barr is a gammaherpesvirus.

The HSV-2 genome is approximately 155 kb, about 15 times larger than that of HIV-1, a size large enough to contain genes that encode for more than 80 proteins. The linear, double-stranded DNA is encapsulated by an icosahedral capsid surrounded by a protein-rich tegument (Figure 6 A). The virus is enveloped harboring at least 11 different surface glycoproteins (Figure 6 B). These glycoproteins are often exposed as complexes at the surface of the viral lipid envelope in form of spikes, an arrangement that enables the virus particle both to attach to and to penetrate into host cells via cell surface receptors such as HS or nectins and to efficiently release from infected cells. Furthermore, many HSV glycoproteins contribute to the virus protective functions such as evasion of host innate and adaptive immunity.

A



B

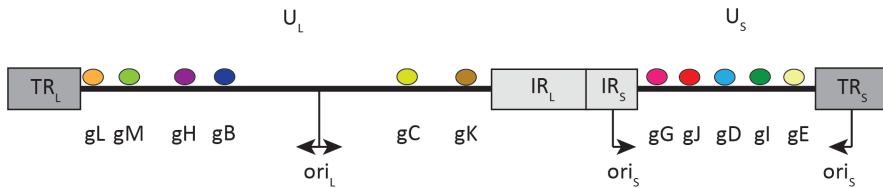


Figure 6. (A) The HSV-2 virion structure with its glycoprotein spikes protruding off the viral membrane. All glycoproteins are marked with colors corresponding to **(B)** the positioning of the genes encoding them in the genomic organization scheme. The genome is composed of two major segments, the unique long (U_L) and the unique short (U_S) regions. TR_L and TR_S define the terminal long and terminal short repeats, respectively. IR_L and IR_S denote the internal repeats (long and short) and ori_S and ori_L define the origin of U_S and U_L replication sites.

Table 2. HSV glycoproteins and their functions

Glycoprotein	Gene	Function
gB	U _L 27	Fusion between viral and cellular membranes
gC	U _L 44	HS-dependent attachment of virions to cells/immune evasion by binding to the C3b component of complement
gD	U _S 6	Initiates cell entry by binding to nectin, HVEM-1, or 3-O-sulfates HS
gE	U _S 8	Cell-to-cell spread/immune evasion by serving as receptor for Fc portion of IgG
gG	U _S 4	Not known but possibly involved in modulation of virus interaction with HS chains, and promotion of virus egress from cell surface ^[72]
gH	U _L 22	Modulation of virus fusion/cell-to-cell spread
gI	U _S 7	Forms complex with gE/cell-to-cell spread
gJ	U _S 5	Inhibits apoptosis in infected cells
gK	U _L 53	Cell egress
gL	U _L 1	Forms complex with gH
gM	U _L 10	Virus particle assembly

1.3.2 HSV-2 entry

HS chains serve as the principal receptors for HSV-2 attachment to cells, an event mediated at least partly by the viral envelope glycoprotein gB2 (gB in HSV-1 is designated gB1) [73, 74]. Experiments using the gB2-deficient virus showed that its interaction with HS was hindered, however when cultured with gB complementing cells the virus was repaired and HS-interaction could take place [75]. Interestingly, the gB protein is required for fusion between the lipid membranes of the cell and the viral envelope, and it is not known whether interaction of this protein with HS chains is of any importance for this process. Studies have shown that depending on the location of contingent mutations in the gB2 cytoplasmic tail, virus might be more or less virulent [76, 77]. Glycoprotein gC, the major protein mediating the interaction between HSV-1 and host cell HS [78, 79], is also responsible for HS-binding of HSV-2 and the gC2-HS interaction was more resistant to NaCl as compared to the gC1-HS binding [80, 81]. Glycoprotein gC of HSV-1 has a mucin-like region on the one-third amino-terminal part, which is lacking in HSV-2 counterpart gC2 [82]. Since modulation of the interaction between GAGs and HSV envelope glycoproteins seems to be dependent on the presence of the mucin-like domain of gC1 [72, 83, 84], this could explain why the binding of gC2 to HS is stronger than that of gC1. Interestingly, in HSV-2 the typical mucin-like domain is present on another glycoprotein, gG2, which is capable of interacting with HSPG or modulating of HSV-2 interaction with HS chains [72]. The gG2-negative virus mutants are infrequent in clinical settings and exhibit lower infectivity [85] suggesting a vital role of the protein in the virus-HS interaction. A proposed binding mechanism between the mucin region of gG2 and cell surface HSPG is based on the fact that the overall negatively charged mucin and HS chains normally repulse each other, a force that could be overcome by patches of positively charged lysine/arginine residues located in or nearby the mucin-like domain of the virus attachment proteins (Figure 7). Thus, an initial repulsion seems to maintain the threshold that prevents non-specific unwanted interactions of the positively charged attachment domain of the viral protein with many different negatively charged components at the cell surface, overpowered by only the high negative charge of HS or CS chains, i.e. the virus receptor molecules.

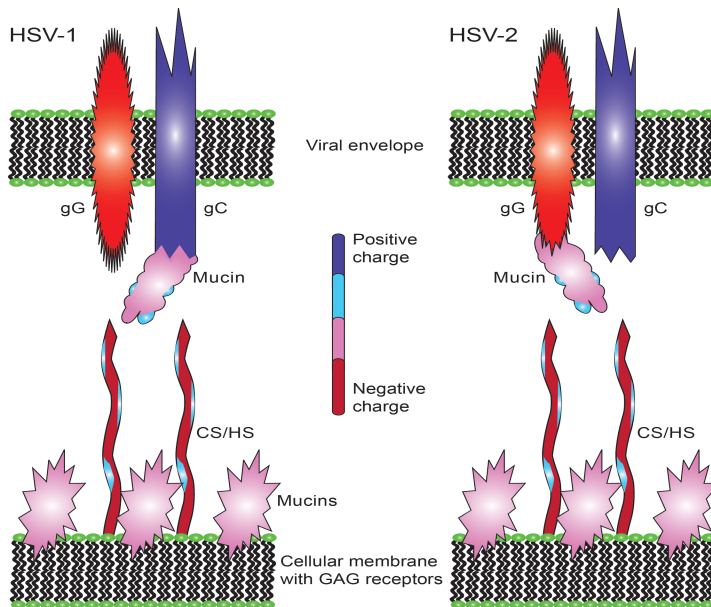


Figure 7. Model of the initial interaction between the mucin-like domain of gC1 and HS in HSV-1 versus HS interaction of gG2 mucin in HSV-2. The mucin-like region on gG2 participates in the binding of gC2 to HS, probably as a modulator.

After attachment of HSV to cell surface HS through the suggested gG/gC interaction, the viral gD component, as shown for HSV-1 but not yet for HSV-2, may bind to a specific stretch of HS containing 3-O-sulfated residues that is part of the antithrombin binding HS pentasaccharide (Shukla et al., 1999; Shukla and Spear 2001) (Figure 8). Furthermore, gD is activated upon binding to additional protein receptors represented by either (i) herpes virus entry mediator (HVEM), (ii) nectin-1 or (iii) nectin-2. These events may trigger conformational changes in gB, and in gH/gL, which initiates direct fusion between lipids of the cell plasma membrane and the viral envelope, and enables insertion of the nucleocapsid into the cell followed by initiation of its replication [86]. An alternate route of HSV entry into the cells is through endocytosis where HSV fuses with the endosomal membrane instead of the plasma membrane of the cell [87] whereupon the nucleocapsid is released and transported into the nucleus.

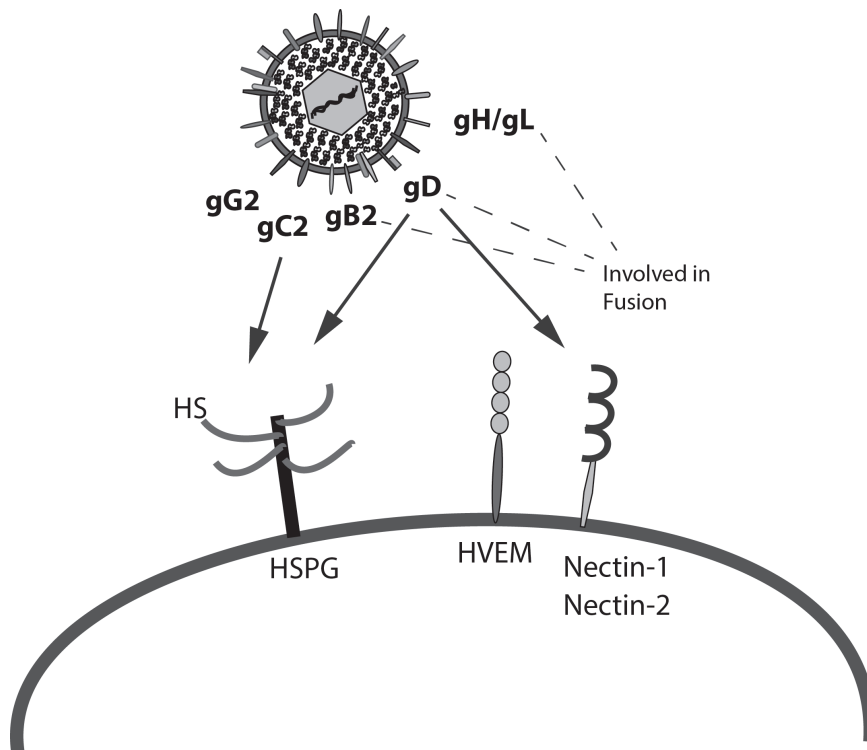


Figure 8. Schematic illustration of the HSV-2 envelope glycoproteins and their cell surface receptors.

1.4 Role of HSPG in HIV-1 and HSV-2 infection

Cell surface HS and chondroitin sulfate (CS) glycosaminoglycans (GAGs) are biologically important molecules with multiple functions exemplified by their participation in cellular processes such as cell adhesion, angiogenesis, neurogenesis, blood coagulation, cell differentiation, wound healing, lipid metabolism and others [88-91]. These carbohydrate chains are expressed on cell surfaces and in extracellular matrices throughout the entire body and are produced in a majority of mammalian cells. In nature, HS/CS chains are commonly conjugated with proteins to form HS or CS proteoglycans (HS/CSPGs). It is noteworthy that HSPGs play a major role in a successful infection of cells of both HIV-1 and HSV-2. When CD4⁺ T cells were treated with heparitinase, an enzyme that degrades HS chains resulting in

removal of the molecule from the cell surface, a significant decrease in HIV-1 binding to and infection of cells was observed [35, 92]. In HSV-2 infection, gB, gC and gD are the principal viral envelope proteins mediating the interaction with HSPGs [74, 81, 93].

1.4.1 Structure and modifications of HSPGs

HS is a typical GAG consisting of polysaccharide entities alternating between uronic acid (D-glucuronic [GlcA] or L-iduronic acid [IdoA]) and D-glucosamine (GlcN) units [94]. HS chains together with a conjugated protein linked via a specific tetrasaccharide sequence constitute the superfamily of HSPGs, which are further divided into sub-families. The two major plasma membrane-bound HSPG families are the glypicans and syndecans [88]. In addition, epicans and betaglycans belong to the minor membrane proteoglycans, and the basement membrane constituting cells express perlecan, agrin and collagen XVIII [95, 96]. It is the GAG (HS or CS) entity of HS/CSPG that is biologically active, and mostly due to the high negative charge of HS or CS chain they interact with basic components of cell surfaces and also with pathogens such as HIV-1 and HSV-2. HS mimetics like muparfostat and PG545 may therefore function as inhibitors of viral infection *in vitro* (as described in Paper I and II) as well as *in vivo* (Paper III).

HS is produced in the Golgi apparatus in a series of synthesis steps. As mentioned above, the HS chain consists of disaccharide entities of glucuronic acid (GlcA) and N-acetylglucosamine (GlcNAc) in a repetitive manner. The tetrasaccharide linker region (GlcA-Gal-Gal-Xyl) anchors this chain to a serine residue of the core protein [97] to form HSPG. The elongation of the HS chain is initiated by the addition of alternating GlcA and GlcNAc catalyzed by the HS co-polymerase. This step is followed by several modifications, the first one being the *N*-deacetylation and the second the *N*-sulfation of GlcNAc resulting in *N*-sulfo-GlcN (GlcNS). Next is the epimerization of GlcA to iduronic acid (IdoA) followed by 2-*O*-sulfation of IdoA, 6-*O*-sulfation of GlcNS and the final but less frequent 3-*O*-sulfation of GlcNS [88, 89, 91, 98-100]. By combining these types of modifications, distinct and specific binding sites for various ligands and viral envelope proteins can be created. For instance, experiments using modified heparin samples revealed that HSV-2 prefers target cells with 6-*O*-desulfated, 3-*O*-desulfated and 2-*O*-desulfated HS as opposed to HSV-1 that rather infects cells rich in IdoA [73] (see Figure 9).

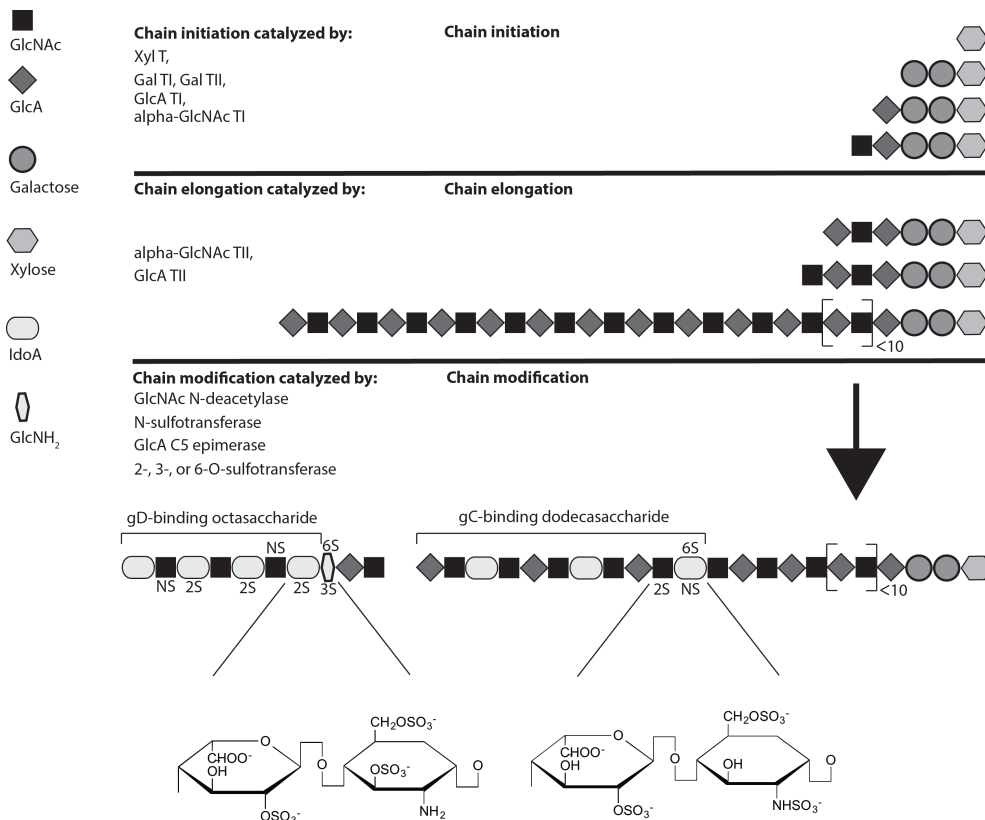


Figure 9. Structure and biosynthesis of viral glycosaminoglycan receptors. The process is initiated by chain elongation, where a xylose is added to the polypeptide backbone, followed by addition of two galactose residues and the addition of the first disaccharide (GlcNAc-Glc) repeat. This step is followed by chain elongation, where several disaccharide repeats are added. The next event in line is chain modification starting with a GlcNAc that is deacetylated followed by sulfation by and IdoA is generated by epimerization of GlcA. A gC- and a gD-binding domain that take part in HSV-1 attachment and prefusion, respectively, are marked. Adapted with permission from Olofsson, S. and Bergström, T. *Ann Med* 2005.

1.5 HIV-1 and HSV-2 transmission

1.5.1 Vaginal HIV-1 transmission

CD4⁺ T lymphocytes appear to be the main cell type targeted by HIV-1 in the vagina but macrophages can also be infected [101, 102]. For infection to occur, a chemokine co-receptor, R5 or X4 (or dual-tropic X4/R5) is needed in addition to the CD4 receptor. R5-using viruses are the most prevalent viral variants during transmission between persons [103, 104] and X4-viruses are associated with rapid AIDS progression and are found only in ~50% of HIV-1 infected individuals [105]. It has been proposed that those patients who remain with a population dominated by R5-using viruses instead harbours viral strains with a.a. changes in the envelope functional domains leading to increased viral fitness [38, 106, 107].

As virus enters the vagina it encounters the cervicovaginal mucus-like fluid present in the lower genital tract, which constitutes a barrier that may block and/or neutralize the HIV infectivity (Figure 10). The X4-tropic viral variants are especially sensitive to this blockage, partly explaining why the R5 viruses are the most common, if not the only variants, that are transmitted between individuals [55]. One explanation for the X4 viruses being more vulnerable to cervicovaginal fluids is that the positively charged a.a.s of the V3 loop are more exposed than in the R5 viruses, which might result in a stronger interaction between X4 viruses and negatively charged proteins and carbohydrates such as HSPG of this excretion. Another possibility is that the polyanionic mucin may bind better to X4- than to the R5-tropic viruses because of the charge interaction. This might hinder further movement and infection of the target cells. The epithelium also serves as a mechanical barrier against HIV infection [108] and the stratified epithelial cells of the lower genital compartment do not seem to be susceptible to infection with HIV-1 since these cells, although expressing the co-receptors, do not produce the required primary receptor CD4 [109]. However, as already mentioned HSPG of syndecans may sequester HIV virions on the surface of these cells and transport them by transcytosis to susceptible immune cells present in the deeper layers of the vaginal epithelium [64, 110].

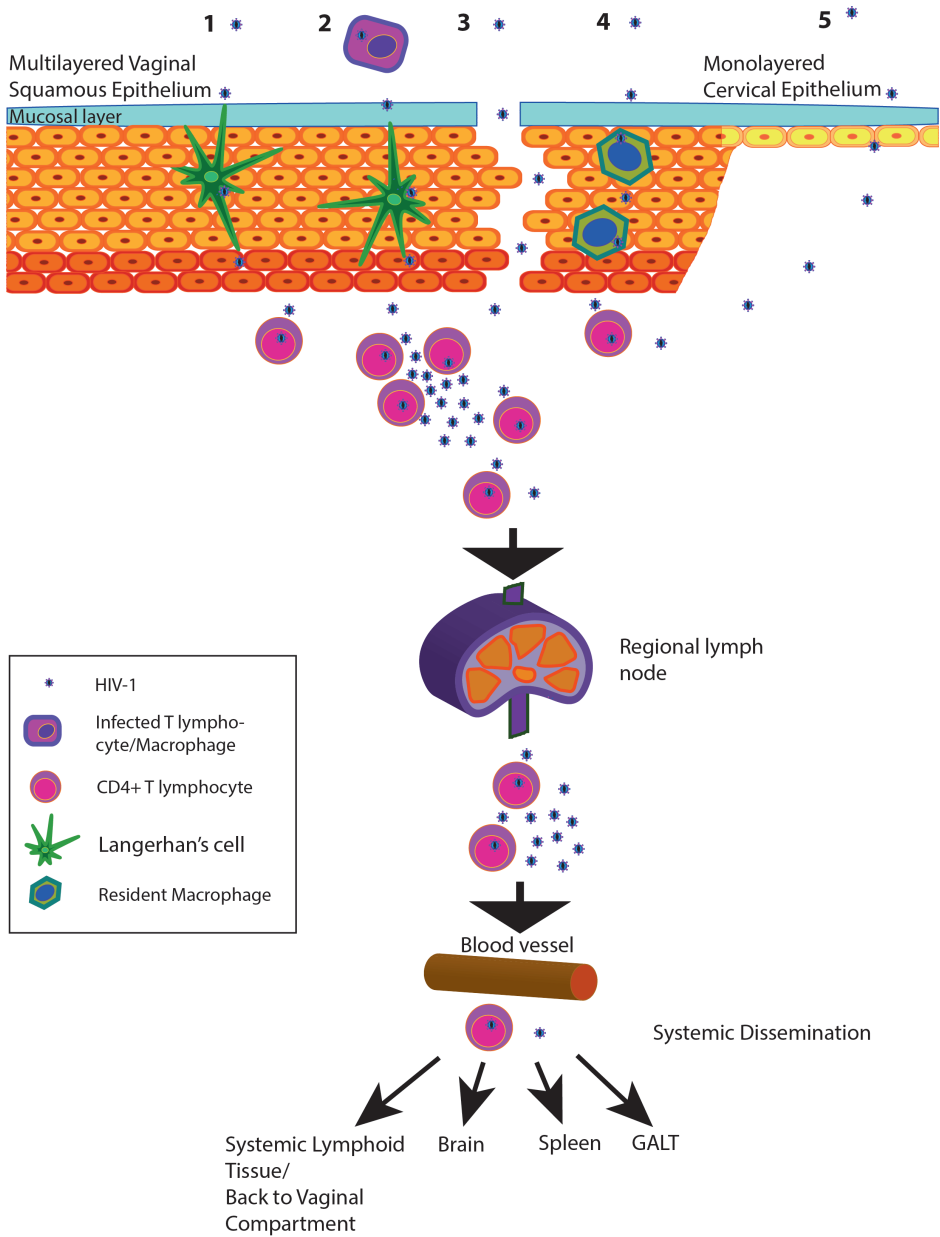


Figure 10. HIV-1 vaginal transmission. The first barrier for the HIV-1 virions (1) or HIV-infected cells (2) during intra-vaginal transmission is the mucosal layer lining the outer surface of the stratified epithelium of vagina or the simple epithelium of cervix (5). This barrier preferentially traps the CXCR4 (X4)- but not the CCR5 (R5)-tropic viral variants, resulting in a predominant prevalence of the latter virus in vivo. This phenomenon, referred to as a gate keeping mechanism, is reviewed by Grivel, J-C et al. (2010). The virions (3) may also reach susceptible cells through the local damages (breaks) to the continuity of the epithelium. Virions that successfully penetrate the mucus barrier and reach the epithelial cells are likely to confront a first line of susceptible target cells such in form of dendrites of Langerhan's cells (1,2). The virions that reach the epithelial cells could also bind to cell surface HSPG syndecans and be transported by transcytosis to macrophages (4). The virus particles derived from Langerhan's cells and/or macrophages may serve as a source of infection of CD4+ T lymphocytes. After extensive replication in primarily T lymphocytes and also in dendritic cells the virus and/or the virus-infected cells can efficiently disseminate into the regional lymph nodes where further replication occurs and progeny are transported to other parts of the body, such as systemic lymphoids, brain and gut associated lymphoid tissue (GALT) through the blood vessels.

The virus may also pass the epithelial cells through cavities, such as microlesions, that can emerge due to other infections, such as HSV-2, or due to sexual intercourse [111] and in that way reach the cells of preference. In addition, soluble factors, such as chemokine SDF-1, produced in epithelial cells, defensins [112-114] microbicidal enzymes, complement and surfactant proteins are all important defence mechanisms against incoming viruses [115]. SDF-1 can bind HIV-1 virions, especially X4-using viruses, and neutralize them [116, 117]. Also some defensins are more prone to inactivate X4 than R5 viruses [118, 119].

The major site for genital transmission of HIV-1 to females seems to be the endocervix, which is the interior part of the cervix. It is covered with a single-layer columnar epithelium, and is therefore not much protected against incoming viruses as opposed to the stratified epithelium that covers the vagina [115, 120, 121]. Moreover, cellular targets for HIV-1, such as T lymphocytes and Langerhans cells are also abundant in this area [122, 123]. The mucus, that covers the epithelial layers of genital tract, is produced by secretory cells located in the cervix. The major components of this fluid are different mucins [124] that, as already mentioned, protect the underlying epithelia from HIV-1 infection. Due to extensive cross-linking, secreted mucin proteins produce an intricate mesh which absorbs numerous water molecules to form a thick and sticky layer of mucus. The mucus can reversibly trap the HIV-1 virions and slow down their movement towards

underlying cells. Viral particles that manage to cross this barrier are likely to encounter the dendrites of Langerhans cells that can protrude through the epithelial layers to the vaginal lumen, thus becoming accessible to HIV-1 virions. The physiological function of these cells is to capture, process, and deliver antigens to the draining lymph nodes where T lymphocytes can neutralize them. Likewise, Langerhan's cells may possibly bind the virus at the vaginal lumen and transport it to the draining lymph nodes [125]. Notably, Langerhans cells express CD4 and R5-, but not X4-co-receptors, which might represent an additional obstacle for the X4-tropic viruses [126]. Furthermore, Langerhan's cells may be infected either through the *cis* mechanism, implying that there is a massive virus production leading to the establishment of infection resulting in long-term viral transmission [127], or through *trans*-infection, which implicates that transmission occurs across viral synapses [128] or via exocytosis of HIV-associated exosomes [129].

The most efficient way for spread of HIV-1 in the body appears to be through the direct cell-to-cell transmission rather than infection of host cells with cell free virus [130-132], which might be relevant to the relatively low risk of HIV acquisition upon contact with infected blood.

1.5.2 HIV-1 pathogenesis

The course of HIV-1 infection can be divided into three phases; the first being referred to as primary or acute infection, the second as sub-clinical or latent state and finally, if infection is untreated, the third phase in form of acquired immunodeficiency syndrome (AIDS).

Acute HIV-1 infection

When the first cells become HIV infected, the infection is established and viral DNA is incorporated into the host cell genome. During the first few weeks following infection, the individual might experience influenza-like symptoms [133]. This stage is associated with a high level of viremia and simultaneously a rapid decline in CD4+ T lymphocytes [134] (Figure 11). Through the lymphoid organs the virus is spread throughout the body and propagates in different organs. Especially the gut-associated lymphoid tissue (GALT) is affected where up to 80% of all CD4+ T cells have been reported to be depleted [135, 136]. Shortly after infection the HIV RNA load reaches a peak where after it declines drastically to a lower level that may be maintained stable for years. This level is referred to as the set point and a high set point is often associated with a faster disease progression. It is

therefore of great importance for a prophylactic drug, such as a topical microbicide, to strongly reduce or completely eliminate the amount of infectious viral particles transmitted during coitus. After a few weeks when the immune response is activated, the CD4+ T lymphocyte count recovers to a certain extent, however seldom to the initial levels if the patient remains untreated.

Sub-clinical phase

The period of primary infection, which normally lasts up to about three months, is followed by a phase of clinical latency. During this period viral replication remains at detectable but relatively low levels but normally increases slowly as the CD4+ T cell count declines in a gradual pace. The length of the latent phase varies between individuals, however the average duration is ten years. Both humoral and cellular immune responses are triggered upon HIV infection and are not only directed towards the virus but also act non-specifically by elevating the levels of inflammatory cytokines and activated immune cells. However, a small number (<1%) of infected persons denoted as elite controllers, are able to keep viral loads at near to undetectable levels and/or maintain the CD4+ T cell count without antiretroviral medication [137].

AIDS stage

The AIDS phase might be defined as the time point at which the level of CD4+ T lymphocytes drops below 200 cells/ μ L and remains low (in North America). Alternatively, the AIDS phase may be based on clinical symptoms, such as characteristic opportunistic infections (www.ecdc.europa.eu). However, the T cell depletion appears not to be caused by direct killing of all such cells but is probably due to the continuously activated immune response, which eventually becomes exhausted and the normal functions are altered [138, 139]. At this stage the immune system is dysfunctional and there is a subsequent susceptibility to opportunistic infections, such as tuberculosis, bacterial or viral pneumonia and toxoplasmosis. In addition, conditions such as non-Hodgkin lymphoma, chronic meningitis and AIDS-associated dementia are common. Establishment of HIV infection in microglia, as well as in other cell populations in the brain, explains the impact of the virus on the central nervous system.

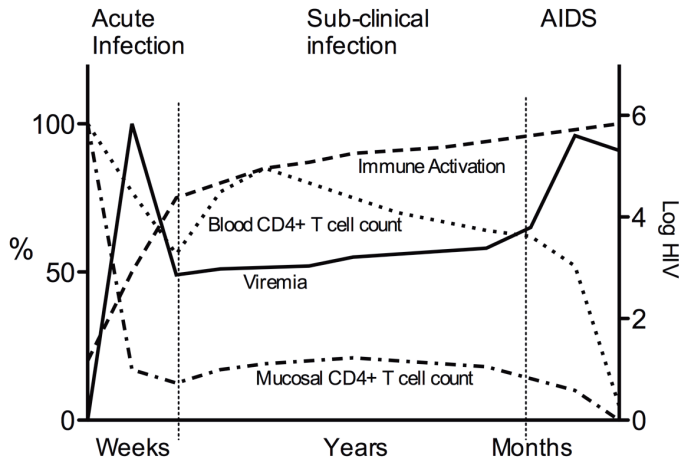


Figure 11. Course of HIV-1 infection [138].

1.5.3 Vaginal HSV-2 infection

Herpes simplex virus type 2 (HSV-2), together with HSV-1 the cause of genital herpes, is also a sexually transmitted infection (STI), which causes recurrent symptomatic [140] or non-symptomatic [141, 142] virus shedding from epithelial cells of the skin and/or genital mucosal surfaces. HSV-2 infection is an ulcerative disease, which is associated with a 2-3-fold increased risk of HIV acquisition [3, 143] and is thus considered as a major obstacle in terms of delimitating the spread of the virus in populations with high HSV-2 prevalence. In addition to a direct lesion of the mucosa, one explanation for this phenomenon might be that the acquired mucosal immune system, as response to a recurrent genital HSV-2 infection, drives the recruitment of HIV target cells such as T lymphocytes to the genital lesion sites [144]. Furthermore, in HIV-1 infected individuals the probability of being infected by HSV-2 is impending and an increased severity of such an infection is more likely to occur as the CD4+ T-lymphocyte cell counts decrease [145-148].

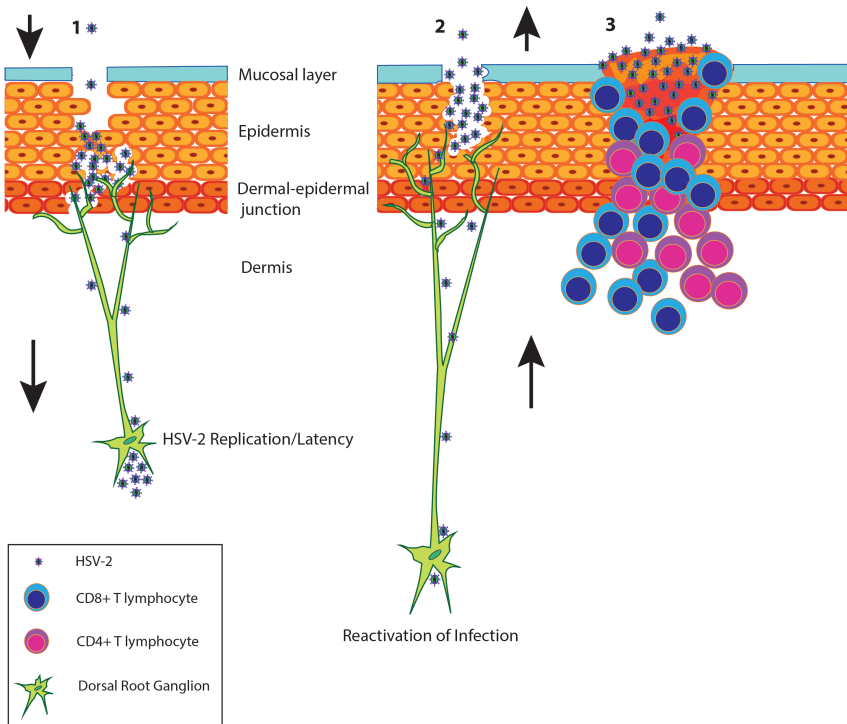


Figure 12. HSV-2 vaginal transmission. (1) Virus enters the epidermis through cracks or microlesions and replicates in the dermal keratinocytes. Virions are spread to cells in close proximity by cell-to-cell spread until reaching the sensory neurons where after they are subsequently transported to dorsal root ganglia. (2) In the neurons viral particles can reside and infection may be reactivated to local replication followed by migration through the sensory neurons back to the epidermal cells and shedding into the vaginal tract. (3) Replication and viral shedding can also occur by infection of CD4+ and CD8+ T lymphocytes recruited to the site lesion site.

Primary HSV-2 infection

Upon HSV-2 entry into the vaginal compartment, the viral particles first encounter the mucosal layers covering the peripheral sites of epithelial cells in the epidermis (Figure 12). The virus can either infect epidermal keratinocytes [149], which leads to rapid cell-to-cell spread to the inner layers of the dermis or enter the epithelium through microlesions caused by coitus or other STIs. In addition, superficial Langerhan's cells of the epidermal cell layers express cell surface entry receptors such as nectin-1 and/or HVEM receptors for HSV

viruses, making them susceptible to infection [150, 151]. Viral replication in epidermal keratinocytes leads to the production of progeny viral particles that egress by cell lysis leading to cell necrosis. These episodes are features of viral infection and replication and are manifested in patients as ulcer lesions.

Neuronal infection, latency and virus reactivation

Following primary HSV-2 infection of epithelial and dendritic cells, the progeny viral particles infect sensory neurons via their free terminals, which are present among the epidermal keratinocyte cells [149, 152]. The whole virions or their capsids are then transported along the axons into the dorsal root ganglia (DRG). The virus can replicate here and continue to spread to nearby neurons. Unlike the lysis of epithelial keratinocytes upon HSV-2 infection, neurons of DRG stay intact creating a possibility for the virus to establish a life-long latency. Viral reactivation can occur at any time point, and recent studies have shown that viral shedding followed by rapid clearance occurs frequently in infected persons regardless of symptomatic or asymptomatic manifestation of infection [153]. In many cases the virus reactivation seems to occur as a response to many different conditions of metabolic and/or environmental stress, such as ultraviolet radiation from sunlight, hormonal influences, other infections, etc. Following reactivation, virions are transported through the sensory neuronal axons, back to the epithelial cells and the mucosa. Here the virus replication and production of progeny virions leads to cell lysis and microulcer or ulcer formation followed by viral shedding into the vaginal lumen. Simultaneously the mucosal immune response is triggered and cytotoxic CD4⁺ and CD8⁺ T lymphocytes and dendritic cells are recruited from the draining lymph nodes and the blood vessels to the site [154]. These T lymphocytes persist at the lesion site for several months, even after the healing process is completed, in order to control the infection but their number decrease over time. If another site becomes HSV-2 infected, the primed T lymphocytes and dendritic cells shift to that site in an attempt to diminish the damage. B cells, natural killer cells and antibodies are also found at lesion sites but their roles are not yet clarified.

Despite the similarity of the two infections in terms of routes of transmission the viruses differ from each other in several functional and biological aspects, which creates obstacles when developing a microbicidal/antiviral compounds active against both viral infections.

1.6 Antiviral treatment and microbicides against HIV-1 and HSV-2

There are no permanent cures or pharmaceutical treatments for use against dual HIV-1 and HSV-2 infections but both are treatable with antivirals that may relieve symptoms and efficiently diminish viral replication. Nucleoside analogue acyclovir [155], which interferes with the HSV viral replication machinery [156], has been used against HSV-1 and HSV-2 infections since the late 1970s as one of the first licensed antiviral drugs and is still in use alongside some closely related derivatives, such as valaciclovir and famciclovir. In the late 1980s, only a few years after the discovery of HIV, the first antiretroviral drug against the virus was approved for clinical use. Nucleoside reverse transcriptase inhibitor (NRTI) zidovudine was primarily used as a single drug, however despite the promising initial rapid inhibition of the viral replication, mono-therapy of this kind was proven insufficient in the long run, allowing drug resistant mutants to appear in a short period of time. At present, treatment with the highly active antiretroviral therapy (HAART) is standard, implying combinations of at least three antiretroviral medications, targeting different events of the viral replication cycle, such as nucleoside/nucleotide reverse transcriptase inhibitors (e.g. tenofovir), non-nucleoside reverse transcriptase inhibitors (NNRTIs)(efavirenz), protease inhibitors (ritonavir), integrase inhibitors (raltegravir) and entry/fusion inhibitors (maraviroc and enfuvirtide).

However, neither curing treatment regimens nor prophylactic vaccines are available to date, despite decades of intense clinical and basic research on both viruses. The high virus turnover leading to high mutation rates during HIV-1 replication, integration of viral DNA into the host genome, intricate viral evasion mechanisms of the host immune system, and other factors all contribute to the explanation to why HIV-1 cure/prophylaxis remain as extremely complicated tasks.

Microbicidal (virucidal) compounds, also referred to as topical pre-exposure prophylaxis (PrEP), that irreversibly may inactivate infectious viral particles, prevent the virus from attaching to and fusing with host cells, hinder the virus from replicating or assisting the local immune defence of genital/rectal tracts, have offered supplementary opportunities to fight transmission of these infections in addition to condoms [157]. It has also been proposed that because of difficulties for women to negotiate male condom use for their own

safety[158-160], microbicides offer alternative means of protection from sexually acquirable diseases. PrEP formulations can be applied topically in different forms, such as gels, creams, foams, vaginal rings, films, in tablet form or as suppositories. Finally an ideal microbicide should protect the host against any possible co-pathogens, such as HIV-1 and HSV-2.

The concept of prophylactic, topical protection against genital pathogens has been established over 20 year ago. One of the first microbicide candidates taken to clinical trials was the detergent-like surfactant nonoxynol 9 spermicide (N9/COL-1492). It was successfully tested *in vitro* against HSV-2 and HIV-1 [161] and *in vivo* in a feline immunodeficiency virus animal model but failed in a Phase III clinical trial in which it appeared that the risk of acquiring HIV-1 infection increased in the group of women using the lead drug [162] probably due to disruption of the protective epithelial barriers in the vaginal tract [163]. Since then numerous substances with various mechanisms of action have been tested as microbicides leading to more than 50 clinical trials of different phases (see www.microbicides.org).

Table 3. Examples of microbicides by mechanism of action [164-166]

Category	Microbicide	Clinical Trial status	Mode of action
Surfactants	Nonoxynol-9	Failed, discontinued phase III	Disrupts the membrane/envelope of pathogens, including host cellular membranes
	Savvy	Failed, discontinued	
Buffers	Buffergel	Completed, no efficacy observed	Maintains genital pH in order to mobilize or keep vaginal defences
	ACIDFORM	Ongoing	
Blockers (sulfated polymers)	Carraguard	Failed, phase III	Inhibits viral attachment/entry into mucosal cells of HIV-1 and HSV-2
	PRO 2000	Failed, phase III	
	Dextrin-2-sulfate	Ongoing phase I/phase II	
	Cellulose sulfate	Failed, phase III	
Co-receptor blockers	Soluble CD4		Prevents fusion between the viral envelope and host cell surface by interfering with co-receptor binding
	Maraviroc inhibitor)	(CCR5 Ongoing	
Antiretroviral agents	Tenofovir inhibitor)	(NRT Ongoing	Blocking different steps of the viral replication cycle

1.6.1 Polyanions as microbicides

Sulfated, anionic, oligo- and polysaccharides such as pentosan polysulfate [167], mannan sulfate [168], fucoidans [169, 170], sulfated galactans [171, 172], xylogalactans [173], calcium spirulan [169] and others (see review by [174]) have long been known inhibitors of HIV-1 and HSV-2 infection. The fact that many such compounds have been extensively studied both *in vitro* and *in vivo* is due to their affinity to the receptor binding sites on the viral proteins, leading to interference with attachment to and/or entry of virus into host cells. The extreme negative charge of these molecules contributes to electrostatic interactions with virions thus abolishing the virus-receptor binding. The longer the saccharide chain and the higher degree of its sulfation, resulting in a higher charge, the more potent the molecule becomes. Despite the advantageous properties, these compounds have poor bioavailability and tissue-penetrating activity and are relatively unstable [174, 175]. In addition some of the substances, such as the HS-related heparin and polysaccharide dextran sulfate, tested in pre-clinical and clinical trials as microbicides against HIV-1 and HSV-2, have turned out to inhibit infection only in a reversible, non-virucidal manner. Dextran sulfate is an efficient HIV and HSV inhibitor *in vitro* however failed to show efficacy in a murine model against HSV infection [176] and in human clinical trials against HIV [177]. A microbicide candidate with irreversible, virus-inactivating capacity is therefore highly desirable. One example is seaweed λ -carrageenan 1T1 or Carraguard, which exhibited virucidal anti-HSV-1 and HSV-2 activities *in vitro* and in mouse models [178]. Similarly, cellulose sulfate and PRO 2000 showed potential as potent topical microbicides in pre-clinical studies and all three compounds have gone through Phase III clinical trials. Disappointingly, all trials were discontinued due to ineffectiveness owing to unknown reasons [179-182]. Although none of the compounds was directly toxic, one could speculate that some of the substances may have interfered with cell-cell contacts in epithelial layers leading to the possibility for virus to traverse these lesions. Need of proper *in vitro*, *ex vivo*, and animal models are thereby crucial for the development of safe and efficient microbicides. In this thesis a potent virucidal compound, P4/PG545, was studied as an inhibitor against HIV-1 *in vitro* (Paper I) and as an antiviral against HSV-2 genital infection in mice locally administered with the lead drug (Paper III).

1.6.2 Microbicide resistance

A topical vaginal or rectal microbicide should be designed to be applied frequently, perhaps as often as several times per day. The risk of developing drug-resistant intra-host viral strains is thereby imminent. This is probably especially true for already existing antivirals and antiretrovirals that are currently being investigated as potential microbicides [166, 183]. Theoretically, resistance might occur either during transmission of drug resistant viral isolates or might emerge due to selection of such strains in the individual using the microbicide following HIV infection [166]. *In vitro* studies have shown that NNRTI UC-781, which is closely related to efavirenz and nevirapine, induces resistance in HIV-1 isolates when passaged in cells with increasing drug concentrations fairly quickly [184, 185]. In addition, selected resistant strains in one of the studies appeared to be highly cross-resistant to several first-line NNRTIs (i.e. delavirdine, nevirapine and efavirenz) but less resistant to recently developed NNRTIs (i.e. lersivirine and etravirine) [185]. Although few human studies have investigated this matter as yet, the NRTI tenofovir has been extensively analyzed for its efficacy in recent clinical trials both as topical and oral prophylactic formulations against HIV-1. In CAPRISA 004, a double-blinded randomized controlled trial, tenofovir was given to South African women in a 1% vaginal gel formulation. There was no evidence of developed drug resistance in the women who acquired HIV-1 infection during this trial [166], however more studies are needed in order to investigate the impact of the dosing frequency on the induction of resistance. Unexpectedly, the drug decreased the acquisition rate of HSV-2 by 51 % despite earlier *in vitro* studies showing no anti-HSV-2 effect [4]. This outcome might be explained by the fact that the drug concentrations used *in vitro* were significantly lower than the concentrations reached in the genital tract when administered topically [4]. The concentrations used in one of the *in vitro* UC-781 studies were 100-fold lower than in the clinical trials thus suggesting that at high concentrations the transmission of such resistant isolates may be of limited significance *in vivo* [184].

In the case of sulfated polysaccharides and their derivatives, it has been reported that they are poor inducers of resistance *in vitro* [173, 186]. Nevertheless, generation of drug resistant mutants *in vitro* has been performed in the past and unveiled important information about the functions of viral attachment and entry processes. Dextran sulfate, a potent inhibitor of both HIV-1 and HSV-2, selected resistant HIV-1 mutant viruses that comprised mutations in the gp120 envelope region, specifically in the V3 loop that decreased the overall net positive charge, a selective advantage for

the virus to escape the negatively charged compound [187]. In contrast, cationic substances such as bicyclams instead selected for HIV-1 mutants with amino acids in the V3 loop that increased the overall net positive charge [188].

In our laboratory, HSV-2 escape mutant viruses were generated after being passaged in cell culture in the presence of increasing concentrations of the sulfated oligosaccharide muparfostat (formerly known as PI-88). Our hitherto published studies revealed that the mucin-like envelope glycoprotein gG2 was targeted by this sulfated oligosaccharide suggesting the involvement of the protein in GAG interactions during virus attachment and entry [72]. In this thesis, we have investigated HIV-1 escape mutants of muparfostat (Paper II) and the antiviral potential for a muparfostat derivative named P4 (or PG545) *in vitro* (Paper I) and in a mouse model (Paper III) as a microbicide against HIV-1 and HSV-2 respectively.

2 AIM

The aim of this study was to develop a microbicide for prophylaxis of male-to-female transmission of HIV infection. The specific aims were the following:

- To discover novel microbicidal compounds for prevention of HIV-1 infection by cell-based screening of a library of sulfated oligosaccharides
- To determine the antiviral and virucidal properties against laboratory strains and clinical isolates of HIV-1, as well as the cytotoxicity, of selected compounds with focus on muparfostat and its derivative cholestanol-coupled muparfostat P4/PG545
- To elucidate the mechanism of action of muparfostat and P4/PG545 against HIV-1 by *in vitro* time-of-addition assays and by generation of viral escape mutants resistant to muparfostat
- To investigate the antiviral and virucidal properties of PG545 in an *in vivo* model of genital HSV-2 infection.

3 MATERIALS AND METHODS

3.1 Compounds

The oligosaccharide muparfostat, originally derived from the yeast *Pichia Holstii*, was chemically modified into the present structure (Fig 1). Initially, hydrolysis of the extracellular phosphomannan polysaccharide from the yeast was performed resulting in a phosphorylated oligosaccharide fraction called PM5, which was there after sulfonated [189-191]. Muparfostat and analogues coupled to lipophilic tails, such as P4/PG545, were prepared by conjugating hydrophobic groups with various chemical properties to the reducing end of the oligosaccharide chain [192]. All compounds were analyzed by ^1H NMR, ^{13}C NMR and mass spectrometry. Substances were dissolved in water and kept frozen at -20°C stocks of 10 mg/mL.

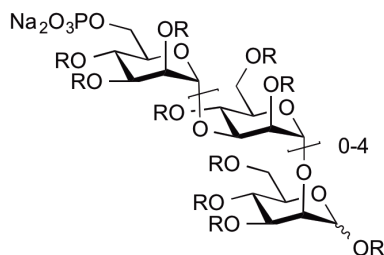


Figure 13. General chemical structure of sulfated oligosaccharide muparfostat.

3.2 Cells, viruses and clinical specimens

HIV-1 experiments with laboratory strain HIV-1^{IIIB} were performed in H9 cells in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (60 $\mu\text{g}/\text{mL}$), streptomycin (100 $\mu\text{g}/\text{mL}$) and polybrene (2.24 $\mu\text{g}/\text{mL}$). Clinical HIV-1 isolates, i.e., R5-tropic and dualtropic X4/R5 viruses, were cultured in peripheral blood mononuclear cells (PBMCs) from healthy blood donors. Cells were stimulated with phytohemagglutinin (2.5 $\mu\text{g}/\text{mL}$) in RPMI supplemented with 10% FCS, penicillin and streptomycin for 3-5 days prior to cultivation in fresh RPMI medium including, interleukin-2 (200 IU/mL), hydrocortisone (5 $\mu\text{g}/\text{mL}$) and polybrene. For experiments with HSV-2, strain 333 [193] was used in cell culture and animal experiments.

Samples of cervical secretions were obtained by swabbing the uterine cervix and were pre-diluted in NaCl solution. Another 10-fold dilution in RPMI medium resulted in a final 1:20 dilution.

3.3 Cell-based assays

3.3.1 Screening and IC₅₀ determination

In vitro screening of potential anti-HIV-1 agents was performed with the use of strain HIV-1^{IIIB} infection of H9 cells. Compounds (100 µg/mL of each) from a library containing muparfostat and lipophile-conjugated analogues were incubated with 100 times the 50% cell culture infective dose (CCID₅₀) of virus for approximately 5 min prior to infection of cells. Cells were monitored daily for cytopathic effect (CPE), i.e. syncytium-formation for 13-14 days, including a change of medium 6 days post-infection. Some cultures did not exhibit any CPE until as late as 10 days post-infection. Thus, the long incubation time proved to be an excellent way of distinguishing lead drugs with weak antiviral effect from the ones with stronger antiviral potencies. Infected cells excluding compound served as controls.

The compound concentration by which the substance was able to inhibit viral infection by 50%, IC₅₀, was determined for muparfostat and several analogues. In this thesis IC₅₀ values of P3, P4/PG545 and P5 are presented. Firstly, 100 × CCID₅₀ of each viral strain was incubated with the compound at different concentrations for 5 min prior to infection of cells. The CPE of cultures was monitored and supernatants were collected on days 6-9. End-point dilutions of virus were also performed regularly alongside the IC₅₀ determinations in order to ensure a more accurate CCID₅₀ due to the variation in culture conditions, such as different batches of medium and FCS and different blood donors. Virus levels were determined by a p24 *in house* ELISA performed on collected cell culture supernatants where optical densities (ODs) were converted into p24 quantities using a standard curve of recombinant p24. IC₅₀ values were determined by plotting the p24 levels against substance concentration. The detection limit of the ELISA was 0.5 ng/mL.

3.3.2 Cell proliferation

The cytotoxicity of the compounds was determined in H9 cells and PBMCs using a commercial cell proliferation kit (Promega). Cells were incubated with 2-fold dilutions of each lead drug in 96-well plates at 37°C for 24 h prior to addition of the commercial reagent. This assay was based on the bio-reduction of MTS tetrazolium compound in viable cells into a colored formazan product, which was measured after 4 h incubation by recording the absorbance (OD) at 490 nm. Cells without compound served as controls. Proliferation activity was expressed as % of cell control for each compound.

3.3.3 Virucidal assay

After the establishment of HIV-1 antiviral activity of muparfostat and derivatives, inactivation assays were performed where the virucidal activity was assessed. This assay was based on the assumption that if a compound binds to the virus particles and permanently inactivate their infectivity (virucidal activity) then upon dilution of this virus-compound mixture to the non-inhibitory concentration of compound, the infectious virus cannot be recovered. Recovery of infectious virus following its incubation with a compound in question is indicative of a lack of virucidal activity. In line with this assumption the test compound and virus were mixed together and incubated at 37°C for 60 min and then diluted 10-fold so that the lead drug reached non-inhibitory concentrations and was then added to cells to measure the residual infectivity of HIV-1. The virus suspensions without compound were processed in an identical manner to serve as controls. If no infectious virus in the highest dilution could be detected by p24 ELISA 13 days post-infection, it was reasonable to assume that the compound had inactivated all infectious virions.

In addition, this assay was performed in the presence of cervical secretions and FCS in order to determine the effect of such components, which may be present during natural vaginal infection, on the compound activity.

3.3.4 “Time-of-addition” assay

The mode of action of the compounds was investigated by “time-of-addition/removal” assays where the respective substance was added to the H9 cell culture at different time points relative to HIV-1_{IIIB} inoculation. If cells were pre-treated with compound and incubated for 2 h at 37°C prior to

washing of cells followed by viral infection, the activity of the compound on cells could be determined. Furthermore, the effect of the compounds on the virus was tested in a pre-attachment step where HIV-1III_B and substance were mixed 5 min prior to addition of cells and incubation for 2 h on ice. The low temperature probably affected the attachment/entry step by inhibiting the conformational changes of gp 120/41, that normally occur at 37°C, and its interaction with the compound by blocking the binding to cellular receptors. In addition, the post-entry step was challenged by a first incubation of virus and cells on ice for 2 h where after the cells were washed and fresh medium supplemented with compound was added to cells prior to incubation for 2 h at 37°C followed by a final wash and medium change. In the final assay the cells were pre-incubated with the virus for 2 h at 37°C where after the compound was added. By this strategy the antiviral effect could be studied when the compound was present outside the cell shortly after viral infection. All cultures were analyzed for p24 antigen contents by the *in house* ELISA at day 13 post-infection and the results were expressed as p24 levels.

3.3.5 Generation of muparfostat-resistant virus and gene sequencing

Viral strain HIV-1III_B was cultured in H9 cells in the presence of increasing amounts (between 12.5 µg/mL to 100 µg/mL) of muparfostat. Cultures were monitored for syncytium-formation at 7 days post-infection and supernatants from cultures with visible CPE were passaged by transferring 75 µL to fresh cells in the presence of the same or elevated lead drug concentration. A total of 24 passages were performed, including limiting dilutions between passages 19-21 where supernatants were diluted 10-fold in medium, cultured in fresh cells where after supernatants from the highest dilution that induced CPE was passaged further. IC₅₀ values of muparfostat were determined as described above.

This very strategy was used in a new set of experiments in order to attempt to generate a P4/PG545-resistant strain. A strategy of starting at a concentration near the IC₅₀ value, then slowly increasing the amount of the substance may be an efficient way of selecting for viruses resistant to virucidal compounds, such as PG545.

Another strategy was applied to the non-virucidal muparfostat in order to apply an even stronger selection pressure on virus than in the first protocol. In this experiment, the starting concentration was 100 µg/mL (several times

higher than the initial concentration) instead of 12.5 µg/mL and passages of supernatants were performed at later time points, 7-14 days, sometimes including change of medium supplemented with lead drug on day 6.

Nucleotide gene sequencing of passaged HIV-1_{IIIB} was performed by lysis and reverse transcriptase PCR of virus obtained from cell culture supernatants into cDNA using primers coding for the viral envelope gene. Five primer pairs were used in order to generate DNA fragments suitable for sequencing and sequences were analyzed by Sequencher 4.9 software.

3.4 Mouse model of vaginal HSV-2 infection

The mouse model described in this thesis has turned out to be very useful for pre-clinical studies of prevention of vaginal HSV-2 infection. Although the genital infection in humans differs from the mice model utilized here, important features can be analyzed since the female mouse exhibits clinical manifestations upon vaginal instillation of HSV-2, such as genital lesions as well as impact on the CNS, including hind limb paralysis and eventually death [194]. The model of intravaginal infection in murines used in this thesis was first described by in 1984 [195]. The vagin

al HSV-2 challenge should ideally be performed after treatment with progesterone since the ability for the virus to infect the mouse through the genital tract decreases with the age of the animal [196].

3.4.1 Vaginal administration of virus and compound

Two types of assays were performed using an intravaginal challenge in a mouse model. In the first, a “time-of-addition” experiment was undertaken, in which a fixed concentration of compound PG545 (500 µg/mL) was administered vaginally at different time points relatively to infection with viral strain HSV-2 333. This experiment was performed in order to elucidate the mode of action of the substance in mice. The other assay involved pre-incubation of virus with different lead drug concentrations (ranging between 20 and 500 µg/mL) where after the mixture was instilled vaginally mimicking the virucidal assay performed in vitro with the viral strain HIV-1_{IIIB}. In all experiments a group of non-PG545 treated mice were included as controls.

At day 3 post-infection, when local viral replication reached its peak, vaginal lavages were collected where after viral DNA levels were determined by real-time PCR and infectious virus quantified by plaque number-reduction assay in GMK AH1 cells. Mice were monitored daily and scored according to the degree of vaginal inflammation and to their general condition. Both survival and disease scores were recorded and plotted as % of surviving mice and of mean disease score, respectively.

3.4.2 Tissue collection and immunohistochemistry

In order to assess the viral loads of HSV-2 in neuronal tissue and to compare completely healthy mice that had recovered from vaginal infection with control mice, tissue samples from the CNS were collected. On day 6 post-infection, when the HSV-2 infection normally has reached neuronal cells, anesthetized animal were infused with 4% formaldehyde and the spine was removed, decalcified and sectioned into pieces including the sacral dorsal root ganglions, where the infection is located, and the spinal cord. The sections were then dehydrated and imbedded in

paraffin and incubated with rabbit antibodies against HSV-2 antigen. Immunoreactions were visualized using liquid DAB and DAKO substrate. The immunohistochemistry preparations were analyzed by Zeiss Axio Image M1 microscope and AxioVision software.

4 RESULTS AND DISCUSSION

HS mimetics, i.e. negatively charged oligosaccharides, such as muparfostat, have been shown to act as potent inhibitors of HIV-1 infection *in vitro* by interfering with the interaction between positively charged domains of the viral envelope and negatively charged host cell surface HS molecules. In previous studies from our laboratory, muparfostat and analogues prevented HSV-2 infection in cell culture in a non-virucidal, manner [83, 84, 197]. However, for a candidate microbicide aimed at prophylaxis of HIV transmission to have inhibition of electrostatic binding as its only mode of action is insufficient since this type of antiviral mechanism tends to be reversible. In this thesis, the activity of muparfostat and several lipophilic-conjugated derivatives synthesized for possible virucidal effects, in particular P4/PG545, were thoroughly examined against HIV-1 *in vitro* and HSV-2 in an *in vivo* murine model.

In **Paper I**, we screened a library of compounds based on muparfostat (see Figure 14), a chemically sulfated oligosaccharide originating from the yeast *Pichia Holstii*.

Muparfostat has been shown to inhibit infection and abrogate cell-to-cell spread of HSV-2 *in vitro*, without displaying virucidal properties [72, 84]. Thus, new analogues were synthesized and modified by addition of various groups, mainly hydrophobic, to the reducing end of the oligosaccharide chain with the aim of improving the antiviral activities. Compounds P3 and P4 both exhibited virucidal capacity against HSV-2 [84] and were tested, as described in Paper I, against laboratory strain HIV-1IIIB and two clinical HIV-1 isolates.

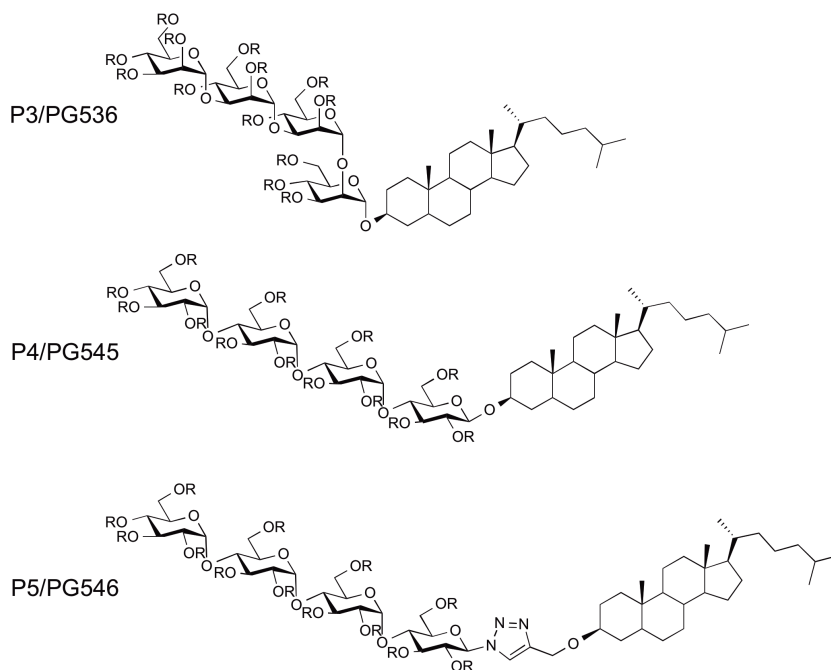


Figure 14. General chemical structure of sulfated muparfostat analogues.

In the present work, compounds P3, P4/PG545 and P5 were all found to be potent inhibitors of infection and irreversibly inactivated the laboratory strain HIV-1III_B. However, there was a difference in the virucidal activity demonstrated against the two clinical isolates. The CCR5-using isolate was more efficiently inactivated by P4 and P5 than the CXCR4/CCR5-dual-tropic virus, while the opposite was true for P3 (see Table 4). Although we cannot explain these differences at present, and only few strains have been studied, the findings suggest that GAG-binding and co-receptor usage might be interdependent.

Table 4. Inactivation assays with HIV-1HIB and clinical isolates of P3, P4 and P5.

Compound	Virus	Final CCID₅₀	Residual Inf. (%)
P3	X4/R5	100	0
		10	0
	R5	100	1
		10	63
P4	X4/R5	100	1
		10	87
	R5	100	0
		10	0
P5	X4/R5	100	0
		10	84
	R5	100	0
		10	0

Also cytotoxicity values differed between the substances, P3 being the least toxic compound. P4 had a lower toxic effect than P5, which may be due to the triazole linker connecting the cholestanyl group with the carbohydrate chain present only in P5. To investigate sensitivity to protein binding/inactivation of the compounds, maintenance of the virucidal potencies was tested in an inactivation assay in the presence of 10% fetal calf serum and cervical secretions. Compound P4 was only slightly affected by cervical secretions, compared to P5 for which the activity drastically dropped. When the effect of fetal calf serum on antiviral potency was tested, both compounds were markedly impaired. Taken together these results suggest that a lipophilic cholestanyl group, β -linked rather than α -linked, to a sulphated maltotetraose or maltotriose, without a triazole linker may be the most appropriate in terms of toxicity, stability in protein-rich environments and virucidal activity against CCR5-tropic viral strains, although these properties ought to be further improved if to be developed as a topical microbicide.

In **Paper I** the mode of action of the cholestanyl-conjugated derivatives was investigated in a time-of-addition assay using compound P3. Substance was added to cell cultures at different time points relative to HIV-1III_B infection.

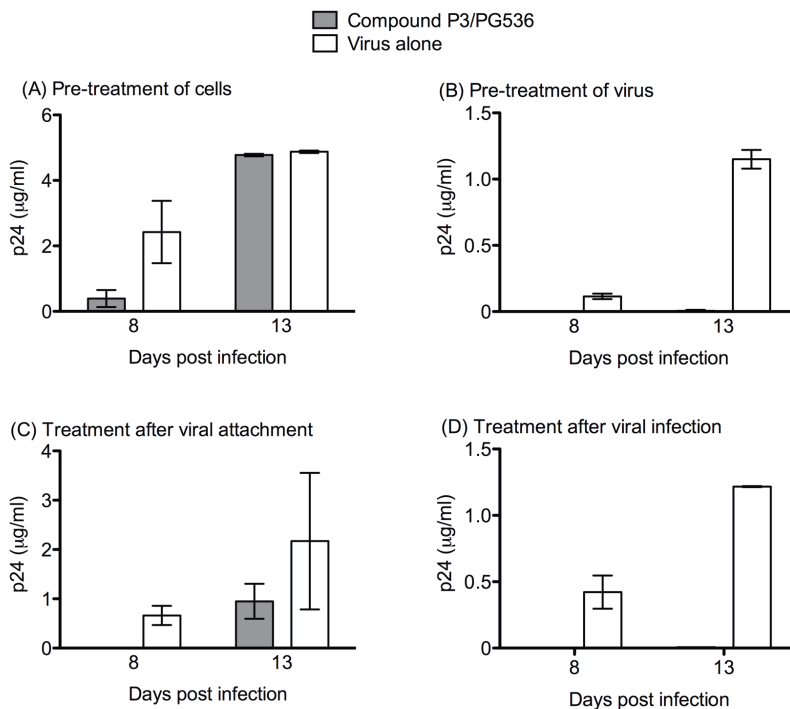


Figure 15. “Time-of-addition” assay with P3/PG536 and HIV-1^{III}B. In (A) H9 cells were incubated with compound for 2 h at 37°C prior to virus infection as opposed to (B) where instead virus was first incubated with compound for 2 h on ice where after the cells were added. In (C) cells and virus were mixed and kept on ice for 2 h prior to addition and incubation with the compound for another 2 h at 37°C and washing. In (D) cells were first mixed with HIV-1^{III}B and incubated at 37°C where after cells were washed and compound P3/PG536 was added. All cultures were monitored for as long as 13 days and supernatants were collected 8 and 13 days post-infection and analyzed by ELISA for p24 antigen quantification. The p24 values were expressed in µg/mL.

The studies were performed to define the phases of the infectious cycle in which the antiviral compound was active. Optimal antiviral effect was achieved when virus was pre-incubated with compound for a few minutes (Figure 15 B) and after viral entry (see Figure 15 D) suggesting interference of the virus-cell attachment step and probably also with egress of newly

produced viral particles. This dual-phase of activity could be explained by the fact that viral entry and egress are closely related processes, often involving similar molecules and even domains both on the virus envelope proteins and cellular membranes. Another finding was the lack of mature virions, as compared to untreated but HIV-1 infected controls, when the effect of P4/PG545 was studied by electron microscopy. In the presence of the drug the virus was possibly restricted from egressing and assembling its envelope properly. When the compound was added after viral attachment but before entry, only partial blocking of the infection was seen, further supporting the hypothesis that the molecule interfered with the attachment step. Interestingly, these results on the antiviral kinetics were supported by *in vivo* data in **Paper III** where P4/PG545 was evaluated in a mouse model of vaginal HSV-2 infection. There we found a 50% survival rate amongst mice administered with the compound just minutes before viral infection and almost 40% of mice survived when treated locally with the substance as long as two hours after infection. Taken together, P4/PG545 fulfilled two important criteria for a prophylactic microbicide, i.e. to block infection at an early stage, and to have a virucidal effect. In addition, the probable effect on egress may suggest that the compound also hold promises as a conventional antiviral substance, since effective anti-influenza drugs such as Tamiflu and Relenza act by blocking virus release [198].

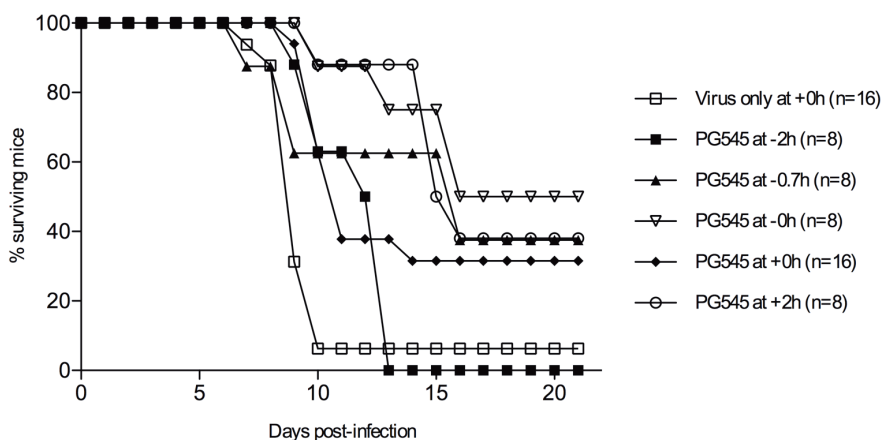


Figure 16. Proportion of survival of vaginally HSV-2 infected mice in a time-of-addition assay of PG545.

An ideal microbicide intended for vaginal/rectal use in connection with coitus should be potent enough to function when applied before or even after sexual intercourse. A shortcoming of the current results of the time-of- addition was that we did not achieve complete protection at any time point. Indeed, the task to abrogate infection by a single dose of a compound is a difficult one. In addition, the viral challenge dose in this murine model was higher by several orders of magnitude than what is estimated during natural infection of HSV-2 in humans. Follow-up studies using a lower virus dose, and drug instillation at time points closer to infection, are underway to attempt to achieve higher survival rates.

In parallel with the findings presented in **Paper I** (Figure 15), in the time-of-addition experiment in **Paper III**, virtually no antiviral effect of compound P4/PG545 could be demonstrated when the compound was instilled 2 hours before vaginal HSV-2 infection. Although pharmacokinetic data are lacking, these results indicate a relatively short biological half-life of the compound *in vivo* as well as *in vitro*. Furthermore, the pronounced antiviral potency when the compound was added immediately before infection, but lack of activity at earlier time points, may support a direct effect on the virus and its attachment domains rather than blockage of cellular sites.

Further support for the concept that PG545 and its components interfered with viral entry was given in **Paper II** where a.a. substitutions in envelope glycoprotein regions of gp120 and gp41 appeared after numerous passages of HIV-1_{IIIB} in the presence of muparfostat. By inducing a selection pressure *in vitro*, escape mutant viral strains may emerge carrying important information about the mode of action of the compound and also which protein regions that are targeted by the substance. In **Paper II**, muparfostat, instead of the virucidal PG545, was chosen based on its moderate antiviral effect, for generating compound-resistant HIV-1 viral variants *in vitro*. The mutants selected for after up to 24 passages displayed 3-4-fold elevated IC₅₀ values compared to wild type and control virus passaged in the absence of the substance. These results indicated that the compound, a relatively weak and non-virucidal inhibitor, was capable of selecting moderately muparfostat-resistant variants. The decrease in sensitivity to the compound was accompanied by mutations in specific domains of gp120 and gp41. Single mutations, leading to a.a. substitutions located in the variable loops V2 and V3, and, in addition, a deletion of a five amino acid stretch (366FNSTW370) in the V4 loop, were detected.

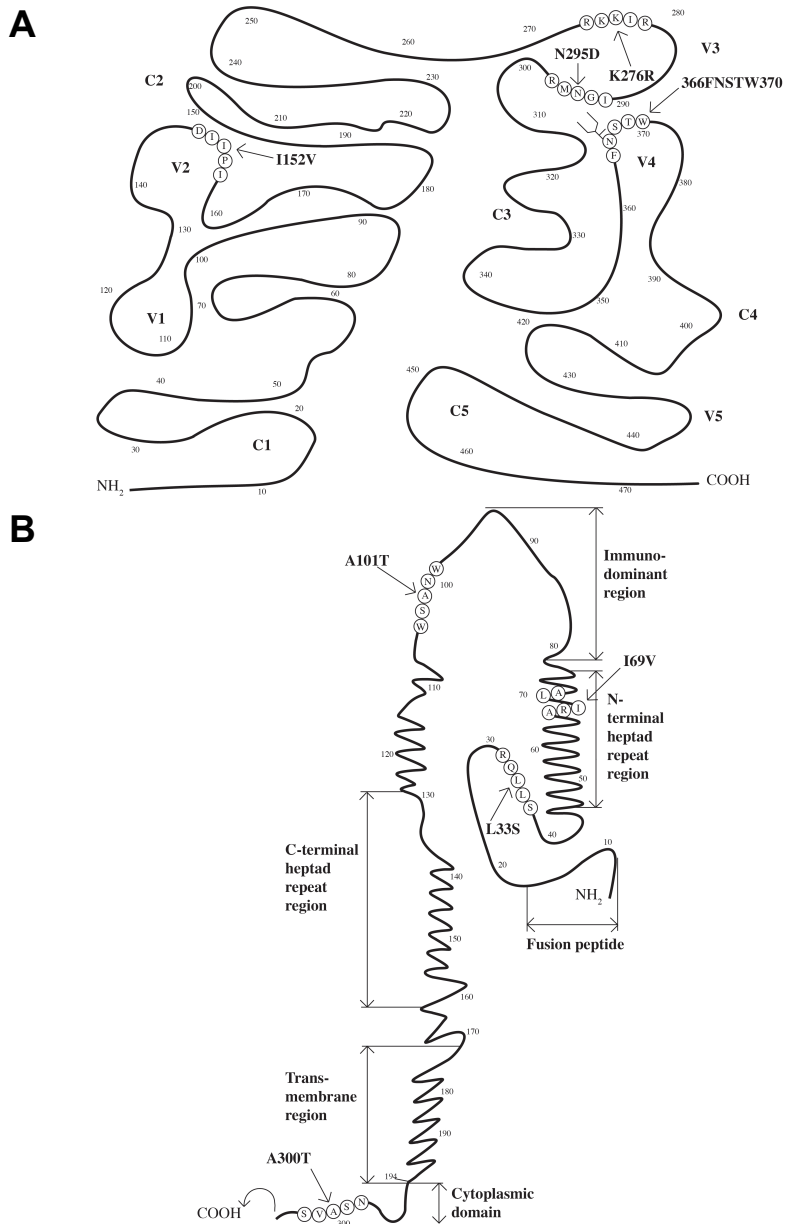


Figure 17. Location of mutations in HIV-1 nucleotide sequences coding for the viral envelope resulting in amino acid substitutions after virus-passaging the presence of muparfostat in (A) the gp120 region and (B) gp41.

The deletion comprises a glycosylation site for an N-linked glycan and is a common alteration in viruses resistant to sulfated polyanions or polycations. This specific deletion has previously been shown after selection with dextran sulfate [187], lipophile-modified cyclodextrin sulfate [199] and bicyclam [188, 200], the latter being a macrocyclic polyamine, which interacts with the X4-receptor. In the muparfostat-passaged virus, this alteration was the only of the three variants found in gp120 that has been associated with antiviral resistance in other studies. Valine at position 152 in the V2 loop of the passaged virus was found in several published sequences [201, 202] but has not been linked to resistance. Due to the location in the hypervariable region of the V2 loop of gp120, this a.a. position is likely to be subjected to variance.

It has been described that the here demonstrated pentamer deletion in V4 often appears in tandem with several amino acid changes in the V3 loop contributing to an altered overall net charge, as in dextran sulfate resistant variants [187]. Those studies show that the positive charge of the V3 loop decreased owing to three a.a. substitutions in the region thus weakening the interaction between the negatively charged receptor domain and the positively charged V3 region. In our study, the a.a. change from lysine to arginine (K276R) is probably of smaller importance since the overall charge in V3 was not altered. On the other hand, arginine has been suggested to act as a more potent HS binder than lysine. The gp41 transmembrane region was also subjected to a.a. substitutions. The change from hydrophobic a.a. leucine to the polar serine was unexpected, and we suggest that this mutation may influence the fusion process in which the heptad repeat regions undergo conformational changes. However, two a.a. substitutions in gp41 of passaged controls were also detected. This indicates that spontaneous alterations in the a.a. sequence, that are not related to development of antiviral resistance, frequently occur during extensive passaging of virus in vitro.

4.1 Preliminary results

We have, in a fourth set of passage experiments, attempted to generate HIV-1 escape mutants resistant to PG545. A cholestanol-conjugated oligosaccharide molecule may exhibit similarities with muparfostat in the mode of action but because of the virucidal properties, which are lacking in muparfostat, there are probably some major differences in the selection process. Previous studies in our laboratory showed that when respiratory syncytial virus was passaged in cell culture in the presence of muparfostat or PG545, the

envelope protein G appeared to be targeted by both compounds. However, mutations leading to a.a. substitutions at different positions were specifically selected for by the respective compound [203].

The PG545 molecule, owing to its irreversible virucidal capacity, either eradicates all virions if the concentration is sufficient, or leaves enough amounts of infectious viral particles in the cell culture to enable them to replicate and expand, but without selecting for any resistant variants. Therefore a cautious approach was applied where, as in Paper II, passages with viral strain HIV-1IIIB in the presence of slowly increasing concentrations of the lead compound in H9 cells were performed. Virus passaged alongside PG545 supplemented cultures, without any compound served as control.

Table 5. Preliminary results.

HIV-1 passaged with muparfostat					
Passage no.	Day	Virus/ supernatant volume	Compound concentration ($\mu\text{g}/\text{mL}$)	Amino acid change in gp120	Amino acid change in gp41
0	0	750	50	wt	wt
1	14	250	50		
2	22	150	100		
3	28	150	100		
4	35	75	200		
5	45	500	200	N295D	wt

In parallel with the PG545 passages, muparfostat was once again passaged in H9 cells (as in Paper II), however this time with a higher starting concentration with the aim of creating a stronger selection pressure and, if

possible, a more substantial increment of IC₅₀ values. After 5 passages, gene sequencing of envelope gene regions gp120 and gp41 was performed using the same primer pairs as described in Paper II.

The outcome after five passages was as follows: no mutations were detected in the PG545-passaged cell cultures compared to control virus and wild type HIV-1III_B, suggesting the number of passages were inadequate and the compound concentrations too low in order to achieve any resistant escape mutants. Further passages are ongoing. Interestingly, after five passages at the higher concentration, the muparfostat-passaged virus displayed one single nucleotide mutation leading to a.a. substitution N->D at the position 295 in the V3 region of HIV-gp120

(Figure 17 A). Asparagine is a polar a.a. with uncharged side-chains whereas aspartic acid is negatively charged. Hence, this amino acid exchange results in a decrement of the overall positive charge in the V3 loop.

The N->D change at position 295 may have a strong impact on the muparfostat resistance. This very mutation has previously been described in dextran sulphate- and bicyclam-resistant viral strains and was associated to increment of IC₅₀ values against these substances [187]. The fact that this mutation did not appear in the mildly muparfostat-resistant viral variants described in **Paper II** might be due to the different strategies and selection pressures employed when generating the escape mutants. Experiments to define the IC₅₀ values to muparfostat of this variant is ongoing. Finally, it should be noted that the decrease in net charge by the N->D alteration after selection with muparfostat is in contrast to a recently reported genetic drift towards increase of cationic a.a. with duration of human infection [38], linked to higher replicative yields of the HIV-1 strains.

Lastly, in **Paper III** an inactivation experiment with PG545 was performed in order to clarify whether the phenomenon of dilution into non-inhibitory concentrations described in Paper I could be achieved in an *in vivo* model. Complete virus inactivation and thereby total protection against HSV-2 clinical manifestation was shown in mice administered with virus pre-incubated with compound at high concentrations (500 and 100 µg/mL). When mice were instilled with HSV-2 pre-treated with only 20 µg/mL, the clinical manifestations were restricted to a mild vaginal infection from which they recovered in a few days. All mice in this group survived and no impact on the CNS was recorded, in contrast to infected controls not receiving any compound. Surprisingly, mice inoculated with HSV-2 and the low-dose of PG545 displayed comparable amount of infectious viral particles as

determined by plaque assay, and HSV-2 DNA viral loads quantified by TaqMan PCR, in dorsal root ganglia as well as in their spinal cords. This was in stark contrast to the differences in clinical outcome, why we performed immunohistochemistry to search for a possible explanation in form of altered distribution of viral antigens. Horizontally sectioned preparations of formaldehyde-fixated spinal cord tissue of sacral regions, including dorsal root ganglia and cauda equina were incubated with rabbit polyclonal antibodies against HSV-2 antigen in order to localize infected cells. No antigen was detected in spinal cord or dorsal root ganglia in any of the animals treated with the highest (100 µg/mL) concentration. In mice treated with the low dose of PG545 (20 µg/mL), the infection appeared to be limited to the sacral dorsal root ganglia and to the neuronal projections within cauda equina. In this group, no virus-infected neuronal cell bodies were detected in the spinal cord as opposed to the non-treated but infected control animals in which sensory neurons of the posterior roots of the grey matter were strongly stained for HSV-2 antigen. The findings indicate a clear border of viral passage between the first order of sensory neurons, including dorsal root ganglia and cauda equina, and the second order of sensory neurons to which the compound-treated virus particles could not enter. We propose that, as denoted in Figure 19, preincubation of HSV-2 with low-dose PG545 induced a block of the virus infection between the first and second order of sensory neurons in the spinal cord, and that this block might explain the favourable clinical outcome in this group of mice.

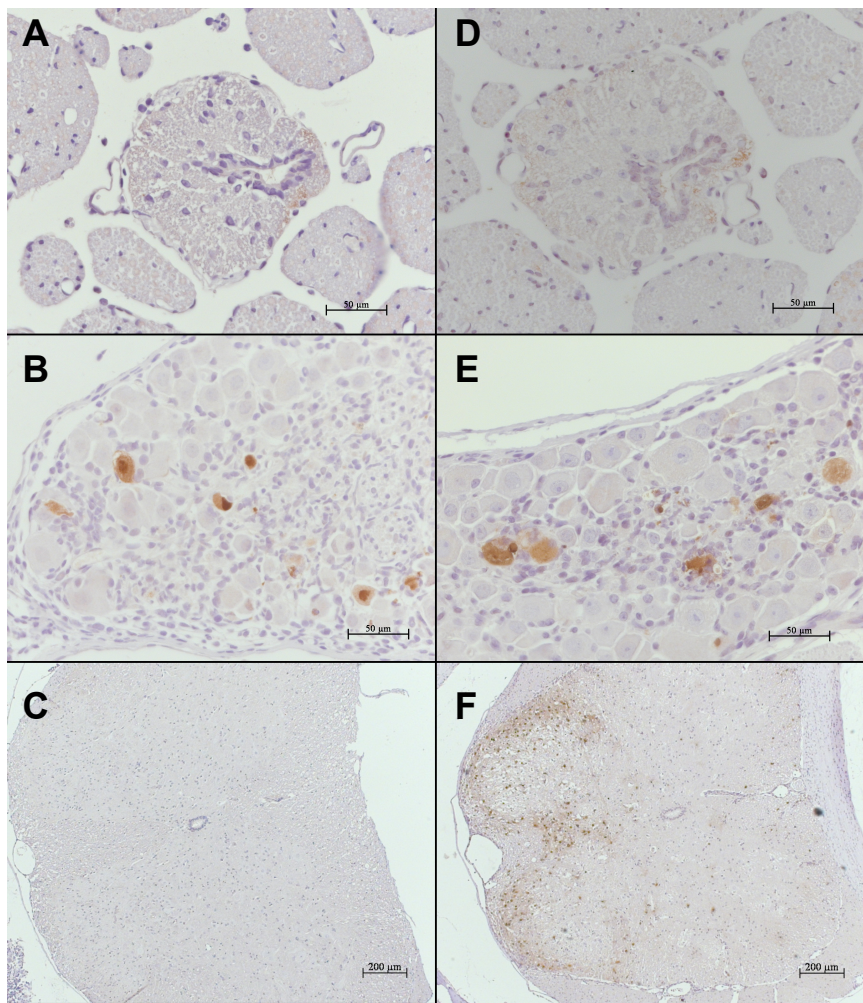


Figure 18. Immunohistochemistry of dorsal root ganglia, spinal cord and cauda equina of mice vaginally infected with HSV-2. Dorsal root ganglia and spinal cord were transversally sectioned and stained for immunohistochemistry with polyclonal rabbit antibodies to HSV. Mice were inoculated vaginally with either (A-C): a HSV-2-compound mixture with a concentration of 20 $\mu\text{g}/\text{mL}$ of PG545 or (D-F): with virus only. Virus-infected cells are stained brown. **A and D:** Cauda equina with HSV-2 antigen present, in both groups, near the fissura mediana anterior in the conus medullaris, close to the central canal. **B and E:** Medium to small size neuronal cell bodies of sensory ganglia stained positive for HSV-2 antigen. **F:** HSV-2 antigen present in cell bodies of second order of sensory neurons located in Lamina I and II of the grey matter of the posterior horns of animals inoculated with virus only. **E:** Absence of viral antigen staining in the spinal cord of mice inoculated with the virus-PG545 mixture, which was in parallel with survival and lack of neurological symptoms seen during this regimen. The results suggest a block of infection of the second order of sensory neurons by the microbicide.

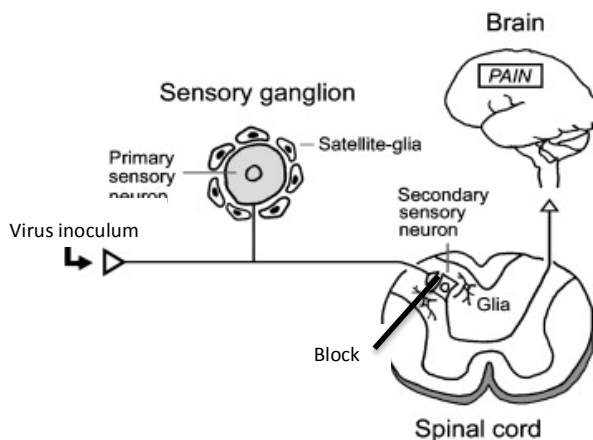


Figure 19. Schematic illustration of the axonal transport of HSV-2 from the periphery, via the first order of sensory neurons to the ganglia, and further to the cell bodies of the second order of sensory neurons located in the posterior horns of the gray matter of the spinal cord. PG545, at a low dose, most likely invoked a block of viral infection at the synaptic level prior to entry of the second order of sensory neurons.

The compiled results from the three papers, together with our preliminary data, indicate that PG545 is a potent virucidal compound, interfering with HIV-1 attachment to host cells and possibly also with viral egress. Analyses of escape mutants, albeit only after selection with muparfostat representing the sulphated carbohydrate component of the complex, clearly indicated that the substance targeted domains of gp120 and gp41 involved in viral entry, and, more specifically, in interaction with cell surface carbohydrate attachment receptors such as HS. PG545 was also effective in a mouse model of vaginal HSV-2 infection, reducing the clinical burden and protecting the CNS from viral invasion and destruction.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This work has generated new knowledge as regards the interaction between the sulfated oligosaccharide muparfostat and the GAG-binding domains of the HIV-1 envelope proteins gp120 and gp41. This compound, when conjugated to cholestanol and other lipophilic tails, exerted potent antiviral and virucidal effect against HIV-1 laboratory strains and clinical isolates *in vitro*. Moreover, the muparfostat-cholestanol conjugate PG545 protected mice against vaginal infection, neurological disease and death when administered locally shortly before or after instillation of HSV-2. When animals were inoculated with a mixture of virus and compound in high dose, all survived and no clinical manifestation was observed. Low-dose treatment resulted in visible genital inflammation, however the nature of the infection differed significantly from that of the control mice. Virtually all animals exhibited a low degree of vaginal infection and all these mice recovered within days without any symptoms of neuronal disease. Since there was a 100% survival rate in the low-dose group, compared to a 100% death rate in the untreated control group, the outcome was clearly beneficial.

A topical prophylactic microbicide active against sexually transmitted microbes, such as HIV-1 and HSV-2, must remain stable in protein rich environments, which are often present in the genital compartment. Since body fluids such as cervical secretion hampered the antiviral activity of PG545, the incentives to continue the chemical modifications of the substance is obvious, in order to improve its stability and bioavailability and to decrease cytotoxicity.

In addition, the animal experiments may be repeated in a way that more closely resembles the situation in humans in form of lower titers of the HSV-2 inoculums, but also by the use of higher concentrations of the microbicides. The next step would be to use a humanized mouse model to test the ability of PG545 to inhibit HIV-1 infection *in vivo*.

Finally, before phase I trials in humans can be initiated, the mode of action and toxicity of these compounds *in vitro* and in animal models needs to be further elucidated both *in vitro* and *in vivo*. These studies should lead to further improvement of the compound by chemical optimization. Given the virucidal property of PG545 demonstrated in the current work, we are confident such efforts are motivated.

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