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Comparison of the antiviral activity of the microbicide candidate griffithsin and its tandemers derivatives against different modes of HIV-1 transmission

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ABSTRACT

Tandemers 2MG, 2MG3, 3MG and 4MG are derivatives of the potent anti-HIV-1 microbicide candidate griffithsin (GRFT). We compared these compounds anti-HIV-1 activity to GRFT using the viruses CAP206.08 and CAAN5342.A2 that have decreased sensitivity to this lectin. The 2MG and 2MG3 tandemers had similar activity to GRFT against cell-free and cell-associated viruses, while 3MG and 4MG were significantly more potent. Furthermore, the restoration of the 234N or 295N glycan in these viruses, known to increase sensitivity to GRFT, also increased sensitivity to 2MG and 2MG3, and not to 3MG and 4MG. In addition, GRFT resistant viruses generated *in-vitro* were equally resistant to 2MG and 2MG3 while they had considerably low resistance to 3MG and 4MG. Lastly, all five compounds showed increased inhibitory activity in seminal and vaginal simulants although the effect was more pronounced in the former. These data support further studies of tandemers as potential microbicides.

1. Introduction

The heterosexual male-to-female transmission of HIV-1 begins when cell-free or cell-associated viruses released into the genital tract infect target cells (Alexandre et al., 2016; Lederman et al., 2006; Stein, 2003). HIV-1 surface glycoprotein gp120 plays the important role of mediating the virus interaction with cellular receptors during entry into these cells (Griffin, 2003; Pandey et al., 2016; Wang et al., 2016). This glycoprotein is heavily glycosylated and contains complex glycans that have terminal sialic acid residues; mannose-rich glycans composed of stretches of 7–9 mannose residues; and hybrids glycans that are a mixture of both (Calarese et al., 2005; Leonard et al., 1990; Panico et al., 2016). On average there are ~24 potential N-linked glycosylation sites on gp120 with complex oligosaccharides occupying 13 of these sites while the remaining sites are occupied by mannose-rich and/or hybrid glycans (Zhu et al., 2000). Glycans account for nearly 50% of gp120 molecular mass (Kwong et al., 1998; Leonard et al., 1990) and may play the critical role of shielding the virus against the host immune response (Wei et al., 2003); although on some occasions they are also points of vulnerability in its defense against this response (Moore et al., 2012). In addition, glycans promote the proper folding of gp120 (Li et al., 1993), and facilitate its interaction with cellular receptors such as DC-SIGN that help spread the infection systemically (Ordanini et al., 2015; Pohlmann et al., 2001a, 2001b). However, these chains of carbohydrates are also targets of many HIV-1 entry inhibitors, some studied for use as microbicides to prevent the heterosexual transmission of the virus (Alexandre et al., 2010a; Alexandre et al., 2012; Balzarini and Van Damme, 2007; Balzarini et al., 2006; Calarese et al., 2003).

Griffithsin (GRFT) is a lectin or carbohydrate-binding molecule isolated from the red algae *Griffithsia sp.* originating from the coastal regions of New Zealand (Mori et al., 2005). GRFT is made of 121 amino acids with a molecular weight of ~13kDa. This lectin has a domain swapped dimer structure in which two β-strands of one monomer combine with 10 β-strands of the other monomer to form a β prism of three four-stranded sheets (Ziolkowska et al., 2006; Ziolkowska and Wlodawer, 2006). Each GRFT monomer (mGRFT) contains three binding sites that have high affinity for mannose residues such as those found on HIV-1 envelope and this compound is one of the most potent microbicide candidates isolated to date (Alexandre et al., 2010a). GRFT has no toxicity to human cells (Kouokam et al., 2011b). We previously showed that it inhibits viral infection with IC_{50} values in the low nanomolar range (Alexandre et al., 2010a, Alexandre et al., 2010, 2012). HIV-1 can develop resistance to GRFT through deletion of glycosylation sites on its envelope (Alexandre et al., 2013). Our previous studies showed that culturing the virus under escalating concentrations of GRFT results in the emergence of strains with significantly reduced sensitivity to this lectin. Glycans that were deleted under GRFT selective pressure were those at position 230, 234, 241, 289, 339, 392 and

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448 (Alexandre et al., 2013).

Tandemers are GRFT derivatives generated by linking monomeric GRFT (mGRFT) in tandem repeats of two (2MG and 2MG3), three (3MG) and four (4MG) units (Moulaei et al., 2015). Two of the main features of tandemers are the increased distance between clusters of mannose residues binding sites on different mGRFT units and the freedom of orientation of these clusters. Previously, we showed that 2MG and 2MG3, differentiated only by the three amino acids linker found between mGRFT in the later, had similar inhibitory activities as the parent lectin; while 3MG and 4MG were significantly more potent (Moulaei et al., 2015). As a follow up to this study we investigated 2MG, 2MG3, 3MG and 4MG inhibitory activities against GRFT resistant viruses generated *in-vitro*. We showed that these viruses were significantly more sensitive to 3MG and 4MG while their sensitivity to 2MG and 2MG3 were reduced and similar to GRFT. We also studied the inhibition of cell-to-cell transmission of the virus with GRFT and its derivatives and showed that 3MG and 4MG were considerably more potent than the parent lectin while 2MG and 2MG3 had the same potency as this compound.

2. Materials and methods

Viruses, cell lines and reagents: HIV-1 subtype C Env Du156.12 and CAP206.8 were amplified from infected individuals in South Africa during the acute or early stage of the infection (Gray et al., 2007; Li et al., 2006). The subtype C virus Du179.14 was also isolated in South Africa from a chronically infected individual (Williamson et al., 2003). HIV-1 subtype B, CAAN5342.A2 was amplified from an acute infection in U.S.A (Li et al., 2005). The transmitted variant envelopes p1054.TC4.1499, QH209.14M.Env.A2, QH359.21M.Env.D1, QG984.21M.Env.A3, and QF495.23M.Env.D1, as well as the pSG3ΔEnv plasmid were obtained from the NIH Reference and Reagent Program (Blish et al., 2009; Keele et al., 2008; Long et al., 2002). The 293T cell line was purchased from the American Type Culture Collection. All cell lines were cultured in DMEM containing 10% fetal bovine serum (FBS) and cell monolayers were disrupted at confluence by treatment with 0.25% trypsin in 1 mM EDTA. Tandemers were a gift from Dr. Barry O'Keefe (NCI, Frederick, MD) while the viral envelope Du156R18 was provided by Prof. Kenneth Palmer (University of Louisville, Louisville, KY).

Site-directed mutagenesis: The 234 and 295 glycosylation sites were introduced in the HIV-1 envelope using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). The confirmation of mutation was achieved by sequencing with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, Foster City, CA) and by resolving on the ABI 3100 automated genetic analyzer.

Selection of GRFT resistant HIV-1 strains: To select GRFT resistant Du179, 1000 $TCID_{50}$ of HIV-1 was grown under escalating concentrations of the lectin (Alexandre et al., 2013). The virus was cultured in 2 mL of 4×10^6 peripheral blood mononuclear cells (PBMC), depleted of $CD8^+$ T cells by means of RosetteSep CD8 depletion cocktail (StemCell Technologies, Vancouver, Canada). The starting concentration of GRFT was the IC_{50} for the inhibition of the virus. A culture without the lectin was included as the experimental control. All cultures were maintained in RPMI 1640 containing 20% FBS and IL-2 (0.05 μg/mL). The virus was passaged every 7 day by transferring 500 mL of the previous culture into fresh CD8 depleted PBMC. The concentration of GRFT was increased whenever the p24 antigen level in the lectin containing culture was similar or higher than the control culture. However, when the level dropped the concentration of the lectin was reduced. After 20 weeks the supernatants of the GRFT and control tissue cultures were genotyped to identify escape mutants (Alexandre et al., 2013).

Single-cycle neutralization assay: The single-cycle neutralization assay was performed as previously described (Alexandre et al., 2010a; Montefiori, 2004). Briefly, a three-fold dilution series of each inhibitor was prepared in 100 μL of DMEM containing 10% FBS (growth medium) per well of a 96 well plate. This was followed by the addition of 50 μL/well of 200 $TCID_{50}$ HIV-1, and 1 h incubation at 37 °C. Then 1×10^4 TZM-bl cells/well/100 μL of growth medium, containing 37.5 μg/mL DEAE dextran, were added before 48h incubation. HIV-1 infection was quantified by measuring the activity of the firefly luciferase. Titers were calculated as the inhibitory concentration that caused 50% reduction (IC_{50}) of relative light unit (RLU) compared to the virus control (wells with no inhibitor) after subtraction of the background (wells without both the virus and the inhibitor).

Inhibition of cell-to-cell transmission of HIV-1: This inhibition was measured as described by Abela et al. (2012) with some modification. Briefly, 2×10^6 293-T cells in 10 mL of growth medium were seeded in a tissue culture dish for 24 h. This was followed by transfection with 4 μg each of pSG3ΔEnv and CAP206.8 or CAAN5342.A2 envelope plasmids using the Fugene 6 transfection reagent (Roche, Basel, Switzerland). After 48 h virus producing 293-T cells were washed with PBS and trypsinized. Then 5×10^3 293-T cells/well/100 μL of growth medium were incubated with a three-fold dilution series of GRFT or tandemer for an hour before addition of 3×10^4 TZM-bl cells/well/150 μL of growth medium. This was followed by the co-culture of the two cell lines for 48 h. The virus infection was quantified by determining the activity of the firefly luciferase. Titers were calculated as the inhibitory concentration that caused 50% reduction of RLU when compared to the virus control after subtraction of the background.

Preparation of vaginal simulant: One liter of solution was prepared by combining 3.51 g NaCl, 1.40 g KOH, 0.22 g $Ca(OH)_2$, 0.018 g bovine serum albumin, 2 g lactic acid, 1 g acetic acid, 0.16 g glycerol, 0.4 g urea and 5.0 g glucose. The pH was adjusted to 4.2 using 6 N HCl (Owen and Katz, 1999).

Preparation of seminal simulant: A one hundred milliliters solution was first prepared by mixing 5.46 mL of 0.123 M sodium phosphate monobasic monohydrate with 49.14 mL of 0.123 M sodium phosphate dibasic anhydrate. Then 813 mg sodium citrate dehydrate, 90.8 mg potassium chloride, 88.1 mg potassium hydroxide, 272 mg fructose, 102 mg glucose anhydrous, 62 mg lactic acid, 45 mg urea and 5.04 mg bovine serum albumin were added. Calcium chloride dehydrate (101 mg + 15.13 mL of water), magnesium chloride hexahydrate (92 mg + 5.13 mL of water) and zinc chloride (34.4 mg + 15.13 mL of water) were each prepared as separate reagents and then slowly added one at a time in that order. The pH was adjusted with 1 N sodium hydroxide to 7.7, and then the solution was sterile filtered and stored frozen at −80 °C (Owen and Katz, 2005).

Inhibition of HIV-1 infection in TZM-bl cells in the presence of vaginal and seminal simulants: Twenty-four hours prior to the assay TZM-bl cells were seeded in a 96-well flat bottom plate at the concentration of 1.5×10^4 cells/well/100 μL of growth medium. Next the inhibitors were diluted half logarithmically in 50% v/v vaginal or seminal simulant or in growth medium alone as the assay control. Then HIV-1 and the diluted test compounds were added to the cells in an equal volume in duplicate for a final simulant concentration of 25%. This was followed by incubation for 48 h at 37 °C and 5% CO_2. Following incubation the inhibition of HIV-1 infection was evaluated by measuring luciferase activity in the assay medium as a read out of virus expression.

3. Results

3.1. Tandemers neutralization of HIV-1 viruses with naturally decreased sensitivity to GRFT

CAP206.8 and CAAN5342.A2 are naturally less sensitive to GRFT (Alexandre et al., 2010a). We previously reported that the concomitant restoration of the 234N and 295N glycosyltion sites on these viruses' envelopes increased the inhibitory potency of GRFT derived tandemers 2MG and 2MG3, while it had no effect on the tandemers 3MG and 4MG (Moulaei et al., 2015). In the current study we follow up on this work to

Table 1
Effects of the 234N and 295N glycans on HIV-1 sensitivity to tandemers.

Inhibitor	Envelope					
	CAP206.8	CAP206/234N	CAP206/295N	CAAN5342.A2	CAAN5342/234N	CAAN5342/295N
	IC_{50} (nM)					
GRFT	8.10 ± 2.70	2.10 ± 1.20 (↓4)	0.11 ± 0.02 (↓73)	20.98 ± 1.22	9.27 ± 2.32 (↓2)	2.53 ± 0.52 (↓8)
2 MG	3.30 ± 0.54	0.30 ± 0.23 (↓11)	0.19 ± 0.05 (↓17)	11.24 ± 2.50	2.93 ± 0.91 (↓4)	1.39 ± 0.77 (↓9)
2 MG3	2.10 ± 0.45	0.91 ± 0.37 (↓2)	0.16 ± 0.03 (↓13)	7.25 ± 2.31	2.15 ± 0.43 (↓3)	0.75 ± 0.26 (↓10)
3 MG	0.14 ± 0.01	0.18 ± 0.06	0.13 ± 0.03	0.22 ± 0.03	0.17 ± 0.04	0.25 ± 0.03
4 MG	0.24 ± 0.06	0.16 ± 0.06	0.15 ± 0.02	0.36 ± 0.01	0.28 ± 0.02	0.20 ± 0.03

The arrows indicate decrease in IC_{50} values implying an increase in sensitivity.

investigate the contribution of each glycosylation site in the increased potency observed for 2MG and 2MG3. To study this we restored the 234N or 295N glycan on HIV-1 CAP206.8 and CAAN5342.A2 envelopes. The two glycosylation sites were added on HIV-1 Env using site directed mutagenesis (Alexandre et al., 2010a). The mutants and their parent viruses were tested against 2MG, 2MG3, 3MG, 4MG, and GRFT using the TZM-bl neutralization assay. The addition of the 234N glycan enhanced CAP206/234N sensitivity to 2MG by 11 fold compared to the wild type virus, while it enhanced that of CAAN5342/234N by 4 fold (Table 1). When the 295 glycosylation site was restored on gp120 the mutant viruses sensitivity to 2MG increased by 17 and 9 fold for CAP206/295N and CAAN5342/295N, respectively (Table 1). A similar trend was observed for 2MG3, where the restoration of the 234 glycosylation site increased CAP206/234N and CAAN5342/234N sensitivity to the lectin by 2–3 fold (Table 1). Furthermore, the addition of the 295N glycan enhanced CAP206/295N and CAAN5342/295N sensitivity to 2MG3 by 13 and 10 fold, respectively, compared to their wild type viruses (Table 1). These increase in potency following the restoration of the 234 or 295 glycosylation site reflected what happened with the parent lectin GRFT (Table 1). However, neither the addition of the 234N nor that of the 295N glycan had an effect on HIV-1 sensitivity to 3MG and 4MG. This suggests that similar to GRFT, each of these glycans is important to 2MG and 2MG3 interaction with HIV-1 envelope, while this is not the case for 3MG and 4MG. These data are also consistent with what we reported on the effect of the concomitant addition of the 234N and 295N glycans on tandemers neutralization of HIV-1 (Moulaei et al., 2015).

3.2. Neutralization of in-vitro generated GRFT resistant viruses with tandemers

Based on data obtained with HIV-1 that naturally have decreased sensitivity to GRFT, we hypothesized that *in-vitro* generated GRFT resistant viruses will also show resistance to 2MG and 2MG3; and no or less resistance to 3MG and 4MG. To show this we tested tandemers inhibition of GRFT resistant viruses generated by growing under escalating concentrations of this lectin. One of the viruses used was Du179 GRFT cl7 that was isolated after 20 passages of the HIV-1 virus Du179 in PBMC when the concentration of GRFT reached ~10 times its starting concentration or concentration at passage zero (Alexandre et al., 2013). At passage 20 Du179 had deletions of the 230, 234, 339, 392, and 448 mannose-rich glycosylation sites as well as deletion of four amino acids at position 400 to 403 (Alexandre et al., 2013). The other virus used in the study was Du156R18, isolated after growing the wild type Du156 in M7-luc cells under escalating concentrations of GRFT for 11 passages (personal communication with Kenneth E. Palmer, Louis Ville University, School of Medicine). Du156R18 could tolerate up to 250 times the starting concentration of the lectin. Growing under escalating concentrations of GRFT resulted in the deletion of mannose-rich glycans at positions 294 and 433 in addition to the deletion of 10 amino acids at position 138 to 147. As shown in Table 2, Du179 GRFT cl7 sensitivity to GRFT, 2MG and 2MG3 was between 116 and 240 fold lower than that of the wild type virus while its sensitivity to 3MG and 4MG was only ~30 fold lower. With Du156R18 the decrease in sensitivity to GRFT, 2MG and 2MG3 ranged from 280 to ~1500 fold compared to the parent Du156 whereas its loss of sensitivity to 3MG and 4MG was significantly less pronounced at only ~10 fold. Taken together this supports the suggestion that HIV-1 interaction with 2MG and 2MG3 is much closer to GRFT than its interaction with 3MG and 4MG.

3.3. Inhibition of cell-to-cell transmission of HIV-1 with tandemers

Since HIV-1 infection can be mediated by both cell-free and cell-associated viruses (Mazurov et al., 2010; Rudnicka et al., 2009; Shattock and Moore, 2003; Sowinski et al., 2008), and given that we already reported on tandemers inhibition of cell-free HIV-1 (Moulaei et al., 2015), we decided to investigate these compounds inhibitory activity against the cell-to-cell transmission of the virus. This was studied using 293-T cells transfected with plasmids expressing HIV-1 pseudoviruses. The 293-T cells were used as donor cells while TZM-bl cells were the target cells. Viruses expressed by 293-T cells were either the subtype C CAP206.8 or the subtype B CAAN5342.A2. The transfer of HIV-1 from the donor to target cells was achieved by co-culturing the two cell lines.

To determine what 293-T cells input/well to use in the inhibition of cell-to-cell transmission assay that will only reflect the lectins inhibitory activity on cell-associated viruses and not cell-free ones, we conducted the following experiment: A two-fold dilution series of 293-T cells, transfected with a plasmid expressing HIV-1 as explained above, was performed in a flat bottom 96 well plate starting with the concentration of 2×10^5 cells/well and ending with 781 cells/well. The cells were cultured for 48 h followed by the transfer of all supernatants from each well to corresponding wells of another flat bottom 96-well plate. Then 3×10^4 TZM-bl cells/well were added to the 96 well plate containing 293-T cells and the one containing their supernatants. Cells were then cultured for 48 h before luminescence readings. The levels of HIV-1 infection between the two plates were compared. For the plate containing transfected 293-T cells co-cultured with TZM-bl cells there was observable HIV-1 infection from 2×10^5 293-T cells/well to 1×10^3 293-T cells/well (Fig. 1 shows the graph for CAP206.8). However, with the plate containing TZM-bl cells cultured with the supernatants, we observed infection only for supernatants derived from 2×10^5 to 1.0×10^5 293-T cells/well (Fig. 1). This implied that below 1.0×10^5 293-T cells/well input, transfected cells did not produce enough cell-free viruses in the supernatant to cause a visible infection of TZM-bl cells. Thus, for the inhibition of cell-to-cell transmission assay we decided to use 293-T cells at the input of 5×10^3 cells/well as at this input all infection observed will be coming from cell-associated viruses.

To investigate tandemers and GRFT inhibition of cell-to-cell transmission of HIV-1, 5×10^3 293-T cells/well were first incubated with a

Table 2
In-vitro generated GRFT resistant viruses sensitivity to tandemers.

Envelope	IC_{50} (nM)				
	2MG	2MG3	3MG	4MG	GRFT
Du179.14 (WT)	0.158 ± 0.096	0.144 ± 0.044	0.124 ± 0.026	0.0934 ± 0.029	0.606 ± 0.14
Du179 GRFT cl7 (R)	35.99 ± 0.44 (↑228)	34.37 ± 0.63 (↑239)	3.86 ± 0.89 (↑31)	2.08 ± 0.63 (↑22)	70 ± 13.010 (↑116)
Du156.12 (WT)	0.0182 ± 0.0013	0.0616 ± 0.0034	0.0794 ± 0.025	0.0887 ± 0.018	0.0324 ± 0.0021
Du156R18 (R)	27.1 ± 11.60 (↑1489)	26.0 ± 15.70 (↑422)	1.11 ± 0.31 (↑14)	0.688 ± 0.20 (↑8)	9.14 ± 5.34 (↑282)

The arrows indicate increase in IC_{50} values implying a decrease in potency.

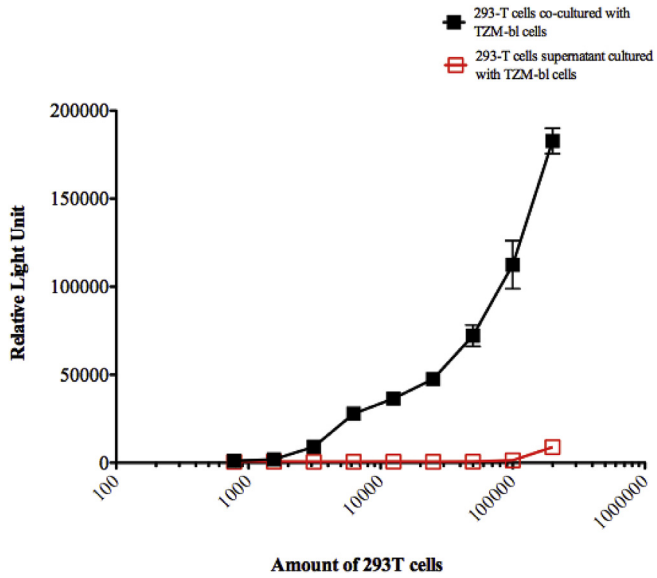

Fig. 1. Determination of 293T cells input for the inhibition of cell-to-cell transmission assay. TZM-bl cells were co-cultured with either the indicated amount of 293 T cells transfected with plasmids expressing HIV-1 pseudovirus CAP206.8 (closed symbols) or with supernatants from these 293 T cells (open symbols). After 48 h HIV-1 infection was determined by measuring luminescence emitted by the cells. Data shown represent the results of two independent experiments, and bars represent the mean plus standard deviations.

three fold dilution series of each lectin before addition of TZM-bl cells at 3×10^4 cells/well. The inhibition of HIV-1 infection was measured after 48 h of co-culture. As shown in Fig. 2, each compound inhibited the two viruses used with comparable potency judging from their IC_{50} values. In addition, the 3MG and 4MG tandemers had stronger inhibitory activity than GRFT; with IC_{50} values that were ~7 fold lower. However, this difference was statistically significant ($p \leq 0.05$) only for 3MG against CAP206.8 (Fig. 2). The inhibition of cell-to-cell transmission with 2MG and 2MG3 was generally similar to GRFT. These data are consistent with what we previously reported for the TZM-bl neutralization assay that uses cell-free viruses (Montefiori, 2004; Moulaei et al., 2015). Next we compared the IC_{50} values of tandemers and GRFT for the inhibition of cell-associated and cell-free HIV-1 in order to determine the mode of transmission that these compounds inhibited the strongest. We observed that they blocked cell-free viruses with significantly lower IC_{50} values than cell-associated ones (Table 3), implying that they were more effective against the former mode of transmission.

3.4. Involvement of the 234N and 295N glycans in tandemers inhibition of cell-to-cell transmission of HIV-1

Given that the addition of the 234N and 295N glycans on HIV-1 envelope had an effect on GRFT, 2MG and 2MG3 inhibitory activity against cell-free viruses, and not on 3MG and 4MG (Table 1), we

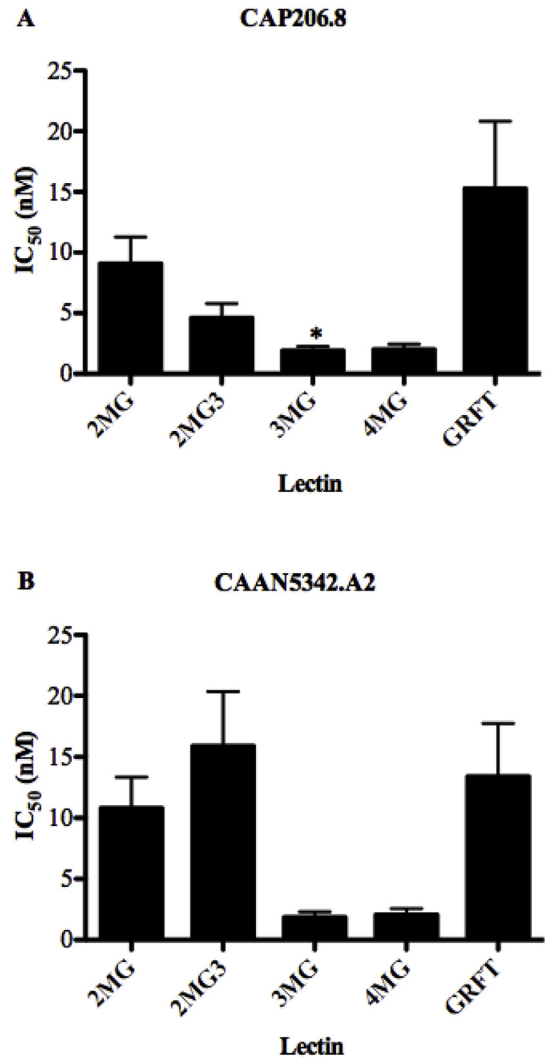

Fig. 2. Tandemers and GRFT inhibition of cell-to-cell transmission of HIV-1. (A) 293T cells expressing HIV-1 pseudovirus CAP206.8 and (B) those expressing CAAN5342.A2 were incubated with the five lectins before co-culture with TZM-bl cells. The inhibition of cell-associated HIV-1 infection was determined by luminescence measurement after 48 h. Bars represent the mean plus standard deviation of three independent experiments. Statistically significant difference ($p \leq 0.05$) is indicated by an asterisks.

investigated whether this was also true for the inhibition of cell-to-cell transmission of HIV-1. We used CAP206.8 and CAAN5342.A2 and their mutant viruses containing the 234N and 295N glycosylation sites restored alone or together. The inhibition of cell-to-cell transmission was carried out as explained above. The addition of the 234N or 295N glycan alone increased the two viruses sensitivity to 2MG, 2MG3 and GRFT between 3 and 18 fold (see the numbers on top of the bars in Fig. 3), while it had no effect on 3MG and 4MG inhibitory activity.

Table 3
Comparison of tandemers and GRFT inhibition of cell-free and cell-associated HIV-1.

Lectin	CAAN5342.A2		CAP206.8	
	IC_{50}			
	Inhibition of cell-free viruses	Inhibition of cell-associated viruses	Inhibition of cell-free viruses	Inhibition of cell-associated viruses
2MG	5.32 ± 1.25	9.54 ± 1.12	0.16 ± 0.02	**10.43 ± 1.67 (↑65)**
2MG3	17.47 ± 0.67	16.76 ± 2.66	0.85 ± 0.15	**5.82 ± 1.53 (↑7)**
3MG	0.45 ± 0.12	**2.08 ± 0.19 (↑5)**[a]	0.20 ± 0.02	**3.1 ± 1.15 (↑16)**
4MG	0.34 ± 0.10	**3.14 ± 1.06 (↑9)**	0.19 ± 0.02	**2.51 ± 0.49 (↑13)**
GRFT	8.93 ± 1.46	13.77 ± 1.32	3.59 ± 1.84	**17.53 ± 2.56 (↑5)**

[a] Where the difference was 3 fold or more is shown in bold and arrows indicate increase in IC_{50} value.

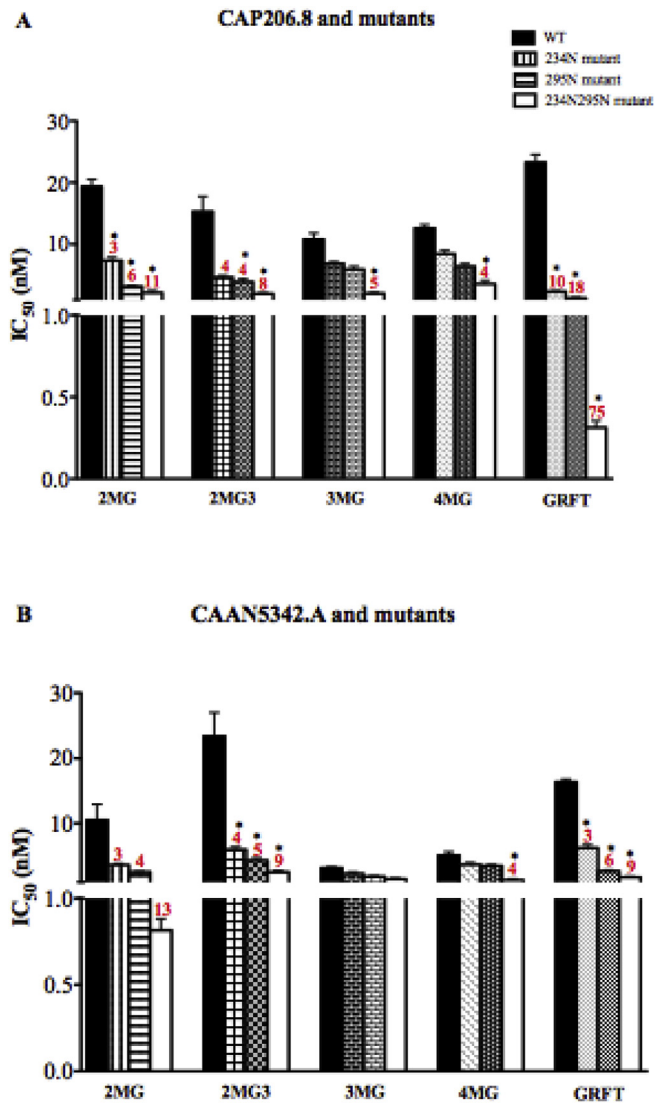

Fig. 3. Comparison of tandemers and GRFT inhibition of cell-to-cell transmission of wild type HIV-1 and their 234N and 295N mutants. The effects of addition of the 234N and 295N glycosylation sites on (A) CAP206.8 and (B) CAAN5342.A2 susceptibility to 2MG, 2MG3, 3MG, 4MG and GRFT inhibition of cell-to-cell transmission. Virus expressing 293T cells were co-cultured with TZM-bl cells in the presence of each lectin. The inhibition of HIV-1 infection was measured after 48 h. The bars represent the mean plus standard deviation of 3 different experiments. The numbers on top of the bars indicate the fold decrease in IC_{50} value resulting from the glycosylation site addition. Statistically significant differences are indicated by asterisks ($p \leq 0.05$).

However, unlike the inhibition of cell-free viruses (Moulaei et al., 2015), the concomitant addition of the 234N and 295N glycans increased cell-associated CAP206.8 and CAAN5342.A2 sensitivity to GRFT and all its four derivatives. This being said, the effect was more pronounced for 2MG, 2MG3 and GRFT, with the viruses increase in sensitivity ranging from 8 to 75 fold, compared to around 4 fold for 3MG and 4MG. In most cases all the observed increase in HIV-1 sensitivity to the lectins were statistically significant ($p \leq 0.05$) (Fig. 3). These data are consistent with those we obtained for the inhibition of cell-free HIV-1 in TZM-bl cells (Table 1) (Moulaei et al., 2015); and suggest that the 234N and 295N glycans also play an important role in the tandemers and GRFT inhibition of cell-associated HIV-1 infection of susceptible cells.

3.5. Investigation of tandemers inhibitory activity in seminal and vaginal simulants

A study by Zirafi *et al.* showed that the presence of semen significantly decreased the inhibitory activity of many HIV-1 microbicide candidates (Zirafi et al., 2014). We investigated whether seminal and vaginal fluids would have a similar effect on GRFT and tandemers. Since suitable microbicides should be inhibitory to transmitted viruses that are present at the initiation of the infection, we tested GRFT and its derivatives against five transmitted HIV-1 variants that included four subtype A and one subtype B virus (Blish et al., 2009; Keele et al., 2008; Long et al., 2002). All the transmitted variants used were isolated during the acute phase and mostly within the first three weeks of the infection. We also used seminal and vaginal simulants for this study, which have been used before by other investigators to mimic seminal and vaginal fluids (Albertini et al., 2009; Hombach et al., 2009; Owen and Katz, 1999, 2005). Free viral particles were incubated with each inhibitor in the presence of either simulant before addition of TZM-bl cells. Contrary to what we observed with nontransmitted HIV-1 variants (Moulaei et al., 2015), GRFT, 2MG, 2MG3, 3MG, and 4MG had comparable inhibitory potency against transmitted viruses (Table 4). Furthermore, all lectins showed increased potency, ranging from 3 to 9 fold, against the five viruses tested in the presence of seminal simulants. However, in most cases GRFT and the tandemers did not have increased inhibitory activity in the presence of vaginal simulant (Table 4). We obtained similar data when we tested GRFT and its derivatives against a laboratory adapted X4 virus, in the presence of vaginal and seminal simulants (data not shown). These data suggest that the anti-HIV-1 activity of GRFT, 2MG, 2MG3, 3MG, and 4MG is not likely to be negatively affected in seminal or vaginal fluid. On the contrary these compounds may become more potent in such environment. In addition, the data indicate that the increase in array of monomeric GRFT in a tandemer may not have an effect on its potency against transmitted HIV-1 variants.

Table 4
Tandemers and GRFT Inhibition of transmitted HIV-1 variants in the presence of seminal and vaginal simulants.

Envelope	Day of isolation post transmission	[a]IC_{50} values (μg/ml)														
		2MG			2MG3			3MG			4MG			GRFT		
		[b]GM	[c]SS	[d]VS	GM	SS	VS	GM	SS	VS	GM	SS	VS	GM	SS	VS
p1054.TC4.1499	**During acute infection**	0,0087	**0,0016**	0,0073	0,023	**0,0050**	0,017	0,0068	**0,0020**	0,0056	0,0029	**0,00031**	0,0020	0,00044	**0,00008**	0,0005
QH209.14M.Env.A2	**14**	0,0005	**0,00015**	0,0004	0,0018	**0,00041**	0,0016	0,001	**0,00021**	0,00082	0,0016	**0,00042**	0,0012	0,00054	**0,00014**	0,001
QH359.21M.Env.D1	**21**	0,0003	**0,000080**	0,0003	0,00028	**0,00006**	0,0003	0,0002	**0,00003**	0,00046	0,0002	**0,000028**	0,00080	0,00033	**0,000092**	0,0003
QG984.21M.Env.A3	**21**	0,015	**0,0020**	0,0094	0,024	**0,0090**	0,029	0,0070	**0,0018**	**0,0026**	0,0069	**0,0016**	**0,0012**	0,00046	**0,00011**	0,0012
QF495.23M.Env.D1	**23**	0,032	**0,005**	**0,010**	0,038	**0,011**	0,040	0,0061	**0,0022**	0,0033	0,0051	**0,0018**	**0,0012**	0,00042	**0,00016**	0,00046

[a] Concentration that inhibited HIV-1 infection by 50% (IC_{50}).
[b] Growth media (GM).
[c] Growth media containing seminal simulant (SS).
[d] Growth media containing vaginal simulant (VS).
[e] In bold are IC_{50} values in simulants that were lower by 3 fold or more than the corresponding IC_{50} values in GM.

4. Discussion

Tandemers 2MG, 2MG3, 3MG and 4MG are derivatives of the HIV-1 microbicide candidate GRFT (Alexandre et al., 2016; Grooms et al., 2016; Kouokam et al., 2011a, 2016). We previously reported that 2MG and 2MG3 inhibition of HIV-1 was similar to GRFT while 3MG and 4MG were significantly more potent (Moulaei et al., 2015). In the current study we followed up on these findings and tested tandemers against GRFT resistance viruses generated *in vitro* as well as viruses that were naturally resistant to this compound. We found that these viruses were similarly resistant to 2MG and 2MG3 while being considerably less resistant to 3MG and 4MG. We also compared the inhibition of cell-to-cell transmission of HIV-1 between tandemers and GRFT and observed that 3MG and 4MG were markedly more potent than 2MG, 2MG3 and GRFT. Lastly, we investigated the five lectins inhibitory activities in the presence of seminal and vaginal simulants and showed that under these conditions their activities were generally increased.

The envelope of the GRFT resistant virus Du179 cl7 has deleted mannose-rich glycans at position 230, 234, 339, 392 and 448, together with the deletion of four amino acids, as a result of growing under escalating concentrations of this lectin (Alexandre et al., 2013). Similarly, GRFT selective pressure resulted in Du156R18 losing four potential N-linked glycosylation sites, including mannose-rich glycans at positions 294 and 433, and incurring a 10 amino acids deletion. The fact that these viruses also showed a decrease in sensitivity to 2MG and 2MG3 that was comparable to GRFT suggests that these three compounds may target the same binding site on HIV-1 envelope. This is in agreement with what we have showed in Table 1 i.e. the restoration of either the 234N or the 295N glycan on HIV-1 envelope had similar effect on GRFT, 2MG and 2MG3 inhibition of the virus. It is also consistent with our previous study that reported that when these two glycosylation sites are restored together they affect GRFT, 2MG, and 2MG3 anti-viral activity the same way (Moulaei et al., 2015). Du179 cl7 and Du156R18 decrease in sensitivity to 3MG and 4MG were markedly less pronounced, consistent with the idea that these two tandemers binding site may be different to GRFT. The high number of mannose residue binding sites on 3MG and 4MG, 9 and 12, respectively (Moulaei et al., 2015), may be allowing these lectins to interact with many more mannose-rich glycans on HIV-1 than GRFT, 2MG, and 2MG3 that only have 6 binding sites each (Alexandre et al., 2013; Moulaei et al., 2015; Ziolkowska et al., 2006; Ziolkowska and Wlodawer, 2006). This in turn may reduce the effects caused by the loss of few glycans.

The finding that GRFT, 2MG, and 2MG3 had similar potency against the cell-to-cell transmission of HIV-1 while 3MG and 4MG were more potent followed the same pattern as what we observed for the inhibition of cell-free viruses (Table 1). However, the fact that all five lectins performed significantly better in inhibiting the cell-free transmission of the virus compared to the inhibition of cell-to-cell transmission (Table 3) is similar to what have been reported for other entry inhibitors that were also found to be more potent against cell-free HIV-1 than cell-associated viruses (Abela et al., 2012; Chen et al., 2007; Ganesh et al., 2004). One possible explanation is that HIV-1 infected cells, such as the 293-T cells used here, have both viral particles and virion-free Envs on their surface (Miranda et al., 2002; Vincent et al., 1999). Thus, this may result in envelop inhibitors targeting the virus also binding these free Envs ultimately reducing the number of inhibitor molecules that are present in solution to inhibit cell-associated HIV-1 infection (Fig. 4 A). It is noteworthy that the reduction of HIV-1 inhibitors activity against the cell-to-cell transmission of infection due to virus-free Envs expressed by virus producing cells has also been reported before (Herschhorn et al., 2011). For the inhibition of cell-free viruses the situation is different as inhibitor molecules do not have virion-free Envs competing with viral particles for binding (Fig. 4 B). Another possibility is that since the cell-to-cell transmission of HIV-1 takes place via virological synapses (Jolly et al., 2004; Jolly and Sattentau, 2004; Sattentau, 2008), these may render it difficult for

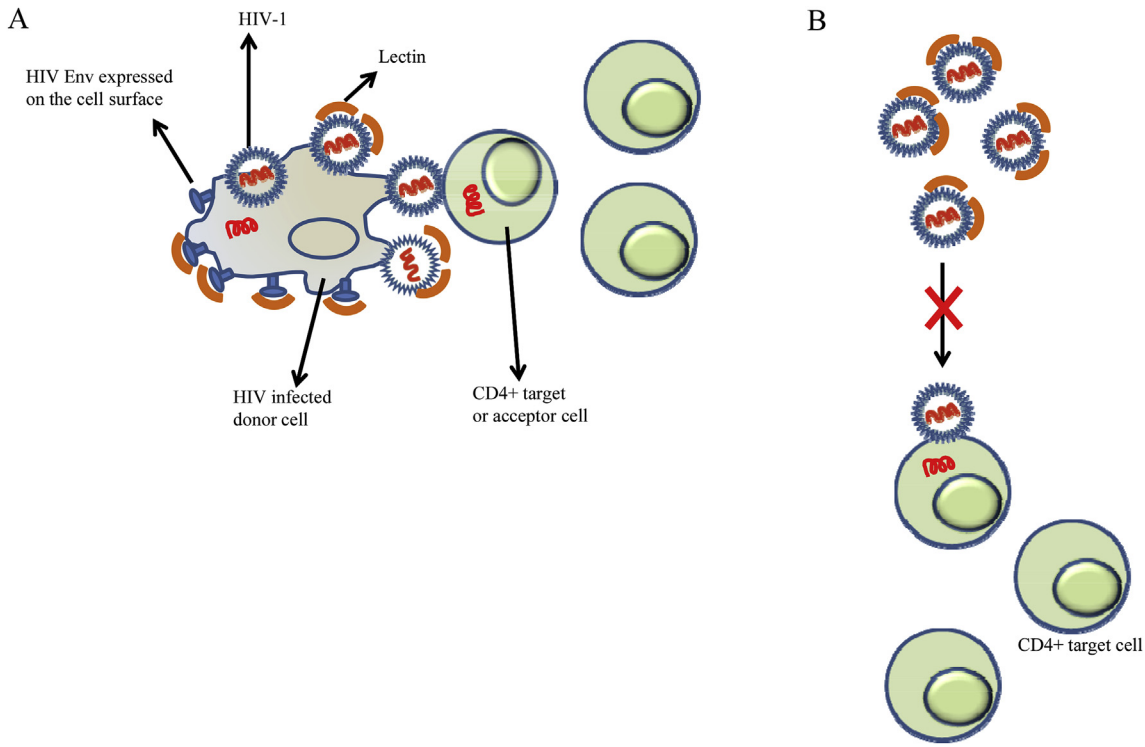

Fig. 4. Illustration of the inhibition of cell-free and cell-associated HIV-1 infection of target cells. (A) Partial inhibition of cell-to-cell transmission of HIV-1 as some envelope inhibitors bound Envs on the cell surface instead of those on the viral envelope. (B) Illustration of how the same amount of the inhibitor is likely to have a stronger effect on similar number of cell-free viruses. Note that the interaction of the lectins with the viral envelope or Env as shown in the Figure is simply for illustration purposes and does not suggest that the five lectins interact with the virus in exactly the same way.

lectins to reach their targets as readily as it would be the case with cell-fee viruses. Lastly, the concomitant restoration of the 234N and 295N glycans increased the inhibitory activity of all four tandemers and GRFT against the cell-to-cell transmission of HIV-1. This is different from what we observed in cell-free viruses where this double restoration had no effect on 3MG and 4MG activity (Moulaei et al., 2015). Therefore, it is likely that for the inhibition of cell-to-cell transmission of HIV-1 the 234N and 295N glycans play a role in 3MG and 4MG binding to the viral envelope while they do not in the inhibition of cell-free viruses. The further implication of this observation is that the interaction of 3MG and 4MG with cell-free and cell-associated viruses may somewhat be different.

Like GRFT, tandemers are potential HIV-1 microbicides destined for use in the female genital tract to prevent the sexual transmission of the virus (Alexandre et al., 2010a; Moulaei et al., 2015). Thus, these compounds have to be active against transmitted variants. Interestingly, our data indicated that against the four subtype A and one subtype B transmitted HIV-1 we tested all four tandemers and GRFT had comparable potency (Table 4). This is different from what we reported in the past, when screening of 2MG, 2MG3, 3MG, 4MG, and GRFT against two subtype A, four subtype B, and five subtype C viruses that were not transmitted variants showed that 3MG and 4MG were markedly more potent (Moulaei et al., 2015). Thus, it is possible that as microbicides GRFT and its derivatives might have similar activity, at least against cell-free viruses given that the study with the transmitted variants was done using this model. Since seminal simulant increased these lectins inhibition of HIV-1, it can be speculated that the presence of human semen may in fact enhance their potency. This is important given that a study by Zirafi *et al.* showed that semen significantly decreased the inhibitory activity of several HIV-1 microbicide candidates (Zirafi et al., 2014), among them the lectin antibody 2G12 that has an overlapping binding site with GRFT (Alexandre et al., 2010a; Calarese et al., 2005; Zirafi et al., 2014). On some occasions we also observed the enhanced inhibition of HIV-1 in vaginal simulant (Table 4) supporting the idea that in the female genital tract the potency of these lectins is likely to be increased.

Tandemers are GRFT derivatives designed with the aim of obtaining stronger HIV-1 inhibitory activity than the parent compound. The current study suggests this can be achieved with tandemers containing more than two arrays of monomeric GRFT. In addition, those made of three monomers may be optimal since the inhibitory activity of 4MG mirrored that of 3MG in nearly all assays we conducted. Lastly, given HIV-1 high genetic barrier to GRFT resistance (Alexandre et al., 2013), it will be interesting to investigate whether this is also the case for 3MG and 4MG. Although, our previous study with GRFT resistant viruses and the current one (Alexandre et al., 2013; Moulaei et al., 2015) suggest that HIV-1 may need to delete different and/or more glycans and amino acids on the envelope in order to show resistance to 3MG and 4MG that is comparable to GRFT.

CRediT authorship contribution statement

Kabamba Alexandre: Formal analysis, Writing - original draft, Supervision. **Kanyane Malatji:** Data curation, Formal analysis, Writing - review & editing. **Takalani Mulaudzi:** Writing - review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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References

Abela, I.A., Berlinger, L., Schanz, M., Reynell, L., Gunthard, H.F., Rusert, P., Trkola, A., 2012. Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies. PLoS Pathog. 8, e1002634.

Albertini, B., Passerini, N., Di Sabatino, M., Vitali, B., Brigidi, P., Rodriguez, L., 2009. Polymer-lipid based mucoadhesive microspheres prepared by spray-congealing for the vaginal delivery of econazole nitrate. Eur. J. Pharmaceut. Sci. 36, 591–601.

Alexandre, K.B., Gray, E.S., Lambson, B.E., Moore, P.L., Choge, I.A., Mlisana, K., Karim, S.S., McMahon, J., O'Keefe, B., Chikwamba, R., Morris, L., 2010a. Mannose-rich glycosylation patterns on HIV-1 subtype C gp120 and sensitivity to the lectins, Griffithsin, Cyanovirin-N and Scytovirin. Virology 402, 187–196.

Alexandre, K.B., Gray, E.S., Mufhandu, H., McMahon, J.B., Chakauya, E., O'Keefe, B.R., Chikwamba, R., Morris, L., 2012. The lectins griffithsin, cyanovirin-N and scytovirin inhibit HIV-1 binding to the DC-SIGN receptor and transfer to CD4(+) cells. Virology 423, 175–186.

Alexandre, K.B., Gray, E.S., Pantophlet, R., Moore, P.L., McMahon, J.B., Chakauya, E., O'Keefe, B.R., Chikwamba, R., Morris, L., 2010. Binding of the mannose-specific lectin, griffithsin, to HIV-1 gp120 exposes the CD4-binding site. J. Virol. 85 (17), 9039–9050.

Alexandre, K.B., Moore, P.L., Nonyane, M., Gray, E.S., Ranchobe, N., Chakauya, E., McMahon, J.B., O'Keefe, B.R., Chikwamba, R., Morris, L., 2013. Mechanisms of HIV-1 subtype C resistance to GRFT, CV-N and SVN. Virology 446, 66–76.

Alexandre, K.B., Mufhandu, H.T., London, G.M., Chakauya, E., Khati, M., 2016. Progress and Perspectives on HIV-1 microbicide development. Virology 497, 69–80.

Balzarini, J., Van Damme, L., 2007. Microbicide drug candidates to prevent HIV infection. Lancet 369, 787–797.

Balzarini, J., Van Laethem, K., Peumans, W.J., Van Damme, E.J., Bolmstedt, A., Gago, F., Schols, D., 2006. Mutational pathways, resistance profile, and side effects of cyanovirin relative to human immunodeficiency virus type 1 strains with N-glycan deletions in their gp120 envelopes. J. Virol. 80, 8411–8421.

Blish, C.A., Jalalian-Lechak, Z., Rainwater, S., Nguyen, M.A., Dogan, O.C., Overbaugh, J., 2009. Cross-subtype neutralization sensitivity despite monoclonal antibody resistance among early subtype A, C, and D envelope variants of human immunodeficiency virus type 1. J. Virol. 83, 7783–7788.

Calarese, D.A., Lee, H.K., Huang, C.Y., Best, M.D., Astronomo, R.D., Stanfield, R.L., Katinger, H., Burton, D.R., Wong, C.H., Wilson, I.A., 2005. Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. Proc. Natl. Acad. Sci. U. S. A. 102, 13372–13377.

Calarese, D.A., Scanlan, C.N., Zwick, M.B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M.R., Stanfield, R.L., Roux, K.H., Kelly, J.W., Rudd, P.M., Dwek, R.A., Katinger, H., Burton, D.R., Wilson, I.A., 2003. Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. Science 300, 2065–2071.

Chen, P., Hubner, W., Spinelli, M.A., Chen, B.K., 2007. Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. J. Virol. 81, 12582–12595.

Ganesh, L., Leung, K., Lore, K., Levin, R., Panet, A., Schwartz, O., Koup, R.A., Nabel, G.J., 2004. Infection of specific dendritic cells by CCR5-tropic human immunodeficiency virus type 1 promotes cell-mediated transmission of virus resistant to broadly neutralizing antibodies. J. Virol. 78, 11980–11987.

Gray, E.S., Moore, P.L., Choge, I.A., Decker, J.M., Bibollet-Ruche, F., Li, H., Leseka, N., Treurnicht, F., Mlisana, K., Shaw, G.M., Karim, S.S., Williamson, C., Morris, L., 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. J. Virol. 81, 6187–6196.

Griffin, S., 2003. Overview of the HIV replication cycle. In: In: Richman, D.D. (Ed.), Human Immunodeficiency Virus International Medical Press, pp. 3:1–3:14.

Grooms, T.N., Vuong, H.R., Tyo, K.M., Malik, D.A., Sims, L.B., Whittington, C.P., Palmer, K.E., Matoba, N., Steinbach-Rankins, J.M., 2016. Griffithsin-modified electrospun fibers as a delivery scaffold to prevent HIV infection. Antimicrob. Agents Chemother. 60, 6518–6531.

Herschhorn, A., Finzi, A., Jones, D.M., Courter, J.R., Sugawara, A., Smith 3rd, A.B., Sodroski, J.G., 2011. An inducible cell-cell fusion system with integrated ability to measure the efficiency and specificity of HIV-1 entry inhibitors. PloS One 6, e26731.

Hombach, J., Palmberger, T.F., Bernkop-Schnurch, A., 2009. Development and in vitro evaluation of a mucoadhesive vaginal delivery system for nystatin. J. Pharmaceut. Sci. 98, 555–564.

Jolly, C., Kashefi, K., Hollinshead, M., Sattentau, Q.J., 2004. HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. J. Exp. Med. 199, 283–293.

Jolly, C., Sattentau, Q.J., 2004. Retroviral spread by induction of virological synapses. Traffic 5, 643–650.

Keele, B.F., Giorgi, E.E., Salazar-Gonzalez, J.F., Decker, J.M., Pham, K.T., Salazar, M.G., Sun, C., Grayson, T., Wang, S., Li, H., Wei, X., Jiang, C., Kirchherr, J.L., Gao, F., Anderson, J.A., Ping, L.H., Swanstrom, R., Tomaras, G.D., Blattner, W.A., Goepfert, P.A., Kilby, J.M., Saag, M.S., Delwart, E.L., Busch, M.P., Cohen, M.S., Montefiori, D.C., Haynes, B.F., Gaschen, B., Athreya, G.S., Lee, H.Y., Wood, N., Seoighe, C., Perelson, A.S., Bhattacharya, T., Korber, B.T., Hahn, B.H., Shaw, G.M., 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc. Natl. Acad. Sci. U. S. A. 105, 7552–7557.

Kouokam, J.C., Huskens, D., Schols, D., Johannemann, A., Riedell, S.K., Walter, W., Walker, J.M., Matoba, N., O'Keefe B, R., Palmer, K.E., 2011a. Investigation of griffithsin's interactions with human cells confirms its outstanding safety and efficacy profile as a microbicide candidate. PloS One 6, e22635.

Kouokam, J.C., Huskens, D., Schols, D., Johannemann, A., Riedell, S.K., Walter, W., Walker, J.M., Matoba, N., O'Keefe, B.R., Palmer, K.E., 2011b. Investigation of griffithsin's interactions with human cells confirms its outstanding safety and efficacy profile as a microbicide candidate. PloS One 6, e22635.

Kouokam, J.C., Lasnik, A.B., Palmer, K.E., 2016. Studies in a murine model confirm the safety of griffithsin and advocate its further development as a microbicide targeting HIV-1 and other enveloped viruses. Viruses 8.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., Hendrickson, W.A., 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393, 648–659.

Lederman, M.M., Offord, R.E., Hartley, O., 2006. Microbicides and other topical strategies to prevent vaginal transmission of HIV. Nat. Rev. Immunol. 6, 371–382.

Leonard, C.K., Spellman, M.W., Riddle, L., Harris, R.J., Thomas, J.N., Gregory, T.J., 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. 265, 10373–10382.

Li, M., Gao, F., Mascola, J.R., Stamatatos, L., Polonis, V.R., Koutsoukos, M., Voss, G., Goepfert, P., Gilbert, P., Greene, K.M., Bilska, M., Kothe, D.L., Salazar-Gonzalez, J.F., Wei, X., Decker, J.M., Hahn, B.H., Montefiori, D.C., 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. 79, 10108–10125.

Li, M., Salazar-Gonzalez, J.F., Derdeyn, C.A., Morris, L., Williamson, C., Robinson, J.E., Decker, J.M., Li, Y., Salazar, M.G., Polonis, V.R., Mlisana, K., Karim, S.A., Hong, K., Greene, K.M., Bilska, M., Zhou, J., Allen, S., Chomba, E., Mulenga, J., Vwalika, C., Gao, F., Zhang, M., Korber, B.T., Hunter, E., Hahn, B.H., Montefiori, D.C., 2006. Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. J. Virol. 80, 11776–11790.

Li, Y., Luo, L., Rasool, N., Kang, C.Y., 1993. Glycosylation is necessary for the correct folding of human immunodeficiency virus gp120 in CD4 binding. J. Virol. 67, 584–588.

Long, E.M., Rainwater, S.M., Lavreys, L., Mandaliya, K., Overbaugh, J., 2002. HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. AIDS Res. Hum. Retrovir. 18, 567–576.

Mazurov, D., Ilinskaya, A., Heidecker, G., Lloyd, P., Derse, D., 2010. Quantitative comparison of HTLV-1 and HIV-1 cell-to-cell infection with new replication dependent vectors. PLoS Pathog. 6, e1000788.

Miranda, L.R., Schaefer, B.C., Kupfer, A., Hu, Z., Franzusoff, A., 2002. Cell surface expression of the HIV-1 envelope glycoproteins is directed from intracellular CTLA-4-containing regulated secretory granules. Proc. Natl. Acad. Sci. U. S. A. 99, 8031–8036.

Montefiori, D.C., 2004. Evaluating neutralizing antibodies againts HIV, SIV and SHIV in luciferase reporter gene assays. In: Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., Strober, W., Coico, R. (Eds.), Current Protocols in Immunology. John Wiley & Sons p12.11.11-12.11.15.

Moore, P.L., Gray, E.S., Wibmer, C.K., Bhiman, J.N., Nonyane, M., Sheward, D.J., Hermanus, T., Bajimaya, S., Tumba, N.L., Abrahams, M.R., Lambson, B.E., Ranchobe, N., Ping, L., Ngandu, N., Abdool Karim, Q., Abdool Karim, S.S., Swanstrom, R.I., Seaman, M.S., Williamson, C., Morris, L., 2012. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. Nat. Med. 18, 1688–1692.

Mori, T., O'Keefe, B.R., Sowder 2nd, R.C., Bringans, S., Gardella, R., Berg, S., Cochran, P., Turpin, J.A., Buckheit Jr., R.W., McMahon, J.B., Boyd, M.R., 2005. Isolation and characterization of griffithsin, a novel HIV-inactivating protein, from the red alga Griffithsia sp. J. Biol. Chem. 280, 9345–9353.

Moulaei, T., Alexandre, K.B., Shenoy, S.R., Meyerson, J., Krumpe, L.R., Constantine, B., Wilson, J., Buckheit, R., McMahon, J.B., Subramaniam, S., Wlodawer, A., O'Keefe, B.R., 2015. Griffithsin tandemers: flexible and potent lectin inhibitors of the human immunodeficiency virus. Retrovirology 12, 6.

Ordanini, S., Varga, N., Porkolab, V., Thepaut, M., Belvisi, L., Bertaglia, A., Palmioli, A., Berzi, A., Trabattoni, D., Clerici, M., Fieschi, F., Bernardi, A., 2015. Designing nanomolar antagonists of DC-SIGN-mediated HIV infection: ligand presentation using molecular rods. Chem. Commun. 51, 3816–3819.

Owen, D.H., Katz, D.F., 1999. A vaginal fluid simulant. Contraception 59, 91–95.

Owen, D.H., Katz, D.F., 2005. A review of the physical and chemical properties of human semen and the formulation of a semen simulant. J. Androl. 26, 459–469.

Pandey, D., Podder, A., Pandit, M., Latha, N., 2016. CD4-gp120 interaction interface - a gateway for HIV-1 infection in human: molecular network, modeling and docking studies. J. Biomol. Struct. Dyn. 1–14.

Panico, M., Bouche, L., Binet, D., O'Connor, M.J., Rahman, D., Pang, P.C., Canis, K., North, S.J., Desrosiers, R.C., Chertova, E., Keele, B.F., Bess Jr., J.W., Lifson, J.D., Haslam, S.M., Dell, A., Morris, H.R., 2016. Mapping the complete glycoproteome of virion-derived HIV-1 gp120 provides insights into broadly neutralizing antibody binding. Sci. Rep. 6, 32956.

Pohlmann, S., Baribaud, F., Doms, R.W., 2001a. DC-SIGN and DC-SIGNR: helping hands for HIV. Trends Immunol. 22, 643–646.

Pohlmann, S., Baribaud, F., Lee, B., Leslie, G.J., Sanchez, M.D., Hiebenthal-Millow, K., Munch, J., Kirchhoff, F., Doms, R.W., 2001b. DC-SIGN interactions with human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus. J. Virol. 75, 4664–4672.

Rudnicka, D., Feldmann, J., Porrot, F., Wietgrefe, S., Guadagnini, S., Prevost, M.C., Estaquier, J., Haase, A.T., Sol-Foulon, N., Schwartz, O., 2009. Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through

polysynapses. J. Virol. 83, 6234–6246.
Sattentau, Q., 2008. Avoiding the void: cell-to-cell spread of human viruses. Nat. Rev. Microbiol. 6, 815–826.
Shattock, R.J., Moore, J.P., 2003. Inhibiting sexual transmission of HIV-1 infection. Nat. Rev. Microbiol. 1, 25–34.
Sowinski, S., Jolly, C., Berninghausen, O., Purbhoo, M.A., Chauveau, A., Kohler, K., Oddos, S., Eissmann, P., Brodsky, F.M., Hopkins, C., Onfelt, B., Sattentau, Q., Davis, D.M., 2008. Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. Nat. Cell Biol. 10, 211–219.
Stein, S.E.G., 2003. Transmission and epidemiology. In: In: Richman, D.D. (Ed.), Human Immunodeficiency Virus International Medical Press, pp. 5:1–5:22.
Vincent, M.J., Melsen, L.R., Martin, A.S., Compans, R.W., 1999. Intracellular interaction of simian immunodeficiency virus Gag and Env proteins. J. Virol. 73, 8138–8144.
Wang, H., Cohen, A.A., Galimidi, R.P., Gristick, H.B., Jensen, G.J., Bjorkman, P.J., 2016. Cryo-EM structure of a CD4-bound open HIV-1 envelope trimer reveals structural rearrangements of the gp120 V1V2 loop. Proc. Natl. Acad. Sci. U. S. A. 113, E7151–E7158.
Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D., Shaw, G.M., 2003. Antibody neutralization and escape by HIV-1. Nature 422, 307–312.
Williamson, C., Morris, L., Maughan, M.F., Ping, L.H., Dryga, S.A., Thomas, R., Reap, E.A., Cilliers, T., van Harmelen, J., Pascual, A., Ramjee, G., Gray, G., Johnston, R., Karim, S.A., Swanstrom, R., 2003. Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. AIDS Res. Hum. Retrovir. 19, 133–144.
Zhu, X., Borchers, C., Bienstock, R.J., Tomer, K.B., 2000. Mass spectrometric characterization of the glycosylation pattern of HIV-gp120 expressed in CHO cells. Biochemistry 39, 11194–11204.
Ziolkowska, N.E., O'Keefe, B.R., Mori, T., Zhu, C., Giomarelli, B., Vojdani, F., Palmer, K.E., McMahon, J.B., Wlodawer, A., 2006. Domain-swapped structure of the potent antiviral protein griffithsin and its mode of carbohydrate binding. Structure 14, 1127–1135.
Ziolkowska, N.E., Wlodawer, A., 2006. Structural studies of algal lectins with anti-HIV activity. Acta Biochim. Pol. 53, 617–626.
Zirafi, O., Kim, K.A., Roan, N.R., Kluge, S.F., Muller, J.A., Jiang, S., Mayer, B., Greene, W.C., Kirchhoff, F., Munch, J., 2014. Semen enhances HIV infectivity and impairs the antiviral efficacy of microbicides. Sci. Transl. Med. 6, 262ra157.