

Griffithsin, Alone and Combined with All Classes of Antiretroviral Drugs, Potently Inhibits HIV Cell-Cell Transmission and Destruction of CD4 $^{\rm +}$ T cells

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Abstract

HIV spreads by cell-free virions but also efficiently through cell-cell mediated contacts. These cell-cell HIV transmission pathways have also been suggested as a mechanism of viral escape from neutralizing antibodies and antiretroviral (ARV) therapy. The carbohydrate-binding agent (CBA) griffithsin (GRFT) inhibits cell-free HIV replication in the pM range (43-630 pM). Here, we evaluated GRFT alone and in combination with compounds of four different classes of antiretroviral drugs (entry inhibitors, reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors)in cell-cell HIV transmission routes and determined the combination index (CI) using the median effect principle. The activities of GRFT and antiretroviral drugs were evaluated in assays of inhibition of giant cell formation, HIV replication and target T cell destruction via light microscopy, multi-parameter flow cytometry and p24 HIV-1 Ag ELISA. GRFT potently inhibits (*i*) giant cell formation between persistently HIV-infected T cells and non-infected CD4⁺ target T cells (EC₅₀: 87 ± 4 pM) and (*ii*) HIV transmission, CD4⁺ T-cell destruction and viral replication through the DC-SIGN mediated pathway (EC₅₀: 25 ± 3 pM).All GRFT/ARV drug combinations displayed synergistic or additive effects (Cl₉₅: 0.30-1.08) on inhibition of cell-cell fusion and on protection against target CD4⁺ T cell destruction. In addition, the GRFT/ARV combinations also potently inhibited short-term (20–24 h) viral replication in T-cells via the DC-SIGN mediated route of transmission. These *in vitro* data are very encouraging for GRFT as an ingredient in a multi-targeted microbicide.

Keywords: Griffithsin; Antiretroviral drugs; Cell-cell; HIV transmission; Flow cytometry combinations; DC-SIGN; Microbicide

Abbreviatons: BanLec: Banana Lectin; CBAs: Carbohydratebinding Agents; CI(s): Combination Index/Indices; DCs: Dendritic Cells; DC-SIGN: Dendritic Cell Specific ICAM-3 Grabbin Nonintegrin; EC_{50} : 50% Effective Concentration; EI(s): Entry Inhibitor(s); GRFT: Griffithsin; INI(s): Integrase Inhibitor(s); mAb: Monoclonal Antibody; NNRTI(s): Non-nucleoside Reverse Transcriptase Inhibitor(s); PI(s): Protease Inhibitor(s); RTI(s): Reverse Transcriptase Inhibitor(s); T20: Enfuvirtide

Background

Genital fluids (e.g. semen and cervico vaginal fluids) from HIV infected people contain both cell-free HIV virions and persistently HIV-infected cells. The dissemination of HIV throughout the human body can occur by cell-free HIV infection and by cell-cell contacts between HIV-infected T cells and non-infected CD4⁺ target T cells at avirological synapse [1,2]. The formation of these synapses starts when high amounts of gp120 expressed on the HIV-infected cells binds to CD4 receptors present on uninfected CD4⁺ target T cells. This results in conformational changes for further co receptor (CXCR4 or CCR5) interactions and finally membrane fusion mediated by gp41 [2,3]. Integrins (e.g. LFA-1) and intercellular adhesion molecule (ICAM) interactions stabilize this complex [2]. This polarized HIV virion budding at the cell-cell contact sites in the presence of lipid rafts increases the infection rate and transmission compared with cellfree HIV [4-6]. Besides cell-cell membrane fusion, recent studies also showed that nanotubes, polysynapses and filopodia are involved in the cell-cell transmission of HIV [7-9].

Acute infection after sexual transmission of HIV is predominated by CCR5 using (R5) viruses [10,11]. Although transmission of CXCR4 using (X4) HIV-1 strains is believed to be rather rare, recent studies clearly demonstrate the presence of X4 viruses during acute infection [12-14].

For therapeutic options or prophylaxis (e.g. microbicide and vaccine) it would be very important to inhibit these HIV cell-cell contacts in addition to inhibiting replication of cell-free virus. The class of carbohydrate-binding agents (CBAs) is described as potent inhibitors of cell-cell virus transmission between persistently HIV-infected cells and non-infected target T cells [15]. They also inhibit capture of HIV by the DC-SIGN (dendritic cell ICAM-3 grabbing non-integrin) receptor and the subsequent transmission to naïve uninfected CD4⁺ T cells [15]. DC-SIGN present on submucosal or intra epithelial DCs seems to play an important role in the transmission of HIV. Upon capture, immature DCs migrate and maturate to the lymph nodes to transmit HIV very efficiently to naive CD4⁺ T cells [16-18]. Together with biomedical and behavioral interventions, antiviral pre-exposure prophylaxis, systemic and/or topically applied, will be very helpful additional tools to reduce the sexual transmission of HIV [17]. As is currently standard of care for systemic treatment of HIV/AIDS infections, microbicidal intravaginal

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ring devices or antiviral gels will presumably consist of a combination of selected anti-HIV drugs.

Here, we focus on griffithsin (GRFT), an unique lectin isolated from the red alga *Griffithsia sp.*, that is described as the most potent and broad-spectrum anti-HIV CBA to date, capable of inhibiting viral replication in the picomolar range [19,20]. We demonstrate that GRFT has a very potent activity in cell-cell HIV transmission assays, alone and in combination with various classes of HIV inhibitors (e.g. entry inhibitors (EIs), reverse transcriptase inhibitors (RTIs), integrase inhibitors (INIs) and protease inhibitors(PIs)) and inhibits the massive depletion of CD4⁺ target T cells. In addition, we also show that dual combinations of GRFT with antiretroviral drugs potently inhibit the efficient and massive short-term viral replication in the DC-SIGN mediated pathway.

Materials and Methods

Compounds

Griffithsin (GRFT; MW= 25.4 kDa) was isolated and purified as described previously [21]. BanLec (MW=30 kDa) was a kind gift from Dr. D.M. Markovitz (University of Michigan, USA). Enfuvirtide (T20; MW=4492 Da) was provided by Dr. E. Van Wijngaerden (UZ Leuven, Belgium). AMD3100 (MW=830 g/mol) was obtained from Sigma-Aldrich (Bornem, Belgium). The mAbs b12, 2F5 and 2G12 were ordered from Polymun Scientific GmbH (Vienna, Austria). Etravirine (TMC-125; MW=435.28 g/mol), raltegravir (MW=482.51 g/mol) and elvitegravir (MW=447.88 g/mol) were obtained from Tibotec (Belgium). Tenofovir (MW=287.21 g/mol) was obtained from Gilead Sciences (Foster City, CA). The naphthalene sulfonated polyanionic compound PRO2000 (MW=~5 g/mol) was a gift from Dr. A.T. Profy (Indevus Pharmaceuticals, Inc. Lexington, MA, USA). The thiocarboxanilide UC-781 (MW=335.5 g/mol) was obtained from Uniroyal Chemical Ltd. (Guelph, Ontario, Canada). The protease inhibitors aquinavir (MW=670.86 g/mol) was kindly provided by Roche Laboratories (Hertfordshire, UK).

Cell cultures and viruses

SupT1, C8166 and non-infected HUT-78/0 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The transfected Raji.DC-SIGN⁺ cells were a gift from Dr. L. Burleigh (Pasteur Institute, Paris France). All cell types were cultured in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) containing 10% fetal calf serum (FCS, Hyclone, Utah, USA) and 1% L-glutamine (Invitrogen). The HIV-1 X4 strain IIIB was obtained from the NIAID AIDS reagent program (Bethesda, MD). The dual-tropic R5/X4 HIV-1 strain HE was originally isolated from a Belgian AIDS patient and further cultured in T cell lines [22].

Generation of persistently HIV-infected T cells

HUT-78/0 cells (5×10⁶ cells) were incubated with high amounts of HIV-1 IIIB (~3×10⁶ pg/ml) for 1.5 h at 37°C, then the cells were resuspended in HUT-78/0 cell culture medium and further cultured for 2 to 3 weeks before being used in co cultivation assays. Viral persistence was determined by giant cell formation after mixture of CD4⁺ SupT1 cells with either HUT-78/0 or HUT-78/IIIB cells.

Cell-cell cocultivation assays (giant cell assays)

Five-fold dilutions of each inhibitor in 100 μ l medium were added in a 96-well plate (BD, Falcon) along with SupT1 cells (1×10⁵ cells/50 μ l). The persistently HIV-infected HUT-78/IIIB cells were washed to remove the presence of cell-free virions and immediately thereafter, the same amount of these persistently infected HUT-78/IIIB cells were seeded and incubated at 37°C. Syncytia formation was first scored microscopically 20-24 h post co cultivation and afterwards the EC₅₀s were measured by flow cytometry (FACSArray, BD). Pictures of giant cell formation over time were generated using the live cell image viewer, JuLITM analyzer (International Medical Products S.A., Brussels, Belgium). SupT1 cell membranes were stained using CellVue^{*} Jade (Polysciences, Inc., Eppelheim, Germany) according to manufacturer's guidelines.

Cell-cell cocultivation combination experiments

Based on EC₅₀s of each inhibitor solely, HIV inhibitors from different classes (EIs, RTIs, INIs and PIs) were combined with the CBA griffithsin (GRFT). Our experimental setup was designed such that the EC₅₀ of each inhibitor was positioned in the middle of its 5-fold dilution range (where possible).

Five-fold dilutions of GRFT (50 μ l) and of each inhibitor (50 μ l) were added in a 96-well plate. Next, 1×10⁵ cells/50 μ l of SupT1 cells were added and immediately mixed withthe persistently infected HUT-78 cells. After 20-24 h of co culture, syncytia formation and EC₅₀s were scored microscopically and the protection of the CD4⁺ SupT1 population was determined by flow cytometry (FACSArray, Beckton Dickinson, San Jose, CA, USA).

Measuring the depletion of CD4⁺ SupT1 cells by flow cytometry

After cocultivation, the target CD4⁺ SupT1 cells were stained with phycoerythrin (PE)-labeled anti-CD28 (anti-CD28-PE; BD), according to a modified method described by Schols et al. [23]. The cells were incubated for 30 min at 4°C with anti-CD28-PE. After extensive washing with PBS containing 2% FCS (PBS/FCS2%), the cells were fixed with a 1% paraformaldehyde solution. Acquisition and analysis occurred on a FACS Array flow cytometer using windows-based FACS Array System Software (BD). Aspecific binding was excluded on the basis of negative control samples of cells incubated with Simul TestTM control (IgG γ 1-FITC/IgG2a-PE) (BD).

HIV-1 HE capture by Raji.DC-SIGN expressing cells (capture assay)

Raji.DC-SIGN cells (5×10^5 cells/200 µl) were added in a 15 ml conical tube (Falcon) together with 200 µl cell culture medium and 100 µl of HIV-1 HE stock ($\sim 3 \times 10^6$ pg/ml of p24 Ag). After 1 h of incubation at 37°C, the cells were thoroughly washed to remove unbound virus to obtain a Raji.DC-SIGN/HE cell suspension of 1×10^5 cells/50 µl. The amount of virus bound to the Raji.DC-SIGN cells was measured by p24 Ag ELISA.

Transmission of HIV-1 captured virus by DC-SIGN to uninfected CD4⁺ target T cells (transmission assay)

Various test compounds were diluted in cell culture medium and added in a 96-well plate together with the CD4⁺ C8166 T cells $(1 \times 10^5$ cells/50 µl). After 1 h of incubation at 37°C, the same numbers of Raji. DC-SIGN/HE cells from the capture assay were co cultivated with the compound pre-treated T cells. Giant cell formation was scored microscopically after 20-24 h of co culture and the HIV-1 p24 Ag levels were also determined as described above.

Combination index (CI) determinations

Combination indices (CIs) were determined using CalcuSyn

software (Biosoft, Cambridge, UK) based on the median effect principle of Chou and Talalay [24]. The obtained CIs represent the kind of drugdrug interactions whereby CI<0.9 are synergistic; 0.9 < CI < 1.1 are additive and CI>1.1 are antagonistic. The CIs at the calculated EC₅₀, EC₇₅ and EC₉₅-level are shown. Figures were made using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Statistical significance was calculated using an unpaired T-test.

Results

Antiviral activity of griffithsin against HIV cell-cell contacts (giant cell assay)

The mixing of persistently HIV-infected T cells (HUT-78/IIIB) with non-infected CD4⁺ target T cells (SupT1) results in massive syncytia or giant cells after 20-24 h (Figures 1 and 2, panels a-c). The first giant cells appear after ~8 h of co culture at 37°C as visualized by the JuLITM analyzer (Figure 1). The maximum number of giant cells is reached at approximately 20 h after the start of the co cultivation. When the target CD4⁺ SupT1 T cells were stained with the green fluorescent Jade dye (λ =478 nm), the migration of these cells into the giant cells could be visualized over time and can be observed as specific green fluorescence inside these syncytia (Figure 1, last panel).

GRFT is very active in inhibiting the fusion between persistently HIV-infected T cells and non-infected target CD4⁺T cells. In figure 2 (panels d-f), a dose-dependent inhibitory effect of GRFT is shown and a mean EC_{so} of 87 ± 4pM is obtained.

Griffithsin/entry inhibitor dual combinations against giant cell formation

In the first set of co cultivation experiments, we evaluated the effects of GRFT in combination with various entry inhibitors (EIs) targeting different steps in the HIV entry process. We combined GRFT with the broad neutralizing anti-gp120 carbohydrate mAb 2G12 [25], the CD4 binding site neutralizing mAb b12 [26], the anti-gp41 neutralizing mAb 2F5 [27], the CXCR4 receptor antagonist AMD3100 [28], the CBA BanLec [29], the fusion inhibitor T20 [30] and the polyanionic compound PRO2000 [31]. The EC₅₀s of GRFT and each entry inhibitor tested, alone and in combination, are shown in table 1. Significant dose-reductions in the EC₅₀ for GRFT were observed in the combination with mAb 2F5, BanLec, T20 and PRO2000 (p<0.05). Each of the evaluated EIs inhibited the giant cell formation dose-dependently, with the exception of 2G12mAb, which had by itself no antiviral effect in this type of cell-cell fusion assay (EC₅₀>100 µg/ml; Table 1 and figure 3A).

The combination indices (CIs) were determined to observe the effect of the GRFT/EI combinations. Most of the GRFT combinations showed synergism at the 50% HIV inhibition level (CI<0.9), with exception of the GRFT/2F5 mAb (CI=1.02) and GRFT/T20 combinations (CI=0.97), which were additive drug pairs (Table 2). Except for the GRFT/BanLec and the GRFT/PRO2000 combinations, a trend in enhancement of synergy with increasing inhibitory concentrations was observed (Table 2). GRFT in combination with PRO2000 showed a moderate synergistic profile at the 3 calculated inhibition levels (50%, 75% and 95%). In the dual CBA combination GRFT/BanLec, rather additive effects were measured at the highest concentration (Table 2). As the 2G12 mAb lacks anti-HIV activity in this assay no CIs could be determined. The observed synergy or additivity with the mAb b12, BanLec and T20, demonstrated by the increase in antiviral potency in combination, can be seen in the leftward shift of the red linein the dosedependent effect curves (Table 2; Figures 3B-D).

Overall, we may conclude that GRFT alone and in combination with the EIs AMD3100, BanLec, T20, PRO2000 and the neutralizing mAbs b12, 2F5 and 2G12profoundly inhibits the fusion between persistently HIV-infected T cells and uninfected CD4⁺ target T cells.

Griffithsin/HIV enzyme inhibitor dual combinations against giant cell formation

Next, we investigated the effects of GRFT in combination with 4 different types of reverse transcriptase inhibitors (RTIs): the nucleotide RTI tenofovir [32] and the non-nucleoside RTIs efavirenz [33], UC-781 [34] and etravirine [35] (Table 3). A significant, up to 5-fold, increase in antiviral activity of GRFT was observed in combination with efavirenz, UC-781 and etravirine. For the combined RTIs, a 2 to 53-fold reduction in EC₅₀-values was obtained, which was significant for tenofovir (p=0.001). Tenofovir at a concentration of 50 µM was never able to completely inhibit giant cell formation (Figure 4A). Similar observations were made with the other used RTIs (data not shown).



Figure 1: Visualization ofgiant cell formation. Persistently HIV-infected HUT-78/IIIB cells were cocultivated with non-infected CD4⁺ target SupT1 T cells at 37°C. Over 24 h of co culture, every 15 minutes, giant cell formation was recorded by the live cell image JuLITM viewer. Images are shown at 0 h, 5 h, 8 h, 11 h, 14 h, 17 h and 20 h. The SupT1 cells were labeled with the green fluorescent CellVue[®] Jade dye kit and after 20 h of coculture the green fluorescent dye of the SupT1 cell membranes is present in the HIV-induced multinucleated giant cells (20 h, last picture panel).



Figure 2: Anti-HIV activity of GRFT in the giant cell assay. The following light microscopic pictures show respectively: persistently HIV-infected HUT-78/IIIB cells (panel a), CD4⁺ target SupT1 T cells (panel b), massive multinucleated giant cells after 24 h of coculture (panel c), dose-dependent protective effect of GRFT in giant cell formation at 0.98 nM (panel d), 0.20 nM (panel e) and 0.04 nM (panel f). Magnification×10/0.25.



Figure 3: Dose-dependent effect curves of GRFT/entry inhibitor combinations in giant cell formation assay. The dose response curves for GRFT/2G12 mAb (A), GRFT/b12 mAb (B), GRFT/T20 (C) and GRFT/BanLec (D) are shown. The black line shows the dose-dependent effect of GRFT. The green line shows the dose-dependent effects of each tested inhibitor (2G12 mAb, b12 mAb, T20 and BanLec) andthe red line shows the effect of the two-drug combinations. Mean values ± SEM up to 4 individual experiments are shown.



Figure 4: Dose-dependent effect curves of GRFT/HIV enzyme inhibitors (e.g. reverse transcriptase, integrase and protease) combinations in T cell-T cell HIV transmission. The dose response curves of GRFTin combination with the reverse transcriptase inhibitor tenofovir (A), the integrase inhibitor rattegravir (B) and the protease inhibitor saquinavir (C) are shown. The black lines show the dose-dependent effects of GRFT on giant cell formation. The green lines show each HIV enzyme inhibitor and the red lines show the effect of the two-drug combinations. Mean \pm SEM up to 3 independent experiments are shown.

At all the calculated HIV inhibition levels (50%, 75% and 95%), the CIs were <1.1, indicating synergistic to additive drug effects (Table 4). The GRFT/efavirenz, GRFT/tenofovir and GRFT/UC-781 combinations showed moderate synergy to synergy with CIs ranging between 0.48 and 0.80 against cell-cell HIV inhibition (Table 4). With the clinically-approved RTI tenofovir, decreasing synergism was observed with increasing drug concentrations, however no antagonism was observed (Table 4, Figure 4A). Surprisingly, the GRFT/etravirine combination resulted in additive effects in this giant cell formation assay (Table 4).

When we combined GRFT with the recently approved class of HIV inhibitors, the integrase inhibitors (INIs) raltegravir [36] and elvitegravir [37], a significant ~15-fold dose reduction was observed for raltegravir (EC₅₀ decreases from 18.7 towards 1.2 nM, Table 3, Figure 4B). A significant 3.6-fold increase in antiviral potency was observed for GRFT when combined with elvitegravir (p = 0.008: Table 3). As shown on figure 4B, at concentrations up to 200 nM of raltegravir, no full protection of giant cell formation was observed.

At the highest concentrations tested, elvitegravir (600 nM) also did not completely inhibit giant cell formation (data not shown). All the calculated inhibitory levels for GRFT, in combination with raltegravir and elvitegravir, were <0.7, thus resulting in synergy (Table 4). For the GRFT/raltegravir combination, an increase in synergy was seen with increasing drug concentrations (Table 4).

Finally, when GRFT was combined with the protease inhibitor (PI) saquinavir [38], no CIs could be determined as saquinavir lacked all anti-HIV activity in this giant cell formation assay (EC₅₀>14.9 μ M; Tables 3 and 4, Figure 4C). A similar observation was also noted for the PI ritonavir (data not shown).

Antiviral activity of GRFT in the DC-SIGN mediated HIV transmission route

Raji.DC-SIGN cells were exposed to the HIV-1 strain HE (Raji. DC-SIGN/HE) and co cultivated with CD4⁺ C8166 T cells. After 24 h massive giant cells were formed (Figure 5, panels a-c). GRFT profoundly and dose-dependently inhibited this process with a mean EC_{50} of 25.3 ± 3.2pM (Figure 5, panels d-f).

Finally, we investigated the effects of GRFT combinations with different classes of antiretroviral drugs on HIV transmission and subsequent viral replication. For these experiments, we pre-incubated the target T cells with antiviral agents for 1 h at 37°C and then added an equal number of HIV-1 HE exposed Raji.DC-SIGN cells.

First, we evaluated the effects of the GRFT/2G12 mAb combination on the HIVDC-SIGN mediated transmission route. The carbohydratebinding anti-gp120 mAb 2G12 inhibited this process dose-dependently with a mean EC₅₀ of 1.8 ± 1.3 µg/ml; a ~9-fold dose reduction (*p* =0.2796) towards 0.21 ± 0.04 µg/ml was observed when combined with GRFT (Figure 6A, left panel). The EC₅₀ for GRFT dropped from 18.4 ± 5.6 pM to 7.2 ± 1.1 pM (*p* =0.1212). At the highest HIV inhibitory levels, synergy was observed (Table 5).

When GRFT was mixed with T20, a non-significant increase in antiviral potency was observed. The EC₅₀s decreased from 20.7 \pm 5.2 pM to 6.4 \pm 1.5 pM (p =0.0574) for GRFT and from 0.048 \pm 0.016 μ M to 0.020 \pm 0.005 μ M (p =0.1676) for T20 (Figure 6A, right panel). At the highest inhibitory concentrations, moderate synergy was observed (Table 5).



Figure 5: Antiviral activity of GRFT in the DC-SIGN mediated HIV transmission pathway. The following light microscopical pictures show respectively: R5/X4 HIV-1 strain HE captured by Raji.DC-SIGN cells (Raji. DC-SIGN/HE; panel a), CD4⁺ target C8166 T cells (panel b), hugegiant cell formation after 24 h of coculture (panel c), dose-dependent protective effect of GRFT against HIV-1 transmission at 0.31 nM (panel d), 0.063 nM (panel e) and 0.013 nM (panel f). Magnification×10/0.25.

With the NNRTI UC-781, at the 3 inhibitory levels, additivity was observed (Table 5). In combination with GRFT, only a significant increase in antiviral potency was observed for UC-781 (EC₅₀, 6.7 ± 0.1 to 1.9 ± 0.3 ; *p*=0.0050).

As shown on the left panel of figure 6B, the NtRTI tenofovir inhibited HIV replication after DC-SIGN transmission dose-dependently with an EC₅₀ of 1.5 \pm 0.6 μ M and decreased to 0.19 \pm 0.08 μ M (p =0.1100) when combined with GRFT. With increasing inhibitory concentrations, a shift from additivity to synergy was observed (Table 5).

Next, we evaluated GRFT in combination with raltegravir against HIV DC-SIGN mediated viral transmission and replication. The EC₅₀ values for single GRFT and raltegravir treatment were 26.5 \pm 2.6 pM and 3.4 \pm 0.6 nM and decreased significantly to 9.9 \pm 0.6 pM (p =0.0033) and 1.18 \pm 0.09 nM (p =0.018), respectively. In combination, no antagonistic effects were observed (Table 5, Figure 6B, middle).

Despite having no activity in the giant cell assay with persistently

HIV-infected T cells, the PI saquinavir was a potent inhibitor of HIV replication at 20-24 h. in the target T cells after co cultivation with DC-SIGN captured virus. Saquinavir inhibited HIV replication in this assay with an EC₅₀ of 38 ± 8 nM. A significant ~12-fold increase in antiviral potency was observed when saquinavir was combined with GRFT (EC₅₀, 3.3 ± 1.5 nM; p =0.0134; Figure 6B, right panel).

Discussion

HIV can infect its CD4⁺ target cells (e.g. macrophages, T cells, DCs) via cell-free HIV particles and/or by virus-infected leukocytes. Multiple studies have shown that HIV spreads very efficient by cell-cell transmission [4,5,39,40]. Although cell-cell interactions are clearly a very common phenomenon *in vivo* (e.g. dendritic cell-T cell, T cell-T cell interactions), HIV-induced syncytia or giant cells seem very difficult to demonstrate *in vivo*. The giant cells are rapidly purged by the immune system; nevertheless a post-mortem study on brain sections of the central nervous system demonstrated the presence of giant cells

Inhibitor	Griffithsin (nM)		Deep reduction	D 40.05	Inhibitor		Dees advetien	D 40.05
	Alone	Combi	Dose reduction	P<0.05	Alone	Combi	Dose reduction	P<0.05
b12mAb (µg/ml)	0.057 ± 0.015	0.019 ± 0.006	3.1	0.058	0.074 ± 0.031	0.038 ± 0.013	1.9	0.323
2F5 mAb (µg/ml)	0.094 ± 0.017	0.024 ± 0.004	4.0	0.016	3.6 ± 1.6	1.2 ± 0.2	3.0	0.200
2G12 mAb (µg/ml)	0.082 ± 0.014	0.070 ± 0.000	1.2	0.487	>100	>100		
AMD3100 (µM)	0.11 ± 0.03	0.040 ± 0.020	2.8	0.111	4.1 ± 3.2	0.50 ± 0.24	8.1	0.332
BanLec (nM)	0.10 ± 0.01	0.043 ± 0.013	2.4	0.012	0.54 ± 0.17	0.15 ± 0.05	3.6	0.073
T20 (µM)	0.10 ± 0.02	0.032 ± 0.001	3.2	0.039	0.098 ± 0.059	0.029 ± 0.011	3.4	0.293
PRO2000 (µM)	0.088 ± 0.008	0.047 ± 0.004	1.9	0.009	0.084 ± 0.003	0.048 ± 0.004	1.8	0.003

^aMean $EC_{50}s \pm SEM$ up to 4 independent experiments

P<0.05 (unpaired T-test), compared to single drug treatment

Table 1: EC_{sn}s^a of GRFT and various entry inhibitors, alone and in combination, in the HIV-1 induced cell-cell assay (giant cell assay).

	CI at varying HIV inhibition		Effe seb		
GRF1+	50% 75% 95%			Enect	
b12 mAb	0.84 ± 0.16	0.72 ± 0.11	0.66 ± 0.12	+++	
2F5 mAb	1.02 ± 0.10	0.85 ± 0.10	0.80 ± 0.20	++	
2G12 mAb	N.A. ^a	N.A. ^a	N.A. ^a	N.A. ^a	
AMD3100	0.60 ± 0.14	0.48 ± 0.12	0.46 ± 0.09	+++	
BanLec	0.88 ± 0.02	0.79 ± 0.12	0.98 ± 0.09	Additivity	
T20	0.97 ± 0.08	0.72 ± 0.05	0.55 ± 0.05	+++	
PRO2000	0.75 ± 0.11	0.72 ± 0.11	0.78 ± 0.17	++	

^aN.A.: not applicable

^bEffect of drug interaction based at Cl_{96%}, whereby Cl<0.9 are synergistic; 0.9<Cl<1.1 are additive and Cl>1.1 are antagonistic. The degree of synergy: 0.85<Cl<0.9: + (slight synergy); 0.7<Cl<0.85: ++ (moderate synergy); 0.3<Cl<0.7: +++ (synergy); 0.1<0.3: ++++ (potent synergy).

Table 2: Combination index (CI) determination of GRFT/entry inhibitor combinations in the HIV-1 induced cell-cell assay (giant cell assay).

Inhibitor	Griffithsin (nM)		Deep reduction	D-0.05	Inhibitor		Dees wednetien	D <0.05
	Alone	Combi	Dose reduction	P<0.05	Alone	Combi	Dose reduction	P <0.05
Reverse transcriptase inhibitors								
efavirenz (nM)	0.094 ± 0.014	0.019 ± 0.003	5.1	0.002	9.7 ± 2.1	4.8 ± 0.7	2.0	0.069
tenofovir (µM)	0.069 ± 0.020	0.064 ± 0.009	1.1	0.860	34.7 ± 4.0	0.66 ± 0.09	52.8	0.001
UC-781 (nM)	0.081 ± 0.009	0.024 ± 0.008	3.3	0.003	3.7 ± 1.1	1.5 ± 0.5	2.5	0.104
etravirine (nM)	0.074 ± 0.015	0.017 ± 0.005	4.4	0.010	1.4 ± 0.5	0.79 ± 0.22	1.8	0.285
Integrase inhibitors								
raltegravir (nM)	0.093 ± 0.015	0.060 ± 0.019	1.5	0.213	18.7 ± 4.1	1.2 ± 0.4	15.2	0.003
elvitegravir (nM)	0.101 ± 0.015	0.028 ± 0.011	3.6	0.008	3.7 ± 1.1	1.7 ± 1.1	2.2	0.252
Protease inhibitors								
saquinavir (µM)	0.080 ± 0.022	0.054 ± 0.004	1.5	0.322	>14.9	>14.9		

^aMean $EC_{50}s \pm SEM$ up to 5 independent experiments.

P<0.05 (unpaired T-test), compared to single drug treatment.

Table 3: EC₅₀s of GRFT and HIV enzyme inhibitors, alone and in combination, in the HIV-1 induced cell-cell assay (giant cell assay).

in a patient who died from AIDS [41] and may contribute to HIV-1 associated dementia [42]. As shown with the live cell image viewer JuLITM analyzer, the first giant cells are formed approximately 8 h post co cultivation. After 20 h the target CD4⁺ SupT1 T cells are almost all fused and engulfed inside the giant cells (Figure 1), demonstrating the strength and importance of cell-cell contacts in the pathogenesis of HIV. The carbohydrate-binding agent (CBA) GRFT, potently inhibited giant cell formation in this type of assay in the pM range (EC₅₀, 87 ± 4 pM; Figure 2). These data are in accordance with observations made by other research groups in comparable performed giant cell assays [19,43].

The attachment receptor DC-SIGN on submucosal or intraepithelial DCs captures various pathogens such as HIV and these DCs transport HIV to the lymph nodes where HIV is efficiently transmitted to naive T cells [17,18]. A recent study also showed that GRFT has an inhibitory effect on DC-SIGN mediated viral capture and subsequent cell-cell transmission in the nM-range [44]. We also demonstrated a very potent anti-HIV activity of GRFT (EC $_{\rm 50}$, 25.3 \pm 3.2 pM) against DC-SIGNmediated viral transmission. The discrepancy between nM potency observed by Alexandre et al. [44] and the pM activity in our assay could be explained by our use of different HIV strains and target T cells (e.g. TZM-bl, C8166 cells) and also by the duration of the assays. Until now, GRFT was only evaluated as a single agent in co cultivation assays and the combination studies described so far were only performed in cellfree HIV replication assays [45,46]. To our knowledge, the anti-HIV activity of GRFT in combination with different classes of antiretroviral drugs has neither been evaluated in the giant cell assay model, nor the DC-SIGN transmission route assay. As the giant cell formation assay is based on the co-cultivation between persistently HIV-infected cells and non-infected CD4⁺ target T cells, multiple replication cycles occur even during a short 20-24 h period.

That EIs as such can be combined in HIV replication assays has previously been reported by us and other research groups [46-49]. However, there is quite some disagreement on the anti-HIV activity of mAbs (e.g. 2G12, b12 and 2F5) and EIs (e.g. T20, AMD3100) in cell-cell HIV transmission models. It has been proven by electron tomography that virological synapse-mediated spread of HIV between T cells is sensitive to entry inhibitors [40]. Our results indicate that all of the evaluated neutralizing mAbs, with exception of 2G12 mAb, interfered with the formation of multinucleated giant cells (Table 1; Figure 3). Comparable observations for the gp41 targeting 2F5 mAb and the anti-carbohydrate binding anti-gp120 mAb 2G12 were seen by Abela et al. [50]. Both these data and our data are in discrepancy with results published by other research groups who found no to weak inhibitory activity of 2F5 mAb [51,52]. The lack of anti-HIV activity in the co cultivation assay of 2G12 mAb is not exactly understood, as 2G12 mAb showed significant antiviral activity in the HIV-1 HE replication assay [53]. We presume that the N-glycan at position 295 (N295) is not sufficiently accessible to the 2G12 mAb during cell-cell contacts. Yee et al. also described a very high IC_{50} for 2G12 mAb of 80 µg/ml [52]. In contrast to the 2G12 mAb, the CD4 binding site (CD4bs) targeting mAb b12 displayed potent activity (IC₅₀: 0.074 μ g/ml) in the giant cell formation assay (Table 1).

GRFT +		CI at varying HIV ir			
	50%	75%	95%	Effect	
Reverse transcriptase in	hibitors	· · · · · · · · · · · · · · · · · · ·			
efavirenz	0.80 ± 0.28	0.48 ± 0.14	0.48 ± 0.08	+++	
tenofovir	0.63 ± 0.11	0.67 ± 0.09	0.76 ± 0.06	++	
UC-781	0.51 ± 0.16	0.49 ± 0.12	0.65 ± 0.06	+++	
etravirine	0.91 ± 0.31	0.87 ± 0.24	1.08 ± 0.12	Additivity	
Integrase inhibitors					
raltegravir	0.67 ± 0.17	0.59 ± 0.15	0.58 ± 0.14	+++	
elvitegravir	0.37 ± 0.13	0.30 ± 0.14	0.64 ± 0.30	+++	
Protease inhibitor					
saquinavir	N.A.ª	N.A. ^a	N.A.ª	N.A.ª	

^aMean Cls ± SEM up to 5 independent experiments are shown. N.A.: not applicable ^bEffect of drug interaction based at $Cl_{95\%}$, whereby Cl<0.9 are synergistic; 0.9<Cl<1.1 are additive and Cl > 1.1 are antagonistic. The degree of synergy: 0.85<Cl<0.9: + (slight synergy); 0.7<Cl<0.85: ++ (moderate synergy); 0.3<Cl<0.7: +++ (synergy); 0.1<0.3: ++++ (potent synergy).

Table 4: Combination index (CI) determination of GRFT/HIV enzyme inhibitor combinations in the HIV-1 induced cell-cell assay (giant cell assay).

GRFT +	CI at varying HIV inhib	CI at varying HIV inhibition ^a				
	50%	75%	95%	Ellect		
Entry inhibitors		· · · · · · · · · · · · · · · · · · ·				
2G12 mAb	0.97 ± 0.24	0.85 ± 0.14	0.65 ± 0.02	+++		
T20	1.13 ± 0.11	0.96 ± 0.07	0.78 ± 0.07	++		
Reverse transcripta	se inhibitors					
UC-781	0.99 ± 0.06	0.95 ± 0.02	1.06 ± 0.01	additivity		
tenofovir	0.92 ± 0.11	0.58 ± 0.12	0.30 ± 0.11	+++(+)		
Integrase inhibitor						
raltegravir	1.08 ± 0.14	1.02 ± 0.11	0.97 ± 0.13	additivity		
Protease inhibitor						
saquinavir	0.79 ± 0.23	0.67 ± 0.15	0.69 ± 0.12	+++		

^aMean values ± SEM out of 2-3 experiments are shown, each performed in duplicate

^bEffect of drug interaction based at Cl_{95%} whereby Cl<0.9 are synergistic; 0.9<Cl<1.1 are additive and Cl > 1.1 are antagonistic. The degree of synergy: 0.85<Cl<0.9: + (slight synergy); 0.7<Cl<0.85: ++ (moderate synergy); 0.3<Cl<0.7: +++ (synergy); 0.1<0.3: ++++ (potent synergy)

Table 5: Combination index determination of GRFT/antiretroviral drug combinations in the DC-SIGN mediated HIV transmission and subsequent replication.

We could also prove that the cell-cell fusion in the co cultivation assay depends on gp41, as we demonstrated that 2F5 mAb and T20 inhibited this process very efficiently. The antiviral activity of T20 is decreased compared to cell-free HIV replication, but still in the lower nM range, as also described by others [50,52,54]. Very strong synergy was observed (CI=0.18) in a cell-cell HIV fusion model between the fusion inhibitors enfuvirtide (T20) (first generation) and sifuvirtide (a second generation gp41 inhibitor) [55]. Synergism of GRFT with T20 was also observed in cell-free HIV replication assays [45].

Approximately a 1000-fold decrease in anti-HIV activity was observed for the CXCR4 antagonist AMD3100 (EC₅₀, ~4 μ M) in a co cultivation assay, compared to its activity in HIV replication assays (EC₅₀, ~5 nM). Presumably the movement and high local density of receptors in virological synapses on the membranes during cell-cell contacts could explain the massive loss of AMD3100 in antiviral activity. However, in both cell-free HIV replications as well as in cell-cell HIV transmission, we observed a synergistic profile between GRFT and AMD3100.

Recently, we showed that GRFT showed a synergistic profile with various other members of the class of CBAs (e.g. BanLec) [46]. However, in the cell-cell co cultivation assay, only additivity between these CBAs was observed. The high expression of viral glycoproteins on the membrane of the persistently-infected T cells may create a less optimal environment (steric hindrance) for the CBAs to gain complete antiviral activity and this could explain the drift from synergy to additivity. For the first time the antiviral activity of BanLec is described in these cell-cell HIV transmission models. BanLec blocked cell-free HIV (EC₅₀, 0.28 - 2.3 nM; [29]) and cell-cell HIV transmission with equal potency (EC₅₀, ~0.5 nM).

Independent of the HIV inhibitory levels (namely $\mathrm{EC}_{\scriptscriptstyle 50},\,\mathrm{EC}_{\scriptscriptstyle 75}$ and EC_{ac}), the CIs of the GRFT/PRO2000 combination varied between 0.72 and 0.78 (Table 2). When this combination was tested against cellfree HIV virus replication in PBMCs against the R5 tropic strains BaL (clade B) and ETH2220 (clade C), moderate synergy was observed as the CI₀₅₀₆ ranged between 0.78 and 0.85 (unpublished observations). Our hypothesis to explain the moderate synergistic activity is that the binding of GRFT to the N-linked glycans present on gp120 can freeze the conformation of gp120, providing PRO2000 with better access to the surface of gp120. This mechanism can also explain the synergy observed between GRFT and b12 mAb in this assay. Alexandre and colleagues claimed that GRFT exposes the CD4bs on gp120 after its interaction with gp120 [56]. On the other hand, polyanionic compounds (e.g. dextran sulfate) are also known as inducers of conformational changes in the viral envelope [57], and could create a better "GRFT-shaped" binding envelope. Combination studies with PRO2000 showed a good synergistic profile with various other types of entry inhibitors (e.g. b12 mAb, T20) against cell-free viral replication at the CI_{90%} levels [48].

RTIs are widely used in the treatment of HIV/AIDS infections and the NtRTI tenofovir was also the first proof-of-concept in the prophylaxis against HIV [58], however these results are difficult to be confirmed in novel clinical trials (e.g. VOICE trial) [59]. Against HIV replication, tenofovir showed a very potent activity (lower μ M ranges) in macrophages and dendritic cells [60]. However its activity in the cocultivation assay was much lower (EC₅₀, ~35 μ M). A study from Sigal et al. [61] confirmed viral replication in the presence of antiretroviral drugs such as tenofovir. We observed a significant ~50-fold reduction in tenofovir concentration effective against cell-cell HIV transmission, in combination with GRFT (Table 3). This combination showed also a



dependent anti-HIV effects of GRFT and the entry inhibitors 2G12 mAb (left panel) and T20 (right panel), alone and in combination in the DC-SIGN pathway. (A) Dosedependent anti-HIV effects of tenofovir (left), raltegravir (middle) and saquinavir (right panel) on HIV replication and transmission alone and in combination with GRFT. Mean values ± SEM out of 3 independent experiments, performed in duplicate are shown.

good synergy against cell-free HIV replication as was described by our research group [45]. The GRFT/tenofovir combination also showed synergy in inhibiting viral replication and transmission via the DC-SIGN route of infection (Table 5).

The NNRTI UC-781 showed a very potent antiviral activity in the co cultivation assay (EC_{50} , 3.7 nM). Due to its high lipophilic properties and high affinity for the RT enzyme, it has been shown to enter intact HIV virions, virus-infected cells and uninfected target T cells and is thus able to inhibit viral replication as well as the cellcell fusion assay [34]. The two other tested NNRTIs efavirenz and etravirine are also lipophilic small molecules, which could interact with the cell membrane to inhibit the giant cell formation. Why the GRFT/ efavirenz combination showed synergy and GRFT/etravirine additivity could presumably be explained by the low solubility and permeability of etravirine. GRFT/efavirenz combinations also showed a synergistic profile against cell-free R5 clade C HIV-1 strain ETH2220 (CIs ranged between 0.75 and 0.59; unpublished results).

Next, we investigated if GRFT could be combined with the INIs raltegravir and elvitegravir. In cell-free HIV replication assays, the GRFT/raltegravir combination showed a moderate synergistic profile (CI_{95%}, 0.84 \pm 0.11, unpublished data). The EC₅₀ of raltegravir in the PBMC HIV replication assay is ~6 nM, a slight decrease in antiviral activity was observed in the co cultivation assay (EC₅₀, 18.7 \pm 4.1 nM). These data are in agreement with other published results [54]. A 1% raltegravir containing gel applied vaginally in macaques challenged with SHIV, 3 hours post-infection, provided significant protection [62]. Elvitegravir, a second integrase inhibitor, is a part of the quadpill (4-in-1 pill, also known as StribildTM) together with tenofovir, emtricitabineand the pharmaco-booster (or CYP3A4 inhibitor) cobicistat. Remarkably, compared to the class of EIs, the evaluated RTIs and INIs are never able to completely inhibit syncytia formation (Figure 4). However, at the three HIV inhibition levels a good synergistic drug profile was observed.

GRFT does not only bind to the glycans on gp120, it also seems to interact with the cell surface of epithelial cells and PBMCs [63]. This interaction has no effect on the trafficking of the INIs through the cell membrane, as synergistic combinations were always observed (Table 4).

The two evaluated PIssaquinavir and ritonavir completely lacked all antiviral activity in the giant cell formation assay (EC₅₀, >14 μ M). Selhorst et al. [54] showed a very potent activity in the lower nM range of these PIs in cell-associated virus, with equal activity against cell-free HIV replication. In order to investigate the effects of antiretroviral drug combinations in cell-cell transmission of HIV, we evaluated our results after 1 day of co culture (e.g. after 20-24 h). The antiviral activity measured by Selhorst et al. [54] was performed 7 days post-infection. Although saquinavir cannot protect the cells from destruction in the cocultivation assay, it very efficiently inhibits viral replication when the target T cells are exposed to HIV captured on DC-SIGN.

Overall, we may conclude that GRFT/antiretroviral drug combinations could be very helpful in the prevention of novel HIV infections as they inhibit the cell-cell contacts between persistently HIV-infected cells and uninfected CD4⁺ target T cells and the DC-SIGN mediated HIV transmission and subsequent replication in naive target T cells. These data are very promising results of the use of GRFT as a microbicidal agent [64], alone or in combination with various classes of antiretroviral drugs. However, regarding these *in vitro* very optimistic results, further *in vivo* experiments are needed.

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