Combinations of Griffithsin with Other Carbohydrate-Binding Agents Demonstrate Superior Activity Against HIV Type 1, HIV Type 2, and Selected Carbohydrate-Binding Agent-Resistant HIV Type 1 Strains

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Abstract

Carbohydrate-binding agents (CBAs) are potential HIV microbicidal agents with a high genetic barrier to resistance. We wanted to evaluate whether two mannose-specific CBAs, recognizing multiple and often distinct glycan structures on the HIV envelope gp120, can interact synergistically against HIV-1, HIV-2, and HIV-1 strains that were selected for resistance against particular CBAs [i.e., 2G12 mAb and microvirin (MVN)]. Paired CBA/CBA combinations mainly showed synergistic activity against both wild-type HIV-1 and HIV-2 but also 2G12 mAb- and MVN-resistant HIV-1 strains as based on the median effect principle with combination indices (CIs) ranging between 0.29 and 0.97. Upon combination, an increase in antiviral potency of griffithsin (GRFT) up to ∼12-fold (against HIV-1), ∼8-fold (against HIV-2), and ∼6-fold (against CBA-resistant HIV-1) was observed. In contrast, HHA/GNA combinations showed additive activity against wild-type HIV-1 and HIV-2 strains, but remarkable synergy with HHA and GNA was observed against 2G12 mAb- and MVN-resistant HIV-1 strains (CI, 0.64 and 0.49, respectively). Overall, combinations of GRFT and other CBAs showed synergistic activity against HIV-1, HIV-2, and even against certain CBA-resistant HIV-1 strains. The CBAs tested appear to have distinct binding patterns on the gp120 envelope and therefore do not necessarily compete with each other's glycan binding sites on gp120. As a result, there might be no steric hindrance between two different CBAs in their competition for glycan binding (except for the HHA/GNA combination). These data are encouraging for the use of paired CBA combinations in topical microbicide applications (e.g., creams, gels, or intravaginal rings) to prevent HIV transmission.

Introduction

THE HIV ENVELOPE GLYCOPROTEIN gp120 is highly glyco-sylated and N-linked glycans account for ∼50% of its molecular mass.[1,2] The high-mannose and hybrid-type N-glycans on gp120 contain α(1,2), α(1,3), and α(1,6) mannose (Man) bridges and are thus considered as interesting targets for mannose-specific carbohydrate-binding agents (CBAs).[3] CBAs are proposed as potential microbicidal agents as they inhibit (1) cell-free HIV replication, (2) cell–cell transmission between HIV-infected and HIV-uninfected CD4$^+$ T cells, (3) capture of HIV by DC-SIGN-expressing cells, and (4) subsequent transmission to CD4$^+$ target T cells.[3]

Natural CBAs are present in various species such as prokaryotes, plants, invertebrates, and vertebrates. The mannose-specific plant lectins *Hippeastrum hybrid* agglutinin (HHA) and *Galanthus nivalis* agglutinin (GNA) predominantly recognize, respectively, α(1,3) or α(1,6)Man and α(1,3)Man structures on gp120.[4] One of the most potent and broad-spectrum plant-derived anti-HIV-1 CBA is BanLec, a jacalin-related CBA isolated from the banana *Musa acuminata*; however, it also showed mitogenic activity.[5] Griffithsin (GRFT), another mannose-specific CBA originally isolated from the red algae *Griffithsia* sp., shows a very potent and broad-spectrum anti-HIV-1 activity in the picomolar range,[6,7] as well as an outstanding safety

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and efficacy profile.[8] The prokaryotic lectin microvirin (MVN) targets α(1,2)Man residues on the viral envelope and showed an anti-HIV-1 activity that is comparable to the well-studied microbicidal drug candidate cyanovirin-N (CV-N), but has a better safety profile.[9] The neutralizing mAb 2G12 was isolated from the blood of an HIV-infected patient and also targets α(1,2)Man structures.[10,11] The discovery that the monoclonal antibody (mAb) 2G12 targets N-linked glycans present on gp120 revealed that these glycans are interesting targets for neutralizing antibodies.[11,12]

Antiretroviral drug resistance is one of the major reasons for anti-HIV therapy failure and the spread of resistant viral strains. To control and eventually diminish the HIV pandemic, novel prevention strategies are necessary. Therefore, self-administered topical preexposure prophylaxis (PrEP) measures such as vaginal and rectal gels, creams, and various intravaginal ring (IVR) systems with sustained or controlled release of substances[13] would be very helpful tools. While gels mainly are being evaluated at the moment for further use,[14,15] studies with an IVR system containing the nonnucleoside reverse transcriptase inhibitor dapivirine also showed a safe and good pharmacokinetic profile.[16–18] Efficient prevention by topical/systemic microbicides will, as for the treatment of HIV infections, likely necessitate a combination of drugs. A silicone IVR loaded with dapivirine and the CCR5 antagonist maraviroc is now undergoing preclinical investigations (MTN 013/IPM 026). The focus on prevention methods against novel HIV infections is mainly on HIV-1. HIV-2 receives less attention but is predominantly found in West-African countries (such as Guinea-Bissau, Ivory Coast), and has also been reported in parts of Europe, America, and Asia as well.[19] Although having lower transmission efficiency and a slower disease progression compared to HIV-1,[20,21] in late stage disease a similarly high mortality is noted.[22] The antiviral therapeutic or prophylactic options against HIV-2 have been less investigated. All first-generation nonnucleoside reverse transcriptase inhibitors (e.g., efavirenz), some protease inhibitors (e.g., amprenavir), and some entry inhibitors (e.g., enfuvirtide) have strongly reduced or no anti-HIV-2 activity.[23]

Here, we focus on the most potent mannose-specific CBAs described to date (Table 1) and investigated whether the combination of two mannose-specific CBAs (in particular GRFT or MVN in combination with other CBAs), recognizing multiple and often different glycans on gp120, can behave synergistically against HIV-1, HIV-2, and CBA-resistant HIV-1 replication.

Materials and Methods

Compounds and monoclonal antibodies

GRFT was isolated and purified as described elsewhere.[24] BanLec was produced in *Escherichia coli* (M. Swanson *et al.*, unpublished observations). MVN was a kind gift from Prof. Dr. Elke Dittmann and Dr. Jan-Christoph Kehr (University of Potsdam, Germany), and the mannose-specific plant lectins HHA and GNA were ordered from E.Y. Laboratories Inc. (San Mateo, CA). The nucleotide reverse transcriptase inhibitor tenofovir was obtained from Gilead Sciences (Foster City, CA). The mAbs 2G12 and b12 were obtained from Polymun Scientific (Vienna, Austria).

TABLE 1. MANNOSE SPECIFICITY OF THE CARBOHYDRATE-BINDING AGENTS USED IN THIS STUDY

CBAs	Molecular weight	Sugar specificity
HHA	50 kDa	α(1,3)-α(1,6)Man
GNA	50 kDa	Manα(1,3)Man
2G12 mAb	∼150 kDa	Manα(1,2)Man
MVN	14.3 kDa	Manα(1,2)Man
BanLec	30 kDa	High-mannose
GRFT	24.4 kDa	High-mannose

CBAs, carbohydrate-binding agents; HHA, *Hippeastrum* hybrid agglutinin; GNA, *Galanthus nivalis* agglutinin; mAb, monoclonal antibody; Man, mannose; MVN, microvirin; BanLec, banana lectin; GRFT, griffithsin.

Cell lines and cell cultures

MT-4 cells were a gift from Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France) and cultured in RPMI-1640 medium supplemented with 10% FCS (Hyclone, Perbio Science, Aalst, Belgium) and 2 mM L-glutamine (Invitrogen, Merelbeke, Belgium) at 37°C in a 5% CO_2 controlled atmosphere.

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated out of buffy coats using Lymphoprep (density: 1.077 g/ml) (Nycomed, Oslo, Norway), obtained from the Blood Transfusion Centre (UZ Leuven, Belgium). PBMCs were cultured in RPMI-1640 containing 10% FCS, 2 mM L-glutamine, and 2 ng/ml interleukin (IL)-2 (Roche Molecular Biochemicals, Indianapolis, IN). The cells were activated with 2 μg/ml PHA (Sigma-Aldrich, Bornem, Belgium) for 3 days before infection with HIV-1 BaL.

Viruses

The HIV-1 R5 strain BaL and X4 strain NL4.3 were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). The *in vitro* generation of the HIV-1 strains NL4.3[2G12res] (2G12 mAb-resistant NL4.3 HIV-1 strain) and NL4.3[MVNres] (microvirin-resistant NL4.3 HIV-1 strain) was described earlier.[9,25] The HIV-2 strain ROD was originally obtained from the Medical Research Council (MRC; London, UK).

Antiviral assays

The anti-HIV-1 and anti-HIV-2 activity of each compound, alone and in combination, in MT-4 cell cultures was determined by a tetrazolium-based colorimetric assay.[26] Briefly, 3-fold dilutions of various test compounds were added in a 96-well plate and preincubated for 20 min at 37°C with MT-4 cells (1×10^6 cells/ml). Next, various concentrations of virus (NL4.3 wild-type, NL4.3[2G12res], NL4.3[MVNres], and HIV-2 ROD) were given depending on the $TCID_{50}$ of the virus stock. Five days postinfection, cytopathic effects (CPE) were scored microscopically and antiviral activity was measured by MTS/PES using a Spectramax 96-well plate reader (Molecular Devices) as described previously.[26,27]

PHA-stimulated PBMCs were resuspended in cell culture medium supplemented with 2 ng/ml IL-2 and seeded (2.5×10^6 cells/ml) in 48-well plates (Iwaki Glass, Iwaki, Japan) containing various concentrations of test compounds. After 20 min of preincubation at 37°C, infection with HIV-1 BaL (500 pg/ml) occurred. IL-2 was added at days 3 and 6

postinfection. Supernatant was collected at day 10 and viral replication was measured using HIV-1 p24 Ag ELISA (Perkin Elmer, Zaventem, Belgium) according to the manufacturer's guidelines.

Synergy analysis

Combination indices (CI) were calculated using the CalcuSyn software (Biosoft, Cambridge, UK) based on the median effect principle of Chou and Talalay[28] whereby CI < 0.9 are being synergistic, 0.9 < CI < 1.1 are additive, and CI > 1.1 are antagonistic.

Results

GRFT/CBA combinations demonstrate potent inhibition against X4 HIV-1 NL4.3 infection

GRFT, MVN, mAb 2G12, BanLec, HHA, GNA, and the CD4 binding site (bs)-targeting mAb b12 were first tested individually, as single agents, and afterward in combination with other CBAs against X4 HIV-1 NL4.3 replication in MT-4 T cell cultures. The 50% effective concentration (EC$_{50}$s) for each agent alone and in combination against HIV-1 replication are shown in Table 2. For GRFT, the EC$_{50}$s were in the lower nM range between 0.048 and 0.075 nM and up to 3-fold and 7-fold reductions in the GRFT and the combined inhibitor concentrations were observed in a two-drug combination study to keep equally potent antiviral activities (Table 2). The most potent synergistic combinations were GRFT/2G12 mAb and GRFT/b12 mAb with combination indices (CIs) of 0.36 ± 0.05 and 0.44 ± 0.08, respectively (Fig. 1A and Table 2). Synergism against X4 HIV-1 NL4.3 was also noticed with GRFT/MVN and GRFT/BanLec, as observed by the leftward shift of the dose-dependent curves in the drug combinations compared to single drug treatment (Fig. 1B and C).

A comparable observation in EC$_{50}$ value decrease and CI determination was made for the CBA MVN (Table 2). Combination with the carbohydrate binding mAb 2G12 and the CD4bs-targeting mAb b12 resulted in potent cooperative synergistic protection with CIs of 0.29 ± 0.04 and 0.56 ± 0.11 for the combinations of MVN/2G12 mAb and MVN/b12 mAb, respectively (Table 2).

When evaluating HHA in combination with GRFT (CI, 0.71 ± 0.13), MVN (CI, 0.96 ± 0.07), or GNA (CI, 1.31 ± 0.14), moderate synergy to nonsynergistic interactions were observed (Table 2 and Fig. 1D).

Synergistic activity of GRFT/CBA combinations against R5 HIV-1 BaL

Next, we investigated the potential synergistic effects of GRFT and other CBAs against the R5 HIV-1 strain BaL in peripheral blood mononuclear cell (PBMC) cultures. GRFT inhibits HIV-1 BaL replication with a mean EC$_{50}$ of 0.20 ± 0.05 nM. In the GRFT/2G12 mAb combination (Fig. 2A), up to 12-fold less GRFT and 2G12 mAb were needed to inhibit HIV-1 BaL replication. Reduced EC$_{50}$s from 0.20 ± 0.05 nM toward 0.014 ± 0.005 nM for GRFT ($p = 0.08$) and from 0.89 ± 0.72 μg/ml towards 0.067 ± 0.02 μg/ml for 2G12 mAb ($p = 0.3$) were observed after combination. When the combination index was determined, a CI value of 0.48 ± 0.12 was observed (Fig. 3). MVN blocked HIV-1 BaL with an EC$_{50}$ of

TABLE 2. ANTI-HIV-1 ACTIVITY (EC$_{50}$s EXPRESSED IN nM) OF TWO-DRUG COMBINATIONS IN MT-4 CELLS INFECTED WITH X4 HIV-1 NL4.3

| Agent 1 | Agent 2 | EC$_{50}$ of agent 1 (nM) | | | | EC$_{50}$ of agent 2 (nM) | | | | CI | Synergy |
		Individual	Combined	Dose reduction	p-value	Individual	Combined	Dose reduction	p-value		
GRFT	2G12 mAb[a]	0.061 ± 0.014	0.026 ± 0.004	2.3	0.0800	4.0 ± 0.8[a]	0.56 ± 0.09[a]	7.1	0.0145	0.36 ± 0.05	+ + +
GRFT	MVN	0.048 ± 0.006	0.023 ± 0.004	2.1	0.0146	17.8 ± 0.9	9.1 ± 1.7	2.0	0.0038	0.63 ± 0.13	+ + +
GRFT	BanLec	0.052 ± 0.008	0.020 ± 0.002	2.6	0.0170	0.20 ± 0.05	0.047 ± 0.006	4.3	0.0345	0.59 ± 0.09	+ + +
GRFT	HHA	0.053 ± 0.003	0.023 ± 0.005	2.3	0.0024	3.9 ± 0.4	2.0 ± 0.4	2.0	0.0165	0.71 ± 0.13	+ +
GRFT	b12 mAb[a]	0.075 ± 0.005	0.027 ± 0.006	2.8	0.0029	0.82 ± 0.26[a]	0.29 ± 0.06[a]	2.8	0.1159	0.44 ± 0.08	+ + +
MVN	2G12 mAb[a]	20.3 ± 3.6	10.1 ± 2.5	2.0	0.0624	2.1 ± 0.6[a]	0.22 ± 0.03[a]	9.2	0.0180	0.29 ± 0.04	+ + + +
MVN	HHA	10.7 ± 4.2	3.0 ± 0.1	3.6	0.1499	4.3 ± 1.2	2.1 ± 0.7	2.1	0.1893	0.96 ± 0.07	Additive
MVN	BanLec	15.40 ± 0.02	9.1 ± 1.4	1.7	0.0459	0.15 ± 0.03	0.06 ± 0.01	2.6	0.0937	0.65 ± 0.01	+ + +
MVN	b12 mAb[a]	16.4 ± 0.4	5.7 ± 1.3	2.9	0.0145	0.54 ± 0.10[a]	0.14 ± 0.03[a]	3.9	0.0208	0.56 ± 0.11	+ + +

[a]Concentrations of the mAbs 2G12 and b12 are expressed in μg/ml. Dose reduction is the reduction in inhibitor concentration after combination compared to single drug treatment. Combination index: CI represented by the mean ± SEM from up to five independent experiments, where by CI < 0.9 indicates synergism, 0.9 < CI < 1.1 indicates additivity, and CI > 1.1 indicates antagonism. The degree of synergy is calculated at the EC$_{95}$ level with +, slight synergism (CI: 0.85–0.90); + +, moderate synergism (CI: 0.70–0.85); + + +, synergism (CI: 0.30–0.70); and + + + +, strong synergism (CI: 0.10–0.30). $p < 0.05$ is considered significant. CI, combination index. A p-value with $p < 0.05$ (unpaired t-test), compared to single drug treatment.
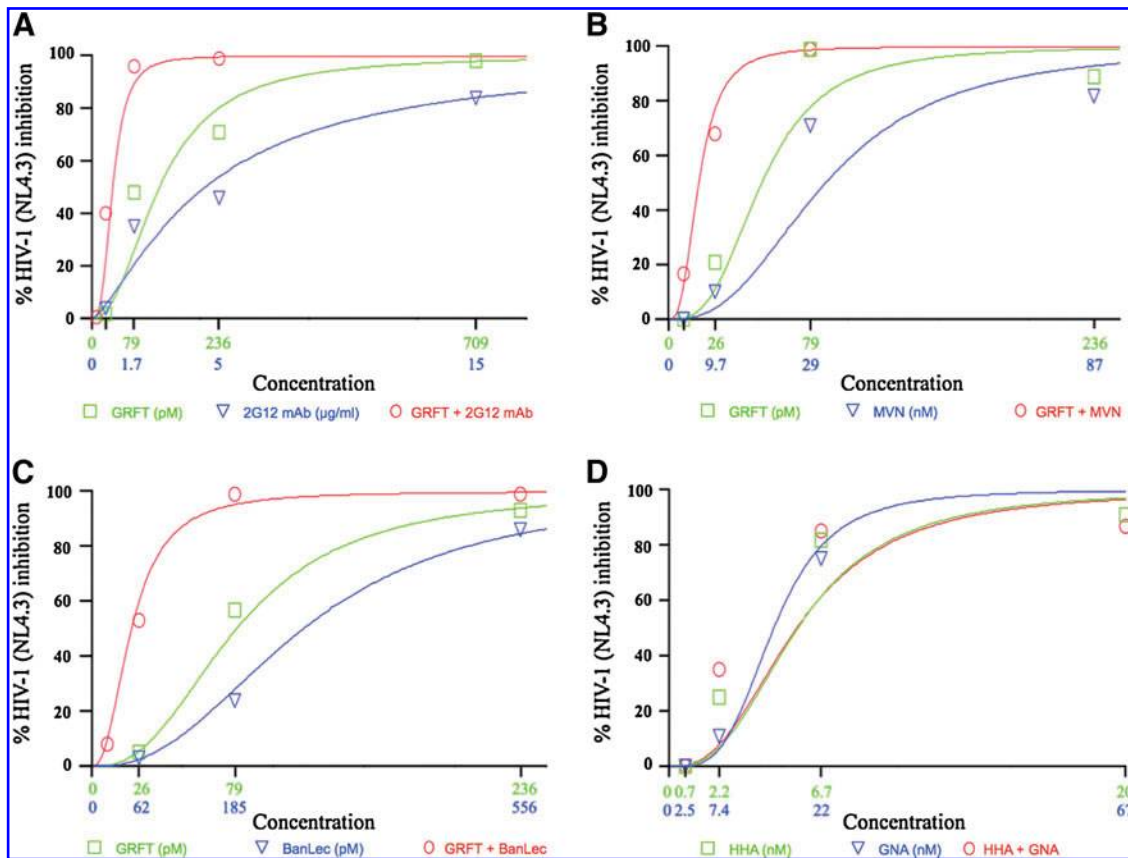
FIG. 1. Dose-dependent effect curves of CBA combinations against X4 HIV-1 NL4.3 replication in MT-4 cells. Prior to infection with X4 HIV-1 NL4.3, MT-4 cells were pre-incubated with GRFT, 2G12 mAb, MVN, BanLec, HHA or GNA alone and in combination for 20 minutes. The dose-response curves for GRFT/2G12 mAb **(A)**, GRFT/MVN **(B)**, GRFT/BanLec **(C)** and HHA/GNA **(D)** are shown. The green squares and blue triangles show the dose-dependent effects of each CBA individually and the red circles their combinations. The decrease in EC_{50}s of each CBA after combination and also the degree of synergism can be seen in the shift to the left of the red line towards the Y-axis. One representative experiment out of 5 separate experiments is shown. CBA, carbohydrate-binding agent.

34.5 ± 9.6 nM. In combination with GRFT, a 2-fold decrease to 19.0 ± 4.3 nM was observed for MVN ($p = 0.2$) and a 3-fold decrease to 0.07 ± 0.02 nM for GRFT ($p = 0.07$) (Fig. 2B). Evaluation of the GRFT/MVN combination showed a moderate synergistic profile (CI value of 0.74 ± 0.07; Fig. 3). A comparable degree of synergism could be observed for the GRFT/BanLec combination (CI, 0.77 ± 0.05); however, its synergistic effect is less pronounced, probably due to a weak but significant mitogenic activity of BanLec on PBMCs (Figs. 2C and. 3).

The observed EC_{50} of HHA against HIV-1 BaL was 76.1 ± 6.5 nM. In combination with GRFT and GNA, additive effects or weak antagonistic effects (CI, 0.97 ± 0.04 and 1.10 ± 0.10, respectively) were observed (Figs. 2D and 3).

Potent anti-HIV-2 activity of GRFT and BanLec alone and in combination with other anti-HIV drugs

No data have been reported on the anti-HIV-2 activity of GRFT and BanLec. Therefore, we evaluated the antiviral activity of GRFT and BanLec as a single agent, and in combination with the CBAs HHA, MVN, and 2G12 mAb against HIV-2 ROD replication in MT-4 cell cultures (Table 3). HIV-2 ROD was susceptible to GRFT and BanLec with a mean EC_{50} of 0.17 ± 0.02 nM and 0.13 ± 0.01 nM, respectively. When GRFT was combined with BanLec or with HHA, a significant

5-fold increase in antiviral activity was noted for GRFT up to 46 pM, whereby both combinations resulted in a synergistic inhibitory profile with CI values varying between 0.42 and 0.84 (Table 3 and Fig. 4A).

Next, we investigated whether the CBAs 2G12 mAb and MVN, which have very weak, if any anti-HIV-2 activity,[9] could enhance the antiviral activity of GRFT on HIV-2 replication. The lack of a dose-dependent effect of 2G12 mAb and MVN makes the calculation of a combination index impossible,[28] but as shown in Fig. 4B and C, neither 2G12 mAb nor MVN seems to have any beneficial effect on the antiviral activity of GRFT. No dose reductions in GRFT concentrations were observed (and vice versa). Finally, when the plant lectins HHA and GNA were combined to inhibit HIV-2 replication, an additive antiviral effect was observed (Fig. 4D and Table 3).

Anti-HIV-2 activity of combinations of GRFT with tenofovir and mAb b12

Additionally, we investigated whether synergism occurred between CBAs and the clinically widely used anti-HIV-1 reverse transcriptase inhibitor (RTI) tenofovir and the CD4bs-targeting mAb b12. As shown in Table 3, combining GRFT and tenofovir increased their antiviral potency and resulted in a pronounced synergistic drug–drug profile with a CI of
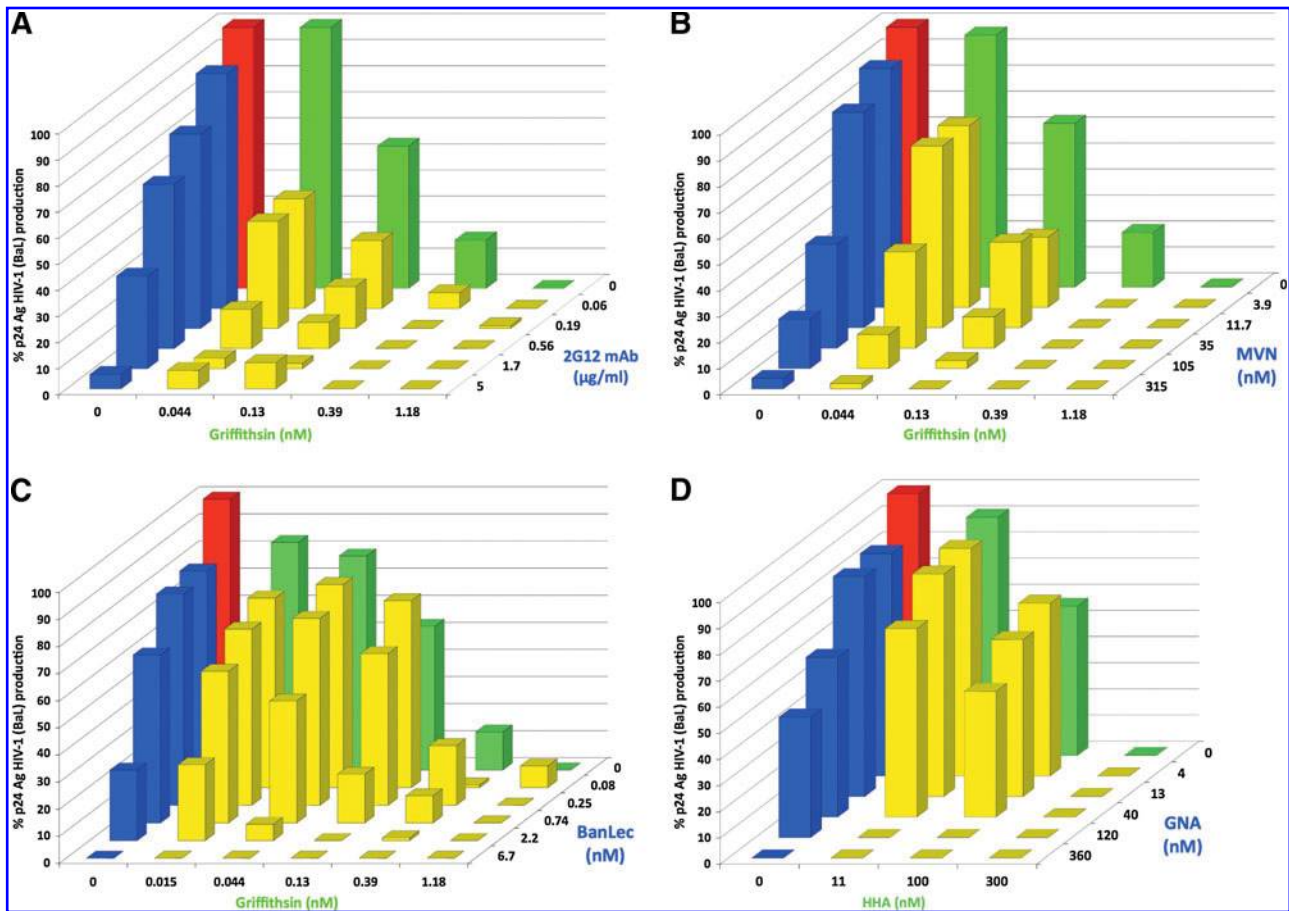
FIG. 2. Dose-dependent effect plots of CBA combinations in PBMCs infected with R5 HIV-1 BaL. PBMCs were infected with R5 HIV-1 BaL after pre-incubation with GRFT, 2G12 mAb, MVN, BanLec, HHA or GNA alone and in combination. Viral replication was measured by p24 HIV-1 Ag ELISA. The green bars represent the dose-dependent effect of GRFT (panels **A–C**) and HHA (panel **D).** The blue bars show the effects of 2G12 mAb **(A)**, MVN **(B)**, BanLec **(C)** and GNA **(D)**. The effect of the dual CBA combinations is represented in yellow and the red bar shows the % p24 Ag HIV-1 production in untreated conditions (positive control). The mean values out of 2–4 separate PBMC donor experiments are shown.
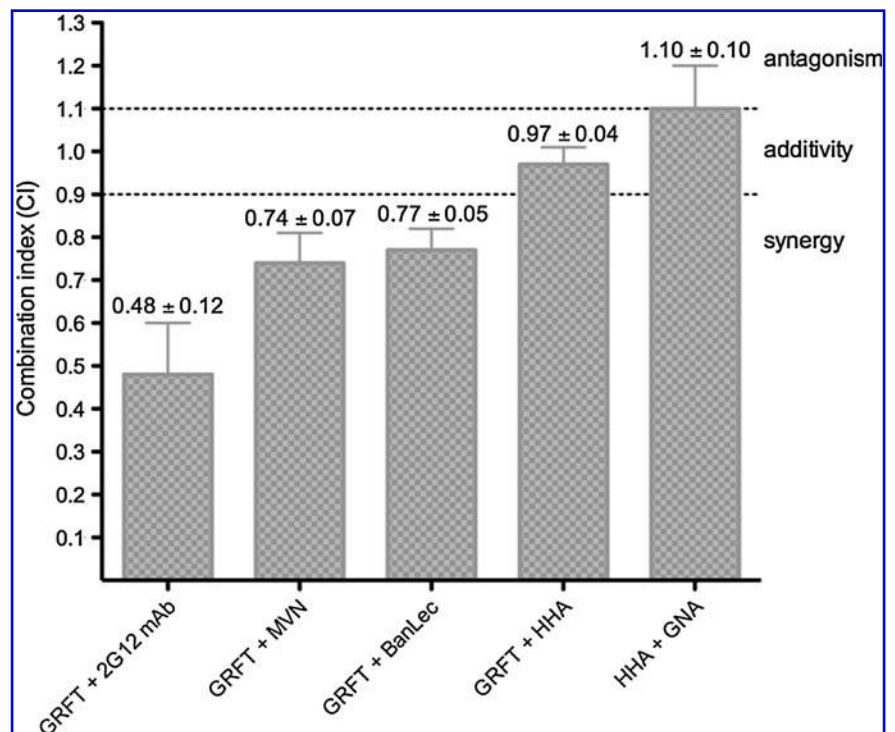
FIG. 3. Combination index (CI) determination in PBMCs after infection with R5 HIV-1 BaL. Synergism, additivity or antagonism of 2-drug combinations were calculated using CalcuSyn, whereby $CI < 0.9$ are synergistic; $0.9 < CI < 1.1$ are additive and $CI > 1.1$ are antagonistic. The GRFT/2G12 mAb combination showed the most potent synergistic interaction, while GRFT/MVN and GRFT/BanLec were moderately synergistic. GRFT/HHA and HHA/GNA combinations showed no synergism. Mean CI-values ± SEM of 2–4 PBMC donors are shown.

TABLE 3. ANTI-HIV-2 ACTIVITY OF GRIFFITHSIN (EC$_{50}$ VALUES IN nM) ALONE AND IN COMBINATION
WITH OTHER ANTI-HIV AGENTS IN MT-4 CELLS

| | | EC$_{50}$ of agent 1 (nM) | | Dose | | EC$_{50}$ of agent 2 (nM) | | Dose | | | |
Agent 1	Agent 2	Individual	Combined	reduction	p-value	Individual	Combined	reduction	p-value	CI	Synergy
GRFT	BanLec	0.19 ± 0.02	0.038 ± 0.005	5.0	0.0017	0.13 ± 0.01	0.089 ± 0.012	1.5	0.0853	0.84 ± 0.08	+ +
GRFT	HHA	0.23 ± 0.06	0.046 ± 0.006	5.0	0.0317	6.4 ± 0.4	3.9 ± 0.5	1.6	0.0200	0.42 ± 0.07	+ + +
GRFT	2G12 mAb[a]	0.13 ± 0.05	0.10 ± 0.02	1.3	0.6388	>50[a]	>50[a]	N.A.		N.A.	
GRFT	MVN	0.11 ± 0.02	0.12 ± 0.05	1.1	0.8999	>262	>262	N.A.		N.A	
GRFT	Tenofovir	0.24 ± 0.02	0.030 ± 0.006	8.0	0.0004	2000 ± 500	1200 ± 200	1.7	0.1969	0.54 ± 0.05	+ + +
GRFT	b12 mAb[a]	0.14 ± 0.02	0.13 ± 0.01	1.1	0.6608	>50[a]	>50[a]	N.A.		N.A	
HHA	GNA	14.0 ± 10.0	1.7 ± 0.4	8.1	0.1992	3.2 ± 1.6	1.7 ± 0.4	1.8	0.3562	1.03 ± 0.07	Additive

[a]Concentrations of the mAbs 2G12 and b12 are expressed in μg/ml. Dose reduction is the reduction in inhibitor concentration after combination compared to single drug treatment. Combination index: CI represented by the mean ± SEM from up to four independent experiments, whereby CI < 0.9 indicates synergism, 0.9 < CI < 1.1 indicates additivity, and CI > 1.1 indicates antagonism. The degree of synergy is calculated at the EC$_{95}$ level with +, slight synergism (CI: 0.85–0.90); + +, moderate synergism (CI: 0.70–0.85); + + +, synergism (CI: 0.30–0.70); and + + + +, strong synergism (CI: 0.10–0.30). A p-value with $p < 0.05$ is considered significant. N.A., not applicable.

0.54 ± 0.05. As observed for the 2G12 mAb, b12 mAb lacks anti-HIV-2 activity and therefore, again, no combination index could be determined.

Superior anti-HIV-1 activity of dual CBA combinations against CBA-resistant HIV-1 strains.

First, we evaluated the effects of paired CBA combinations against HIV-1 NL4.3^{2G12res}, lacking the N-glycan on position

295 (N295) of gp120.[25] GRFT inhibited this strain with a mean EC$_{50}$ of 0.60 ± 0.07 nM, while for HHA and BanLec an EC$_{50}$ of 0.28 ± 0.04 nM and 0.24 ± 0.07 nM was noticed, respectively (Table 4). In both combinations, similar (synergistic) combination indices were observed: CI of 0.64 ± 0.10 for GRFT/HHA and 0.62 ± 0.15 for GRFT/BanLec (Table 4). When the combination of GRFT with 2G12 mAb was evaluated, no significant changes in the effective GRFT concentrations were noticed. Due to the lack of a dose-dependent effect of 2G12
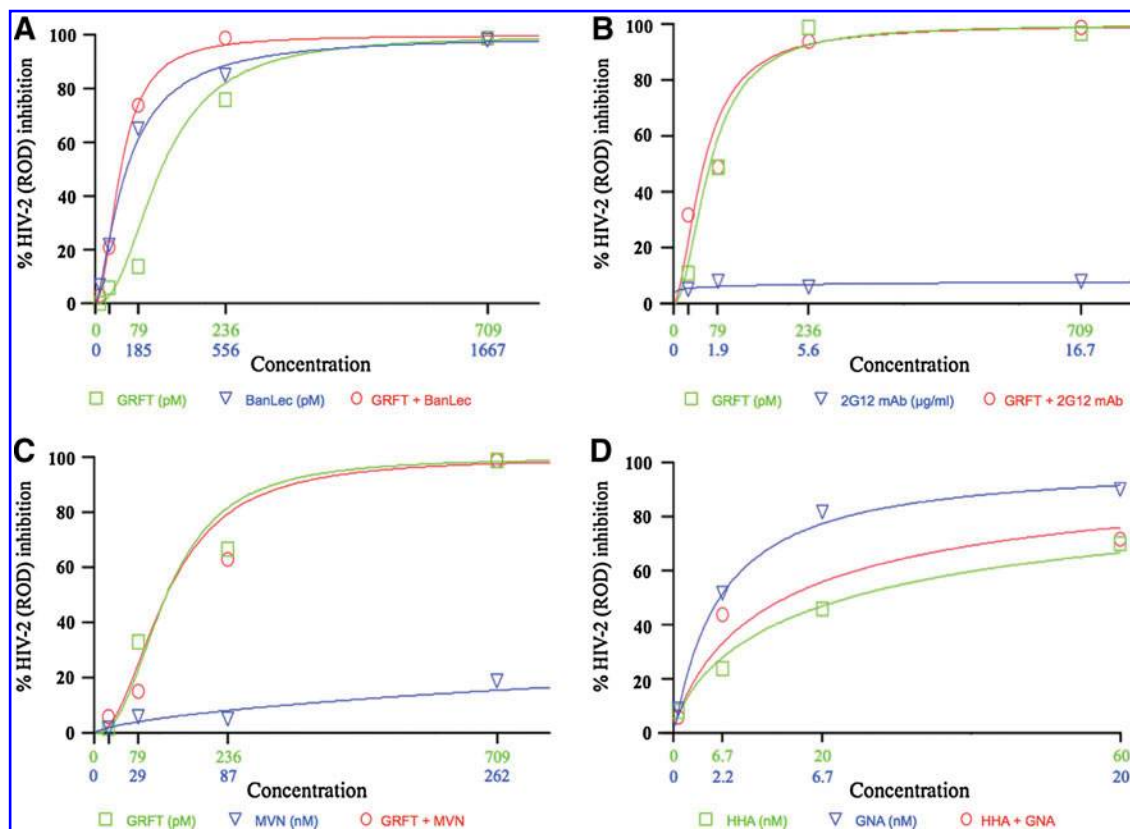

FIG. 4. Dose-dependent effect curves of CBA combinations on HIV-2 replication. Dose-dependent anti-HIV-2 effects for GRFT/BanLec (A), GRFT/2G12 mAb (B), GRFT/MVN (C) and HHA/GNA (D) are shown. The green squares and blue triangles, and the red circles indicate the antiviral activity of each CBA individually and their combination, respectively. The observed leftwards shift is an indication for synergistic drug interactions. One representative experiment out of 4 is shown.

TABLE 4. POTENT SYNERGISM OF SELECTED DUAL CARBOHYDRATE-BINDING AGENT COMBINATIONS AGAINST HIV-1 NL4.3^{2G12res} AND NL4.3^{MVNres} IN MT-4 CELLS

Mutant HIV-1	CBA 1	CBA 2	EC$_{50}$ of CBA 1 (nM)		Dose reduction	p-value	EC$_{50}$ of CBA 2 (nM)		Dose reduction	p-value	CI	Synergy
			Individual	Combined			Individual	Combined				
NL4.3^{2G12res}	GRFT	BanLec	0.53±0.01	0.14±0.02	3.7	<0.0001	0.24±0.07	0.11±0.01	2.2	0.1388	0.62±0.15	+++
	GRFT	2G12 mAb[a]	0.74±0.19	0.46±0.13	1.6	0.3526	>50[a]	>50[a]	N.A.		N.A.	+++
	GRFT	HHA	0.53±0.05	0.14±0.02	3.8	0.0023	0.28±0.04	0.12±0.02	2.3	0.0237	0.64±0.10	+++
	HHA	GNA	0.46±0.07	0.19±0.03	2.5	0.0103	0.43±0.07	0.17±0.04	2.5	0.0166	0.64±0.09	+++
NL4.3^{MVNres}	GRFT	BanLec	0.89±0.17	0.32±0.05	2.8	0.0169	0.94±0.30	0.20±0.03	4.6	0.0511	0.42±0.04	+++
	GRFT	2G12 mAb[a]	0.35±0.33	0.38±0.35	1.1	0.9548	>50[a]	>50[a]	N.A.		N.A.	
	GRFT	HHA	1.14±0.31	0.18±0.04	6.4	0.0223	3.8±0.6	0.68±0.15	5.7	0.0025	0.34±0.16	+++
	HHA	GNA	12.9±2.1	2.3±0.6	5.6	0.0407	5.1±1.7	2.3±0.6	2.2	0.2657	0.49±0.06	+++

[a]Concentration of 2G12 mAb is expressed in μg/ml. Dose reduction is the reduction in inhibitor concentration after combination compared to single drug treatment. Combination index: CI represented by the mean±SEM from up to four independent experiments, whereby CI<0.9 indicates synergism, 0.9<CI<1.1 indicates additivity, and CI>1.1 indicates antagonism. The degree of synergy is calculated at the EC$_{95}$ level with +, slight synergism (CI: 0.85–0.90); ++, moderate synergism (CI: 0.70–0.85); +++, synergism (CI: 0.30–0.70); and ++++, strong synergism (CI: 0.10–0.30). A p-value with p<0.05 is considered significant. N.A., not applicable.

mAb against the 2G12-resistant virus, a calculation of the CI was not possible (Table 4). Remarkably, when HHA was combined with GNA against the NL4.3^{2G12res} virus, a significant 2.5-fold increase in antiviral potency was noted for both CBAs, resulting in a synergistic cooperative drug–drug interaction profile (CI, 0.64±0.09; Fig. 5A and Table 4).

Finally, we investigated whether synergy occurred against HIV-1 NL4.3^{MVNres} that contains four N-glycan deletions on gp120 at positions N295, N339, N386, and N396.[9] As shown in Table 4, a comparable, but more potent synergistic combination profile was observed against MVN-resistant HIV-1, with CI-values <0.50, and synergistic activity was found again for the HHA and GNA combination (Fig. 5B and Table 4).

Discussion

An effective microbicide must have (1) a potent and preferentially broad-spectrum antimicrobicidal activity, (2) limited to no toxicity on the vaginal or rectal epithelial cell layer, and (3) a good pharmacokinetic/dynamic profile.[29] Disruption of the epithelial layer increases the risk for sexually transmitted diseases such as HIV and herpes simplex virus type 2 (HSV-2).[30] From all the diverse CBAs described so far, GRFT is presumably the most potent and broad-spectrum anti-HIV-1 entry inhibitor with an outstanding safety and efficacy profile.[6,8] Recombinant forms of GRFT, with no loss of antiviral activity, can also be easily produced in *E. coli* and in the *Nicotiana benthamiana* plant.[24] Also BanLec ranks among the most potent anti-HIV-1 lectins reported so far.[5] Although GRFT and BanLec have been reported to suppress a wide variety of HIV-1 strains and clinical isolates, the HIV-2 inhibitory activity of GRFT and BanLec has not previously been reported. When the efficacy of GRFT was evaluated against HIV-2 (ROD) replication, a range of EC$_{50}$ values between 110 and 240 pM was obtained (Table 3). These values of antiviral activity were only 2- to 3-fold higher than those observed for inhibition of HIV-1 (NL4.3), since the EC$_{50}$s for HIV-1 (NL4.3) varied between 48 and 75 pM (Table 2). When the anti-HIV-2 activity of BanLec was compared with HIV-1, no significant differences in inhibitory concentrations were observed (EC$_{50}$ of 0.20±0.05 nM for HIV-1 and 0.13±0.01 nM for HIV-2; Tables 2 and 4). Thus, both GRFT and BanLec efficiently suppress both HIV-1 and HIV-2 in the picomolar range.

In the search for an effective, safe, acceptable, and affordable microbicide, many potential microbicidal candidates were tested alone or in combination with other (classes of) antiretroviral drugs.[31,32] However, no investigations were performed on the effects on HIV replication when two drugs belonging to the functional class of CBAs would be combined. To evaluate synergistic drug–drug interactions, some research groups propose to use "single-round" instead of "multiple-round" infection assays to evaluate synergy.[33–35] Recently a paper by Ketas *et al.* was published that suggested a modification of the classic synergy calculation by the Chou and Talalay method as they used single-round infection and a nonlinear calculation model.[33] Here, we used the multiple-round virus infection to mimic more the real viral life cycle and replication abilities in a CD4$^+$ T cell line and PBMCs.

It was interesting to observe synergistic anti-HIV activity when GRFT was combined with any other CBA, irrespective of the coreceptor tropism of the virus (X4 or R5) or the cell type
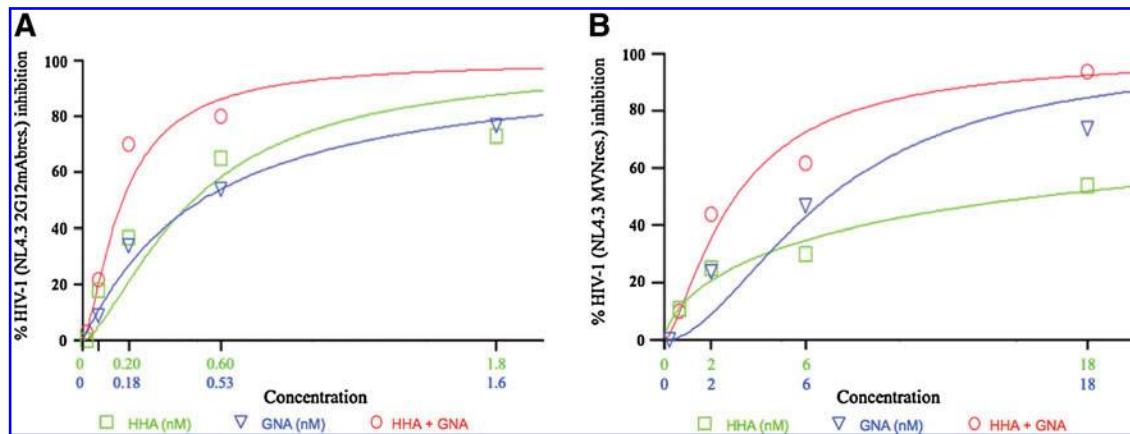
FIG. 5. Synergy of HHA/GNA against NL4.3[2G12res] and NL4.3[MVNres] HIV-1 strains. Dose-dependent effects of HHA/GNA combinations against NL4.3[2G12res] **(A)** and NL4.3[MVNres] **(B)**. The green triangles show the HHA concentration, the blue squares the GNA concentration and the red circles their combined effect. Their increase in antiviral potency and thus leftwards shift indicates in a synergistic profile. One representative experiment out of 4 separate experiments is shown.

(MT-4, PBMC). Such synergistic potential of GRFT was also kept against mutant HIV-1 strains that were (partially) resistant to other CBAs. BanLec in combination with GRFT (or MVN) also resulted in synergy. Although BanLec and GRFT lost some of their antiviral activity ($\sim$10-fold) against the HIV-1 NL4.3[2G12res] and NL4.3[MVNres] strains compared to the original wild-type HIV-1 NL4.3 virus (Tables 2 and 4), they proved to be so exquisitely potent that they kept pronounced antiviral activity against the drug-resistant virus strains and, when combined, synergy is still observed. Based on these data, GRFT as well as BanLec or any nonmitogenic derivative thereof could be an interesting lead molecule for further combined microbicidal development.

CV-N and MVN are both CBAs isolated from Cyanobacteria and have a similar gp120 sugar binding specificity, directed against Manα(1,2)Man residues. CV-N has both potent anti-HIV-1 and anti-HIV-2 activity, but also strong cytokine-stimulatory and mitogenic activity.[36,37] On the other hand, MVN has comparable anti-HIV-1 activity to CV-N but a much higher safety profile.[9,38] Therefore we used MVN instead of CV-N for exploring dual CBA combinations. In all, except one (GRFT/HHA), dual MVN/CBA combinations, synergistic activity was observed (Table 2 and Fig. 2B). The additivity between GRFT and HHA can possibly be explained by the sharing of a similar epitope on gp120 by both CBAs.

Strong synergism was observed between GRFT and many other lectin CBAs as discussed above but also when combined with the carbohydrate-binding mAb 2G12 (CI, 0.29±0.04), although 2G12 mAb has a much lower antiviral activity as a single agent compared to GRFT. Remarkably, while rather low-dose reductions were observed in most of the evaluated dual CBA combinations, the 2G12 mAb had a 9-fold dose reduction in MT-4 cells and even up to 13-fold in PBMCs, which could have potential benefits when it would be used in a combined microbicidal gel application. These synergy data are in agreement with recently published data on pseudo-typed HIV-1 using MVN and CV-N in combination with 2G12 mAb.[38] As also observed in Fig. 1A, the broadly neutralizing 2G12 mAb did not completely block viral replication even at high doses and thus may lead to the rapid appearance of resistant virus. In fact, deletion of one specific glycan mutation at position 295

(N295) in gp120 proved sufficient to display full resistance to 2G12 mAb.[25] Also, it has been demonstrated that the binding of 2G12 mAb to gp120 can be blocked by MVN and BanLec but not vice versa as 2G12 mAb does not block the binding of MVN or BanLec to gp120.[5,9] These findings can be explained by the assumption that the lectin CBAs can bind to several sugars on gp120, whereas 2G12 mAb recognizes only one well-defined sugar epitope. The incomplete suppression of virus replication by 2G12 mAb and the subsequent risk of resistance development are other arguments for combining 2G12 mAb with a lectin CBA. Our data revealed not only full virus suppression in such CBA/2G12 mAb drug combination experiments, but also a pronounced synergistic antiviral activity. The observed synergy can have multiple explanations, i.e., targeting of multiple but distinct gp120 (N-glycan) epitopes by different CBAs, different time points of CBA interaction during the gp120/gp41-CD4/CXCR4/CCR5 binding and fusion process, and/or conformational changes in gp120 upon binding of one CBA causing exposure of previously less available epitopes allowing a more efficient binding by the other CBA. The synergy/additivity between the CBAs can also be explained by subtle differences in the CBA sugar specificity.

With the non-CBA mAb b12, targeting the CD4 binding site on gp120, synergism occurred in combination with MVN and GRFT (Table 2). Alexandre *et al.* showed that GRFT interacts, among others, with the N-glycan at position 386 (N386) on gp120, and exposes the CD4 binding site by binding to this glycan. This allows a more tight interaction of the GRFT-bound gp120 with the mAb b12, which may explain the synergistic activity.[39] It could therefore be possible that a similar mechanism of action is responsible for the observed synergy between MVN and the non-CBA mAb b12. Our generated HIV-1 NL4.3[MVNres] with a deleted N-linked glycan on position 386 indeed demonstrated that N386 is a crucial anchoring point for MVN.[9]

For actinohivin, a broadly neutralizing prokaryotic lectin, a tight interaction with the envelope protein of SIV was demonstrated by surface plasmon resonance technology, but it lacked anti-SIV activity in several viral replication assays.[40] These data revealed that binding of CBAs to gp120 glycans may occur, but these are not always necessarily in neutralizing viral replication.

Based on these findings, we investigated whether the CBAs 2G12 mAb and MVN, which both lack anti-HIV-2 activity, could influence the anti-HIV-2 activity of GRFT by binding to gp120 and triggering conformational changes in the envelope that may influence GRFT binding and eventual antiviral efficacy or vice versa. However, no increase or decrease in the anti-HIV-2 effect of GRFT was seen in the paired drug combinations (Fig. 4B and C). Neither did binding of GRFT to gp120 induce conformational changes throughout gp120 that led to a gain of anti-HIV-2 activity for MVN or 2G12 mAb.

In contrast to most other paired CBA combinations, no synergy was observed between HHA and GNA against the wild-type HIV-1 strains NL4.3 (X4) and BaL (R5) and against HIV-2 ROD (Figs. 1–4 and Table 3). Instead, against the NL4.3[2G12res] and NL4.3[MVNres] HIV-1 strains, HHA/GNA clearly showed synergistic interactions (Fig. 5 and Table 4). Both HHA and GNA are tetrameric plant lectins with a rather high molecular weight (50 kDa). These agents recognize structurally comparable N-glycans on gp120, namely $\alpha(1,3)$ and $\alpha(1,6)$Mannose residues,[3] which seems to result in an additive combinatory drug profile. Surprisingly, HIV-1 NL4.3[2G12res] virus was strikingly more susceptible to the CBAs HHA and GNA than wild-type virus.[25] This peculiar phenomenon is again shown here when both CBAs were combined, resulting in a ~10-fold decrease of HHA concentration from 3.8 nM to 0.37 nM and a 35-fold decrease of GNA concentration from 15 nM to 0.43 nM (Tables 2 and 4). The deleted N-glycans in the mutant gp120 of the CBA-resistant virus strains may create "holes" in the N-glycan shield and/or conformational changes on the surface of gp120 resulting in a better "binding site" for certain high-molecular-weight CBAs such as HHA and GNA to allow synergy.

The microbicide 1% tenofovir gel study (CAPRISA 004) revealed a reduced transmission of HIV-1 but also of HSV-2 by 39% and 51%, respectively.[41] The latest results of the VOICE (Vaginal and Oral Interventions to Control the Epidemic) study (MTN-003) were less optimistic. The oral tenofovir tablet and tenofovir gel arms of VOICE were dropped following interim reviews of data that determined neither product was effective in the women assigned to those study groups. Adherence seemed to be the major problem, although many other questions remain.

A recent study has shown that tenofovir inhibits HSV-2 infections by targeting the herpetic DNA polymerase,[42] which was made possible by the high local tenofovir concentrations that were afforded by the topical drug delivery. A microbicide drug with such a dual mechanism of antiviral (i.e., HIV and HSV) action is beneficial in terms of clinical application since HSV-2 is considered as an important copathogen that may accelerate HIV transmission in HIV-exposed individuals. Interestingly, besides its potent anti-HIV activity, GRFT also inhibits HSV-2 (K.E. Palmer *et al.,* unpublished observations). Since we could show that tenofovir acts synergistically with various CBAs including GRFT, MVN, and BanLec,[43,44] a GRFT/tenofovir combination gel may become a very attracting microbicide formulation to diminish HIV-1 and HIV-2 but also HSV-2 transmission worldwide. Additionally, due to the potent anti-HCV activity of GRFT and other CBAs, such agents could even be able to reduce the number of novel (co)infections with HCV.[45,46] Within the scope of the topical PrEP perspective, the CBAs have the benefit of their broad antiviral activity, and by strongly binding to the glycans on gp120 as a virucide they target HIV in the lumen of the vagina before genital tissue penetration can occur. In addition, they have also the advantage of a high genetic barrier to resistance and do not need cellular uptake as well as activation/modification to gain anti-HIV activity (e.g., the RTI tenofovir). Even the important DC-SIGN-mediated pathway (HIV capture and transmission to susceptible CD4$^+$ T cells) can be completely blocked by this class of inhibitors.[3,47]

The first trials in macaques using gel-formulated CV-N applied vaginally and rectally showed promising results as it potently inhibited SHIV transmission.[48,49] More recently, a 63% reduction in SHIV transmission was observed when macaques were treated with a CV-N-expressing *Lactobacillus* strain.[50] Given the many failures in clinical trials of polyanionic antivirals (e.g., nonoxynol-9 and PRO2000),[51,52] which also target HIV in the lumen of the vagina, the class of CBAs (especially GRFT) could contribute to novel perspectives in the field of PrEP.

In conclusion, our study has shown synergistic antiviral (HIV-1/HIV-2) activity when dual combinations of CBAs were exposed to virus-infected cell cultures, irrespective of the nature of the virus (X4, R5) and cell type (MT-4, PBMC). Also, virus strains containing N-glycan deletions in their envelope often maintain high sensitivity to the synergistic CBA combinations. These data are very encouraging in the search for the development of efficient drug pairs to be combined for microbicide treatment.

Acknowledgments

Author Disclosure Statement

No competing financial interests exist.

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