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Short Communication

Synergistic *in vitro* anti-HIV type 1 activity of tenofovir with carbohydrate-binding agents (CBAs)

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ABSTRACT

Tenofovir, a well-known and highly prescribed anti-HIV-1 drug for the treatment of HIV/AIDS infections, has recently also shown its effectiveness as a potential microbicide drug in the prevention of HIV transmission.

Here, we evaluated the combination of tenofovir with various members of the class of carbohydrate-binding agents (CBAs) targeting the glycans on the viral envelope gp120 for their anti-HIV efficacy. The tenofovir/CBA combinations predominantly showed synergistic antiviral activity using the median effect principle.

These findings illustrate that combination of tenofovir with CBAs may increase the antiviral potency of the individual drugs and reducing the risk on potential side-effects.

1. Introduction

Introduction of highly-active antiretroviral therapy (HAART) significantly decreased the mortality rate in human immunodeficiency virus (HIV)-infected individuals (Perelson et al., 1997). However, HAART only results in suppression and not elimination of the virus. Therapy failure is often due to drug resistance and can result in transmission of multidrug resistant HIV strains, making prevention of HIV transmission a very important aim in the combat against HIV/AIDS. Most HIV infections occur by sexual intercourse and because of cultural/social aspects (e.g. religion, polygamy) or sexual violence, women cannot always protect themselves or force the male partner to condom use, which still is the best prevention method.

Microbicides can be very helpful tools being self-administering prophylactic agents. Many attempts have been made to impede HIV transmission by topical microbicides. Many potential microbicide candidates during the last decade failed throughout diverse clinical trials due to toxicity and/or ineffectiveness (Karim et al., 2009; Lacey et al., 2010; Skoler-Karpoff et al., 2008; Van Damme et al., 2008, 2002). The most promising microbicide candidate, PRO2000, was found to be safe, but unfortunately failed in a recent clinical study (Karim et al., 2009; McCormack et al., 2010). Like systemic antiretroviral therapy, pre-exposure prophylaxis of HIV transmission by microbicides very likely needs to be also a combination of drugs targeting different HIV replication steps.

The anti-HIV activity of tenofovir was first described in 1993 (Balzarini et al., 1993) and in 2001 it was approved by the Food and Drug Administration (FDA) as tenofovir disoproxil fumarate (Viread®) for treatment of HIV/AIDS infections. This was later followed by the clinical introduction of fixed dose-combinations designated Truvada® and Atripla® (Borroto-Esoda et al., 2006; Feng et al., 2009). Tenofovir is a highly prescribed anti-HIV drug that very recently turned out to be an interesting microbicide candidate. Indeed, the South-African CAPRISA 004 trial involving ~900 women showed that a 1% vaginal tenofovir gel was safe and significantly reduced the HIV incidence (Abdool Karim et al., 2010). While tenofovir had proven its effectiveness for systemic treatment of HIV/AIDS infections since many years, topical use in the prophylaxis of sexual HIV transmission was never shown before. In addition, the 1% tenofovir gel reduced genital herpes infections by 51% (Abdool Karim et al., 2010).

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Carbohydrate-binding agents (CBAs), targeting N-linked oligosaccharides on the viral envelope protein gp120, were previously described as potent inhibitors of HIV infection and transmission *in vitro* and can be considered as potential candidates for microbicide application (Balzarini, 2007).

Here, we evaluated combinations of tenofovir with the CBAs *Hippeastrum hybrid* agglutinin (HHA), *Galanthus nivalis* agglutinin (GNA), *Urtica dioica* agglutinin (UDA), *Nicotiana tabacum* agglutinin (nictaba), pradimicin-S (PRM-S), microvirin (MVN), the banana lectin (BanLec) and the anti-carbohydrate mAb 2G12 in MT-4 cell

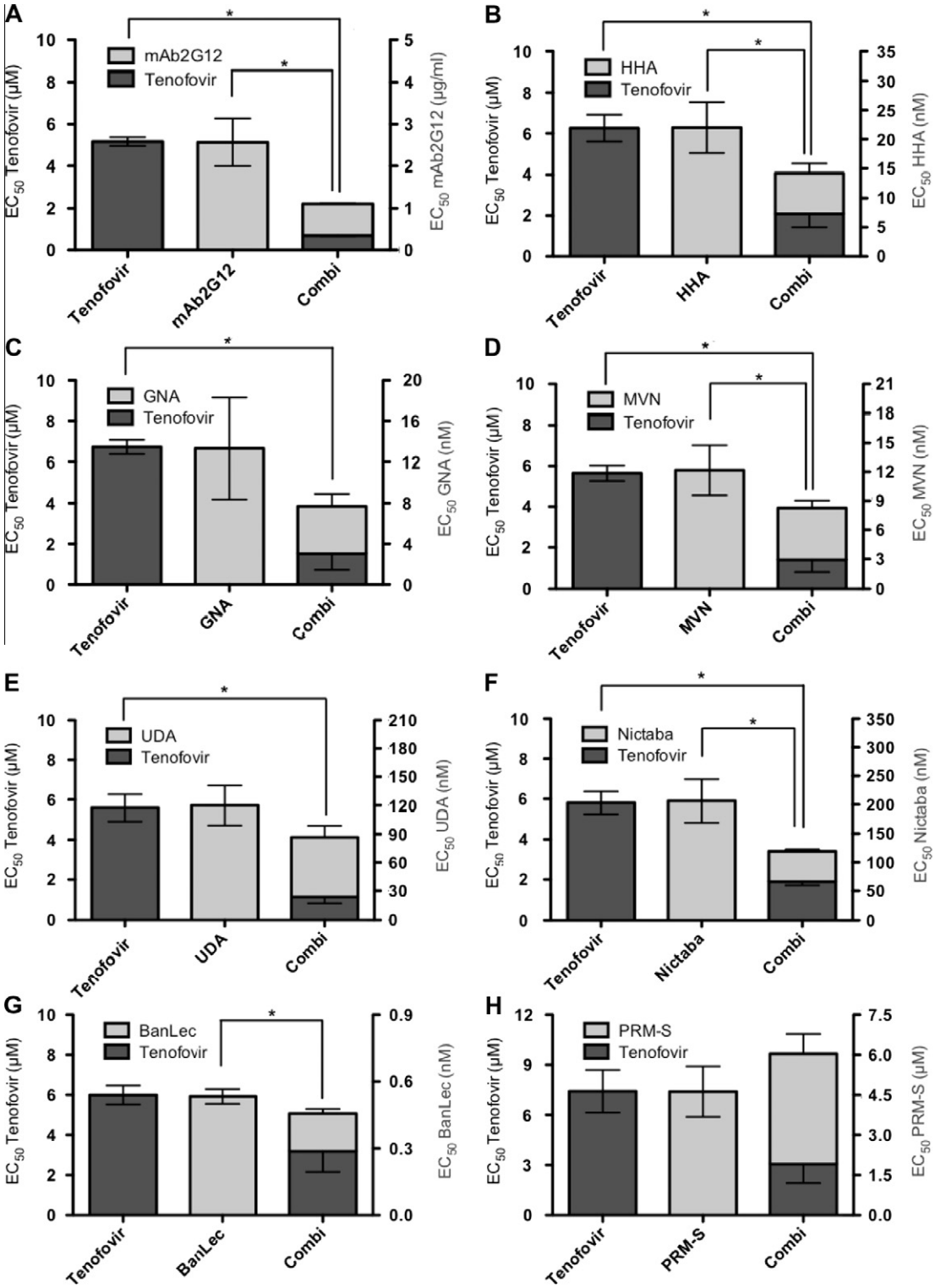

Fig. 1. MT-4 cells (1×10^6 cells/ml) were infected with HIV-1 NL4.3 (1.000 pg/ml) in the presence of various concentrations of each compound separately and afterwards in a 1:1 fixed drug ratio combination as described previously (Vermeire et al., 2004). Fifty percent effective concentrations (EC_{50}) of tenofovir (black bars) and various CBAs (gray bars) alone and in combination (black + gray bars) for inhibition of HIV-1 NL4.3 infection in MT-4 cells are shown. Mean ± SD is shown of 3–6 individual experiments. $^*p < 0.05$ (unpaired T-test), compared with single drug treatment.

cultures against HIV-1 X4 NL4.3 and in PBMCs against HIV-1 R5 BaL infection. While tenofovir is active at the reverse transcription step inside the virus-infected cell, CBAs interfere with the viral entry process. We hypothesize that combination of both classes of HIV inhibitors should result in synergistic interactions and thus better prevention against sexual transmission of HIV. Previous combination studies with tenofovir and various protease and reverse transcriptase inhibitors showed no antagonistic activity (Mulato and Cherrington, 1997).

The methods used for single drug antiviral and combination assays were in detail described earlier (Vermeire et al., 2004) and synergism analysis was performed using CalcuSyn software (Biosoft, Cambridge, UK), based on the median effect principle (Chou and Talalay, 1984) where combination index (CI) values between 0.1 and 0.9 are synergistic, between 0.9 and 1.1 are additive and >1.1 are antagonistic. Fig. 1 was made using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).

The EC_{50}-values for tenofovir and various CBAs, alone and in combination, are shown in Table 1. The EC_{50} for tenofovir varied between 5.2 and 7.4 μM when used as single drug. A significant decrease in tenofovir concentration was observed after combination with mAb 2G12, HHA, GNA, MVN, UDA and nictaba ($p < 0.05$). Significant reductions were noted for the mAb 2G12 and the CBAs HHA, MVN, nictaba and BanLec ($p < 0.05$).

First, we evaluated the combination of tenofovir with the broad-neutralizing anti-gp120 carbohydrate mAb 2G12, which targets the high-mannose type glycans present around the V3/V4 loop of HIV-1 gp120 (Scanlan et al., 2002). The EC_{50}s for tenofovir and the mAb 2G12 decreased significantly with respectively 7.5- and 3.4-fold ($p < 0.05$) (Table 1 and Fig. 1A). CI was then calculated to reveal whether synergistic, additive or antagonistic effects occurred after combination. All CI-values were <1 at the three calculated EC_{50}, EC_{75} and EC_{95} levels (Table 2). We can conclude that the combination of tenofovir with the mAb 2G12 results in pronounced synergistic effects.

For microbicidal applications, CBAs could be considered as more attractive candidates than the mAb 2G12. An ideal microbicide must target all clades of HIV and currently, genetic spreading of HIV subtypes and the appearance of recombinant forms occur worldwide (Perrin et al., 2003). In South-East Africa, predominantly clade C HIV infections occur, while in Western-Europe and America, mainly clade B circulates (Spira et al., 2003). Several CBAs are described to inhibit a broad variety of HIV-1 clades (group M), while mAb 2G12 inhibits only members of clades A and B (Balzarini et al., 2004a; Huskens et al., 2007). Viral resistance against mAb 2G12 in cell culture occurs very quickly compared to all other CBAs (Balzarini et al., 2004b; Huskens et al., 2007). Therefore, tenofovir was combined with the mannose-specific CBAs HHA and GNA. A significant 3-fold decrease in EC_{50}-values was observed after combination with HHA ($p < 0.05$) (Table 1 and Fig. 1B). As seen with the mAb 2G12, CI determination showed all values <1, indicating synergistic effects (Table 2). A comparable synergistic profile was observed with the combination tenofovir/GNA, with significant changes for tenofovir (Tables 1 and 2 and Fig. 1C). It is generally accepted that CBAs preferentially target the glycans present on gp120. However, cellular pre-incubation of the cells for 24 h with certain CBAs, followed by washing and virus infection of these cells still resulted in an inhibition of HIV replication (Balzarini et al., 2004a), indicating "coating" effects by CBAs on the cell membrane. Although this phenomenon could possibly interfere with the cellular uptake of tenofovir, such potential antagonistic effects were never observed.

Cyanovirin-N is perhaps the most extensively studied lectin for microbicide application, however it displayed severe cellular toxicity and strong mitogenic properties (Balzarini et al., 2006; Huskens et al., 2008). MVN is another lectin isolated from cyanobacteria with comparable anti-HIV-1 activity. Both compounds share 33% homology in their primary sequence, but MVN shows a much better *in vitro* safety profile (Huskens et al., 2010). Therefore, MVN was combined with tenofovir and a significant decrease in EC_{50}s was noted after combination ($p < 0.05$) (Table 1 and Fig. 1D) and CI determination showed a moderate synergistic interaction (Table 2).

UDA is derived from *Urtica dioica* (stinging nettle) rhizomes and prefers binding to GlcNAc oligomers (Balzarini et al., 2005), whereas nictaba is produced by the tobacco plant, only after treatment with jasmonates. Nictaba binds high-type mannoses and complex N-glycans (including GlcNAc oligomers) (Lannoo et al., 2006, 2007) and shows potent broad-spectrum anti-HIV-1 activity (manuscript in preparation). In combination with UDA and nictaba, EC_{50}s dropped significantly for tenofovir and nictaba ($p < 0.05$) (Table 1 and Fig. 1E and F). All CI-values were <1, indicating a good synergistic inhibitory profile (Table 2).

Next, we evaluated the interaction between tenofovir and BanLec, a recently described potent anti-HIV lectin isolated from bananas with potential as a microbicide (Swanson et al., 2010). The EC_{50}s for tenofovir and BanLec decreased 2-fold after combination, which was significant for the banana lectin ($p < 0.05$) (Table 1 and Fig. 1G). In line with the other evaluated CBAs, this drug combination also resulted in synergy (Table 2).

Table 1
Mean 50% effective concentrations (EC_{50}s) of tenofovir and various entry inhibitors alone and in combination at equipotent ratio for HIV-1 NL4.3 infection in MT-4 cells.[a]

	EC_{50} individual drug		EC_{50} combination	
	Tenofovir (μM)	Inhibitor	Tenofovir (μM)	Inhibitor
mAb 2G12 (μg/ml)	5.2 ± 0.4	2.6 ± 1.0	0.69 ± 0.02*	0.77 ± 0.02*
HHA (nM)	6.3 ± 1.3	22 ± 9	2.1 ± 1.3*	7 ± 3*
GNA (nM)	6.7 ± 0.6	14 ± 8	1.5 ± 1.4*	4.6 ± 1.9
MVN (nM)	5.6 ± 0.9	12 ± 6	1.4 ± 1.3*	5.4 ± 1.9*
UDA (nM)	5.6 ± 1.2	120 ± 40	1.1 ± 0.6*	60 ± 20
Nictaba (nM)	5.8 ± 1.0	210 ± 70	1.9 ± 0.3*	50 ± 10*
BanLec (nM)	6.0 ± 0.8	0.53 ± 0.06	3.2 ± 1.8	0.17 ± 0.03*
PRM-S (μM)	7.4 ± 1.8	4.6 ± 1.3	3.1 ± 1.6	4.1 ± 1.1
AMD3100 (nM)	6.3 ± 1.8	24.7 ± 19.9	2.0 ± 0.5*	10.6 ± 7.8

[a] MT-4 cells (1×10^6 cells/ml) were seeded in a 96-well plate and pre-incubated for 30 min with various concentrations of test compounds. Then HIV-1 NL4.3 was added and 5 days post-infection, cytopathic effect was scored microscopically and EC_{50} values were determined by the MTS/PES method (Vermeire et al., 2004). Mean EC_{50}s ± SD from 4–6 independent experiments are shown.
* $p < 0.05$ (unpaired T-test), compared with single drug treatment.

Table 2
Combination indices at varying X4 HIV-1 NL4.3 inhibition levels for the combinations of tenofovir in two-drug assays.

Inhibitor	Combination index[a]			Synergy[b]
	50%	75%	95%	
mAb 2G12	0.50 ± 0.14	0.46 ± 0.15	0.41 ± 0.18	+++
HHA	0.75 ± 0.17	0.69 ± 0.16	0.63 ± 0.18	+++
GNA	0.63 ± 0.10	0.56 ± 0.10	0.47 ± 0.11	+++
MVN	0.80 ± 0.15	0.79 ± 0.16	0.80 ± 0.21	++
UDA	0.72 ± 0.15	0.67 ± 0.16	0.59 ± 0.20	+++
Nictaba	0.53 ± 0.07	0.41 ± 0.11	0.32 ± 0.16	+++
BanLec	0.79 ± 0.11	0.69 ± 0.09	0.56 ± 0.12	+++
PRM-S	1.05 ± 0.30	1.00 ± 0.28	0.98 ± 0.33	additive
AMD3100	0.74 ± 0.13	0.67 ± 0.08	0.60 ± 0.11	+++

[a] Combination indices (CI) represented by the mean value ± SD from 3–6 independent experiments in MT-4 cells infected with HIV-1 NL4.3. CI < 0.9 indicates synergism; 0.9 < CI < 1.1 is additive; CI > 1.1 indicates antagonism.
[b] Synergy calculated at the EC_{95}-level: 0.85 < CI < 0.9: + (slight synergism); 0.7 < CI < 0.85: ++ (moderate synergism); 0.3 < CI < 0.7: +++ (synergism); 0.1 < CI < 0.3: ++++ (potent synergism).

Whereas the above-mentioned CBAs are all proteins, we tested also whether synergism occurred with PRM-S, a water-soluble low molecular weight non-peptidic CBA, having potent anti-HIV activity *in vitro* (Balzarini et al., 2010). Physicochemical studies with PRM-S have shown stability at low pH levels (e.g. vaginal pH) and high temperatures (e.g. an advantage for storage of microbicides in tropical regions) for several days, as also shown earlier for the mannose-specific CBAs HHA and GNA (Balzarini et al., 2010, 2004a). No significant reductions were seen after combination of tenofovir with PRM-S (Table 1 and Fig. 1H). Compared to all other combinations tested, an additive effect was observed (Table 2).

As a control for inhibition of X4 viruses, we included the combination of tenofovir and the CXCR4 antagonist AMD3100. A significant decrease in tenofovir concentration was noted ($p < 0.05$) (Table 1) and as seen with almost all the CBAs tested, combination resulted in synergism (CI, 0.60 ± 0.11; Table 2).

As sexual transmission of HIV is primarily mediated by R5 viruses, we evaluated the combinations of tenofovir with several CBAs also in PBMCs infected by the HIV-1 R5 BaL strain. The EC_{50}-values of tenofovir, mAb 2G12, HHA, GNA, MVN and maraviroc, alone and in combination, against HIV-1 BaL in PBMCs are shown in Table 3, as well as their significant changes ($p < 0.05$). The most potent synergistic effects (at the 95% level) were found for the combinations tenofovir/mAb 2G12 (CI, 0.18 ± 0.02) and tenofovir/MVN (CI, 0.24 ± 0.15) (Table 4). Synergism was also observed for tenofovir in combination with HHA and GNA and with the CCR5 antagonist maraviroc (Table 4).

Overall, entry of HIV is an important target in the prevention of HIV transmission. HIV entry is a complex process consisting of several consecutive steps of virus envelope/cell receptor interactions and envelope conformational changes (Tilton and Doms, 2010). Two-drug combination studies with the CD4 down-modulating agent CADA, have shown synergistic anti-HIV activity with different classes of all approved ARV drugs (including tenofovir) (Vermeire et al., 2004). For prophylaxis against sexual transmission of HIV, CBAs need to be applied in a gel formulation. The first successful clinical study with the tenofovir gel and here described our synergistic observations with various members among the class of antiviral CBAs *in vitro*, suggests that a topical microbicide tenofovir/CBA containing gel could be very promising. Tenofovir lacks antiviral activity (>100 μM) in a co-cultivation assay between persistently HIV-infected cells and non-infected $CD4^+$ T cells and is also not able to block HIV capture to DC-$SIGN^+$ cells. In contrast, the CBAs are potent inhibitors in these antiviral assays (Balzarini, 2007). Various *in vitro* studies to obtain an appropriate gel formulation as well as *in vivo* toxicity and pharmacokinetic/dynamic studies still need to be performed and are going to be crucial for a successful microbicide application (Doncel and Clark, 2010).

Table 3
EC_{50}-values of tenofovir (μM) and various CBAs alone and in combination obtained in PBMCs after infection with R5 HIV-1 BaL.[a]

	EC_{50} drug alone		EC_{50} drug combination[b]	
	Tenofovir	Inhibitor	Tenofovir	Inhibitor
mAb 2G12 (μg/ml)	1.9 ± 0.2	0.03 ± 0.01	0.2 ± 0.2*	0.02 ± 0.01
HHA (nM)	0.30 ± 0.26	80 ± 10	0.18 ± 0.10	8 ± 3*
GNA (nM)	1.2 ± 0.2	150 ± 30	1.0 ± 0.6	40 ± 30
MVN (nM)	0.22 ± 0.18	40 ± 20	0.23 ± 0.17	5 ± 2*
maraviroc (nM)	1.65 ± 0.74	5.1 ± 2.5	0.52 ± 0.07	0.96 ± 0.40

[a] PBMCs (2.5×10^6 cells/ml) were seeded in a 48-well plate and infected with HIV-1 R5 BaL (1,000 pg/ml of p-24 HIV-1 Ag) in the presence of various concentrations of test compounds. After 10 days, viral replication was measured using p24 HIV-1 Ag ELISA (Perkin–Elmer). Mean EC_{50}-values ± SD of 3–4 donors are shown.

[b] Two-drug combination at a 1:1 ratio of both anti-HIV drugs (EC_{50} of tenofovir/EC_{50} of inhibitor).

* $p < 0.05$ (unpaired T-test), compared with single drug treatment.

Table 4
Combination indices at varying R5 HIV-1 BaL inhibition levels for the combinations of tenofovir in two-drug assays.

Inhibitor	Combination index[a]			Synergy[b]
	50%	75%	95%	
mAb 2G12	0.28 ± 0.05	0.23 ± 0.02	0.18 ± 0.02	++++
HHA	1.22 ± 0.93	0.66 ± 0.25	0.39 ± 0.05	+++
GNA	1.13 ± 0.02	0.82 ± 0.01	0.56 ± 0.04	+++
MVN	2.09 ± 1.69	0.66 ± 0.35	0.24 ± 0.15	++++
maraviroc	0.70 ± 0.02	0.60 ± 0.03	0.53 ± 0.12	+++

[a] CI < 0.9 indicates synergism; 0.9 < CI < 1.1 is additive; CI > 1.1 indicates antagonism.

[b] Synergy calculated at the EC_{95}-level: 0.85 < CI < 0.9: + (slight synergism); 0.7 < CI < 0.85: ++ (moderate synergism); 0.3 < CI < 0.7: +++ (synergism); 0.1 < CI < 0.3: ++++ (potent synergism).

Conflict of interest

No conflict of interest is declared.

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