Rapid Communication

Synergistic activity profile of griffithsin in combination with tenofovir, maraviroc and enfuvirtide against HIV-1 clade C

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Abstract

Griffithsin (GRFT) is possibly the most potent anti-HIV peptide found in natural sources. Due to its potent and broad-spectrum antiviral activity and unique safety profile it has great potential as topical microbicidal component. Here, we evaluated various combinations of GRFT against HIV-1 clade B and clade C isolates in primary peripheral blood mononuclear cells (PBMCs) and in CD4+ MT-4 cells. In all combinations tested, GRFT showed synergistic activity profile with tenofovir, maraviroc and enfuvirtide based on the median effect principle with combination indices (CI) varying between 0.34 and 0.79 at the calculated EC95 level. Furthermore, the different glycosylation patterns on the viral envelope of clade B and clade C gp120 had no observable effect on the synergistic interactions.

Overall, we can conclude that the evaluated two-drug combination increases their antiviral potency and supports further clinical investigations in pre-exposure prophylaxis for GRFT combinations in the context of HIV-1 clade C infection.

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Introduction

The broad genetic diversity of human immunodeficiency viruses makes it very difficult to generate an effective vaccine (McElrath and Haynes, 2010). While HIV-1 clade C dominates the HIV-1 epidemic worldwide with >50% of the total viral infections, predominately in Southern Africa, India and other parts of Asia, HIV-1 subtype B is mainly found in (Western-) Europe and in the Americas, accounting around 10% of the total infections (Buonaguro et al., 2007). In areas where they have been recently introduced, like Brazil, Clade C viruses become rapidly established, and have started to replace the previously dominant Clade B epidemic (de Oliveira et al., 2010). The standard combination antiretroviral therapy does not cure HIV, making prevention of novel infections of extreme importance. A high number of the new HIV infections occur through heterosexual intercourse.

Therefore, self-administered topical microbicides (e.g. vaginal gel or intravaginal ring devices) could be very helpful tools to reduce the infection rates. In the last decade, many microbicidal clinical trials failed due to safety problems or lack of efficacy (Lacey et al., 2010; McCormack et al., 2010; Van Damme et al., 2008). However, recently a 1% tenofovir gel product showed partial efficacy in prevention of HIV-1 transmission (Abdool Karim et al., 2010). As for systemic HIV treatment, the most effective HIV prophylaxis strategy will involve combinations of various antiretroviral drugs. Viral entry is considered an important target in combating HIV/AIDS infections and thus entry inhibitors are proposed as candidates for microbicidal applications. The gp41 fusion peptide inhibitor enfuvirtide (Fuzeon) was the first clinically approved entry inhibitor and has clearly proven its effectiveness (Kilby et al., 1998; Kilby et al., 2002). The only HIV coreceptor inhibitor, the CCR5 antagonist maraviroc (Selzentry), was approved for the treatment of HIV infections, in combination with other antiretroviral drugs (MacArthur and Novak, 2008).

At present, griffithsin (GRFT) is possibly the most potent and broad-spectrum HIV entry inhibitor as it inhibits viral replication at pM levels by specifically targeting multiple mannose-rich residues present on the viral envelope protein gp120 (Emau et al., 2007; Mori et al., 2005). GRFT has a molecular weight of 12.7 kDa and was originally isolated from the red alga Griffithsiella sp. (Mori et al., 2005). It contains 121 amino acids and has a unique dimeric structure (Ziółkowska et al., 2006). Production of recombinant forms of GRFT in E. coli and in the Nicotiana benthamiana plant did not alter its potent anti-HIV activity (IC50: 0.043–0.63 nM) nor its safety profile compared with native isolated GRFT (CC50: >1000 nM) (Mori et al., 2005; O’Keefe et al., 2009). Besides inhibiting HIV-1 subtype B strains, GRFT was also able to potently inhibit HIV-1 clade A and clade C viruses.
isolated from blood and cervico-vaginal samples (Alexandre et al., 2010; O’Keefe et al., 2009). Glycosylation patterns between different HIV-1 clades vary (Zhang et al., 2004) and thus play an important role in the antiviral activity of carbohydrate-binding agents (CBAs). Molecular studies with GRFT have shown that introduction of the glycosylation sites N234 and N295 in HIV-1 clade C virus increased its potency (Alexandre et al., 2010).

Here, we describe that GRFT acts synergistically with tenofovir, maraviroc and enfuvirtide in PBMCs against R5 HIV-1 clade B BaL. We observed synergistic effects with the combination of GRFT/tenofovir and GRFT/maraviroc on 3 different R5 clade C HIV-1 clinical isolates (DJ259, ZAM18 and ETH2220) with different glycosylation patterns. Additionally, synergistic interactions were also observed in MT-4 cells against X4 HIV-1 NL4.3 with GRFT/tenofovir, GRFT/AMD3100 and GRFT/enfuvirtide. These data show that combination of GRFT with tenofovir, maraviroc or enfuvirtide increases antiviral potency and support further clinical research on these combinations as potential microbicide also in the context of HIV-1 clade C infections.

### Results

#### GRFT is synergistic with tenofovir, maraviroc and enfuvirtide against clade B HIV-1 R5 strain BaL in PBMCs

First, we determined the 50% effective concentrations (EC₅₀) of GRFT, tenofovir, maraviroc and enfuvirtide alone, followed by the two-drug combinations GRFT/tenofovir, GRFT/maraviroc and GRFT/enfuvirtide against HIV-1 R5 clade B BaL replication. The EC₅₀ of each inhibitor alone and in combination are shown in Table 1. Overall, GRFT inhibits HIV-1 BaL with an average EC₅₀ of 0.18 nM and significant reductions (p<0.05) in GRFT concentrations were noted after combination with tenofovir (p = 0.048), maraviroc (p = 0.0094) and enfuvirtide (p<0.0001). A statistical significant difference was also calculated for enfuvirtide in combination with GRFT (p = 0.0032) using the unpaired T-test when compared to single drug treatment (Table 1). As shown in Fig. 1, combination of GRFT with each inhibitor tested, resulted in a more efficient inhibitory profile against HIV-1 replication as measured by p24 HIV-1 core Ag ELISA. Combination indices (CI) were determined to evaluate the type of drug–drug interactions, where CI<0.9 are synergistic, 0.9<CI<1.1 are additive effects and CI>1.1 are antagonistic. The obtained CI values varied between 0.44 and 0.53, resulting thus in synergy at the calculated EC₉₅ level for each combination (Table 2).

#### Synergistic anti-HIV-1 activity of GRFT with tenofovir and maraviroc against various clade C clinical isolates in PBMCs

The number of clade C HIV-1 infections dominates the number of clade B infections in Africa. Consequently, an effective microbicide must

### Table 1

<table>
<thead>
<tr>
<th>Clade</th>
<th>Inhibitor</th>
<th>EC₅₀ individual drug</th>
<th>EC₅₀ combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clade B/BaL</td>
<td>Tenofovir</td>
<td>0.16±0.02</td>
<td>1.3±0.09</td>
</tr>
<tr>
<td></td>
<td>Maraviroc</td>
<td>0.17±0.02</td>
<td>6.6±1.9</td>
</tr>
<tr>
<td></td>
<td>Enfuvirtide</td>
<td>0.21±0.01</td>
<td>41.0±1.7</td>
</tr>
<tr>
<td>Clade C/DJ259</td>
<td>Tenofovir</td>
<td>0.11±0.01</td>
<td>0.95±0.19</td>
</tr>
<tr>
<td></td>
<td>Maraviroc</td>
<td>0.34±0.30</td>
<td>1.6±0.9</td>
</tr>
<tr>
<td>Clade C/ZAM18</td>
<td>Tenofovir</td>
<td>0.61±0.01</td>
<td>0.53±0.37</td>
</tr>
<tr>
<td></td>
<td>Maraviroc</td>
<td>1.8±0.6</td>
<td>4.4±1.7</td>
</tr>
<tr>
<td>Clade C/ETH2220</td>
<td>Tenofovir</td>
<td>0.59±0.06</td>
<td>0.72±0.11</td>
</tr>
<tr>
<td></td>
<td>Maraviroc</td>
<td>0.69±0.19</td>
<td>3.1±1.5</td>
</tr>
</tbody>
</table>

* 50% effective concentration for HIV-1 infection in PBMCs after single drug treatment. Mean EC₅₀±SEM of 2–4 donors are shown.

* 50% effective concentration after two-drug combination at equipotent ratio (1:1).

* p<0.05 (unpaired T-test), compared to single drug treatment.
after combination with maraviroc against DJ259 (CI, 0.67±0.08). (Table 3 and Fig. 2). A comparable synergistic interaction was also found between GRFT and tenofovir against the 3 isolates tested: DJ259 (CI, 0.70±0.10), ZAM18 (CI, 0.76±0.10) and ETH2220 (CI, 0.79±0.02)

CI calculations showed synergy against various clade C HIV-1 clinical isolates after GRFT/tenofovir and GRFT/maraviroc combination treatment. (Table 3) 

Stronger synergy was observed against ZAM18 and ETH2220 with CI values of 0.51±0.14 and 0.59±0.24, respectively (Table 3 and Fig. 2).

Table 3

<table>
<thead>
<tr>
<th>HIV strains Combi</th>
<th>Synergy⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DJ259</td>
</tr>
<tr>
<td>GRFT/tenofovir</td>
<td>++(+).</td>
</tr>
<tr>
<td>GRFT/maraviroc</td>
<td>++</td>
</tr>
</tbody>
</table>

Stronger synergy was observed against ZAM18 and ETH2220 with CI values of 0.51±0.14 and 0.59±0.24, respectively (Table 3 and Fig. 2).

Discussion

The proof-of-concept trial of a 1% tenofovir vaginal gel product (CAPRISA 004) for the prevention of HIV infection in women opened novel perspectives in the field of microbical research (Abdool Karim et al., 2010). However, like systemic treatment of HIV/AIDS infections, single drug microbicides are perhaps not potent enough and could also increase the risk of transmission of resistant viruses, making combinations of at least 2 different antiretroviral drugs with broad-spectrum anti-HIV activity the best prevention strategy against viral transmission Klasse et al. (2008). An effective microbicide must also have a good cervicovaginal safety and pharmacokinetic/dynamic profile (Doncel and Clark, 2010). Griffithsin is not mitogenic and showed no toxicity in rabbit vaginal irritation tests as well as no induction of pro-inflammatory cytokines in human cervical explants.
at 2 μM (O’Keefe et al., 2009). We also found no induction of any cytokines nor cellular activation markers in human PBMCs at 2 μM using the Bio-plex Human Cytokine 27-plex assay (unpublished data).

GRFT belongs to the class of carbohydrate-binding agents (CBAs) and is, to our knowledge, the most potent CBA described for inhibiting HIV infection and replication in the pM range (Alexandre et al., 2010; Mori et al., 2005; O’Keefe et al., 2009). CBAs are attractive microbicidal candidates as they target HIV at 4 different intervention steps, namely (i) inhibition of cell-free virus replication and (ii) inhibition of syncytium formation between uninfected CD4+ T cells and HIV-infected T cells, (iii) inhibition of capture by DC-SIGN and (iv) transmission of captured virus to uninfected CD4+ T cells (Balzarini, 2007).

Certain sexually transmitted diseases (such as Chlamydia or Herpes Simplex Virus) can increase the risk on HIV transmission (Abu-Raddad et al., 2008; Baeten et al., 2007). The results of the CAPRISA 004 trial showed that tenofovir also reduced the genital herpes infections (Abdool Karim et al., 2010). In addition to its activity against HIV-1, GRFT is also active against HSV-2 (Palmer et al., manuscript in preparation). Given the established efficacy of tenofovir vaginal gel against HSV-2 acquisition, a GRFT/tenofovir combination microbicidal becomes an appealing product to combat HIV transmission.

Here, we showed that the potent anti-HIV CBA GRFT can be combined with the nucleotide reverse transcriptase inhibitor (RTI), tenofovir, the CCR5 HIV co-receptor antagonist, maraviroc and the gp41 fusion inhibitor enfuvirtide and that all combinations are synergistic against clade B (mainly present in the Americas, Australasia and Europe) and clade C, which accounts for approximately 60% of the HIV-1 infections, worldwide (mainly in Africa and the Indian subcontinent, with expanding epidemics elsewhere). By combining GRFT with an entry inhibitor targeting ensuing steps in the entry process or with a RTI, a broader range of (multi-drug resistant) HIV subtypes can be blocked.

GRFT acts as a virucide by targeting multiple glycans present on gp120 (Mori et al., 2005). Alexandre et al. (2010) showed that the N-glycosylations on position 295 (N295) and 234 (N234) are crucial binding sites for GRFT and their absence decreased its antiviral potency. The HIV-1 strains NL4.3 (clade B) and DJ259 (clade C) both contain these N-linked glycosylations. Bal. (clade B) lacks N234, ZAM18 (clade C) lacks N295 and ETH2220 (clade C) lacks both N-glycosylations. The complete amino acid sequences are available in GenBank with GenBank ID: AAB60578, BA97458, ABC55874, BAH97494 and Q75008 resp. (www.ncbi.nlm.nih.gov/protein). In our experiments performed in PBMCs, GRFT lost almost no activity against the three different clade C isolates compared to the well-described and widely used laboratory strain clade C Bal. (Table 1).

A strong hallmark of the class of CBAs is their high genetic barrier to resistance and excellent pharmacocinetic properties (e.g. resistant to high temperature and low pH) (Balzarini et al., 2004; Huskens et al., 2010). Griffthsin maintained its potent antiviral activity in both acidic and alkaline pH (Emau et al., 2007) and we recently showed that tenofovir can be combined with various other CBAs in vitro against R5 and X4 HIV-1 (Féfrir et al., 2011). In vivo studies using a tenofovir/GRFT gel combination are planned to evaluate various antiviral efficacies in preparation for human clinical studies.

Maraviroc is a specific CCR5 interacting small chemical molecule with potent activity against R5 viruses (Dorr et al., 2005). For systemic HIV treatment, maraviroc is used in combination with other antiretroviral drugs and various studies have shown that it also has potential for microbicidal applications. An open-labeled pharmacokinetic study in healthy women showed that orally administered maraviroc concentrates in the female genital tract (Dumond et al., 2009). A recent study using maraviroc in a gel-based formulation protects rhesus macaques from infection with CCR5-tropic SHIV (Veazey et al., 2010). GRFT shows synergistic interactions with maraviroc against clade B and clade C HIV-1 strains (Tables 2 and 3), making a GRFT/maraviroc containing gel or controlled-release intravaginal ring device good candidates for HIV prevention studies.

Although sexual transmission of HIV mainly occurs by R5 viruses, presumably because of lower replication “fitness” of X4 viruses, we investigated potential synergistic interactions against X4 viruses. A study with macaques showed that X4 viruses can also be transmitted vaginally (Miller and Hu, 1999). We used CD4+ MT-4 cells, which are CCR5− and CXCR4+, therefore in these experiments maraviroc was replaced by the specific CXCR4 antagonist AMD3100 (Schols et al., 1997). GRFT showed synergistic interactions in all combinations tested against X4 HIV-1 NL4.3 (Table 5). Potent synergistic interactions between enfuvirtide/AMD3100 in PBMCs were also observed by other research groups (Tremblay et al., 2000).

Enfuvirtide showed potent anti-HIV activity, a safe pharmacokinetic profile and became the first approved peptide entry inhibitor for the treatment of HIV/AIDS infections (Kirby et al., 1998, 2002). Enfuvirtide is mainly used in non-African countries, so we only evaluated the combination with GRFT against HIV-1 BaL. Here we also demonstrated that GRFT has synergistic interactions with enfuvirtide against HIV-1 clade B. Since its discovery, novel derivatives of enfuvirtide have been generated (such as T-1249 and T-2635). An advantage of these fusion inhibitors compared with co-receptor antagonists is their tropism independent antiviral activity. Macaques that received a T-1249-containing vaginal gel showed inhibition of SHIV replication (Veazey et al., 2008) and this peptide showed a superior antiviral profile compared with enfuvirtide. Resistance analysis of this second generation peptide revealed that mutations occur at position 38 (V38) in gp41 and resulted in enfuvirtide cross-resistance. However, a third generation peptide, T-2635, retained its antiviral activity (Eggink et al., 2008). These data and our observations show that enfuvirtide (and its later generation peptides) could also be useful in the prevention of HIV transmission.

Overall, due to its potent and broad-spectrum anti-HIV activity as well as its synergistic interactions with tenofovir, maraviroc and enfuvirtide, we believe that GRFT has a great potential as microbicide in the prevention of sexual transmission of HIV when co-formulated in appropriate gel or solid dosage form, or in a sustained-release intravaginal ring device. All GRFT-antiretroviral combinations tested showed a synergistic profile, with improved antiviral potency. The observed synergy for the GRFT/enfuvirtide and the GRFT/maraviroc combinations may be a reflection of their common target, viral entry, but the agents are targeting subsequent steps during the entry process. As we showed previously with several other members of the CBA family (e.g. microvirin and Hippiestrum Hybrid agglutinin), also GRFT has no negative effects on the cellular uptake and processing of the RTI tenofovir. Synergy may enable reductions in effective dose of each component, with corresponding decreases in side-effect profiles, and even reduction in the product costs. Our data strongly support further translation research on these GRFT-antiretroviral combinations as potential microbicides.

Materials and methods

Cells and cell culture

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 containing 10% FCS (Hyclone, Perbio Science, Aalst, Belgium) and 2 mM l-glutamine (Invitrogen, Merelbeke, Belgium) at 37 °C and 5% CO2. Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated out of a buffy coat obtained from the Blood Transfusion Center (U.Z. Leuven, Belgium). PBMCs were also cultured RPMI-1640 supplemented with 10% FCS, 2 mM l-glutamine and 2 mg/ml IL-2 (Roche Molecular Biochemicals, Indianapolis, USA) and activated with 2 μg/ml PHA (Sigma-Aldrich, Bornem, Belgium) for 3 days before antiviral testing.
Viruses

HIV-1 CCR5-tropic (R5) laboratory strain Bal. (clade B) and HIV-1 CXCR4-tropic (X4) NL4.3 (clade B) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). The R5 primary clinical HIV-1 isolates DJ259, ZAM18 and ETH2220 (all clade C) were kindly provided by Dr. J.L. Lathey (BBI Biotech Research Laboratories, Inc., Gaithersburg, MD).

Compounds

GRFT was isolated and purified as described elsewhere (O’Keefe et al., 2009). Enfuvirtide was a gift from Prof. Dr. E. Van Wijngaerden (U.Z. Leuven, Belgium). AMD3100 and maraviroc were provided by Dr. G. Bridger (at that time by AnorMED Inc., Langley, British Columbia, Canada). Tenofovir was obtained from Dr. A. Holy (Prague, Czech Republic).

Antiviral assays and combination experiments

Methodology of antiretroviral assays for single drug testing and their combinations at various ratios were in detail described earlier (Vermeire et al., 2004). Briefly, 3-fold dilutions of test compounds were added in a 96-well plate and then MT-4 cells (5 × 10^5 cells/well) containing already cell culture medium with 3% fetal bovine serum (FBS) were added in a 96-well plate and then MT-4 cells (5×10^4 cells/well) containing 2 ng/ml IL-2 and then seeded in 48-well plates containing 2 ng/ml IL-2 and then seeded in 48-well plates containing 2 ng/ml IL-2 and then seeded in 48-well plates containing already cell culture medium with 3-fold drug dilutions. After 20 min of pre-incubation, cells were infected with 1000 pg/ml of p24 HIV-1 Ag of HIV-1 NL4.3. Cytotoxic effects (CE) were scored microscopically at day 5 and antiviral activity was measured by MTS/PEs method as described (Vermeire et al., 2004).

PHA-activated PBMCs were resuspended in cell culture medium containing 2 ng/ml IL-2 and then seeded in 48-well plates (5 × 10^5 cells/well) containing already cell culture medium with 3-fold fold dilutions. After 20 min of pre-incubation, 500 pg/ml of HIV-1 p24 HIV-1 Ag (Bal. DJ259, ZAM18 or ETH2220) was added. IL-2 was added after 3 and 6 days post-infection. At day 10, supernatant was collected and viral replication was measured by p24 HIV-1 Ag ELISA (Perkin Elmer, Zaventem, Belgium).

Combination indices (CI) were calculated using CalcuSyn software (Biosoft, Cambridge, UK) based on the median effect principle of Chou and Talalay (1984). CI-value <-0.9 indicates synergism, 0.9<CI<1.1 indicates additive effects and CI>1.1 indicates antagonism.

Acknowledgments

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