Feglymycin, a unique natural bacterial antibiotic peptide, inhibits HIV entry by targeting the viral envelope protein gp120

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ABSTRACT

Feglymycin (FGM), a natural *Streptomyces*-derived 13mer peptide, consistently inhibits HIV replication in the lower µM range. FGM also inhibits HIV cell-to-cell transfer between HIV-infected T cells and uninfected CD4$^+$ T cells and the DC-SIGN-mediated viral transfer to CD4$^+$ T cells. FGM potently interacts with gp120 (X4 and R5) as determined by SPR analysis and shown to act as a gp120/CD4 binding inhibitor. Alanine-scan analysis showed an important role for L-aspartic acid at position 13 for its anti-HIV activity. In vitro generated FGM-resistant HIV-1 IIIB virus (HIV-1 IIIB^{FGMres}) showed two unique mutations in gp120 at positions I153L and K457I. HIV-1 IIIB^{FGMres} virus was equally susceptible to other viral binding/adsorption inhibitors with the exception of dextran sulfate (9-fold resistance) and cyclotriazadisulfonamide (> 15-fold), two well-described compounds that interfere with HIV entry. In conclusion, FGM is a unique prototype lead peptide with potential for further development of more potent anti-HIV derivatives.

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Introduction

Despite enormous costs on effective HIV/AIDS prevention campaigns, every day more than 7000 novel human immunodeficiency virus (HIV) infections are occurring and approximately 34 million people are estimated to live with HIV in 2011 (http://www.who.int/hiv/data/en/index.html). Although there are already more than 25 Food and Drug Administration (FDA)-approved antiretroviral

(ARV) drugs, HIV resistance to these ARV drugs has raised to high levels. Therefore, continued efforts in discovering new drugs and developing new HIV prevention methods (e.g., microbicides) are still needed. An interesting target for HIV drug development is still the HIV entry pathway. HIV entry into its target cells starts with the binding of the heavy glycosylated envelope protein, gp120, with the cellular CD4 receptor resulting in conformational changes inside the viral envelope for subsequent interactions with the chemokine receptors CCR5 and/or CXCR4. Interaction of gp120 with these HIV co-receptors results in insertion of gp41 into the target membrane and formation of the hairpin structure necessary for membrane fusion (Tilton and Doms, 2010).

At present, enfuvirtide (T20, Fuzeon®) is the only licensed peptidic HIV entry fusion inhibitor. This synthetic peptide of 36 amino acids interacts with the heptad repeat 1 (HR1) domain region of gp41 and inhibits the hairpin structure formation necessary for virus-cell fusion (Matthews et al., 2004). It is self-administered twice daily subcutaneously in patients infected with multidrug resistant HIV-1 (Joly et al., 2010). Due to its unique mechanism of action, no cross-resistance with other ARV drugs has been observed (Matthews et al., 2004). Novel generations of HIV-1 fusion inhibitory peptides with more potent antiviral activity and better pharmacokinetics are in development (Dwyer et al., 2007; Eggink et al., 2011).

Developing drugs inhibiting the HIV entry pathway are not only important for treatment of HIV-infected persons; they are also candidates for the prevention of HIV transmission. At the moment, the development of antiviral compounds in mainly gel-based formulations for microbicide application for vaginal/rectal use is a thriving research area. Many challenges are still remaining and it will be crucial to understand the exact mechanisms of HIV infections through mucosal pathways (Hladik and Doncel, 2010). In recent years we and other research groups focused on natural occurring peptides and proteins isolated from microorganisms (e.g., *Cyanobacteria* and *Streptomyces sp.*) with anti-HIV activity. For example, cyanovirin-N isolated from the cyanobacteria *Nostoc ellipsosporum* has a broad-spectrum anti-HIV activity by targeting the *N*-linked glycans on gp120 (Boyd et al., 1997). Recently, microvirin, closely related to cyanovirin-N, was isolated from the cyanobacteria *Microcystis aeruginosa* and showed a comparable antiviral activity, but a much higher safety profile (Huskens et al., 2010). Different kinds of *Streptomyces*-derived peptides (e.g., the class of chloropeptins and siamycins) were described as HIV entry/fusion inhibitors that demonstrated broad anti-HIV activity (Constantine et al., 1995; Detlefsen et al., 1995; Lin et al., 1996; Matsuzaki et al., 1997; Tsunakawa et al., 1995).

Here, we focus on a novel *Streptomyces*-derived peptide, called feglymycin (FGM). FGM is a unique natural peptide of 13 amino acids (MW, 1900.9 Da) originally isolated from *Streptomyces* sp. DSM11171 (Vértesy et al., 1999). It mainly contains unusual amino acids such as 4-hydroxyphenylglycine (Hpg) and 3,5-dihydroxy-phenylglycine (Dpg) (Fig. 1) and was recently fully chemically synthesized by a sophisticated peptide synthesis strategy (Dettner et al., 2009). X-ray crystallography has shown that FGM is able to form a double stranded anti-parallel β-helical dimer (Bunkóczi et al., 2005). Here, we investigated its anti-HIV activity profile, its mode of action and succeeded in generating in vitro an HIV-1 IIIB^{FGMres} virus that appeared to have 2 unique mutations in the viral gp120, not reported so far by any other known entry inhibitor.

Results

Broad-spectrum anti-HIV activity profile of feglymycin

First, we evaluated the activity of feglymycin (FGM) against cell line-adapted HIV-1 strains (NL4.3, IIIB, HE) and against the HIV-2 strain ROD in susceptible CD4^{+} T cell lines (MT-4 and C8166 cells). As shown in Table 1, FGM could inhibit infection of these HIV strains with an EC$_{50}$ in the lower μM-range (EC$_{50}$: 0.8–3.2 μM). Then, the anti-HIV activity of FGM was tested against two HIV-1 strains and several HIV-1 clinical isolates from different clades in PHA-activated PBMCs (or T cell blasts). We found that FGM inhibited infection of the CXCR4-using (X4) NL4.3 and the CCR5-using (R5) BaL HIV-1 strains in lower μM ranges (EC$_{50}$s:

2.4–4.3 μM). Monocyte-derived macrophages (MDM) were also protected against HIV-1 BaL infection by FGM (Table 1). FGM was found active against clinical HIV-1 isolates representing members of different viral clades (A–D, A/E and G) with mean EC$_{50}$s varying between 0.5 μM and 6.7 μM (Table 2).

The antiviral activity of FGM was also tested against in vitro generated mutant HIV-1 strains: virus resistant to the gp41 fusion inhibitor T20 (HIV-1 NL4.3^{T20res}), virus resistant to the carbohydrate-binding anti-gp120 mAb 2G12 (HIV-1 NL4.3^{2G12res}) and virus resistant to the CXCR4 antagonist AMD3100 (HIV-1 NL4.3^{AMD3100res}). Comparable antiviral activity against these resistant viruses was observed (EC$_{50}$s: 0.9–2.4 μM; Table 1).

Next, the anti-HIV activity of FGM against an increasing series of virus inputs of NL4.3 starting at ∼100 TCID$_{50}$ (normally used in our MT-4 antiviral assays) up to 5000 TCID$_{50}$ was evaluated and compared with the activity of the gp41 fusion inhibitor T20 and the anti-gp120 mAb 2G12. Increasing the viral input up to 50-fold

Table 1
Antiviral activity (EC$_{50}$ in μM) profile of feglymycin (FGM) against cell-line-adapted and several entry inhibitor resistant HIV strains in various cell types.

Cells	Virus strains	Tropism	EC$_{50}$ of FGM (μM)
MT-4	HIV-1 NL4.3	X4	0.9
MT-4	HIV-1 IIIB	X4	3.2
MT-4	HIV-1 HE	X4/R5	0.8
MT-4	HIV-2 ROD	X4/R5	1.2
C8166	HIV-1 IIIB	X4	1.9
PHA-activated PBMCs[a]	HIV-1 NL4.3	X4	2.4
PHA-activated PBMCs[a]	HIV-1 BaL	R5	4.3
MDM[b]	HIV-1 BaL	R5	2.9
MT-4	HIV-1 NL4.3^{T20res}	X4	0.9
MT-4	HIV-1 NL4.3^{2G12res}	X4	2.2
MT-4	HIV-1 NL4.3^{AMD3100res}	X4	2.4

[a] PHA-activated PBMCs: Phytohemagglutinin-activated peripheral blood mononuclear cells.
[b] MDM: monocyte-derived macrophages. Mean EC$_{50}$s of at least 3 independent experiments are shown.

Table 2
Broad-spectrum anti-HIV-1 activity (EC$_{50}$ in μM) of feglymycin (FGM) against clinical isolates representing different HIV-1 clades of group M.

	UG273 R5	US2 R5	ETH2220 R5	UG270 X4	ID12 R5	RU570 R5
	Clade A	Clade B	Clade C	Clade D	Clade A/E	Clade G
FGM	2.8	0.5	1.8	6.7	3.1	5.2

Mean EC$_{50}$s out of at least 2 individual PHA-activated PBMC donor experiments are shown. Co-receptor tropism (R5/X4) of the viruses was determined as described (Princen et al., 2004).
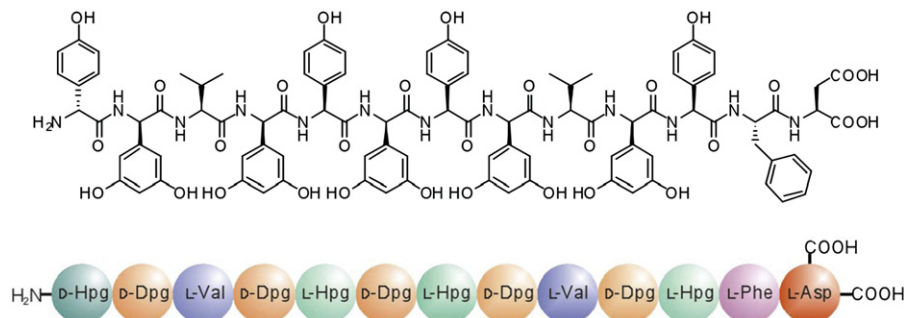

Fig. 1. Structure of the 13mer peptide antibiotic feglymycin (FGM).

(the highest virus input feasible in this assay) resulted, surprisingly, in a very consistent antiviral activity of FGM (only ~4-fold loss of activity). In contrast, T20 and 2G12 are quite sensitive to the viral input and both agents lost completely their antiviral activity when increasing the amount of virus input (Table 3).

Finally, we investigated if FGM has a broader antiviral activity and tested it against dengue virus, hepatitis C virus and herpes simplex virus type 1 and type 2. No activity for FGM was detected against these viruses up to 26.3 μM (or 50 μg/ml) (data not shown).

Antiviral activity of alanine derivatives of feglymycin (ala-FGM)

To gain more insights in the position of crucial amino acids involved in the anti-HIV activity of FGM various alanine derivatives (ala-FGM) were synthesized. As shown in Fig. 2, the substitution of L-aspartic acid at position 13 (L-Asp[13]) with a neutral alanine (L-ala[13]-FGM) reduces the antiviral activity of FGM ~6.5-fold compared to native FGM. Other alanine substitutions had no effect on the potency of the anti-HIV activity of FGM.

Feglymycin inhibits viral entry

To gain more insight in the mechanism of action of FGM, time-of-drug addition (TOA) experiments were performed. Therefore, MT-4 cells were infected with X4 HIV-1 IIIB and, at different time points after infection (ranging from 0 h to 26 h and after 31 h), FGM and several reference compounds were added. When a compound is added after its antiviral target becomes operative, no inhibition of viral replication will be observed. The polyanionic binding/adsorption inhibitor dextran sulfate 8000 (DS8000; 421 μM or 100 μg/ml) lost its antiviral activity completely when added 1 h after infection (Fig. 3). The CXCR4 antagonist AMD3100 (6 μM or 5 μg/ml) became completely ineffective when added 2 h after infection while reverse transcriptase inhibitors (RTI) (e.g., nevirapine at 2 μg/ml or 7.5 μM) and protease inhibitors (PI) (e.g., ritonavir at 2 μg/ml or 2.8 μM) kept their anti-HIV activity

Table 3
Antiviral activity (EC_{50}) of feglymycin (FGM), T20 and mAb 2G12 against a series of viral inputs ($TCID_{50}$) of HIV-1 NL4.3 in MT-4 cells.

Agents	Viral input ($TCID_{50}$)					Fold decrease[a]
	100	500	1000	2500	5000	
FGM (μM)	0.66	1.8	1.8	2.8	2.5	**4**
T20 (nM)	9.7	24.4	104	424	>1113	>**115**
2G12 mAb (μg/ml)	0.51	2.1	3.4	>20	>20	>**39**

[a] Fold decrease in antiviral activity calculated as: highest EC_{50}/lowest EC_{50}. Mean EC_{50}s of at least 2 independent experiments are shown.
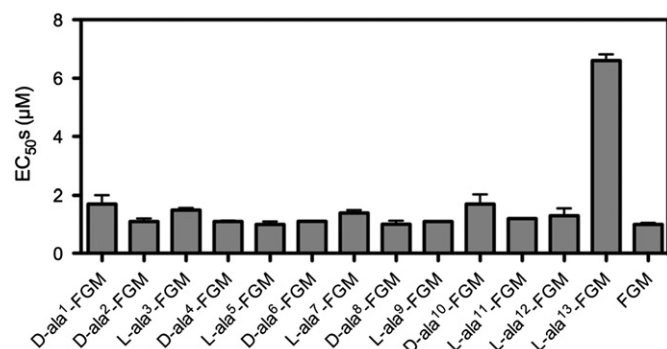

Fig. 2. Antiviral activity of FGM alanine derivatives (ala-FGM) against X4 HIV-1 NL4.3. Mean EC_{50}s ± SEM out of at least two independent experiments are shown.
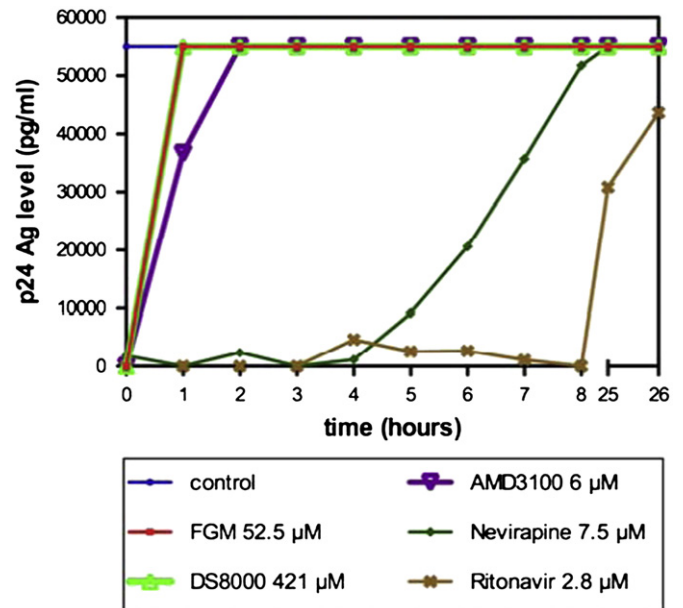

Fig. 3. Time-of-drug addition experiments whereby MT-4 cells were infected with X4 HIV-1 IIIB and FGM and various compounds with diverse well-described antiviral inhibition modes were added at different time points in a range from 0 h to 26 h post-infection.

4 h and 8 h post-infection, respectively (Fig. 3). The inhibitory curve of FGM (tested at 100 μg/ml or 52.5 μM) coincides with DS8000 and its antiviral activity disappeared when added 1 h post-infection (Fig. 3). So these data suggests that FGM, like dextran sulfate, acts as an early viral binding/adsorption inhibitor.

Inhibition of syncytia formation by feglymycin

CD4^{+} T cells cannot only be infected with free virions but also very efficiently through cell-to-cell contacts with HIV-infected CD4^{+} T cells. Consequently, we investigated if FGM was able to inhibit the giant cell or syncytia formation in a co-cultivation assay between persistently HIV-infected CD4^{+} T cells (HUT-78/IIIB cells) and non-infected CD4^{+} target T cells (SupT1 cells). Fig. 4A shows a light microscopic view of this assay and at 10.5 μM FGM completely inhibits the appearances of giant cells or syncytia. FGM prevented the appearances of giant cells dose-dependently with a mean EC_{50} of 5.4 μM (Fig. 4B). A comparable activity could be observed for the CXCR4 antagonist AMD3100 (mean EC_{50}: 4 μM) and the gp41 fusion inhibitor T20 (mean EC_{50}: 3 μM) (Fig. 4B). Important to mention here is that the reference compounds AMD3100 and T20 lost at least 1000-fold of their inhibitory activity in these cell-to-cell viral transmission assays compared to their nM activity reported by us and many other research groups in the cell-free virus replication assays, whereas the antiviral activity of FGM in this assay remained almost unchanged (Table 1). Another included reference compound, the RTI tenofovir, showed as expected no inhibitory effect at 50 μM (Fig. 4B). FGM also inhibited syncytia formation when HUT-78/ROD (HIV-2) cells were co-cultured with CD4^{+} T cells (mean EC_{50}: 17.1 μM, Fig. 4C), although a ~3-fold loss of activity was observed compared to HUT-78/IIIB. However, AMD3100 and T20 showed even no activity in the HIV-2 ROD mediated co-cultivation assay (Fig. 4C).

Feglymycin interferes with CD4/gp120 interaction

Based on our TOA experiments and syncytia formation assays, FGM inhibits an early step of the HIV replication cycle. The initial

A

B HUT-78/IIIB + SupT1 cells
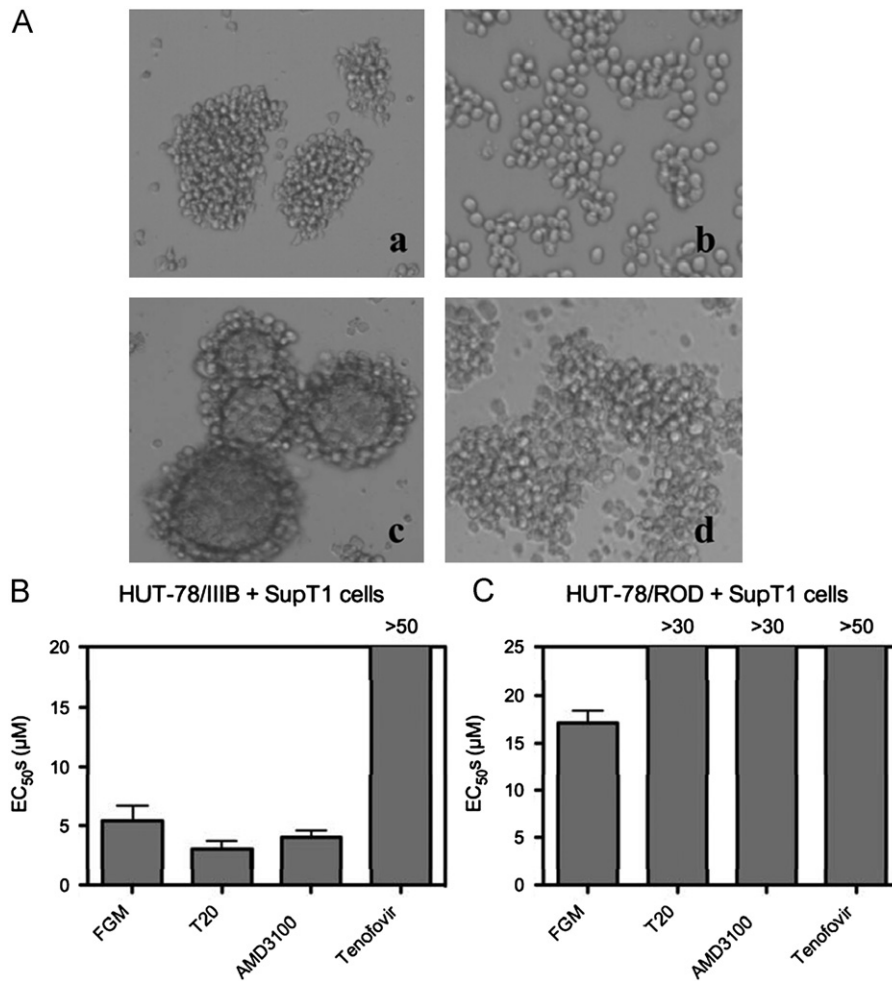
C HUT-78/ROD + SupT1 cells

Fig. 4. (A) Light microscopic view of the syncytia formation assay after 24 h of co-cultivation between persistently infected HUT-78/IIIB cells and non-infected CD4$^+$ SupT1 cells. Upper panels: (a) uninfected CD4$^+$ SupT1 cells and (b) persistently infected HUT-78/IIIB cells. Lower panels: (c) giant cell formation after co-cultivation of SupT1 cells with HUT-78/IIIB cells and (d) complete inhibition of syncytia formation in the presence of 10.5 μM FGM. Persistently infected HUT-78/IIIB (HIV-1) cells (B) and HUT-78/ROD (HIV-2) cells (C) were co-cultivated with SupT1 cells in the presence of FGM, T20, AMD3100 and tenofovir. The bars represent the mean EC$_{50}$-values ± SEM. Values > 30 and > 50 indicate an EC$_{50}$ above 30 μM and 50 μM, respectively.

step of HIV entry is the binding of gp120 to the CD4 receptor and we investigated if FGM interferes with this process using a sCD4/HIV-1 ELISA and a flow cytometric cellular virus binding assay. Human soluble CD4 (sCD4) was coated on a 96-well plate and exposed to HIV-1 NL4.3 virus in the presence of test compounds. FGM inhibited significantly the binding of HIV-1 NL4.3 on the coated CD4 with a mean EC$_{50}$ of 4.4 μM ($p=0.002$), as determined using a sCD4/HIV-1 NL4.3 binding ELISA (Fig. 5A). The anti-CD4 mAb RPA-T4 and the polyanionic compound PRO2000 were used as control agents and inhibited also significantly the binding of HIV-1 with CD4 with mean EC$_{50}$s of 3.7 μg/ml for RPA-T4 and 0.33 μM for PRO2000. As expected, the RTI tenofovir had no effect in this sCD4/HIV-1 binding assay (EC$_{50}$ > 10 μM).

In another experimental setup, we analyzed if FGM could inhibit the binding of HIV-1 NL4.3 virus particles on cell surface expressed CD4 using CD4$^+$ SupT1 T cells (Fig. 5B). The amount of virus bound on surface CD4 was detected using the anti-gp120 mAb 9205. In the presence of 10.5 μM FGM, virus binding was abrogated for 74.5% (MFI of 12.2). The polyanionic compound, PRO2000, here included as a control compound, inhibited HIV binding by 94.3% (MFI of 6.1) at 4 μM. The CXCR4 antagonist AMD3100 at 24.1 μM had no effect on virus binding to CD4$^+$ SupT1 T cells, as described previously (Schols et al., 1997) (Fig. 5B). These data clearly demonstrate that FGM interferes with the CD4/gp120 binding process.

Kinetic analysis of the interaction of feglymycin with the viral envelope protein gp120

A more quantitative way to determine if agents bind to the HIV-1 envelope is the use of surface plasmon resonance (SPR) technology (Hoorelbeke et al., 2010). In these experiments, binding properties of FGM, D-ala^6-FGM and L-ala^{13}-FGM were evaluated towards HIV-1 gp120 IIIB (X4) and to HIV-1 gp120 YU2 (R5). Both ligands were covalently immobilized on CM5 sensor chips and 8200 RU and 9626 RU of density was obtained, respectively. Two-fold serial dilution series covering a concentration range of 7.8–125 nM for FGM and D-ala^6-FGM and 62.5 nM to 1000 nM for L-ala^{13}-FGM were applied to the gp120's immobilized on a CM5 sensor chip. A 1:1 Langmuir kinetic fit was applied to obtain the in real time measured association rate constant k_a (k_{on}, on-rate) and dissociation rate constant k_d (k_{off}, off-rate) and the affinity constant K_D (ratio k_d/k_a) (Table 4; Fig. 6). As depicted in Fig. 6 (panels a and b) and Table 4, FGM interacts potently to gp120 from the X4 strain IIIB as well as the R5 strain YU2, with K_D values of 6.1 ± 0.2 nM and 2.2 ± 0.3 nM, respectively. For D-ala^6-FGM, a loss in K_D value of 4-fold (X4 IIIB) and 6-fold (R5 YU2) was observed (Table 4; Fig. 6, panels c and d). When we compared the peptide L-ala^{13}-FGM with native FGM, a 8-fold and 13-fold loss in K_D was measured with 50.6 ± 1.5 nM and 29.5 ± 1.3 nM for HIV-1X4 IIIB and HIV-1 R5 YU2, respectively (Table 4; Fig. 6, panels e and f).
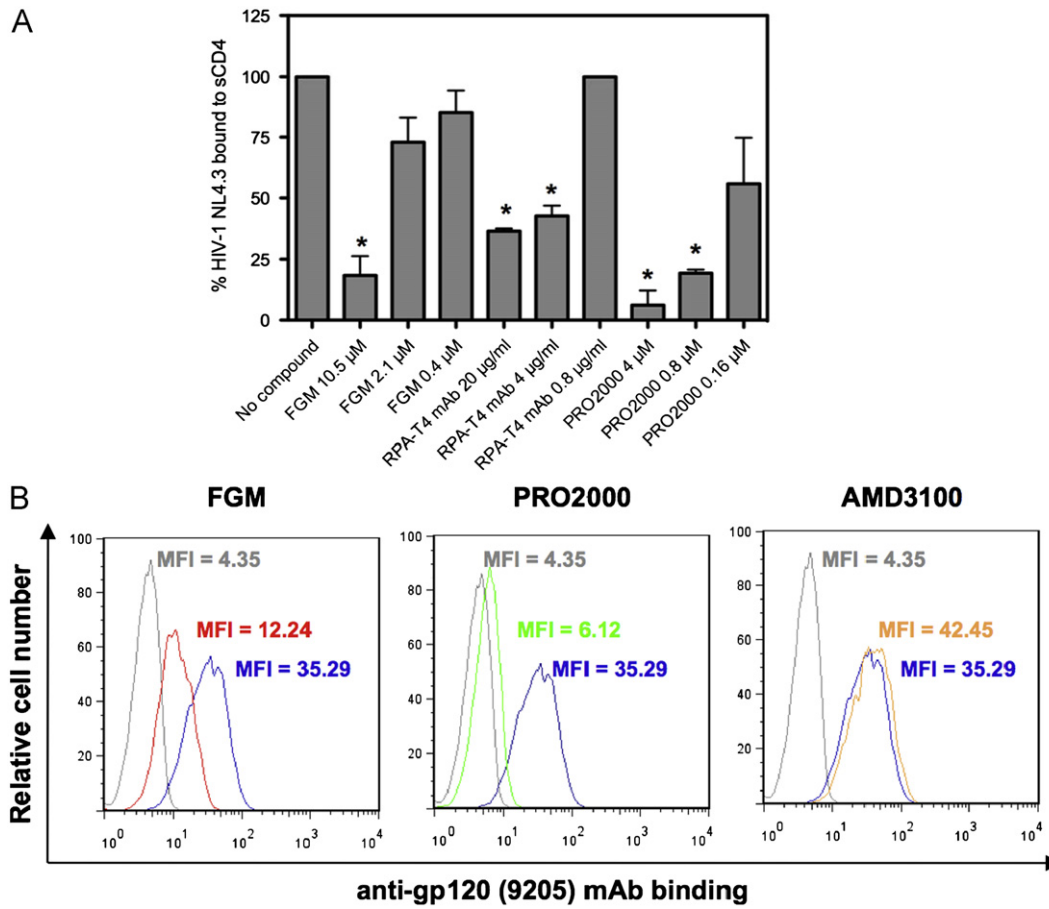
Fig. 5. FGM inhibits the binding of HIV-1 to CD4. (A) The binding of HIV-1 NL4.3 to sCD4 in the presence of FGM, anti-CD4 mAb RPA-T4 and PRO2000 was measured using a sCD4/HIV-1 binding ELISA. The mean percentages ± SEM of virus binding to sCD4 from at least 3 independent experiments are shown. Statistical significance whereby $p < 0.05$ is indicated by an asterisk (∗) according to an unpaired T-test. (B) CD4$^+$ SupT1 T cells were incubated with HIV-1 NL4.3 in the presence of 10.5 μM FGM, 4 μM PRO2000 and 24.1 μM AMD3100, and virus binding was determined by flow cytometry using the anti-gp120 mAb 9205. The blue histograms represent virus binding in the absence of compound. Virus binding in the presence of FGM, PRO2000 and AMD3100 is shown in red, green and orange, respectively. The grey histograms show background fluorescence. The mean fluorescence intensity (MFI) is shown. One representative experiment out of 2 is shown.

Table 4
Kinetic data for the interaction of FGM, D-ala^6-FGM and L-ala^{13}-FGM with immobilized HIV-1 envelope proteins gp120 IIIB (X4) and gp120 YU2 (R5).

	K_D (nM)	K_{on} (1/M.s)	K_{off} (1/s)
FGM			
gp120 IIIB (X4)	6.1 ± 0.2	(4.95 ± 0.36) E+05	(3.03 ± 0.14) E-03
gp120 YU2 (R5)	2.2 ± 0.3	(5.47 ± 0.72) E+05	(1.20 ± 0.05) E-03
D-ala^6-FGM			
gp120 IIIB (X4)	23.0 ± 0.7	(7.92 ± 0.39) E+05	(1.82 ± 0.11) E-02
gp120 YU2 (R5)	12.1 ± 0.9	(4.89 ± 0.44) E+04	(5.92 ± 0.36) E-03
L-ala^{13}-FGM			
gp120 IIIB (X4)	50.6 ± 1.5	(6.54 ± 0.19) E+04	(3.37 ± 0.12) E-03
gp120 YU2 (R5)	29.5 ± 1.3	(8.03 ± 0.30) E+04	(2.37 ± 0.09) E-03

K_D, affinity constant; k_{on}, association rate constant; k_{off}, dissociation rate constant.

Effect of feglymycin on the capture of HIV by DC-SIGN-expressing cells and on the subsequent virus transmission to CD4$^+$ T cells

DC-SIGN on dendritic cells (DCs) plays also a pivotal role in the transmission of HIV to CD4$^+$ T cells (Hladik and McElrath, 2008). We investigated if FGM prevented capture of HIV-1 (strain HE (X4/R5)) by DC-SIGN using Raji cells transfected with DC-SIGN (Raji.DC-SIGN$^+$ cells). HIV-1 was pre-exposed to FGM, PRO2000 or HHA for 30 min before virus was administered to Raji.DC-SIGN$^+$ cells (Fig. 7A). FGM did not inhibit the binding of HIV-1 to DC-SIGN (EC$_{50}$ > 5.3 μM)

($p > 0.05$). The polyanionic compound PRO2000 also lacked any activity in this type of assay (EC$_{50}$ > 2 μM) ($p > 0.05$). In contrast, the plant lectin HHA inhibited significantly the capture of HIV-1 on DC-SIGN (mean EC$_{50}$: 0.02 μM; $p=0.048$), which is in agreement with our previously published data on HHA and its inhibition of the capture of X4 HIV-1 IIIB on Raji.DC-SIGN$^+$ cells (Balzarini et al., 2007).

We also examined if FGM was able to inhibit giant cell formation of virus-captured on DC-SIGN$^+$ cells with CD4$^+$ T cells. In the absence of inhibitors, massive giant cells were formed 24–48 h post co-cultivation (Fig. 7B, panels a–c). When FGM was added at the start of co-cultivation (and thus virus was already captured on Raji.DC-SIGN$^+$ cells), FGM was able to inhibit the subsequent syncytia formation dose-dependently (mean EC$_{50}$: 2.60 ± 0.03 μM; Fig. 7B, panel d) comparable even to PRO2000 (mean EC$_{50}$: 0.23 ± 0.03 μM). HHA inhibited this process very efficiently (mean EC$_{50}$: 43 ± 11 nM; Fig. 7B, panel e). Thus, FGM inhibits virus binding and transmission to CD4$^+$ T cells, but does not influence the capture of virus to the DC-SIGN receptor as such as this interaction is mainly carbohydrate-dependent due to the high number of glycans on gp120.

Selection and mutation determination of feglymycin resistant HIV-1 IIIB (HIV-1 IIIB^{FGMres})

To decipher more in depth the mechanism of action of FGM, we also investigated the development of HIV-1 resistance to FGM. We therefore cultured X4 HIV-1 IIIB in the CD4$^+$ T cell line C8166 in
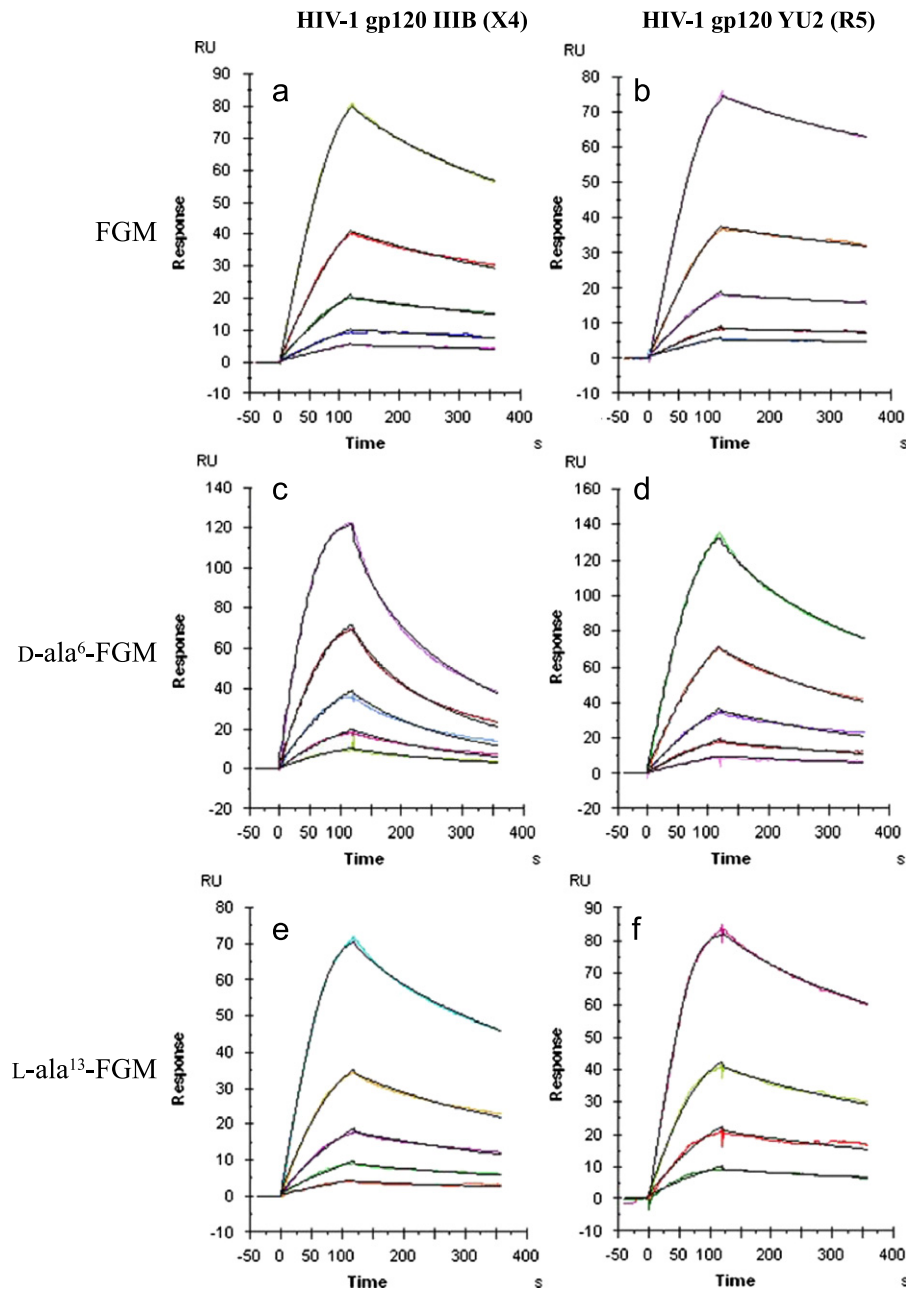
Fig. 6. Kinetic analysis of the interactions of FGM, D-ala[6]-FGM and L-ala[13]-FGM with immobilized HIV-1 gp120 IIIB (panels a, c and e) and HIV-1 gp120 YU2 (panels b, d and f). Serial 2-fold analyte dilutions covering a concentration range from 7.8 nM to 125 nM for FGM and D-ala[6]-FGM and 62.5 to 1000 nM for L-ala[13]-FGM were injected over the surface of the immobilized gp120. The experimental data (colored curves) were fit using the 1:1 binding model (black lines) to determine the kinetic parameters. The data are a representative experiment out of three independent experiments.

the presence of sub-optimal concentrations of FGM (Fig. 8A). Subcultivations were performed on a weekly basis and when strong virus-induced cytopathic effects (CPE) were observed microscopically, FGM concentrations were slightly increased. At different time points, virus was analyzed for changes in amino acids in the *env* sequence and compared to the sequence of wild-type HIV-1 IIIB, which has been cultured in parallel but in the absence of compound. It took between 20 and 30 passages of FGM selection to gain an I153I/L mixed mutation in gp120, resulting in a ~5-fold decrease in antiviral activity. Eight passages later, the mixed mutation became a pure mutation I153L and a novel mixed mutation K457K/I arose, resulting in a ~7-fold decrease in FGM sensitivity. Finally, after 44 passages resistant virus (HIV-1 IIIB[FGMres]) was able to grow in the presence of 26.3 μM (or

50 μg/ml) FGM. Interestingly, only two specific mutations in gp120 of the resistant virus were observed, namely I153L in the V2 loop region and K457I in the C5 loop region, and no amino acid changes were observed in gp41.

Sensitivity of HIV-1 IIIB[FGMres] virus to various other classes of entry inhibitors

The susceptibility of HIV-1 IIIB[FGMres] to various other EIs was investigated and compared with their antiviral activity against wild-type HIV-1 IIIB. As can be seen in Fig. 8B, the virus binding inhibitor dextran sulfate (DS5000) and the CD4 down-modulating compound, cyclotriazadisulfonamide (CADA), showed a ~9-fold and >15-fold decrease in antiviral activity, respectively. Other
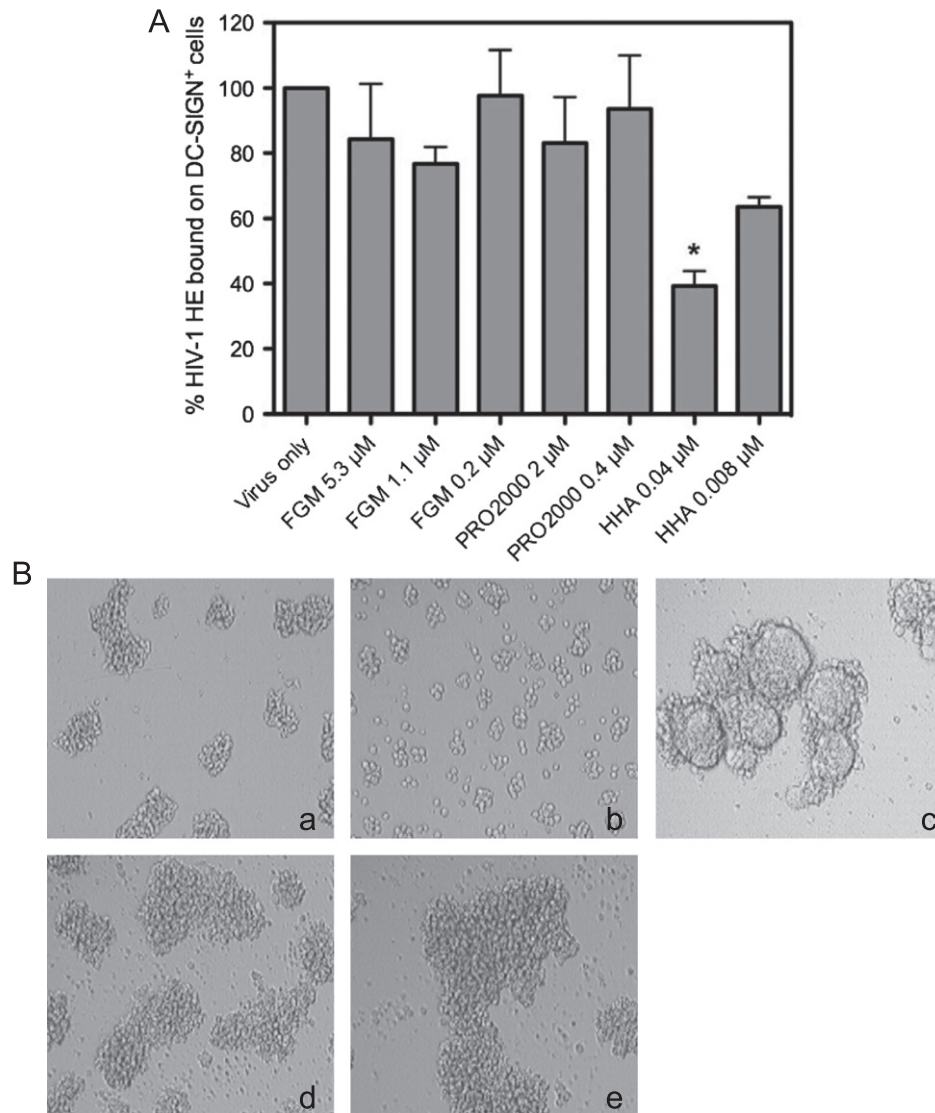
Fig. 7. (A) Raji.DC-SIGN$^+$ cells were incubated with HIV-1 HE in the presence of various concentrations FGM, PRO2000 and HHA. The effect on virus capture was determined by the use of p24 HIV-1 Ag ELISA. Grey bars represent mean values ± SEM. Statistical significance whereby p < 0.05 is indicated by an asterisk (∗) according to an unpaired T-test. (B) Microscopic view of syncytia formation after 24 h of co-cultivation of Raji.DC-SIGN$^+$ cells pre-exposed to HIV-1 HE (Raji.DC-SIGN$^+$/HE) and CD4$^+$ C8166 T cells in the presence or absence of FGM and HHA. Upper panels: (a) C8166 T cells, (b) Raji.DC-SIGN$^+$/HE and (c) giant cell formation after co-cultivation of Raji.DC-SIGN$^+$/HE and C8166 cells. Lower panels: protective effect of FGM (5.3 μM) when added only at the time of the co-cultivation (d) and protective effect of HHA (0.2 μM) when given at the time of co-cultivation (e).

evaluated EIs such as PRO2000, HHA, T20, AMD3100 and b12 mAb were equally active against the HIV-1 IIIB^{FGMres} virus as the wild-type HIV-1 IIIB. We evaluated several of the ala-scan peptides such as L-ala^5-FGM and D-ala^6-FGM against HIV-1 IIIB^{FGMres} virus and observed that they also lost all antiviral activity (EC$_{50}$ > 25 μM).

Discussion

The entry process of HIV can still be considered as an attractive target for ARV therapy. Each step of this process, binding/adsorption, (co)-receptor binding, membrane fusion and viral uncoating can offer possibilities for the development of unique classes of EIs.

Here, we report the antiviral activity and mechanism of action of the antibiotic peptide feglymycin (FGM). Due to its unique structure, FGM can be considered as a prototype peptide of a novel class inside the group of the viral EIs. FGM showed in vitro a potent and consistent broad-spectrum ARV activity against various HIV-1 clinical isolates representing different clades as well as T cell line-adapted strains of HIV-1 and HIV-2 and this independent of their co-receptor tropism (Tables 1 and 2). In addition to the inhibition of infection of target CD4$^+$ T cells by cell-free virus, FGM was also able to prevent cell-to-cell virus transmission between HIV-infected and non-infected CD4$^+$ T cells (Fig. 4). Human semen not only contains cell-free virus but also cell-associated virus and evidence has accumulated that seminal infected leucocytes can cross the vaginal barrier and reach permissive target cells within the epithelium or submucosal tissue (Coombs et al., 2003; D'Cruz and Uckun, 2006; Lederman et al., 2006; Sallé et al., 2010). Developing a microbicide blocking this pathway of cell-to-cell HIV transmission can be of great importance. In the VOICE (Vaginal and Oral Interventions to Control the Epidemic) study, a large phase IIb clinical trial designed to determine whether the tenofovir gel could prevent transmission of HIV, no significant protective effect was observed (Celum, 2011). This lack of inhibition could be due to cell-associated HIV transmission and a recent study showed that viral
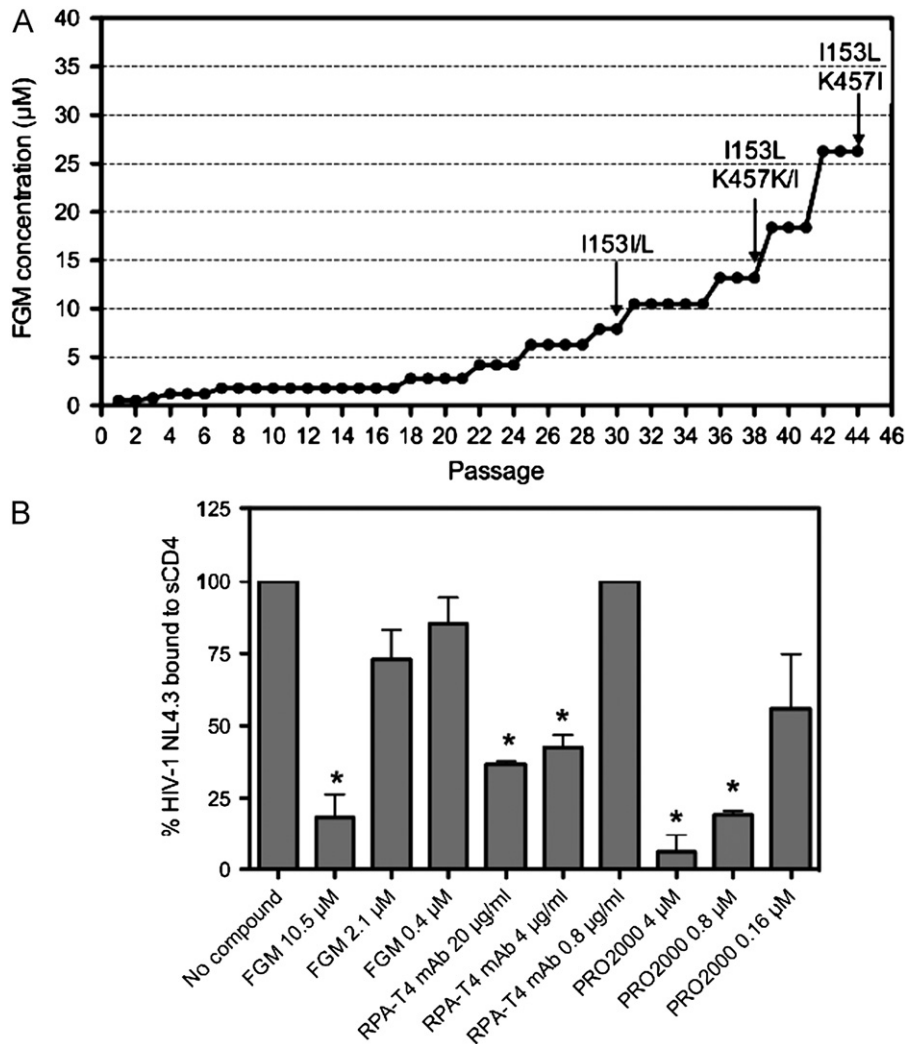
Fig. 8. (A) Selection of HIV-1 IIIB FGM-resistant virus (IIIB[FGMres]). The HIV-1 IIIB virus was cultured in C8166 cells in the presence of sub-optimal concentrations of FGM, and the drug concentration was increased when full CPE was observed. The time points where infected cell culture supernatant was taken for *env* gene characterization and their appeared mutations are shown (arrows). (B) Cross-resistance profile of HIV-1 IIIB[FGMres] to the entry inhibitors FGM, DS5000, PRO2000, HHA, T20, CADA, AMD3100 and b12 mAb. The fold-increase in EC$_{50}$s compared with wild-type HIV-1 IIIB virus is shown. One representative experiment out of 2 is shown.

replication still can occur despite treatment with tenofovir due to virus cell-to-cell transmission (Sigal et al., 2011). Because FGM also inhibits HIV cell-to-cell transmission it could have a potential advantage as microbicide candidate. No inhibitory effects of FGM were observed when tested against other classes of enveloped viruses such as herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2), dengue virus (DENV) and hepatitis C virus (HCV) (data not shown). Thus, although limited broad-spectrum antiviral data are available it demonstrates that FGM shows an antiviral activity profile with specificity for HIV.

To study the contribution of a specific amino acid to the stability and function of FGM, alanine scanning mutagenesis was performed. Substitution of L-aspartic acid at position 13 (L-Asp[13]) by alanine reduces the antiviral activity ∼7-fold (Fig. 2). This decrease in antiviral activity of L-ala[13]-FGM compared to native FGM is correlated with a ∼8-fold loss in affinity for gp120, measured as affinity constant (K_D) by SPR technology (Table 4). The substitution of other amino acids has no effect on the antiviral activity compared to the natural FGM. X-ray crystallography showed that the linear 13mer amino acid peptide FGM could also form dimers (Bunkóczi et al., 2005). L-Asp[13] contributes to the creation of stable hydrogen bridges in the helical dimer structure of FGM. The change to L-Ala[13] might result in loss of

stabilizing hydrogen bridges and a less distinct secondary structure of FGM in solution and as consequence a lower antiviral activity. The substitution of L-Asp[13] by an alanine also affects the net charge of the peptide, which could lead to reduced electrostatic interactions with its target protein. In addition, nearly all amino acids of FGM are repeated at least once in the sequence. Therefore, the deletion of a single amino acid side chain may be well tolerated regarding the antiretroviral activity of this peptide. This should enable the synthesis of structurally less complex FGM derivatives with similar or even increased biological activity.

As shown here FGM acts at a very early step in the virus replication cycle, the viral binding/adsorption process. Based on time-of-drug addition experiments, FGM interferes with this entry process at the same moment as the polyanionic class of compounds dextran sulfate and PRO2000, but earlier then the CXCR4 antagonist AMD3100. So, we could already assume that FGM inhibits virus binding to the CD4 receptor and that it did not interact with the HIV fusion-uncoating process. When we investigated virus attachment in more detail, FGM acted in a comparable manner as the anti-CD4 mAb RPA-T4 and PRO2000 in the sCD4/HIV-1 binding ELISA and in a virus binding assay on CD4^{+} T cells measured by flow cytometry (Fig. 5). Additionally, when we used SPR technology whereby gp120 was immobilized on a

CM5 biosensor chip, the interaction between CD4 and gp120 was blocked by FGM (data not shown). RPA-T4 mAb targets the D1 region of the cellular CD4 receptor and blocked, as FGM, the binding of HIV-1 to human sCD4. However, when we incubated T cells with FGM, no down-regulating effect on the CD4 receptor was observed, in contrast to RPA-T4 and the CD4 down-modulating agent CADA (data not shown and Vermeire et al., 2009). Also, no direct interaction of FGM with the HIV cellular co-receptors was measured as FGM could not block the intracellular Ca^{2+} signaling induced by CXCL12 and CCL3L1 in CXCR4- and CCR5-transfected cell lines, respectively (data on file).

PRO2000 is described to be an inhibitor of CD4/gp120 binding by interacting with gp120 and CD4 (Rusconi et al., 1996; Scordi-Bello et al., 2005). FGM could also inhibit the binding of HIV to $CD4^+$ T cells. However, more potent inhibition of the virus binding with PRO2000 was measured: 94.3% inhibition with 4 μM PRO2000 and 74.5% inhibition with FGM at 10.5 μM. The stronger antiviral activity of PRO2000 in HIV replication assays and the surface plasmon resonance (SPR) studies showed that PRO2000 interacted with high affinity on (soluble) gp120 of X4 and R5 viruses (K_D: 2–3 nM) (Scordi-Bello et al., 2005 and our own unpublished data). When SPR-based kinetic analysis was performed with FGM for binding to gp120, comparable affinity constants were recorded with mean K_D-values of 6.1 nM for X4 gp120 IIIB and 2.2 nM for R5 gp120 YU2 (Fig. 6 and Table 4).

To get more insight in the mechanism of action of FGM against HIV, an FGM-resistant virus was selected (Fig. 8). Finally, at passage 44 of HIV-1-infected cell cultures, two specific mutations were found: I153L and K457I, resulting in a ~14-fold increase in the EC_{50}-value of FGM (Table 1 and Fig. 8). Mutation I153L is located in the V2 region of gp120, which is localized proximal to the CD4 binding domain and is also a part of the bridging sheet (Kwong et al., 1998; Zolla-Pazner, 2004). Additionally, Wu et al. (2009) showed that certain mutations inside the V1/2 loop can influence the flexibility of gp120 and thus the binding of the mAb b12, recognizing the CD4 binding site in the C4 region on gp120. K457I is located in the C5 region, near the CD4 binding site, and can be involved in stabilizing the CD4 binding conformation site on gp120. The small molecule, BMS378806, was described as a specific CD4/gp120 inhibitor by targeting gp120 and the BMS378806-resistant virus contained also two mutations in gp120 (M426L in the C4 region and M475I in the C5 region) (Lin et al., 2003; Wang et al., 2004). The observation that the resistance mutation K457I for FGM is in the same region as the M475I BMS378806 mutation is another indication for its antiviral mode of action. These two mutations in gp120 are unique for FGM and no cross-resistance was observed with the class of CBAs such as HHA and 2G12 mAb. This was expected, as FGM does not target the N-glycans present on gp120, as it does not inhibit HIV binding to $DC-SIGN^+$ cells and that it kept its antiviral activity against the HIV-1 NL4.3^{2G12res} virus (Table 1). The CBA class of HIV inhibitors have also activity against other classes of viruses such as DENV and HCV (Alen et al., 2009; Bertaux et al., 2007). As already mentioned and in contrast with the CBAs, FGM was not able to inhibit the capture of virions to DC-SIGN (Fig. 7). In vitro studies have shown that polyanionic compounds such as PRO2000 and dextran sulfate also do not inhibit the capture of HIV to DC-SIGN, while the CBAs potently inhibited this process (Balzarini et al., 2007). Interestingly, approximately a ~9-fold increase in EC_{50} was observed for the polyanionic compound dextran sulfate (DS5000) against the HIV-1 IIIB^{FGMres} virus. Dextran sulfate resistant virus showed ~7 mutations throughout various regions in gp120 (such as V1/2) (Esté et al., 1997) and can be considered as a much more aspecific anti-HIV compound compared to BMS378806. The appearance of DS5000 cross-resistance further suggests that FGM acts as virus binding/adsorption inhibitor. The lack of cross-resistance with the

polyanionic compound PRO2000 can be explained by the additional mechanisms of action of PRO2000. Our research group showed earlier that PRO2000 could also interact with the CXCR4 chemokine receptor while DS5000 does not (Huskens et al., 2009).

Many other described anti-HIV peptides targeting the entry process are mainly active not at binding but at the fusion event. The gp41 fusion inhibitor T20 was equally active against the HIV-1 IIIB^{FGMres} virus as the wild-type virus. Also, no difference in antiviral activity of FGM was noticed against HIV-1 NL4.3^{T20res} virus (Table 1). FGM and T20 interfere with different stages of the HIV entry process and the most commonly observed HIV-1 mutations for resistance to T20 are located in the HR1 region of gp41 at positions 36 (G36S) and 38 (V38M/A), although changes in gp120 also can appear and influence the activity of T20 (Matthews et al., 2004). If we compare FGM to an earlier described Streptomyces-derived peptide, siamycin I, a complete different mechanism of antiviral action is observed. Siamycin I is a tricyclic peptide containing 21 amino acids that acts as fusion inhibitor and does not inhibit the CD4/gp120 interaction (Lin et al., 1996 and our unpublished data). As FGM, siamycin I inhibited viral replication in the cell-free virus assay as well as giant cell formation in a co-cultivation assay. The siamycin-resistant virus showed 6 amino acid changes throughout gp120 and gp41 (Lin et al., 1996). Virus-inhibitory peptide, or VIRIP, is also a peptide and it represents a small piece of a larger protein normally found in our blood, alpha1-anti-trypsin. This peptide also differs in its mode of action with FGM as VIRIP blocks HIV infection by binding to the viral protein gp41 and preventing fusion between the viral envelope and the cell membrane, as T20 does (Münch et al., 2007).

AMD3100 targets specifically the cellular CXC-chemokine CXCR4 receptor (Schols et al., 1997) and the HIV-1 IIIB^{FGMres} virus was still equally sensitive to AMD3100 (Fig. 8). The HIV-1 NL4.3^{AMD3100res} virus also showed multiple mutations in various regions in gp120 (De Vreese et al., 1996), however, no loss of antiviral activity was observed when FGM was tested against this HIV-1 NL4.3^{AMD3100res} (Table 1). Somewhat surprisingly, the CD4 down-modulating agent CADA lost its antiviral activity against HIV-1 IIIB^{FGMres} virus.

During sexual intercourse, vaginal/cervical micro-abrasions play a pivotal role in the transmission of HIV. DC-SIGN$^+$ DCs in the mucosal tissues play an important role in the defense against pathogens, however several viruses (such as HIV) can hijack the attachment receptor DC-SIGN on DCs and efficiently transmit HIV towards naive T cells in the lymph nodes (Hladik and McElrath, 2008). The role of DC-SIGN in the transmission of HIV remains still controversial. Granelli-Piperno et al. have shown that DC-SIGN is not required for transmission of HIV and subsequently stimulation of $CD4^+$ T cells (Granelli-Piperno et al., 2005), while Geijtenbeek et al. (2000) described that the presence of DC-SIGN increased HIV infection . While FGM was not able to inhibit virus capture to DC-SIGN, it inhibited subsequent viral transmission and infection of $CD4^+$ T cells. The effect of FGM in the transmission assay can be explained also by efficiently inhibiting the trans-infection of HIV to the T cells by interfering with the gp120 attachment on the cellular CD4 receptor and this further strengthens its potential as microbicide candidate (Wu and KewalRamani, 2006). Despite a lot of intensive research and several clinical trials, the ideal and reasonable priced microbicide still has to be developed. Manufacturing T20 is a very complicated process of more than 100 steps, which is reflected in a high production price (Bray, 2003). For FGM, it is possible to use the bacterial producer in a fermentative process for large-scale production. Likewise, fermentative processes are widespread in the synthesis of anti-biotics, e.g., tetracycline, erythromycin, vancomycin and penicillin are all natural products that are gained from fermentative processes (Elander, 2003).

It is an important approach that especially women can prevent themselves from HIV infection by using an effective vaginal/rectal microbicide gel. An important feature for further microbicide application and development is the observed consistent anti-HIV activity of FGM regardless of viral input (Table 3). No significant decrease in activity was noticed when the viral input was even up to 50-fold higher, while the gp41 fusion inhibitor T20 and the carbohydrate-binding gp120 mAb 2G12 lost completely their activity with higher viral input. This shows that FGM interacts completely different with the viral envelope protein compared to other described entry inhibitors. The lack in the titration of FGM inhibition could suggest a non-saturable effect.

In conclusion, FGM is to our knowledge the only peptide described so far that inhibits the gp120/CD4 interaction, while many other peptide entry inhibitors (e.g., siamycin, T20, VIRIP and CBAs) do not. The results of the alanine scan indicate that FGM might also serve as a lead-structure for further development of even smaller inhibitory peptides against HIV.

Materials and methods

Test compounds/peptides

Feglymycin (FGM) and its alanine derivatives (ala-FGM) were synthesized as described previously (Dettner et al., 2009). The CXCR4 antagonist AMD3100 was obtained from Dr. G. Bridger (at that time at AnorMED, Langley, Canada). Enfuvirtide (T20) was a kind gift from Dr. E. Van Wijngaerden (UZ Leuven, Belgium). Nevirapine and ritonavir were ordered from Boehringer Ingelheim GmbH (Germany) and Abbot (USA), respectively. Tenofovir was a gift from Dr. A. Holý (Prague, Czech Republic). The polyanionic compound PRO2000 was obtained from Dr. A. Profy (Indevus Pharmaceuticals Inc., Lexington, MA, USA) and the dextran sulfates MW 5000 and 8000 were ordered from Sigma-Aldrich (Bornem, Belgium). HHA was ordered from E.Y. Laboratories (San Mateo, CA, USA). CADA was a kind gift from Dr. T.W. Bell (University of Nevada, Reno, Nevada). The anti-gp120 mAbs b12 and 2G12 were purchased by Polymun Scientific (Vienna, Austria). The anti-CD4 mAb RPA-T4 was ordered by eBioscience (San Diego, CA, USA).

Cell lines and cell cultures

MT-4 cells were obtained from Dr. L. Montagnier (at that time at the Pasteur Institute, Paris, France), while the HUT-78, SupT1 and C8166 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HUT-78 cells were infected with HIV-1 IIIB and HIV-2 ROD and cultured for 2–3 weeks to become persistently infected. Raji.DC-SIGN$^+$ cells were a gift from Dr. L. Burleigh (Pasteur Institute, Paris, France). All cell lines were cultured in RPMI-1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, Utah) and 1% L-glutamine (Invitrogen).

Peripheral blood mononuclear cells (PBMCs) were isolated out of buffy coats from healthy donors, derived from the blood transfusion Center (UZ Leuven, Belgium), by density gradient centrifugation. PBMCs were cultured in RPMI-1640 supplemented with 10% FCS, 1% L-glutamine and stimulated with 2 µg/ml PHA (Sigma-Aldrich) for 3 day at 37 °C before further use in antiviral assays as PHA-activated PBMCs (T cell blasts).

Monocyte-derived macrophages (MDM) were prepared as follows: PBMCs (2×10^6 cells/ml) were seeded in a 48-well plate (Costar 3548) in 1 ml RPMI-1640 medium with 10% FCS and incubated for 1 week at 37 °C. Afterwards, the non-adherent cells were gently mixed and removed from the adherent cell layer. The cells were carefully washed and this washing step was repeated after 6 day of incubation at 37 °C. The adherent cells were then infected with HIV-1 R5 BaL.

Viruses

X4 HIV-1 strains NL4.3 and IIIB were obtained from the NIAID AIDS reagent program (Bethesda, MD). R5 HIV-1 BaL and HIV-2 ROD were obtained from the Medical Research Council (MRC, London, UK). The dual-tropic (R5/X4) strain HE was originally isolated from a Belgian AIDS patient and later on cultured in various T cell lines (Pauwels et al., 1990). The clinical isolates were kindly provided by Dr. J. L. Lathey (then at BBI Biotech Research Laboratories, Gaitherburg, MD). Their co-receptor usage was determined by us using the astroglioma U87.CD4.CXCR4 and U87.CD4.CCR5 transfected cells.

The NL4.3 strains resistant to AMD3100, 2G12 mAb and T20 were generated earlier (Huskens et al., 2007; De Vreese et al., 1996).

Antiviral replication assays

The antiretroviral assays in MT-4 cells and PBMCs have been described in detail earlier (Vermeire et al., 2004). Briefly, MT-4 (50 µl; 1×10^6 cells/ml) or C8166 cells (50 µl; 2×10^5 cells/ml) were pre-incubated for 30 min at 37 °C with test compounds (100 µl) in a 96-well plate (Falcon). Next, virus (NL4.3, IIIB, HE, ROD) was added according to the TCID$_{50}$ of the viral stock. Cytopathic effect (CPE) was scored microscopically 5 day post-infection, and EC$_{50}$-values were determined using the MTS/PES method (Vermeire et al., 2004). PHA-stimulated PBMCs (200 µl; 5×10^5 cells/ml) were also pre-incubated for 30 min with test compounds (250 µl) in the presence of 2 ng/ml IL-2 (Roche Applied Science) and afterwards 1000 pg p24/ml virus (50 µl) was given. At days 3 and 6 post-infection, again 2 ng/ml IL-2 was added. Ten days post-infection, supernatant was collected for p24 Ag HIV-1 ELISA (Perkin Elmer, Zaventem, Belgium) according to manufacturers' guidelines. The MDM cells were seeded in a 48-well plate in 1 ml medium. After removal of 800 µl of cell culture medium (RPMI-1640 supplemented with 10% FCS and 1% L-glutamine), 250 µl of test compounds was added. Each concentration was tested in triplicate. After an incubation of 30 min at 37 °C, 1000 pg/well of HIV-1 R5 BaL was added. Three weeks post-infection, supernatant was collected and evaluated by p24 HIV-1 Ag ELISA.

Cell-cell co-cultivation assays

Various concentrations of test compounds (100 µl) were added in a 96-well plate along with SupT1 cells (1×10^5 cells/50 µl). Next, 50 µl of HUT-78 cells persistently infected with either HIV-1 IIIB (HUT-78/IIIB) or HIV-2 ROD (HUT-78/ROD) were seeded at the same amount. Syncytia formation was scored microscopically 20–24 h post-co-cultivation at 37 °C.

Time-of-drug addition studies

MT-4 cells (1×10^6 cells/ml) were infected with HIV-1 IIIB at a multiplicity of infection (MOI) of 0.5. Test compounds were added at different time points in a range from 0 h to 26 h post-infection. After 31 h, virus production was measured using p24 HIV-1 Ag ELISA. The reference compounds were added at 100 times their EC$_{50}$, as obtained in MT-4 cells by the MTS/PES assay.

HIV-1 capture by Raji.DC-SIGN$^+$ cells and subsequent transmission to uninfected CD4$^+$ target T cells

Raji.DC-SIGN$^+$ cells (200 µl; 2.5×10^6 cells/ml) were added to a 30 min pre-incubated mixture at 37 °C of 100 µl HIV-1 HE

($\sim 3.1 \times 10^6$ pg p24/ml) and 200 μl medium or various concentrations of test compounds. After 2 h incubation at 37 °C, samples were extensively washed with PBS and lyzed with triton-X100/PBS buffer for p24 Ag detection by p24 HIV-1 Ag ELISA. Raji.DC-SIGN$^+$ cells were exposed to HIV-1 HE for 1 h at 37 °C. Meanwhile, 100 μl of various concentrations of test compounds were added in a 96-well plate and pre-incubated with C8166 target T cells (50 μl; 2×10^6 cells/ml). Unbound virus was removed by extensive washing and the same amount of Raji.DC-SIGN$^+$ cells were added to the 96-well plate, already containing the C8166 cells. After 24 h, giant cell formation was scored microscopically.

Soluble CD4/HIV-1 binding ELISA

Human soluble CD4 (sCD4; ImmunoDiagnostics, Inc., Woburn, MA, USA) (25 ng/ml) was coated on 96-well Maxi-Sorp plates (Fisher Scientific, Erembodegem, Belgium) using freshly prepared 50 mM carbonate buffer pH 9.6 for 1 h at 37 °C. Subsequently, PBST (phosphate buffered saline, pH 7.4 with 0.05% Tween-20) containing 2% milk powder was used overnight at 4 °C to block the wells. The next day, HIV-1 NL4.3 was pre-treated with 500 μM aldrithiol-2 (Sigma-Aldrich) for 1 h at 37 °C. The coated plates were washed 3 times with PBST before various concentrations of test compounds and virus (5×10^4 pg p24/ml) were added. After 1 h incubation at 37 °C, the plates were extensively washed with PBST and incubated for 1 h at room temperature with D7324, a primary sheep anti-gp120 antibody (Aalto Bio Reagents, Dublin, Ireland). After washing, a secondary alkaline phosphatase-labeled donkey-anti-sheep antibody (DaS-AP; Sigma-Aldrich) was incubated under the same conditions. Color development occurred after washing and substrate solution addition (SigmaFAST^{TM} p-nitrophenyl-phosphate (PNPP; Sigma-Aldrich)). Absorbance was determined at 405/620 nm using the Safire 2 plate reader (Tecan, Männedorf, Switzerland).

Surface plasmon resonance (SPR) analysis

Recombinant gp120 proteins from X4 HIV-1 (IIIB) and R5 HIV-1 (YU2) (ImmunoDiagnostics Inc., Woburn, MA) were covalently immobilized on the carboxymethylated dextran matrix of a CM5 sensor chip in 10 mM sodium acetate, pH 4.0, using standard amine coupling chemistry. The exact chip densities are given in the Results section. A reference flow cell was used as a control for non-specific binding and refractive index changes. All interaction studies were performed at 25 °C on a Biacore T200 instrument (GE Healthcare, Uppsala, Sweden). The test agents were serially diluted in HBS-P (10 mM HEPES, 150 mM NaCl and 0.05% surfactant P20; pH 7.4), supplemented with 10 mM Ca^{2+}, covering a concentration range between 62.5 nM and 500 nM by using two-fold dilution steps. Samples were injected for 2 min at a flow rate of 45 μl/min and the dissociation was followed for 5 min. One duplicate sample and several buffer blanks were used as a positive control and for double referencing, respectively. The CM5 sensor chip surface was regenerated with 1 injection of 50 mM NaOH. The experimental data were fit using the 1:1 binding model (Biacore T200 Evaluation software 1.0) to determine the binding kinetics.

HIV-1 NL4.3 binding to CD4$^+$ SupT1 cells

First, 200 μl of test compounds were diluted in a 15 ml polypropylene tube (Falcon). Subsequently, CD4$^+$ SupT1 cells (2.5×10^6 cells/ml) were added at an equal volume. Then, high amounts of HIV-1 NL4.3 stock (100 μl; $\sim 2.8 \times 10^6$ pg p24/ml) was given and incubated at room temperature for 2 h. After extensive washing with PBS/FCS2% (PBS supplemented with 2% FCS), the samples were incubated with 200 μl anti-gp120 mAb (NEA-9205,

NEN, Boston, MA) (500 ng/ml 9205 diluted in PBS/FCS2%) solution for 30 min at 4 °C. A secondary goat-anti-mouse PE labeled (GaM-PE; Invitrogen) antibody (1/100 diluted) was added after washing and incubated for 30 min at 4 °C. As a control for aspecific background staining, the cells were stained with GaM-PE only. Finally, the samples were washed and fixed with 1% p-formaldehyde solution and virus binding was determined by flow cytometry (FACSCalibur, BD Biosciences) and analyzed using CellQuest (BD Biosciences) and Flowjo software (Tree Star, San Carlos, CA). Virus binding is expressed in mean fluorescence intensity (MFI) values. Percentage inhibition was determined after subtracting the MFI of the background staining.

Selection of FGM resistant HIV-1 IIIB (IIIB^{FGMres})

HIV-1 IIIB was added to C8166 cells (2.5×10^5 cells/ml) in 24-well plates in the presence of 0.5 μM of FGM. Every 5 day, viral replication was scored microscopically and when full CPE was observed, replicated virus was passaged to uninfected C8166 T cells in the presence of increasing FGM concentrations. After 44 passages, virus (HIV-1 IIIB^{FGMres}) was collected that was able to grow in the presence of 50 μg/ml (or 26.3 μM) FGM.

Genotyping of the HIV-1 env region

Viral RNA was extracted from cell culture supernatants using QIAamp Viral RNA minikit (Qiagen, Hilden, Germany). The genotyping of the gp120 and gp41 genes were determined as described earlier (Van Laethem et al., 2005).

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