Effect of human cathelicidin and fragments on HIV-1 enzymes

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Key Messages

1. Cathelicidins are small cationic antimicrobial peptides. Cathelicidin LL-37 and its fragments inhibit HIV replication. Whether there is any inhibitory effect on enzymes essential to the HIV life cycle is not known. Therefore, human cathelicidin LL-37 and its fragments were investigated for their ability to inhibit HIV reverse transcriptase, protease, and integrase.
2. Human cathelicidin LL-37 and its fragments LL13-37 and FK-16 inhibited HIV-1 reverse transcriptase dose-dependently, with respective IC_{50} values of 15, 7, and 70 μM.
3. The three peptides inhibited HIV-1 protease with weak potency, achieving 20 to 30% inhibition at 100 μM. The mechanism of inhibition was a protein-protein interaction as revealed by surface plasmon resonance.
4. The peptides did not inhibit translocation of HIV-1 integrase, labelled with green fluorescent protein, into the nucleus.
5. The peptides were not toxic to human peripheral blood mononuclear cells.

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Introduction

Multicellular organisms produce antimicrobial peptides for defence against microorganisms. One of these defence peptides is cathelicidin.[1] Cathelicidins are small cationic peptides widely distributed in tissues and bodily fluids, and were first found in bovine neutrophils.[2] They display antimicrobial anti-inflammatory and immunomodulatory activities.

Only an 18-kDa cathelicidin (hCAP18) is found in humans. Serine proteases catalyse the cleavage of hCAP18 to produce a cathelin domain and an antimicrobial peptide LL-37, which has diverse chemostatic activities towards monocytes, T cells, neutrophils, and mast cells. It stimulates interleukin-8 secretion, mast cell release of histamine, angiogenesis, and wound healing, while exerting no toxicity on lung epithelial cells when tested up to 111 μM.

Novel therapeutic agents that prevent the transmission of HIV are needed. In mammals, defensins and cathelicidins are the two main types of antimicrobial peptides. Among cathelicidins, LL-37, protegrin-1, and indolicidin have anti-HIV activity. LL-37 is the only cathelicidin found in humans and can be cleaved in vivo into active fragments that are detected in human skin and sweat.

Human cathelicidin LL-37 and its fragments inhibit HIV-1 replication.[3] However, it is not known whether it and its fragments also inhibit enzymes essential to the life cycle of the HIV (HIV reverse transcriptase, HIV protease, and HIV integrase). We therefore investigated the human cathelicidin LL-37 and its fragments to determine their ability to inhibit HIV reverse transcriptase, protease, and integrase. Representative examples of at least three experiments are reported.

Methods

This study was conducted from November 2009 to January 2011. LL-37 and its fragments LL13-37 and FK-16 were synthesised by the solid-phase method using Fmoc-chemistry. HIV reverse transcriptase activity was conducted using a commercially available ELISA kit (Boehringer).

For HIV integrase inhibitory activity, 24 hours before transfection, cultured HeLaTet-Off Advanced cells were seeded onto a culture dish in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum. The EGFP-C-IN expression vector was transfected into HeLaTet-Off Advanced cells using Lipofectamine 2000 reagent. The medium was removed 5 hours after transfection. Fresh medium containing the test compound was added at a final concentration of 10 μM DMSO was used as control. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes. Transfection results were analysed with a confocal microscope.

For HIV protease inhibitory activity, HIV-1 protease cDNA was cloned in pET3b, and transformed into *Escherichia coli* BL21(DE3)pLysS. HIV-1 protease expression was induced by IPTG. The expressed proteins (found predominantly as inclusion bodies) were analysed by SDS-PAGE. Bacterial

colonies which expressed a high level of 11 kDa HIV-1 protease were chosen for preparation of large-scale cultures. HIV-1 protease was purified from cultures. HIV-1 protease activity was assayed by cleavage of a fluorogenic substrate Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg. After incubation at 37ºC for 2 hours, the fluorescence intensity in each well was measured with an excitation wavelength at 340 nm and an emission wavelength at 490 nm.

A BIAcore 3000 surface plasmon resonance biosensor was used to measure the kinetic parameters of the interaction. Cathelicidin or its fragment (1nM) was covalently linked to the dextran on the surface of a CM5 sensor chip via primary amino groups using the Amine Coupling Kit (Pharmacia) at a flow rate of 5 µL/min, 25ºC. A range of 0 to 240 nM of HIV-1 reverse transcriptase in PBS was injected at a flow rate of 5 µL/min, at 25ºC, onto the cathelicidin or its fragments immobilised on the sensor chip surface. The binding surface was regenerated by 2M NaCl between sample injections. A control experiment was carried out similarly on an uncoupled sensor chip surface.

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy donors by density gradient using Ficoll-Paque Plus PBMCs ($1x10^5$) that were incubated with LL-37, LL 13-37, and FK-16 at 37ºC for 24 hours. Then, a [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] solution was added and the plates were incubated for further 4 hours and then centrifuged. The supernatant was removed and dimethyl sulfoxide added to dissolve the formazan at the bottom of the wells. Ten minutes later, absorbance at 590 nm was determined.

Results

After exposure for 24 hours at 37ºC to various concentrations (0.98-125 µM) of LL-37, LL13-37, and FK-16, the viability of human PBMCs fluctuated from >80% to 100%, indicating minimal toxicity.

All three peptides exhibited a dose-dependent suppressive action on HIV-1 reverse transcriptase activity as disclosed by ELISA. The fragment LL13-37 was slightly more potent (IC_{50}=7 µM) than LL-37 (IC_{50}=15 µM). The fragment FK-16 was less potent (IC_{50}=70 µM) than LL-37 and LL13-37.

The actions of the three peptides on HIV-1 protease are shown in Figure 1. The positive control pepstatin exerted a potent inhibitory action, achieving around 60% inhibition at a concentration of 10 µM. In contrast, the inhibition elicited by LL-37 (about 22%), LL13-37 (about 30%), and FK-16 (about 30%), all at 100 µM, was much less marked.

The positive control compound X (a proprietary ring compound isolated from a plant) was capable of inhibiting the translocation of HIV-1 integrase, labelled with green fluorescent protein, from the cytoplasm to the nucleus, because green fluorescence associated with the integrase was located outside the nucleus. The nucleus was stained red by propidium iodide. A merger showed the distinct separation of the red and green colours (data not shown).

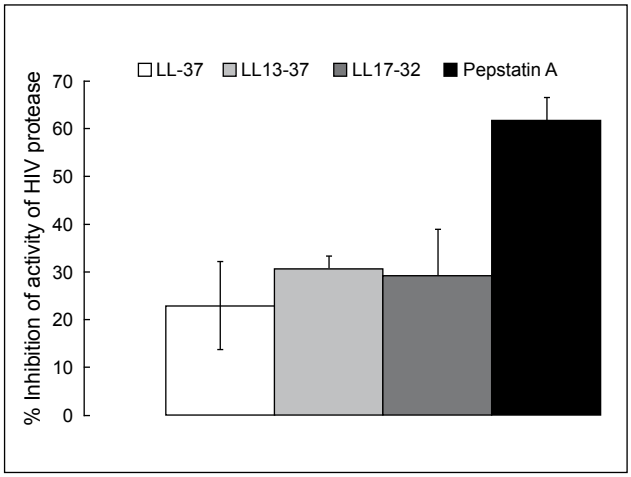

Fig 1. Inhibitory effect of human cathelicidin LL-37 (100 µM), and its fragments LL 13-37 (100 µM), 17-32 (100 µM), and positive control Pepstatin A (10 µM) on HIV-1 protease

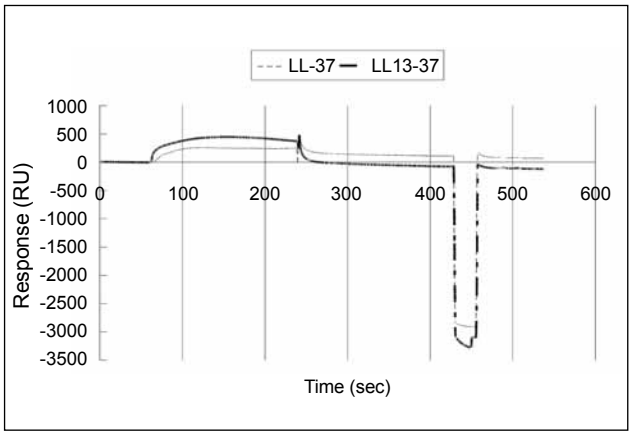

Fig 2. Assay of binding between HIV-1 protease (1 µM) and LL-37/LL13-37 (1 µM) using surface plasmon resonance (Biacore). 1 µg was immobilised on the surface of a CM5 sensor chip. HIV-1 protease was injected at a flow rate of 5 µL/min, starting at 60 seconds. The signals observed from 60 to 240 seconds represented protein-protein interaction between LL-37 and HIV-1 integrase. Washing with buffer from 240 to 420 seconds resulted in baseline response. Regeneration by washing with 2M NaCl resulted in a negative peak which appeared midway between 400 and 500 seconds. The results indicated protein-protein interaction between HIV-1 integrase and LL-37/LL13-37

After exposure to LL-37, LL13-37, and FK-16, all at 50 μM concentration, the distribution of green fluorescence was similar to that in the control (data not shown). Green fluorescence revealing the location of HIV-1 integrase was detected in the nucleus, indicating that LL-37, LL13-37, and FK-16 lacked the ability to inhibit the translocation of HIV-1 integrase from the cytoplasm into the nucleus. Thus, it appears that among the three key HIV-1 enzymes, LL-37 and its fragments exhibited the most prominent inhibitory effects on HIV-1 reverse transcriptase; inhibition on HIV-1 protease was less marked while there was no inhibition of the translocation of HIV-1 integrase into the nucleus.

In the test of interaction between HIV-1 protease and LL-37/LL13-37 using surface plasmon resonance, the positive response recorded between 60 and 240 seconds indicated a protein-protein interaction between HIV-protease and LL-37/LL13-37 (Fig 2). Hence, this interaction contributed to the inhibitory effect of LL-37 and LL 13-37 on the activity of HIV-1 protease.

Discussion

The antiviral activity of cathelicidins is well established. Human cathelicidin LL-37 and porcine cathelicidin protegrin-1 decrease lentiviral and retroviral vector infectivity. LL-37 lowers herpes simplex virus type 1 and adenovirus 19 titres. Corneal and conjunctival epithelial cells express LL-37 to protect against viral and bacterial ocular infections. LL-37 has been demonstrated to be virucidal and attacks the vaccinia viral envelop.

Cathelicidin is associated with HIV. Cervicovaginal secretions from Kenyan sex workers with sexually transmitted bacterial infections had heightened levels of LL-37, which were linked to escalated HIV acquisition.[4]

Human cathelicidin is expressed in human epididymal epithelium. It is detected in seminal plasma, and attached to sperm, indicating a role in fertilisation and in the antibacterial integrity of the male reproductive tract. Thus LL-37 may constitute a mechanism for post-coital protection against infection.

The ability to inhibit translocation of HIV-1 integrase from the cytoplasm to the nucleus has greater significance than inhibitory activity on the enzyme in view of the nuclear site of action of the enzyme. Neither cathelicidin LL-37 nor its fragments could inhibit integrase translocation. In the same assay a natural product, designated as compound X, was capable of impeding the process of integrase translocation.

LL-37 and its fragments produce the greatest inhibitory effect on HIV-1 reverse transcriptase. The peptides reduce the activity of HIV-1 protease but the potency of inhibition is low compared to that of the aspartyl protease inhibitor pepstatin. The mechanisms of inhibition of HIV enzymes by homologous enzymes and natural products have been elucidated. HIV-1 protease lowers HIV-1 reverse transcriptase activity by the protein-protein interaction. Polysaccharopeptide from the medicinal mushroom *Coriolus* versicolor reduces the activity of HIV-1 reverse transcriptase by means of a mixed competitive and non-competitive mechanism.

Although the ranking of HIV-1 reverse transcriptase inhibitory activity is LL13-37>LL-37>FK-16, the ranking of HIV-1 protease inhibiting activity is LL13-37≈FK-16>LL-37. LL13-37 is more potent than LL-37 in inhibiting HIV-1 reverse transcriptase and protease. It can be deduced that the first 12 N-terminal amino acid residues in LL-37 do not contribute to the inhibitory activity of LL-37 on the two HIV-1 enzymes. This is noteworthy, because LL13-37 is shorter and its chemical synthesis is easier. In contrast, LL13-32 displays a weaker HIV-1 reverse transcriptase inhibitory potency than LL-37 and higher HIV-1 protease inhibitory activity than LL-37. Thus, peptide fragments 14-16 and 33-36 have some contribution to the HIV-1 reverse transcriptase inhibitory activity of LL-37, but do not play a crucial role in its HIV-1 protease inhibitory activity. Thus, LL13-37 seems to be better than FK-16 in its potential applicability. It remains to be elucidated whether the same ranking of potency applies to the inhibitory effect of these cathelicidin peptides on HIV. The mechanism of inhibition of HIV-1 protease by LL-37 and LL13-37 has been demonstrated by surface plasmon resonance to be a protein-protein interaction. It is highly likely that an analogous mechanism of inhibition operates with regard to HIV-1 reverse transcriptase. None of the three cathelicidin peptides tested could inhibit nuclear translocation of HIV-1 integrase. The differential activity of cathelicidin peptides towards the three key HIV-1 enzymes is reminiscent of similar observations on some antifungal proteins and milk proteins.

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