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Anti-Human Immunodeficiency Virus Type 1 Activities of Antimicrobial Peptides Derived from Human and Bovine Cathelicidins▽

Guangshun Wang,[1]* Karen M. Watson,[2] and Robert W. Buckheit, Jr.[2]

The Structure-Fun Laboratory, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, Nebraska 68198,[1] and ImQuest BioSciences, Inc., 7340 Executive Way, Suite R, Frederick, Maryland 21704[2]

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From among 15 human cathelicidin LL-37-derived peptides, FK-13 was identified as the smallest peptide active against human immunodeficiency virus (HIV) and GI-20 had the highest therapeutic index, which was twice that of LL-37. BMAP-18, which is derived from bovine cathelicidin BMAP-27, possessed a therapeutic index similar to that of GI-20. Peptide sequence order, helical structures, and aromatic residues are important in HIV inhibition.

AIDS has become the fourth leading cause of death worldwide, and the majority of human immunodeficiency virus (HIV) infections are acquired through heterosexual intercourse. The United Nations estimates that there are now 40 million people living with HIV infection or AIDS. Thus, it is urgent that novel therapeutic and preventative agents, including topical microbicides that block the sexual transmission of HIV, be developed (5, 26). Antimicrobial peptides are ancient and potent host defense molecules in nearly all forms of life (3, 12, 37). More than 870 such peptides have been registered in the updated antimicrobial peptide database (http://aps.unmc.edu/AP/main.html) (33). However, only a few have been subjected to antiviral assays (8, 12). Known examples are brevinin-1 (35), caerin 1.1, caerin 1.9, and maculatin 1.1 (27), dermaseptin S4 (15), esculentin 2P and ranatuerin 2P (7), and the magainins (17) from amphibians and cecropin A and melittin from insects (28). In mammals, defensins and cathelicidins are the two major classes of antimicrobial peptides. While the three types of defensins, the α-, β-, and θ-defensins, contain several β strands, which are further stabilized by three pairs of disulfide bonds, the cathelicidins vary in both their sequences and their three-dimensional structures (usually extended or α-helical structures). Another important difference is that there are at least 10 different defensins in humans, but only one cathelicidin (LL-37) has been identified (29, 36). All human α-defensins and human β-defensin-3 inhibit HIV infection (11), but the θ-defensins are more effective (9, 10, 18, 31, 32). Cathelicidins have been shown to have effects on bacteria, fungi, and viruses (36). Among them, LL-37 (2), protegrin-1 (25), and indolicidin (16, 23) have been demonstrated to have anti-HIV activities. As the only cathelicidin in humans, LL-37 can be cleaved in vivo into active fragments. While KS-30, KR-20, and RK-31 were identified in human sweat, LL-23, KS-27, and LL-29 were detected in human skin (19, 34). Furthermore, several laboratories have identified active fragments within LL-37 by synthesizing peptides corresponding to different regions (4, 20, 21, 29). Using nuclear magnetic resonance spectroscopy, we previously identified a minimally antimicrobial and anticancer region corresponding to residues 17 to 29 (FK-13 in Table 1) (13). Here, we report on the anti-HIV activities of 20 synthetic peptides (>95% purity; Genemed Synthesis, Inc.) derived from human and bovine cathelicidins.

Inhibition assays for determination of the anti-HIV cytopathic effect were conducted as described previously (6). Briefly, serially diluted peptides were added to a 96-well round-bottom microtiter plate in triplicate. CEM-SS cells at a concentration of 2.5×10^3 cells per well and HIV type 1_{IIIB} (HIV-1_{IIIB}) at the appropriate predetermined titer were sequentially added to the microtiter plate. The cultures were incubated at 5% CO_2 and 37°C for 6 days. Following the incubation, the microtiter plates were stained with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide dye to evaluate the efficacy and toxicity of the test compound(s). By using the Microsoft Excel program, the 50% effective concentration for inhibition of virus replication (EC_{50}), the concentration that reduced cell viability by 50% (TC_{50}), and a therapeutic index (TI; which is equal to TC_{50}/EC_{50}) were obtained. Consistent with the findings of Bergman et al. (2), we found that synthetic LL-37 is active against HIV-1_{IIIB} (Table 1). However, LL-23 and KR-20, the naturally occurring N- and C-terminal fragments of LL-37, respectively, showed no effect on the virus even at a high peptide concentration of 100 μg/ml (in μM in Table 1), suggesting that the HIV-active region of LL-37 is located in the middle region. Indeed, SK-21, which is composed of LL-37 with 8 residues truncated from each end, was active. We predict that naturally occurring LL-37 fragments, such as KS-27, LL-29, KS-30, and RK-31, inhibit HIV, as they all contain the sequence of SK-21. Inactive terminal fragments LL-23 and KR-20 were apparently not produced to protect humans from HIV or retrovirus infection, but they are bactericidal (19, 34).

* Corresponding author. Mailing address: Eppley Cancer Institute, Room ECI 3018, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805. Phone: (402) 559-4176. Fax: (402) 559-4651. E-mail: gwang@unmc.edu.

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TABLE 1. Anti-HIV activities of human cathelicidin LL-37-derived peptides in CEM-SS cells[a]

Peptide name	Peptide sequence[b]	EC_{50} (μM)	TC_{50} (μM)	TI
LL-37	LLGDLLRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	1.6	18.4	11.5
LL-23	LLGDLLRKSKEKIGKEFKRIVQR	>35.4	>35.4	
KR-20	KRIVQRIKDFLRNLVPRTES	>40.5	>40.5	
SK-21	SKEKIGKEFKRIVQRIKDFLR	10.8	22.5	2.08
FK-13	FKRIVQRIKDFLR	3.4	10.4	3.1
Retro-FK-13	RLFDKIRQVIRKF	>58.1	33.7	
KR-12	KRIVQRIKDFLR	>63.5	>63.5	
GF-17	GFKRIVQRIKDFLRNLV	0.98	8.9	9.1
$GF\text{-}17_{d1}$[c]	GFKRIVQR**I**KDFLRNLV	>47.5	22.7	
$GF\text{-}17_{d2}$[c]	GFKRIVQR**I**KDF**L**RNLV	>47.5	>47.5	
GI-20	GIKEFKRIVQRIKDFLRNLV	1.08	22.7	21
$GI\text{-}20_{X17}$	GIKE**X**KRIVQRIKDFLRNLV	>40.6	7.3	
$GI\text{-}20_{W17}$	GIKE**W**KRIVQRIKDFLRNLV	7.4	23.6	3.2
$GI\text{-}20_{Q16}$	GIK**Q**FKRIVQRIKDFLRNLV	0.91	13.7	15.1
$GI\text{-}20_{EF}$	GIKEFKR**E**F**Q**RIKDFLRNLV	1.6	9.9	6.2

[a] Although the standard errors from multiple antiviral assays are not provided, they were, on average, less than 10% of the respective mean EC_{50} or TC_{50}.
[b] In the sequence of LL-37, the region used to engineer an optimal anti-HIV peptide (GI-20) is underlined. For all sequences, mutated residues are in boldface, and X represents phenylglycine.
[c] The incorporation of D-amino acids is indicated by d followed by the number of D-amino acids (in boldface).

Our previously identified 13-residue antibacterial peptide FK-13 (13) also displayed an effect on HIV (Table 1). When F-17 (as numbered in LL-37; Table 1) was removed, the resultant KR-12 peptide lost its activity, indicating that F-17 is essential and that FK-13 represents the minimal anti-HIV region of human LL-37. Furthermore, reversal of the FK-13 sequence (retro-FK-13) led to a peptide that was inactive against HIV, although the peptide retained its antibacterial activity (14). Thus, there is no correlation between the antibacterial and the anti-HIV activities of these peptides. To obtain peptide templates with improved TIs, additional peptides were designed on the basis of the sequence of FK-13. GF-17 (i.e., FK-13 plus the NLV segment) had a TI slightly less than that of LL-37, whereas GI-20 (30), obtained by adding GIKE to the N terminus of GF-17, had a TI twice that of LL-37. We found that the incorporation of D-amino acids into GF-17 at position 24 ($GF\text{-}17_{d1}$) or both position 24 and position 28 ($GF\text{-}17_{d2}$) disrupted the activity of the peptide, but the cytotoxicity of the peptide to human cells decreased with an increase in the number of D-amino acids (Table 1). Since D-amino acid incorporation tends to reduce helicity (13, 22), the putative helical structure of GF-17 might be important for anti-HIV activity. In the case of GI-20, a change of F-17 to phenylglycine ($GI\text{-}20_{X17}$ in Table 1) led to the loss of activity. Likewise, a change of F-17 to W ($GI\text{-}20_{W17}$ in Table 1) also reduced the anti-HIV activity of the peptide. These results further substantiated the essential role of F-17 of LL-37 in inhibiting HIV infection. Interestingly, when E-16 of GI-20 was replaced by Q-16 ($GI\text{-}20_{Q16}$ in Table 1), the peptide became more toxic to human cells without any change in the anti-HIV activity observed. Thus, E-16 endowed selectivity to the peptide. Since F-17 is critical for the anti-HIV effect and E-16 modulates selectivity, we also tested the effects of this amino acid pair by substituting I19V20 for E19F20 ($GI\text{-}20_{EF}$ in Table 1). While the antiviral efficacy of the resulting peptide was reduced only slightly, cellular toxicity increased twofold relative to that of GI-20. Clearly, the effect of F overrode the effect of E in this case. Hence, both the anti-HIV efficacy and the cellular toxicity of GI-20 are subjected to modulation, laying the basis for peptide engineering.

To obtain additional peptide templates, we also evaluated the anti-HIV activity of a few peptides (Table 2) based on BMAP-27. BMAP-27 is a 27-residue bovine cathelicidin peptide with the potential to form an α-helical conformation followed by a hydrophobic tail (24). To identify the region within BMAP-27 active against HIV, we first deleted the hydrophobic tail to obtain BMAP-18. BMAP-18 was found to be more active against HIV than GI-20, but it was also more toxic to human cells than GI-20, although the overall TI of BMAP-18 was slightly better (Table 2). Further deletion of the three residues from the C terminus of BMAP-18 disrupted both the antiviral and the cytotoxic effects of the resulting peptide (BMAP-15). Subsequent mutational studies revealed that changing F-6 and F-10 of BMAP-18 to phenylglycines slightly reduced the anti-HIV activity of BMAP-18. The peptide became inactive when the two phenylalanines were changed to

TABLE 2. Anti-HIV activities of bovine cathelicidin BMAP-27-derived peptides in CEM-SS cells[a]

Peptide name	Peptide sequence[b]	EC_{50} (μM)	TC_{50} (μM)	TI
BMAP-18	GRFKRFRKKFKKLFKKIS	0.35	8.45	24.1
$BMAP\text{-}18_{P9}$	GRFKRFRK**P**FKKLFKKIS	3.20	18.9	5.9
$BMAP\text{-}18_{X6X10}$	GRFKR**X**RKK**X**KKLFKKIS	0.68	10.2	15
$BMAP\text{-}18_{I6L10}$	GRFKR**I**RKK**L**KKLFKKIS	>44.0	2.79	
BMAP-15	GRFKRFRKKFKKLFK	>49.6	>49.6	

[a] Although the standard errors from multiple antiviral assays are not provided, they were, on average, less than 10% of the respective mean EC_{50} or TC_{50}.
[b] Mutated residues are in boldface, and X represents phenylglycine.

isoleucine/leucine residues, suggesting that the aromatic rings of BMAP-18 are critical for its anti-HIV activity. Also, the antiviral effect was decreased when K-9 of BMAP-18 was replaced with a proline residue. The introduction of a proline (1, 10) could distort the helical structure of the peptide (24), which might also be critical for anti-HIV activity.

In conclusion, our evaluation of 20 synthetic peptides derived from LL-37 and BMAP-27 led to the identification of the most important regions in both human and bovine cathelicidins active against HIV. Peptide sequence order, aromatic phenylalanine residues, and potential helical structures were found to play important roles in blocking HIV-1 infection. Because GI-20 and BMAP-18 have TIs superior to the TI of LL-37, they may now be used as templates for the engineering of novel anti-HIV microbicides.

REFERENCES

1. **Barlow, D. J., and J. M. Thornton.** 1988. Helix geometry in proteins. J. Mol. Biol. **201:**601–619.
2. **Bergman, P., L. Walter-Jallow, K. Broliden, B. Agerberth, and J. Soderlund.** 2007. The antimicrobial peptide LL-37 inhibits HIV-1 replication. Curr. HIV Res. **5:**410–415.
3. **Boman, H. G.** 2003. Antibacterial peptides: basic facts and emerging concepts. J. Intern. Med. **254:**197–215.
4. **Braff, M. H., M. A. Hawkins, A. Di Nardo, B. Lopez-Garcia, M. D. Howell, C. Wong, K. Lin, J. E. Streib, R. Dorschner, D. Y. Leung, and R. L. Gallo.** 2005. Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. J. Immunol. **174:**4271–4278.
5. **Buckheit, R. W., Jr.** 2001. Non-nucleoside reverse transcriptase inhibitors: perspectives on novel therapeutic compounds and strategies for the treatment of HIV infection. Expert Opin. Investig. Drugs **10:**1423–1442.
6. **Buckheit, R. W., Jr., T. L. Kinjerski, V. Fliakas-Boltz, J. D. Russell, T. L. Stup, L. A. Pallansch, W. G. Brouwer, D. C. Dao, W. A. Harrison, R. J. Schultz, et al.** 1995. Structure-activity and cross-resistance evaluations of a series of human immunodeficiency virus type-1-specific compounds related to oxathiin carboxanilide. Antimicrob. Agents Chemother. **39:**2718–2727.
7. **Chinchar, V. G., J. Wang, G. Murti, et al.** 2001. Inactivation of frog virus 3 and channel catfish virus by esculentin-2P and ranatuerin-2P, two antimicrobial peptides isolated from frog skin. Virology **288:**351–357.
8. **Cole, A. M.** 2005. Antimicrobial peptide microbicides targeting HIV. Protein Pept. Lett. **12:**41–47.
9. **Cole, A. M., T. Hong, L. M. Boo, T. Nguyen, et al.** 2002. Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proc. Natl. Acad. Sci. USA **99:**1813–1818.
10. **Cordes, F. S., J. N. Bright, and M. S. Sansom.** 2002. Proline-induced distortions of transmembrane helices. J. Mol. Biol. **323:**951–960.
11. **Hazrati, E., B. Galen, W. Lu, W. Wang, Y. Ouyang, M. J. Keller, R. I. Lehrer, and B. C. Herold.** 2006. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. J. Immunol. **177:**8658–8666.
12. **Jenssen, H., P. Hamill, and R. E. Hancock.** 2006. Peptide antimicrobial agents. Clin. Microbiol. Rev. **19:**491–511.
13. **Li, X., Y. Li, H. Han, D. Miller, and G. Wang.** 2006. Solution structures of human LL-37 fragments and NMR-based identification of a minimal membrane-targeting antimicrobial and anticancer region. J. Am. Chem. Soc. **128:**5776–5785.
14. **Li, X., Y. Li, A. Peterkofsky, and G. Wang.** 2006. NMR studies of aurein 1.2 analogs. Biochim. Biophys. Acta **1758:**1203–1214.
15. **Lorin, C., H. Saidi, A. Belaid, et al.** 2005. The antimicrobial peptide dermaseptin S4 inhibits HIV-1 infectivity in vitro. Virology **334:**264–275.
16. **Marchand, C., K. Krajewski, H. Lee, S. Antony, A. A. Johnson, R. Amin, P. P. Roller, M. Kvaratskhelia, and Y. Pommier.** 2006. Covalent binding of the natural antimicrobial peptide indolicidin to DNA abasic sites. Nucleic Acids Res. **34:**5157–5165.
17. **Matasnic, V. C. A., and V. Castilla.** 2004. Antiviral activity of antimicrobial cationic peptides against Junin virus and herpes simplex virus. Int. J. Antimicrob. Agents **23:**382–389.
18. **Munk, C., G. Wei, O. O. Yang, A. J. Waring, et al.** 2003. The theta-defensin, retrocyclin, inhibits HIV-1 entry. AIDS Res. Hum. Retrovir. **19:**875–881.
19. **Murakami, M., B. Lopez-Garcia, M. Braff, R. A. Dorschner, and R. L. Gallo.** 2004. Postsecretory processing generates multiple cathelicidins for enhanced topical antimicrobial defense. J. Immunol. **172:**3070–3077.
20. **Nagaoka, I., S. Hirota, F. Niyonsaba, M. Hirata, Y. Adachi, H. Tamura, S. Tanaka, and D. Heumann.** 2002. Augmentation of the lipopolysaccharide-neutralizing activities of human cathelicidin CAP18/LL-37-derived antimicrobial peptides by replacement with hydrophobic and cationic amino acid residues. Clin. Diagn. Lab. Immunol. **9:**972–982.
21. **Nell, M. J., G. S. Tjabringa, A. R. Wafelman, R. Verrijk, P. S. Hiemstra, J. W. Drijfhout, and J. J. Grote.** 2006. Development of novel LL-37 derived antimicrobial peptides with LPS and LTA neutralizing and antimicrobial activities for therapeutic application. Peptides **27:**649–660.
22. **Papo, N., and Y. Shai.** 2004. Effect of drastic sequence alteration and D-amino acid incorporation on the membrane binding behavior of lytic peptides. Biochemistry **43:**6393–6403.
23. **Robinson, W. E., B. McDougall, D. Tran, and M. E. Selsted.** 1998. Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. J. Leukoc. Biol. **63:**94–100.
24. **Skerlavaj, B., R. Gennaro, L. Bagella, L. Merluzzi, A. Risso, and M. Zanetti.** 1996. Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities. J. Biol. Chem. **271:**28375–28381.
25. **Steinstraesser, L., B. Tippler, J. Mertens, E. Lamme, H. Homann, M. Lehnhardt, O. Wildner, H. Steinau, and K. Uberla.** 2005. Inhibition of early steps in the lentiviral replication cycle by cathelicidin host defense peptides. Retrovirology **2:**1–12.
26. **Turpin, J. A.** 2002. Considerations and development of topical microbicides to inhibit the sexual transmission of HIV. Expert Opin. Investig. Drugs **11:**1077–1097.
27. **VanCompernolle, S. E., R. J. Taylor, K. Oswald-Richter, et al.** 2005. Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. J. Virol. **79:**11598–11606.
28. **Wachinger, M., A. Klenschmidt, D. Winder, et al.** 1998. Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. J. Gen. Virol. **79:**731–740.
29. **Wang, G.** 2007. Tool developments for structure-function studies of host defense peptides. Protein Pept. Lett. **14:**57–69.
30. **Wang, G.** 2008. NMR studies of a model antimicrobial peptide in the micelles of SDS, dodecylphosphocholine, or dioctanoylphosphatidylglycerol. Open Magn. Reson. J. **1:**9–15.
31. **Wang, W., A. M. Cole, et al.** 2003. Retrocyclin, an antiretroviral theta-defensin, is a lectin. J. Immunol. **170:**4708–4716.
32. **Wang, W., S. M. Owen, D. L. Rudolph, A. M. Cole, T. Hong, A. J. Waring, R. B. Lal, and R. I. Lehrer.** 2004. Activity of alpha- and theta-defensins against primary isolates of HIV-1. J. Immunol. **173:**515–520.
33. **Wang, Z., and G. Wang.** 2004. APD: the antimicrobial peptide database. Nucleic Acids Res. **32:**D590–D592.
34. **Yamasaki, K., J. Schauber, A. Coda, H. Lin, R. A. Dorschner, N. M. Schechter, C. Bonnart, P. Descargues, A. Hovnanian, and R. L. Gallo.** 2006. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. FASEB J. **20:**2068–2080.
35. **Yasin, B., M. Pang, J. S. Turner, et al.** 2000. Evaluation of the inactivation of infectious herpes simplex virus by host-defense peptides. Eur. J. Clin. Microbiol. Infect. Dis. **19:**187–194.
36. **Zanetti, M.** 2004. Cathelicidins, multifunctional peptides of the innate immunity. J. Leukoc. Biol. **75:**39–48.
37. **Zasloff, M.** 2002. Antimicrobial peptides of multicellular organisms. Nature **415:**359–365.