De Novo Design of Antiviral and Antibacterial Peptides with Varying Loop Structures

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Abstract

Although the rate of new HIV infections has been declining, AIDS continues to be one of the leading causes of death worldwide. The lack of an effective HIV vaccine makes it necessary to develop alternative strategies, such as the development of topical microbicides, to prevent transmission. Antimicrobial peptides represent promising microbicide candidates. Previously, we succeeded in enhancing the anti-HIV activities of several peptides that form helical structures based on the bioinformatic results learned from the antimicrobial peptide database. This study showed that Lys-to-Arg alterations also improved the HIV inhibitory activity of thanatin which is known to form a β-hairpin structure. Using a previously reported de novo designed HIV inhibitory peptide GLR-19 as the starting template, loop structures of varying sizes were generated by restraining a disulfide bond at different positions. The thanatin-mimicking constructs are referred to as GLRC peptides since they are composed of only four amino acid residues G, L, R, and C. While GLRC-2, the peptide with a medium-sized loop structure, was most potent against HIV-1 and HSV-2, GLRC-3, with the small loop structure, was most potent against Escherichia coli K12. Thus, the efficacy of the GLRC peptides is microbe dependent. Further terminal sequence truncation of GLRC-2 reduced antimicrobial activity against both viruses and bacteria. It appears that the high antiviral potency of GLRC-2 is related to high hydrophobicity, although a wide-range correlation is lacking. In addition, GLRC-2, which is more active against viruses, is also more resistant to the action of chymotrypsin. Therefore, GLRC-2, a novel peptide that acquired not only higher stability but also higher anti-HIV activity than the GLR-19 template, serves as the starting point for additional rounds of peptide engineering.

Keywords: Antimicrobial peptides; HIV-1; Peptide stability; Microbicides

Introduction

Since the report of the first human immunodeficiency virus (HIV) case in 1981, the acquired immunodeficiency syndrome (AIDS) has become the fourth leading cause of death worldwide. The United Nations estimates that there are now 40 million people living with HIV/AIDS. Thus, it is urgent to develop novel therapeutic and preventative agents to reduce the transmission of HIV. Because the majority of HIV infections are acquired through heterosexual intercourse, it would be helpful if measures can be taken to stop or prevent the sexual transmission of HIV [1,2].

We have been focusing on identifying potent anti-HIV candidates derived from naturally occurring antimicrobial peptides (AMPs). These peptides have been proven to have antimicrobial effects on a broad range of bacteria, viruses, fungi, and parasites [3-6]. Select amphibian and piscine AMPs are known to reduce infectivity of channel catfish virus (CCV), frog virus 3 (FV3), and HIV [7,8]. In addition, AMPs with anti-HIV activity have also been discovered from other sources, which include melittin and cecropin from insects [9], cathelicidins and defensins from mammals [5,10-13], and cyclotides (i.e. circular peptides) from plants [14]. We previously identified the most potent as well as the minimal anti-HIV fragments within human cathelicidin LL-37 [13]. A follow-up study showed that LL-37 fragments inhibited HIV reverse transcriptase in vitro [15]. It is also likely that the antiviral effect of vitamin D is associated with the expression of human LL-37 in vivo [16]. We also found that BMAP-18, a fragment derived from bovine cathelicidin BMAP-27, is a HIV-1 inhibitor (EC50 = 0.83 μg/ml) with a therapeutic index (TI) of 24 [13]. To identify additional templates, we have evaluated 30 peptides selected from the antimicrobial peptide database [17,18]. The results of that peptide screening led to the identification of 10 HIV inhibitory peptides with EC50 values in the range of 1.0 to 10 μg/ml [19]. This study extends the results of our previous studies by evaluating AMPs with different loop structures. These peptides were designed using GLR-19 as a template. GLR-19 was previously de novo designed based on the antimicrobial peptide database [17,19]. We also compared the stability of these peptide scaffolds to the action of chymotrypsin. The results of the current study are reported herein.

Materials and Methods

Peptides

All the peptides were chemically synthesized using the solid-phase method and purified by HPLC to >95% (Genemed Synthesis, Inc., TX).

Anti-HIV assays

Anti-HIV-1 cytopathic effect (CPE)-inhibition assays were performed as described [20]. In brief, 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-1IIIB at the appropriate pre-determined titer were sequentially added to the microtiter plate. The cultures were incubated at 5% CO_2/37°C for six days. Following the incubation, the microtiter plates were stained with XTT tetrazolium dye to evaluate the efficacy and toxicity of the test compound(s). Using 4-parameter curve fitting, values of EC50 (50% inhibition of virus replication), and TC_{50} (50% reduction in cell viability), as well as a therapeutic index (TI = TC_{50}/EC_{50}), were determined for each peptide.

Antibacterial assays

The antibacterial activity of the peptides was analyzed using the standard approach of microdilution [21]. The bacterial strains used were Escherichia coli K12 and Bacillus subtilis. In brief, small bacterial cultures were grown overnight. Fresh cultures were inoculated with a small aliquot of the overnight culture and incubated at 37oC until the optical density A600 of the culture reached ~0.5. The bacterial culture (90 µl each) was then diluted to an A600 of 0.001 and partitioned into a 96-well plate with ~10^5 colony-forming units (CFU) per well. The cells were then treated with 10 µl of the peptide at different concentrations, allowing the minimum inhibitory concentration (MIC) measurement for each. The assay was duplicated and repeated multiple times. The plate was then further incubated at 37oC overnight (~16 hrs) and read at 630 nm on a ChroMate 4300 microplate reader (GMI, MN).

High performance liquid chromatography (HPLC)

The retention time of the peptide was measured on a Waters HPLC system equipped with an analytical reverse-phase Waters symmetry C8 column (150 × 3.9 mm). The peptide was eluted by a gradient of acetonitrile (containing 1% TFA) from 5% to 95% at a flow rate of 1 ml/min. The peptide peak was detected by UV at 254 nm.

Electrophoresis assays of peptide stability

Peptide (0.5 mM) and chymotrypsin (molar ratio 40:1) were mixed and incubated at 37oC in 10 mM PBS buffer (pH 8.0). An aliquot (20 µl) of the reaction was taken at different hours and immediately mixed with 10 µl of SDS loading buffer to terminate the action of the protease. Aliquots of the reactions were subsequently analyzed using 18% stacking tricine SDS-PAGE. The bands were stained by Coomassie Brilliant Blue R250 (Fisher). Images of the gels were taken and digitized in Photoshop 6.0 for band quantification.
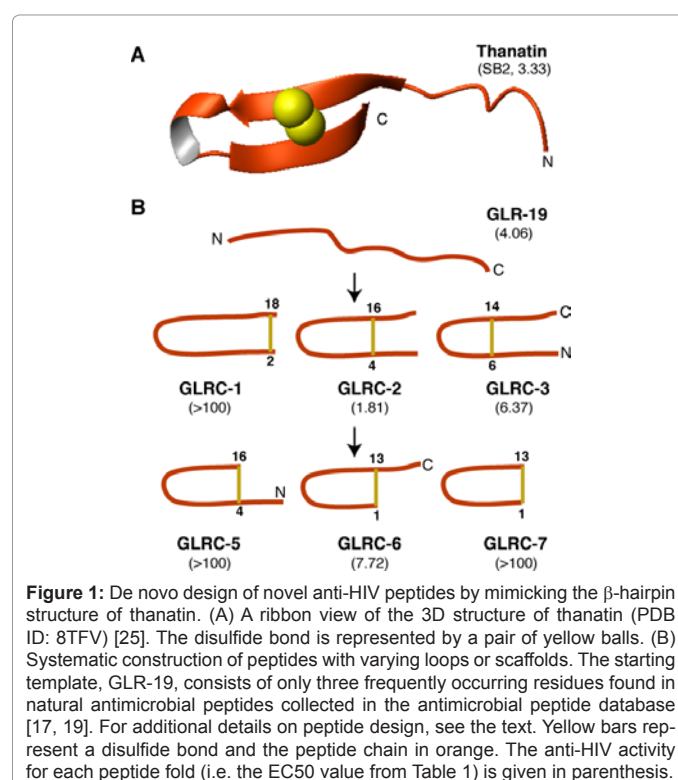
Results

Engineering of HIV inhibitory peptides based on an AMP with a b-sheet structure

Our previous studies focused on linear peptides from which several HIV inhibitory candidates have been identified. Those peptides such as the GI-20 and BMAP-18 have a tendency to form amphipathic helical structures, especially when in complex with membranes [21-23]. In this study, BAMP-18 was included as a control and it showed a similar efficacy in Table 1 as reported previously [13]. To expand the conformation space, we shifted our focus to AMPs with a µ-sheet structure. Thanatin is a natural AMP isolated from the insect Podisus maculiventris (i.e., the spined soldier bug) [24]. This peptide was chosen because it has a simple hairpin structure with a single disulfide bond (C11-C18) between the two strands (Figure 1A) [25]. Two derivatives of thanatin were obtained. In the structure-based peptide 1 (SB1), the last residue Met of thanatin was substituted with Ile to reduce possible oxidation of the peptide. This peptide demonstrated

activity against HIV-1 but poor activity against HSV-2 (Table 1). To enhance the anti-HIV activity of SB1, residues K3, K4, and K17 were converted to arginines based on previous findings from the AMP database [17,18]. SB2, the arginine analog of SB1, indeed showed higher activity against HIV-1. However, these changes deprived SB1 of the weak activity against HSV-2 (Table 1). Since there was no increase in cytotoxicity, the reduced EC50 value of SB2 directly translated into a greater TI (Table 1). The TI of SB2 reached 30, which is slightly greater than those (TI = 21-25) determined for GI-20 (derived from human LL-37), BMAP-18 (derived from cattle cathelicidin BMAP-27), and an arginine analog of dermaseptin S9 [13,19].

De novo design of peptides with various loop structures

We designed additional peptides using GLR-19 as the starting sequence. This peptide is a de novo designed peptide which is known to be inhibitory to HIV-1 [17,19]. We found that GLR-19 also showed antiviral activity against HSV-2 (Table 1). To mimic the hairpin structure of thanatin, we introduced a disulfide bond at different positions within GLR-19 (Figure 1). In each case, the incorporation of a disulfide bond into GLR-19 was at the expense of two leucines, which were replaced by cysteines. Initially, a disulfide bond (yellow lines in Figure 1) was positioned between residues 2 and 18 (C2-C18) to generate a large loop structure. This disulfide bond was shifted to generate additional peptides with a reduced loop size (Figure 1). We referred to these disulfide bond-linked peptides as GLRC peptides since their sequences are composed of only G, L, R and C amino acid residues. GLRC-1, the peptide with the large 17-residue loop, yielded an EC50 greater than 100 µg/ml, which was the highest concentration evaluated in antiviral assays [13]. GLRC-2, however, the peptide with a middle-sized 13-residue loop, demonstrated the highest potency (EC50 = 1.81 µg/ml). When the size of the loop was furthered reduced to obtain GLRC-3, antiviral activity was reduced. In parallel antiviral assays, GLRC-2 also showed the best activity against HSV-2 compared

Figure 1: De novo design of novel anti-HIV peptides by mimicking the β-hairpin structure of thanatin. (A) A ribbon view of the 3D structure of thanatin (PDB ID: 8TFV) [25]. The disulfide bond is represented by a pair of yellow balls. (B) Systematic construction of peptides with varying loops or scaffolds. The starting template, GLR-19, consists of only three frequently occurring residues found in natural antimicrobial peptides collected in the antimicrobial peptide database [17, 19]. For additional details on peptide design, see the text. Yellow bars represent a disulfide bond and the peptide chain in orange. The anti-HIV activity for each peptide fold (i.e. the EC50 value from Table 1) is given in parenthesis.

Peptide	Amino acid sequence	# of AA	S-S bond	HIV-1IIIB in CEM-SS cells			HSV-2MS in Vero cells		
				EC_{50}^a (µg/ml)	TC_{50} (µg/ml)	TI	EC_{50} (µg/ml)	TC_{50} (µg/ml)	TI
BMAP-18	GRFKRFRKK FKKLFKKIS	18		0.83	19.8	23.9	27.2	>100	>3.7
SB1	GSKKPVPIIYC NRRTGKCQRI	21	C11-C18	13.1	>100	>7.6	64.9	>100	>1.5
SB2	GSRRPVPIIYC NRRTGRCQRI	21	C11-C18	3.33	>100	>30	>100	>100	---
GLK-19	GLKKLLGKLL KKLGKLLLK	19		>47.5	25.1	<0.5	n.e.[b]	n.e.	
GLR-19	GLRRLLGRLL RRLGRLLLR	19		4.06	17.8	4.4	2.32	12.4	5.3
GLRC-1	GCRRLLGRLLRRLGRLLCR	19	C2-C18	>100	59.2	<1.0	30	>100	>3.3
GLRC-2	GLRCRLGRLLRRLGRCLLR	19	C4-C16	1.81	17.8	9.83	5.43	31.8	5.9
GLRC-3	GLRRLCGRLGRRLCRLLLR	19	C6-C14	6.37	18.5	2.9	15	62.3	4.2
GLRC-4	GCRRLCGRLGRRLCRLLCR	19	C2-C18 C6-C14	69.3	>100	>1.44	64.7	>100	>1.5
GLRC-5	GLRCRLGRLLRRLGRC	16	C4-C16	>100	59.5	<1.00	n.e.	n.e.	
GLRC-6	CRLGRLLRRLGRCLLR	16	C1-C13	7.72	41.9	5.43	n.e.	n.e.	
GLRC-7	CRLGRLLRRLGRC	13	C1-C13	>100	>100	---	n.e.	n.e.	
GLRC-8	GLRCLRLRGRLRLGRCLLR	19	C4-C16	>100	31	<1.00	n.e.	n.e.	

Table 1: Antiviral activities of de novo designed peptides. aAlthough not provided in the table, the standard error from multiple antiviral assays is on average less than 10% of the respective mean EC50 or TC50 values. bn.e., not evaluated.

Peptide name	Net charge	# of R or K	Pho%	HPLC t_R (min)	Boman index	*E. coli* MIC (µM)	*B. subtilis* MIC (µM)
GLK-19	7	7K	47	9.776	-0.43	10[a]	n.e.[b]
GLR-19	7	7R	47	9.263	3.01	>120	n.e.
GLRC-1	7	7R	47	7.452	3.4	30	>120
GLRC-2	7	7R	47	12.344	3.4	30	>120
GLRC-3	7	7R	47	10.101	3.4	7.5	>120
GLRC-4	7	7R	47	9.601	3.78	60	>120
GLRC-5	7	6R	43	9.373	3.39	60	>120
GLRC-6	7	6R	50	9.395	3.47	30	60
GLRC-7	6	5R	46	8.734	3.88	120	>120
GLRC-8	8	7R	47	9.18	3.4	15-30	>120

Table 2: Effects of peptide scaffolds on their bacterial inhibitory activities. a. Data were taken from ref. [19]. bn.e., not evaluated.

to both GLRC-1 and GLRC-3 (Table 1). These results highlight the importance of the C4-C16 disulfide linkage in GLRC-2. Consistent with this observation, GLRC-4 was also determined to be inactive when two different disulfide bonds (C2-C18 and C6-C14) were applied in the peptide. However, the GLRC peptides with disulfide bonds all had reduced activity against HSV-2 compared to the linear template GLR-19 (Table 1). The possibility that linear AMPs are generally more active than their disulfide counterparts against HSV-2 remains to be tested.

Next, we further mimicked the scaffold of SB2 by removing either the N- or the C-terminal residues outside of the loop (Figure 1). When the C-terminal segment LLR was truncated, the resulting peptide GLRC-5 became inactive. In contrast, the peptide GLRC-6 remained active after the N-terminally exposed residues, GLR, of GLRC-2 were removed. Another peptide (GLRC-7) was obtained by removing both the N- and C-terminal segments outside of the loop of GLRC-2. Like GLRC-5, GLRC-7 was inactive. These results suggest that the C-terminal LLR segment of GLRC-2 is important for anti-HIV activity. We also evaluated if the sequence of the central fragment between the two Cys residues of GLRC-2 played a role in antiviral activity. To address this question, we altered the sequence order without changing the amino acid composition. To our surprise, the resulting peptide GLRC-8 was found to be inactive (Table 2). We conclude that both the

order of the central sequence and the C-terminal region of GLRC-2 play an important role in HIV inhibition.

Because these GLRC peptides are novel, we also evaluated their antibacterial activity using representative laboratory strains. Overall, these peptides showed poor activity against Gram-positive B. subtilis (Table 2). In the case of Gram-negative E. coli K12, the most active peptide (MIC = 7.5 µM) was GLRC-3, the peptide with the smallest loop (Figure 1). Peptides GLRC-1, GLRC-2, GLRC-6, and GLRC-8 showed similar anti-E. coli activity (MIC = 30 µM), indicating that the N-terminal segment GLR of GLRC-2 is dispensable for antibacterial activity. Interestingly, the least active peptide was determined to be GLRC-7, the shortest GLRC peptide with a 13-residue loop structure. Thus, the C-terminal segment of GLRC-2 appears to be important also for bactericidal activity.

Peptide parameters that influence activity

To provide further insight into the antimicrobial activities of these peptide constructs, we measured the retention times of the GLRC peptides by reverse-phase HPLC (Table 2). The retention time is related to peptide hydrophobicity [23]. Other parameters such as net charge, hydrophobic residues% (pho% in Table 3), and Boman index [18] (i.e. protein binding potential) [3] were also calculated for the

GRLC peptides using the prediction interface of the AMP database (http://aps.unmc.edu/AP/main.html) [18] and are included in Table 3. While it is not obvious why GRLC-3 is most active against E. coli, the high potency of GLRC-2 against HIV-1 is likely to be due to high hydrophobicity of the peptide, which has the longest retention time (tR) (Table 2).

Stability of the GLRC peptides to chymotrypsin

We also compared the stability of the GLRC peptides to the action of proteases such as chymotrypsin. Figure 2 shows the percentage of intact peptide after protease treatment at 37oC for 4 hrs. For comparison, SB1 was also tested and was found to have 23% intact peptide remaining at 4 hrs. Compared to SB1, the linear template GLR-19, with 15.5% intact peptide remaining, was less stable (Figure 2). Restraining the GLR-19 polypeptide chain with disulfide bonds had a significant impact on peptide stability. First, we determined that peptide stability was related to the loop size: GLRC-2 > GLRC-1 > GLRC-3 (Figure 1). Thus, GLRC-2, with a medium-sized loop structure, acquired improved stability (>60% intact peptide) compared to SB1 (23% intact peptide, Figure 2). The stability of GLRC-2 increased by 4.4 fold relative to GLR-19. Note that GLRC-4, with two disulfide bond patterns as in GLRC-1 (C2-C18) and GLRC-3 (C6-C14), did not gain additional stability to the protease, further indicating the importance of the C4-C16 disulfide bond. Second, although GLRC-2 and GLRC-8 share the medium-sized loop, they had drastically different stability. It is likely that the sequence order in the loop determines the extent of overall packing of the peptide chain. Third, the segment outside of the loop appeared to be critical for GLRC-2 stability. In particular, removal of the N-terminal segment was detrimental, leading to complete degradation of the peptide in four hours. The C-terminal segment was less important with about 50% of the peptide remaining at 4 hrs. This is in contrast to the importance of the C-terminal segment for anti-HIV activity. Nevertheless, it is interesting to note that GLRC-2, which demonstrated the greatest activity against HIV-1 (Table 1), was also the most stable to protease compared to the other GLRC peptides (Figure 2).
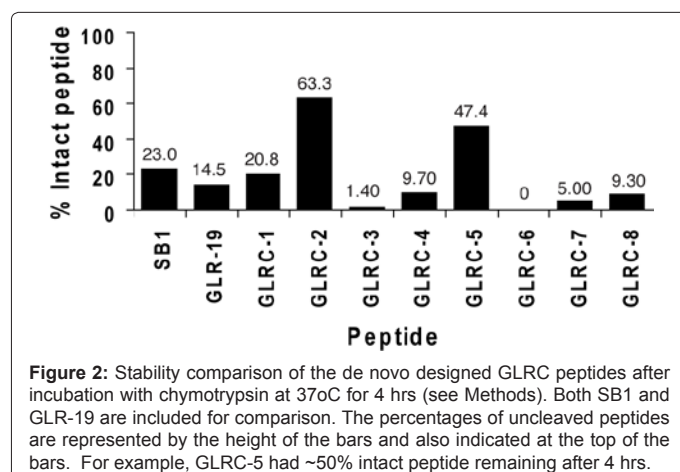
Discussion

In order to identify AMP candidates as lead products for the development of topical microbicides, we have continued our efforts to search for potent anti-HIV peptides. In our previous studies [13,19], different engineering strategies were adopted. These included database screening, sequence shuffling, and database-aided antiviral activity

Figure 2: Stability comparison of the de novo designed GLRC peptides after incubation with chymotrypsin at 37oC for 4 hrs (see Methods). Both SB1 and GLR-19 are included for comparison. The percentages of uncleaved peptides are represented by the height of the bars and also indicated at the top of the bars. For example, GLRC-5 had ~50% intact peptide remaining after 4 hrs.

enhancement [26]. After activity enhancement, several anti-HIV peptides in the helical family were obtained with TIs >20. The current study identified a β-hairpin peptide SB2 with a TI = 30 by using the same Lys-to-Arg mutational strategy [19]. It seemed that this strategy might be applicable to the improvement of anti-HIV activity of AMPs with different structural scaffolds.

The establishment of the antimicrobial peptide database facilitated bioinformatic analysis of natural AMPs from a defined family or source [17,18]. For example, the frequently occurring residues from amphibian AMPs were identified as G, L, A, and K. The peptide, GLK-19 (Table 1), was previously de novo designed using only G, L, and K residues. GLK-19 was found to be active against Gram-negative E. coli but not Gram-positive Staphylococcus aureus [17]. Because only AMPs with antiviral activity are known to have an R/K ratio greater than one in our database, GLR-19 was designed by converting all lysines to arginines. While GLK-19 showed no HIV-1 inhibitory activity at a peptide concentration of 100 μg/ml, GLR-19 was found to be active against both HIV-1 [19] and HSV-2 (Table 1). In this study, we have designed a series of peptides with loop structures based on the linear peptide GLR-19 (Figure 1). We demonstrated that the anti-HIV activity of the peptide was also dependent on the loop size. While GLRC-2 with a medium 13-residue loop was the most potent against HIV-1, GLRC-3 with a small 9-residue loop was the most active against E. coli. It is evident that the optimal design of an antimicrobial peptide is microbe dependent.

Because these de novo designed peptides have a relatively simple amino acid composition, we also investigated possible structure-activity correlations. In the case of E. coli, there was no clear correlation between peptide activity and any physical parameters listed in Table 2, including net charge, the number of Lys or Arg, percentage of hydrophobic residues, Boman index [18] (or protein binding potential [3], and HPLC retention time (hydrophobicity). For the GLRC peptides, however, we noticed that GLRC-7, with a minimal number of positively charged residues, was the least active against Gram-negative E. coli, whereas GLRC-6, with the highest hydrophobic content (50%), is the only peptide that was moderately active against Gram-positive B. subtilis (Table 2). These observations are in line with the current knowledge on AMPs in general [26]. It was also difficult to find any correlation between anti-HIV activity and these peptide properties. These observations indicate that multiple factors determine the biological activity of these AMPs. However, for the three most active GLRC peptides, their HIV inhibitory activities were found to be proportional to hydrophobicity as measured by HPLC (Tables 1 & 2). In particular, the higher activity of GLRC-2 corresponded with its higher hydrophobicity, although this correlation could not be extended to all the peptides in Table 1. Craik and colleagues noticed that, among the HIV inhibitory plant cyclotides, cycloviolacin Y5, isolated from the traditional Chinese herb Viola yedoensis, was the most hydrophobic and also the most potent [27]. They proposed that cycloviolacin Y5 acted on viral membranes. Whether GLRC-2 acts by the same mechanism remains to be established. Interestingly, GLRC-2 also showed higher stability to the action of chymotrypsin compared to the other members of the GLRC peptide family as well as to its linear template GLR-19 (Figure 2).

In summary, we have demonstrated the enhancement of anti-HIV activity of linear AMPs that tend to form amphipathic helical structures using the Lys-to-Arg mutational strategy [19]. We illustrated here that anti-HIV activity of SB1, a thanatin analog with a β-hairpin fold, could also be improved by the same strategy [19]. Our current study has

expanded our knowledge of the relationship between AMP structure and anti-HIV activity. In addition to peptide length, sequence order, charge, amino acid type, and structure [13,19], peptide scaffolds or folds also play a pivotal role (Tables 1 & 2). Evaluation of peptides with varying scaffolds is important because of their different susceptibilities to proteases (Figure 2). This study led to the discovery of a novel peptide GLRC-2 that demonstrated both greater anti-HIV activity and greater stability to chymotrypsin over the GLR-19 template. These results should be helpful for our future design of highly potent anti-HIV peptides with desired stability. In addition, our studies may also contribute to the identification of novel antimicrobial agents with activity against other viral and bacterial pathogens.

Acknowledgements

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