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Identification of Novel Human Immunodeficiency Virus Type 1-Inhibitory Peptides Based on the Antimicrobial Peptide Database▽

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To identify novel anti-HIV-1 peptides based on the antimicrobial peptide database (APD; http://aps.unmc.edu/AP/main.php), we have screened 30 candidates and found 11 peptides with 50% effective concentrations (EC_{50}) of <10 μM and therapeutic indices (TI) of up to 17. Furthermore, among the eight peptides (with identical amino acid compositions but different sequences) generated by shuffling the sequence of an aurein 1.2 analog, two had a TI twice that of the original sequence. Because antiviral peptides in the database have an arginine/lysine (R/K) ratio of >1, increases in the Arg contents of amphibian maximin H5 and dermaseptin S9 peptides and the database-derived GLK-19 peptide improved the TIs. These examples demonstrate that the APD is a rich resource and a useful tool for developing novel HIV-1-inhibitory peptides.

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/AIDS. Thus, it is urgent to develop novel therapeutic and preventative agents. Effective HIV vaccines are not yet available, making the development of topical microbicides that prevent the sexual transmission of HIV desirable (2, 16). Naturally occurring antimicrobial peptides (AMPs) are potent host defense molecules in all life forms (8, 9, 23). More than 1,500 such peptides have been registered in the antimicrobial peptide database (APD; http://aps.unmc.edu/AP/main.html) (19, 20). Prior to this study, a search of the database found entries for 68 peptides with known HIV-inhibitory activity. Some typical examples are melittin and cecropin (17), polyphemusin-derived T22 (21), LL-37 (1, 18), α-, β-, and θ-defensins (5, 9), cyclotides (7), and indolicidin (15). To date, less than 5% of the AMPs in the database have been evaluated, and thus, we hypothesize that the APD constitutes a useful resource for identifying novel HIV-inhibitory peptides. To test this hypothesis, we illustrate database-aided identification of new anti-HIV peptides using three different approaches: database screening, peptide sequence shuffling, and database-based peptide design.

All the peptides (>95% pure) were purchased from Genemed Synthesis, Inc., TX. The well-accepted and highly standardized cytopathic effect (CPE) inhibition assay with CEM-SS cells was utilized as the primary screening assay to evaluate the efficacies and toxicities of compounds with unknown anti-HIV activities (3). The titer of the laboratory strain HIV-1_{IIIB} used is equivalent to a 50% tissue culture infective dose of approximately 1,000 infectious units per ml and results in an in-well multiplicity of infection of 0.0005 to 0.001 (14). CEM-SS cells are human T4 lymphoblastoid cells that have been cloned for virus-induced syncytial and fusogenic sensitivity following infection with HIV-1_{IIIB} (6, 11, 12). Both HIV-1_{IIIB} and the CEM-SS cells were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 5% penicillin-streptomycin, and 5% L-glutamine. CEM-SS cells at a concentration of 2.5×10^3 cells per well and HIV-1_{IIIB} at the above-mentioned predetermined titer were sequentially added to 96-well round-bottom microtiter plates. Serially diluted peptides were added to the plates in triplicate. Each peptide was evaluated at six concentrations ranging from 0.32 to 100 μg/ml. The cultures were incubated at 5% CO_2 and 37°C for 6 days. Following incubation, the microtiter plates were stained with XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] dye (Sigma-Aldrich, St. Louis, MO) to evaluate the efficacies and toxicities of the test compounds (22). By using Microsoft Excel, the 50% effective concentration (EC_{50}; the concentration causing 50% inhibition of virus replication), the 50% toxic concentration (TC_{50}; the concentration causing 50% reduction in cell viability), and the therapeutic index (TI; the TC_{50}/EC_{50} ratio) were calculated for each peptide. For Tables 1 to 3, the EC_{50}s and TC_{50}s were converted into micromolar values based on peptide molecular weights.

Thirty peptides (Table 1) were selected for anti-HIV evaluation with the aid of the search interface of the database. Peptides with the following known properties were rejected: (i) length of >25 residues, because it is less expensive to synthesize shorter peptides; (ii) charge of <0, as anionic peptides tend to be inactive; (iii) Cys content, since peptides with multiple disulfide bonds are more costly to synthesize; (iv) toxicity to mammalian cells, since this is an undesired peptide property; (v) synthetic origin; and (vi) known anti-HIV activity,

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TABLE 1. Findings from database-based anti-HIV-1_{IIIB} peptide screening

Name[a] (source)	Peptide sequence (no. of residues)[i]	EC_{50} (μM)[j]	TC_{50} (μM)[j]	TI
AZT		0.009	>0.5	>55.6
D70 (frog)	GWFDVVKHIA**KRF**-NH_2 (13)	2.99	10.6	3.55
Metalnikowin I (insect)	VDKPDYRPRPRPPNM (15)	>54.4	>54.4	
Ranatuerin 9 (frog)	FLFPLITSFLSKVL (14)	16.7	34.6	2.07
Mastoparan M (insect)[b]	INLKAIAALAKKLL (14)	>67.6	>67.6	
D74 (insect)[c]	I**K**WKKLL**R**AAK**R**IL-NH_2 (14)	>57.6	1.3	<0.02
Hyposin-5 (frog)	FRPALIVRTKGTRL (14)	>61.4	>61.4	
D76 (frog)[d]	FFG**K**VLKLI**R**KI**F**-NH_2 (13)	0.63	6.78	10.8
Apidaecin IA (insect)	GNNRPVYIPQPRPPHPRI (18)	>47.4	>47.4	
Drosocin (insect)[e]	GKPRPYSPRP**T**SHPRPIRV (19)	>45.5	>45.5	
PGLa (frog)	GMASKAGAIAGKIAKVALKAL-NH_2 (21)	>50.8	>50.8	
Clavanin B (tunicate)	VFQFLGRIIHHVGNFVHGFSHVF (23)	7.1	37.1	>5.18
Buforin II (toad)	TRSSRAGLQFPVGRVHRLLRK (21)	>41.1	>41.1	
Styelin A (tunicate)	GFGKAFHSVSNFAKKHKTA-NH_2 (19)	>48.5	>48.5	
Ponericin L2 (insect)	LLKELWTKIKGAGKAVLGKIKGLL (24)	1.4	24.7	17.6
Spinigerin (insect)	HVDKKVADKVLLLKQLRIMRLLTRL (25)	3.05	>33.3	>10.9
Piscidin 3 (fish)	FFHHIFRGIVHVGKTIHRLVTG (22)	2.1	6.9	3.3
Pseudin 1 (frog)	GLNTLKKVFQGLHEAIKLINNHVQ (24)	35.7	>36.8	>1.03
Misgurin (fish)	RQRVEELSKFSKKGAAARRRK (21)	>40.0	>40.0	
D88 (frog)[f]	NLVSGLIEARKYLEQLHRKLKN**R**KV (25)	>33.3	>33.3	
Lycotoxin I (spider)	IWLTALKFLGKHAAKHLAKQQLSKL (25)	>35.2	2.4	<0.07
Parasin I (fish)	KGRGKQGGKVRAKAKTRSS (19)	>50.0	>50.0	
Brevinin-2 related (frog)	GIWDTIKSMGKVFAGKILQNL-NH_2 (21)	1.65	7.42	4.49
Maculatin 1.3 (frog)	GLLGLLGSVVSHVVPAIVGHF-NH_2 (21)	4.0	8.02	2.01
Latarcin 3a (spider)	SWKSMAKKLKEYMEKLKQRA (20)	32.7	>40.3	>1.23
D94 (bacteria)[g]	GAWKNFWSSLRKGFYDGEAGRAIRR (25)	>34.1	>34.1	
Ascaphin-8 (frog)	GFKDLLKGAAKALVKTVLF-NH_2 (19)	1.2	2.9	2.4
Desertcolin 1 (frog)	GLADFLNKAVGKVVDFVKS-NH_2 (19)	>49.9	>49.9	
Melectin (insect)	GFLSILKKVLPKVMAHMK-NH_2 (18)	4.34	7.75	1.79
D98 (frog)[h]	SLSRFL**R**FLKIVY**RR**AF-NH_2 (17)	0.83	8.6	10.4
Isracidin (cow)	RPKHPIKHQGLPQEVLNENLLRF (23)	>36.2	>36.2	

[a] The peptides were chosen from the APD (20). D70 is a mutant in which the last three residues of uperin 7.1 were changed from SAV to KRF. AZT, zidovudine.
[b] C-terminal amidation is absent.
[c] A peptide mutant of polybia-MPI with the following mutations: D2K, D8R, and Q12R.
[d] A peptide mutant of temporin-PTa with S4K, P10R, and L13F mutations.
[e] Residue T11 is not O glycosylated.
[f] The sequence of this peptide corresponds to chain A of distinctin with residue C23 changed to R.
[g] The sequence of this peptide corresponds to chain A of plantaricin JK.
[h] A mutant of temporin-LTc with three mutations: S7R, P14R, and P15R.
[i] The number of amino acid residues in the peptide is indicated in parentheses. C-terminal amidation is represented by NH_2. Mutated residues are listed in bold.
[j] The standard error of results from multiple antiviral assays is on average less than 10% of the respective mean EC_{50} or TC_{50}.

since criteria v and vi prevent duplicated efforts. In addition, we selected a representative member when a family of peptides had similar sequences. Of the 30 peptides selected (Table 1), 13 originated in amphibians, 8 in insects, 3 in fish, 2 in spiders, 2 in tunicates, 1 in cows, and 1 in bacteria. As detailed in the footnotes to Table 1, D70, D74, D76, and D98 are mutants of uperin 7.1, polybia-MPI, temporin-PTa, and temporin-LTc, respectively. The mutations were made in an attempt to enhance peptide activity based on the findings of our previously reported study (18). Eleven of the 30 peptides were found to have EC_{50}s of <10 μM. These include D70, D76, clavanin B, ponericin L2, spinigerin, piscidin 3, brevinin-2 related, maculatin 1.3, ascaphin-8, melectin, and D98. These peptides can be further grouped based on their TIs. Of these 11 peptides, D76, D98, ponericin L2, and spinigerin have a TI of >10. Ponericin L2 and spinigerin displayed toxicity to human cells at 25 μM or higher concentrations and may be utilized as starting templates for developing HIV-inhibitory microbicides. The HIV-inhibitory activity of these peptides may be further improved by optimization of amino acid composition and sequence shuffling (see below).

We define peptides with EC_{50}s greater than the highest concentration tested (100 μg/ml; micromolar concentrations are given in Table 1) as inactive against HIV-1 (18). These peptides are metalnikowin I, mastoparan M, D74, hyposin-5, apidaecin IA, drosocin, PGLa, buforin II, styelin A, misgurin, D88, lycotoxin I, parasin I, plantaricin JK (chain A), desertcolin 1, and isacidin. Of these 16 peptides, 3 peptides of insect origin (metalnikowin I, apidaecin IA, and drosocin) are rich in prolines. These antibacterial peptides can cross bacterial membranes and associate with heat shock proteins (13). Buforin II can cross bacterial membranes and bind to DNA (4). The lack of efficacy of these peptides against HIV-1 may be attributed to the absence of such molecular targets in the virus as well as the human cell. This information may be utilized as one of the criteria for peptide selection from the database.

Next, we chose Trp-containing aurein 1.2 (peptide B1 in Table 2) as a template (10) to evaluate the effect of sequence shuffling on anti-HIV-1 activity. Peptides B2 to B8 were generated by rearranging the 13 residues of peptide B1 but preserving the potential of the sequence to form an amphipathic helix. In addition, each peptide was designed by mimicking a

TABLE 2. Effects of sequence shuffling on HIV-1_{IIIB}-inhibitory activity of peptides in CEM-SS cells[a]

Peptide name	Peptide sequence	EC_{50} (μM)	TC_{50} (μM)	TI	Model peptide
B1	GLFDIIKKIAESW	11.7	37.0	3.2	Aurein 1.2
B2	GLWEKIDKFASII	>65.8	>65.8		Caerin 3.2
B3	GIIDIAKKLFESW	20.1	>65.8	>3.3	Uperin 2.7
B4	GWFDIIKKIASEL	10.7	38.9	3.6	Uperin 7.1
B5	GIFDKLAKEISIW	>65.8	>65.8		Brevinin-2DYd
B6	GIWSDLAEIIKKF	11.4	>65.8	>5.8	Ponericin W3
B7	GFLDIIEKIAKSW	10.5	>65.8	>6.3	Ranatuerin 3
B8	GWLKKIESIIDAF	29.5	>65.8	2.2	Cecropin

[a] The standard error of results from multiple antiviral assays is on average less than 10% of the respective mean EC_{50} or TC_{50}. All the group B peptides are C-terminally amidated.

natural peptide in the database so that at least a few residues at the N terminus of the peptide are shared with the model peptide (listed in the last column of Table 2). For example, peptide B2, with GLWE as the N-terminal sequence, mimics the sequence pattern of caerin 3.2 from amphibians. The group B peptides were also subjected to HIV-1 inhibition evaluation. Peptides B1, B4, B6, and B7 showed similar antiviral EC_{50}s. In particular, peptides B1 and B4 had essentially identical EC_{50}s, TC_{50}s, and TIs. This is not surprising since B1 and B4 are very similar in sequence pattern. B4 was obtained primarily by swapping L2 and W13 of peptide B1. Peptides B6 and B7 showed improved TIs as a consequence of lower toxicity to human cells. These two peptides are promising candidates for future development. Peptides B3 and B8 are less interesting owing to simultaneous reductions in activity against the virus and toxicity to human cells. Finally, peptides B2 and B5 were rejected because they were not found to be efficacious against HIV-1. Hence, peptide sequence shuffling led to the identification of B6 and B7 with improved TIs. Evidently, sequence shuffling has the potential to expand the sequence space for HIV-inhibitory peptides.

As a third approach, we attempted to improve the anti-HIV-1 activity of AMPs based on database findings. Previous statistical analysis of AMPs in the database led to the identification of frequently occurring amino acids (frequency, >10%) in different kingdoms (19). For example, Gly and Cys residues are abundant in plant peptides, while amphibian peptides are rich in Gly, Ala, Leu, and Lys residues. Further investigation reveals that the antiviral peptides have a higher average percentage of arginines than the antibacterial, antifungal, or anticancer peptides (Fig. 1), in which the percentage of Lys residues is always higher than that of Arg residues. Although there is some overlap between the different groups, a similar trend was observed for the original 525 peptides (20), suggesting that the peptide composition is important for activity. This observation may be useful for enhancing the anti-HIV activity of AMPs. Maximin H5 is an anionic peptide and was found to be inactive against HIV-1_{IIIB} (Table 3). However, the peptide became active against HIV-1 when the three acidic Asp residues were converted to basic Arg residues. Consistent with this observation, we found that GF-17, a human LL-37-derived peptide active against HIV-1 (18), lost its anti-HIV-1 activity when an acidic version of the peptide was synthesized (Table 3). These examples suggest that positively charged residues in AMPs play an important role in HIV-1 inhibition.

To illustrate the importance of Arg residues, we synthesized a dermaseptin S9 (DRS S9) mutant by replacing three lysines with arginines (Table 3). While wild-type DRS S9 showed very weak activity against HIV-1, the virus-inhibitory activity of the arginine mutant DRS S9r3 was high, with the mutant having a TI of ~26. As another example, we tested the idea using a database-derived peptide, GLK-19, which consists of only residues Gly, Leu, and Lys (19). Although the peptide showed higher activity against *Escherichia coli* K-12 than human LL-37, it displayed no activity against HIV-1. After all Lys residues were changed to Arg, however, the resulting peptide (GLR-19) became active against HIV-1 (Table 3). These examples indicate that increases in the arginine contents of AMPs may improve the potencies of the peptides against HIV-1. Future mutagenesis studies will identify which arginine residues are critical for viral inhibition.

In summary, this study documents HIV-1-inhibitory activity analysis of 46 synthetic peptides derived from the APD. This work demonstrates database-aided anti-HIV-1 peptide identification by a variety of strategies. First, a select set of peptides from the APD can be directly screened, allowing for the identification of HIV-1-inhibitory peptide templates. Of the 30 peptides screened, 11 were found to have EC_{50}s in the range of 0.63 to 10 μM (Table 1). Second, additional peptides active

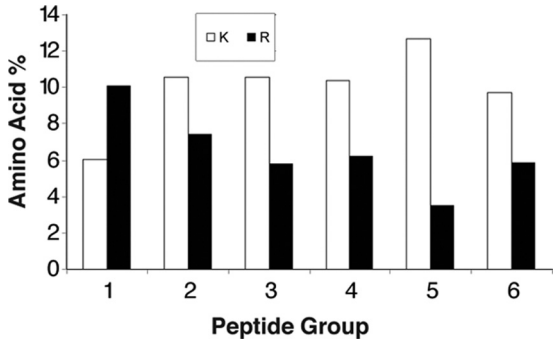

FIG. 1. Average Lys (K) and Arg (R) residue contents in groups of AMPs. Each of the peptide groups (antiviral, antifungal, anticancer, and antibacterial peptides and peptides toxic to mammalian cells) was obtained by searching the APD (http://aps.unmc.edu/AP/main.html). Groups: 1, 80 antiviral peptides; 2, 405 antifungal peptides; 3, 82 anticancer peptides; 4, 1,035 antibacterial peptides; 5, 157 peptides that are toxic to mammalian cells; and 6, all 1,405 peptides analyzed. The average amino acid contents in each group were obtained by initiating the statistical analysis program at the end of the peptide output. Detailed data for the original 525 peptides were reported in our database paper (20).

TABLE 3. Effects of charge and cationic residues on HIV-1_{IIIB}-inhibitory activity of AMPs and their analogs in CEM-SS cells[a]

Peptide name	Peptide sequence (no. of residues)[b]	EC_{50} (μM)	TC_{50} (μM)	TI
Maximin H5	ILGPVLGLVSDTLDDVLGIL (20)	>49.4	>49.4	
Maximin H5r3	ILGPVLGLVS**R**TL**RR**VLGIL (20)	2.2	8.7	3.9
GF-17	GFKRIVQRIKDFLRNLV-NH_2 (17)	0.76	10.8	14.2
GF-17−	GF**N**E**I**VQ**DI**EDFL**Q**NLV-NH_2 (17)	>25.1	>25.1	
DRS S9	GLRSKIWLWVLLMIWQESNKFKKM (24)	31.6	>32.9	>1.04
DRS S9r3	GLRS**R**IWLWVLLMIWQESN**R**FK**R**M (24)	1.25	>32.1	>25.7
GLK-19	GLKKLLGKLLKKLGKLLLK (19)	>47.5	25.1	<0.5
GLR-19	GL**RR**LLG**R**LL**RR**LG**R**LLL**R** (19)	4.4	25.7	5.8

[a] The standard error of results from multiple antiviral assays is on average less than 10% of the respective mean EC_{50} or TC_{50}.
[b] The number of residues in the peptide is indicated in parentheses. Mutated residues in the sequence are in bold.

against HIV-1 can be generated via sequence shuffling (i.e., rearrangement of the amino acids) of a peptide template from the APD. Of the eight sequence-shuffled peptides, two peptides were found to have nearly doubled TIs compared to the original aurein 1.2 analog (Table 2). Third, database findings may also be utilized to improve the TI of the peptide. Higher Arg/Lys ratios in antiviral peptides (20) inspired us to enhance the HIV-1-inhibitory activity of AMPs in several cases by increasing the Arg content. In particular, the TI of a DRS S9 mutant reached ~26. This work resulted in an increase in HIV-1-inhibitory AMPs in the APD from 68 to 83. All these examples prove that the APD (19, 20) provides a rich library for engineering novel anti-HIV-1 peptides.

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