Structural and Functional Characterization of Membrane Fusion Inhibitors with Extremely Potent Activity against Human Immunodeficiency Virus Type 1 (HIV-1), HIV-2, and Simian Immunodeficiency Virus

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ABSTRACT T-20 (enfuvirtide) is the only membrane fusion inhibitor available for the treatment of viral infection; however, it has low anti-human immunodeficiency virus (anti-HIV) activity and a low genetic barrier for drug resistance. We recently reported that T-20 sequence-based lipopeptides possess extremely potent *in vitro* and *in vivo* efficacies (X. Ding, Z. Zhang, H. Chong, Y. Zhu, H. Wei, X. Wu, J. He, X. Wang, Y. He, 2017, *J Virol* 91:e00831-17, <https://doi.org/10.1128/JVI.00831-17>; H. Chong, J. Xue, Y. Zhu, Z. Cong, T. Chen, Y. Guo, Q. Wei, Y. Zhou, C. Qin, Y. He, 2018, *J Virol* 92:e00775-18, <https://doi.org/10.1128/JVI.00775-18>). Here, we focused on characterizing the structure-activity relationships of the T-20 derivatives. First, a novel lipopeptide termed LP-52 was generated with improved target-binding stability and anti-HIV activity. Second, a large panel of truncated lipopeptides was characterized, revealing a 21-amino-acid sequence core structure. Third, it was surprisingly found that the addition of the gp41 pocket-binding residues in the N terminus of the new inhibitors resulted in increased binding but decreased antiviral activities. Fourth, while LP-52 showed the most potent activity in inhibiting divergent HIV-1 subtypes, its truncated versions, such as LP-55 (25-mer) and LP-65 (24-mer), still maintained their potencies at very low picomolar concentrations; however, both the N- and C-terminal motifs of LP-52 played crucial roles in the inhibition of T-20-resistant HIV-1 mutants, HIV-2, and simian immunodeficiency virus (SIV) isolates. Fifth, we verified that LP-52 can bind to target cell membranes and human serum albumin and has low cytotoxicity and a high genetic barrier to inducing drug resistance.

IMPORTANCE Development of novel membrane fusion inhibitors against HIV and other enveloped viruses is highly important in terms of the peptide drug T-20, which remains the only one for clinical use, even if it is limited by large dosages and resistance. Here, we report a novel T-20 sequence-based lipopeptide showing extremely potent and broad activities against HIV-1, HIV-2, SIV, and T-20-resistant mutants, as well as an extremely high therapeutic selectivity index and genetic resistance barrier. The structure-activity relationship (SAR) of the T-20 derivatives has been comprehensively characterized, revealing a critical sequence core structure and the target sites of viral vulnerability that do not include the gp41 pocket. The results also suggest that membrane-anchored inhibitors possess unique modes of action relative to unconjugated peptides. Combined, our series studies have not only provided drug candidates for clinical development but also offered important tools to elucidate the mechanisms of viral fusion and inhibition.

KEYWORDS HIV-1, HIV-2, fusion inhibitor, T-20, lipopeptide

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Host cell infection with human immunodeficiency virus (HIV) requires fusion of viral and cellular membranes, which is mediated by the envelope (Env) glycoproteins on the surface of the virion (1, 2). The surface subunit gp120 is responsible for receptor binding, which triggers huge conformational changes in the Env complex and releases the noncovalently associated transmembrane subunit gp41. In the fusion process, the N-terminal fusion peptide of gp41 protrudes and inserts into target cell membrane, leading to an extended, membrane-bridging prehairpin structure. Subsequently, the C-terminal heptad repeats (CHR) of gp41 pack in an antiparallel fashion into the internal hydrophobic grooves created by trimeric N-terminal heptad repeat (NHR) coiled coils to adopt a stable six-helix bundle (6-HB) structure that drives membrane merger. Peptides derived from either NHR (N peptide) or CHR (C peptide) of gp41 can bind the prehairpin intermediate to interfere with the structural arrangements, thus inhibiting Env-mediated cell fusion and viral entry in a dominant negative fashion (3–5). Prominently, the crystal structure of 6-HB formed by N and C peptides (N36/C34) revealed a hydrophobic deep pocket on the C-terminal portion of the NHR helices, which is penetrated by the pocket-binding domain (PBD) from the N terminus of the CHR helices (6–8). Since its discovery, the deep pocket of gp41 has been considered an ideal target site for anti-HIV agents (9–12).

As a 36-amino-acid peptide drug, T-20 (enfuvirtide) was identified in the early 1990s and approved for clinical use in 2003 (13–16). Disappointedly, the clinical acceptance of T-20 has been severely limited by its weak antiviral activity and short half-life (90 mg, twice daily), as well as a low genetic barrier to inducing drug resistance (17–19). In sequence structure (Fig. 1), T-20 has an 8-amino-acid tryptophan-rich motif (TRM) at its C terminus, which is considered a “lipid-binding domain” to anchor the inhibitor to the target cell membrane, but it lacks an N-terminal PBD that is critical for high-affinity binding. Therefore, T1249 was designed as a second-generation fusion inhibitor by incorporating both the TRM and PBD sequences, but unfortunately its clinical development was terminated due to formulation difficulties (20, 21). Since then, the C peptide C34 has been widely used as a designing template, because it is considered a core CHR sequence and has the PBD sequence (6, 8, 22). Indeed, the resulting C34 derivatives, such as SC34EK, sifuvirtide, and T2635, possessed improved activities in inhibiting both wild-type and T-20-resistant viruses (23–26). By adding the M-T hook structure to the PBD, we developed several highly potent short peptides, such as MTSC22EK, HP23, and 2P23, which mainly target the deep pocket rather than the T-20 resistance sites (27–29).

To overcome the intrinsic problems of a peptide drug, there are considerable efforts to develop lipopeptide-based viral fusion inhibitors, which are considered to bind preferentially with target cell membranes where fusion occurs, thus raising the local concentration of the inhibitors (30–34). As a leading lipopeptide, C34-Chol was developed by adding a cholesterol group to the C terminus of C34, which showed greatly enhanced anti-HIV potency and *in vivo* half-life (31). By modifying the short peptides HP23 and 2P23 with different lipids (fatty acid, cholesterol, sphingolipids), we previously developed LP-11 and LP-19 with dramatically increased *in vitro* and *in vivo* antiviral activities and stability (35, 36). To explore the mechanisms of action of lipopeptides and to develop new HIV-1 fusion inhibitors with different modes of action, we recently generated T-20- and T1249-based lipopeptides (LP-40 and LP-46) by replacing their TRM sequences with a fatty acid group, resulting in increased anti-HIV activities (37, 38). Very recently, we modified LP-40 by introducing the intrahelical salt bridge-prone and HIV-2/simian immunodeficiency virus (SIV) sequences, resulting in LP-50 and LP-51, which could inhibit HIV-1, HIV-2, and SIV isolates at very low picomolar concentrations and suppress viral loads to undetectable levels in acutely and chronically simian-human immunodeficiency virus (SHIV)-infected rhesus monkey models (39). As we discussed, LP-50 and LP-51 are the most potent and broadly active HIV-1/2 and SIV fusion inhibitors, which can be developed for clinical use and serve as tools to explore the mechanisms of viral entry and inhibition (39). Actually, we are still being excited by the fact that the antiviral activity of T-20 could be enhanced more than

RESULTS

Generation of a novel lipopeptide with further increased binding and inhibitory activities. By refining the structure and function of the T20-based lipopeptide LP-40, we recently developed LP-50 and LP-51, which showed dramatically increased α -helicity, binding stability, and anti-HIV activity. Later, the crystal structure of T1249-based lipopeptide LP-46 was determined, revealing the importance of its N-terminal sequence in the binding of inhibitor (37), and we thus decided to further optimize LP-51 by replacing its three N-terminal residues with the corresponding residues from LP-46, resulting in a new inhibitor termed LP-52 (Fig. 1). First, we determined its secondary structure and binding stability by circular dichroism (CD) spectroscopy. As shown in Fig. 2, LP-52 displayed a typical α -helical conformation similar to LP-51, but its thermostability was slightly enhanced. Interestingly, the α -helical content and thermostability of LP-52 in the presence of a target mimic N peptide (N39) were significantly increased. While the LP-51/N39 complex showed 54% α -helices with a melting temperature (T_m) value of 72°C, the LP-52/N39 complex had 64% α -helices with a T_m value of 79°C.

FIG 2 Secondary structure and binding stability of T-20 derivatives. The α -helicity (A) and thermostability (B) of the isolated inhibitors and the α -helicity (C) and thermostability (D) of inhibitors in complexes with the NHR-derived target mimic peptide N39 were determined by CD spectroscopy. The final concentration of each peptide in PBS was 10 μ M. The α -helical contents and T_m values are shown in parentheses (NA, not applicable for calculation). The experiments were repeated two times, and representative data are shown.

Meanwhile, we compared the anti-HIV activities of LP-52 and its templates by three functional approaches. As shown in Fig. 3, LP-52 exhibited slightly increased activity in inhibiting HIV-1_{HXB2} Env-mediated cell-cell fusion and HIV-1_{NL4-3} pseudovirus-mediated cell entry, with 50% inhibitory concentrations (IC_{50} s) of 13 and 4 pM, respectively; however, LP-52 inhibited a panel of replication-competent HIV-1 strains with different phenotypes at a mean IC_{50} of 9 pM, which was 3.2-fold lower than that of LP-51 (29 pM) and 1,622-fold lower than that of T-20 (15,141 pM). Especially, LP-52 inhibited the HIV-1 isolates NL4-3 (X4 tropic), SG3 (X4 tropic), JRCSF (R5 tropic), and 89.6 (R5X4 tropic) more efficiently. Therefore, the sequence optimization greatly improved the binding stability and anti-HIV activity of inhibitors.

Identification of a core sequence structure for binding and antiviral activities.

We next focused on exploring the structure-activity relationship (SAR) of the newly designed T-20 derivatives. First, a large panel of N- and C-terminally truncated lipopeptides was designed and characterized for their binding and anti-HIV activities. As shown in Fig. 4, deletion of three C-terminal residues (LEK) from LP-40 and LP-50 resulted in dramatic losses in both the binding and inhibitory potencies, as shown by LP-53 and LP-54, indicating their importance for LP-40 and LP-50. Interestingly, the LEK motif also played a critical role for the binding of LP-52, as indicated by a significantly reduced T_m (from 79 to 63°C) from LP-55 and LP-56, but its deletion had a minor effect on the anti-HIV activity. Here, introducing a flexible linker (AEEA) between the peptide sequence and the C16 group of LP-56 had no appreciable functionalities. As evidenced by LP-57, LP-58, and LP-59, LP-52 could not tolerate a further C-terminal truncation. Similarly, deletion of two or four N-terminal residues significantly impaired LP-50 (Fig. 4, see LP-60 and LP-61); however, such deletions did weaken the binding of LP-52 but had little effect on its antiviral activity (see LP-62, LP-63, and LP-65). Surprisingly, LP-64, which resulted from the deletion of three N-terminal residues from LP-52, showed relatively lower anti-HIV activity than LP-65. Here, LP-64 and LP-65 had the same T_m value (72°C), but the extreme N-terminal lysine might interfere with the α -helicity of a

FIG 3 Inhibitory activities of T-20 derivatives. Percentages of inhibition by T-20, LP-40, LP-50, LP-51, and LP-52 of HIV-1_{HXB2} Env-mediated cell-cell fusion (A), HIV-1_{NL4-3} pseudovirus-based virus entry (B), and infections of the replicative viruses HIV-1_{JRC5F} (C), HIV-1_{89.6} (D), HIV-1_{R3A} (E), HIV-1_{NL4-3} (F), HIV-1_{SG3} (G), and HIV-1_{LAI} (H), as well as vesicular stomatitis virus (I), were determined. The experiments were performed in triplicate and repeated three times. Percentages of inhibition of the peptides and IC_{50} s were calculated.

6-HB formed by inhibitor and N39. As demonstrated by LP-66, LP-67, and LP-68, LP-52 could also not tolerate a further N-terminal truncation. Therefore, we synthesized LP-69 by removing both the N-terminal WEQK motif and the C-terminal LEK motif, resulting in a lipopeptide that was only 21 residues in length but still displayed high binding and antiviral activities, thus representing a core sequence structure for the T-20 lipopeptide derivatives.

Addition of the PBD enhances binding but attenuates anti-HIV activity. The pocket-binding domain (PBD) located at the N terminus of C peptides has been largely used for designing novel HIV fusion inhibitor peptides (3–5). In consideration of T-20-based lipopeptides that had no PBD sequence and thus did not target the gp41 pocket, we were interested to know whether the PBD sequence could further increase the antiviral activity of new inhibitors. Thus, a panel of lipopeptides was designed with or without the pocket-binding and M-T hook residues (LP-70, -71, -72, -73, -74, -75). Compared to the parental lipopeptide LP-50 or LP-51, these inhibitors exhibited significantly reduced activities in inhibiting viral fusion, entry, and infection, especially LP-74, which contains a full PBD, and LP-75, which contains both the PBD and M-T hook sequences (Fig. 4).

Inhibitor	Sequence structure	Length	CD data			Anti-HIV activity (IC ₅₀ , pM)		
			N peptide	Helix (%)	T _m (°C)	HXB2 fusion	NL4-3 entry	JRC5F infection
T-20	YTSLIHSLIEESQNNQKEQELLELDK WASLWNVF	36	N39	49	44	24633 ± 2467	10825 ± 1354	4503 ± 384
LP-40	YTSLIHSLIEESQNNQKEQELLELDK (C16)	28	N39	58	51	361 ± 60	392 ± 73	384 ± 33
LP-50	YTSLIEELIKKSEEQKKNEEELKKLEK (C16)	28	N39	56	63	21 ± 3	7 ± 1	23 ± 2
LP-51	LEANIEELLKKAEEQKKNEEELKKLEK (C16)	28	N39	54	72	21 ± 10	6 ± 1	27 ± 5
LP-52	WEQ KIEELLKKAEEQKKNEEELKKLEK (C16)	28	N39	64	79	13 ± 1	4 ± 0	5 ± 1
LP-53	YTSLIHSLIEESQNNQKEQELLEK (C16)	26	N39	54	43	2949 ± 400	9968 ± 2224	21352 ± 1876
LP-54	YTSLIEELIKKSEEQKKNEEELKK (C16)	25	N39	50	47	149 ± 48	301 ± 26	1796 ± 340
LP-55	WEQ KIEELLKKAEEQKKNEEELKK (C16)	25	N39	51	63	14 ± 2	8 ± 1	12 ± 1
LP-56	WEQ KIEELLKKAEEQKKNEEELKK-AEEA-K (C16)	26	N39	43	63	12 ± 2	9 ± 1	11 ± 2
LP-57	WEQ KIEELLKKAEEQKKNEEELKK (C16)	23	N39	59	43	213 ± 21	178 ± 38	1917 ± 440
LP-58	WEQ KIEELLKKAEEQKKNEK (C16)	21	N39	38	NA	143000 ± 19911	110900 ± 3715	107033 ± 9473
LP-59	WEQ KIEELLKKAEEQKK (C16)	18	N39	27	NA	>900000	240800 ± 23649	210477 ± 25537
LP-60	SLIEELIKKSEEQKKNEEELKKLEK (C16)	26	N39	39	58	55 ± 6	48 ± 6	62 ± 27
LP-61	IEELIKKSEEQKKNEEELKKLEK (C16)	24	N39	54	59	85 ± 9	43 ± 9	126 ± 63
LP-62	EQKIEELLKKAEEQKKNEEELKKLEK (C16)	27	N39	57	73	15 ± 1	5 ± 1	12 ± 1
LP-63	QKIEELLKKAEEQKKNEEELKKLEK (C16)	26	N39	48	70	17 ± 5	6 ± 2	13 ± 2
LP-64	K IEELLKKAEEQKKNEEELKKLEK (C16)	25	N39	40	72	128 ± 27	36 ± 1	25 ± 2
LP-65	I EELLKKAEEQKKNEEELKKLEK (C16)	24	N39	63	72	14 ± 3	8 ± 3	7 ± 2
LP-66	EELLKKAEEQKKNEEELKKLEK (C16)	23	N39	64	60	1008 ± 248	434 ± 71	1109 ± 193
LP-67	LLEQAEEQKKNEEELKKLEK (C16)	21	N39	50	48	3417 ± 419	627 ± 154	1527 ± 565
LP-68	AEEQKKNEEELKKLEK (C16)	17	N39	53	60	>250000	>250000	>250000
LP-69	I EELLKKAEEQKKNEEELKK (C16)	21	N39	45	51	239 ± 16	125 ± 27	157 ± 29
LP-70	INNYTSLIEELIKKSEEQKKNEEELKKLEK (C16)	31	N39	45	69	25 ± 3	21 ± 4	30 ± 10
LP-71	IEEYTKKIEELIKKSEEQKKNEEELKKLEK (C16)	31	N39	57	69	27 ± 2	25 ± 3	55 ± 5
LP-72	VRYLEANIEELLKKAEEQKKNEEELKKLEK (C16)	31	N39	43	74	23 ± 3	28 ± 1	24 ± 9
LP-73	VEELEKKIEELLKKAEEQKKNEEELKKLEK (C16)	31	N39	87	80	33 ± 6	19 ± 3	42 ± 2
LP-74	VEEWEKKI IEEYTKKIEELIKKSEEQKKNEEELKKLEK (C16)	38	N44	87	>98	110 ± 10	59 ± 11	59 ± 32
LP-75	EW TVEEWEKKI IEEYTKKIEELIKKSEEQKKNEEELKKLEK (C16)	41	N44	85	>98	100 ± 22	65 ± 8	86 ± 26
LP-11	EW TVEEWEKKI IEEYTKKIEELIKK-PEG8-K (C16)	24	N36	100	>98	931 ± 68	201 ± 41	253 ± 40
LP-19	EW TVEEWEKKV EELEKKIEELK-PEG8-K (C16)	24	N36	93	>98	296 ± 45	95 ± 18	92 ± 11
LP-46	WQ WEQKI -----TALLEQAQIQQEKNEYELQKLDK (C16)	31	N44	89	86	88 ± 4	50 ± 8	44 ± 7
LP-48	WEQKI -----TALLEQAQIQQEKNEYELQKLDK (C16)	28	N44	45	78	83 ± 9	47 ± 6	37 ± 4
C34-C16	WMEWDREIN NYTSLIHSLEESQNNQKEQELLEK (C16)	35	N36	96	77	247 ± 28	65 ± 25	109 ± 13
C34-Chol	WMEWDREIN NYTSLIHSLEESQNNQKEQELLEK-GSG-C (Chol)	38	N36	84	80	316 ± 62	24 ± 2	37 ± 5

FIG 4 Structure-activity relationship of T-20 derivatives. The α -helicity and binding thermostability of diverse inhibitors were determined by CD spectroscopy, with the final concentration of each peptide at 10 μ M in PBS. The inhibitory activities of inhibitors on HIV-1_{HXB2} Env-mediated cell-cell fusion, HIV-1_{NL4-3} pseudovirus entry, and HIV-1_{JRC5F} infection were determined. The TRM, M-T hook, and PBD residues in the inhibitors are marked in purple, green, and red, respectively. The representative inhibitors and their corresponding results are marked in blue. AEEA, 8-amino-3,6-dioxoactanoic acid; C16, palmitic acid. The antiviral experiments were performed in triplicate and repeated at least three times. Data are expressed as means $\pm$ standard deviations (SD).

For comparison, we also determined the binding and antiviral activities of several previously reported inhibitors, including LP-11 and LP-19, which specifically target the gp41 pocket site, and LP-46, which was derived from T1249. As shown in Fig. 4, these pocket-binding inhibitors had much higher binding stabilities but relatively low anti-HIV activities. Interestingly, deletion of partial PBD residues (WEQ) from LP-46 markedly reduced its α -helicity and thermostability but did not impair its antiviral activity, as indicated by LP-48. Furthermore, we synthesized two C34-based lipopeptides, C34-C16 and C34-Chol. Similarly, their antiviral activities were significantly lower than those of newly designed inhibitors, such as LP-50, LP-51, LP-52, and LP-52's truncated versions (LP-55 and LP-65). Actually, the shortest lipopeptide, LP-69, exhibited an anti-HIV activity comparable to that of diverse PBD-focused inhibitors, but their binding thermostabilities differed dramatically. Taken together, our results suggest that the pocket-binding ability is not required for T-20 sequence-based lipopeptides, as illustrated in Fig. 5.

Characterizations of the binding and inhibitory activities of the template peptides. To facilitate our understanding of the structural and functional properties of

FIG 5 Schematic illustration of the NHR-CHR interactions and the binding sites of diverse inhibitors. (A) A helical wheel model illustrating the interactions of the NHR and CHR helices of gp41. (B) A hairpin model illustrating the interacting residues between the NHR and CHR sequences. The dashed black lines indicate the interactions between the residues located at the "e" and "g" positions in the NHR and the "a" and "d" positions in the CHR, respectively. The pocket-forming sequence (PFS) in the NHR and the pocket-binding domain (PBD) in the CHR are marked in blue and red, respectively. The peptide inhibitors are depicted as lines to express their positions and sequences as well as the corresponding binding sites.

new membrane fusion inhibitors with extremely potent anti-HIV activity, a panel of unconjugated template peptides was synthesized and characterized by CD spectroscopy and antiviral experiments. Consistent with our previous studies, the template peptide for LP-40 (designated P-40) had no appreciable binding or inhibitory activities (Fig. 6); however, sequence optimization by introducing the EK motifs and the HIV-2/SIV sequences sharply improved both the binding stability and the antiviral potency, as indicated by the templates for LP-50 (P-50), LP-51 (P-51), and LP-52 (P-52). In comparison, P-52 showed much better binding and inhibition than P-50 and P-51, providing a sequence basis for their lipopeptide derivatives. The binding and inhibitory activities of all the C- and N-terminally truncated template peptides markedly decreased, except that P-64 showed an inhibitory potency similar to that of P-52 on HIV-1_{JRCSE} infection. Unlike the lipopeptides shown in Fig. 4, P-64 was also more active than P-65. Here, P-69, the template for the lipopeptide with a core sequence (LP-69), had no detectable inhibitory activities at a concentration as high as 750 nM. Also, conversely, the longer template peptides possessing the PBD sequence showed the most robust binding and inhibition, verifying that the pocket-binding ability is highly important for unconjugated peptide inhibitors. In summary, the data presented have further provided new insights into the SAR of diverse HIV fusion inhibitors.

Template	Sequence	Length	N peptide	CD data		Inhibition data (IC ₅₀ , nM)		
				Helix (%)	T _m (°C)	HXB2	NL4-3	JRCSF
P-40	YTSLIHSLIEESQKQKNEQELLELDK	28	N39	22.3	NA	>2000	>2000	>2000
P-50	YTSLIEELIKKSEEQKKNEEELKKLEK	28	N39	58.3	43.6	35.6 ± 1.3	191.1 ± 57.2	21.8 ± 3
P-51	LEANIEELLKKAEEQKKNEEELKKLEK	28	N39	46.2	45.1	10.2 ± 0.7	24.9 ± 2.9	45.4 ± 10.7
P-52	WEQKIEELLKKAEEQKKNEEELKKLEK	28	N39	47.6	61.1	0.9 ± 0.1	1.9 ± 0.6	1.9 ± 0.4
P-54	YTSLIEELIKKSEEQKKNEEELKK	25	N39	21.9	NA	>750	>750	>750
P-55	WEQKIEELLKKAEEQKKNEEELKK	25	N39	37.4	42.1	14.2 ± 1.8	16.9 ± 2	32.3 ± 3.9
P-57	WEQKIEELLKKAEEQKKNEEELKK	23	N39	19	NA	>750	>750	>750
P-58	WEQKIEELLKKAEEQKKNEEK	21	N39	19	NA	>750	>750	>750
P-59	WEQKIEELLKKAEEQKKK	18	N39	17.8	NA	>750	>750	>750
P-60	SLIEELIKKSEEQKKNEEELKKLEK	26	N39	37.9	35.1	>750	591.4 ± 65.2	>750
P-62	EQKIEELLKKAEEQKKNEEELKKLEK	27	N39	52.7	48.1	25.3 ± 2.6	44.2 ± 2.6	22.1 ± 3.9
P-63	QKIEELLKKAEEQKKNEEELKKLEK	26	N39	46.8	46.1	16.1 ± 2.5	24 ± 2.2	24.4 ± 2.8
P-64	KIEELLKKAEEQKKNEEELKKLEK	25	N39	54.3	48.1	7.6 ± 1.3	9.3 ± 0.6	10.4 ± 2.8
P-65	IEELLKKAEEQKKNEEELKKLEK	24	N39	55.3	46.1	17.9 ± 2.2	25.7 ± 1.3	25.8 ± 1.9
P-69	IEELLKKAEEQKKNEEELKK	21	N39	36.6	NA	>750	>750	>750
P-70	INNYTSLIEELIKKSEEQKKNEEELKKLEK	31	N39	52.8	48.1	8.5 ± 0.5	36.5 ± 6.4	49.7 ± 5.7
P-71	IEEYTKKIEELIKKSEEQKKNEEELKKLEK	31	N39	53.8	49.1	2.1 ± 0.2	9.1 ± 3.2	38.7 ± 7.6
P-72	VRYLEANIEELLKKAEEQKKNEEELKKLEK	31	N39	51.9	52	4.1 ± 0.9	16.2 ± 3.4	15.9 ± 1.2
P-73	VEELEKKIEELLKKAEEQKKNEEELKKLEK	31	N39	49.9	58.1	2 ± 0.2	5.6 ± 0.7	8.2 ± 1.3
P-74	WEEWEKKIEEYTKKIEELIKKSEEQKKNEEELKKLEK	38	N44	100.3	>98	0.4 ± 0.1	0.8 ± 0.1	1.4 ± 0.4
P-75	EMTWEEWEKKIEEYTKKIEELIKKSEEQKKNEEELKKLEK	41	N44	95.2	>98	0.4 ± 0.1	0.9 ± 0.2	0.6 ± 0.1

FIG 6 Structural and functional characterization of template peptides. The α -helicity and thermostability of diverse template peptides were determined by CD spectroscopy, with the final concentration of each peptide at 10 μ M in PBS. The inhibitory activities of peptides on HIV-1_{HXB2} Env-mediated cell-cell fusion, HIV-1_{NL4-3} pseudovirus entry, and HIV-1_{JRCSF} infection were determined. The representative peptides and their corresponding results are marked in blue. The antiviral experiments were performed in triplicate and repeated three times. Data are expressed as means $\pm$ standard deviations (SD).

LP-52 and its truncated versions are extremely potent inhibitors of divergent HIV-1 subtypes. We next sought to determine the inhibitory activities of LP-52 and several truncated lipopeptides (LP-55, LP-61, LP-64, LP-65, and LP-69) on divergent HIV-1 subtypes. Here, T-20 and P-52 were also included as controls. A large panel of primary HIV-1 Envs that represent the genetic and antigenic diversities of the global AIDS epidemic, including subtypes A (3), B (8), B' (4), C (7), G (1), A/C (1), A/E (5), and B/C (6), were assembled to generate pseudoviruses (Table 1). In a single-cycle infection assay, LP-52 inhibited various HIV-1 pseudotypes bearing Envs from primary HIV-1 isolates, with a mean IC₅₀ of 17 pM, which was 2,050-fold lower than that of T-20 (34,858 pM) and 1,208-fold lower than that of P-52 (20,541 pM). Consistently, the truncated inhibitors LP-55, LP-61, LP-64, and LP-65 maintained highly potent activities, as indicated by their mean IC₅₀s at 34, 144, 58, and 35 pM, respectively. The minimum inhibitor LP-69 had a mean IC₅₀ of 1,391 pM, which was 24-fold greater than that of T-20. Compared to LP-52, the C-terminally truncated LP-55 and the N-terminally truncated LP-65 showed only about 2-fold decreases in potencies, but both C- and N-terminally truncated LP-69 showed 81-fold decreased potency, suggesting that either the WEQ motif or the LEK motif is required to generate the superpotent inhibitors. Therefore, these results demonstrate that LP-52 is the most potent inhibitor against divergent HIV-1 subtypes, but its truncated versions LP-55 and LP-65 also possess extremely high potencies.

LP-52 is the most potent inhibitor of T-20-resistant mutant viruses, and both its N- and C-terminal motifs play critical roles. We previously reported that LP-50 and LP-51 were potent inhibitors of HIV-1 mutants conferring high resistance to T-20 and LP-40. Here, we were interested to know the activities of LP-52 and its truncated versions. Thus, T-20-sensitive and -resistant pseudoviruses were constructed and used in single-cycle infection assays. As shown in Table 2, LP-52 showed notable improvements over LP-50 and LP-51 in inhibiting both the wild-type NL4-3 that carried a natural T-20-resistant mutation (G36D) and a panel of T-20-resistant mutants. For example, LP-52 inhibited NL4-3_{I37T} with an IC₅₀ of 0.01 nM, which was 40-, 106-, and 61,848-fold

TABLE 1 Inhibitory activities of LP-52 and its truncated lipopeptides on divergent HIV-1 subtypes^a

Primary Env	Subtype	Mean IC ₅₀ ± SD (pM)							
		T-20	P-52	LP-52	LP-55	LP-61	LP-64	LP-65	LP-69
92RW020	A	7,671 ± 127	7,854 ± 163	29 ± 8	41 ± 14	122 ± 7	67 ± 4	17 ± 7	2,269 ± 166
92UG037.8	A	3,413 ± 240	9,257 ± 414	18 ± 4	20 ± 8	96 ± 6	38 ± 4	20 ± 5	419 ± 16
398-F1_F6_20	A	18,150 ± 1,749	13,819 ± 1,719	7 ± 5	11 ± 1	72 ± 27	8 ± 2	6 ± 1	590 ± 39
PVO	B	70,820 ± 1,595	46,294 ± 4,712	31 ± 4	122 ± 8	91 ± 15	43 ± 13	25 ± 1	1,922 ± 262
pREJO4541	B	56,356 ± 1,948	74,401 ± 372	6 ± 1	36 ± 11	169 ± 43	21 ± 2	18 ± 3	1,904 ± 14
SF162	B	22,041 ± 6,310	19,951 ± 3,628	16 ± 4	25 ± 2	48 ± 4	102 ± 3	30 ± 3	337 ± 30
JRFL	B	9,310 ± 441	20,839 ± 269	57 ± 8	45 ± 10	51 ± 11	73 ± 14	43 ± 8	230 ± 29
SC422661.8	B	16,288 ± 262	6,155 ± 735	8 ± 2	51 ± 12	149 ± 21	42 ± 9	52 ± 11	2,112 ± 86
AC10.0.29	B	3,171 ± 460	15,349 ± 3,116	10 ± 1	8 ± 2	22 ± 4	51 ± 20	7 ± 2	280 ± 15
TRO.11	B	6,612 ± 618	64,530 ± 7,414	18 ± 3	20 ± 1	273 ± 47	29 ± 3	31 ± 3	2,065 ± 119
X2278_C2_B6	B	5,823 ± 230	2,315 ± 388	6 ± 3	1 ± 0	95 ± 21	19 ± 4	12 ± 2	483 ± 22
B01	B'	86,817 ± 3,349	47,825 ± 1,940	12 ± 1	19 ± 2	1234 ± 270	73 ± 6	71 ± 18	2,915 ± 147
B02	B'	9,823 ± 763	23,081 ± 530	16 ± 4	35 ± 5	113 ± 12	90 ± 17	24 ± 5	1,343 ± 33
B04	B'	5,501 ± 207	9,958 ± 598	8 ± 2	28 ± 9	156 ± 34	72 ± 21	25 ± 3	2,414 ± 219
43-22	B'	26,933 ± 5,255	4,105 ± 85	6 ± 1	2 ± 0	25 ± 5	35 ± 5	14 ± 2	269 ± 89
Du156	C	15,089 ± 927	9,142 ± 598	5 ± 1	15 ± 2	40 ± 5	15 ± 2	5 ± 1	448 ± 23
ZM53 M.PB12	C	24,268 ± 2,179	10,597 ± 1,218	15 ± 2	21 ± 3	92 ± 16	116 ± 12	27 ± 2	1,536 ± 390
CAP210.2.00.E8	C	131,894 ± 12,407	38,487 ± 1,763	23 ± 3	223 ± 33	286 ± 21	72 ± 20	100 ± 7	1,806 ± 118
CAP45.2.00.G3	C	131,799 ± 14,374	25,568 ± 2,133	9 ± 4	6 ± 1	9 ± 1	20 ± 1	6 ± 1	198 ± 39
CE703010217_B6	C	41,842 ± 7	40,667 ± 3,431	9 ± 2	27 ± 6	127 ± 3	41 ± 5	18 ± 5	2,138 ± 69
HIV_25710-2.43	C	13,851 ± 174	39,309 ± 3,149	17 ± 1	46 ± 2	165 ± 14	47 ± 1	40 ± 4	1,672 ± 227
CE1176_A3	C	8,503 ± 148	12,510 ± 1,398	13 ± 1	12 ± 1	58 ± 10	47 ± 5	11 ± 1	1,061 ± 48
X1632-S2-B10	G	13,896 ± 2,342	16,334 ± 816	27 ± 5	5 ± 1	118 ± 25	44 ± 4	34 ± 1	1,693 ± 89
246_F3_C10_2	A/C	37,650 ± 2,087	8,986 ± 226	12 ± 1	11 ± 1	40 ± 7	64 ± 17	16 ± 3	238 ± 20
AE03	A/E	11,025 ± 1,915	4,612 ± 227	11 ± 1	6 ± 1	132 ± 9	54 ± 8	41 ± 10	647 ± 92
GX11.13	A/E	23,558 ± 1,894	6,498 ± 393	9 ± 1	14 ± 3	28 ± 3	58 ± 8	13 ± 1	576 ± 97
SHX335.24	A/E	44,513 ± 5,526	98,670 ± 7,823	16 ± 7	19 ± 4	48 ± 5	46 ± 16	12 ± 1	345 ± 45
CNE8	A/E	26,929 ± 3,027	5,655 ± 504	7 ± 1	13 ± 2	589 ± 75	112 ± 10	316 ± 17	1,165 ± 23
CNE55	A/E	23,512 ± 2,297	10,132 ± 671	12 ± 4	2 ± 0	33 ± 13	16 ± 3	11 ± 3	236 ± 50
CH64.20	B/C	32,282 ± 1,431	6,931 ± 106	27 ± 1	28 ± 4	41 ± 2	75 ± 25	33 ± 2	258 ± 12
CH070.1	B/C	164,035 ± 1,016	46,517 ± 3,953	42 ± 6	114 ± 16	205 ± 42	189 ± 17	42 ± 9	3,138 ± 9
CH110	B/C	32,398 ± 1,969	2,993 ± 92	13 ± 3	16 ± 7	46 ± 2	34 ± 6	8 ± 1	674 ± 23
CH119.10	B/C	14,754 ± 4,581	3,529 ± 155	18 ± 0	8 ± 1	29 ± 6	103 ± 3	23 ± 2	326 ± 4
CH120.6	B/C	49,844 ± 2,400	20,797 ± 902	11 ± 2	98 ± 33	97 ± 12	49 ± 12	25 ± 2	1,590 ± 611
BJOX002000.03.2	B/C	29,664 ± 4,728	12,222 ± 1,304	35 ± 6	42 ± 2	132 ± 14	61 ± 2	36 ± 5	1,337 ± 342
Mean		34,858	20,541	17	34	144	58	35	1,391

^aThe assay was performed in triplicate and repeated three times.

more potent than LP-51 (0.4 nM), LP-50 (1.06 nM), and T-20 (618.48 nM), respectively; LP-52 inhibited NL4-3_{V38A/N42T} with an IC₅₀ of 1.22 nM, which was 9-, 62-, and >1,844-fold more potent than LP-51 (10.54 nM), LP-50 (75.21 nM), and T-20 (>2,250 nM), respectively. In sharp contrast, the C-terminally truncated LP-55, the N-terminally truncated LP-64 and LP-65, and the C- and N-terminally truncated LP-69 exhibited dramatically decreased activities in inhibiting diverse T-20-resistant viruses.

HIV-1 and HIV-2/SIV share only ~50% genetic similarity, including the sequences across the peptide binding site in the NHR of gp41 (40). Therefore, we further examined the inhibitory activities of LP-52 and truncated lipopeptides on several HIV-2 and SIV isolates. As shown in Table 2, LP-52 was also an extremely potent inhibitor of two HIV-2 isolates (HIV-2_{ROD} and HIV-2_{ST}) and two SIV isolates (SIV₂₃₉ and SIV_{PBJ}), with IC₅₀s of 0.06, 0.34, 0.01, and 0.04 nM, respectively. Interestingly, LP-55 and LP-69 exhibited dramatically reduced activities in inhibiting HIV-2 and SIV isolates, with IC₅₀s at comparable levels, whereas LP-64 and LP-65 retained activities similar to those of LP-52. These results suggested that the C-terminal LEK motif critically determines the activity of LP-52 in the inhibition of HIV-2 and SIV isolates. Combined, these results demonstrate that LP-52 is a highly potent inhibitor of T-20-resistant mutants and that both its N- and C-terminal motifs are critical determinants.

LP-52 can efficiently bind and accumulate in the target cell membrane. We also conducted experiments to verify the binding capacity of LP-52 with the target cell

TABLE 2 Inhibitory activity of LP-52 and its truncated lipopeptides on T-20-resistant HIV-1 mutants and HIV-2 and SIV isolates^a

Env	Mean IC ₅₀ ± SD (nM)							
	T-20	LP-50	LP-51	LP-52	LP-55	LP-64	LP-65	LP-69
T-20 sensitive								
HIV-1								
NL4-3 _{D36G}	9.12 ± 0.58	<0.01	<0.01	<0.01	<0.01	0.04 ± 0	<0.01	0.31 ± 0.01
T-20 resistant								
HIV-1								
NL4-3 _{WT}	90.18 ± 1.52	0.06 ± 0.01	0.02 ± 0	<0.01	0.11 ± 0	0.08 ± 0.02	0.15 ± 0.03	2.01 ± 0.22
NL4-3 _{I37T}	618.48 ± 71.35	1.06 ± 0.24	0.40 ± 0.04	0.01 ± 0	1.65 ± 0.2	1.43 ± 0.15	1.04 ± 0.11	41.41 ± 0.28
NL4-3 _{V38A}	708.62 ± 21.46	8.94 ± 1.2	1.24 ± 0.44	0.37 ± 0.02	4.77 ± 0.67	17.66 ± 1.62	9.63 ± 1.13	157.47 ± 15.62
NL4-3 _{V38 M}	1,026.82 ± 29.71	1.53 ± 0.07	0.41 ± 0.1	0.20 ± 0.02	3.05 ± 0.35	8.92 ± 1.15	6.96 ± 2.27	89.86 ± 10.57
NL4-3 _{Q40H}	1,456.5 ± 153.6	4.22 ± 0.09	0.55 ± 0.04	0.1 ± 0.01	8.2 ± 1.16	8.96 ± 1.19	5.59 ± 0.91	631.03 ± 31.58
NL4-3 _{N43K}	729.12 ± 79.37	9.81 ± 0.78	1.03 ± 0.21	0.31 ± 0.01	23.44 ± 0.99	21.71 ± 0.58	5.51 ± 0.52	1,255.27 ± 226.28
NL4-3 _{G36S/V38 M}	466.42 ± 36.48	1.53 ± 0.07	0.48 ± 0.03	0.13 ± 0.02	1.18 ± 0.18	8.54 ± 0.99	3.57 ± 0.58	22.57 ± 3.15
NL4-3 _{I37T/N43K}	>2,250	136.12 ± 5.32	12.07 ± 1.61	1.86 ± 0.21	189.34 ± 9.43	140.75 ± 1.77	88.63 ± 17.67	12,130 ± 345.47
NL4-3 _{V38A/N42T}	>2,250	75.21 ± 8.22	10.54 ± 1.29	1.22 ± 0.09	44.81 ± 5.65	115 ± 11.82	69.93 ± 4.73	1,815.67 ± 478.35
HIV-2/SIV								
HIV-2 _{ROD}	263.68 ± 14.96	0.09 ± 0.01	0.09 ± 0	0.06 ± 0	137.77 ± 16.95	0.23 ± 0.04	0.11 ± 0.02	152.28 ± 58.37
HIV-2 _{ST}	829.01 ± 65.71	5.81 ± 0.32	0.84 ± 0.11	0.34 ± 0.05	10.67 ± 1.93	0.75 ± 0.12	0.43 ± 0.1	9.83 ± 1.6
SIV ₂₃₉	490.8 ± 24.5	0.06 ± 0.01	0.03 ± 0.01	0.01 ± 0	0.19 ± 0.11	0.10 ± 0.01	0.07 ± 0.01	0.56 ± 0.03
SIV _{PBJ}	648.49 ± 16.75	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0	10.61 ± 0.75	0.11 ± 0.01	0.04 ± 0	11.91 ± 3.67

^aThe assay was performed in triplicate and repeated three times.

membrane. First, LP-52 and several control inhibitors were preincubated with TZM-b1 cells, followed by thorough washing to remove unbound peptides, and the virus was then added to initiate infection. The antiviral activities of the residual inhibitors that survived the washing steps were measured by HIV-1_{NL4-3} pseudovirus-based single-cycle infection assays. As shown in Fig. 7A, the inhibitory activity of LP-52 could be largely sustained; in sharp contrast, the unconjugated peptides P-52, T-20, C34, and 2P23 dramatically reduced their activities after the washing. Further, the stability of the membrane-bound LP-52 was functionally examined by determining its sustained anti-HIV activities at different time points after washes. Promisingly, LP-52 fully retained its anti-HIV activity, even though infection was initiated after washing steps over 24 h (Fig. 7B and C).

To physically visualize LP-52 that bound to the cell membrane, we labeled LP-52 and P-52 with fluorescein isothiocyanate (FITC). Similar to the functional assays described above, the resulting FITC-LP-52 and FITC-P-52 were preincubated with target cells, followed by thorough washing, and their membrane-binding abilities were observed by fluorescence microscopy. As shown in Fig. 8A, FITC-LP-52 accumulated in the cell surface in a dose-dependent manner, whereas FITC-P-52 could not be observed at a concentration as high as 50 μM. Similarly, the membrane-bound LP-52 was highly stable over 24 h (Fig. 8B). Therefore, LP-52 can maintain its extremely potent and long-lasting anti-HIV activity through binding with the target cell membrane.

LP-52 can efficiently bind to HSA. A lipopeptide is considered to be able to interact with human serum albumin (HSA), thus improving its *in vivo* stability, but little information is available for HIV fusion inhibitors. Here, we also analyzed the binding ability of LP-52 with HSA by two methods. First, FITC-LP-52 and FITC-P-52 were directly added to an ELISA plate that was precoated with HSA. After thorough washing, the fluorescence intensities of sustained FITC-labeled inhibitors were measured. As shown in Fig. 9A, FITC-LP-52 bound to HSA in a dose-dependent manner, whereas FITC-P-52 was largely removed by washing. Second, we determined the binding abilities of LP-52 and P-52 with HSA by an ELISA-based method, in which a mouse monoclonal antibody reacting with both LP-52 and P-52 was developed and used for detection. As shown in Fig. 9B, LP-52 showed dose-dependent binding, but P-52 had no appreciable binding function. Taken together, our results demonstrated that the lipid moiety can mediate the binding abilities of LP-52 with both the target cell membrane and HSA, which

FIG 7 Binding ability of LP-52 with the target cell membrane. (A) LP-52 and control inhibitors were preincubated with TZM-b1 cells, followed by thorough washes, and their sustained antiviral activities were measured by an HIV-1_{NL4-3} pseudovirus-based single-cycle infection assay. (B) The sustained antiviral potency of LP-52 bound to the target cells was determined at different time points after washes. (C) Percentages of inhibition of LP-52 administered with the wash and with no wash were compared. LP-52, P-52, T-20, C34, and 2P23 were administered at 10, 10, 200, 40, 10 nM, respectively. The experiments were performed three times, and data are expressed as means $\pm$ standard deviations (SD).

would render a dramatically increased virus-inhibitory activity and an extended *in vivo* half-life.

LP-52 has an extremely high therapeutic selectivity index and genetic resistance barrier. We also evaluated the cytotoxicity of T-20 derivatives, including T-20, LP-40, LP-50, LP-51, and P-52. As shown in Table 3, all the inhibitors obtained a very high 50% cytotoxic concentration (CC_{50}) in each of three cell lines (TZM-bl, MT-4, and HEK293T) and human peripheral blood mononuclear cells (PBMC), suggesting their extremely high therapeutic selectivity indexes (CC_{50}/IC_{50} ratio). As noticed, LP-40 and the template peptide P-52 demonstrated less cytotoxic effects than T-20, but addition of the C16 fatty acid group resulted in slightly increased cytotoxicity, in a comparison of their CC_{50} values.

To gain insight into the mechanisms of action of our newly designed inhibitors and their resistance profiles, we also dedicated our efforts to select HIV-1 mutants

FIG 8 Visualization of LP-52 bound to the target cell membrane. (A) Different concentrations of FITC-labeled LP-52 (FITC-LP-52) and P-52 (FITC-P-52) were preincubated with TBM-b1 cells for 30 min, followed by washes, and the fluorescence intensities of membrane-bound inhibitors were observed under a confocal microscope. Results for FITC-P-52 used at 1 μ M (a) and 50 μ M (b) and FITC-LP-52 used at 1 μ M (c), 2 μ M (d), 5 μ M (e), 10 μ M (f), 25 μ M (g), and 50 μ M (h) are shown. (B) FITC-LP-52 and FITC-P-52 at 10 μ M were preincubated with TBM-b1 cells, followed by washes, and the fluorescence intensities of membrane-bound inhibitors were observed at different time points after the washes. Results for FITC-P-52 at 5 min (a) and 24 h (b) after washes and FITC-LP-52 at 5 min (c), 10 min (d), 25 min (e), 1.5 h (f), 12 h (g), and 24 h (h) after washes are shown.

resistant to LP-50, LP-51, and LP-52, but so far our experiments have failed. Indeed, it was considerably difficult to passage the virus (HIV-1_{NL4-3}) on MT-4 cells in the presence of such potent inhibitors at a concentration of greater than 100 pM; however, the concentration of the control peptide T-20 could be easily increased to as high as 10,000 nM. Taken together, these results suggest that the new inhibitors

FIG 9 Binding ability of LP-52 with human serum albumin. (A) Different concentrations of FITC-LP-52 and FITC-P-52 were incubated with human serum albumin (HSA) precoated in the ELISA wells, followed by washes, and the fluorescence intensity levels were directly measured by a fluorescence reader. (B) Different concentrations of LP-52 and P-52 were incubated with precoated HSA, followed by washes, and bound inhibitors were detected by a mouse anti-LP-52/P-52 monoclonal antibody and HRP-conjugated anti-mouse IgG.

possess extremely low cytotoxicity and high genetic barriers to inducing drug resistance.

DISCUSSION

In the present study, we first developed LP-52, a novel lipopeptide membrane fusion inhibitor with extremely potent inhibitory activity against divergent HIV-1, HIV-2, and SIV isolates. Then, we focused on exploiting the structure-activity relationships (SAR) of the T-20 derivatives to gain insights into their mechanisms of action. While a core sequence structure as illustrated by LP-69 was identified to be essential, addition of the gp41 pocket-binding domain (PBD) could not increase rather than reduce the antiviral capacities of new inhibitors, suggesting the target sites of viral vulnerability to inhibition. We also demonstrated that both the N- and C-terminal motifs of LP-52 played critical roles in inhibiting T-20-resistant HIV-1 mutants and HIV-2 and SIV isolates. Also, importantly, LP-52 was verified to have abilities to bind the target cell membrane and human serum albumin at a dose-dependent manner, and it exhibited extremely low cytotoxicity and a high genetic barrier to resistance. Therefore, this study is very

TABLE 3 Cytotoxicity of T-20 derivatives^a

Inhibitor	CC ₅₀ ± SD (μM)			
	TZM-bl cells	MT-4 cells	HEK293T cells	PBMC
T-20	159.87 ± 8.79	171.73 ± 23.13	216.23 ± 33.08	81.23 ± 4.59
LP-40	800.8 ± 62.29	366.3 ± 119.08	408 ± 40.1	212.6 ± 25.71
LP-50	106.4 ± 2.72	138.37 ± 6.55	166.77 ± 35.33	40.01 ± 4.63
LP-51	434.03 ± 90.38	135.07 ± 32.3	130.07 ± 15.27	46.83 ± 4.65
LP-52	112.9 ± 2.21	94.06 ± 4.97	88.86 ± 6.68	114.8 ± 12.68
LP-61	193.63 ± 13.92	164.47 ± 37.69	140.43 ± 10.7	46.43 ± 2.6
LP-65	256.23 ± 14.58	213.4 ± 59.59	208.43 ± 22.9	49.95 ± 2.97
P-52	916.6 ± 42.46	770.63 ± 131.46	649.07 ± 37.03	97.19 ± 20.46

^aThe assay was performed in triplicate and repeated three times.

significant for developing novel anti-HIV drugs and for understanding the mechanisms of viral fusion and inhibition.

Like many other studies, we previously focused on the PBD sequence to develop HIV fusion inhibitor peptides. In the early stage, we discovered that the sequences preceding the PBD can dramatically enhance the binding and inhibition of a CHR-derived C peptide (26, 41). Later, we identified the M-T hook structure that critically determined both the binding and antiviral activities (42–47), and with it, we designed short peptides and their lipid derivatives, which specifically target the gp41 pocket site and possess greatly improved anti-HIV activities and *in vivo* half-lives (27–29, 35, 36). However, we have also been attracted by the mechanisms of T-20, which are complicated by its target sites located at the NHR helix, the CHR helix, the fusion peptide, and the transmembrane domain (TMD) of gp41 (13, 48–50), as well as the coreceptor binding site of gp120 (50–52). Thus, we have also dedicated our efforts to explore the structural and functional characteristics of T-20. For the first time, we determined the crystal structures of T-20 and its lipid derivative LP-40 in complexes with a target mimic peptide, which revealed their critical binding motifs underlying the mechanisms of action (38). We are also working to develop T-20 sequence-based fusion inhibitors in terms of their different binding sites and inhibitory modes compared to pocket-binding-based inhibitors. It was very surprising that two T-20 lipopeptide derivatives (LP-50 and LP-51) could inhibit divergent HIV-1 isolates at extremely low picomolar IC_{50} s and suppress viral replication at undetectable levels in SHIV-infected rhesus monkey models. In this study, we were again surprised by LP-52, which showed further improvements in both binding stability and anti-HIV activity. To our knowledge, LP-52 is the most potent and broadly active HIV-1/2 and SIV fusion inhibitor reported to date.

It is very exciting to review the design process of LP-52. Deletion of the C-terminal tryptophan-rich motif (TRM) from T-20 fully abolished its binding and inhibitory activity, as indicated by P-40; however, replacement of the TRM with a fatty acid group resulted in LP-40 with prominent improvements. Further, LP-50 was generated by introducing the intrahelical salt bridge-facilitated amino acids into the solvent-accessible sites of LP-40, and LP-51 was generated by incorporating the HIV-2/SIV-derived amino acids into the target binding positions of LP-50. From the structural insights of a T1249-based lipopeptide (LP-46), the present study delivered LP-52 by optimizing the N-terminal sequence of LP-51. Independently, the binding and anti-HIV activities of the unconjugated template peptides could be sharply improved by sequence optimization, as indicated by P-40, P-50, P-51, and P-52. Even though it was shortened by 8 amino acids, P-52 possessed much higher potent activities than T-20 in the inhibition of viral fusion, entry, and infection. By considering these findings together, we realize that the design of T-20 sequence-based superpotent inhibitors requires both lipid conjugation and sequence optimization strategies, which synergistically coordinate the potent anti-HIV activity. From T-20 or its inactive version, P-40, to the extremely potent LP-52, the design process represents a huge success and an encouraging story.

In this study, the structural and functional relationships of T-20 derivatives have been comprehensively characterized by a large panel of conjugated and unconjugated peptides. A core sequence structure that critically determines binding stability and antiviral activity was identified, and it has only 21 amino acids, as indicated by LP-69. Obviously, the presence of either the WEQK motif in the N terminus (LP-55) or the LEK motif in the C terminus (LP-65) was important to maintain the extremely high potency of LP-52, and both of these motifs were required in the efficient inhibition of T-20-resistant HIV-1 mutants as well as HIV-2 and SIV isolates. More meaningfully, our studies found that the pocket-binding ability is not necessary for this class of inhibitors, because the addition of pocket-binding sequences did not increase but rather decreased their anti-HIV activities, even if the binding stabilities could be dramatically enhanced, as demonstrated by LP-74 and LP-75. In comparison, short-peptide inhibitors that mainly target the gp41 pocket site (LP-11, LP-19) as well as C34- and T1249-based inhibitors that contain the PBD sequence did have extremely high binding stabilities, but their anti-HIV activities were significantly lower than that of diverse T-20 derivatives.

Therefore, our studies imply that the site of viral vulnerability to inhibition is located upstream of the NHR helices and pocket-binding ability is not necessary to produce such potent fusion inhibitors.

The relationships between the membrane-anchoring lipopeptides and unconjugated template peptides have also provided insights into the structural and functional properties of new inhibitors. Obviously, while the antiviral activity of lipopeptides was attenuated by the addition of the PBD sequence, the N-terminally extended template peptides exhibited significantly increased potencies, as indicated by P-74 and P-75. Interestingly, an N-terminal lysine residue might interfere with the binding and inhibition of LP-64, as its deletion resulted in more active LP-65; however, an inverse effect was observed between the templates P-64 and P-65. Similar to the relationship of LP-40 and P-40, while LP-69 maintained a high antiviral potency (with its IC_{50} of ~ 1 nM), its template, P-69, had no detectable inhibitory activity. These results suggest that membrane-anchoring lipopeptides and unconjugated peptides possess different mechanisms of action that need to be characterized in more detail. To exploit the mechanisms of action of lipopeptide inhibitors, several previous studies analyzed their cell membrane-binding functions by applying functional assays (30, 31). In addition, the present studies have offered direct evidence for the accumulation of LP-52 on the cell membrane by physically visualizing FITC-labeled inhibitors. It is also the first time that the binding ability of lipopeptide-based HIV fusion inhibitors with human serum albumin has been validated. Combined with our characterization of the cytotoxicity and genetic barriers to inducing resistance of these inhibitors, all of the results will help in the development of novel drugs that target the viral membrane fusion step. We believe that the new inhibitors reported here would circumvent some limitations inherent to T-20 therapy, such as a frequent high dosage, drug resistance, and injection site reactions.

MATERIALS AND METHODS

Cell lines and reagents. The following reagents were obtained through the AIDS Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl cells, which stably express CD4 and CCR5 along with endogenously expressed CXCR4, from John C. Kappes and Xiaoyun Wu; HL2/3 cells, which contain stably integrated copies of the HIV-1 molecular clone HXB2/3gpt that express high levels of HIV Gag, Env, Tat, Rev, and Nef proteins, from Barbara K. Felber and George N. Pavlakis; the Panel of Global HIV-1 Env Clones from David Montefiori; a panel of infectious HIV-1 molecular clones, including pNL4-3 from Malcolm Martin, pLAI.2 from Keith Peden, pSG3.1 from Sajal Ghosh, Beatrice Hahn, and George Shaw, pYK-JRCSF from Irvin SY Chen and Yoshio Koyanagi, and p89.6 from Ronald G. Collman; the infectious HIV-2 ST molecular clone from Beatrice Hahn and George Shaw; the infectious HIV-2 ROD molecular clone (pROD10) from the Centre for AIDS Reagents, NIBSC, United Kingdom. Two plasmids encoding SIV Envs (pSIVpbj-Env and pSIV239-Env) were kindly provided by Jianqing Xu at the Shanghai Public Health Clinical Center, Fudan University, China. HEK293T cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in complete growth medium that consisted of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and $1 \times$ MEM nonessential amino acids (Gibco/Invitrogen, USA) and were maintained at 37°C in 5% CO₂.

Peptide synthesis and lipid conjugation. Peptides were synthesized on rink amide 4-methylbenzhydrylamine (MBHA) resin using a standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) method, as described previously (36). For lipopeptides, each template peptide contains a lysine residue at its C terminus with a 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) side chain-protecting group, enabling the conjugation of a fatty acid group requiring a deprotection step in a solution of 2% hydrazinehydrate-*N,N*-dimethylformamide (DMF) (36); C34-Chol was produced by chemoselective thioether conjugation between the peptide precursor that has a C-terminal cysteine residue and the cholesterol derivative, as described previously (31). For fluorescein (FITC)-labeled peptides, FITC was conjugated to the N terminus of the peptides through a 6-amino-hexanoic acid (AHX) linker. All peptides were N-terminally acetylated and C-terminally amidated, and they were purified by reverse-phase high-performance liquid chromatography (HPLC) to more than 95% homogeneity, followed by characterization with mass spectrometry.

CD spectroscopy. The α -helicity and binding thermostability of a CHR-derived peptide or lipopeptide inhibitor with an NHR-derived target mimic peptide (N39, N44, or N36) were determined by circular dichroism (CD) spectroscopy, as described previously (47). Briefly, an inhibitor was diluted in phosphate-buffered saline (PBS; pH 7.2) or double-distilled, deionized water (ddH₂O) and incubated at 37°C for 30 min in the presence or absence of an equal molar concentration of an N peptide. CD spectra were acquired on a Jasco spectropolarimeter (model J-815) using a 1-nm bandwidth with a 1-nm step resolution from 195 to 270 nm at room temperature. Spectra were corrected by subtracting a solvent blank. The α -helical content was

calculated from the CD signal by dividing the mean residue ellipticity (θ) at 222 nm by the value expected for 100% helix formation ($-33,000 \text{ degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$). Thermal denaturation was performed by monitoring the ellipticity change at 222 nm from 20°C to 98°C at a rate of 2°C/min, and T_m (melting temperature) was defined as the midpoint of the thermal unfolding transition.

Cell fusion assay. The inhibitory activity of fusion inhibitors on HIV-1_{HXB2} Env-mediated cell-cell fusion was measured using a reporter gene assay based on the activation of an HIV long terminal repeat (LTR)-driven luciferase cassette in TZM-bl cells (target) by HIV-1 tat from HL2/3 cells (effector), as described previously (27). In brief, TZM-bl cells were plated in 96-well plates (1×10^4 /well) and incubated at 37°C overnight. Target cells were cocultured with HL2/3 cells (3×10^4 /well) for 6 h at 37°C in the presence or absence of a test peptide at graded concentrations. Luciferase activity was measured using luciferase assay reagents and a luminescence counter (Promega, Madison, WI, USA).

Single-cycle infection assay. The inhibitory activity of fusion inhibitors on divergent HIV-1 subtypes and two SIV isolates was determined by a pseudovirus-based single-cycle infection assay as described previously (53). Briefly, a pseudovirus was generated via the cotransfection of HEK293T cells with an Env-expressing plasmid and a backbone plasmid that contains the Env-defective, luciferase-expressing HIV-1 genome (pSG3Δenv). Culture supernatants were harvested 48 h after transfection, and 50% tissue culture infectious doses (TCID₅₀) were determined in TZM-bl cells. To measure the inhibitory activity of inhibitors, peptides were prepared in 3-fold dilutions, mixed with 100 TCID₅₀ of viruses, and then incubated for 1 h at room temperature. The mixture was added to TZM-bl cells (10^4 /well), and the cells were incubated for 48 h at 37°C. Luciferase activity was measured as described above.

Inhibition of replicative HIV-1 and HIV-2 isolates. The inhibitory activity of fusion inhibitors on a panel of replication-competent HIV-1 (NL4-3, LAI, SG3, JR-CSF, 89.6, R3A) and HIV-2 (ROD, ST) isolates was determined as described previously (35). Briefly, viral stocks were prepared by transfecting viral molecular clones into HEK293T cells. Culture supernatants were harvested 48 h posttransfection, and the TCID₅₀ in TZM-bl cells were quantified. One hundred TCID₅₀ of viruses were used to infect TZM-bl cells in the presence or absence of serially 3-fold diluted inhibitors. Cells were harvested 2 days postinfection and lysed in reporter lysis buffer, and luciferase activity was measured.

Visualization of LP-52 bound to the target cell membrane. TZM-bl cells (10^5 /well) were plated on coverslips and incubated at 37°C overnight. On the next day, fluorescein (FITC)-labeled LP-52 (FITC-LP-52) or P-52 (FITC-P-52) was diluted and added to the cells, followed by incubation at 37°C for 30 min. TZM-bl cells were then fixed with freshly prepared 4% paraformaldehyde for 15 min and washed three times with PBS. Images were captured with a laser confocal microscope (Leica Microsystems, Leica, Germany).

Binding ability of LP-52 with HSA. The binding ability of LP-52 with human serum albumin (HSA) was determined by two methods. First, 5% HSA in PBS was coated onto the wells of a 96-well microtiter plate (Costar, Corning, NY) and incubated at 4°C overnight. After washing with PBS, FITC-labeled LP-52 (FITC-LP-52) or P-52 (FITC-P-52) at graded concentrations was added and incubated at 37°C for 1 h. The wells were then washed three times with PBS, and the fluorescence intensity levels were directly measured by a Multimode Reader. Second, an enzyme-linked immunosorbent assay (ELISA) was established for detection. Briefly, 5% HSA in 0.1 M carbonate buffer (pH 9.6) was coated onto a 96-well polystyrene plate at 4°C overnight. After washing, LP-52 or P-52 was added to the wells and incubated at 37°C for 1 h, followed by three washes with PBS-T (PBS containing 0.1% Tween 20). Then, a mouse anti-LP-52/P-52 monoclonal antibody (developed in our laboratory) was added and incubated at 37°C for 1 h. After three washes with PBS-T, the bound antibodies were detected by horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma). The reaction was visualized by the addition of 3,3',5,5'-tetramethylbenzidine (TMB) substrate, and the absorbance at 450 nm (A_{450}) was measured by an ELISA plate reader (Bio-Rad).

Cytotoxicity of peptide and lipopeptide inhibitors. The cytotoxicity of fusion inhibitors on TZM-bl, MT-4, HEK293T, and human peripheral blood mononuclear cells (PBMC) was measured using a CellTiter 96 AQ_{ueous} One solution cell proliferation assay (Promega). In brief, 50-μl volumes of peptides at graded concentrations were added to cells, which were seeded on a 96-well tissue culture plate (1×10^4 cells per well). After incubation at 37°C for 2 days, 20 μl of CellTiter 96 AQ_{ueous} One solution reagent was pipetted into each well and further incubated at 37°C for 2 h. The absorbance was measured at 490 nm using a SpectraMax M5 microplate reader.

Selection of LP-52-resistant HIV-1 mutants. The *in vitro* selection of HIV-1 mutants resistant to fusion inhibitors was performed as described previously (54). HIV-1_{NL4-3} stocks were generated by transfecting HEK293T cells. MT-4 cells were seeded at 1×10^4 in RPMI 1640 medium containing 10% FBS on 12-well plates. The virus was used to infect the cells in the presence or absence of a diluted fusion inhibitor (LP-50, LP-51, LP-52 or T-20). Cells were incubated at 37°C with 5% CO₂ until an extensive cytopathic effect was observed. Culture supernatants were harvested and used for the next passage on fresh MT-4 cells with a 1.5- to 2-fold increase in peptide concentrations.

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