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Generation of a long-acting fusion inhibitor against HIV-1†

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AIDS has evolved from a fatal infectious disease to a manageable chronic disease under the treatment of anti-AIDS medications. HIV fusion inhibitors with high activity, low side effects and strong selectivity are promising drugs against HIV. Only one fusion inhibitor is currently approved, thereby highly active long-acting fusion inhibitors need to be developed for long-term AIDS treatment. Here, we synthesised MT-SC22EK (a small HIV fusion inhibitor) derivatives containing 1–2 staples to improve its stability. Antiviral activity studies showed that MT-SC22EK-2 with two staples exhibited potent inhibitory activity against HIV-1 standard strains and Chinese epidemic strains, and at the same time, MT-SC22EK-2 presented strong anti-T20 resistance. Surprisingly, MT-SC22EK-2 possessed excellent protease stability with a half-life of 3665 min. MT-SC22EK-2 is a potential HIV fusion inhibitor considered as a long-acting anti-HIV drug candidate.

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Introduction

The acquired immunodeficiency syndrome (AIDS) is a global epidemic caused by human immunodeficiency virus (HIV) attacking the human immune system, and then resulting in a progressive decline of immune function.¹ HIV can be divided into two main types, HIV-1 (the main causative agent for the spread of AIDS) and HIV-2, according to the difference in prevalent regions. In order to restrain the AIDS pandemic, scientists have made a great deal of effort to develop vaccines and drugs against HIV-1.² Compared to HIV-1 vaccines still in the research phase, anti-HIV drugs have become the main means for AIDS treatment. The advent of reverse transcriptase inhibitors and protease inhibitors has effectively controlled the HIV-1 levels of patients, thereby reducing the AIDS death rate.³ However, HIV-1 can't be completely eliminated from patients by using current anti-HIV-1 drugs.⁴ So AIDS has evolved from a fatal infectious disease to a manageable chronic disease. Furthermore, the long-term use of reverse transcriptase inhibitors and protease inhibitors has led to serious side effects and the emergence of resistant strains. HIV-1 fusion inhibitors as anti-HIV-1 drugs with a clear target, strong anti-virus activity, and high target selectivity overcome the shortcomings of the above two classes of drugs.⁵ T20 was approved by the FDA in 2003 as the first peptide fusion inhibitor,

but the shortcomings of T20 resistance and short half-life (requiring two injections a day) have limited its application in long-term treatment of AIDS.⁷ Therefore, highly active long-acting HIV-1 fusion inhibitors need to be developed to reduce the frequency of medication for improving the quality of life of AIDS patients in the current AIDS treatment.⁸

A number of new highly active peptide fusion inhibitors targeting gp41 (HIV-1 surface glycoprotein, regulating virus and cell fusion processes) are constantly discovered to improve bioactivity and resist T20 resistance.⁹ MT-SC22EK, a small fusion inhibitor with 24 amino acids, shows potent anti-HIV-1 activity, because it contains not only the α -helical structure stabilized by hydrogen bonds and salt bridges, but also the M-T hook structure, allowing MT-SC22EK to enhance its binding to the target.¹⁰ At present, there is no report on the half-life improvement of MT-SC22EK.

Replacing protease cleavage sites with D-amino acids (resistant to proteases) is a conventional method to improve the stability of peptides towards protease hydrolysis.¹¹ But this method is not suitable for the situation when the protease cleavage sites are the key amino acids involved in the target interaction, because the key amino acids replaced will diminish the bioactivity of peptides. Protease cleavage site protection is another important method that just overcomes the shortcomings of the previous strategy, using a given structure such as an α -helix macrocycle to protect the protease cleavage sites from exposure to protease.¹² An additional advantage of protease cleavage site protection is that it can also enhance the biological activity of the peptides.¹³ The all-hydrocarbon stapling strategy, developed by Verdine,¹⁴ is a representative of protease cleavage site protection. The main idea of

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stapling is the introduction of two non-natural amino acids containing a terminal olefin at the i , $i + 4$ or i , $i + 7$ positions of the peptide to give an all-hydrocarbon staple by ring closing olefin metathesis (RCM) reaction, allowing the peptides to form a stable α -helical structure. The stapling strategy has been used successfully to improve the protease stability of cancer-associated peptides,¹⁵ Wnt signaling pathway regulatory peptides,¹⁶ and HCV and HIV inhibitory peptides.¹⁷

In this study, we incorporated all-hydrocarbon staples into MT-SC22EK for the first time. We found that the insertion of staples could not only improve the anti-HIV-1 activity of MT-SC22EK but also significantly strengthen the stability of MT-SC22EK against protease. MT-SC22EK-2, an ultra-stable and highly active molecule, was obtained by insertion of two staples. This work provides a long-acting and highly active drug candidate for the future long-term treatment of AIDS.

Results and discussion

We utilized the stapling strategy to prolong the protease stability of the small HIV fusion inhibitor MT-SC22EK, making it a long-acting drug candidate (Fig. 1). The MT-SC22EK/T21 complex structure indicated that Met626, Trp631, Ile635, Tyr638, Ile642, Leu645, Ile646 and Ser649 in MT-SC22EK are the key amino acids which directly interacted with T21.¹⁰ In order to protect the key amino acids from protease degradation, we chose the positions near the key amino acids (633 & 637 and 640 & 644) to insert 1–2 staples. We designed the insertion of a single staple at the 640 & 644 or 633 & 637 position of MT-SC22EK to generate MT-SC22EK-1A and 1B, respectively, and the insertion of double staples at the position above MT-SC22EK to generate MT-SC22EK-2.

MT-SC22EK and three stapled MT-SC22EKs were prepared by using Fmoc solid-phase peptide synthesis (SPPS)¹⁸ on the 0.06 mmol scale. The synthetic route for preparing stapled MT-SC22EKs as MT-SC22EK-2 for example is shown in Fig. 2, while the synthetic routes for preparing the other peptides are available in the ESI.† Natural amino acids were incorporated into the peptide using HCTU and DIPEA as coupled reagents, while side chains carrying alkene amino acids (Fmoc-S₅-OH) were incorporated using HATU, HOAt and DIPEA as

coupled reagents. All synthetic peptides were amidated at the C-terminus and acetylated at the N-terminus. After mass spectrometry identification and HPLC purification, white MT-SC22EK and stapled MT-SC22EKs were obtained with more than 95% purity (the isolated yield for MT-SC22EK 29%, MT-SC22EK-1A 20%, MT-SC22EK-1B 18%, and MT-SC22EK-2 14%).

To confirm whether the synthesized stapled MT-SC22EK peptides retained the ability to inhibit HIV-1 infection, we validated the half maximal inhibitory concentration (IC₅₀) of the peptides against HIV-1 pseudovirion standard strains and Chinese epidemic strains. CNE6, CNE11, CNE15, CNE30, CNE5 and CNE55 belong to the Chinese epidemic strains, while sf162, JRFL and HXB2 belong to the standard strains. After co-incubation with different concentrations of MT-SC22EK and stapled MT-SC22EK peptides, respectively, HIV-1 pseudovirions containing the luciferase reporter gene were used to infect Ghost(3)X4R5 cells. The ability of the HIV-1 pseudovirions to infect Ghost(3)X4R5 cells was assessed by measuring the reporter luciferase activity. The IC₅₀ values of inhibition of HIV-1 pseudovirion-infected cells by MT-SC22EK and stapled MT-SC22EK peptides are presented in Table 1. We found that MT-SC22EK exhibited nanomolar inhibitory activity against diverse HIV-1 variants, which was consistent with the view that it was a potent fusion inhibitor. Compared to MT-SC22EK, MT-SC22EK-2 had 2–48-fold enhanced inhibitory activity against 9 HIV-1 variants. However, MT-SC22EK-1A and 1B, respectively, exhibited 1–4-fold increased activity for CNE6, CNE5 and HXB2, and 1–5-fold for CNE15, CNE30, CNE5 and HXB2 compared to MT-SC22EK. MT-SC22EK-2 was the most active stapled MT-SC22EK inhibitor. These results demonstrated that the simultaneous insertion of double staples at the 633 & 637 and 640 & 644 positions could significantly improve the inhibitory activity of MT-SC22EK against HIV-1 pseudovirions, while the insertion of a single staple only at the 633 & 637 or 640 & 644 positions could improve its activity against a fraction of HIV-1 pseudovirions.

To understand why MT-SC22EK-2 was more potent than the unmodified peptide, we determined the three dimensional structure of the MT-SC22EK-2/T21 (NHR derived peptides from gp41) complex. High quality complex crystals were obtained by the sitting-drop vapor-diffusion method in 0.1 M magnesium chloride, 0.1 M HEPES sodium salt (pH 7.5) and 30% PEG 400 at 18 °C. In Fig. 3A, MT-SC22EK-2 formed a typical 6-HB structure with T21, indicating that the target of MT-SC22EK-2 was the gp41 NHR of HIV-1. As anticipated, both the 633 & 637 and 640 & 644 positions of MT-SC22EK-2 formed the all-hydrocarbon staples, which were exposed to the solvent exposure surface (Fig. 3A). These staples observed in the MT-SC22EK-2/T21 complex provided a structural basis for the formation of two correct staples in MT-SC22EK-2. To our knowledge, the MT-SC22EK-2/T21 complex structure is the first reported crystal structure containing double all-hydrocarbon staples.

To elucidate the impact of insertion of two staples into MT-SC22EK-2 on its binding to T21, we anchored MT-SC22EK

Fig. 1 Structural optimization of MT-SC22EK via hydrocarbon stapling.

Fig. 2 The synthetic route to MT-SC22EK-2. Grubbs I catalyst (579944) was purchased from Sigma-Aldrich.

Table 1 Inhibitory activity of MT-SC22EK and its stapled peptides against HIV-1 strains

Pseudovirus	Subtype	IC ₅₀ (nM)			
		MT-SC22EK	MT-SC22EK-1A	MT-SC22EK-1B	MT-SC22EK-2
CNE6	B'	10.3 ± 1.0	10.4 ± 2.4	12.5 ± 0.2	1.2 ± 0.2
CNE11	B'	5.9 ± 0.3	8.8 ± 0.1	7.2 ± 0.6	2.6 ± 0.8
CNE15	BC	7.5 ± 2.4	14.0 ± 1.0	3.3 ± 1.4	1.8 ± 0.8
CNE30	BC	22.3 ± 3.5	71.7 ± 27.9	16.2 ± 4.7	7.7 ± 0.9
CNE5	CRF01_AE	35.0 ± 12.0	27.7 ± 8.2	6.8 ± 0.6	3.6 ± 0.2
CNE55	CRF01_AE	12.9 ± 2.1	82.3 ± 2.6	20.3 ± 9.3	5.3 ± 1.7
sf162	B	2.9 ± 1.0	14.4 ± 5.6	3.3 ± 0.4	1.0 ± 0.1
JRFL	B	7.3 ± 6.9	8.7 ± 2.3	8.6 ± 1.5	0.6 ± 0.2
HXB2	B	2.4 ± 1.0	0.5 ± 0.0	0.4 ± 0.1	0.05 ± 0.03

Data are shown as means ± SD.

Fig. 3 (A) The 6-HB structure formed by MT-SC22EK-2/T21 (PDB ID: 5ZPW). The T21 trimer is in grey. MT-SC22EK-2 peptides are in green. (B) Comparison of the structures of MT-SC22EK-2 (green) and MT-SC22EK (cyan, PDB ID: 3VU6).

to the MT-SC22EK-2/T21 complex (Fig. 3B). Previous work has shown that the M-T hook structure of MT-SC22EK played a crucial role in the inhibitor interaction with its target. We also observed a hook structure formed by the Met626 and Thr627 in MT-SC22EK-2. Surprisingly, the side chain of Met626 in MT-SC22EK-2 was closer to Trp571 and Leu568 in T21 than that of MT-SC22EK. In addition, the conformations of the key amino acids Trp631, Tyr638, Leu645, Ile646 and Ser649 of MT-SC22EK-2 exhibited some observable differences, compared to those of MT-SC22EK. The insertion of staples into MT-SC22EK-2 could cause conformational

changes that promoted its interaction with the target, which would explain the enhanced inhibitory activity of MT-SC22EK-2 against HIV-1.

T20 resistance is a challenge that newly developed HIV-1 fusion inhibitors must solve.¹⁹ To investigate the effect that the insertion of double staples has on the activity of MT-SC22EK against T20-resistant HIV-1 variants, we tested the antiviral activities of MT-SC22EK-2 and MT-SC22EK using single and double mutant T20-resistant HIV-1NL-4-3 pseudovirions. As seen in Table 2, the IC₅₀ values of MT-SC22EK and MT-SC22EK-2 against nine mutant strains of HIV-1 NL-4-

3 pseudoviruses were 2.82–48.14 nM and 0.84–5.27 nM, respectively. These data implied that double staples could enhance the ability of MT-SC22EK to resist T20-resistant strains. A possible reason for the activity increase of MT-SC22EK-2 against T20-resistant strains was that the insertion of double staples caused the conformational changes to promote the interaction of MT-SC22EK-2 with T20-resistant strains. For the T20-sensitive D36G mutant strain, the inhibitory activities of MT-SC22EK and MT-SC22EK-2 were higher than that of the wild type, indicating that MT-SC22EK and MT-SC22EK-2 were insensitive to the D36G mutant. In addition, we found that the V38A-N42T and V38A mutant strains resulted in 0.5 and 0.8-fold resistance for MT-SC22EK-2, suggesting that MT-SC22EK-2 was sensitive to the V38A mutant while MT-SC22EK did not possess such a property. These results strongly supported that MT-SC22EK-2 is a potential anti-HIV-1 drug candidate to overcome the T20-resistance.

Having confirmed that the stapled MT-SC22EKs could effectively inhibit the HIV-1 pseudovirions, we next explored the protease stability of the stapled MT-SC22EKs. We chose chymotrypsin which could specifically recognize aromatic amino acids (Trp, Tyr and Phe) for protease digestion, according to the characteristics of the MT-SC22EK sequence. T20, MT-SC22EK and its stapled peptides (25 μ M) were respectively digested in a chymotrypsin-containing phosphate buffer (0.5 ng μ L⁻¹, pH 7.4) at 30 °C. The kinetic degradation curves of the five peptides were obtained by conducting HPLC analysis of the residual amount of the peptide at different time intervals (Fig. 4A). The half-life of T20, MT-SC22EK, MT-SC22EK-1A and 1B was calculated to be 17 min, 125 min, 495 and 705 min, respectively, while the value for MT-SC22EK-2 was 3665 min. The distinct difference in half-life among MT-SC22EK and its stapled peptides suggested that the insertion of staples into MT-SC22EK could significantly improve its chymotrypsin resistance. Compared to T20, the only approved HIV-1 peptide fusion inhibitor currently, MT-SC22EK-2 displayed a remarkable 216-fold enhancement of half-life. Such excellent stability made MT-SC22EK-2 a potential candidate for long-acting HIV-1 fusion inhibitors.

To explain why MT-SC22EK-2 has excellent chymotrypsin stability, we evaluated the α -helicity of MT-SC22EK and its

Fig. 4 (A) Degradation curves of T20, MT-SC22EK and its stapled peptides in the chymotrypsin digestion. (B) Circular dichroism spectra of MT-SC22EK and its stapled peptides in phosphate buffer (pH 7.4) at 25 °C.

stapled peptides using a Jasco-715 circular dichroism (CD) spectrometer. The final concentration of each sample was 50 μ M. The collected ellipticity of MT-SC22EK and its stapled peptides under far-UV irradiation is shown in Fig. 4B. MT-SC22EK and its stapled peptides all showed a strong positive absorption peak at 195 nm and two negative absorption peaks at 208 and 222 nm in the CD spectrum. The positions of these three absorption peaks were consistent with those of the characteristic absorption peaks of a typical α -helical structure,²⁰ indicating that MT-SC22EK and its stapled peptides formed an α -helical structure. The relative percent helicity of the four peptides at 222 nm was calculated using the helicity equation.²¹ The helicity value of MT-SC22EK, MT-

Table 2 Inhibitory activity of MT-SC22EK and its stapled peptides against T20-resistant HIV-1 variants

Pseudovirus	T20		MT-SC22EK		MT-SC22EK-2	
	IC ₅₀ (nM)	<i>n</i> -Fold	IC ₅₀ (nM)	<i>n</i> -Fold	IC ₅₀ (nM)	<i>n</i> -Fold
WT	209.4 ± 44.0	1	2.48 ± 0.33	1	1.58 ± 0.16	1
D36G	6.1 ± 2.4	0.03	5.77 ± 1.06	2.3	2.17 ± 0.83	1.4
I37T	636.0 ± 158.0	3	6.49 ± 2.51	2.6	2.51 ± 1.00	1.6
V38A	2219.3 ± 437.0	10.6	3.79 ± 1.23	1.5	1.33 ± 0.25	0.8
V38M	1182.3 ± 323.3	5.6	5.60 ± 0.84	2.3	1.64 ± 0.38	1.0
Q40H	1058.5 ± 264.3	5.1	2.82 ± 0.62	1.1	1.76 ± 0.16	1.1
N43K	1929.0 ± 311.9	9.2	48.14 ± 6.81	19	5.27 ± 0.44	3.3
I37T/N43K	>2250	>10.7	31.08 ± 13.83	13.0	3.43 ± 0.78	2.2
G36S/V38M	550.7 ± 54.9	2.6	8.53 ± 0.46	3.4	3.38 ± 0.80	2.1
V38A/N42T	>2250	>10.7	2.93 ± 0.81	1.2	0.84 ± 0.10	0.5

Data are shown as means ± SD. *n*-Fold values mean the ratio of peptides to T20-resistant and WT virus (HIV-1_{NL4-3}).

SC22EK-1A and 1B was 52.4%, 54.2% and 79.9%, respectively. Interestingly, the helicity value of MT-SC22EK-2 was calculated to be 103%, a two-fold increase compared to that of MT-SC22EK. The insertion of double staples at the 633 & 637 and 640 & 644 positions in MT-SC22EK significantly increased the helicity of the peptide. Comparing the α -helicity values of MT-SC22EK and its stapled peptides, it was found that the insertion of staples could improve the helicity of MT-SC22EK, and the number and position of staples could affect the helicity. We speculated that the superior helical structure protected the cleavage site of MT-SC22EK-2 from exposure to protease which slowed the kinetics of hydrolysis, resulting in significant protease resistance.

Conclusions

In this work, singly and doubly stapled MT-SC22EKs were synthesized by using the stapling strategy. Compared to MT-SC22EK and singly stapled MT-SC22EK, doubly stapled MT-SC22EK (MT-SC22EK-2) showed the highest anti-HIV-1 activity. The reason for the increased activity of MT-SC22EK-2 might be that the insertion of two staples leads to MT-SC22EK-2 forming a preferential conformation to strengthen the inhibitor–target interaction. Amazingly, MT-SC22EK-2 not only presented a higher ability against T20-resistant HIV-1 variants than MT-SC22EK but also displayed a markedly prolonged half-life of 3665 min in chymotrypsin digest. The above activity and stability data indicated that MT-SC22EK-2 would be a promising long-acting anti-HIV-1 drug candidate. Collectively, our studies and those of others^{15–17,22} on the insertion of staples into small HIV fusion inhibitors would provide a valuable reference for the future design and synthesis of long-acting inhibitors for other viruses.

Conflicts of interest

The authors declare no competing interests.

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