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Research strategies of the N-peptide fusion inhibitor: a promising direction for discovering novel antivirals

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ABSTRACT AIDS, caused by HIV-1, is a devastating condition that severely compromises the human immune system, often resulting in fatal consequences. The primary therapeutic approach for AIDS involves a combination of multiple agents, known as "cocktail therapy," aimed at maximizing and sustainably suppressing viral replication within patients. The ongoing discovery of novel compounds and the establishment of innovative research strategies have become the mandatory path to provide increasingly effective treatment options for AIDS. Peptide-based fusion inhibitors, exemplified as enfuvirtide, are able to target the six-helix bundle fusion core in HIV-1 envelope protein and function during the early stage of viral invasion. However, the prolonged and intensive use of enfuvirtide in clinical settings has posed significant challenges, including the emergence of drug resistance. N-peptide fusion inhibitors, whose sequences are different from enfuvirtide, exhibit potential anti-HIV-1 activity and inhibition of drug-resistant strains through the advanced coiled-coil conformation and are expected to serve as novel peptide inhibitors in the iteration of enfuvirtide. This paper provides a comprehensive summary of N-peptide fusion inhibitor research and development (R&D) to date, with the aim of providing investigators with prospective ideas for exploring antivirals.

KEYWORDS virus, research strategies, antivirals, N-peptide fusion inhibitor

As is known to all, the human immunodeficiency virus (HIV) could attack CD4$^+$ T lymphocytes in the immune system, which can lead to infection with a variety of diseases (1). HIV is classified into HIV-1 and HIV-2 based on genetic differences, and the predominant strain currently prevalent worldwide is the HIV-1 strains. In 1981, scientists identified the first case of acquired immunodeficiency syndrome (AIDS) caused by HIV-1, subsequently proven to be a highly lethal viral infection (2). Until today, the latest "2023 Progress Report on the Global AIDS Response - The Path to an End" published by the United Nations Programme on HIV/AIDS (UNAIDS) (3) shows that AIDS has become a chronic infectious disease that can be controlled, but not fully cured. Meanwhile, according to the UNAIDS survey, by 2023, there will still be more than 39 million people living with HIV. Although UNAIDS has set the ambitious goal of ending AIDS as a public health threat by 2030 in alignment with the Sustainable Development Goals (SDGs), the global HIV/AIDS epidemic remains a critical challenge. The development of novel drugs, especially those targeting drug-resistant strains and latent viral reservoirs, remains an indispensable priority in the fight against HIV/AIDS.

The primary treatment for AIDS is highly active antiretroviral therapy (HAART), also referred to as "cocktail therapy," which involves the simultaneous administration of three to four antiretroviral drugs, aiming to maximize the suppression of HIV-1 replication and reduce its viral load. Due to the different action mechanisms of multiple medicines, it is difficult for the virus to develop resistance to all medicines at the

same time, thus maintaining therapeutic effectiveness for a longer time (4). Antiviral medicines currently in use include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase inhibitors (INSTIs), chemokine receptor antagonists (CRAs), adhesion inhibitors (AIs), and fusion inhibitors (FIs). Of these, fusion inhibitors are effective at the stage of viral invasion into host cells, and listed agents show advantages in fast onset of action, high safety, strong efficacy, and low metabolic drug–drug interactions (5). Therefore, fusion inhibitors show great potential toward further exploitation. HIV-1 invasion into host cells is mainly based on the membrane fusion process (Fig. 1). First, HIV could recognize and bind to the host cell surface receptor, then trigger conformational change of envelope proteins. The N-terminal fusion peptide of transmembrane subunit in envelope proteins is then anchored to the host cell membrane. Afterward, the key N-terminal heptads repeat (NHR, or HR1) region of the transmembrane subunit is spontaneously assembled to form the coiled-coil structure (N-trimer), and the downstream C-terminal heptads repeat (CHR, or HR2) region immediately folds backward to interact with the NHR region, eventually forming the stable six-helix bundle (6-HB) (6). Then, HIV genetic material enters into the host cell through the fusion core. Theoretically, blocking the HIV-1 membrane fusion process is an effective strategy against viral infection; the 6-HB structure is an ideal target. Peptide-based fusion inhibitors could target the 6-HB structural domain and prevent virus-host cell membrane fusion (7).

Customarily, peptide-based fusion inhibitors are categorized as C-peptide and N-peptide on the basis of sequence origins and different binding targets that prevent 6-HB formation. Currently, enfuvirtide (T20) is the first marketed C-peptide fusion inhibitor against HIV (9, 10). However, in the clinical application process, T20 has several shortcomings such as a high level of resistance development, short half-life *in vivo*, and rapid renal clearance, which significantly limits the clinical application (11–13). Therefore, it is necessary for researchers to search for novel fusion inhibitors that can address the T20 clinical application problems. However, researchers are currently focusing on mainly C-peptides derived from the HIV-1 envelope CHR region, which still cannot compensate fully for the T20 shortcomings (14–17). The N-peptide fusion inhibitor, with a sequence entirely distinct from the listed peptide segments, originates from the N-terminal repeat sequence of the HIV-1 envelope protein gp41 and interacts with the CHR region downstream of the NHR region to disrupt the endogenous 6-HB structure through coiling, thereby preventing virus-target cell membrane fusion and exhibiting notable antiviral activity (18, 19). Researchers have developed N-peptide fusion inhibitors with higher biological activity and metabolic stability by employing strategies such as amino acid substitution and heterologous peptide bond crosslinking, demonstrating good inhibitory activity in experiments. Importantly, the improved stability and high inhibition to drug-resistant strains of N-peptides offer the future solutions to the current clinical dilemma as drug candidates (20). With the deepening research into the fusion mechanism of HIV-1 and the development of novel N-peptide fusion inhibitors, there is potential for achieving more efficient and broader-spectrum anti-HIV therapies in the future, providing a theoretical basis for the optimized design of targeted peptide
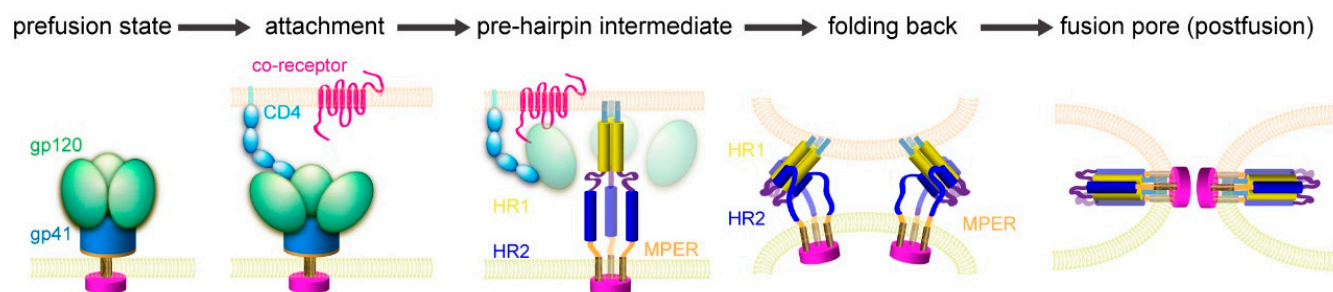

FIG 1 Membrane fusion process of HIV invading host cells (8).

inhibitors. This article summarizes the N-peptide research progress and development strategies to date, aiming to provide crucial guidance for the discovery of novel anti-HIV-1 agents.

BIOACTIVE CONFORMATION FOR N-PEPTIDE FUSION INHIBITORS: 1α-HELIX OR 3α-HELICES?

Previous studies demonstrated that the N-peptide could inhibit HIV-1 membrane fusion via mainly two mechanisms (Fig. 2) (19, 21). One mechanism is that N-peptide as 1α-helix (we called $N_{1\alpha}$-peptide) could admix into natural NHR region assembly, forming heterologous N-trimer to prevent NHR region→6-HB deformation process; another mechanism is that N-peptide as 3α-helices self-assembly (called $N_{3\alpha}$-peptide) could interact with the natural CHR region to disrupt CHR region→6-HB deformation process. The above mechanisms bring up a key issue: whether to design potential N-peptide fusion inhibitors with a 1α-helix or a 3α-helices as the active structure? Notably, under physiological or, e.g., phosphate-buffered saline (PBS) solution conditions, isolated natural $N_{1\alpha}$-peptides are less stable, prone to aggregation and precipitation, and unable to bind targets as the 1α-helix conformation, and thus have lower antiviral activity than C-peptides. In contrast, the $N_{3\alpha}$-peptides were able to mimic the coiled-coil conformation (N-trimer) during the deformation of the NHR region to 6-HB and showed significant antiviral activity with high fitness to the target (22). Currently, researchers have successively discovered many pioneer $N_{3\alpha}$-peptides and established a wealth of modification research strategies, mainly including a site-mutagenesis strategy for assembling the coiled-coil bioactive conformation, a self-assembly strategy with chimeric tool peptides, a strategy for small-molecule skeleton stapled N-multimers, a strategy for constructing covalent bond among coiled-coil helices, and so on. The $N_{3\alpha}$-peptides obtained through the above strategies essentially showed high stability, nanomolar-level antiviral activity, potential ability against drug-resistant strains, and good pharmacokinetic characteristics, which are suitable as antiviral candidates for further discovery.
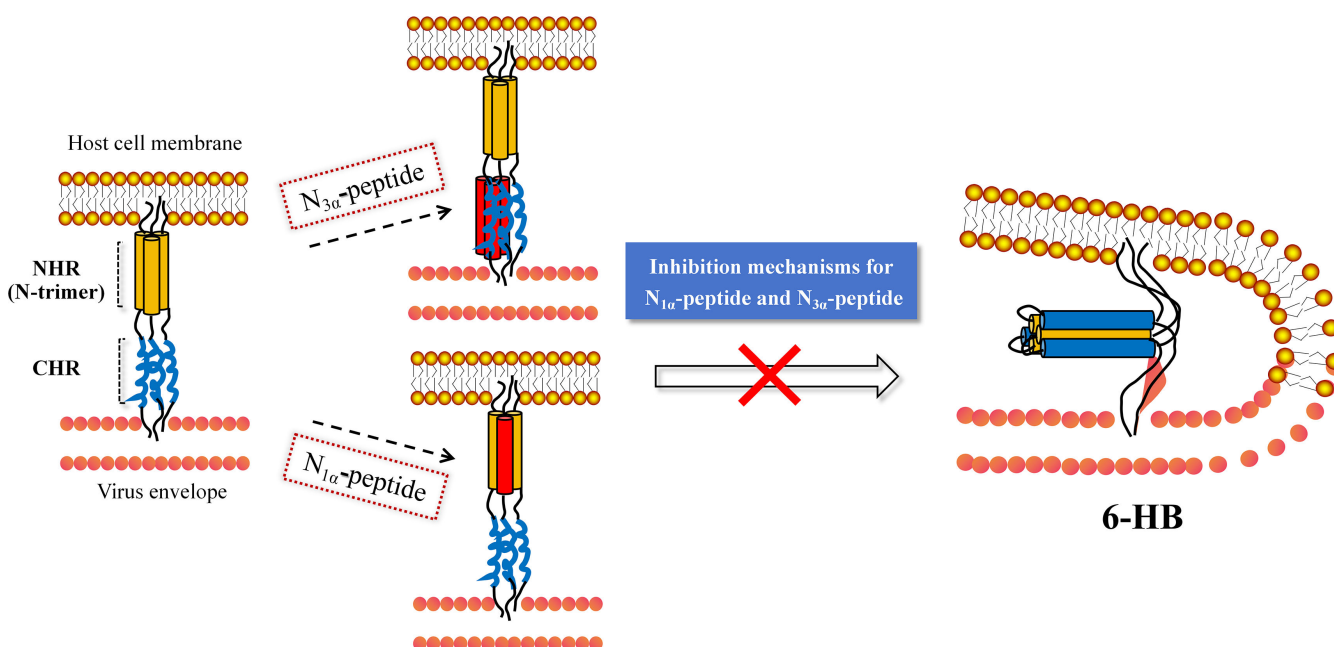

FIG 2 $N_{1\alpha}$ interacts at NHR.

SITE-MUTAGENESIS STRATEGY FOR ASSEMBLING THE COILED-COIL BIOAC-TIVE CONFORMATION

As the commonly utilized approach for structure optimization, the site-mutagenesis strategy holds a pivotal position in the peptide-based R&D realm. In the peptide body, all amino acid residues with differentiated side chain motifs and physicochemical properties together determine the overall conformation and biological activity. The site-mutagenesis strategy precisely utilizes the aforementioned principle, achieving fine-tuned regulation of peptide structure and function through precise modulation of specific amino acid residues (23). The site-mutagenesis strategy precisely regulates the structure and function of peptides by replacing specific amino acid sites. Applied to the $N_{3\alpha}$-peptides investigation, regular site mutations enable the assembly of N-trimer mimics conformation. In 2002, Bewley et al. (24) employed the natural N36 as a template and introduced mutations at the "e" and "g" positions of its amino acid residues, resulting in $N36_{Mut(e, g)}$ (Fig. 3). Analytical ultracentrifugation and circular dichroism (CD) spectroscopy confirmed that $N36_{Mut(e, g)}$ can self-assemble into an N-trimer structure and effectively inhibit HIV-1 infection (half maximal inhibitory concentration [IC_{50}] = 308 nM). In order to further investigate the site mutation design pattern, Dwyer et al. (25) replaced the isoleucine residue at the "g" position in T865 with alanine residue, and then boldly replaced the residue of "a, d" sites with the residue originated from the natural convoluted helix of respiratory syncytial virus (Fig. 3), and then the substituted T865RSV_AA unexpectedly showed a very high stability and enhanced potency against HIV-1. The results discussed above not only verify the effectiveness of the site-mutagenesis strategy in forming N-peptide coiled-coil and improving the inhibitory activity, but also lay the foundation for self-assembly strategy with chimeric tool peptides, constructing covalent bonds among coiled-coil helices, or other strategies. Moreover, the site-mutagenesis strategy has shown great potential for peptide design against other viruses, such as influenza viruses (IAVs) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (26–28).
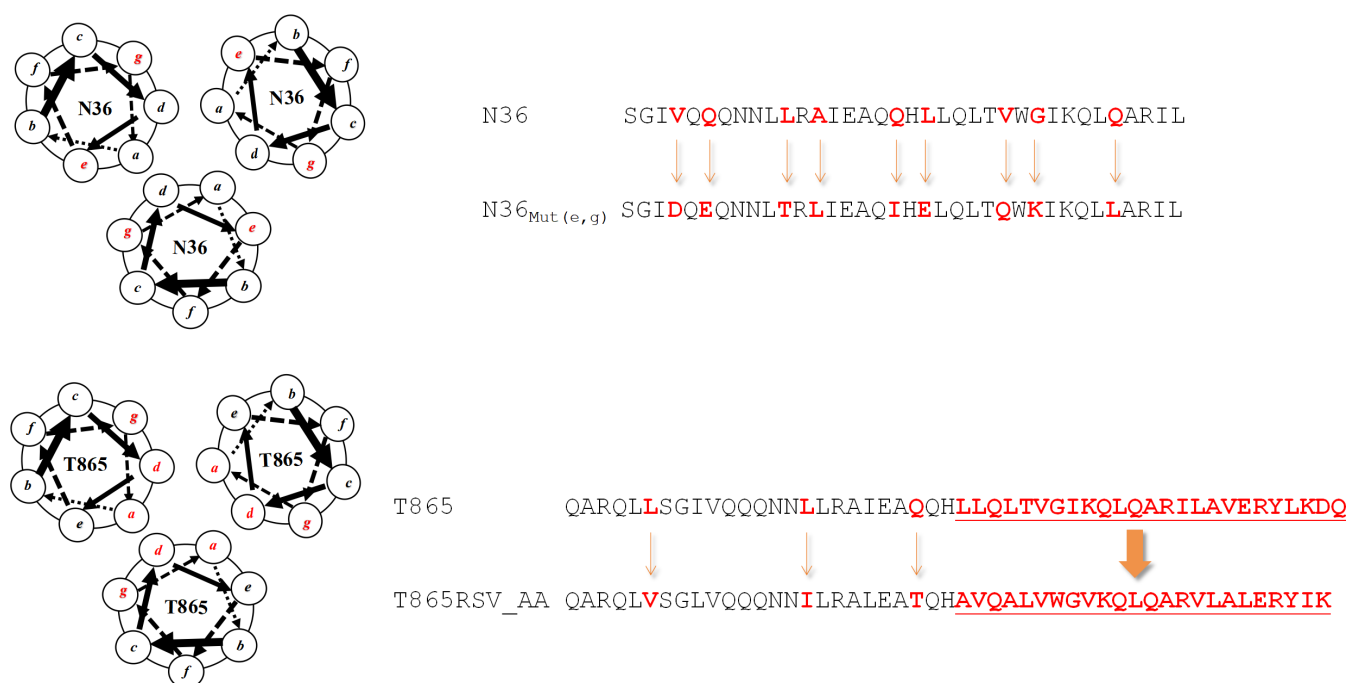

FIG 3 Sequence of designed N-peptides based on site-mutagenesis strategy.

SELF-ASSEMBLY STRATEGY WITH CHIMERIC TOOL PEPTIDES

The coiled-coil structure is a superhelical structure found in many natural proteins and has become an ideal model for protein research as repetitive and regular structure (29). In 1953, Crick et al. (30) first proposed the coiled-coil structure, as a superhelical structural domain, which consists of two or more α-helices twisted parallel or antiparallel to each other through hydrophobic interactions. The peptide sequences that compose the coiled-coil structure are presented as having the heptad-repeat principle, with each repeat sequence region generally containing seven amino acid residues, known as the heptad-repeat (HR) region (Fig. 4). In the HR region, amino acid residue sites are labeled with "*a, b, c, d, e, f, g*" in order, in which the "*a, d*" sites are mostly hydrophobic amino acid residues, such as leucine residues, isoleucine (Ile) residues, etc., which hydrophobically assemble the coiled-coil core; the "*e, g*" sites are located on the outside of the coiled-coil core, and most of them are polar charged amino acid residues, such as lysine (Lys) residue, glutamic acid (Glu) residue, etc., which can maintain the stability of coiled-coil structure through electrostatic interaction at the outer side (31).

The coiled-coil assembly method can improve protein/peptide stability, whereas natural N-peptides after isolation cannot self-assemble to form a 3α-helical structure. In view of the above complementary and synergistic properties, the researchers attempted to chimeraize coiled-coil tool peptides' natural N-peptide sequences in the hope of obtaining novel N-peptides with potential antiviral activity. In 1998, Suzuki et al. (18) designed a tool peptide with four heptad repeats, named *isoleucine zipper* (*IZ*). In the *IZ* sequence, Ile residues are located in "*a*" and "*d*" positions for assembling coiled-coil in the hydrophobic manner. Subsequently, Glu and Lys residues are located in "*e*" and "*g*" positions for creating stable electrostatic interactions among helices, and Glu and alanine residues are fixed at "*b*" and "*c*" positions. Ultimately, the *IZ* sequence is finalized as *YGG(IEKKIEA)$_4$*. Experimentally, *IZ* was demonstrated to be an ideal tool peptide capable of assisting in the coiled-coil formation with the N17 sequence from the HIV-1 NHR region, and the combined *IZ*N17 exhibited nanomolar level anti-HIV-1 activity and superior thermal stability. In 2001, Eckert et al. (19, 32) obtained another tool peptide with the sequence *RMKQIEDKIEEILSKQYHIENEIARIKKLIGER*, named *IQ*, which could assemble into 3α-helices the same as *IZ*. *IQ* could embed into N17, N23, and N36 sequences from the HIV-1 NHR region, yielding a series of novel N-peptides, called *IQ*N17, *IQ*N23, and *IQ*N36 (Fig. 5), which also exhibit nanomolar level anti-HIV-1 activity. The aforementioned examples demonstrate that tool peptides provide remarkable supplementary functionalities for N-peptides to effectively exert antiviral activity. Besides *IZ* and *IQ*, Yang and Chen et al. (20, 33, 34) discovered the third tool peptide, named *Fd*, with sequences from Foldon protein in T4 phage fibronectin (Fig. 5). The anti-HIV-1
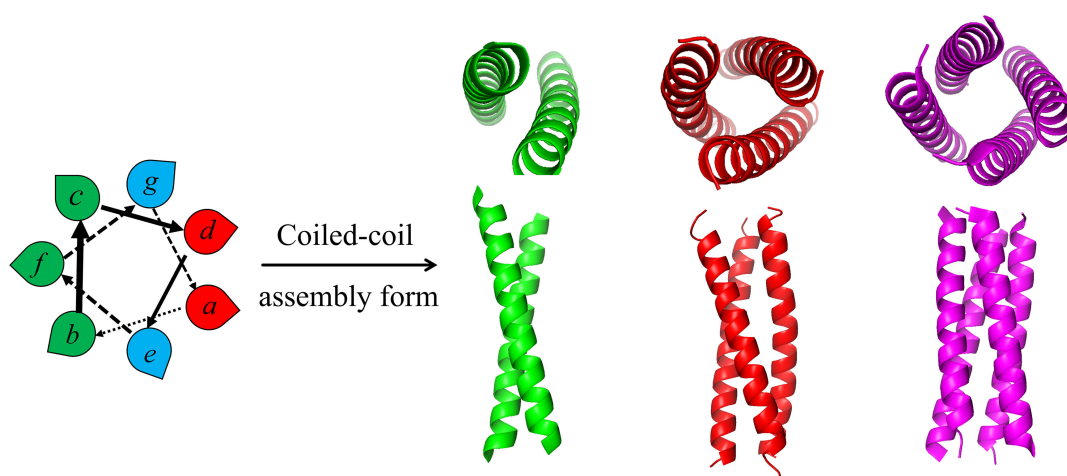

FIG 4 Common assembly forms of the coiled-coil.

Tool peptides：

IZ: IKKEIEAIKKEQEAIKKKIEAIEK
IQ: RMKQIEDKIEEIESKQKKIENEIARIKK
Fd: GYIPEAPRDGQAYVRKDGEWVLLSTFL

Sequences from NHR region of HIV-1, EBOV, SARS-CoV-2：

N17: LLQLTVWGIKQLQARIL
N23: IEAQQHLLQLTVWGIKQLQARIL
N28: IEAQQHLLQLTVWGIKQLQARILAVERY
N36: SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL
N39: LRELANTTTKALQLFLRATTEERTFSLINRHAIDFLLTR
HR1M:ENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVV

Chimerized N-peptides：

*IZ*N17: IKKEIEAIKKEQEAIKKKIEAIEK━━LLQLTVWGIKQLQARIL
*IQ*N17: RMKQIEDKIEEIESKQKKIENEIARIKK━━LLQLTVWGIKQLQARIL
*IQ*N23: RMKQIEDKIEEIESKQKKIENEIARIKK━━LIEAQQHLLQLTVWGIKQLQARIL
*IQ*N36: RMKQIEDKIEEIESKQKKIENEIARIKK━━LISGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL
N28*Fd*: IEAQQHLLQLTVWGIKQLQARILAVERY━━GYIPEAPRDGQAYVRKDGEWVLLSTFL
N36*Fd*: SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL━━GYIPEAPRDGQAYVRKDGEWVLLSTFL
ebo*IZ*N39*IQ*:GHMDIKKEIEAIKKEQEAIKKKIEAIEKE━━LRQLANETTQALQLFLRATTELRTFSILNRKAIDFLLQR━━IMI
               KQIEDKIEEIESKQKKIENEIARIKKLIGERY
HR1S*Fd*: ENQKLIANQFNSAIGKIQDSLSSTASALG━━GYIPEAPRDGQAYVRKDGEWVLLSTFL
HR1M*Fd*: ENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVV━━GYIPEAPRDGQAYVRKDGEWVLLSTFL
HR1L*Fd*: ENQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALNTLVKQ━━GYIPEAPRDGQAYVRKDGEWVLLSTFL

FIG 5 Assigned NHR sequences of different viruses (cords represent tool peptides and key sequences chimerized by covalent bond).

experiment results indicated that chimeric N-peptides, named N28Fd and N36Fd, were 99 nM and 39 nM, respectively, and antiviral potency aligned with the previously mentioned observed trend: the anti-HIV-1 activity of natural N-peptide stays at the micromolar level, whereas the anti-HIV-1 activity of the chimeric N-peptide could reach the nanomolar level.

Chimeric tool peptides such as *IZ, IQ,* and *Fd* exhibit broad potential applications in viral fusion inhibitors, not only in the HIV-1 field but also in other virus studies. Drawing inspiration from the anti-HIV-1 N-peptide designing, Clinton et al. (35) innovatively constructed the anti-Ebola virus (EBOV) N-peptides by embedding the coiled-coil tool peptides *IQ* and *IZ* in the EBOV N-peptide C-terminus, named ebo*IZ*N39*IQ* (Fig. 5), with stable coiled-coil structure, based on the similarities in fusion mechanisms between EBOV and HIV-1. The ebo*IZ*N39*IQ* not only successfully mimicked the target helical structure but also exhibited exceptional thermal stability. To further confirm whether ebo*IZ*N39*IQ* can adopt the active conformation N-trimer in the pre-hairpin intermediate, the researchers conducted detailed studies on the binding of ebo*IZ*N39*IQ* to native target using surface plasmon resonance analysis techniques. The results demonstrated tight binding affinity with 14 nM dissociation constant between the native target and ebo*IZ*N39*IQ*, comparable to the interaction strength of HIV-1 N-peptides and native target, thereby strongly evidencing effective presentation of the ebo*IZ*N39*IQ* N-trimer structure. Similarly, aiming to develop peptide fusion inhibitors against SARS-CoV-2, Bi et al. (36) fused the trimerization motif Fd to SARS-CoV-2 NHR-derived peptide, finally obtaining novel N-peptides (Fig. 5). These N-peptides (named HR1S*Fd*, HR1M*Fd*, and HR1L*Fd*) indeed formed stable trimers and showed dramatically increased antiviral activity and thermostability compared with the natural HR1 from the NHR region. Moreover, HR1M*Fd* showed broad-spectrum inhibitory activity against various SARS-CoV-2 pseudoviral mutants, SARS-CoV pseudovirus, and Middle East respiratory syndrome coronavirus (MERS-CoV) pseudovirus.

Therefore, based on the self-assembly strategy with chimeric tool peptides, the novel N-peptide could self-assemble into 3α-helices structure and thus bind tightly

to the target, significantly improving stability, antiviral activity, and viral mutation adaptability. However, the current small variety of tool peptides is far from matching the lead compound, and more tool peptides are needed to support the development of candidate antivirals in the future.

STRATEGY FOR SMALL-MOLECULE SKELETON STAPLED N-MULTIMERS

Currently, the small-molecule skeleton plays a crucial role in biochemistry and drug design, as they could not only serve as the cornerstone for stable structures, but also endow new functions and characteristics through specific chemical modifications (37). In the fusion inhibitors field, small-molecule skeletons are the crucial design tool with plasticity and modifiability, and researchers developed a method to staple N-multimers forming 3α-helices structure through small-molecule skeleton rigidity to exert antiviral efficiency. In 2010, Nakahara et al. (38) connected a novel small-molecule skeleton with three equal-length branching junctions with ester aldehyde groups to the N-terminal cysteine of the natural peptide N36 in aqueous solution and finally obtained the novel N-peptide, named triN36e (Fig. 6). CD experiments revealed that triN36e possesses α-helix structure, with significantly higher helix content compared to N36. In the anti-HIV-1 experiment, compared with N36, triN36e showed more than threefold increase in antiviral activity and a 30-fold increase in binding capacity, demonstrating the feasibility of small-molecule skeleton stapled N-multimers strategy. In 2024, Wu et al. (39) used Kemp's triacid (KTA) skeleton to immobilize natural peptide N51 to get trimeric peptide $KTA(N51)_3$ (Fig. 6), which exhibited 10-fold enhancement in the inhibitory activity compared to N51. In order to explore the generalizability of the small-molecule skeleton strategy, this team prepared tribromoacetylated scaffold, linking to N51, yielding a series of novel N-peptides, all of them possessing effective antiviral activities and virus mutant adaptability. It is worth mentioning that the small-molecule skeleton stapled multimers strategy can also be applied in the C-peptide fusion inhibitors. Nomura's team (40) obtained a novel C-peptide triC34e by selectively coupling C3 template to natural peptide C34, which has 100-fold more antiviral activity than single C34. Based on the small-molecule skeleton stapled N-multimers strategy, it is possible to rigidly constrain three homologous natural N-peptides to get one macromolecule; the
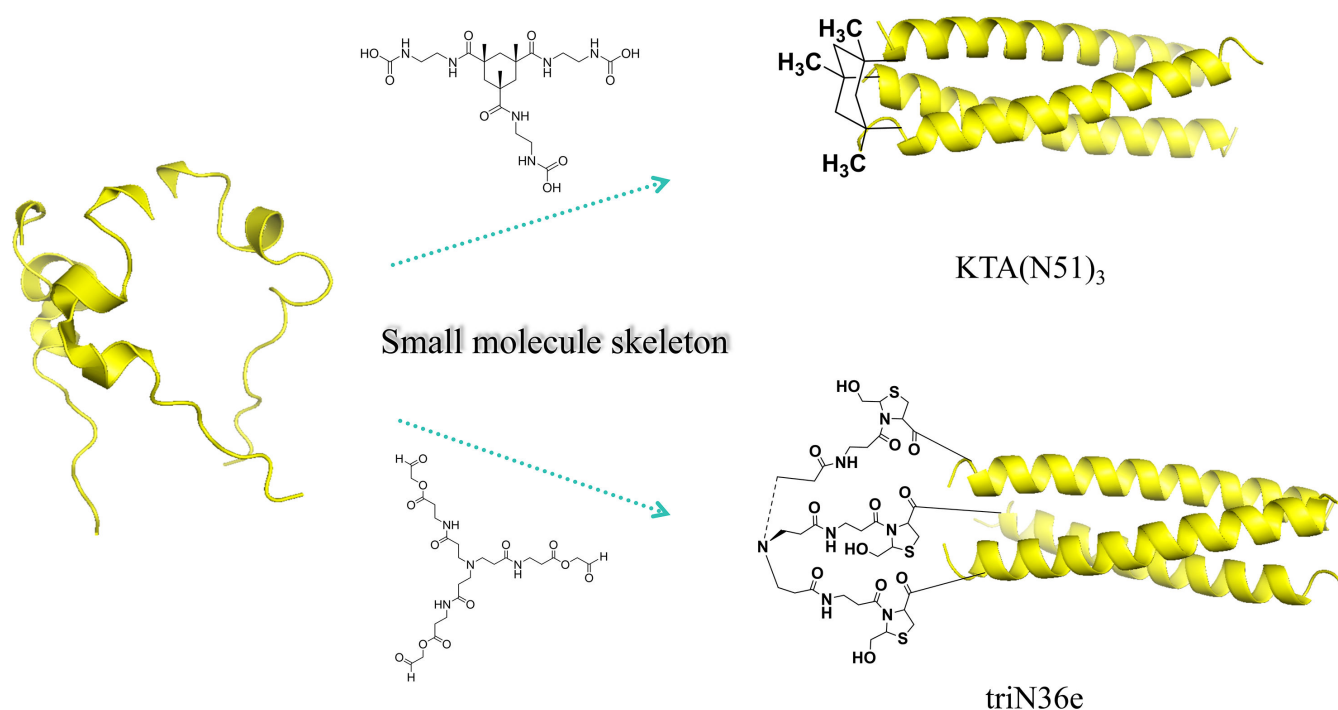

$KTA(N51)_3$

Small molecule skeleton

triN36e

FIG 6 Schematic diagram of small-molecule skeleton stapled N-multimers.

small-molecule skeleton is positioned at the N-peptide terminus, allowing N-peptide to assemble into coiled-coil structure with high freedom degrees. In addition, this strategy may play a broad role in the fusion inhibitor investigation such as C-peptide.

STRATEGY FOR CONSTRUCTING COVALENT BOND AMONG COILED-COIL HELICES

By comparison with the C-peptides derived from the CHR region, peptides derived from the NHR region generally require the construction of N-trimer mimetics to exhibit potential inhibitory effects. Chemistry-driven covalent bond bundling coiled-coil provides another well-established approach for N-peptide designing. The introduction of covalent bonds not only prefolds the N-peptide to minimize the conformational entropy loss when interacting with the CHR region, but also improves the thermal stability, protease stability, and *in vitro* metabolic stability of the N-peptide (41). Disulfide bonds, as covalent linkages, are extensively utilized in protein/peptide design. Disulfide bonds can form rigid lock-arm structures within or between protein/peptide molecules, thereby significantly enhancing structural integrity and thermal stability. Given these advantages, researchers have introduced disulfide bonds into N-peptides to stabilize their active conformations (42). Louis et al. (43) firstly introduced cysteine residues at specific positions in the N-peptide sequences to establish interchain disulfide bonds by oxidation reaction and ultimately obtained $N35_{CCG}$-N13 and $N34_{CCG}$ with disulfide bond arm (Fig. 7), which inhibited HIV-1 envelope protein-mediated cell-cell fusion at nanomolar level. Considering that the antiviral activity of *IZ*N17 is somewhat limited by the self-association equilibrium, Bianchi et al. (44) introduced cysteine residues at the *IZ*N17 sequence terminus, which self-associated into the coiled-coil in solution, and the interhelical cysteine residues were also oxidized to form disulfide bonds, finally obtaining a novel N-peptide named $(CCIZN17)_3$ (Fig. 7). Experimental results showed that $(CCIZN17)_3$ exhibited remarkable thermodynamic stability with T_m >90°C in 2 M GdnHCl

Sequences from NHR region of HIV-1：
```
N13:  SGIVQQQNNLLRA
N17:  ------------------LLQLTVWGIKQLQARIL
N34:  SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQAR
N35:  SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQCCGRI
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Disulfide-bond peptides：
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N35CCG-N13: SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQCCGRISGIVQQQNNLLRA
N34CCG:     ----SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQCCGR
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(CCIZN17)3: (CCGGIKKEIEAIKKEQEAIKKKIEAIEKLLQLTVWGIKQLQARIL)3
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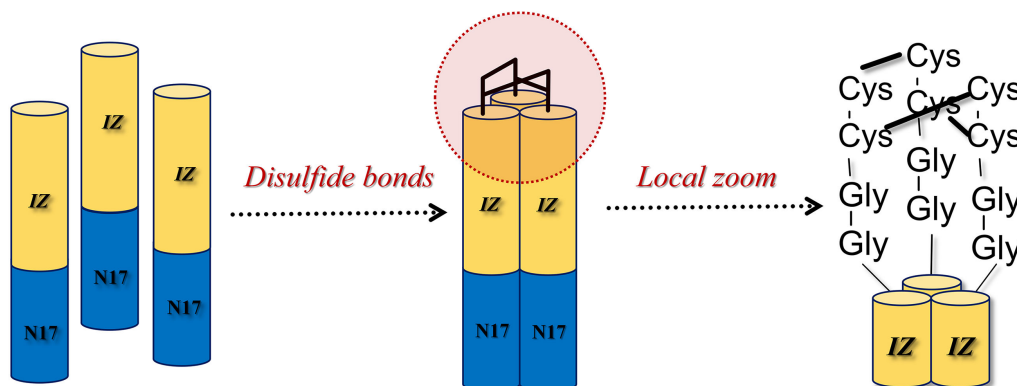

FIG 7   Schematic diagram of disulfide bond stapled N-peptides.

solution (compared to 61.5°C for *IZ*N17), and inhibitory activity against HIV-1 reached 40 pM–380 pM. In addition, (CC*IZ*N17)$_3$ was further efficacious in neutralizing acute viral infections in peripheral blood mononuclear cells and exhibited a broad spectrum of HIV-1, including R5, X4, and R5/X4 strains. Overall, the anti-HIV-1 potency of (CC*IZ*N17)$_3$ exceeded that of T20 as well as monomeric *IZ*N17. The above findings mean that disulfide bond for bundling coiled-coil structure is a feasible strategy, not only because of the relatively easy synthetic method but also because of the substantial improvement in stability and activity of novel N -peptides. It is worth noting that disulfide bonds also have certain drawbacks, such as being easily degraded by glutathione reductase or oxidoreductase, which may affect the N-peptide activity *in vivo* to some extent. Nevertheless, disulfide bonds still provide promising ideas for N-peptide design (45).

The isopeptide bond, as a specialized covalent bond present in bacterial hyphae, consists of the non-alpha amino or carboxyl group of an amino acid condensed with the carboxyl or amino group of another amino acid. The isopeptide bond is able to significantly maintain the bacterial hyphae integrity when the bacteria are exposed to severe mechanical, thermal, and protein hydrolysis stress conditions. In comparison to disulfide bonds, isopeptide bonds have higher thermodynamic stability, chemical stability, and pH tolerance. These excellent properties make isopeptide bonds a handy tool for protein structure modification, especially for the N-peptides with poor physical and chemical stability and requiring coiled-coil active conformations (46–49).

Wang et al. (22) pioneered the innovative application of isopeptide bonds to N-peptide. First, Wang et al. designed the tool peptides 3HR and 4HR (consisting of the sequences of *IQQIEQK IHHIEQR IQQIEQR IQQIEQR* aligned from "*a*" to "*g*" positions), which are capable of self-assembling into triple helices, and combined with natural peptide sequences derived from the HIV-1 NHR region to form the N-peptide primary sequences. Spatially, the carboxyl group of the Glu residue at the "*e*" position in one helical peptide could form an intermolecular salt bridge with the amino group of the Lys residue at the "*g*" position in the neighboring helical peptide, which provides a precondition for the isopeptide bond creation. Then, Wang et al. skillfully designed the Glu carboxyl thioesterification at the one-helix "*e*" position, forming an isopeptide bond with the Lys amino group at another-helix "*g*" position in PBS solution by acyl transfer reaction (Fig. 8). The isopeptide-bonded N-peptides were not completely denatured at 90°C in stability experiments and were comparable to T20 in anti-HIV-1 activity. As reported in another publication (50), this same group successfully synthesized another isopeptide-bonded N-peptide based on natural NHR sequences by using N36 as the lead compound and introducing isopeptide bonds with site mutation and thioesterification modifications. These novel N-peptides also exhibited excellent physical and chemical stability and anti-HIV-1 activity. Subsequently, many researchers have continued to delve deeper and deeper to elucidate the isopeptide bond formation rules.

Wang et al. (51) further investigated the applied isopeptide bonds to N-peptides and systematically summarized three major rules of isopeptide bond bundling coiled-coil. (i) Isopeptide bond construction at tool peptides at different sites: Wang et al. used IZN17 sequence as a template and obtained (*IZ*N17L)$_3$ by cross-linking the Glu at the *4* position of one helix with the Lys at the *9′* position of another adjacent helix to form an isopeptide bond; obtained (*IZ*N17M)$_3$ by cross-linking the Glu at the *4+7* position of one helix with the Lys at the *9′+7′* position of another adjacent helix; obtained (*IZ*N17R)$_3$ by cross-linking the Lys at the *4+7+7* position of one helix with the Glu at the *9′+7′+7′* position of another adjacent helix. In summary, the Glu residues at *4*, *4+7*, and *4+7+7* positions within the tool peptide sequence could react with the Lys residues at *9′*, *9′+7′*, and *9′+7′+7′* positions in the parallel sequence, respectively, leading to the formation of three pairs of isopeptide bonds among helices. (ii) Truncating tool peptides: since the tool peptide only acts as an assembly role, it can be shortened in terms of the synthesis difficulty and production cost considerations while ensuring the N-peptide antiviral ability. Wang et al. selected (IZN17R)$_3$ as a template and shortened 7, 10, and 14 amino acid residues from the IZ N-terminal, respectively, and were still able to construct isopeptide bonds and
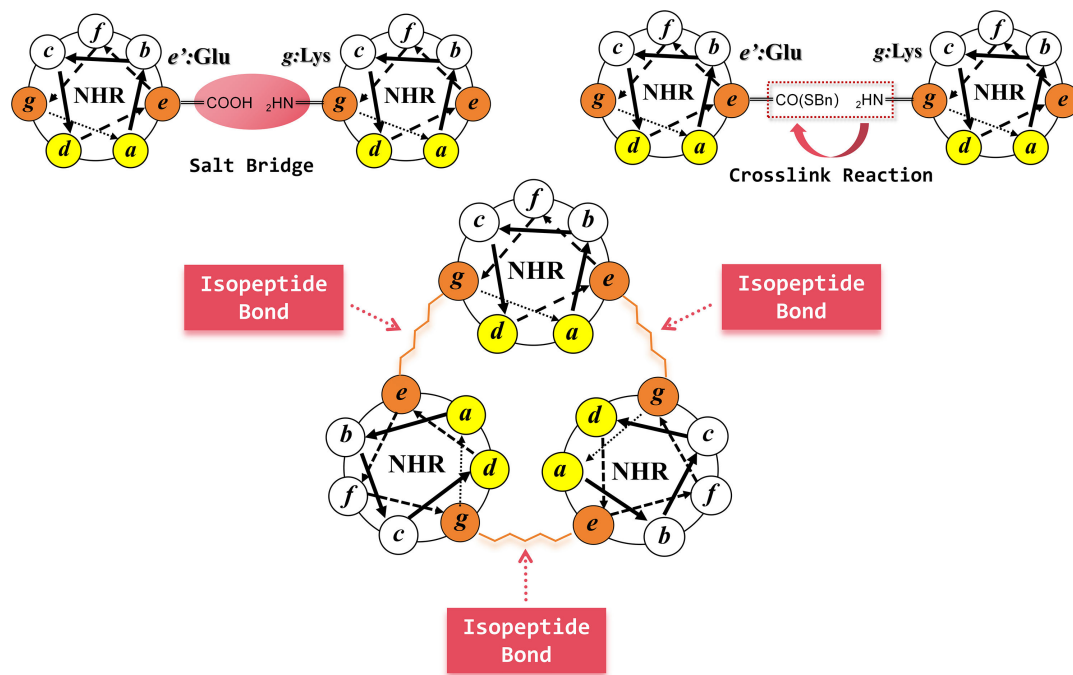
FIG 8 Schematic diagram of isopeptide bond stapled N-peptides.

obtain $(IZ17N17)_3$, $(IZ14N17)_3$, and $(IZ10N17)_3$ with nanomolar anti-HIV-1 activity. (iii) Extending peptide sequences from the natural NHR region: to further enhance the binding affinity toward the target and inhibitory activity of N-peptides, it is necessary to increase the natural sequence length in N-peptide primary structure. Wang et al. selected the shorter peptide $(IZ14N17)_3$ and $(IZ10N17)_3$ as templates and extended seven natural amino acid residues at the N17 N-terminal and C-terminal to obtain $(IZ14N24N)_3$, $(IZ10N24N)_3$, $(IZ14N24C)_3$, and $(IZ10N24C)_3$. All of these showed higher antiviral activity and stability compared to the positive control. On the basis of the above studies, our team even achieved artificial N-peptides by employing isopeptide bonds (Fig. 9) (52). The most active compound, IZNP02QE, surpassed the positive control by demonstrating remarkable nanomolar-level inhibitory activity against HIV-1.

It is promising that the isopeptide bond bundling coiled-coil strategy can also be applied to the development of other viral fusion inhibitors. Zheng et al. (21) selected a 21-amino-acid residue fragment from the NHR region of MERS-CoV S2 subunit as a template and constructed an N-trimer structural model by forming isopeptide bonds. Compared with unmodified monomer, the novel N-peptide did not show the aggregation and had stronger thermal stability, and the anti-MERS-CoV activity was comparable to that of positive control. However, the current synthesis process of isopeptide bond is relatively complicated, which limits N-peptide development to some extent. Therefore, the synthetic process needs to be continuously explored and optimized in the future to provide more powerful support for N-peptides.

OTHER STRATEGIES

The biotechnological recombinant technique has achieved "protein/peptide custom manufacturing" by enabling DNA precise manipulation (53). The researchers were able to translate endogenous NHR sequences into proteins by DNA translation, which were able to spontaneously assemble as a multimeric form (containing N-trimer), thus exhibiting significant antiviral capabilities. Louis et al. (43, 54) first successfully obtained the N35CCG-N13 protein by using recombinant technology, which consists of natural N35 and N13 forming N-trimer mimics structure by head-to-tail linkage. Louis et al. further skillfully ligated N36 and N35, originating from the HIV-1 NHR region, with C28
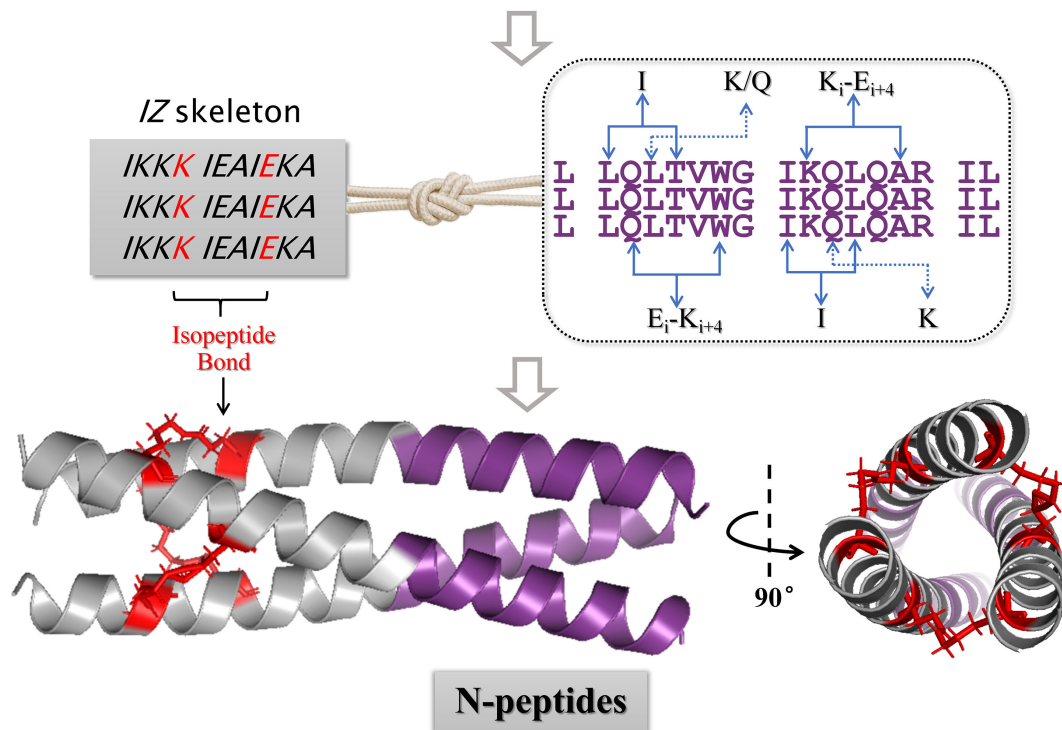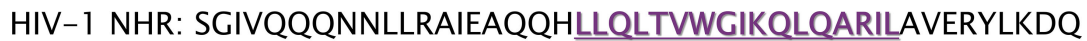
FIG 9 Schematic N-peptide design based on constructing isopeptide bonds (52).

originating from the CHR region and yielded the recombinant protein NCCG-gp41, which exhibited the potent inhibitory ability by HIV-1 envelope-mediated cell-cell fusion (IC$_{50}$ = 16.1 nM), flush with the control C34. Utilizing biotechnological recombinant techniques, the N-trimer structure construction has also been achieved. Based on the ability of NHR and CHR to form six-advanced helix bundles, the design of supercoiled macromolecules beyond the N-trimer is also possible.

Root et al. (55) designed a distinctive supercoiled protein, named 5-Helix, which is composed of three native N40 and two native C38. Arguably, 5-Helix is composed of three NHRs and two CHRs, which precisely provide a high-affinity binding site for the gp41 CHR region (Fig. 10). Under physiological conditions, 5-Helix is able to spontaneously fold into a pentamer that specifically binds to the CHR region to form the 6-HB mimics, which exhibit nanomolar levels of antiviral activity for inhibition of HIV-1 envelope-mediated cell-cell fusion. Through biotechnological recombinant techniques, peptides can be upgraded into macromolecular proteins that contain N-peptide sequences, thereby effectively exerting antiviral activity. The ability to artificially create proteins containing the N-trimer structure by biological recombination is an emerging strategy in recent years, which has been applied in the design of peptide-based fusion inhibitors for EBOV, MERS-CoV, and SARS-CoV-2, etc. (56–58).

SUMMARY AND PROSPECT

Over the years, the key peptides to block the early membrane fusion process of HIV-1 invasion into host cells have made significant progress, thanks to the efforts of researchers who have gone before and after (59, 60). At present, peptide-based fusion inhibitors have also been intensively studied in other viruses such as SARS-CoV-2, MERS-CoV, and IAVs, etc., and even the peptide EK1 has been entered into the clinic against SARS-CoV-2, thereby demonstrating broad development prospects and significant application significance. We need to discover more and more peptide-based new chemical
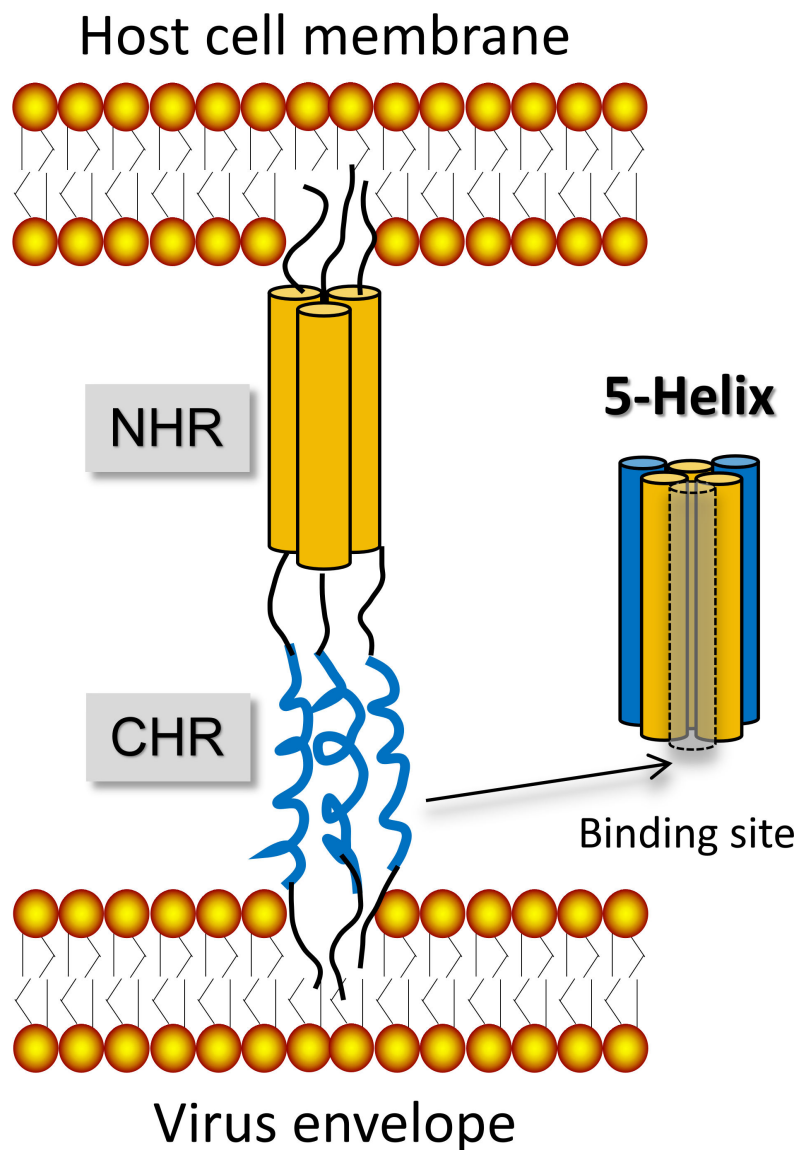
FIG 10 Schematic diagram of 5-Helix binding to the target.

entities and establish entirely novel R&D strategies to lay the foundation for the future of peptide-based fusion inhibitors. Given the difficulties in the clinical application of current fusion inhibitors (such as T20), N-peptides with a unique active structure act on the target to exhibit excellent antiviral activity, serving as the main focus for future development with high expectations. This article summarizes the research strategies for novel N-peptides to date, offering valuable insights to researchers in this field. We also call for more researchers to join the peptide-based fusion inhibitors R&D in the future to support the treatment of viral infectious diseases.

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