α-Helix Mimetics

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Inhibition of HIV-1 Fusion by Hydrogen-Bond-Surrogate-Based α Helices**

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Entry of HIV-1 into its target cells to establish an infection is mediated by viral envelope glycoprotein (Env) and cell-surface receptors (CD4 and a coreceptor, such as CXCR4 or CCR5).[1] The mature Env complex is a trimer, with three gp120 glycoproteins associated noncovalently with three membrane-anchored gp41 subunits. Binding of gp120/gp41 to cellular receptors triggers a series of conformational changes in gp41 that ultimately leads to the formation of a postfusion trimer-of-hairpins structure and membrane fusion.[2–4] The core of the postfusion trimer-of-hairpins structure is a bundle of six α helices: three N-peptide helices form an interior, parallel, coiled-coil trimer, while three C-peptide helices pack in an antiparallel manner into hydrophobic grooves on the coiled-coil surface (Figure 1).[2–4] The N-peptide region features a hydrophobic pocket targeted by C-peptide residues W628, W631, and I635.[5] Peptides and synthetic molecules that bind to the N-terminal hydrophobic pocket and inhibit the formation of the six-helix bundle have been shown to effectively inhibit gp41-mediated HIV fusion.[6–11] Herein, we describe the structure–activity relationships of hydrogen-bond-surrogate-derived α helices that inhibit HIV fusion in cell culture. Our work suggests that hydrogen-bond-surrogate (HBS) helices can potentially function as in vivo inhibitors of protein–protein interactions involved in viral fusion.

HBS α helices are obtained by replacing an N-terminal main-chain *i* and *i*+4 hydrogen bond with a carbon–carbon bond through a ring-closing metathesis reaction (Figure 2).[12–14] The hydrogen-bond surrogate preorganizes an α turn and stabilizes the peptide sequence in an α-helical conformation. We have shown that hydrogen-bond-surrogate α helices adopt stable α-helical conformations from a variety of short peptide sequences.[15] We have also demonstrated that these artificial α helices can target their expected protein receptor with high affinity.[16]

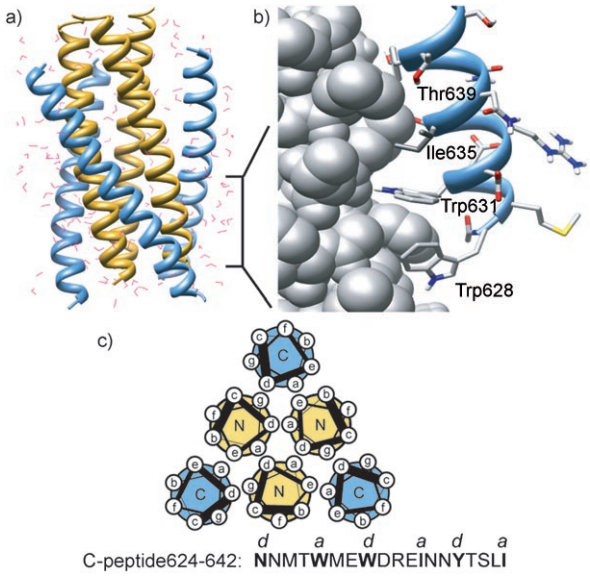

Figure 1. a) The HIV-1 gp41 core six-helix bundle with the N region in gold and the C region in blue. b) Interaction of C-peptide residues W628, W631, and I635 with the N-peptide (PDB code: 1AIK).[2] c) Helical wheel diagram showing interactions between C- and N-peptide coiled-coil domains.

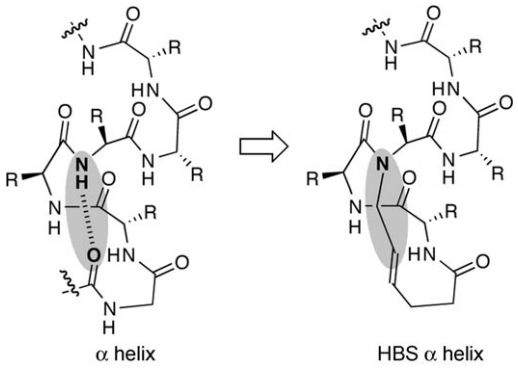

Figure 2. Hydrogen-bond-surrogate (HBS) α helices feature a carbon–carbon bond in place of an *i* and *i*+4 hydrogen bond. R = amino acid side chain.

We began our gp41-targeting studies by mimicking the 14-residue C-peptide derived from gp41 that contains residues W628, W631, and I635 (**1**, Table 1). This sequence would be expected to bind the gp41 hydrophobic pocket based on

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

Table 1: Binding affinities and cell-fusion-inhibition properties of peptides and HBS α helices.

Compound	Sequence[a]	K_d [μM][b]	EC_{50} [μM][c]
C-peptide	NNMTWMEWDREINNYTSLI	–	–
1	AcMTWMEWDREINNYT	37.4 ± 14.8[d]	–
2	XMTWMEWDREINNYT	46.6 ± 14.6[d]	≫200
3	XWAAWDKKI	>500	≫200
4	XAAAWEEWDKKI	>500	≫200
5	XWAAWDREINNYT	>500	≫200
6	XMTWEEWDKKIEEYT	7.50 ± 1.70	≫200
7	XEMAWEEWDKKIEEYT	146 ± 47.3	≫200
8	XNEMTWEEWDKKIEEYT	<5.00[e]	≫200
9	XMTWEEWDKKIEEYTKKI	<5.00[e]	42.7 ± 7.50
10	AcMTWEEWDKKIEEYTKKI	<5.00[e]	≫200

[a] X: pentenoic acid residue; Ac: acetyl. Residues that occupy *a* or *d* positions in the heptad are shown in bold. [b] Binding affinity for IZN17 as calculated from a fluorescence polarization assay.[17] [c] Inhibitory activity of peptides in a gp41-mediated cell–cell fusion assay monitoring syncytia formation.[18] [d] Precise measurements of the K_d value were not possible because the peptide aggregates at high concentrations. [e] The competitive binding assay (see the Supporting Information) does not allow accurate estimates of K_d values much lower than binding values of the fluorescent probe.

the crystal structure (Figure 1b)[2] but has been previously shown to be ineffective in cell–cell fusion assays.[8]

We utilized a previously described fluorescence polarization assay to determine the in vitro binding affinity of unconstrained peptides and HBS helices for a stable model of the gp41 N-terminal three-strand coiled-coil, IZN17, which contains the binding site for residues W628, W631, and I635.[17] A fluorescein-labeled peptide, containing residues 628–641 of gp41, was used as a probe. The competitive displacement of this probe by HBS helices afforded the dissociation constant (K_d) value for each peptide (Table 1 and Figure 3b).[11] Details of the fluorescence polarization assay are included in the Supporting Information. The fluorescein-labeled C-peptide derivative (suc-MTWMEWDREINNYTC^{Flu}; suc: succinimidyl) bound IZN17 with a K_d value of 24 μM, within the range of previously reported values.[8,11] The competition assay provided a binding affinity of 37 μM for peptide **1**, which is also in the range of reported values, although aggregation of the peptide made it difficult to obtain accurate K_d values.

Circular dichroism spectroscopy suggested that **1** is unstructured or very weakly helical in 10% TFE in PBS.[8] We conjectured that stabilization of this peptide in helical conformation with the HBS approach may increase its helicity and affinity for IZN17. HBS α helix **2** is roughly four times more helical than **1** (Figure 3a) but does not bind the target protein with higher affinity (Table 1). This outcome suggests that the aggregation state of the peptide is influencing the binding affinity for the target. Our results with the short peptide and constrained helix mirror those observed by Kim and co-workers,[8] and this prompted us to design a small library of HBS α helices to determine sequences that bind IZN17 with higher affinity. A representative selection of HBS helices prepared in this project is shown in Table 1.

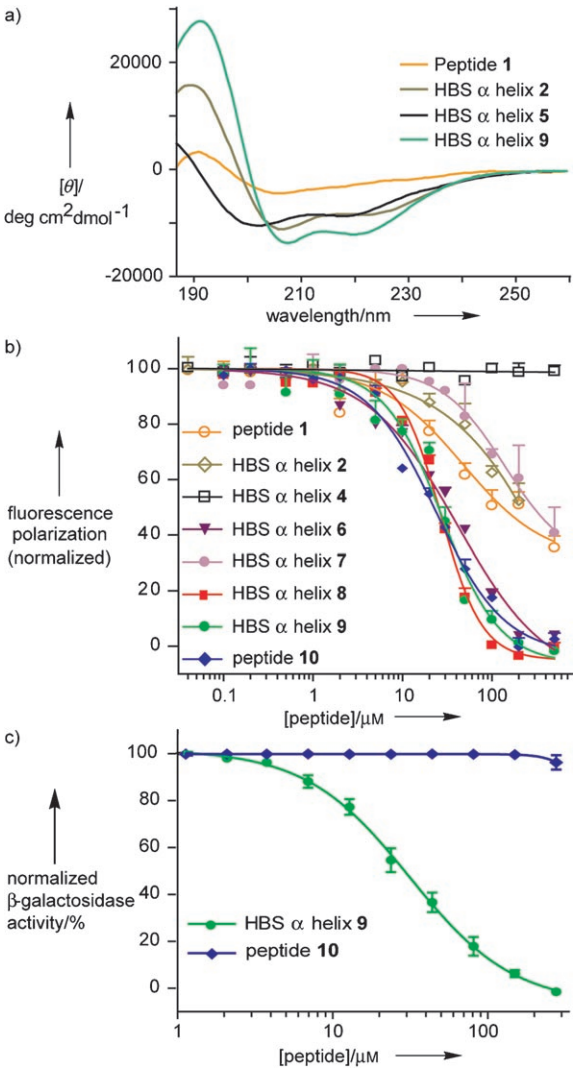

Figure 3. a) Circular dichroism spectra of **1**, **2**, **5**, and **9** in 10% trifluoroethanol (TFE) in phosphate-buffered saline (PBS). b) Determination of peptide binding to IZN17 by a fluorescence polarization assay. c) Inhibition of gp41-mediated cell–cell fusion by HBS α helix **9** and peptide **10**.

The limited solubility of short C-peptides in aqueous solution has been proposed as a key reason for their inactivity.[19] To improve the solubility and helicity of constrained C-peptides, we incorporated charged residues at positions not expected to be involved in binding to IZN17, based on the work of Otaka, Fujii, and co-workers.[19] HBS α helix **3** is the shortest peptide designed to contain the Trp/Trp/Ile motif necessary for binding. Two lysine residues were added within this sequence to improve the solubility of the peptide. We designed HBS **4** such that the Trp/Trp/Ile motif would lie outside of the HBS constraint, in order to probe the potential steric effects of this constraint. Pairs of glutamic acid and lysine residues were placed at the *i* and *i*+4 positions to further stabilize the α-helical conformation through potential salt-bridging interactions.[20,21] Neither of these two HBS helices bound IZN17, a result suggesting that more contact points are needed to target the coiled-coil complex. HBS **5**

contains Y638 in addition to the Trp/Trp/Ile residues; Y638 occupies the *d* position in the heptad and is expected to directly contact the N-terminal three-strand coiled-coil (Figure 1c). The HBS **5** sequence is similar to that of the parent compounds **1** and **2** but is missing the N-terminal M626 and T627 residues. This compound also showed negligible affinity for IZN17, which was surprising because we had expected **5** to bind IZN17 with affinity similar to that observed for **2**. CD spectroscopy suggests that **2** and **5** are equally helical (Figure 3a). The low binding affinity of **5** relative to that of **2** highlights the potential role of T627, which occupies the *g* position in the heptad and was not projected to contribute significantly to binding (Figure 1c). Reintroduction of the M626 and T627 residues and incorporation of charged residues at positions not expected to be involved in binding provided HBS **6**, which bound IZN17 with a higher affinity than the parent HBS α helix **2**. The role of T627 in the binding of HBS helices is also highlighted by HBS **7**, in which this residue is replaced with an alanine residue.[22] HBS **7** bound IZN17 with 20-fold lower affinity than **6**.

HBS helices **8** and **9** were prepared to explore the effect of additional contact points (residues that occupy *a* or *d* positions in the heptad) on the amino and carboxy ends. HBS helices **8** and **9** both include five residues that occupy *a* or *d* positions in the heptad and bind IZN17 with K_d values < 5 μM. The values for **8** and **9** represent the lower limits in the present 1ZN17 assay because competitive-binding analysis does not allow accurate estimates of K_d values much lower than the binding affinity of the fluorescent probe (see the Supporting Information).[23] Although the 1ZN17 assay did not provide accurate values for the best HBS helices, it allowed us to qualitatively evaluate various constructs described herein. We decided that a combination of the 1ZN17 binding assay and a gp41-mediated cell–cell fusion assay would provide a better gauge of the effectiveness of HBS helices as inhibitors of HIV fusion.[18]

Cell–cell fusion (that is, syncytium formation) was assayed by coculturing CHO[HIVe] (clone 7d2) cells expressing HXB2 envelope and Tat[24] with U373-MAGI cells in the presence of different concentrations of the peptide inhibitors. Cell fusion allows the expression of nuclear β-galactosidase from the U373-MAGI indicator cell line and can be quantitated by monitoring β-galactosidase activity.

Inhibition of gp41-mediated cell fusion by short peptides is a challenging feat and has only been accomplished with a handful of synthetic peptides.[8,11,18] We found that only HBS α helix **9** inhibited cell fusion, with an EC_{50} value of 43 μM (Figure 3c).[25] This value is comparable to those measured for side-chain-constrained α helices,[8] cyclic D-peptides,[18] aromatic foldamers,[9] and β-peptide foldamers.[11] The other HBS α helices, **2**–**8**, did not provide any hint of cell-fusion inhibition at concentrations up to 200 μM. Unconstrained peptide **10**, which bound IZN17 with a similar affinity to that of **9**, remained ineffective in the cell-culture assay. This result potentially reflects the proteolytic instability of the unconstrained peptide, as stabilization of peptides in α-helical conformation is expected to enhance their resistance to proteases.[26] We have previously reported improvements in the proteolytic stability of HBS α helices as compared to that of their unconstrained counterparts.[16]

In summary, through rational design and synthesis, we have developed an artificial α helix, **9**, that inhibits gp41-mediated cell fusion. As the formation of coiled-coil assemblies is a prerequisite for the fusion of several classes of viruses to their host cells,[27] this work suggests that HBS helices may be effective scaffolds for the generation of small-molecule inhibitors or antigens against these viruses.[28,29]

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