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De Novo Design of Helical Peptides to Inhibit Tumor Necrosis Factor-α by Disrupting Its Trimer Formation†

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A computational strategy was used to design helical peptides that can bind to tumor necrosis factor-α (TNFα) dimers to prevent the active trimer formation. Three designed peptides showed TNFα inhibition at the cellular level. Chemical crosslinking and mass spectrometry studies verified that these peptides function by breaking TNFα trimers.

Tumor necrosis factor-α (TNFα) is an important therapeutic target for treating autoimmune disorders such as rheumatoid arthritis and asthma,[1] which functions in a trimeric form. Its receptors, TNFR1 and TNFR2 form homo-oligomers.[2] Each TNFα trimer may interact with three TNFR trimers or dimers, which in turn bind with other TNFα trimers to eventually form a large two-dimensional cluster.[2] It has been suggested that the formation of the TNFα-TNFR cluster is essential for signal amplification.[3] To inhibit the biological function of TNFα, two possible ways have been explored: to directly block its binding with the receptor or to disassemble its active trimer. Antibodies and fused receptors have been successfully developed to directly bind to TNFα trimers to block its function, which are now clinically used for treating related diseases.[4] However, the immunogenicity of these proteins may interfere with efficacy and even lead to serious and sometimes life-threatening complications.[5] To avoid these drawbacks, peptide-based drug candidates have been explored as potential TNFα inhibitors, but only at the discovery stage at the moment.[6] On the other hand, several recently discovered small-molecules were found to inhibit TNFα through breaking the trimer and binding to its dimer.[7, 8, 9] However, none of these candidates has reached clinical application, due to the low potency and high cell toxicity.[9] In fact, due to the large size and shallow shape of protein-protein interfaces, designing small-molecule modulators of protein-protein interaction remains challenging.[10] Peptide therapeutics, which have low immunogenicity and cell toxicity, could be an alternative choice.[11]

Regulation of protein function by altering the oligomerization states of the target protein is a strategy that has started to attract increasing attention.[12] For example, phenothiazines have been discovered to disrupt S100A4 activity by inducing S100A4 to oligomerize into pentamers.[13] Another example is on HIV integrase (IN). Short peptide inhibitors derived from an IN's cellular-binding protein, LEDGF/p75, blocked the DNA-binding of IN by shifting the IN oligomerization equilibrium from the active dimer toward the inactive tetramer.[14] SPD304, a small-molecule that binds TNFα dimer to prevent the formation of active trimers, is of course another successful case.[7] The discovery of these protein assembly modulation molecules that selectively stabilize certain quaternary structures over others offers a new perspective for drug design. In most cases, modulation of protein self-assembly states requires the binder molecule to target protein-protein interfaces, which is difficult, especially for small-molecules, as protein-protein interfaces are usually large and flat. As a result, compared to small molecules, peptides, which can be considered as 'mini proteins', may be better candidates for modulating protein oligomerization states, as validated in a few previous studies.[15] More specifically, helical peptides can be promising modulators as helical motifs are often involved in protein-protein recognitions.[16]

Currently, most peptide-based therapeutics are derived from known native interactions, either via modification of native peptide ligands or from structure-based computational design.[17] In comparison, *de novo* designing peptide binders for a chosen target clearly opens more opportunities. Since helical motifs are often involved in protein-protein recognitions[16] and we have plenty of knowledge about its folding and binding, helical peptide can be a good starting point for *de novo* peptide design. Recently, we reported a computational *de novo* design method for helical peptide binders. This method has been successfully applied to design peptide inhibitors that can directly bind to TNFα trimer to block its interaction with the receptor. In the present study, we aimed to design peptides that can disassemble TNFα trimer by stabilizing its dimeric structure. To be specific, we aimed to find peptides that can replace one of the three monomers of TNFα as illustrated in Fig. 1a.

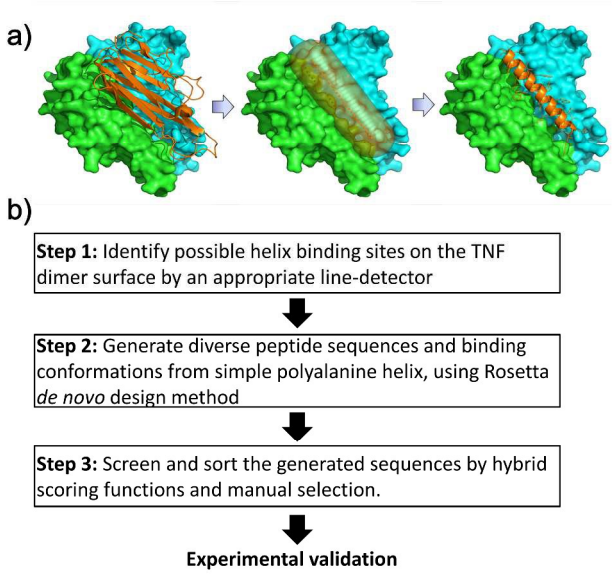

Fig. 1 Design of helical peptides that can block TNFα trimer formation by binding to TNFα dimer. a) TNF dimer (green and cyans surface) and monomer (orange on the left) interaction was blocked by de novo designing a single helical peptide (orang on the right). The The initial binding positions of the helix were determined by systematically searching the crevice surface of TMP dimer using a capsule (in the middle) sketchily representing the helix b) Computational design scheme.

The previously reported computational protocol[18] was applied to *de novo* design TNFα-dimer-binding peptides (Fig. 1b). In this work, six TNFα dimer structures including all of two neighboring chains in the two TNFα trimer crystal structure (1TNF[19] and 1A8M[20]) were used as the target (see details in ESI). The inhibitor peptides were designed to have 30 or 25-residues to fit the length of crevice between the two protomers of the dimer (~45 Å). First, the helical peptides were sketchily represented by a capsule (Figure 1a). The capsule was systematically translated and rotated on the crevice surface of TNFα dimer. Geometrical matching between the capsule and the crevice, as well as the hydrophobicity of attached target surface were evaluated at each position (details were given in ESI). We assumed that the designed peptide should take over as much as hydrophobic crevice surface to compete with TNF monomer. Figure S1 shows the best positions with both good geometrical matching and high hydrophobicity for 30-residue and 25-residue helix, respectively. Then, Rosetta program (version 2.3.0)[21] was used to generate sequences and binding conformations from initial TNF dimer- peptide structures (the initial structures were illustrated in Figure S2 and details about the structure preparation and Rosetta command line are described in SI). For either the 25-residue or 30-residue peptide, a total of 7,200 sequences (Fig. S2) and corresponding binding structure models were obtained from the Rosetta evolution trajectories. After that, a set of scoring functions, containing both the helical peptide stability evaluation and peptide-TNFα dimer interaction energy estimation, were used for virtual peptide screening. Peptide sequences that demonstrated low folding probability, high aggregation probability, low helix propensity, poor Rosetta energy, small contact size, or poor hydrophobic packing were eliminated according to the respective scoring parameter (screening conditions were listed in Table S2). The remaining sequences were ranked using a hybrid scoring function containing all of the above scoring parameters (ESI, Section 4). Tables S3 and S4 list the top 50 30-residue sequences (named DLH) and top 50 25-residue sequences (named DSH), respectively. Eventually, three 30-residue sequences and three 25- residue sequences (Fig. S3) were manually selected from the top- ranking 50 sequences for experimental validation.

To test whether the selected peptides display inhibitory effects on TNFα as designed, we first performed a surface plasmon resonance (SPR)-based *in vitro* screening for candidates that were able to bind with TNFα using GST-peptide fusions.[22] Next, active sequences were synthesized and tested for their inhibitory activities in cell-based NF-κB activation assay. Their ability to disrupt TNFα trimers was verified using chemical crosslinking and mass spectrometry.[7, 23]

We used a SPR-based method for the *in vitro* screening for peptides binders, where TNFα molecules were immobilized on a CM5 sensor chip. We followed a previously reported literature to prepare the chip surface so that a certain amount of TNFα dimers can be ensured.[22] Then, each of the selected peptide sequences fused with GST was injected as analytes. GST alone was used as a control. Four out of the six sequences, DLH01, DLH08, DSH01 and DSH03, showed significant binding with TNFα, while GST alone did not display any observable interaction (Fig. 2b). In subsequent studies, label-free chemically synthesized peptides were used to eliminate any possible interference from GST. Circular dichroism (CD) spectra of these peptides confirmed their α-helical secondary structures (Fig. 2c).

a)

Name	Length	Sequence
DLH01	30 aa	LREQRLKEHLQEIQIFEQWLQIFQEQFQKF
DLH08	30 aa	DAEDELKRNWQEIQEFQQILQMFFEWWQKD
DSH01	25 aa	RQQEELYKKWLQEFWKWFQIALQLD
DSH03	25 aa	QEKWFAEQYARMVLEWIEKELKESL

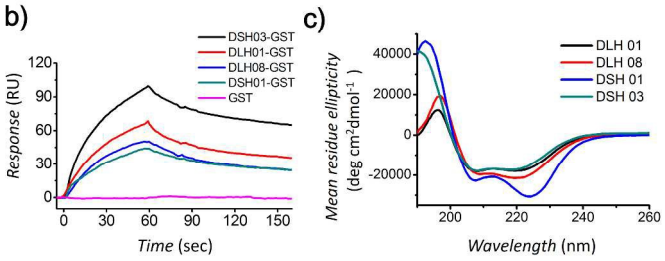

Fig. 2 Characterization of the designed peptides. a) Peptide sequences. Key residues for TNFα recognition are labelled in red (see also: Fig. S3 and Fig. S6). b) BIAcore binding assay curves of GST fusion proteins with immobilized TNFα. c) CD spectra of the synthesized peptides.

To ascertain peptide-TNFα interaction after elimination of the GST-tag, we measured the dissociation constants (K_D) using SPR kinetic assay (Fig. 3). The K_D of all four peptides are around 0.5 μM (Table S5), with DLH08 being the strongest binder with a K_D of 0.39 ± 0.02 μM.

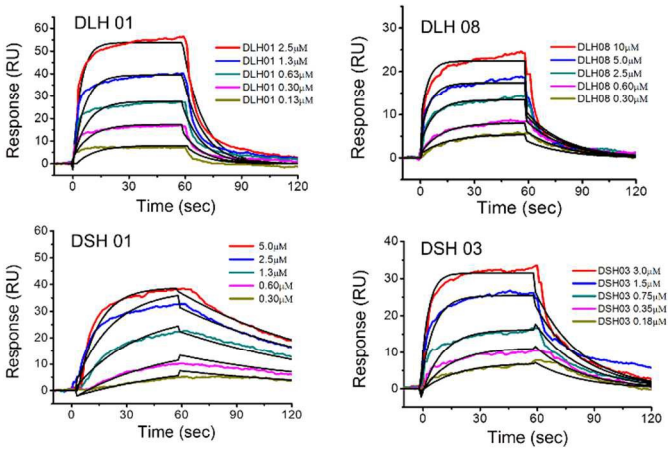

Fig. 3 Biacore kinetic binding assay. Colored curves stand for original data while black curves represent fitted data. The dissociation constants were fitted and summarized in Table S5.

We next investigated the ability of the peptides to inhibit TNFα signaling in cells. The TNFα induced NF-κB activation was measured using the Dual-Glo Luciferase assay system. TNFα and each selected peptide were incubated for 6 hrs and the mixture was then used to stimulate the HEK293T cells transfected with the NF-κB reporter system. The luciferase luminescence signal, which positively correlates with the amount of functional TNFα, was measured. Three of the four binding peptides, DLH08, DSH01 and DSH03, inhibited TNFα function in a dose-responsive manner. The half maxi-mum inhibitory concentration (IC_{50}) values ranged from 12 to 33 μM (Fig. 4). DLH08 was discovered to be the most potent inhibitor with an IC_{50} of 11.9 ± 0.4 μM. DLH01 did not show any activity in the cell assay. This was likely because of its hydrophobicity which caused non-specific binding with the components in the culture media.

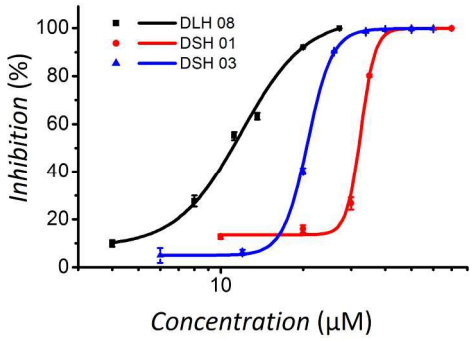

Fig. 4 Dose-response curves of cell luciferase assay. DLH01 has no detect-able activity in this assay. The IC_{50} values were fitted by the Hill model: DLH08 (blue triangle), 11.9 ± 0.4 μM; DSH01 (red circle), 32.6 ± 0.6 μM; and DSH03 (black square), 20.7 ± 0.4 μM. The data are reported as means ± errors from three independent experiments.

To verify the binding modes of the peptides with TNFα, we first checked whether the peptides can indeed break TNFα trimers as designed. We performed chemical cross-linking in combination with SDS-PAGE to reveal the change of TNFα oligomerization states upon peptide interaction. Cross-linking of TNFα alone by disuccinimidyl suberate (DSS) confirmed the dominance of protein trimers in solution (Fig. 3a, lane 2). In the presence of peptides, the ratio of TNFα trimer decreased significantly; accordingly, there was an obvious increase of the relative amount of TNFα dimer and monomer (Fig. 5a, lanes 3-5). As a control, the peptide, TBHa31, which was previously reported to bind with TNFα trimer, was also tested.[18] As expected, no reduction of TNFα trimers was observed (Fig. 3a, lane 6). Mass spectrometry also showed a tendency of trimer to dimer transition after incubation with the peptides (Fig. 5b). Both the cross-linking and mass spectrometry experiments confirmed that, as expected, the designed peptides can break TNFα trimers and shift its oligomerization equilibrium to the dimer side.

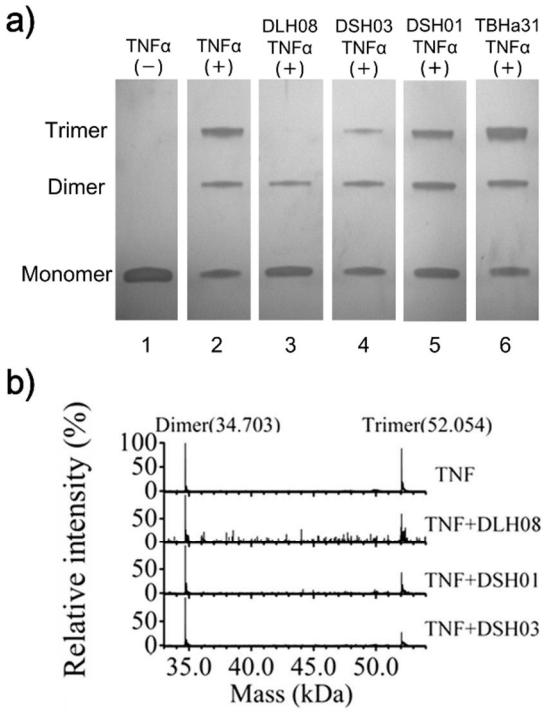

Fig. 5 Chemical cross-linking and mass spectrometry assay for DLH08. a) Chemical cross-linking results detected by SDS-PAGE. (+) indicates the addition of DSS, while (-) suggests no DSS was added. In lane 2 where only TNFα was present, the amount of cross-linked trimer is much more than that of dimer; in comparison, in the presence of DLH08 (lane 3), DSH03 (lane 4), or DSH01 (lane 5), cross-linked TNFα dimer is more than the trimer; as a control, in the presence of TBHa31 (lane 6), cross-linked trimer is more than the dimer. b) Quadrupole-time-of-flight mass spectra of TNFα with and without peptide mixed. The peaks of TNFα dimer and trimer are at 34.703kD and 52.054 kD, respectively. Intensity value is relative to dimer peak intensity in each spectrum. The relative ratio of trimer/dimer decreased upon peptide incubation.

In addition, we mutated the three key interaction residues, E12, F16 and L20, as predicted by the peptide-TNFα dimer complex model and the conservation analysis (Fig. S3), to Ala in turn, and tested their influence on the peptide inhibition ability using the cell assay (Fig. 6). All three mutants displayed reduction of inhibitory activity. Taken together, these experiments suggest that the peptides bind to the TNFα dimer as designed.

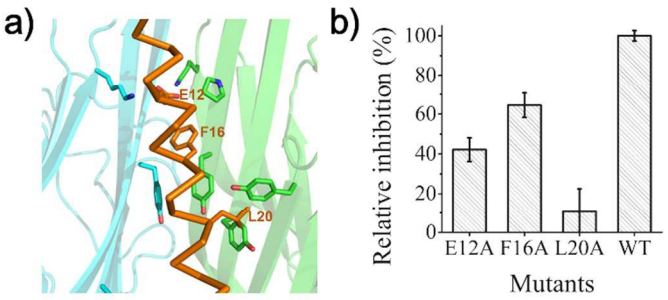

Fig. 6 Mutagenesis study. a) Interaction analysis of the mutated residues (orange sticks) of DLH08 (orange ribbon) with TNFα dimer (in green, cyan). b) Inhibitory activity of single-mutated DLH08 relative to native DLH08, measured by cell luciferase assay.

DLH08, the most potent peptide inhibitor discovered in this study, showed comparable inhibitory activity as SPD304, one of the strongest small molecule TNFα inhibitors reported so far.[7] The IC_{50} for SPD304 obtained in the side-by-side cell assay was about 15 μM (Fig. S4). Noticeably, all these peptides displayed no cell toxicity even at concentrations higher than 300 μM. In comparison, 50% of cells died when treated with 30 μM of SPD304 (Fig. S5). The high potency and low cytotoxicity makes DLH08 a potential drug candidate for anti-TNFα therapies.

Conclusions

We have computationally identified an α-helical peptide binding site in the TNFα dimer structure and de novo designed peptides that can block the TNFα trimer formation and inhibit its biological activity. Four of the six experimentally tested peptides showed binding with TNFα in the SPR assay and three of them showed considerable high activity in the cellular luciferase assay. With an IC_{50} of 11.9 ± 0.4 μM, the strongest peptide, DLH08 displayed comparable inhibitory to SPD304, the best known compound that binds to TNFα dimer. With no observable cell cytotoxicity, these peptides have great potential to be further developed as therapeutic agents targeting TNFα.

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Notes and references

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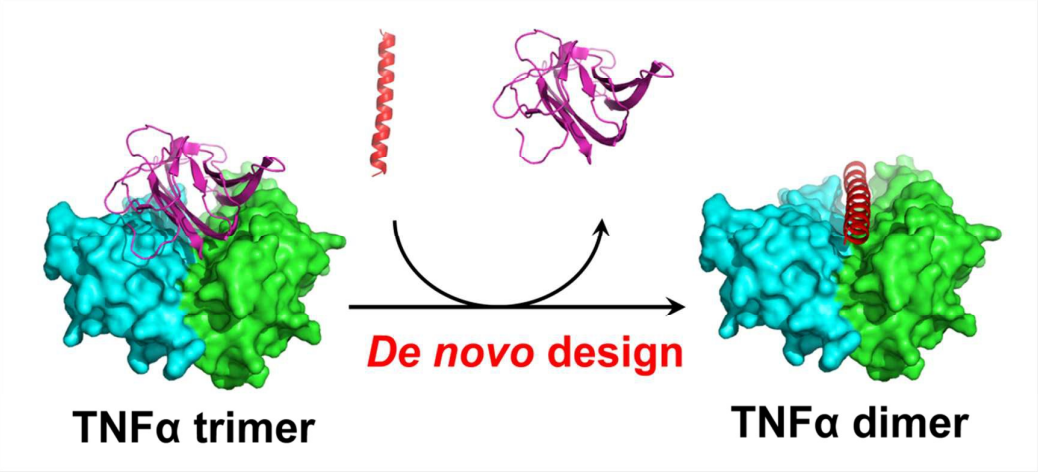

Helical peptide TNFα inhibitors were designed by targeting its dimer structure.