Therapeutic Peptides

Deutsche Ausgabe: DOI: 10.1002/ange.201603230
Internationale Ausgabe: DOI: 10.1002/anie.201603230

A Cyclized Helix-Loop-Helix Peptide as a Molecular Scaffold for the Design of Inhibitors of Intracellular Protein–Protein Interactions by Epitope and Arginine Grafting

Daisuke Fujiwara, Hidekazu Kitada, Masahiro Oguri, Toshio Nishihara, Masataka Michigami, Kazunori Shiraishi, Eiji Yuba, Ikuhiko Nakase, Haeri Im, Sunhee Cho, Jong Young Joung, Seiji Kodama, Kenji Kono, Sihyun Ham, and Ikuo Fujii**

Abstract: *The design of inhibitors of intracellular protein–protein interactions (PPIs) remains a challenge in chemical biology and drug discovery. We propose a cyclized helix-loop-helix (cHLH) peptide as a scaffold for generating cell-permeable PPI inhibitors through bifunctional grafting: epitope grafting to provide binding activity, and arginine grafting to endow cell-permeability. To inhibit p53–HDM2 interactions, the p53 epitope was grafted onto the C-terminal helix and six Arg residues were grafted onto another helix. The designed peptide cHLHp53-R showed high inhibitory activity for this interaction, and computational analysis suggested a binding mode for HDM2. Confocal microscopy of cells treated with fluorescently labeled cHLHp53-R revealed cell membrane penetration and cytosolic localization. The peptide inhibited the growth of HCT116 and LnCap cancer cells. This strategy of bifunctional grafting onto a well-structured peptide scaffold could facilitate the generation of inhibitors for intracellular PPIs.*

The design of inhibitors of intracellular protein–protein interactions (PPIs) remains challenging in chemical biology and drug discovery.[1] To act as an intracellular PPI inhibitor, the inhibitor must exhibit high binding affinity for the target proteins, high resistance to proteolytic degradation, and high stability in the reducing environment found inside cells. In addition, it must be taken up by the cell, penetrate and cross the cell membrane, and be released into the cytosol. One of the methods used to generate such inhibitors is protein grafting on a relatively small protein scaffold.[2] A scaffold capable of comprehensively fulfilling all of these requirements would enable the design of intracellular PPI inhibitors. Herein, we propose a cyclized helix-loop-helix (cHLH) peptide as a suitable scaffold for the design of intracellular PPI inhibitors by using a bifunctional grafting approach involving "epitope grafting" and "arginine grafting".[2,3]

Grafting strategies based on protein scaffolds have been developed for the design of novel functional proteins.[2,3] In epitope grafting, the critical binding residues of proteins, such as the BH3-heilcal region of Bclx2 or the HIV-1-inhibiting epitope, are transferred onto a protein scaffold to provide binding activity.[1] Avian pancreatic polypeptide (aPP), the GCN4 leucine zipper, and Apamin have been used as protein scaffolds onto which epitopes can be grafted.[2] In arginine grafting, polyarginine residues are installed onto the surfaces of GFP and aPP, and these grafted proteins showed cell permeability.[3] In this work, a combination of these two grafting strategies was applied to cHLH to design cell-permeable PPI inhibitors.

We have previously designed a helix-loop-helix (HLH) peptide as a scaffold for generating PPI inhibitors.[4] The HLH peptide YT1 consists of two α-helices stabilized by hydrophobic interactions between leucine residues embedded on the helix–helix interface (Figure 1). The peptide YT1 retains its secondary structure when solvent-exposed amino acids outside the C-terminal helix are randomly substituted with

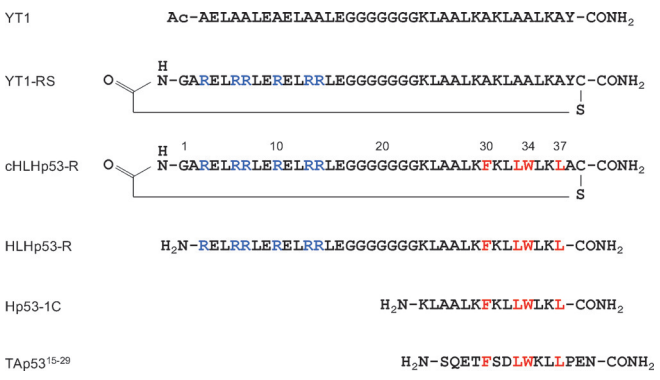

Figure 1. Structures of the cHLHp53-R peptide, its derivatives, and the control peptides.

[*] Dr. D. Fujiwara, H. Kitada, M. Oguri, T. Nishihara, Dr. M. Michigami, Dr. K. Shiraishi, Prof. S. Kodama, Prof. I. Fujii
Department of Biological Science
Graduate School of Science
Osaka Prefecture University
1-1, Gakuen-cho, Naka-ku, Osaka 599-8531 (Japan)
E-mail: fujii@b.s.osakafu-u.ac.jp
Dr. E. Yuba, Prof. K. Kono
Department of Applied Chemistry
Graduate School of Engineering
Osaka Prefecture University
1-1, Gakuen-cho, Naka-ku, Osaka 599-8531 (Japan)
Dr. I. Nakase
N2RC, Osaka Prefecture University
1–2, Gakuen-cho, Naka-ku, Osaka 599-8570 (Japan)
H. Im, S. Cho, Dr. J. Y. Joung, Prof. S. Ham
Department of Chemistry, Sookmyung Women's University
Hyochangwongil 52, Yongsanku, Seoul, 140-742 (Korea)
E-mail: sihyun@sm.ac.kr

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201603230.

a variety of amino acids.[4b] We then constructed phage-display libraries of the HLH peptide, and obtained ligands and inhibitors for the granulocyte-colony stimulating factor receptor (G-CSFR), ganglioside GM1, and Aurora kinase A.[5] Furthermore, an IgG-Fc-binding HLH peptide was successfully designed by using a protein grafting strategy.[6] In this work, we attempted to design an intracellular PPI inhibitor for the p53–HDM2 interaction, based on the HLH scaffold. The p53 tumor suppressor protein is a transcription factor that regulates a major pathway for protecting cells from malignant transformation.[7] Under non-stressed conditions, p53 binds with HDM2 and is inactivated. In response to stress, p53 dissociates from HDM2 and its cellular level is elevated, which leads to cell-cycle arrest or apoptosis. HDM2 is overproduced in many human tumor cells to reduce the level of p53.[8] Therefore, we hypothesized that a cell-permeable HLH peptide capable of specifically binding to HDM2 would disrupt the p53–HDM2 interaction and reactivate the p53 pathway to inhibit tumor-cell growth.[9,10]

The crystal structure of the p53–HDM2 interface shows the steric complementarity between the HDM2 cleft and the hydrophobic face of the p53 transactivation domain ($TAp53^{15–29}$). The TA domain is folded into an α-helical structure. A hydrophobic triad (Phe^{19}, Trp^{23}, Leu^{26}) inserts deeply into the HDM2 cleft, and the side chain of Leu^{22} packs against the side of the HDM2 cleft to provide additional van der Waals contacts.[8] Based on this structural information, we first grafted Phe^{19}, Leu^{22}, Trp^{23}, and Leu^{26} onto the C-terminal helix of the scaffold peptide YT1 to generate a HLH inhibitor for the p53–HDM2 interaction. The grafted HLH peptide should mimic the α-helix of the TA domain of p53. Second, to confer cell permeability, we mutated the six solvent-exposed alanine residues in the N-terminal helix to arginines to generate the cationic peptide HLHp53-R (Figure 1). This poly-Arg-grafted peptide should accumulate on the cell surface and enter the cells.[3] Third, we linked the N and C termini with a thioether bond, which should be stable under the reducing conditions found inside cells, to provide the cyclized peptide cHLHp53-R. The cyclization should stabilize the α-helical structure and improve the proteolytic resistance. All of the peptides were synthesized by solid-phase methods. For the cyclization, a thioether linkage was formed between *N*-chloroacetylglycine at the N terminus and cysteine at the C terminus (Figure 1). Peptide cleavage from the resin, deprotection with TFA, and cyclization in aq. NH_4HCO_3 afforded the cHLH peptides.

We first examined the structure–binding relationship of cHLHp53-R and its derivatives for HDM2 (Table 1). The binding affinity was determined by surface plasmon resonance (SPR) assays using the N-terminal domain of HDM2 ($HDM2^{17–125}$), [11] which has been shown to participate in interaction with p53,[8] as the immobilized ligand (Figure S2 in the Supporting Information). The α-helical structure was evaluated by circular dichroism (CD) spectroscopy.[12] The grafted cyclic peptide cHLHp53-R showed strong binding affinity for HDM2 ($K_d = 10$ nM) and the linear peptide HLHp53-R showed a moderate binding affinity ($K_d =$ 364 nM). The K_d values for the C-terminal half peptide Hp53-C and $TAp53^{15–29}$ were estimated to be 1.9 μM and 2.8 μM, respectively. In the CD spectra, cHLHp53-R showed higher α-helical content than that of the non-cyclized peptide HLHp53-R, thus suggesting that cyclization stabilizes the secondary structure. Peptides, Hp53-C and $TAp53^{15–29}$ showed no CD evidence for α-helical conformations. We thus observed a correlation between the binding affinity and the α-helical contents. The binding affinity of cHLHp53-R was improved by 40- and 210-fold compared to HLHp53-R and Hp53-C, respectively. These results suggest that the structural constraint of the grafted epitope on the cHLH scaffold lowers the entropy cost of binding.[13] In a control experiment, peptides without the binding epitopes (YT1 and its poly-Arg derivative YT1-RS) showed no binding with $HDM2^{17–125}$.

Table 1: Chemical properties of peptide cHLHp53-R and its derivatives.

Peptide	α helicity[a] [%]	K_d[c] [nM]	IC_{50}[e] [nM]
YT1	52	–[d]	–[f]
YT1-RS	75	–[d]	–[f]
cHLHp53-R	63	10.4 ± 2.3	36.5 ± 6.5
HLHp53-R	28	364 ± 71	592 ± 75
Hp53-C	–[b]	1890 ± 90	> 1000
$TAp53^{15–29}$	–[b]	2800 ± 740	660 ± 88

[a] The helical content of each peptide was determined from mean residue CD at 222 nm, $[\theta]_{222}$ (deg cm^2 dmol^{-1}), for 20 μM peptide in phosphate-buffered saline (PBS).[12] [b] A typical spectrum showing α-helical structure was not detected. [c] The K_d values of the peptides were measured by SPR. [d] No binding was observed. [e] The IC_{50} values were estimated from the inhibitory activity against the $TAp53^{15–29}$–$HDM2^{17–125}$ interaction by SPR. [f] No inhibition was observed.

As expected from its binding affinity, the grafted peptide cHLHp53-R acts as a tight-binding inhibitor of the p53–HDM2 interaction (Table 1). The inhibitory activities of the peptides were further estimated by SPR-based competitive assays, in which different concentrations of individual peptides were pre-incubated with $HDM2^{17–125}$ and applied to immobilized-$TAp53^{15–29}$ on a sensor chip (Figure S2).[14] The half-maximal inhibitory concentration (IC_{50}) values for cHLHp53-R, HLHp53-R, Hp53-1C, and $TAp53^{15–29}$ were 36 nM, 592 nM, > 1 μM, and 660 nM, respectively. No inhibition was observed for the scaffolds without the binding epitope (YT1 and YT1-RS).

To understand the structural characteristics of the unbound form of cHLHp53-R and its binding complex with HDM2, we performed fully atomistic, explicit-water molecular dynamics (MD) simulations (Figure 2). For the unbound form of cHLHp53-R, the antiparallel monomeric α-helical structure was observed with 67% helicity, which is in agreement with the α-helical contents determined by CD spectroscopy (Figure S1). The leucine residues were positioned at the helix-loop-helix interface and the grafted hydrophobic residues (Phe^{30}, Leu^{33}, Trp^{34}, and Leu^{37}) were solvent exposed. Based on the MD simulations, the N-terminal helix (residues 2–16) of cHLHp53-R showed salt bridges between the glutamic acid and arginine residues (Glu^4–Arg^7 and Glu^{11}–Arg^{14}), which stabilized its helical conformation.

To investigate the stable complex structure of cHLHp53-R with HDM2, we performed a molecular docking simulation followed by MD simulations (see the Supporting Information

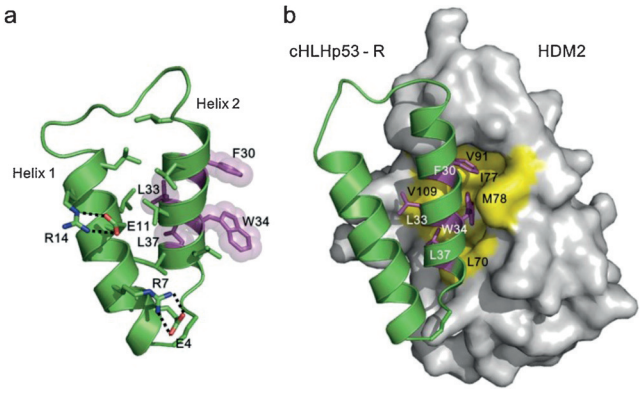

Figure 2. Simulated structures of the cHLHp53-R peptide in unbound form (a) and bound with HDM2 (b). Salt bridges in the N-terminal helix are shown as dotted lines and the grafted residues (Phe^{30}, Leu^{33}, Trp^{34}, Leu^{37}) in the C-terminal helix are shown in magenta. The binding interface in HDM2 is shown in yellow.

for detailed computational methods). Upon complexation, the overall fold of cHLHp53-R was maintained with an increased helicity of 73%. Upon complexation, the main binding mode between cHLHp53-R and HDM2 was hydrophobic interactions between the grafted hydrophobic residues in the C-terminal helix (residues 24–38) of cHLHp53-R and the hydrophobic pocket of the HDM2 cleft (Figure 2b). The binding interface between p53 and HDM2 was fairly well conserved in the cHLHp53-R/HDM2 structure, thus indicating that the bifunctional grafting strategy with the cHLHp53-R peptide led to the successful generation of a PPI inhibitor.[8a]

To further characterize the molecular origin and binding affinity of the cHLHp53-R /HDM2 complex, we computed thermodynamic factors, that is, free energy (f), which is the sum of solvation free energy (G_{solv}) and the total internal energy (E_u): $f = E_u + G_{solv}$. The binding free energy upon complexation of cHLHp53-R and HDM2 was computed to be $-125.5\ kcal\,mol^{-1}$ in aqueous environments. Since the total charge of cHLHp53-R was $+6$ and that of HDM2 is $+5$, the internal energy change upon complexation is highly repulsive ($\Delta E_u = +270.1\ kcal\,mol^{-1}$). This is compensated by the solvation free energy change (ΔG_{solv}) of $-395.6\ kcal\,mol^{-1}$. Based on site-directed thermodynamics analysis of the binding free energy,[15] it was evident that the epitope residues (Phe^{30}, Leu^{33}, Trp^{34}, and Leu^{37}) of cHLHp53-R contribute most distinctively to the binding free energy upon complexation (Figure S4). The binding interface of HDM2 for cHLHp53-R is mostly hydrophobic (Leu^{70}, Leu^{73}, Ile^{77}, Met^{78}, Leu^{82}, Ile^{90}, Val^{91}, Phe^{107}, Val^{109}, and Ile^{115}), and these residues are also involved in recognizing p53 protein in the p53/HDM2 complex. Thermodynamic analysis based on the simulated structure for cHLHp53-R and its binding complex with HDM2 revealed the binding mode and the main contributors upon complexation.

Peptides are generally susceptible to proteolysis, and that is a drawback for their use as therapeutics. Conformational constraint of the peptides based on the cHLH scaffold should provide high stability against proteolysis. To examine the proteolytic stability of the grafted peptides, we determined their half-lives ($t_{1/2}$) in the presence of intracellular protease cathepsin G: the remaining peptides in the proteolysis were monitored by reversed phase (RP)-HPLC.[16] As expected, the unstructured $TAp53^{15-29}$ was easily degraded, with a $t_{1/2}$ value of 4 min, while the grafted peptides cHLHp53-R, HLHp53-R, and Hp53-1C resisted proteolysis, with $t_{1/2}$ values of more than 200 h, 26.7 h, and 25.8 h, respectively (Figure S6). The proteolytic stability of cHLHp53-R and its derivatives thus showed a similar pattern to the α-helical content. The Arg-grafted peptide YT1-RS, a control in the bioassay, also showed proteolytic resistance, with a $t_{1/2}$ value of 60.3 h. As expected from the stability against cathepsin G, cHLHp53-R also showed high stability in human serum, with $t_{1/2} > 103$ h (Figure S7).

The grafted peptide should penetrate cell membranes and enter the cytosol to inhibit the p53–HDM2 interaction. We examined the ability of cHLHp53-R to penetrate a representative cancer cell line (HeLa cells) by observing fluorescently labeled cHLHp53-R with live-cell confocal microscopy (Figure 3a). Tetramethylrhodamine was covalently attached to

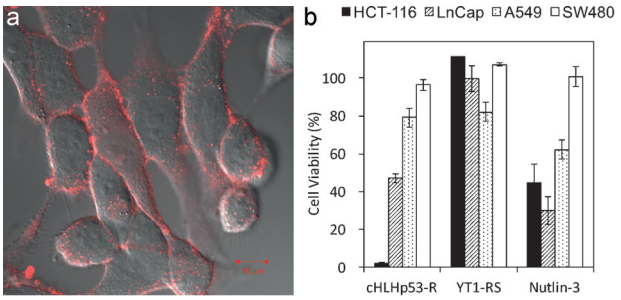

Figure 3. The cellular activity of peptide cHLHp53-R. a) Cell membrane penetration of cHLHp53-R was observed in Hela cells treated with 10 μM TMR-labelled cHLHp53-R within a few minutes. Scale bar: 10 μm. b) Growth inhibition of cancer cells was tested with 25 μM peptide or Nutlin-3 by WST-1 assay.

the N-terminal helix of cHLHp53-R to provide the fluorescent peptide TMR-cHLHp53-R, which has a K_d value of 6 nM (Supporting Figure S5). HeLa cells attached to a glass-bottom dish were treated with TMR-cHLHp53-R (10 μM) and observed. Figure 3a shows efficient cellular uptake and cytosolic diffusion of TMR-HLHp53-R in HeLa cells within a few minutes without any morphological changes to cells following treatment. This suggests that arginine grafting effectively leads to the accumulation of the peptide on cell membranes, thereby leading to induction of cell membrane penetration without cytotoxicity during short periods of application.

Having confirmed the cell membrane permeability of the peptide, we next examined the effect of the peptides on the viability of cancer cells by using the WST-1 assay (Figure 3b). Cancer cells such as HCT116, LnCap, and A549 carry wild-type p53 and SW480 cells carry mutant p53.[9] 4,000 cells per well in a 96-well plate were incubated with serial dilutions of cHLHp53-R, YT1-RS, or Nutlin-3, a well-known inhibitor for the p53–HDM2 interaction. Both Nutlin-3 and cHLHp53-R

showed antiproliferative activity in the HCT116 and LnCap cells and led to moderate growth inhibition in the A549 cells, but no growth inhibition was seen in the SW480 cells. The peptide YT1-RS, which has no binding epitope, showed no growth inhibition for any of the cancer cells. These observations suggest that cHLHp53-R suppresses the growth of cancer cells by inhibiting the p53–HDM2 interaction and not as a result of cytotoxicity caused by the polyarginine moiety on the N-terminal helix.

In summary, cHLHp53-R exhibited high binding affinity for HDM2, inhibitory activity for the p53–HDM2 interaction, and proteolytic stability against the intracellular protease cathepsin G. In addition, this peptide showed cell membrane penetration and p53-dependent antiproliferative activity in cells. In conclusion, the cHLH peptide scaffold is expected to be a potent scaffold for generating intracellular PPI inhibitors. Further optimization of arginine grafting onto the cHLH peptide could help increase the rate of diffusion into the cytosol and/or the rate of efficient escape from early endosomes.[3c] This work demonstrates that grafting both epitope and arginine onto well-structured scaffold peptides can facilitate the generation of intracellular PPI inhibitors and therapeutic leads.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 25242068, and Samsung Science and Technology Foundation under Project Number SSTF-BA1401-13.

Keywords: cell-penetrating peptides · epitope grafting · helix-loop-helix peptides · inhibitors · protein–protein interactions

How to cite: *Angew. Chem. Int. Ed.* **2016**, *55*, 10612–10615
Angew. Chem. **2016**, *128*, 10770–10773

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Received: April 2, 2016
Revised: June 7, 2016
Published online: July 28, 2016

