Development of cell penetrating peptide-based ubiquitin ligase ligand for the development of PROTAC

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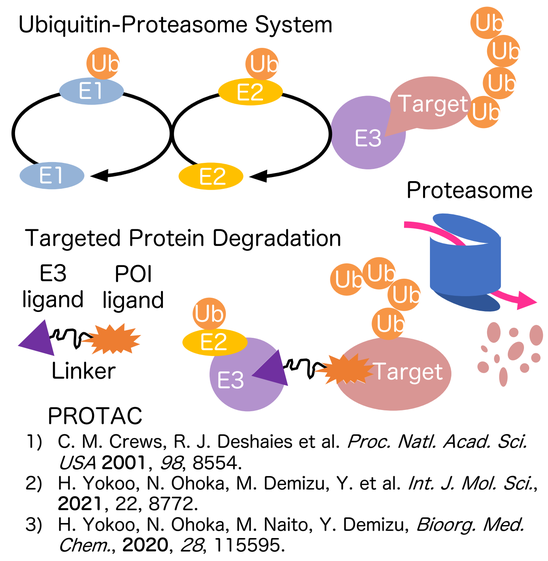
OHidetomo Yokoo[1], Zhou Dongrui[1,2], Yosuke Demizu[1,2]
([1]National Institute of Health Sciences, Japan, [2]Yokohama City University, Japan)

P1.234

Abstract PROTAC (PROteolysis TArgeting Chimeric molecule) [1], which induces ubiquitination and degradation of protein of interest by recruiting ubiquitin ligase (E3) to target proteins, is expected to be a novel therapeutic modality. PROTAC is a chimeric molecule that combines the E3 ligand with the target protein ligand via an appropriate linker, and inhibits protein function through its degradation. We have developed peptide-based PROTAC by incorporating cell penetrating peptides and modifications like stapling structures to enhance cell membrane permeability [2, 3]. This molecule was expected to expand targets to include proteins considered undruggable by conventional small molecule-based drugs. However, low cell membrane permeability remains a problem for the development of PROTAC. Therefore, in this study, we enhanced the cell membrane permeability to the E3 ligand, a commonly used moiety in PROTACs. We designed a peptide-based E3 ligand moiety by conjugating it with cell penetrating peptides, including oligoarginine, endosomal escape domains (EEDs), and oligo-sarcosines, and attached a small molecule-based fluorophore as a model ligand for target proteins to the designated E3 ligand.

The designed peptides were synthesized through solid-phase synthesis, and their cell penetrating ability was evaluated by detecting the fluorescence from the fluorophore conjugated to the peptide. We found that controlling the introduction and sequence of oligoarginine, the EED and oligo-sarcosine could improve the efficiency of intracellular delivery of peptide-based E3 ligands. Therefore, the obtained E3 ligands were applied to PROTAC, in which peptide ligands called peptidomimetic estrogen receptor modulators (PERMs) for estrogen receptor α were introduced into the obtained E3 ligand peptides. Their degradation activities were evaluated by western blotting. As a result, the PROTAC with oligoarginine and the EED showed higher degradation activity than that of the parent peptide, suggesting that the developed E3 ligand peptide would be useful for the development of PROTAC.



1) C. M. Crews, R. J. Deshaies et al. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 8554.
2) H. Yokoo, N. Ohoka, M. Demizu, Y. et al. *Int. J. Mol. Sci.*, **2021**, 22, 8772.
3) H. Yokoo, N. Ohoka, M. Naito, Y. Demizu, *Bioorg. Med. Chem.*, **2020**, *28*, 115595.

Design

The following peptides were designed by introducing VHL peptide ligand (ALAPYIP) as a model E3 ligase for VHL and fluorescent substance 5(6)-FAM as a model for small molecule target protein ligands.

R9 : 5(6)-FAM-GRRRRRRRRR-NH_2
VHL : 5(6)-FAM-βAla-ALAPYIP-NH_2
VHLR9 : 5(6)-FAM-βAla-ALAPYIP-R_9-NH_2
VHLSarnR9 : 5(6)-FAM-βAla-ALAPYIP-$(Sar)_n$-R_9-NH_2
R9VHLSarn : 5(6)-FAM-βAla-R_9-ALAPYIP-$(Sar)_n$-NH_2
R9VHLEEDSarn : 5(6)-FAM-βAla-R_9-ALAPYIP-Ahx-GFWFG-$(Sar)_n$-NH_2
EEDVHLSarnR9 : 5(6)-FAM-βAla-GFWFG-Ahx-ALAPYIP-$(Sar)_n$-R_9-NH_2
EEDVHLR9Sarn : 5(6)-FAM-βAla-GFWFG-Ahx-ALAPYIP-R_9-$(Sar)_n$-NH_2

Ahx; 6-aminohexanoic acid

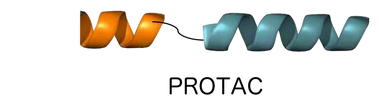
Design of PROTAC

PROTAC1 : H-RILRCLLQ-G_3-ALAPYIP-NH_2

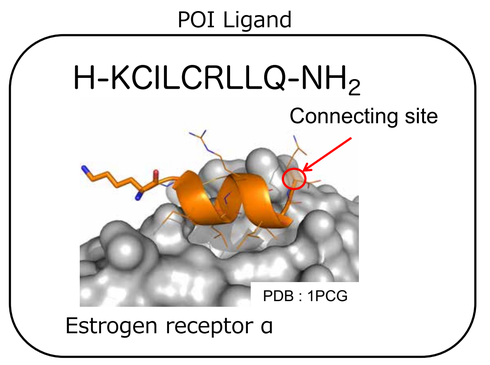
PROTAC1-R_9 : H-RILRCLLQ-G_n-ALAPYIP-R_9-NH_2 (n = 1 – 6)

H-RILRCLLQ-G_3-GFWFG-Ahx-ALAPYIP-$(Sar)_n$-R_9-NH_2 (n = 0, 5, 10, 20)

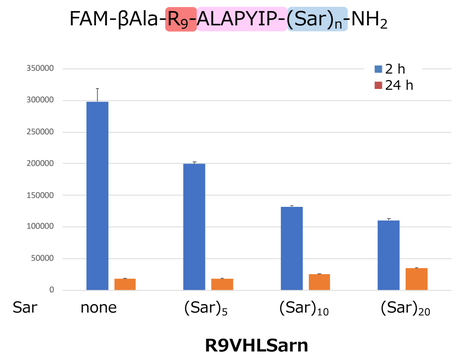
EED-PROTAC1-Sarn-R_9



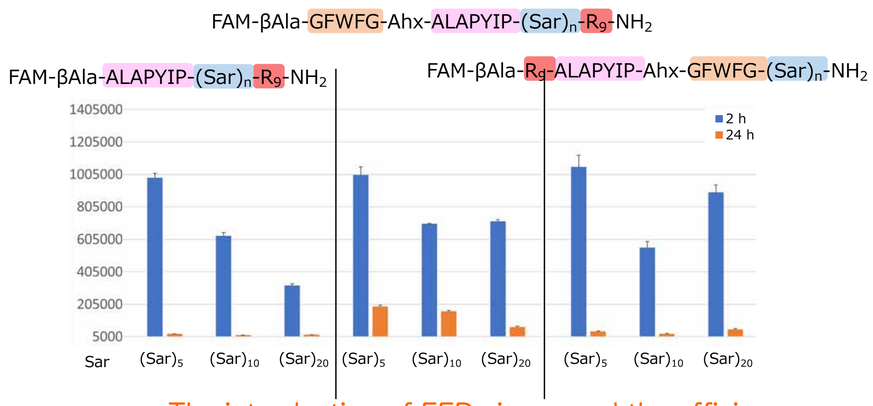
The E3 ligand was bound to sites facing outside that are unlikely to inhibit the binding to the target protein.



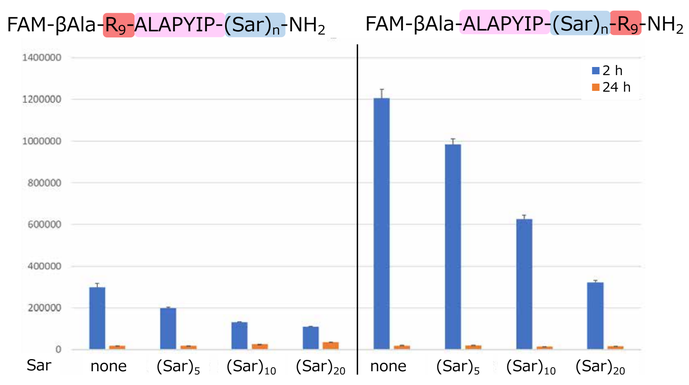
Cell-penetrating efficiency



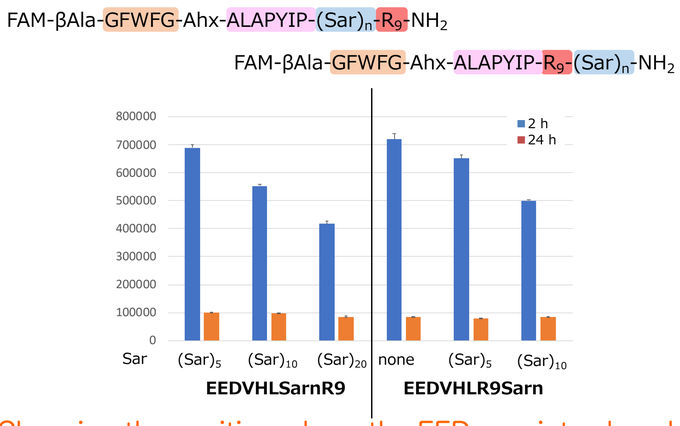
Uptake efficiency decreased as Sar elongated.



The introduction of EEDs increased the efficiency of uptake, especially for peptides containing Sar.



The uptake efficiency was greatly improved by introducing R_9 at the C-terminus and Sar as a linker.



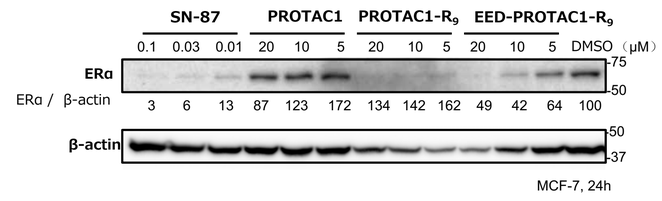
Changing the position where the EED was introduced did not significantly change the uptake efficiency.

Degradation activity

H-RILRCLLQ-G_3-ALAPYIP-NH_2

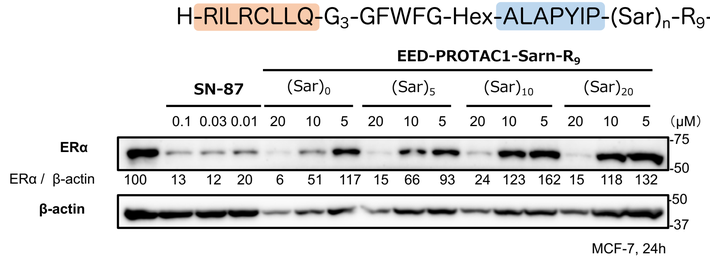
H-RILRCLLQ-G_3-ALAPYIP-R_9-NH_2

H-RILRCLLQ-G_3-GFWFG-Ahx-ALAPYIP-R_9-NH_2



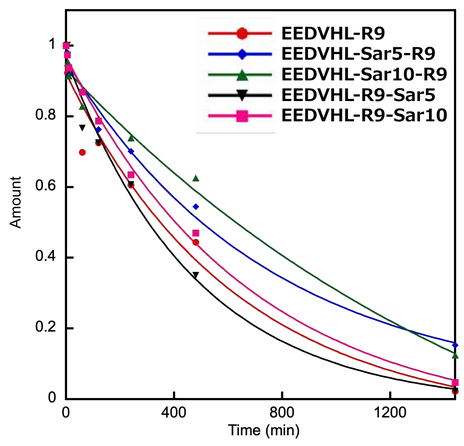
The introduction of cell penetrating peptides and EEDs resulted in higher degradation activity than simply peptide PROTAC.

H-RILRCLLQ-G_3-GFWFG-Hex-ALAPYIP-$(Sar)_n$-R_9-NH_2



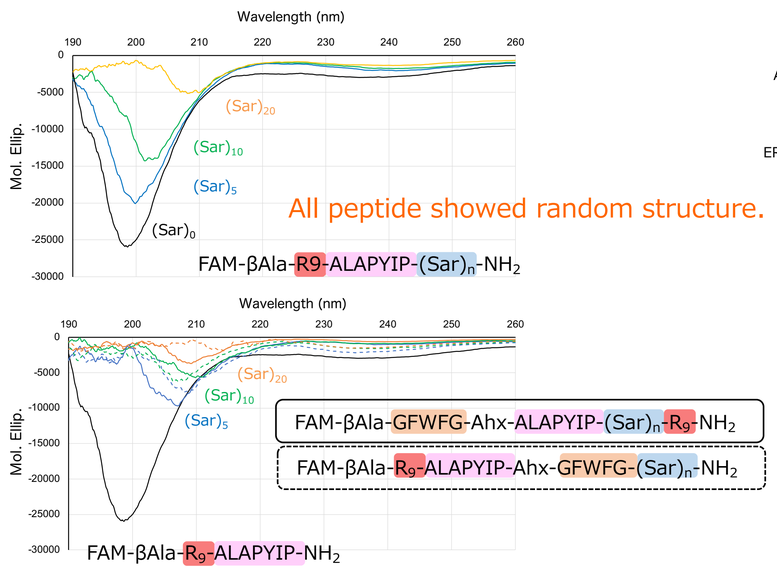
The peptide-based PROTAC with Sar also showed degradation activity.

Protease resistance



The introduction of Sar showed a slight increase in protease resistance.

CD sprectra



All peptide showed random structure.

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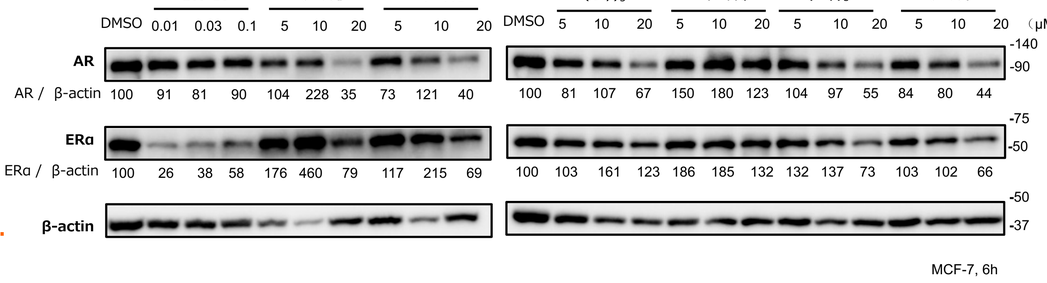
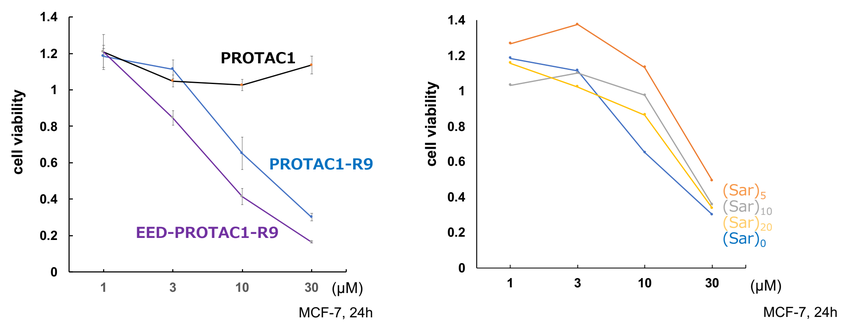
H-RILRCLLQ-G_n-ALAPYIP-R_9-NH_2



Among PROTAC sequences with different linker lengths, PROTAC with Gly_6 linker was highly active. Furthermore, the PROTAC degraded ERα and AR, The developed peptide will be useful as a model PROTAC.

Cell viability

H-RILRCLLQ-G_3-GFWFG-Ahx-ALAPYIP-$(Sar)_n$-R_9-NH_2



PROTAC with R_9 showed cell growth inhibitory effect on MCF-7 cells, and PROTAC with Sar showed similar activity.

Conclusion

- ✓ We designed and synthesized peptides with R_9, EED , and Sar.
- ✓ We found that the cellular uptake efficiency changed depending on the presence or absence of R_9, EED, and Sar and their order and that optimizing the sequences could improve the uptake.
- ✓ We designed and synthesized PROTAC using the obtained cell penetrating peptides.
- ✓ The peptide-based PROTAC with highly cell penetrability that is R_9 showed high activity.
- ✓ We developed a series of cell penetrating E3 ligand peptides that will be useful for optimizing degradation activity.