Investigation of intracellular delivery of NuBCP-9 by conjugation with oligoarginines peptides

（細胞膜透過性 oligoarginines ペプチドによるアポトーシス誘導性 NuBCP-9 ペプチドの細胞内送達に関する研究）

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[Purpose]
Pro-apoptotic peptides are promising alternatives to chemotherapeutic drugs because they could induce cancer cell death effectively and specifically. Bcl-2 is a well-known target for inducing apoptosis. NuBCP-9 peptide has been reported to interact with Bcl-2 to induce apoptosis. However, many Bcl-2-targeted peptides are impermeable to cell. Cell penetrating peptides (CPPs) are commonly used vectors for intracellular delivery of peptides due to simpler synthesis and conjugation methods with high uptake efficiency. Oligoarginines (Rn) are commonly used CPPs. When utilizing R8 for delivering NuBCP-9, not only promoted uptake efficiency but also enhanced non-specific cytotoxicity (unrelated with Bcl-2) have been reported. Due to Rn with different length possess different uptake efficiency, it is worthy to investigate the influence of NuBCP-9 conjugation on uptake efficiency of different Rn. Furthermore, related to such enhancement of uptake efficiency, the uptake mechanism is still undiscussed.

In this study, I firstly investigated the influence of NuBCP-9 conjugation of Rn (n = 0, 6, 8, 10, 12 and 14) on the cellular uptake, distribution and uptake pathways in MDA-MB-231 cells. Besides, effective biological based therapy not only depends on the cellular uptake of therapeutic agent via delivery vectors but also ability to escape from endosomes to reach intracellular target for eliciting its effect. For finding suitable length of Rn to deliver NuBCP-9 into cells then interact with Bcl-2, the cytotoxicity including membrane disrupted effect and apoptosis that caused by NuBCP-9-Rn conjugates were evaluated for confirming the efficient cytosolic delivery of peptides.

[Methods]
Fmoc (9-fluorenylmethyloxy-carbonyl) solid phase peptide synthesis method was utilized for producing crude unlabeled Rn and NuBCP-9-Rn (n = 0, 6, 8, 10, 12 and 14) conjugates peptides, and RP-HPLC was utilized for purifying. For investigating the uptake of peptides, the peptides were labelled with fluorescein-5-maleimide (F5M) by thiol-maleimide click reaction. The purity and molecular weight of peptides were confirmed with RP-HPLC and MALDI-TOF-MS. The cellular uptake level and subcellular distribution of peptides were analyzed under flow cytometry and confocal microscopy (CLSM). The influence of three typical uptake inhibitors and low temperature were also evaluated. The cell viability was evaluated using WST-8 assay. Membrane disruption effect and induced apoptosis level were also evaluated with LDH assay and Annexin V-FITC/ Propidium Iodide (PI) staining, respectively.

[Results and Discussion]
1. Investigation of cellular uptake, distribution and uptake pathways of Rn and NuBCP-9-Rn conjugates in MDA-Mb-231 cells
1.1. Cellular uptake and distribution of Rn and NuBCP-9-Rn conjugates
In MDA-MB-231 cells, NuBCP-9-Rn (n= 6, 8, 10, 12 and 14) conjugates showed
significantly increased uptake than Rn with same numbers of arginine at 10 μM (Fig.1A). Under CLSM, NuBCP-9 conjugation promoted the cytoplasmic diffusion of peptides at 10 μM (Fig.1B). The NuBCP-9-R10 conjugate exhibited the strongest cellular fluorescence signal, suggesting R10 was the most effective length for uptake of NuBCP-9 at 10 μM.

![Fig.1](image)

**Fig.1** NuBCP-9 conjugation promoted the uptake of Rn. NuBCP-9-R10 conjugate was the most effective one. 
(A) Cellular uptake level (B) Cellular distribution with 10μM Rn or NuBCP-9-Rn conjugates treatment

1.2. **Investigation of uptake mechanism of NuBCP-9-R8 conjugate by amino acid replacement of NuBCP-9**

Replacing N-terminal phenylalanine (FA-R8: FSRSLHSLA-R8) or C-terminal leucine (AA-R8: ASRSLHSLA-R8) of NuBCP-9-R8 (FSRSLHSLL-R8) or FA-R8 with alanine significantly suppressed the uptake, respectively. It suggests both of those distal hydrophobic amino acids replacement play a role in uptake enhancement of NuBCP-9-R8 conjugate than R8 at 10 μM.

1.3. **Uptake pathways of Rn and NuBCP-9-Rn conjugates in MDA-MB-231 cells**

In the presence of clathrin-mediated endocytosis inhibitor chlorpromazine or macropinocytosis inhibitor EIPA, Rn and NuBCP-9-Rn conjugates entry were suppressed significantly in MDA-MB-231 cells. When treatment dose of peptides was increased from 2 to 10 μM, the inhibited effect of EIPA treatment was increased. Treatment of 4°C significantly suppressed the uptake of peptides, especially for NuBCP-9-Rn conjugates. It suggests the uptake of Rn and NuBCP-9-Rn conjugates partly mediated by clathrin-mediated endocytosis and macropinocytosis.

2. **Investigation of cytotoxicity of Rn and NuBCP-9-Rn conjugates in MDA-MB-231 cells**

2.1. **Cell viability of MDA-MB-231 cells under Rn and NuBCP-9-Rn conjugates treatment**

As the length of Rn increased, the cytotoxicity of NuBCP-9-Rn conjugates were increased in MDA-MB-231 cells (Fig.2A), which had limited correlation with uptake level (Fig.1A). It suggests that the Bcl-2-based mechanism of internalized peptides was not the only source of NuBCP-9-Rn conjugates-induced cytotoxicity.

2.2. **Membrane disrupted effect caused by Rn and NuBCP-9-Rn conjugates**

In Fig.2B, following conjugation with NuBCP-9, LDH release increased with increasing length of Rn. NuBCP-9-R12 and NuBCP-9-R14 conjugates induced severe membrane damage at 10 μM. It suggests that R12 and R14 may not be suitable choices for conjugation with NuBCP-9 in this context.
NuBCP-9-Rn conjugates showed Rn length-dependency cytotoxicity, but limited relationship with uptake level (B) NuBCP-9 conjugation lead severe membrane disruption with R12 and R14.

2.3. Induced apoptosis level of NuBCP-9-R8 and NuBCP-9-R10 conjugates treatment in MDA-MB-231 cells

In Fig. 3, R10 treated cells and the negative control group cells showed similar levels of apoptosis. NuBCP-9-R10 conjugate induced more apoptosis and necrosis than NuBCP-9-R8 conjugate at 10 μM. It suggests the higher level of apoptosis induced by NuBCP-9-R10 conjugate may be related to its relatively high cellular uptake level.

| Concentration (µM) | Cell Viability % |
|--------------------|------------------|
| 0 | NuBCP-9-R4 |
| 5 | NuBCP-9-R6 |
| 10 | NuBCP-9-R8 |
| 15 | NuBCP-9-R10 |
| 20 | NuBCP-9-R12 |
| 25 | NuBCP-9-R14 |

Fig. 3 NuBCP-9-R10 conjugate induced higher apoptosis levels than NuBCP-9-R8 conjugate.

[Conclusion]

In this study, I analyzed the uptake characteristics and cytotoxicity of Rn and NuBCP-9-Rn conjugates in MDA-MB-231 cells. NuBCP-9 conjugated with Rn significantly promoted the cellular uptake of Rn in MDA-MB-231 cells, and NuBCP-9-R10 conjugate possessed highest uptake partly mediated by clathrin-mediated endocytosis and macropinocytosis. Besides, NuBCP-9-R10 conjugate induced high levels of apoptosis in MDA-MB-231 cells, which reflected the efficient cytosolic delivered level of peptides. Overall, NuBCP-9-R10 conjugate is the most suitable compound for pro-apoptotic therapeutic application of NuBCP-9. The information in this study will be valuable in the design of proapoptotic peptide conjugated with oligoarginines for anti-cancer therapy.

[基礎となった学術論文]

Wei Wang, Tadaharu Suga, Masayori Hagimori, Naotaka Kuroda, Yuki Fuchigami, and Shigeru Kawakami. Investigation of intracellular delivery of the NuBCP-9 by conjugation with oligoarginines peptides in MDA-MB-231 cells. *Biol. Pharm. Bull.*, 2018, in press.