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Drug Delivery

Cell-Penetrating Homochiral Cyclic Peptides as Nuclear-Targeting Molecular Transporters**

*Deendayal Mandal, Amir Nasrolahi Shirazi, and Keykavous Parang**

The intracellular delivery of biologically active cargos by employing linear cell-penetrating peptides (CPPs) has been previously reported.[1] Conjugation to linear cationic CPPs, such as TAT (trans-acting activator of transcription; a peptide derived from the HIV-1 transactivator protein),[2,3] penetratin, antennapedia, or oligoarginine, efficiently enhances the cellular uptake through different mechanisms.[4] The cellular uptake and internalization of many CPPs along with the conjugated cargo occurs predominantly by an endocytic pathway[5] that involves macropinocytosis, a caveolae pathway, clathrin-mediated endocytosis, or lipid-raft dependent endocytosis.[6] Endosomal uptake represents a major challenge in targeted intracellular drug delivery since some compounds are trapped in endosomes and cannot reach the biological targets in the cytoplasm or nucleus. Thus, strategies that promote endosomal escape or avoid endosomal routes are required for improving bioavailability.

Moreover, the nuclear delivery of cell-impermeable and water-insoluble molecules remains a major challenge. The nucleus is a desirable target because the genetic information of the cell and transcription machinery resides there. To date, most approaches for nuclear delivery of compounds have taken advantage of covalent conjugation, which requires release of the cargo from the conjugate and/or endosomal escape.[7]

There is therefore a need to develop alternative stable peptide carriers that avoid endosomal pathways and/or covalent conjugation. Compared to linear peptides that are susceptible to hydrolysis by endogenous peptidases, cyclic peptides are enzymatically more stable. The cell-penetrating properties and application of homochiral L-cyclic peptides in drug delivery remain unexplored.

Previous studies on linear CPPs by our research group and others indicated that an optimal balance of positive charge and hydrophobicity is required for interactions with the cell membrane and deep penetration into the lipid bilayer.[3,4,8–10] Herein, we report the design and evaluation of amphipathic homochiral L-cyclic peptides for potential applications as CPPs and/or as molecular transporters of bioactive compounds.

Eleven cyclic peptides, namely $[WR]_4$, $[FK]_4$, $[AK]_4$, $[EL]_4$, $[RFEF]_2$, $[EK]_4$, $[ER]_4$, $[FR]_4$, $[RFE]_3$, $[WR]_3$, and $[WR]_5$ (Scheme 1), which contain L-amino acids, were synthesized by employing 9-fluorenylmethyloxycarbonyl (Fmoc) based peptide chemistry. The selection of the cyclic peptides was based on the presence of hydrophobic residues (e.g., W, F, L) and charged residues (e.g., K, R, E). We hypothesized that an optimal amphipathic cyclic peptide that contains appropriate residues, and that undergoes intermolecular and intramolecular interactions can act as a CPP and/or entrap and deliver a bioactive compound intracellularly.

To examine the potential application of the cyclic peptides as molecular transporters, a model experiment was performed with lamivudine ((−)-2′,3′-dideoxy-3′-thiacytidine, 3TC). 3TC is a nucleoside reverse transcriptase inhibitor that blocks HIV-1 and hepatitis B virus replication.[11] The efficient cellular uptake of 3TC is critical for effective antiviral activity. To monitor the molecular transport ability of the cyclic peptides, a carboxyfluorescein derivative of 3TC (F-3TC)[12] was synthesized.

The cellular uptake of the fluorescently labeled 3TC (F-3TC) was examined in the leukemia CCRF-CEM cell line in the presence or absence of cyclic peptides. After 1 h incubation at 37 °C, the cells were treated with trypsin to remove the cell-surface-bound drug. The cellular uptake of F-3TC was monitored by fluorescence-activated cell sorting (FACS; Figure 1 a) and fluorescence microscopy (Figure 1 b). The cyclic peptides did not exhibit any cytotoxicity by using an MTT assay at an experimental concentration of 50 μM in four different cell lines, namely CCRF-CEM, HT-29, MDA-MD-468, and SK-OV-3, thus showing consistent results (Figure S12).

FACS and fluorescence microscopy showed significantly higher fluorescence signals in the cells treated with F-3TC-loaded $[WR]_4$ and $[WR]_5$ compared to those treated with other F-3TC-loaded cyclic peptides and with F-3TC alone, thus suggesting that the uptake of F-3TC is facilitated by $[WR]_n$ ($n = 4,5$) and is dependent on nature of amino acids. F-3TC-loaded $[WR]_5$ exhibited a cellular uptake that was approximately five times higher than that of F-3TC alone (Figure 1 a).

Phosphopeptides are valuable probes for studying phosphoprotein–protein interactions because these peptides mimic the interactions between the negatively charged phosphate group of phosphoproteins and positively charged amino acids in the binding pockets of a number of proteins.[13] Studying negatively charged phosphopeptides in cellular systems is challenging because these peptides do not readily

[*] Dr. D. Mandal, A. Nasrolahi Shirazi, Prof. K. Parang
Department of Biomedical and Pharmaceutical Sciences
University of Rhode Island
41 Lower College Road, Kingston, RI 02881 (USA)
E-mail: kparang@uri.edu

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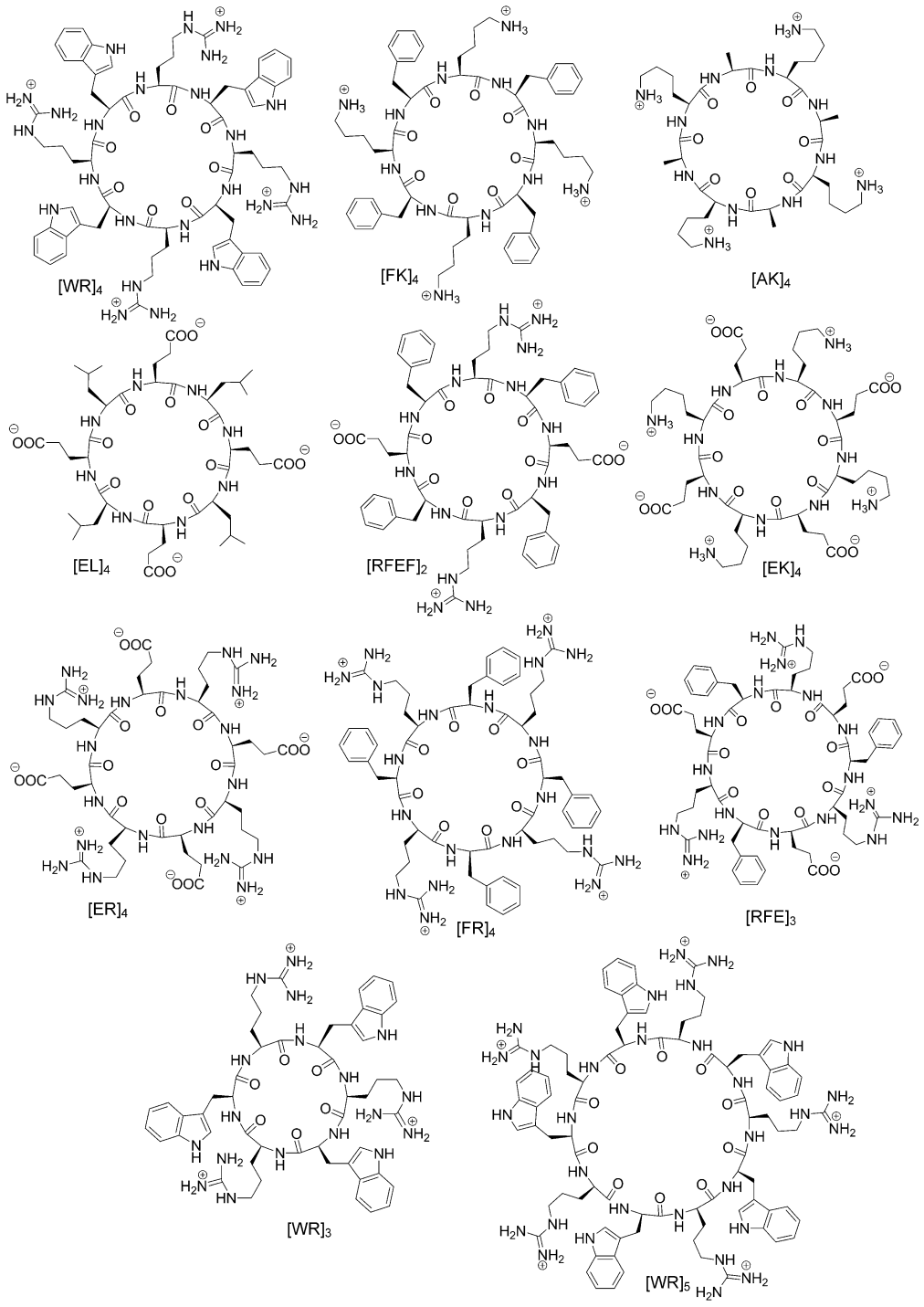

Scheme 1. Chemical structures of synthesized cyclic peptides. A = alanine, E = glutamic acid, F = phenylalanine, K = lysine, L = leucine, R = arginine, W = tryptophan.

cross cellular membranes. The pTyr-Glu-Glu-Ile (GpYEEI) peptide template has been previously studied as an optimal binding sequence for the Src SH2 domain of Src kinase.[14] The potential of cyclic peptides for the delivery of fluorescently labeled GpYEEI (F-GpYEEI)[9a] was investigated by using flow cytometry. The cellular uptake of F-GpYEEI was monitored in the presence of cyclic peptides in breast carcinoma BT-20 cells (Figure S9 in the Supporting Information). Among all cyclic peptides, the cellular uptake of F-GpYEEI was enhanced at least 3.7 times by $[WR]_4$, thus suggesting that this cyclic peptide may function as a delivery tool for F-GpYEEI.

Further investigation of the effect of serum in cellular uptake studies was required to determine whether the cell-based models closely relate to in vivo studies. Thus, the cellular uptake of F-GpYEEI was investigated in presence of $[WR]_4$ and different concentrations of serum in SK-OV-3 cells. FACS analysis showed that in the presence of 10% serum, $[WR]_4$-mediated uptake of F-GpYEEI is at least 4.5 times higher than that of F-GpYEEI alone (Figure S10).

To confirm the cellular uptake of cyclic peptides that contain alternating arginine and tryptophan residues (e.g., $[WR]_n$ $n = 4,5$), fluorescently labeled conjugates of the peptides, F-$[W_5R_4K]$ and F-$[W_4R_3K]$, were synthesized and their cellular internalization was studied. Synthesis of fluorescently labeled peptides was accomplished by replacing one arginine unit with lysine, and conjugation of the side chain of lysine with carboxyfluorescein (FAM) through a short linker, β-alanine, in a multistep synthesis. Incubation of F-$[W_5R_4K]$ or F-$[W_4R_3K]$ with human leukemia cells (CCRF-CEM) showed significantly higher cellular uptake when compared with the corresponding fluorescently labeled linear peptide F-KR_3W_4, and FAM, thus suggesting the cyclic nature of the peptide is critical for the enhanced cellular permeability (Figure 2).

To investigate whether the translocation of F-$[W_5R_4K]$ is cell-specific, the cellular uptake of the peptide (10 μM) was

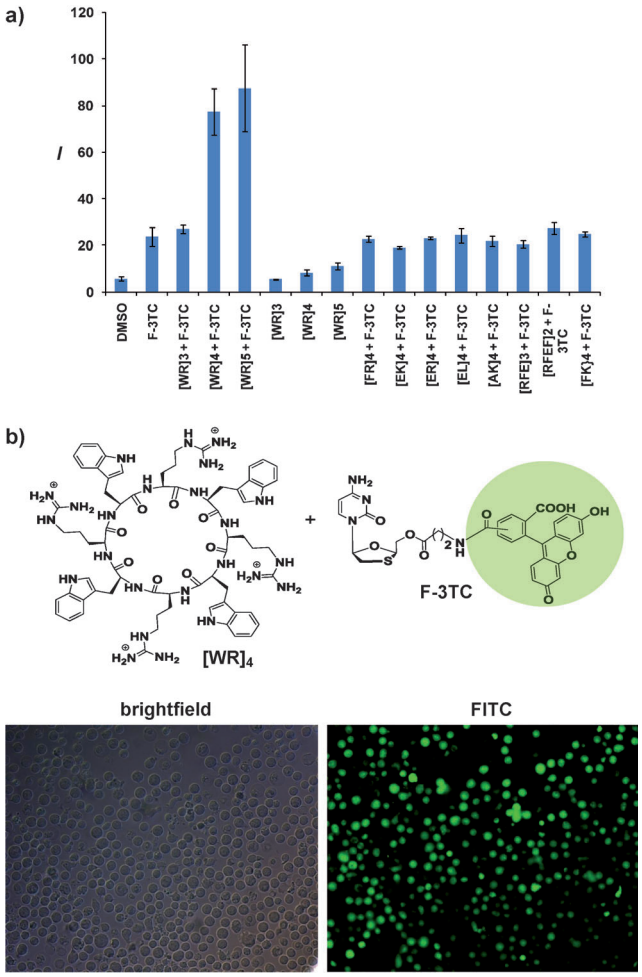

Figure 1. a) Cellular uptake studies for F-3TC alone (10 μM) and in the presence of cyclic peptides (50 μM); b) fluorescence microscopy image of F-3TC (10 μM) uptake by CCRF-CEM cells in the presence of $[WR]_4$ (50 μM).

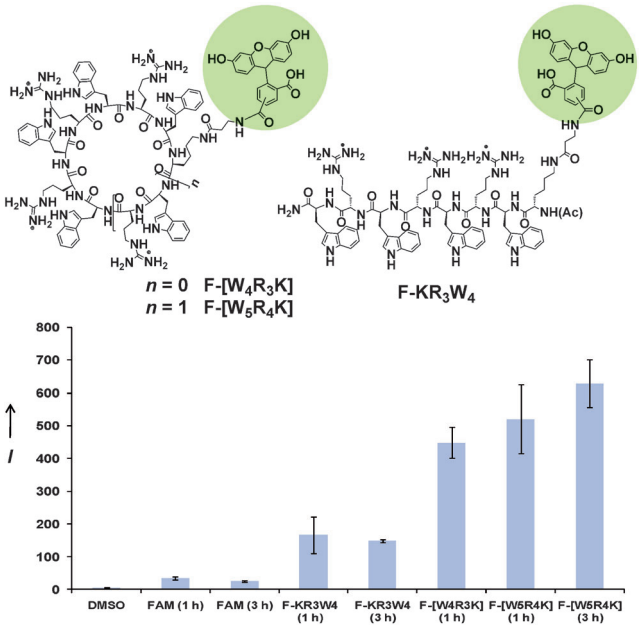

Figure 2. FACS analysis of cellular-uptake assays of F-$[W_4R_3K]$ and F-$[W_5R_4K]$ in CCRF-CEM cells compared with a fluorescently labeled linear peptide F-KR_3W_4 and FAM.

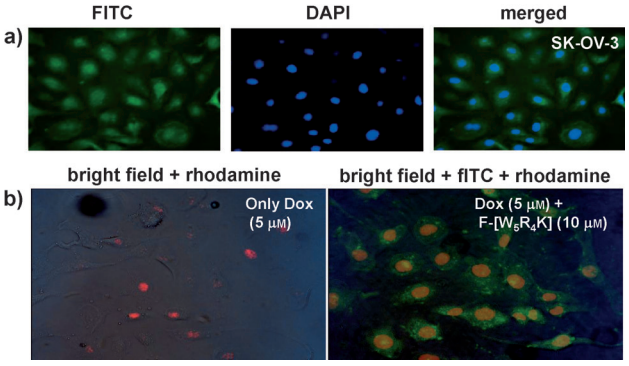

Figure 3. Cellular uptake of F-$[W_5R_4K]$ (1 h incubation, 10 μM) in SK-OV-3 cells a) alone and b) in the presence of Dox. FITC = fluorescein isothiocyanate, DAPI = 4′,6-diamidino-2-phenylindole.

examined in human ovarian adenocarcinoma (SK-OV-3), colon adenocarcinoma (HT-29), and breast carcinoma (MDA-MB-468) at 37 °C. Interestingly, the cells showed significant fluorescence mainly in the nucleus (as shown by FITC and DAPI; Figure 3a). The green (FITC) and blue (DAPI) colors are result of the fluorescence of F-$[W_5R_4K]$ and the stained nuclei, respectively. The green areas overlap with the blue regions, thus suggesting a highly efficient translocation and accumulation in the nucleus. Similar results were obtained when using unfixed cells, thus eliminating the chance of any artificial uptake that arises from fixation of cells.

When doxorubicin (Dox, 5 μM) was incubated with SK-OV-3 cells for 1 h, minimal cellular uptake and nuclear localization was observed. On the other hand, in the presence of F-$[W_5R_4K]$ (10 μM), significant uptake of both Dox and the fluorescently labeled peptide was observed in nucleus as shown by fluorescence microscopy, thus suggesting that the cyclic peptide acts as a molecular transporter of Dox (Figure 3b).

Confocal microscopy showed also nuclear localization of the cyclic peptide (F-$[W_5R_4K]$) versus the corresponding linear peptide (F-KR_3W_4), thus confirming that the cyclic nature of the peptide is critical for the enhanced cellular permeability (Figure 4).

The cellular uptake of the fluorescently labeled peptides was found to be concentration and time-dependent, and rapid even after 5 min (Figure 2, Figures S7 and S8). Rapid internalization and localization of F-$[W_5R_4K]$ to the nucleus in SK-OV-3 cells were also observed at 4 °C or in the presence of sodium azide, which inhibits energy-dependent cellular uptake (Figure 5). F-$[W_5R_4K]$ did not show any significant difference in cellular uptake in CCRF-CEM cells in the presence of different endocytic inhibitors, such as methyl β-cyclodextrin, chlorpromazine, chloroquine, and nystatin, thus ruling out clathrin-mediated or caveolae-mediated endocyto-

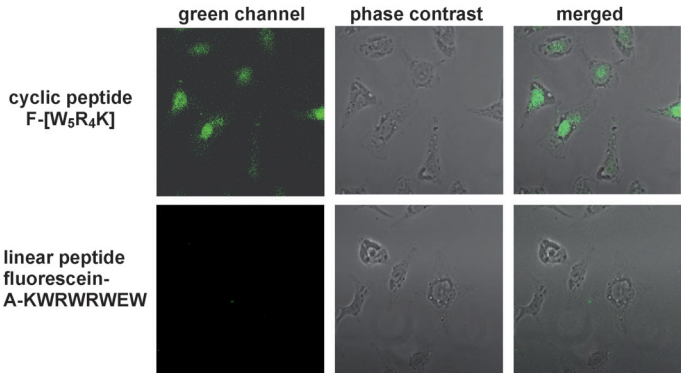

Figure 4. Cellular uptake of cyclic peptide F-[W_5R_4K] (10 μM) and linear peptide F-KR_3W_4 in live SK-OV-3 cells after 1 h incubation imaged by using confocal microscopy.

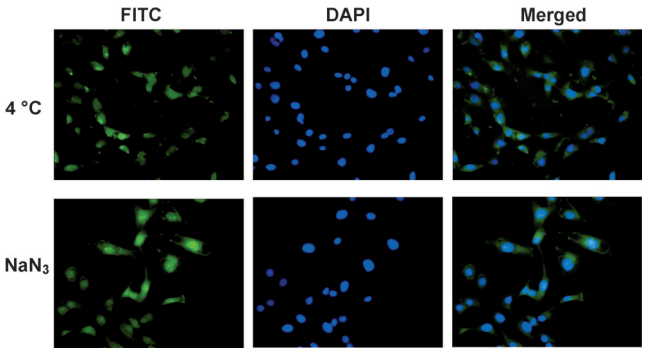

Figure 5. Cellular uptake of F-[W_5R_4K] (10 μM) in SK-OV-3 cells at 4 °C or in the presence of NaN_3 after 1 h incubation.

sis, and macropinocytosis[6] after 1 h incubation (Figure S11). These results suggest that the cellular uptake does not require endosome-derived vesicles. This is a key advantage to the endocytotic entry for known CPPs that require endosomal escape to passage the conjugate out of the resulting endosome.

In a phase-transfer experiment[15] using chloroform and water, negatively charged palmitoyloleoylphosphatidylserine (POPS) promoted the transfer of water soluble F-[W_5R_4K] into the hydrophobic organic phase, probably through charge neutralization and interactions with negatively charged and hydrophobic residues of the phospholipid, respectively, while zwitterionic lipid palmitoyloleoylphosphatidylcholine (POPC) was ineffective (Figure 6). Similarly, the amphipathic nature of $[WR]_n$ ($n=4,5$) and interactions of arginine and tryptophan residues with the corresponding negatively charged phospholipids and hydrophobic residues in the lipid bilayer may have played a role for initial entry into the cell membrane. Hydrophobic interactions between tryptophan residues and the lipids result in possible distortion of the outer phospholipid monolayer, this leading to peptide internalization and enhanced cellular uptake of the cargo.

Dox and $[WR]_4$ were selected as a model drug and a transporter, respectively, in order to investigate complex formation. Isothermal titration calorimetry (ITC) was used to determine the binding constant between the cyclic peptide

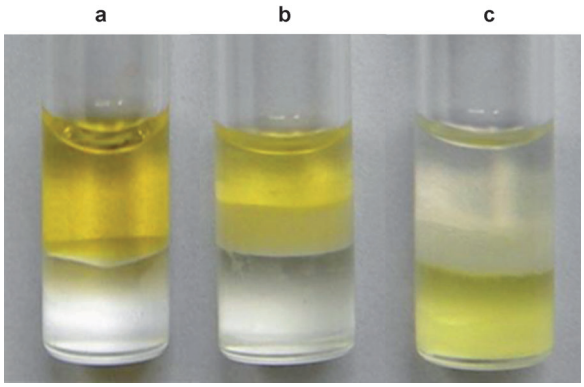

Figure 6. Phase-transfer studies of F-[W_5R_4K] with negatively charged POPS and zwitterionic POPC ($CHCl_3$ (lower layer), water (upper layer)): a) $CHCl_3$/F-[W_5R_4K], b) POPC (in $CHCl_3$)/F-[W_5R_4K], c) POPS (in $CHCl_3$)/F-[W_5R_4K].

and the drug. The ITC measurements were carried out at 30 °C by adding Dox (5 mM) to $[WR]_4$ (100 μM) solution. The integrated heat data were fitted by using different binding models. Application of the equation for two and four sequential binding events afforded the best fits among all the binding models provided by the software. In addition to fitting curves derived by using different models, the chi-squared (χ^2) value was used as the primary criterion for the goodness of fit. The χ^2 values produced by two sites and four sites sequential binding models were 2.7×10^3 and 1.8×10^3, respectively. Considering the smaller χ^2 value, an acceptable fit corresponds to four sequential binding events, with binding constants $K_1=5.9\times10^5\,M^{-1}$, $K_2=1.9\times10^4\,M^{-1}$, $K_3=6.8\times10^3\,M^{-1}$, and $K_4=2.8\times10^2\,M^{-1}$, respectively (Figure S13 and Table S1). These data suggest that Dox and $[WR]_4$ form a complex possibly through multistep intermolecular interactions.

Further mechanistic investigation was conducted to determine the intracellular fate of the drug–peptide complex. The fluorescein-labeled phosphopeptide F-GpYEEI and $[WR]_5$ were mixed for 30 min at room temperature. Briefly, SK-OV-3 cells were incubated with the preformed complex of F-GpYEEI and $[WR]_5$ and the intracellular location of the phosphopeptide was monitored by using confocal microscopy. The image confirmed significant uptake of F-GpYEEI in the presence of the cyclic peptide. Most of the fluorescently labeled phosphopeptide translocated into the nucleus in the presence of $[WR]_5$, while the labeled phosphopeptide alone did not show any cellular uptake (Figure S15). These data suggest that the nuclear translocation mediated by the cyclic peptide is not exclusive to Dox and initial complex formation is critical for nuclear delivery.

In conclusion, we have reported nuclear-targeting cyclic peptides that are capable of molecular transport. To the best of our knowledge, $[WR]_4$ and $[WR]_5$ represent the first reported cyclic CPPs with nuclear targeting and noncovalent molecular transport capabilities. Preliminary mechanistic investigation shows that 1) the cellular uptake is independent of endocytosis and 2) cyclic peptides $[WR]_4$ and $[WR]_5$ form a complex with the cargo for efficient cellular delivery. The

present results provide insights for generation of a new class of nuclear delivery transporters.

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