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Cysteine and Arginine-Rich Peptides as Molecular Carriers

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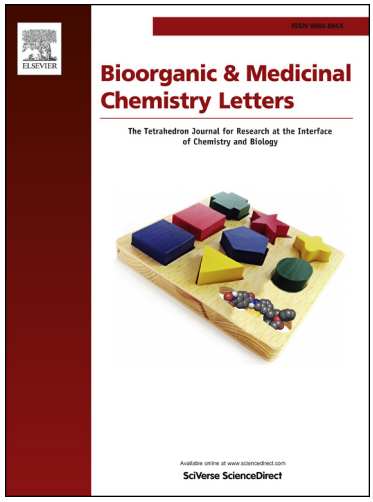

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Graphical Abstract

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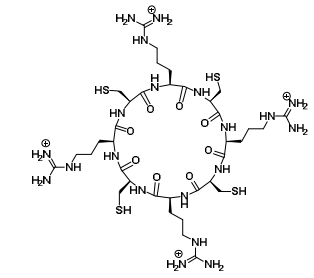
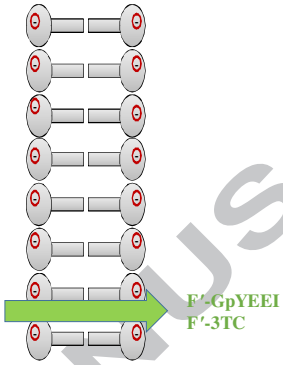


Fluorescence-labeled phosphopeptide (F′-GpYEEI)
Fluorescence-labeled lamivudine (F′-3TC)



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Cysteine and arginine-rich peptides as molecular carriers

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ABSTRACT

A number of linear and cyclic peptides containing alternative arginine and cysteine residues, namely linear $(CR)_3$, linear $(CR)_4$, linear $(CR)_5$, cyclic $[CR]_4$, and cyclic $[CR]_5$, were synthesized. The peptides were evaluated for their ability to deliver two molecular cargos, fluorescence-labeled cell-impermeable negatively charged phosphopeptide (F′-GpYEEI) and fluorescence-labeled lamivudine (F′-3TC), intracellularly in human leukemia cancer (CCRF-CEM) cells. We investigated the role of cyclization and the number of amino acids in improving the transporting ability of the peptides. The flow cytometry studies suggested that the synthesized peptides were able to work efficiently as transporters for both cargos. Among all compounds, cyclic $[CR]_4$ was found to be the most efficient peptide in transporting the cargo into cells. For instance, the cellular uptake of F′-3TC (5 μM) and F′-GpYEEI (5 μM) was enhanced by 16- and 20-fold, respectively, in the presence of cyclic $[CR]_4$ compared to that of the parent compound alone. The mechanism of F′-GpYEEI uptake by cells was found to be energy-independent. The results showed that the number of amino acids and their cyclic nature can impact the efficiency of the peptide in transporting the molecular cargos.

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The cell membrane is composed of a phospholipid bilayer.[1] The bilayer forms an efficient barrier system between intracellular and extracellular aqueous regions. Besides phospholipids, there are also other components present in the structure of the plasma membranes including glycolipids and cholesterol. Several proteins in phospholipid bilayer are responsible for selective transporting of cargos. Moreover, the flexibility of the cell membrane is handled through an interaction of double bonds and free fatty acid chains.[2] The intracellular delivery of highly hydrophobic, poorly water-soluble, and negatively-charged compounds is a challenging task because of the presence of negatively-charged phospholipid bilayer.

To enhance the intracellular concentration of cell impermeable compounds, various classes of drug delivery systems (DDS) have been developed and examined during last two decades.[3] The discovery and development of a new drug molecule are costly and cumbersome. Thus, improving the cellular delivery of known active compounds using DDS to enhance their biological activity and/or offer controlled release or targeted delivery is a subject of major interest.

Long-circulating macromolecular carriers, such as peptide-based molecules, can exploit the 'enhanced permeability and retention' effect on the site of action.[4] Among various classes of peptide-based DDS, cell-penetrating peptides (CPPs) have been broadly used due to their unique properties such as non-viral nature, biocompatibility, and cell permeability into the plasma membrane.[5] The ability of CPPs has been examined for the delivery of various types of cargos including small drugs [6-9] and large-molecule based therapeutics.[10]

Positively charged amino acids such as arginine and lysine are the main residues in the structure of the majority of CPPs. These amino acids provide positive charges on the surface of CPPs and facilitate electrostatic interactions with negatively charged molecules, such as heparin and phospholipids within the cell membrane.

In addition to the ability of CPPs to penetrate into the cell membrane, the loading and release processes of molecular cargos inside the cell are particularly important in the overall functionality of the system. Thus, researchers tend to avoid obstacles in loading and release processes by taking advantage of non-covalent approaches. This method offers simple loading procedure and smooth intracellular release of the intact cargo.[11,12]

Technically, the sequence of amino acids can modify the molecular interactions, such as electrostatic and hydrophobic interactions between the cargo and the peptide. An optimized orientation could improve the loading capacity of the system by providing appropriate regions to entrap the molecular cargos. Thus, a new arrangement of amino acids in peptide-cargo complex can have an impact on the cell permeability and enhance the drug uptake by cells.

Previously, we have reported the synthesis and evaluation of a class of homochiral cyclic peptides with arginine and tryptophan namely $[WR]_4$ and $[WR]_5$ as nuclear-targeting CPPs.[13] These peptides and peptide-capped gold nanoparticles showed a significant efficiency to transport different cargos into cells.[14,15] Our investigations revealed that the existence of a balance between hydrophobic and positive charge properties of peptides was critical for their function as molecular transporters and DDS. Cyclic peptides also offer higher stability in serum compared to the corresponding linear ones.[16]

Cysteine-rich peptides have been previous used as cellular delivery agents.[17] It has been previously reported that a cysteine-rich decapeptide, named CyLoP-1 (Cytosol Localizing Peptide-1) was able to cross the cell membrane readily. This cysteine-rich peptide exhibited the potential for cytoplasmic distribution besides vesicular localization and proficient cellular uptake at low-micro-molar concentrations.[17a] Thus, cysteine appears to contribute in peptide cell permeability.

Herein, we report the preparation of cyclic and linear peptides containing an alternative sequence of cysteine and arginine residues and their evaluation as intracellular molecular transporters. We hypothesized that the presence of cysteine and arginine residues would provide an appropriate combination of amino acids for facilitating the cell permeability of the cargo molecules.

Five cyclic and linear peptides containing alternative sequence of cysteine and arginine amino acids namely $c[CR]_4$, $c[CR]_5$, $l(CR)_3$, $l(CR)_4$, and $l(CR)_5$ (Figure 1) were synthesized. Brackets and parenthesis show cyclic and linear peptides, respectively. The peptide synthesis was performed employing 9-fluorenylmethyl oxycarbonyl (Fmoc)-based chemistry according to our previously reported procedure.[13,18]

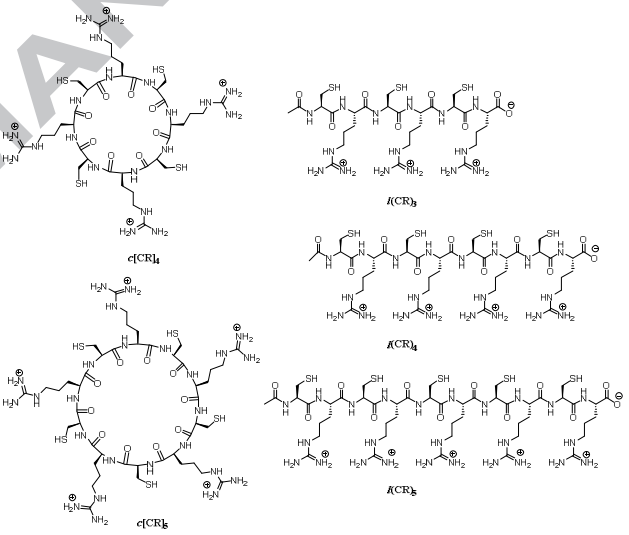
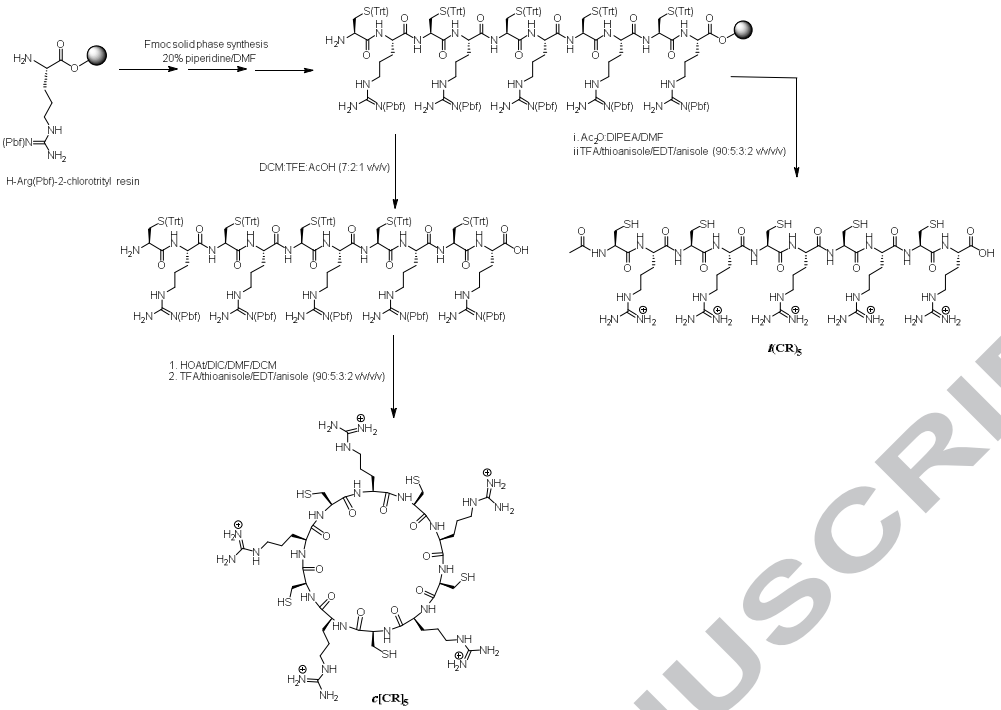


Figure 1. Chemical structures of synthesized peptides.

The synthesis procedure of linear $l(CR)_5$ and $c[CR]_5$ is shown in Scheme 1 as representative examples. The linear peptide containing ten alternative cysteine and arginine amino acids (CRCRCRCRCR) was assembled on the heterogeneous H-Arg(Pbf)-2-chlorotrityl resin. The Fmoc group at the *N*-terminal of the last coupled amino acid was deprotected using piperidine in DMF (20% v/v) followed by capping using acetic anhydride. The resin was washed and completely cleaved in the presence of reagent R containing TFA/thioanisole/1,2-ethanedithiol (EDT)/anisole (90:5:3:2 v/v/v/v) to afford $l(CR)_5$.



Scheme 1. Solid-phase synthesis of *l*(CR)$_5$ and *c*[CR]$_5$.

For the synthesis of the cyclic peptide, the peptide containing protected side chain was cleaved from the trityl resin in the presence of a cocktail composed of acetic acid/trifluoroethanol (TFE)/dichloromethane (DCM) (1:2:7 v/v/v) at room temperature. The side chain-protected linear peptide was used for further cyclization reaction. To cyclize the linear peptide, 1-hydroxy-7-azabenzotriazole (HOAt, 4 equiv) and 1,3-diisopropyl- carbodiimide (DIC, 4.5 equiv) were used in a mixture of DMF/DCM (5:1 v/v) solvents. The reaction was performed for 12 h to obtain the protected cyclic peptide. After the cyclization reaction was complete, the side chain protecting groups were removed by using a cleavage cocktail reagent R containing TFA/thioanisole/EDT/anisole (90:5:3:2 v/v/v/v) for 2 h to yield *c*[CR]$_5$. Both crude peptides were precipitated using cold diethyl ether (75 mL, Et_2O). The purification of the peptides was carried out by using reversed-phase Hitachi HPLC (L-2455) on a Phenomenex Prodigy 10 µM ODS reversed-phase column (2.1 cm × 25 cm). Pure peptides were obtained in the form of white powder after lyophilization.

Circular dichroism (CD) was employed to compare the secondary structure between the linear and cyclic peptides. Two model peptides *l*(CR)$_5$ and *c*[CR]$_5$ were selected for this investigation since they contain an equal number of amino acids. All spectra were corrected for the baseline and the background by subtraction of the blank. CD results showed that *l*(CR)$_5$ (50 µM) exhibited a different CD pattern when compared to that of *c*[CR]$_5$ (50 µM).

l(CR)$_5$ showed two minimum peaks at 204 and 228 nm, suggesting that the carrier does not form a classic secondary structure (Figure 2). However, the CD results of *c*[CR]$_5$ showed a pattern slightly different than a random-coil as shown by a minimum at 210 nm. This has been reported that peptides holding random coil secondary structure, show a maximum and a minimum peak at ~ 220 nm and ~ 198 nm. This classic pattern could be shifted due to several reasons including π-π* transition of amide bond.[19,20] These data suggest that although these two peptides have similar number of amino acids, they form different secondary structures. These results confirm that the cyclization can change the conformation of the peptides.

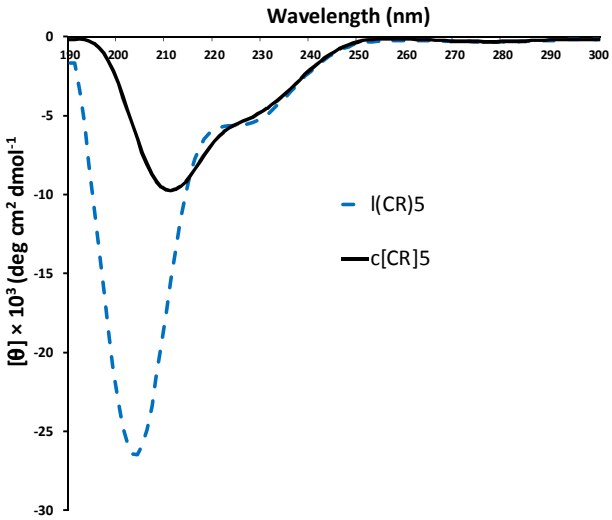


Figure 2. CD spectra of *l*(CR)$_5$ and *c*[CR]$_5$ at 50 µM.

The cytotoxicity of all synthesized peptides was evaluated in human ovarian (SK-OV-3) and leukemia (CCRF-CEM) cancer cells at different concentrations (50 and 100 µM) after 24 h incubation time (Figure 3). None of the peptides showed significant toxicity at 50 µM. However, among all peptides, *c*[CR]$_5$ was found to be more toxic compared to others as CCRF-CEM and SK-OV-3 cells showed 78% and 82% viability,

respectively, in the presence of 100 µM of the peptide. No significant difference in toxicity profile between linear and cyclic peptides was observed at 50 µM showing that the cyclic nature did not alter the cytotoxicity. Based on the cytotoxicity assay results, the non-toxic concentration of 50 µM of peptides was used for further cell-based studies.

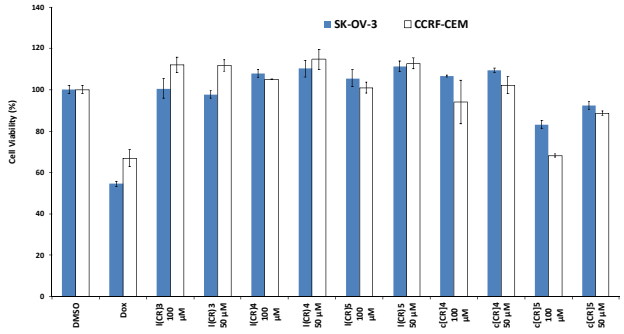


Figure 3. Cytotoxicity assay of the peptides (50 and 100 µM) in CCRF-CEM and SK-OV-3 cells after 24 h.

After a non-toxic concentration of peptides was determined, all peptides were evaluated for their molecular transporting activity. The effect of peptides on the intracellular uptake of molecular cargos was examined using a flow cytometry method. Two different model cargos, anti-HIV drug lamivudine, and phosphopeptide GpYEEI were selected at a low concentration for the cellular delivery investigations. Lamivudine ((–)-2′,3′-dideoxy-3′-thiacytidine, 3TC) is a potent nucleoside reverse transcriptase inhibitor that blocks HIV-1 and hepatitis B virus replication.[21] However, this drug suffers from low cellular uptake because of high polarity. Phosphopeptides have been used as reagent probes to mimic phosphoproteins involved in signal transduction pathway for protein–protein interactions.[22,23] Although phosphopeptides are valuable probes, their use is challenging in cellular studies due to their limited cellular uptake.

The cellular uptake of fluorescence-labeled 3TC (F′-3TC, 5 µM) was measured in the presence and absence of peptides (50 µM) in CCRF-CEM cells after 3 h. As it is shown in Figure 4, the cellular uptake of F′-3TC was enhanced upon mixing with $l(CR)_4$, $l(CR)_5$, $c[CR]_4$, and $c[CR]_5$ by 6-, 10-, 16-, and 12-fold, respectively, compared to that of F′-3TC alone in CCRF-CEM cells after 3 h of incubation. Among all tested peptides, $l(CR)_3$ did not enhance the cargo's uptake, and $c[CR]_4$ showed the highest efficiency in improving the cellular uptake. These data suggest that these peptides can be used as efficient transporters for F′-3TC.

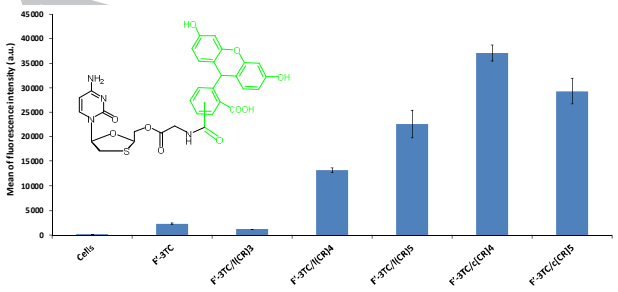


Figure 4. Cellular uptake of fluorescence-labeled F′-3TC (5 µM) after 3 h incubation with cyclic and linear peptides.

As it is shown in Figure 4, the ring size of the peptide also contributes to the delivery efficiency. Two cyclic peptides namely $c[CR]_4$ and $c[CR]_5$ (50 µM) containing eight and ten alternative arginine and cysteine residues were examined for their ability to transport F′-3TC in a comparative assay. It was found out that the cellular uptake of F′-3TC was increased in the presence of $c[CR]_4$ and $c[CR]_5$ by 16 and 12-fold, respectively, compared to that of the F′-3TC alone after 3 h of incubation. These results indicate that although $c[CR]_4$ is composed of less number of amino acids compared to $c[CR]_5$, it has a higher ability to transport a fluorescence-labeled anti-HIV drug presumably, possibly due to the optimal interactions with the cargo and the phospholipid bilyaer.

Different results were obtained when comparing the linear peptides $l(CR)_3$, $l(CR)_4$, and $l(CR)_5$. As shown in Figure 4, $l(CR)_5$ improved the cellular uptake of F′-3TC by 10-fold. However, $l(CR)_3$ and $l(CR)_4$ improved the cellular uptake of F′-3TC by only 0.5- and 6-fold, respectively. These data suggest that the higher number of amino acids in linear peptides can enhance the delivery efficiency of the linear peptides possibly because of a greater number of positively-charged arginine residues. For cyclic peptides, the orientation of peptides for efficient interactions with cargo and the cell membrane appears to be the critical factor in determination of the cellular uptake.

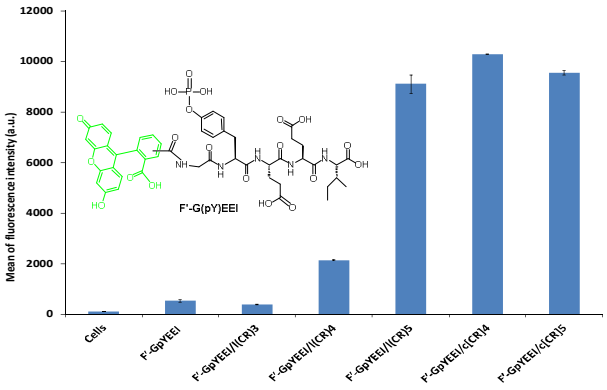


Figure 5. Cellular uptake of fluorescence-labeled F′-GpYEEI (5 µM) after 3 h incubation with cyclic and linear peptides.

In order to investigate the potential of CR peptides to carry other types of cargos, the cellular uptake of a fluorescence-labeled phosphopeptide namely F′-GpYEEI was examined in the presence of the peptides and alone. The cellular uptake of F′-GpYEEI in CCRF-CEM cells was improved by 4, 17, 20, and 18-fold, respectively, when they mixed with $l(CR)_4$, $l(CR)_5$, $c[CR]_4$, and $c[CR]_5$ compared with that of the phosphopeptide alone after were 3 h incubation. As it was shown in Figure 5, $l(CR)_3$ was unable to enhance the cellular uptake of F′-GpYEEI possibly because this number of positively-charged amino acids are not sufficient for the delivery of molecular cargos into cells. It was observed that $c[CR]_4$ was again the most efficient among all tested peptides, presumably due to the optimized interactions with the cargo and the cell membrane. These results were consistent with the data for the delivery of F′-3TC in the presence of $c[CR]_4$ as described above.

Determination of the mechanism(s) of the drug uptake by cells has become important to optimize or design the next generation of carriers. One of the major mechanisms of peptide-mediated drug delivery is endocytosis. Endocytosis includes several

pathways such as micropinocytosis, phagocytosis, and receptor-mediated endocytosis (RME). RME also contain clathrin-mediated, caveolae-mediated pathways.[24]

To get a better understanding of the internalization pathway, the cellular uptake of F′-GpYEEI + *c*[CR]$_4$ (5:50 μM) mixture was tested in the presence of multiple endocytic inhibitors, such as methyl-β-cyclodextrin (β-CD), chlorpromazine (CP), 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIA), and chloroquine (Cq) in CCRF-CEM cells. A similar incubation time (3 h) was used for this assay.

As it is shown in Figure 6, the cellular uptake of F′-GpYEEI was not changed significantly when CP and CQ were used. However, in the presence of β-CD and sodium azide (NaN_3), the uptake was reduced by 20% and 19%, respectively. Sodium azide is used to block the ATP production by oxidative phosphorylation. This data suggest that the ATP depletion can be responsible in the internalization process. Furthermore, β-CD removes the cholesterol out of the structure of the cell membrane and interferes with fluid phase and clathrin-mediated endocytosis.[25] Thus, these two pathways partially contribute for the cellular uptake. The incubation of cells at 4 °C did not block the uptake of the cargo.

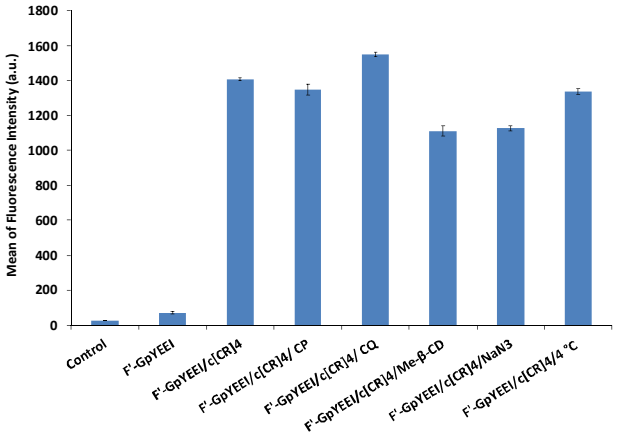


Figure 6. Cellular uptake of F′-GpYEEI (5 μM) and c[CR]$_4$ (50 μM) in the presence of endocytic inhibitors and sodium azide in CCRF-CEM cells after 3 h.

We have previously used isothermal calorimetry (ITC) and modeling techniques to investigate the interaction of cargos with a similar class of cyclic peptides containing tryptophan and arginine residues.[13,26] The first step was the interaction between the cargo (lamivudine or phosphopeptide) and the carrier peptide. We have previously shown that electrostatic interactions between the positively charged arginine in the structure of the peptide and the negatively charged phosphate group in the structure of the phosphopeptide. Furthermore, it was discovered that this class of peptides could undergo self-assembly and aggregation in an organized matter.[27] Thus, drugs can be entrapped by the self-assembled structures. The second step was the interaction between the carrier-cargo complex with the cell membrane. As described in above, the arginine residues in the peptide provide positive charges on the surface of CPPs and facilitate electrostatic interactions with negatively charged phospholipids within the cell membrane. Thus, it was expected that similar interactions to occur between this class of peptides with cargo molecules and cell membrane.

In conclusion, a new peptide-based carrier system composed of cysteine and arginine was discovered for the delivery of an anti-HIV drug and a cell impermeable phosphopeptide. The cellular uptake of F′-GpYEEI and F′-3TC was enhanced in the presence of these peptides. The number of amino acids, cyclic nature, and ring size were found to contribute to the intracellular delivery efficiency. These data confirm CPPs containing cysteine and arginine residues as molecular transporters.

Acknowledgments

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Supplementary Data

Supplementary data (cyclic and linear peptide synthetic procedures and additional supporting data including mass spectra) associated with this article can be found in the online version.

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