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A host-rotaxane derivatized with carboxylic acids efficiently delivers a highly cationic fluoresceinated peptide

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Abstract—A cleft-[2]rotaxane (CR2+2−) was derivatized with carboxylic acids to enhance the intracellular delivery of a highly cationic or anionic pentapeptide. CR2+2− delivers the fluorescein (Fl) tagged peptide Fl-KKALR to a greater amount than Fl-QEAVD, and at a higher concentration, a greater amount than Fl-AVWAL. The level of delivery is largely temperature and ATP independent, suggesting that the Fl-peptide·CR2+2− complexes pass through the cellular membrane without requiring active cell-mediated processes. This study shows that selective delivery of peptides is possible by using a suitably derivatized host-rotaxane as the transporter.

Peptides hold great promise as therapeutic agents. Their potential, however, is severely limited by the inability of most peptides to passively pass through cellular membranes because of their amide bonds and the existence of any polar or charged side chains. Although several methods have been developed recently to deliver materials into cells, most rely on endocytosis for membrane passage, and thus the delivered material needs to be covalently or noncovalently prepackaged often resulting in highly cationic devices which can result in high toxicity.[1–8] Herein we show for the first time that a host-rotaxane can be derivatized with carboxylic acids (CR2+2−, Fig. 1), which gives it an overall small charge of 1+ in buffered water (pH 7.3), and still be an efficient intracellular delivery agent that operates independently of cellular energy. CR2+2− can deliver a greater amount of a highly cationic, fluoresceinated (Fl) pentapeptide (Fl-KKALR) into cells than a Fl-peptide containing apolar (Fl-AVWAL) or negatively charged side chains (Fl-QEAVD). Apparently, interactions between the carboxylates of CR2+2− and the cationic side chains of Fl-KKALR promote membrane passage. These findings show that the selective delivery of small peptides of all charges is possible by using a suitably derivatized host-rotaxane as the transporter.

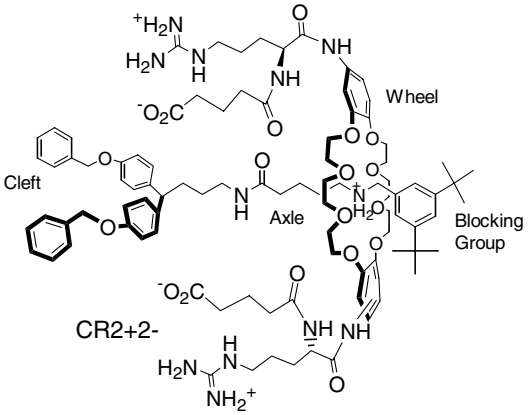

Figure 1. Cleft-[2]rotaxane CR2+2− delivers the Fl-peptides into cells.

Rotaxanes are compounds with an interlocked wheel and axle with blocking groups on the ends of the axle (Fig. 1).[9] We converted them into host-rotaxanes (HRs) by using a synthetic host as one of the blocking groups.[10] Using fluorescein-labeling, we recently showed that a HR delivers Fl-pentapeptides into cells.[11] A comparison of cellular fluorescence showed that, not surpris-

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ingly, Fl-AVWAL, which contains apolar side chains, is delivered into cells with 1.5 times greater efficiency than Fl-KKALR and 2.2 times more efficiently than Fl-QEAVD. To be effective delivery devices, transporters also need to selectively deliver targeted peptides that contain charged side chains. The goal of this research program was to design a transporter that would selectively deliver a highly charged peptide over an uncharged peptide. This is not trivial considering that apolar compounds generally form more stable complexes in water and are more lipophilic than charged compounds. We predicted that adding functional groups to the transporter that are complementary to the charged side chains would result in a greater amount of that peptide being delivered.

CR2+2− was derivatized with carboxylic acids and guanidines (Supporting Information) to promote association with Fl-KKALR or Fl-QEAVD, respectively, through salt-bridge formation. Fl-AVWAL was also used as a guest. The peptides were presented as carboxamides (Fl-peptide$CONH_2$). Intramolecular interactions are possible between the guanidinium and carboxylate ions of CR2+2−, which could weaken intermolecular interactions in the Fl-peptide·CR2+2− complexes. The arginines were utilized because of their likely importance in cellular transport.[6] Selective complex formation should lead to selective delivery if transport occurs through a cell-passive mechanism. Large differences in stability may be needed if the permeability of the complex controls the level of transport. In this case, the delivery efficiencies would be Fl-AVWAL > Fl-KKALR > Fl-QEAVD since the overall charge of the Fl-peptide·CR2+2− complexes, in water at pH 7.3, would be 1−, 2+, and 3−, respectively. The overall charge is based on the fluorescein moiety of the Fl-peptide existing as a dianion.[12]

The ability of the CR2+2− to deliver the Fl-peptides was initially screened using fluorescence microscopy. Figure 2 shows that high and moderate fluorescence is observed in cells exposed to CR2+2− (20 μM) and Fl-AVWAL (10 μM) or Fl-KKALR (10 μM). Cells exposed to CR2+2− (20 μM) and Fl-QEAVD (10 μM), however, were only weakly fluorescent, similar to the background fluorescence obtained from exposure to the Fl-peptides (10 μM) alone. Calcein blue AM (cbAM, Invitrogen) and propidium iodide (PI) were added to confirm low toxicity and high viability of cells exposed to CR2+2− and peptides.

Flow cytometry was used to quantify the relative levels of Fl-AVWAL, Fl-KKALR, Fl-QEAVD, cbAM, and PI within the cells. The threshold for background fluorescence was set using cells incubated with a Fl-peptide (10 μM) alone, as previously described.[13] Generally, increasing the concentration of CR2+2− results in a greater amount of Fl-peptide delivered (Table 1 and Fig. 3), which is consistent with a noncovalent complex forming between the transporter and a Fl-peptide during the delivery process. The delivery level of Fl-KKALR appears to peak at a concentration of 40 μM. The amount of Fl-QEAVD delivered slowly rises and

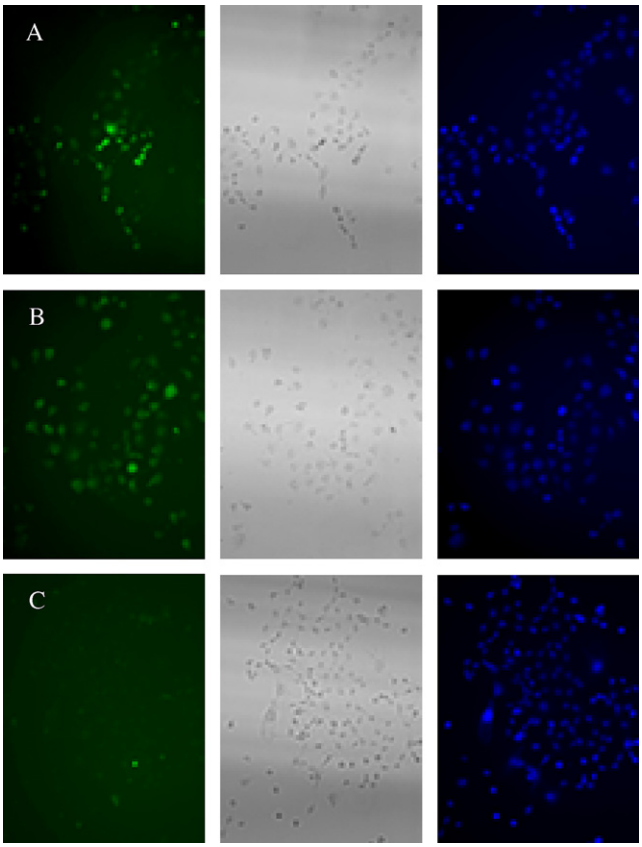

Figure 2. Fluorescence microscopy of COS 7 cells incubated with CR2+2− (20 μM) and (A) Fl-AVWAL (10 μM), (B) Fl-KKALR (10 μM), and (C) Fl-QEAVD (10 μM). Fl-peptide (peptide uptake; left panels), white light (middle panels), and calcein blue AM fluorescence (viability; right panels). Original magnification: 100×.

appears to maximize at 25% of cells being penetrated. Thus, CR2+2− more efficiently transports Fl-KKALR than Fl-QEAVD at the concentrations used in the assays. Fl-AVWAL and Fl-KKALR are delivered in increasing amounts until a concentration of 40 μM is reached. At this concentration of CR2+2−, a dramatic reduction in the delivery of Fl-AVWAL is seen.

We have previously observed lower than expected percentage of fluorescent cells after Fl-AVWAL (10 μM) delivery in an assay performed at 4 °C.[14] Fl-AVWAL or its complex with an HR appeared to precipitate on the surface of the cells in that assay, which may be caused by poor solubility. The Fl-AVWAL·CR2+2− complex has a single charge in the buffered solution and the peptidic residues are apolar. Increasing the concentration Fl-AVWAL will result in a greater concentration of the Fl-AVWAL·CR2+2− complex, which could precipitate at a high concentration. Thus, CR2+2− more efficiently delivers Fl-KKALR than Fl-AVWAL at the higher concentrations of the components.

A high proportion of calcein blue positive cells (80–98%) was observed in the assays, demonstrating that the CRs and Fl-peptides are minimally or not toxic at these concentrations. Less than 3% of the cells were dead, according to the level of PI observed within the cells. Calcein

Table 1. Quantification of Fl-peptide uptake in COS 7 cells by flow cytometry[a]

Condition	Peptide[b]	[CR2+2−] (μM)	%Fl-peptide	%cbAM[c]
rt	Fl-AVWAL	40	25	95
	Fl-AVWAL	20	73	86
	Fl-AVWAL	10	54	84
	Fl-AVWAL	5	31	82
	Fl-AVWAL	0	5	86
	Fl-KKALR	40	84	82
	Fl-KKALR	20	61	84
	Fl-KKALR	10	43	88
	Fl-KKALR	5	30	98
	Fl-KKALR	0	5	92
	Fl-QEAVD	40	23	80
	Fl-QEAVD	20	18	92
	Fl-QEAVD	10	12	81
	Fl-QEAVD	5	8	90
	Fl-QEAVD	0	5	89
Depleted ATP[d]	Fl-AVWAL	10	53	81
	Fl-KKALR	20	65	93
4 °C	Fl-AVWAL	10	30	83
	Fl-KKALR	20	55	85

[a] All transport assays were performed in PBS (pH 7.3) for 1 h.
[b] [Fl-peptide] = 10 μM.
[c] [Calcein blue AM] = 1 μM.
[d] The assay solutions contained 2−deoxyglucose and NaN_3.

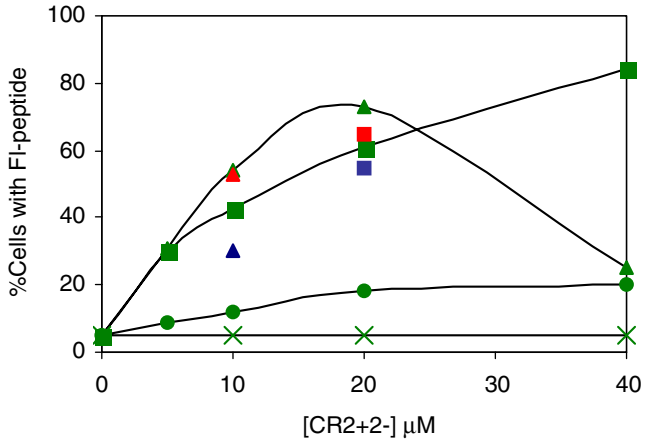

Figure 3. Saturation of fluorescence intensity is observed as the concentration of CR2+2− is raised. CR2+2− and Fl-AVWAL (10 μM) (triangles), Fl-KKALR (10 μM) (squares), or Fl-QEAVD (10 μM circles at room temperature (green), with depleted ATP (red), and at 4 °C (blue). The Fl-peptide line (×'s) represents all the peptides used in this assay, and it was set at a 5% level.[11] The lines show the trends in the plots.

blue/propidium iodide and Fl-peptide fluorescence were independent variables. Membrane integrity was also verified by measuring the amount of enzyme released from the cells during the assay. The level of lactate dehydrogenase (LDH, CytoTox-One Integrity Assay, Promega) released into the solution was 7–9% for untreated cells and cells exposed to the various reagents.

In our design of selective delivery agents, we assumed that the transport process occurs through a cell-passive mechanism that requires the complexes to pass through the membrane. Some highly argininated peptides, however, enter cells through endocytosis.[15] To determine whether endocytosis is the major pathway for cellular entry, the assays were repeated at 4 °C or by using an established ATP-depleting cocktail of 2−deoxyglucose and NaN_3 to deplete the cellular energy.[15] A similar level of transport was observed for the Fl-peptides under the energy-depleted conditions, except for the 44% reduction in the transport level of Fl-AVWAL at 4 °C. As discussed above, we have previously observed a reduction in Fl-AVWAL delivery at 4 °C, and this is likely due to a decreased solubility rather than the involvement of endocytosis.

To verify that depleting the energy of the cells does not alter the transport mechanism, the location and intensity of the intracellularly delivered Fl-peptides were observed under 400× magnification. COS 7 cells were grown on microscope slides. After exposing the cells to the various reagents, the unfixed cells were examined by fluorescence microscopy. Cells exposed to Fl-KKALR showed sharply defined cellular compartments (Fig. 4A–C). Fl-AVWAL was localized more uniformly throughout the cell (Fig. 4D–F). The distribution and the fluorescence intensity of the Fl-peptides do not change for cells lacking energy-dependent pathways. These observations combined with the flow cytometric results show that endocytosis is not the major pathway for the delivery of materials into cells. A cell-passive, rotaxane-dependent mechanism is more likely followed.

The low percentage of cells containing PI, the high percentage of cells with cbAM, and the low amount of LDH released into the assay solutions indicate membrane leakage does not account for transport efficiency. Furthermore if CR2+2− caused membrane leakage, increasing its concentration should increase the percentage of fluorescent cells until cell death occurs. Pore formation is not consistent with the data shown in Figure 3. The observed concentration dependency can be explained by a complex being formed between CR2+2− and a Fl-peptide during the delivery process.

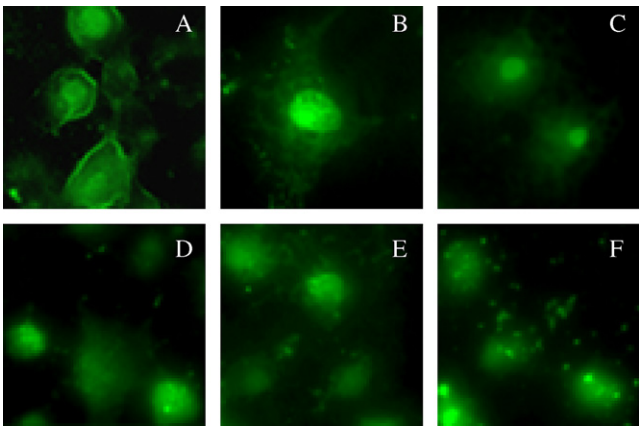

Figure 4. Fluorescence photomicrographs showing representative examples of COS 7 cells exposed to CR2+2− (20 μM) and Fl-KKALR (10 μM) (A–C) and Fl-AVWAL (10 μM) (D–F) at rt (A and D), 4 °C (B and E), and depleted ATP (C and F). Original magnification: 400×.

Table 2. Association constants (M^{-1}) for CR2+2− complexes[a]

Solvent	Fl-AVWAL	Fl-KKALR	Fl-QEAVD
Water[b]	2.7×10^4	3.0×10^3	5.0×10^3
DMSO	1.5×10^5	9.0×10^4	4.9×10^4

[a] The assays were performed at room temperature, the standard deviation is less than 10% for each K_A.
[b] 98% water (PBS 1 mM, pH 7.3)/2% DMSO.

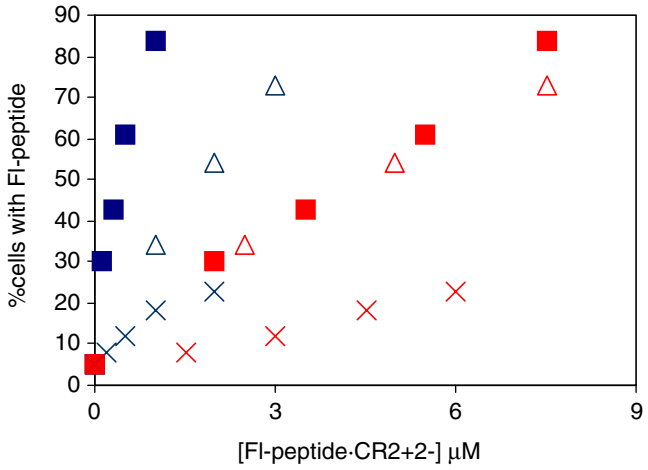

Figure 5. Concentration dependency for transport levels Fl-KKALR (squares), Fl-AVWAL (triangles, [CR2+2−] 40 µM is excluded), and Fl-QEAVD (×'s). Blue indicates the concentration of a complex in water and red DMSO. Standard deviation for both data sets is less than 10%.

Differences in the delivery levels of peptides can arise from the superior solubility (aqueous phase or membrane) or stability of its CR2+2−·Fl-peptide complex as compared to other complexes.

We determined the association constants (K_A's, Supporting Information) for the Fl-peptide·CR2+2− complexes in water (PBS, pH 7.3) and DMSO. These solutions are used to represent the extracellular domain and interface of cells, which is the region of the phosphate head groups, respectively. As expected, the most stable complex in water occurs for the binding of the most apolar peptide Fl-AVWAL (Table 2). The Fl-KKALR·CR2+2− and Fl-QEAVD·CR2+2− complexes are weaker and have similarly sized K_A's. Interaction with the side chains of these peptides requires the removal of water molecules, resulting in a large desolvation penalty for complexation. The same trend in stability for guest binding is observed in DMSO. The magnitude of K_A's, however, is significantly larger in DMSO than water, which is consistent with a diminished desolvation penalty.

Using the K_A's derived in water and DMSO, the concentration of the various Fl-peptide·CR2+2− complexes can be derived. Linear correlations exist between the concentrations of a Fl-peptide·CR2+2− complex versus the percentage of cells that contain that Fl-peptide, excluding the data point for cells exposed to 40 µM CR2+2− and Fl-AVWAL (Fig. 5). The slopes of the lines for a complex are greater in water than DMSO. If the amount of a complex in water dictates the amount of Fl-peptide that enters the cells, Fl-KKALR is delivered more efficiently than Fl-AVWAL. If complex strength in a DMSO environment is a better indicator of transport, then Fl-KKALR and Fl-AVWAL are delivered in a similar amount. In either case, the Fl-QEAVD·CR2+2− complex has the lowest permeability. The ability of CR2+2− to deliver Fl-KKALR as well as or better than Fl-AVWAL is remarkable considering that Fl-AVWAL has apolar side chains and the overall charge of its complex with CR2+2− is one less than the Fl-KKALR·CR2+2− complex in water (pH 7.3).

Here we show that a cleft-[2]rotaxane derivatized with carboxylic acids and guanidines on the wheel can more efficiently deliver a highly cationic peptide over peptides containing negative charges or apolar side chains. In the presence of a high concentration of CR2+2−, the percentage of fluorescent cells was approximately four times greater for cells exposed to Fl-KKALR than to Fl-QEAVD or Fl-AVWAL. We are currently designing transporters that will selectively deliver other peptides with various side chains into cells with the long-term goal of efficiently delivering therapeutic peptides into targeted cells.

Acknowledgments

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Supplementary data

Supplementary Information: includes the synthesis of CR2+2− and the methods used to determine K_A's. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.07.013.

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