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Investigation of the Intracellular Delivery of Fluoresceinated Peptides by a Host-[2]Rotaxane

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Abstract: The development of methods to transport peptides into cells via a passive mechanism would greatly aid in the development of therapeutic agents. We recently demonstrated that an impermeable fluoresceinated pentapeptide enters the cytoplasm and nucleus of COS 7 cells in the presence of a host-[2]rotaxane by a mechanism that does not depend on an active cell-mediated process. In this report, we further investigate the ability of the host-[2]rotaxane to deliver peptides possessing a wide range of polarities (negatively charged, positively charged, polar, and apolar side chains) into live cells. Only in the presence of the host-[2]rotaxane were the Fl-peptides taken up by COS 7 and ES2 cells. Flow cytometry experiments demonstrated that the level of delivery is largely temperature and adenosine 5′-triphosphate (ATP) independent, and the membranes remain intact. Although the level of transport does depend upon the nature of the side chains, it does not correlate with calculated LogD values, indicating that an additional interaction with the host-[2]rotaxane is modifying the permeability properties of the peptide. The amount of Fl-peptides transported from an aqueous phase into a chloroform phase in the presence of the host-[2]-rotaxane correlates with the intensity of cellular fluorescence. Extraction and U-tube studies show that the Fl-peptide can be released from its complex with the host-[2]rotaxane into an aqueous phase, and the host-[2]rotaxane can transport a greater than a stoichiometric amount of an Fl-peptide through a $CHCl_3$ layer. These studies demonstrate the utility of the host-[2]rotaxane in delivering peptides of all polarities across a cell membrane.

Introduction

Membranes surround cells to not only act as a barrier but also to house the biological compounds that control the influx and efflux of materials necessary for cell survival. The phospholipids are arranged as a bilayer, displaying charged groups at the external and internal aqueous phases and aliphatic chains in the membrane interior. These disparate environments keep most compounds from passing through membranes unaided. It has long been established that the permeability of a compound depends on a balance between its affinity for water and its affinity for the highly apolar lipid environment.[1−3] Compounds with charged or polar groups form strong noncovalent bonds with water. These bonds need to be broken for the compound to enter the membrane, which reduces its permeability. The hydrophobic effect, on the other hand, drives molecules from the aqueous phase into the apolar environment of the membrane. Thus, the presence of aliphatic or aromatic groups enhances a compound's permeability. Octanol−water partition coefficients (LogP or LogD) have been derived to predict the permeabilities of compounds.[4] Positive values mean a compound has a high permeability whereas negative values mean a low permeability. The studies of peptides have shown that permeability predictions should also consider the interface that separates the aqueous phase from the lipid phase. Sargent and Schwyzer proposed that the property of the interface could act as a catalyst in the interaction between amphiphilic peptides and receptors, and their theory has been invoked to explain the permeability of some peptides.[5]

Although peptides hold great promise as therapeutic agents, they display biological instability and poor bioavailability. A major component of poor bioavailability is the inability of most peptides to passively pass through cellular membranes. The creation of peptidomimetics has addressed some problems of instability. Passage through membranes, however, still remains a significant barrier. Peptides rely on their amide backbone and their side chains for structure and function. The amino acids display a wide range of side chains. Negatively or positively charged and polar groups favor an aqueous environment,

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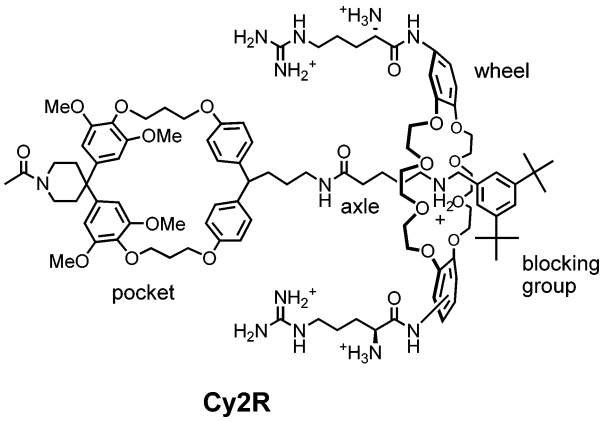
whereas their alkyl chains and aromatic rings favor a lipid layer. Changing hydrophilic groups to improve membrane permeability could detract from their desired function. The other possible solution to improve permeability would be to incorporate amino acids with apolar side chains to a peptide. Besides the possibility of disrupting function, Conradi and co-workers have shown that adding Phe, for example, does not enhance the ability of a peptide to travel across Caco-2 cell membranes, which grow as a monolayer and are used as a model system for permeability through the intestine.[6,7] For every Phe added to the peptide chain, another amide moiety is also added. Calculations have shown that an approximate transfer energy of 6 kcal/mol is needed to transfer an amide bond from water to hexane.[8] Extensive research has shown that out of the following features of uncharged peptides, chain length, lipophilicity, and amide bond, removing water molecules from the amide bond is the principle deterrent to passive membrane passage.[8−10]

Recently, several types of peptides have shown the ability to pass through membranes. Some cyclic peptides can cover their amides through intramolecular H-bonds and can pass through model membranes.[11] Correctly folded acyclic peptides can pass through Caco-2 cell monolayers[12] and liposomal membranes.[13] The ability of the acyclic peptides to change their conformations enables passage, which may by aided by the environment of the interface.[5] Various peptide vectors have been extensively studied because not only do they pass through membranes, but they can also carry compounds with them. Most commonly, these are oligopeptides containing multiple basic amino acids, especially arginines, for example, Tat protein[14,15] and oligo-arginines,[16−19] or are amphiphilic with stretches of basic and hydrophobic amino acids.[20,21] Recent investigations, however, have shown that most of these cell-penetrating peptides do not enter cells passively but enter through endocytosis.[22,23]

We have recently created a novel intracellular delivery method using host-rotaxanes as the delivery agent.[24,25] Transport efficiency is not greatly affected by lowering the temperature to 4 °C or by depleting the level of adenosine 5′-triphosphate (ATP).[25] These results suggest that endocytosis is not the major pathway for cellular entry. Host-rotaxanes comprise an arginine-derivatized wheel, an axle, and an aromatic-rich pocket, as one blocking group (Figure 1). The pocket and the wheel provide the recognition and binding domain for various guests. This domain needs to adjust its conformation to maintain tight guest association during the changes in environment that occur during the passage through the cell membranes.[25] In our original investigation of host-[2]rotaxanes, a series of oligopeptides were available that were based on known CAM kinase II inhibitors.[26] Exposing cells to cyclophane-[2]rotaxane (Cy2R, Figure 1) and the highly charged Fl-KKALRAQEAVDAL-CO_2H peptide (Fl-CAM) resulted in fluorescent cells.[27] To more fully explore the potential of Cy2R to deliver Fl-peptides, a series of Fl-pentapeptides of all polarities with sequences roughly based on CAM were purchased and tested (Table 1). The carboxyl terminus of the pentapeptides were converted to carboxamides, and they were attached to fluorescein isothiocyanate through the amino terminus (HO_2C-Fl-NHC(S)NH-peptide-$CONH_2$).

This study shows that the Fl-peptides are delivered into the cells at levels higher than expected according to their calculated distribution coefficients. Intracellular transport does not rely upon cellular processes. The levels of propidium iodide (PI) within the cells and lactate dehydrogenase (LDH) in the solutions were not detected significantly above background levels after the assays. These results demonstrate membrane integrity for cells exposed to the reagents. Model transport studies were performed to further explore the delivery process. While these studies do not reflect the complexity of cellular membranes, the results of the model study show Cy2R can



Cy2R

Figure 1. Schematic of the cyclophane-[2]rotaxane (Cy2R) indicating the wheel, axle, blocking group, and pocket.

Table 1. Permeability Properties of Fl-Peptides

peptide	dominant polarity of the side chains	LogD calculated[a]
Ac-KKALRAQEAVDAL-NH_2	all polarities	−11.8
Ac-KKALR-NH_2	cationic	−6.84
Ac-QEAVD-NH_2	anionic	−6.38
Ac-AQEAV-NH_2	anionic/polar	−4.59
Ac-AQSAV-NH_2	polar/apolar	−2.85
Ac-AVWAL-NH_2	apolar	0.86

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transport the Fl-peptides from an aqueous phase into a $CHCl_3$ layer at concentrations used in the cellular assay through noncovalent complex formation. The amounts of the Fl-peptides delivered into $CHCl_3$ and into the cells are correlated. Thus, a complex formation step may be involved in the passive mechanism used for intracellular delivery. U-tube assays demonstrate that Cy2R can release an Fl-peptide into an aqueous phase from a liquid membrane and can deliver a greater than a stoichiometric amount of an Fl-peptide through this liquid membrane. At this time, we do not know if Cy2R delivers a greater than stoichiometric amount of Fl-peptides into the cells or if Cy2R delivers the Fl-peptides into the nucleus or other cellular compartments. Current studies with a rhodamine-tagged transporter are underway to address these issues.

Results and Discussion

Determining the Cellular Transport Efficiencies of Cy2R. To obtain a measure on the likelihood of cellular delivery, LogD values for the peptides were calculated using parameters developed by Tao et al.[28] (Table 1). They showed that calculated LogD values strongly correlate with observed LogD values for a series of *N*-acetyl-pentapeptide amides in octanol and buffered water (pH 7.35). This method does not provide a value for fluorescein, but since all of the peptides are fluoresceinated, the calculated LogD values provide for a trend in delivery levels. According to the calculated LogD values, only the AVWAL peptide should be permeable to a small extent. Because fluorescein has low membrane permeability, the actual LogD values are most likely lower than the ones calculated. Thus, Fl-AVWAL should actually have a negative LogD value.

The optimal concentration of Cy2R for guest delivery was determined in part through cell viability assays using trypan blue. Trypan blue is taken up by dead cells, which are detected using light microscopy. Suspended cells were combined with Cy2R at different concentrations for 1 h at room temperature. Trypan blue analysis revealed that 1×10^5 cells exposed to 5 μM Cy2R is not cytotoxic (6% cell death and untreated cells also gave 6% cell death). Doubling the concentration of Cy2R to 10 μM gives about 10% dead cells. Once a nontoxic ratio of cells to Cy2R was determined, the assay was performed for plated cells. The delivery assays were performed with adhered cells to enable the delivery of the Fl-peptide to be visually monitored through fluorescence microscopy. Plated cells (1×10^5 cells per well) were exposed to various combinations of the reagents used in the assays for 1 h. Exposing cells to Cy2R (5 μM) and Fl-AVWAL (10 μM), which was chosen as the representative Fl-peptide, produced approximately the same percentage of cell death as untreated cells (5% to 4%, respectively). To ensure cell viability in the delivery assays, 1×10^5 plated cells were exposed to 5 μM of Cy2R and 10 μM of Fl-peptide.

We used fluorescence microscopy analysis to determine whether the optimal concentration of 5 μM of Cy2R and 10 μM of Fl-AVWAL resulted in intracellular delivery. The assay procedure has been described in detail previously.[25] Briefly, COS 7 or ES2 cells were incubated with Cy2R and an Fl-peptide in a phosphate-buffered saline (PBS) solution pH 7.4. After an hour, aliquots of the assay solutions were analyzed for the presence of LDH using the commercially available CytoTox-ONE Integrity Assay (Promega). The cells were washed thoroughly. Calcein blue AM (Invitrogen) and PI were added to each well to demonstrate cell viability or potential cytotoxicity and membrane integrity, respectively, after incubation with Cy2R and Fl-peptides. Cells were examined via inverted fluorescence microscopy at 100× magnification.

As expected, only a very weak level of fluorescence is observed for cells exposed to the Fl-peptides alone (Figure 2G). Cells exposed to Cy2R (5 μM) and Fl-AVWAL (10 μM), however, resulted in high levels of fluorescent cells (Figure 2A). Cells were exposed to the other Fl-peptides (10 μM) and Cy2R (5 μM). Although the total number of fluorescent cells and the overall brightness were generally less than what was observed for Fl-AVWAL, there were focal areas of live cells that had equivalent brightness (Figure 2B−F). Transport levels depend on the polarity of the side chains. For example, CAM has the smallest calculated LogD value and it showed the least number of fluorescent cells as viewed by inverted microscopy. Interestingly, Fl-KKALR showed a large number of fluorescent cells (Figure 2B) even though its calculated LogD value is also very small. Strong calcein blue fluorescence was observed in the majority of cells regardless of treatment. Only a few cells were stained with propidium iodide.

Flow cytometry was used to quantify the relative levels of the Fl-peptides, calcein blue, and propidium iodide within the cells. Thresholds and gates were set as described in the Experimental Section and were applied to each sample. Cy2R efficiently delivered Fl-AVWAL into a large percentage of the cells (ca. 80%, Figure 3 and Table 2). Fl-KKALR and Fl-AQSAV are delivered into a reduced number of cells (ca. 60%). These similar values are surprising considering that the peptide portion of Fl-KKALR is highly charged whereas the peptide portion of Fl-AQSAV is uncharged. Peptides with one and two negative charges were transported into a lower number of cells (33−38%, Table 2). Apparently, Cy2R can cover two negative charges of a peptide (QEAVD) as well as it can cover one negative charge (AQEAV). Fl-CAM has the same number of negative charges in buffered water as does Fl-QEAVD, but it has three positive charges. Since Fl-CAM is delivered into around 33% of the cells, it appears that the number of negative charges dictates the level of delivery. A high proportion of calcein blue positive cells (range: 86−96%) was observed, demonstrating that Cy2R and the Fl-peptides at these concentrations have a low level of toxicity. Only a small percentage of the cells (≤%8) contained propidium iodide. A similar percentage was observed for cells exposed to the Fl-peptides alone. Calcein blue, propidium iodide, and peptide fluorescence were independent variables.

To determine the cellular location of the delivered Fl-peptides, COS 7 cells were grown on microscope slides. Live cells were exposed to Cy2R (5 μM) and an Fl-peptide (10 μM) in PBS for 1 h at 20 °C. Unfixed cells were examined by fluorescence microscopy (400×). The Fl-peptides were seen in the cytoplasm and the nucleus of COS 7 cells (Figure 4). The fluorescence intensity of the cytoplasm and the nucleus is slightly different for the Fl-peptides. Interestingly, the fluorescence intensity for cells exposed to Fl-KKALR and Cy2R is observed to be about equally distributed in the cytoplasm and the nucleus. For cells exposed to the other Fl-peptides and Cy2R, generally a more intense fluorescence is observed in the nucleus. Short sequences

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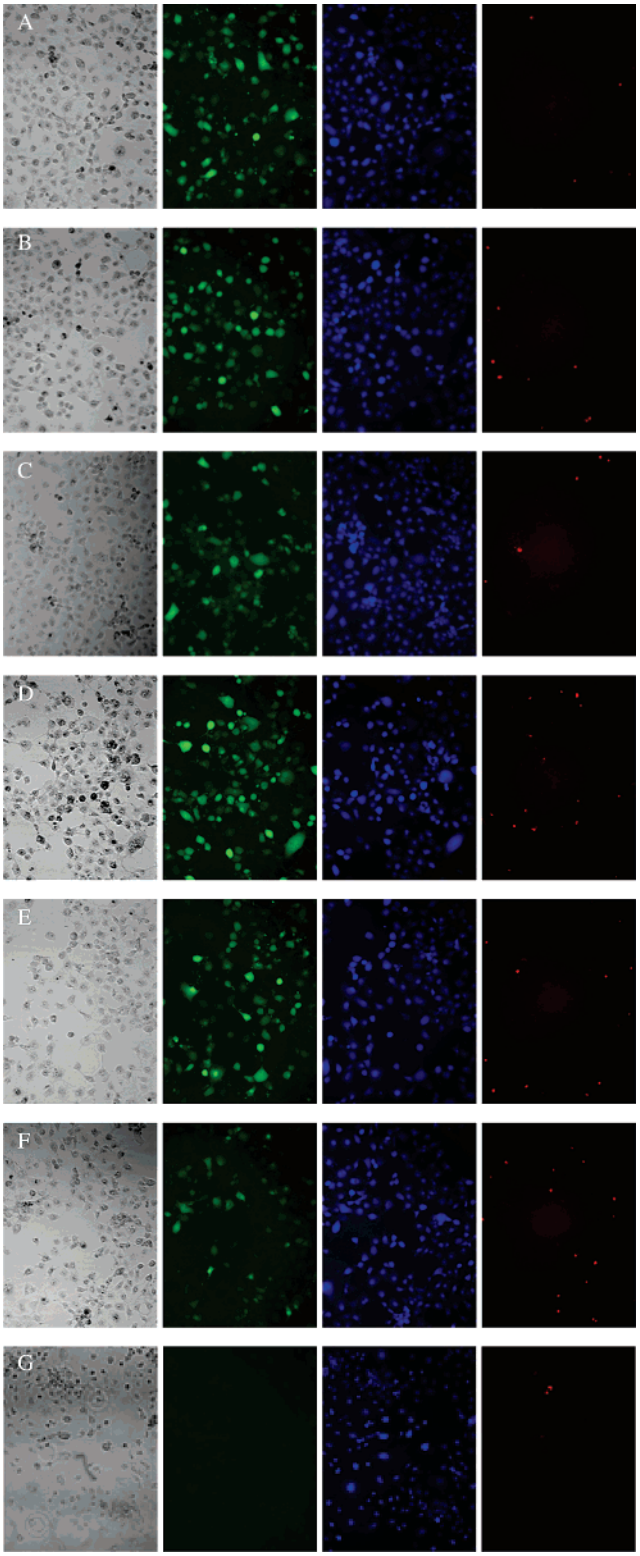

Figure 2. Fluorescence microscopy of COS 7 cells incubated with the Fl-peptides (10 μM) alone or with Cy2R (5 μM). Cells were viewed for fluorescence (from left to right: white light, FITC (Fl-peptide uptake), calcein blue fluorescence (viability), and propidium iodide (decreased membrane integrity)). (A) Cy2R and Fl-AVWAL, (B) Cy2R and Fl-KKALR, (C) Cy2R and Fl-AQSAV, (D) CY2R and Fl-AQEAV, (E) CY2R and Fl-QEAVD, (F) Cy2R and Fl-CAM, and (G) an example of an Fl-peptide (original magnification: 100×).

with highly basic side chains are known as nuclear targeting sequences.[29] Thus, of all the Fl-peptides, we expected Fl-KKALR to reside in the nucleus. Since small molecules (<9 nm in diameter), such as the Fl-peptides, Cy2R, or the various Cy2R·Fl-peptide complexes, can pass into the nucleus through the nuclear pore complex by passive diffusion,[30] we do not know at this time if Cy2R is involved in transport into the nucleus.

Investigating the Cellular Transport Mechanism. The cellular transport mechanism was investigated with COS 7 cells. To determine whether endocytosis was the predominant mechanism of intracellular transport, additional experiments were performed at 4 °C or by reducing the cellular ATP with 6 mM 2-deoxy-D-glucose (2DG) and 10 mM sodium azide to deplete the energy-dependent cellular pathways.[23,31−41] Since some cell death was caused by these procedures (assessed by cell detachment and loss of calcein blue fluorescence), further analysis was restricted to live cells that remained attached to the plates. Cells were incubated at 4 °C or with the ATP-depleting cocktail prior to the addition of Cy2R and subsequent Fl-peptide. The cells were observed via inverted fluorescence microscopy (100×) and then were trypsinized and assessed by flow cytometry. Treatment with 2DG and azide resulted in a 40% reduction in the delivery of Fl-AVWAL and Fl-CAM (Table 3). The delivery of the other Fl-peptides was less affected. At 4 °C, however, the delivery efficiency was enhanced by 20−30% for the Fl-peptides, as compared to room temperature, except for Fl-AQSAV, which remained about the same, and Fl-AVWAL, which showed a 20% reduction. Since Fl-AVWAL and Fl-CAM showed the greatest reduction in delivery levels with energy-depleted cells, these experiments were repeated for cells grown on microscope slides and were observed under 400× magnification. Examination of the live cells exposed to energy-depleted conditions and normal conditions (Figure 5) reveals a similar location and intensity of the fluorescence. These results indicate that endocytosis is not the major pathway for cell entry of the Fl-peptides.

Measuring Membrane Integrity. Another possible means for intracellular entry is Cy2R forming nontoxic holes in the membrane[42] and the Fl-peptides entering through these holes. Propidium iodide was used in the assay to measure membrane integrity. The inability of PI to enter less than 5% of the cells that were exposed to the Fl-peptides or to Cy2R and Fl-peptides indicates that the cell walls were intact (Table 2). The ability of Cy2R to deliver rhodamine was also investigated. Rhodamine is similar in size to fluorescein, but it contains a cationic charge. Cy2R binds rhodamine weaker than fluorescein[25] in buffered water (pH 7.4) and DMSO ($K_{\text{rhodamine,DMSO}} = 4 \times 10^4$

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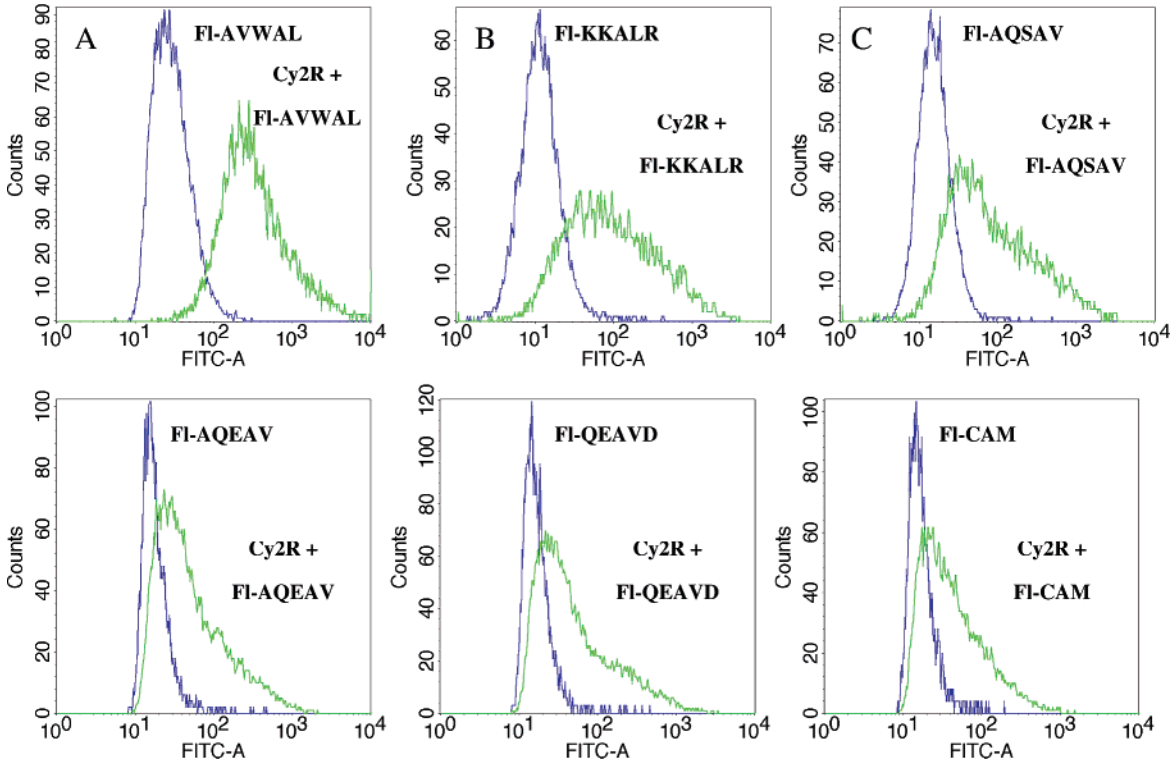

Figure 3. Relative fluorescence of COS 7 cells exposed to the Fl-peptides alone (10 μM) or with Cy2R **1** (5 μM) for 1 h at 20 °C as measured by flow cytometry.

Table 2. Quantification of Uptake into Cells as Measured by Flow Cytometry

compound[a]	%Fl-peptide COS 7 cells	%cbAM COS 7 cells[b]	%PI COS 7 cells[c]	%Fl-peptide ES2 cells	%cbAM ES2 cells	%PI ES2 cells
Fl-AVWAL + Cy2R	82	91	3	74	95	3
Fl-AQSAV + Cy2R	64	93	2	56	93	4
Fl-KKALR + Cy2R	57	90	3	54	94	3
Fl-AQEAV + Cy2R	38	91	4	43	96	4
Fl-QEAVD + Cy2R	34	92	4	36	95	4
Fl-CAM + Cy2R	31	91	5	34	96	3
Fl-peptide[d]	5	95	2	5	96	3
Cy2R	2	86	6	2	87	8

[a] [Cy2R] = 5 μM, [Fl-peptide] = 10 μM, all transport assays were performed in PBS (pH 7.4) for 1 h. [b] [Calcein blue AM] = 1 μM. [c] Propidium iodide. [d] Average values obtained for cells exposed to only the Fl-peptides, %Fl-peptide was set at 5% for each Fl-peptide.

M^{-1}, $K_{\text{rhodamine, water}} < 1 \times 10^3\ M^{-1}$, $K_{\text{fluorescein,DMSO}} = 7.7 \times 10^5\ M^{-1}$, and $K_{\text{fluorescein,water}} = 5.3 \times 10^4\ M^{-1}$). A similarly weak, background level of rhodamine was observed in cells that were exposed to rhodamine alone (0.5 μM) or to rhodamine (0.5 μM) and Cy2R (5 μM) (Supporting Information). Fluorescein (0.5 μM) enters COS7 cells in the presence of Cy2R (5 μM).[24] This suggests that an impermeable compound needs to form a tight complex with Cy2R to more efficiently enter cells.

Membrane integrity was also demonstrated by calcein blue (cbAM). CbAM is a short-term, fluorescent indicator of live cells and remains in the cells for several hours. Within the usual 2 h needed to prepare and analyze the cells in our assay, the intensity of the blue fluorescence does not change noticeably. Furthermore, untreated cells and cells containing the Fl-peptides are analyzed first by flow cytometry to set the gates. Cells containing Cy2R and an Fl-peptide are then examined. The level of cbAM is usually within a few percentage points throughout the samples. Another measure of material released was obtained

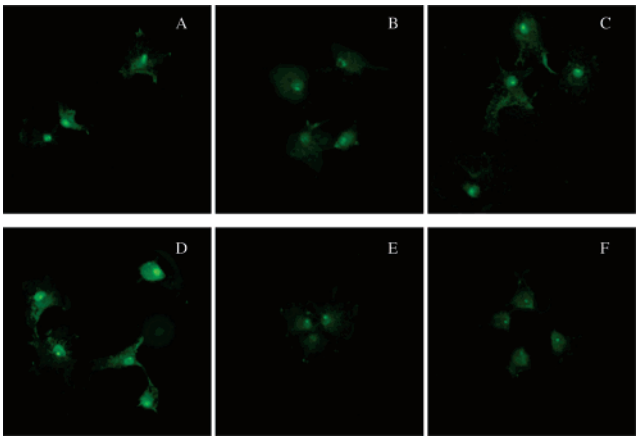

Figure 4. Fluorescence photomicrographs showing representative examples of COS 7 cells exposed to Cy2R (5 μM) and an Fl-peptide (10 μM): (A) Fl-AVWAL, (B) Fl-KKALR, (C) Fl-AQSAV, (D) Fl-AQEAV, (E) Fl-QEAVD, and (F) Fl-CAM. (Original magnification: 400×).

by comparing the level of LDH in the assay solutions (CytoTox-ONE Integrity Assay). The fluorescence signal produced for cells (COS 7 or ES2) exposed to a 0.1% Triton X-100 solution was set at 100% (Figure 6). Untreated cells and cells exposed to the Fl-peptides showed fluorescence intensities in the range of 9−13%. Cells exposed to Cy2R or Cy2R and Fl-peptides ranged from 15% to 17%, except for ES2 cells exposed to Cy2R and Fl-CAM, Fl-QEAVD, or Fl-AQEAV, which had values of 19−20%. Because the latter Fl-peptides are delivered in the least amounts, a positive correlation does not exist between the delivery efficiencies and LDH levels.

Investigating Whether Cy2R and Fl-Peptide Assembly Is Involved in Transport. Only a very weak correlation exists between the number of fluorescent cells of each Fl-peptide versus the calculated LogD values (r = 0.68, Figure 7).

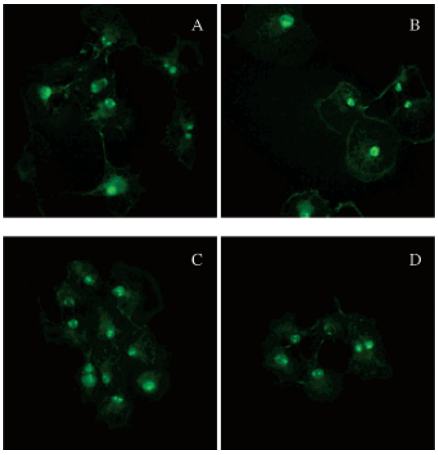

Figure 5. Fluorescence photomicrographs showing representative examples of COS 7 cells exposed to Cy2R (5 μM) and (A) Fl-AVWAL (10 μM) at 4 °C, (B) Fl-AVWAL (10 μM) with depleted ATP, (C) Fl-CAM (10 μM) at 4 °C, and (D) Fl- CAM (10 μM) with depleted ATP.

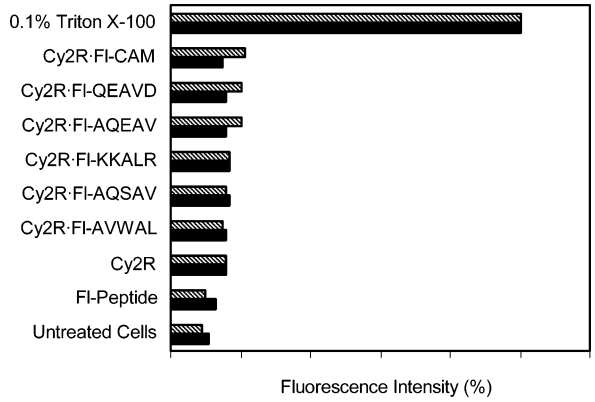

Figure 6. LDH levels for COS 7 (solid bars) and ES2 (stripe bars) cells. The fluorescence signal for cells exposed to 0.1% Triton X-100 for 10 min was set at 100%. Cells exposed to the Fl-peptides had very similar LDH levels, and so the values were averaged. Untreated cells contained 0.2% DMSO, which existed in the assay solutions. Standard deviation is less than 1% for COS 7 and 2% for ES2 cells.

Excluding the data for Fl-AVWAL, the variables are no longer correlated ($r = 0.39$). This suggests that Cy2R interacts with some of the impermeable portions of the peptides in the Fl-peptides during the delivery process. Arginines can be permeable when linked together in small peptides. The carboxylates of the guests can be made more permeable through several processes. Covering a negatively charged group means that either a strong salt bridge or a proton exchange reaction, which produces neutral species, occurs. According to ab initio and semiempirical calculations, neutral complexes are more likely formed since they are more stable than the zwitterionic pair in a solvent-free environment, such as the lipid portion of membranes.[43] Another possibility is that the fluorescein moiety undergoes lactonization through its carboxylic acid upon protonation.[44] If this event occurs, one formal charge of an Fl-peptide would be neutralized.

We know that fluorescein and the Fl-peptides do not enter cells unless in the presence of a host-rotaxane. Cellular membranes appear to be intact during intracellular delivery. These results suggest that a complex is formed between Cy2R and an Fl-peptide during delivery. Association constants were obtained to determine if complex stability during the transport process is responsible for the observed differences in the level of Fl-peptides within the cells. The three main domains are the extracellular aqueous phase, the interface, and the lipid portion of the membrane. Weak association in any one of the phases or the exposure of insoluble groups could lead to the observed differences in delivery. The association constants (K_A's) of Cy2R·Fl-peptide complexes were measured using fluorescence quenching assays in an aqueous solution (98% PBS, pH 7.4/2% DMSO) and DMSO (Table 4). Many of these Fl-peptides contain multiple acidic or basic side chains. Since the charge of the peptide or the fluorescein moiety as it passes through the lipid tails is not known, we could not set the appropriate ionization state of the Fl-peptides in $CHCl_3$. Thus, extraction and U-tube cell experiments were used as model systems (see below) to further investigate membrane passage.

A range of K_A's are observed for complexes in the aqueous solutions. The largest K_A is observed for the complex of Fl-AVWAL and the smallest is observed for the complex of Fl-CAM. These results are consistent with the hydrophobic effect,[45,46] where the apolar side chains of Fl-AVWAL are driven into the aromatic pocket of Cy2R. The complex strength for these peptides is consistent with the observed transport level: Fl-AVWAL showed around 80% fluorescent cells and Fl-CAM showed around 33% fluorescent cells. Complex strength in water, however, does not appear to be the only determinant in the level of transport. The complexes of Fl-KKALR and Fl-AQSAV have similarly small K_A's in the aqueous solution as Fl-CAM, but they are delivered into cells more efficiently (ca. 60% versus ca. 33% fluorescent cells, respectively). The Fl-peptides have much larger K_A's for the complexes in DMSO, representing the polarity experienced at the interface,[47] than in the aqueous solution. A second large K_A was observed in the binding assays (Supporting Information) and was assigned to the 2:1 host-to-guest complex ($K_{A(2)}$ in Table 4). Since $K_{A(1)}$ and $K_{A(2)}$ for the Fl-AQSAV and Fl-CAM complexes have similar values, Job plot analysis was performed. The results verify the existence of 2:1 complexes for these Fl-peptides (Supporting Information). We do not know whether 2:1 complexes are formed at the cellular interface. Neither the aqueous phase nor the interface appears to differentiate the level of transport. Even though the transport levels do not correlate with $LogD_{calc}$ values, the binding results suggest that the lipid domain is the deciding factor.

Modeling of Fl-Peptide Transport through Extraction Experiments. The level of fluorescent cells does not correlate with the calculated LogD values and with the K_A's obtained for complexes in water and DMSO. This suggests that Cy2R interacts with a portion of the peptides as the Fl-peptides pass through the lipid layer. Extraction assays were performed to obtain experimental distribution constants. These constants would represent the delivery of the Fl-peptides from the aqueous

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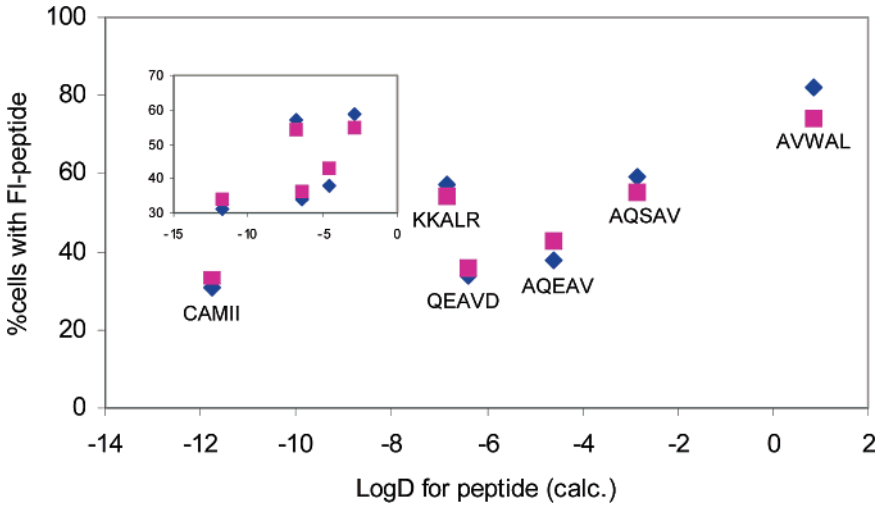

Figure 7. A very weak correlation ($r = 0.68$) exists for the total fluorescence observed in cells (COS 7 diamonds and ES2 squares) exposed to Cy2R and an Fl-peptide, as determined by flow cytometry, and the calculated LogD values[28] (at neutral pH) for the corresponding peptide portion of the Fl-peptides. The insert shows the data without Fl-AVWAL, which results in a plot of scattered data points ($r = 0.39$).

Table 3. Flow Cytometric Analysis of Intracellular Delivery of Fl-Peptides by Cy2R after ATP and Temperature Reduction[a]

	ATP depleted		4 °C	
peptide[b]	%Fl-peptide cells	%cbAM cells	%Fl-peptide cells	%cbAM cells
Fl-AVWAL	49	72	65	85
Fl-AQSAV	59	93	60	77
Fl-KKALR	53	96	68	76
Fl-AQEAV	26	76	51	72
Fl-QEAVD	25	76	45	72
Fl-CAM	19	78	46	74

[a] Assay was performed in PBS (pH 7.4) for 1 h and [Cy2R] = 5 μM. [b] [Fl-peptide] = 10 μM.

Table 4. Association Constants for Cy2R−Guest Complexes[a]

	water[b]	DMSO	
guest	K_A	$K_{A(1)}$[c]	$K_{A(2)}$[d]
Fl-AVWAL	140	140	40
Fl-KKALR	18	320	60
Fl-AQSAV	21	210	120
Fl-AQEAV	29	320	60
Fl-QEAVD	30	190	50
Fl-CAM	17	210	160

[a] The assays were performed at room temperature, the standard deviation is less than 10% for each K_A, and the values have been divided by 1×10^3. [b] 98% water (PBS 10 mM, pH 7.4)/2% DMSO. [c] K_A for Cy2R·Fl-peptide complex. [d] K_A for $(Cy2R)_2$·Fl-peptide complex.

extracellular domain into the lipid layer of a membrane. The same concentration of Cy2R and an Fl-peptide in a 1:1 ratio (37 μM each) were added to the aqueous phase (1.5 mL, PBS, pH 7.4), which was on top of a $CHCl_3$ phase (1.5 mL). After vigorously stirring for 3 h, the amount of Fl-peptide and Cy2R in each phase was determined using high-performance liquid chromatography (HPLC) analysis. After each assay, the solutions were completely removed and the vial was washed with DMSO and was analyzed to confirm that the components did not precipitate.

We expected that the complexes would exist at a measurable concentration in both the aqueous and the $CHCl_3$ phases, which would lead to a set of relative LogD values. However, after extracting the materials for 3 h, there was no Cy2R observed in the aqueous phase. Fl-peptides were observed in the $CHCl_3$ phase. Because the Fl-peptides are not observed in $CHCl_3$ without Cy2R, the amount of an Fl-peptide observed in the $CHCl_3$ phase is equal to the amount of its complex with Cy2R ([Cy2R·Fl-peptide]). For the assay with the same concentration of components (40 μM), a very weak correlation ($r = 0.68$) is observed when the percentages of the Fl-peptides delivered into $CHCl_3$ (Table 5, row 1) are plotted against the total percentages of the fluorescent cells (data not shown). Removing Fl-AVWAL from this plot, however, drops the correlation coefficient to 0.20.

The same extraction assay was repeated except that the amount of Cy2R and Fl-peptide was adjusted to give the same concentration of the [Cy2R·Fl-peptide] complex. This study is a more direct method of determining the ability of the complexes to pass into the lipid. A moderate correlation is observed in the plot of the amount of Fl-peptide in the $CHCl_3$ phase against the total percentages of the fluorescent cells ($r = 0.85$, Figure 8). More importantly, this correlation coefficient is only slightly reduced with the removal of Fl-AVWAL from the plot ($r = 0.78$). This correlation is better than the one obtained for the plot using calculated LogD values ($r = 0.68$), especially when Fl-AVWAL is removed from that plot ($r = 0.39$, Figure 7). Furthermore, the highly charged Fl-KKALR and the polar Fl-AQSAV lie in a similar position in the plot of the complexes versus cellular fluorescence. These Fl-peptides have very dissimilar LogD values, and thus, they are not together in the LogD versus cellular fluorescence plot (Figure 7). The correlation between the observed fluorescence intensity of the cells and the amount of complex delivered into $CHCl_3$ suggests that Fl-peptide·Cy2R complexes form during intracellular delivery and that these complexes exist at a similar concentration. The weak correlation between cell intensity and LogD suggests that Cy2R either forms strong noncovalent bonds with the impassable portions of the peptides or the carboxylates are chemically modified via one or more proton-transfer reactions. The peptides, however, still maintain some unique side chain properties within the complex, and these features control the ability of the complex to pass through the membrane.

To determine whether a protonation step is necessary for transport into $CHCl_3$, Cy2R was added to a pH 12 solution of water, which was on top of a $CHCl_3$ layer, and then Fl-AQEAV

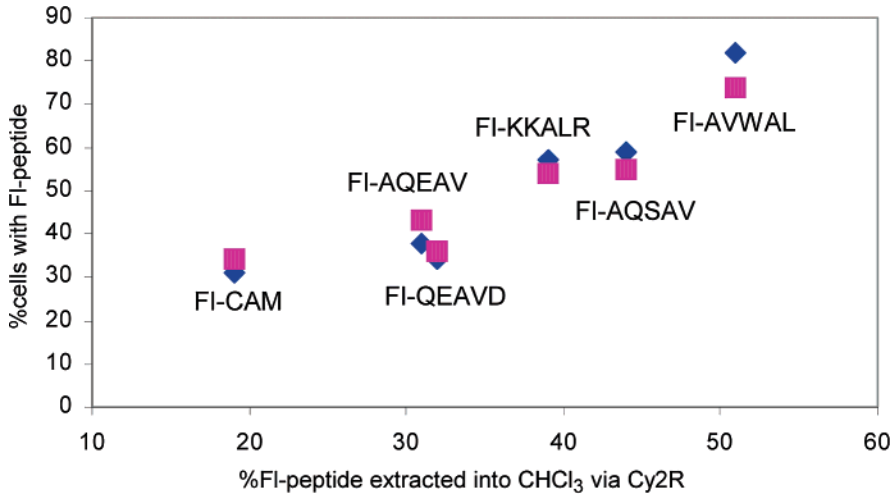

Figure 8. A moderate correlation ($r = 0.85$) exists for the total fluorescence observed in cells (COS 7 diamonds and ES2 squares) exposed to Cy2R and an Fl-peptide, as determined by flow cytometry, and the percent of an Fl-peptide transported into $CHCl_3$ by Cy2R. The data without Fl-AVWAL still produces a moderate correlation ($r = 0.78$).

Table 5. Extraction of the Fl-Peptides into $CHCl_3$ by Cy2R

	%Fl-peptide extracted into $CHCl_3$					
initial conditions in aqueous phase[a]	Fl-AVWAL	Fl-KKALR	Fl-AQSAV	Fl-AQEAV	Fl-QEAVD	Fl-CAM
[Cy2R] = [Fl-peptide] = 37 μM	93	12	27	21	18	8
[Cy2R·Fl-peptide] = 20 μM	51	39	44	31	32	19
[Fl-peptide] = 25 μM	ND[b]	ND	ND	ND	ND	ND

[a] PBS 10 mM, pH 7.4. [b] ND means not detected.

Table 6. Results Obtained From the U-Tube Cell Experiments

	day 1		day 3		day 9	
Ratio Cy2R:Fl-AQEAV[a]	source phase	receiving phase	source phase	receiving phase	source phase	receiving phase
1:1.5	13[b]	0.2	7.9	1.7	5.8	7.1
1:4.5	51	0.6	43	6.4	23	15
1:9.0	100	7	91	20	66	44

[a] Cy2R = 13 μmol. [b] Amount of Fl-AQEAV in the aqueous layers is given in μmol.

was added immediately afterward. After extracting these materials for 24 h, Fl-AQEAV was not found in the $CHCl_3$ phase. This suggests that a protonation step is required for transport. Cy2R binds Fl-AQEAV in pH 12 and pH 7.4 buffered water with similar magnitudes ($K_A = 1.9 \times 10^4$ and 2.9×10^4 M^{-1}, respectively). Thus, a lack of transport is not caused by a lack of complex formation. To determine if protonation is solely responsible for transport, Fl-AVWAL was used in an extraction assay with acidic water (pH = 1) and $CHCl_3$. Fl-AVWAL was chosen as the representative Fl-peptide in this experiment because it has the most permeable side chains in a membrane and is the most soluble in $CHCl_3$ of the Fl-peptides used in this study. Only 3% of Fl-AVWAL was found in the $CHCl_3$ phase via HPLC analysis. HPLC was used to determine the amount of Fl-AVWAL since fluorescein is known to convert to a nonfluorescent lactone when protonated. These results suggest that complexation and proton transfer are both required for the intracellular delivery of the Fl-peptides.

According to these results, Cy2R would need to be reprotonated to continue the transfer cycle. Extraction assays were performed with Cy2R placed into the $CHCl_3$ phase in its basic form (transferred from a pH 12 solution) or its acidic form (transferred from a pH 7.4 solution) and Fl-AQEAV was added to the aqueous phases. At pH 12, the ammonium ions of Cy2R should be deprotonated ($pK_a = 9.0$ for $-NH_3^+$ of arginine). These studies model the transport step of Cy2R leaving the lipid layer, binding Fl-AQEAV, and transporting it to the lipid layer. As expected, the basic form of Cy2R does not transport Fl-AQEAV from the basic aqueous phase (pH 12) into $CHCl_3$ within 24 h (Figure 9A). The acidic form of Cy2R in the $CHCl_3$ phase, however, binds and transfers Fl-AVWAL from a neutral aqueous solution (pH 7.4) into the $CHCl_3$ phase (Figure 9B). After extracting these materials for 24 h, 26% of Fl-AQEAV was found in the $CHCl_3$ phase, which closely matches the previous value of 31% that was obtained by extracting a preformed complex in the aqueous phase (Table 5). Importantly, the extraction of Fl-AQEAV in a pH 7.4 aqueous solution with the basic form of Cy2R in a $CHCl_3$ layer results in the transport of 24% of the Fl-AQEAV into the $CHCl_3$ phase after 24 h (Figure 9C). These results show that the transport cycle is possible where a deprotonated host-rotaxane can interact with an aqueous phase, be reprotonated, and transfer more material.

Modeling of Transport Using U-Tube Cells. The fluorescence assays, which were used to obtain the K_A's, showed that complexation quenches the fluorescence of the Fl-peptides. Some cells, on the other hand, were intensely fluorescent. This suggests that some of the Fl-peptides are released from Cy2R once the complex enters the cells. U-tube assays were performed to determine the possibility of this process: complex formation, membrane passage, and release of an Fl-peptide. The possible

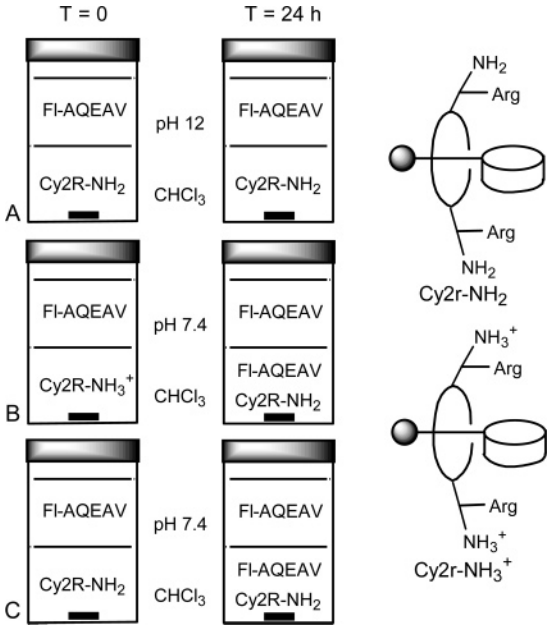

Figure 9. Extraction assays that show an acidic proton is necessary for Cy2R to transport Fl-AQEAV from an aqueous phase into $CHCl_3$.

return of Cy2R to the extracellular domain was also explored. As with the extraction experiments, $CHCl_3$ was chosen as the liquid membrane, and Fl-AQEAV was chosen as the model Fl-peptide. The U-tube cell contains two aqueous phases (source and receiving) separated by the $CHCl_3$ phase. Materials are added to the source phase, and the amount of materials in this phase, in the $CHCl_3$ phase, and in the receiving phase is determined at set time periods using HPLC analysis. We found that Fl-AQEAV alone does not enter the $CHCl_3$ phase, and Cy2R does not reside in the aqueous phase at equilibrium. UV−vis analysis was used to verify the amount of Fl-AQEAV in the aqueous phases. The experiments were designed to determine the extent to which Fl-AQEAV is released into the receiving phase (mimic of the intracellular domain) and the ability of Cy2R to return to the source phase to repeat the transport cycle.

Fl-AQEAV and Cy2R were placed in a 1:1 ratio into the receiving phase. After 9 days of light agitation, we observed by eye that an equivalent intensity of green existed in both aqueous phases. The $CHCl_3$ phase was colorless. HPLC analysis revealed that 21% of Fl-AQEAV existed in the source and receiving phases, so its distribution was equilibrated. About 50% of Fl-AQEAV remained in the $CHCl_3$ phase. Since it is not soluble in $CHCl_3$, Fl-AQEAV existed in a 2:1 host−guest complex [$(Cy2R)_2$·Fl-AQEAV] or some higher order aggregate that remains soluble. After removing both aqueous phases, buffer was added to both sides of the $CHCl_3$ phase, and the solutions were lightly agitated for three more days. Only 2% more of Fl-AQEAV was released to the aqueous phases. Thus, the host−guest complex is very stable in $CHCl_3$ and limits the amount of released Fl-AQEAV. Both aqueous phases were removed, new aqueous phases (pH 7.4) were added, and another equivalence of Fl-AQEAV (18 μmol) was added to the source phase. After 9 days, 36% of Fl-AQEAV was in the source phase and 11% of Fl-AQEAV was in the receiving phase. The $CHCl_3$ phase contained an approximate 1:1 ratio of host to guest. Although the transfer process is less efficient, this study shows that additional material can be delivered once the equilibrium is altered by removal of material in the receiving phase. This would apply to a case when Cy2R transfers compounds into cells that are removed from the intracellular space by being bound to biological materials or by being consumed.

U-tube experiments were preformed with 1.5, 4.5, and 9-fold equivalences of Fl-AQEAV, as compared to Cy2R, in an attempt to deliver a greater than stoichiometric amount of material. As the amount of Fl-AQEAV increases, its rate of transport and the amount of it delivered increase (Table 6). A greater than a stoichiometric amount of Fl-AQEAV, as compared to the limiting amount of Cy2R, is observed to be transported with a 9:1 ratio of Fl-AQEAV to Cy2R at 3 and 9 days. Although the transport rates are slow in the U-tube experiments, the transport rate in cells is most likely much faster. The $CHCl_3$ phase was only gently agitated to keep the source and receiving phases from coming into contact. Furthermore, volume of the cellular membrane is a lot less than the volume of the $CHCl_3$ phase. These results show that host-rotaxanes may release an Fl-peptide once it passes through a membrane. Furthermore, the transport of a greater than a stoichiometric amount of material into a cell is possible. Future experiments will be designed to determine the stoichiometry of the components by using a transporter linked to rhodamine and a peptide linked to fluorescein.

Conclusion

According to the results obtained from the extraction and U-tube assays, Cy2R resides within the membrane environment of cells. The presence of Cy2R should affect membrane integrity, and at a certain concentration, should become cytotoxic. The trypan blue assays show that the concentrations of Cy2R and an Fl-peptide used in the delivery assay are not toxic. At higher concentrations of Cy2R, greater cell death occurs. Thus, a balance most likely exists between Cy2R disrupting the membrane enough to aid in peptide delivery and the production of holes. At the concentrations used in this assay, LDH and calcein blue are not significantly released from within the cells. PI and rhodamine do not enter the cells above background levels. Fl-peptides are found within cells for the delivery assays performed at 4 °C. For some Fl-peptides, a greater number of fluorescent cells were found at 4 °C as compared to room temperature. Leaky membranes appear not to be the reason for peptide entry. The model studies demonstrate that the transport level of the various Fl-peptided·Cy2R complexes into $CHCl_3$ correlates with the observed fluorescence intensity of the cells. The U-tube experiments show that an Fl-peptide can be transported from an aqueous source phase, through a $CHCl_3$ phase, and can be released into an aqueous receiving phase. The results of these experiments show that a cyclophane-[2]rotaxane is required for the intracellular delivery of a series of Fl-peptides, and it likely associates with the Fl-peptide during the delivery process. We are currently designing experiments to further investigate the transport process, which includes using solid-supported membranes and delivering other materials into cells.

Experimental Section

Intracellular Transport Assays. Assays were performed as described.[25] Briefly, cells were grown in six-well culture plates in DME with 10% fetal calf serum until they had reached ca. 70% confluency (approximately 1×10^5 cells per well). After 24 h, the growth media

was removed from the wells, and the cells were washed twice with phosphate-buffered saline (PBS, 11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl, pH 7.4). For the assays performed at room temperature, PBS buffer (1 mL) was added to each well. Cy2R and Fl-peptides were added from DMSO stock solutions to keep the amount of DMSO less than 0.2% (v/v) of the total volume. A baseline level of fluorescence was obtained from wells containing Fl-peptide, and other wells contained no reagents except DMSO (referred to as untreated cells). After rocking the wells at room temperature (RT) for 1 h in the dark, aliquots of the assay solutions were removed and tested for LDH release, according to the protocol provided by Promega. Three aliquots were removed from each well and were tested for LDH. Cells in one well were exposed to 0.1% Triton-X 100 for 10 min to give a reference fluorescence level for lysed cells. An LDH value of 18% was derived for COS 7 cells exposed to Cy2R for 3 h. There is only a small rise in the fluorescent signal (16−18%) over 1.5 h, which shows that the membranes remain stable to the release of the enzyme. There is also a rise in the LDH level for untreated cells (11−13%) during this time period.

The cells were thoroughly washed. Calcein blue AM solution (1 μM in PBS) and 2 μL of a 50 μg/mL propidium iodide solution were added to each well. After 10 min, cells were observed by inverted fluorescence microscopy (100× magnification). The cells were then harvested with trypsin versene (0.4 mL of a solution containing 0.25% trypsin, 0.01% EDTA incubated at 37 °C for 10 min) for flow cytometry. The cells were pelleted via centrifugation (5 min at 600*g*). After removing the PBS solution, the cells were resuspended in FACS buffer (1% fetal bovine serum in PBS), and the proportion of fluorescent cells was measured using two-color flow cytometry. The same thresholds were used as in the previous studies.[25] To determine the level of PI, all events were analyzed. These assays were performed twice to give a standard deviation less than 5% for the values given in Table 2.

We investigated whether the order of addition of Cy2R and an Fl-peptide affects the transport efficiency. Cells were exposed to Cy2R 30 min prior to the addition of Fl-AQSAV to the cells, to Cy2R and Fl-AQSAV at the same time, and to a premixed solution of Cy2R and Fl-AQSAV. Flow cytometric analysis showed that the order of addition does not matter in terms of the Fl-peptide within the cells (61, 65, and 67%, respectively), cell viability (cbAM: 98, 95, and 97%, respectively), and membrane integrity (PI: 1, 1, and 2%, respectively). The LDH levels were also consistent (17, 16, and 18%, respectively).

For cells assayed at 4 °C, the plates were stored at 4 °C for 15 min before the addition of Cy2R, and the above procedure was followed with the plates maintained at 4 °C except for the final wash step, which was performed at room temperature. For the depletion of cellular ATP, the cells were preincubated with 2-deoxy-D-glucose (6 mM) and sodium azide (10 mM) in PBS for 1 h, as described.[23] Cy2R and an Fl-peptide were added to the solution containing 2-deoxy-d-glucose and sodium azide, and the above assay procedure was followed.

Determination of fluoresceinated peptide localization in cells was achieved by high magnification microscopy. COS 7 cells were grown on 11 × 11 mm glass coverslips (Corning) in a six-well culture plate with one slide per well. Cells were exposed to Cy2R and an Fl-peptide in the culture plate, as described above. The coverslips were then mounted onto microscope slides with Permafluor aqueous mounting medium. The unfixed cells were examined at 400× magnification by fluorescence microscopy.

Determination of Association Constants. Fluorescence quenching assays were performed to obtain the association constants. DMSO was freshly distilled and stored over molecular sieves (3 Å) prior to making the stock solutions and performing the assays. The water solution was buffered with PBS (10 mM) at pH 7.4. The assay involved placing 2.7 mL of one of these solutions into a 3.5 mL cuvette. Guests were added from a DMSO stock solution to give a final concentration of 3.0×10^{-6} M in buffered water or 1×10^{-6} M in DMSO. Multiple aliquots of a Cy2R stock solution in DMSO were added to the cuvette to give Cy2R concentrations starting at approximately 0.3-fold lower than that of an Fl-peptide's concentration and ending at the point where significant quenching of the fluorescent signal occurs. The total change in volume caused by addition of an Fl-peptide and Cy2R was less than 2%. The fluorescence spectra were recorded and analyzed after each addition of a Cy2R. Plots of the changes observed in the quenching assays were fitted using a nonlinear least-squares procedure to derive K_A and ΔF_{max} values.[48] The assays in water were duplicated, giving a standard deviation of less than 10% of the value obtained for the association constant.

Examination of the binding plots in DMSO revealed a second equilibrium constant. Benesi-Hildebrand analysis was performed to obtain both equilibrium constants. The initial concentration of an Fl-peptide was set at 1×10^{-7} M, and Cy2R was added in 1 μL aliquots to give concentrations of 1×10^{-6} M to 1×10^{-5} M. Since $K_{A(1)}$ and $K_{A(2)}$ are close in value for the Cy2R·Fl-AQSAV complex and for the Cy2R·Fl-CAM complex, Job plots were performed, and the results verify the existence of the 2:1 Cy2R to Fl-peptide complexes (Supporting Information).

U-Tube and Extraction Experiments. HPLC and UV−vis analyses were used to determine the amount of materials in the various solutions of the assays. Calibration curves were created for each compound and for some complexes to obtain molar extinction coefficients for the UV−vis experiments and to convert the peak areas to moles for the HPLC experiments. The extraction solutions were vigorously stirred, and the $CHCl_3$ phases of the U-tube cells were lightly agitated to ensure that the two aqueous solutions did not come into contact. Cy2R and an Fl-peptide were added to the appropriate solvent from DMSO stock solutions. The total percentage of DMSO was less than 0.1%, which is a similar value as used in the cellular delivery assays. For comparison experiments, a single $CHCl_3$ or aqueous solution was made and divided into aliquots to perform the assays. The extraction assays contained 1.5 mL of $CHCl_3$ or octanol and 1.5 mL of an aqueous solution. The U-tube cells contained 0.5 mL for the source, receiving, and $CHCl_3$ phases. For assays performed at pH 7.4, the PBS solution was used. The acidic and basic solutions were set at the appropriate pH value using a potentiometer. At the end of an assay, the pH of the aqueous solution was checked. After the solutions were removed for analysis via HPLC, the existence of any precipitated materials was measured by washing the vials or U-tubes with DMSO and then by analyzing the contents of the wash solution with HPLC analysis. A small percentage (<2%) of Fl-AQEAV precipitated in the U-tube assays.

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Supporting Information Available: Fluorescence microscopy pictures of ES2 cells exposed to Cy2R and Fl-peptides, Cy2R only, Triton X-100, and rhodamine alone or with Cy2R, binding titrations and Job plots for assays in DMSO. This material is available free of charge via the Internet at http://pubs.acs.org.

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