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Determining the Binding and Intracellular Transporting Abilities of a Host-[3]Rotaxane

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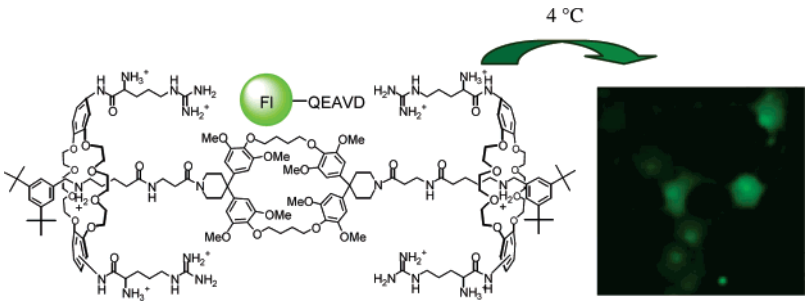

The cellular permeability of compounds can be enhanced in the presence of a host-[2]rotaxane (HR). The effective concentration of an HR is limited by the stoichiometry of the complex formation of the HR and the delivered compound. We speculate that a complex forms between the HR and a guest during membrane passage. To further explore the relationship between guest binding and guest delivery and to obtain more efficient delivery devices, we present, in this report, the first example of a cyclophane-[3]-rotaxane (Cy3R), which has two wheels and a cyclophane as a blocking group. The properties of Cy3R were compared to a new cyclophane-[2]rotaxane (Cy2R) that has the same cyclophane pocket as Cy3R but only a single wheel. The second wheel of Cy3R can form additional noncovalent bonds, e.g., salt bridges, cation−π interactions or aromatic−aromatic interactions, with appropriately functionalized guests. We show by flow cytometric analysis that Cy3R transfers Fl-AVWAL (76%) and to a lesser degree Fl-QEAVD (26%) into live cells. The level of Fl-peptide within a cell is concentration dependent and largely temperature and ATP independent, suggesting that a Cy3R·Fl-peptide complex passes through the cellular membrane without requiring active cell-mediated processes. Cy2R, on the other hand, forms weaker complexes and requires a higher concentration to transfer materials into cells. These results demonstrate that the addition of a second wheel on a rotaxane can improve guest binding in various solvents and hence delivery through cellular membranes.

Introduction

Proteins rely on highly organized recognition domains to perform the various functions necessary for cell survival. Modifying proteins to perform other functions or to operate in non-native environments, however, can be problematic because function requires the protein to be correctly folded. Denaturation can result from alterations in solvent, pH, or temperature. To overcome these shortcomings, mimetic proteins are being extensively pursued. A conceptually simple solution would be to replace the unstable parts of a protein with a synthetic scaffold. These mimetics would be cheap to produce, stable to changes in the environment, and their function could be readily modified by attaching different amino acids. Amino acids, their side chains, or short peptides have been attached to cyclodextrins,[1−5] cyclophanes,[6−8] sugars,[9,10] or steroids and bile acids.[11−17]

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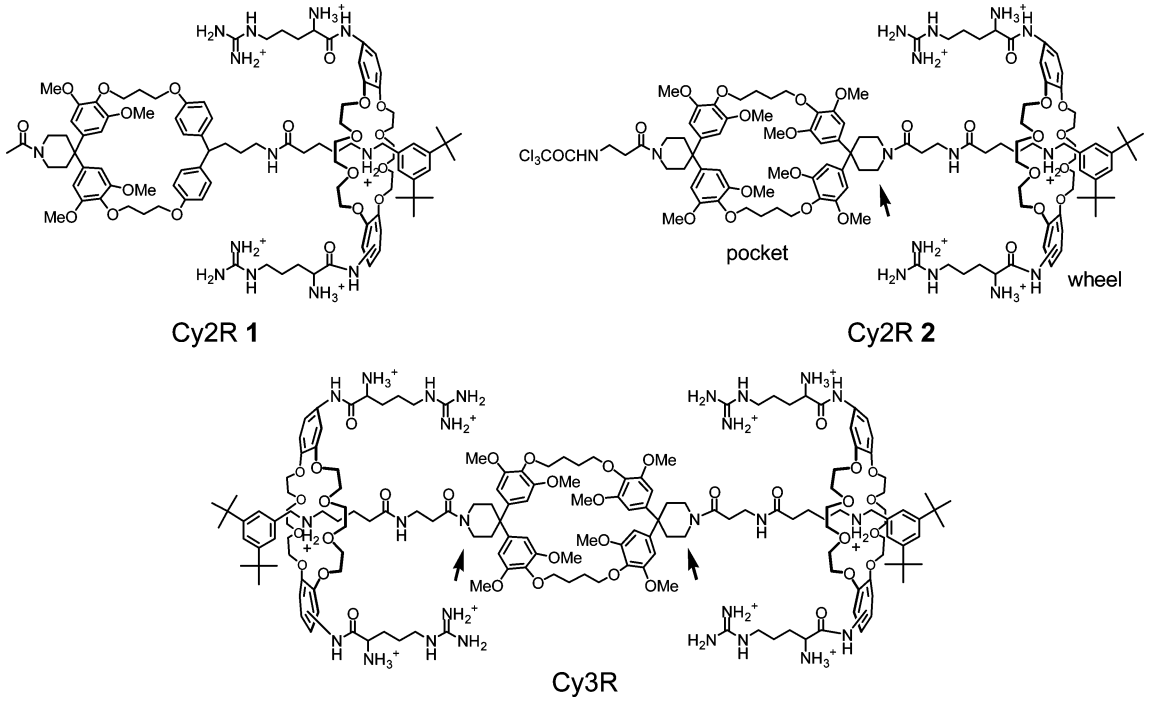

FIGURE 1. The host-rotaxanes used in the studies. Cy2R **2** and Cy3R differ from Cy2R **1** by having a larger pocket and an additional piperidinyl ring (indicated by an arrow) that give the pockets greater rigidity, but moves the wheel further away from the pocket.

Synthetic scaffolds, being formed of covalent bonds, are incapable of aligning the recognition elements in the same spatial arrangement as proteins, which use a myriad of noncovalent bonds to obtain their unique structures. Newer protein mimetics, such as the template assembled synthetic proteins (TASPs),[18−25] are more protein-like, relying on oligo- or polypeptides attached to scaffolds to form secondary structures. As mimetics become more similar to proteins, however, they will develop the same problems as proteins, such as incorrect folding, instability to changes in the environment, and high production costs. Thus, one option available for mimetic construction is the use of covalent bonds, which are stable, but give a nonoptimal orientation of functional groups. Another possibility is the creation of assemblages through noncovalent bonds, which give a greater variation of functional group orientation, but are less stable. A third choice is now available with the successful demonstration of protein mimetics that are formed from interlocked molecules.

Rotaxanes comprise a class of interlocked molecules containing a wheel threaded onto an axle with blocking groups on the ends to keep the wheel from unthreading.[26−33] We converted rotaxanes into protein mimetics by swapping a blocking group with an aromatic rich pocket (cleft,[34] cyclophane,[34] or calix[4]-arene[35]). The binding domain of these host-rotaxanes (HRs) is divided between the pocket and the wheel (Figure 1), which is a dibenzyl-24-crown-8 ring derivatized with arginines. Tight complexes are formed with guests of various sizes and polarities in DMSO, water, $CHCl_3$, and mixed solvent systems. The HRs form different conformations in different solvents.[36] In an aqueous environment, the aromatic moieties of the wheel and the pocket tend to assemble. This "closed" conformation produces an apolar binding domain that forms strong interactions with apolar moieties of a guest. In an apolar environment, the dominant "open" conformation has the wheel residing over the ammonium ion of the axle.

In the presence of HRs, some impermeable materials can be brought into cells.[34,36,37] We have shown that efficient transfer of material depends on the rotaxane forming the different

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conformations in response to changes in the environment that occur during the transfer process.[36] The concentrations of the components have to be at least in the low micromolar range to obtain an observable amount of intracellular material. This minimal concentration is consistent with magnitude of the K_A's for the complexes in buffered water (pH 7.3), DMSO, and $CHCl_3$, which range from 2×10^4 to 9×10^5 M^{-1}. These solutions were used to represent the polarities of the aqueous domain, cellular surface, and lipid portion of cells, respectively.[38] We also observed a slight concentration dependence for the amount of material that entered the cells.[36] These results suggest that the HRs are acting as transporters, whereby an HR forms a noncovalent complex with an impermeable material and carries it across the membrane. Such a transport process would depend on the ability of an HR to form a complex in the environments that impede delivery.

Complex formation depends on the intrinsic attraction between an HR and a guest and the concentration of the components. To more fully explore the necessity of complex formation for delivery, a host-[3]rotaxane (Cy3R, Figure 1) and Cy2R **2** were created. The HRs contain a larger, more rigid cyclophane pocket than for Cy2R **1**[34] to potentially produce more stable complexes. Cy3R contains a second wheel. We envisioned that the additional aromatic rings of the second wheel would stabilize guests in an aqueous environment, and that the additional arginine moieties would more efficiently cover polar or charged groups of a guest as it passes through the membrane. The arginines could also enhance the effective molarity of the host-rotaxane at the cell surface through their attraction to the phosphates on the cell surfaces. These HRs should form complexes with substantially different stabilities, and these differences should be reflected in the amount of material brought into cells if transport occurs.

Herein, we compare the abilities of Cy3R and Cy2R **2** to bind highly charged and apolar guests in various solvents (buffered water, DMSO, and MeCN) and determine whether an observed binding selectivity correlates to more material being brought into COS 7 cells. Fl-AVWAL and Fl-QEAVD were initially chosen to test transfer. Fl-AVWAL has apolar side chains. The Cy3R·Fl-AVWAL complex should be more stable than the Cy2R **2**·Fl-AVWAL complex in water because of the additional aromatic surfaces of Cy3R. Fl-QEAVD contains negatively charged side chains. The additional arginine moieties of Cy3R should make additional salt bridges with the negatively charged side chains. Thus, the Cy3R·Fl-QEAVD complex should be more stable than the Cy2R **2**·Fl-QEAVD complex in apolar solvents or environments. Buffered water, DMSO, and MeCN were chosen to represent the environments outside the cells, at the cell surface, and within the lipid membrane, respectively. MeCN was chosen instead of $CHCl_3$, which was used in earlier studies, to increase the solubility of some of the highly charged guests. The interaction strength for charged or polar groups diminishes from water to DMSO to MeCN. Thus, we predicted that apolar guests would be bound the strongest in buffered water and charged guests would be bound the strongest in MeCN. A comparison is also made between the new HRs and Cy2R **1**.

We discovered that Cy3R can form substantially more favorable complexes than Cy2R **2**. Cy3R efficiently transports both Fl-peptides into cells, whereas Cy2R **2**, surprisingly, does not. We initially thought that the properties of Cy2R **2** and Cy2R **1** would be similar because Cy2R **2** is a modified version of Cy2R **1**. Cy2R **2**'s pocket is slightly larger and contains an additional piperidinyl ring (Figure 1). Also contrary to our expectations, we found that more stable complexes exist for some of the charged and polar guests in DMSO than in MeCN. The results of these experiments suggest that the open and closed conformations are important features of Cy3R and Cy2R **2**. Having two wheels, Cy3R can exist in an open conformation for one wheel and a closed conformation for the other (an open−closed conformation). These dominant conformations are most likely responsible for the unexpected binding energy and, consequently, responsible for the different transporting abilities of Cy3R and Cy2R **2**. We found that for HRs that form a very stable complex with a guest in DMSO, which has a similar polarity as the cell surface, significantly enhanced amounts of guest peptide enters into cells in the presence of the HR.

Results

Design of the Host-Rotaxanes and Experimental Methods. Of proteins, host-rotaxanes most closely mimic antibodies. Antibodies can contain small binding domains and use a few to all of six hypervariable peptide loops (complementary determining regions) to bind a ligand.[39−41] We envisioned the pocket of the host-rotaxane being a mimic of the shallow groove and a functionalized dibenzyl-24-crown-8 ring mimicking a hypervariable loop. Fluorescein was chosen as the initial guest since its association with a host can be monitored by fluorescence quenching assays, and cellular delivery can be monitored

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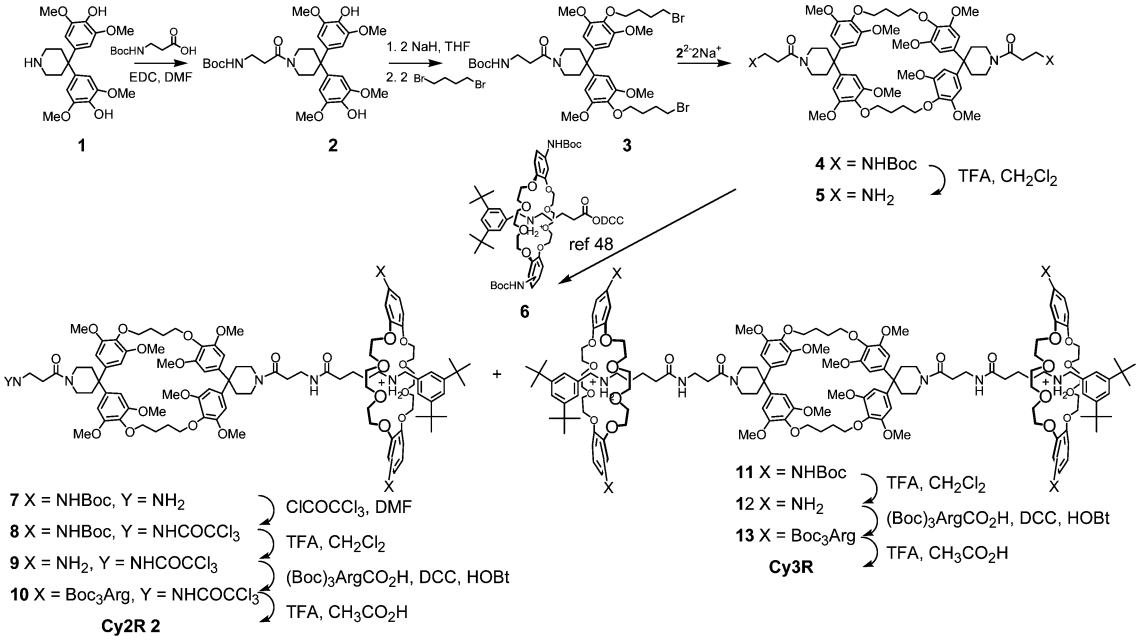
SCHEME 1



via fluorescence microscopy. Therefore, the host-rotaxanes contain recognition groups that are similar to the side chains found in antibodies that bind fluorescein. For example, fluorescein is bound by antibody 4-4-20 in an aromatic slot and makes contact with three tyrosines and two tryptophan side chains.[42] Arginine residues contact the negatively charged enolate oxygen atom and the carboxylate of fluorescein or fluorescein derivatives.[42,43]

Considering that one face of Cy2R **1** interacts with a guest, there are five aromatic rings (four in the cyclophane and one in the crown ether) and one arginine residue available for guest recognition. The methoxy derivatized rings of Cy2R **1** give it a deep, polarizable aromatic pocket.[44,45] Benzylic rings were used on the axle side of the pocket to enable the recognition groups of the wheel and the pocket to form a tight pocket to bind small guests. The pocket of Cy2R **1** is not rigid, however, and thus it is not optimized for guest association. The benzylic rings could collapse, blocking a guest from the pocket. Cy3R and Cy2R **2** contain octamethoxy cyclophanes with piperidinyl rings on both ends to obtain more open, rigid pockets (Figure 1). The size of the pockets was also increased by two $-CH_2-$ units. Diederich showed that these cyclophanes are ideal for binding aromatic guests.[45−47] A series of guests were chosen to determine the extent to which the additional aromatic surfaces and arginine moieties of the Cy3R can contribute to a host−guest complex. Because most host−guest complexes form in the low micromolar range, fluorescent guests were used to enable association constants (K_A's) to be obtained through fluorescence quenching assays. Fl-peptides and fluorescein were also chosen to test the ability of the host-rotaxanes to deliver materials into cells.

Synthesis of the Host-Rotaxanes. The construction of the cyclophane pocket was based on the known cyclophane chemistry[45] (Scheme 1). The DCC-rotaxane method[48] was used to construct the rotaxane. β-Alanine was attached to the piperidinyl ring to provide a primary amine, which was coupled to DCC-rotaxane **6** after cyclophane **5** was formed. A mixture of [2]rotaxane **7** and [3]rotaxane **11** (30 and 55% yields, respectively) was obtained by adding 2 equiv of DCC-rotaxane **6** to cyclophane **5**. The primary amine of the [2]rotaxane was protected with trichloroacetate. This protecting group is stable to the reagents needed to attach and subsequently deprotect the Boc_3-arginines. It also can be selectively removed to enable the conversion of Cy2R **2** to additional host-[3]rotaxanes using the DCC-rotaxane method.

The Complexation of Highly Charged Guests. Ac-Trp-CO_2H, Ac-Glu-Trp-Glu-$CONH_2$, Ac-Glu-Trp-Arg-$CONH_2$, and Ac-Arg-Trp-Arg-$CONH_2$ were used as guests to determine the advantage the additional arginine moieties of Cy3R have on guest recognition. Since the hydrogen bonding ability of the solvents used in the assays increases from MeCN to DMSO to H_2O, the strength of a salt bridge between the glutamic acid residue of these guests and a guanidinium moiety of a wheel should be the strongest in MeCN and the weakest in H_2O. Theoretical calculations have shown that the strength of cation$-\pi$ interactions is solvent independent.[49] Thus, similarly stabilizing cation$-\pi$ interactions could exist between the arginine side chains and the aromatic rings of the HRs and the guests in the solvents used in this study. The indole side chain of the guests will form favorable aromatic−aromatic interactions with the HRs. In water, desolvation of the indole ring of the guests and the apolar surfaces of an HR will drive association in accordance with the hydrophobic effect.[50,51]

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TABLE 1. Association Constants (K_A, M^{-1})[a] for HR−guest Complexes Obtained through Fluorescence Quenching Assays

guest	solvent	$K_{A,Cy3R}$[b]	$K_{A,Cy2R\ 2}$[c]	$K_{A,Cy3R}/K_{A,Cy2R\ 2}$
Ac-Trp-CO_2H	water[d]	19	18	1.0
	DMSO	17	7	2.3
	MeCN[e]	55	32	1.7
Ac-Glu-Trp-Glu-$CONH_2$	water	18	16	1.1
	DMSO	21	16	1.3
	MeCN	490	33	15
Ac-Glu-Trp-Arg-$CONH_2$	water	17	15	1.1
	DMSO	18	11	1.6
	MeCN	42	25	1.7
Ac-Arg-Trp-Arg-$CONH_2$	water	3	ND	> 3
	DMSO	11	ND	>11
	MeCN	1	ND	>1
Ac-Trp-CO_2Me	water	26	17	1.5
	DMSO	44	17	2.6
	MeCN	21	13	1.6
Ac-Trp-Phe-OMe	water	21	16	1.4
	DMSO	18	18	1.0
	MeCN	22	5	4.3
Ac-Trp-Phe-Phe-OMe	water	49	22	2.2
	DMSO	14	4	3.4
	MeCN	13	7	1.9
Fl-Ala-Val-Trp-Ala-Leu-$CONH_2$	water	51	16	3.2
	DMSO	410 (100)[f]	150 (38)	2.5
	MeCN	97	24	4.0
Fl-Gln-Glu-Ala-Val-Asp-$CONH_2$	water	26	13	2.0
	DMSO	400 (20)	92 (35)	4.3
	MeCN	>300 (70)	35	>9
H_2N-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-CO_2H (hormone **1**)	water	26	15	1.4
	DMSO	43	10	4.2
	MeCN	880	483	1.8
CO_2H-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (hormone **2**)	water	14	17	0.8
	DMSO	23	10	2.3
	MeCN	INS	INS	−
fluorescein	water	42	29	1.5
	DMSO	40	37	1.0
	MeCN	41	27	1.5
pyrene	water	283	270	1.0
	DMSO	24	12	2.0
	MeCN	92	39	2.4

[a] The assays were performed at room temperature, the standard deviation is less than 10% for each K_A, and the tabulated K_A values have been divided by 1×10^3. [b] K_A for Cy3R complexes. [c] K_A for Cy2R **2** complexes. [d] 98% water (phosphates 1 mM, pH 7.0)/2% DMSO. [e] 98% MeCN/2% DMSO. [f] Parenthetical values are the K_A's for two HRs bound to a guest.

In a buffered solution (1 mM phosphate, pH 7.0), similar K_A's are observed for these guests bound to Cy3R and Cy2R **2** except for Ac-Arg-Trp-Arg-$CONH_2$, which has a significantly lower K_A (Table 1). The more stable complexes for Ac-Trp-CO_2H, Ac-Glu-Trp-Glu-$CONH_2$, and Ac-Glu-Trp-Arg-$CONH_2$ could be a result of a salt bridge that forms between a guanidinium moiety of an HR and a guest's carboxylate, which does not exist for Ac-Arg-Trp-Arg-$CONH_2$. Another possibility is that greater energy is required to desolvate the arginine side chains of Ac-Arg-Trp-Arg-$CONH_2$ as compared to the carboxylates of the other guests that occurs during the binding event. Host−guest complexation is similar to the transport of amino acids from an aqueous phase into octanol. Water/octanol distribution parameters of amino acids are available and can be combined to calculate the distribution parameters of the tripeptides.[52] Calculated log *D* values show that the tripeptides are highly hydrophilic and that the desolvation penalty for removing water molecules is actually slighter larger for Ac-Glu-Trp-Glu-$CONH_2$ than for Ac-Arg-Trp-Arg-$CONH_2$. Apparently, salt-bridge formation with the HR is strong enough to overcome this penalty. Because the K_A's for the HRs bound to the carboxylic guests are approximately equivalent, the second wheel of Cy3R does not contribute to the binding free energy of the complexes. On the other hand, there is at least a 3-fold larger K_A for the association of Ac-Arg-Trp-Arp-$CONH_2$ with Cy3R as compared to Cy2R **2**. The second wheel of Cy3R stabilizes the complex, most likely through an additional cation−π interaction between a guanidinium moiety of the guest and an aromatic ring of the wheel (see Supporting Information for molecular modeling picture of this complex).

To determine whether salt bridges would form between the guests and both wheels of Cy3R in apolar environments, such as cell surfaces and lipid layers, complexation was investigated in DMSO and MeCN. The properties of DMSO and MeCN are similar except that DMSO is a moderately strong H-bond acceptor and MeCN is a poor H-bond acceptor.[53,54] DMSO is also known to interact strongly with proteins.[55] The guest's carboxylates were presented as tetramethyl ammonium salts to enhance their solubilities. Most K_A's derived for complexes in DMSO are similar to the ones obtained in the aqueous solution. Cy3R is more selective for Ac-Arg-Trp-Arg-$CONH_2$, as compared to Cy2R **2**, in DMSO than in water (11-fold vs 3-fold, respectively). This selectivity could arise through the formation of more stable cation−π interactions in DMSO than in water or because Cy3R is able to form a better binding conformation (see Discussion). As expected, the largest K_A's and greatest binding selectivity are observed for the association of the carboxylic guests in MeCN, which interacts weakly with charged groups. More importantly, a 15-fold larger K_A is seen for the Cy3R·Ac-Glu-Trp-Glu-$CONH_2$ complex as compared to Cy2R **2**·Ac-Glu-Trp-Glu-$CONH_2$ complex (Figure 2A; see Supporting Information for a molecular modeling picture). This result is consistent with the second wheel of Cy3R forming an additional salt bridge with a Glu side chain of Ac-Glu-Trp-Glu-$CONH_2$.

The Complexation of Aromatic Guests. A series of compounds with extensive aromatic surfaces were used as guests to determine the extent to which the second wheel of Cy3R contributes to binding of aromatic compounds. For the series Ac-Trp-OMe, Ac-Trp-Phe-OMe, Ac-Trp-Phe-Phe-OMe in the buffered solution, only the Cy3R·Ac-Trp-Phe-Phe-OMe complex shows a significantly larger K_A in water (Table 1). This result is consistent with the hydrophobic effect.[50,51] Removing water molecules from aromatic surfaces and back to the bulk provides a large favorable entropic term for the association of aromatic rings. The fact that the tripeptide Ac-Trp-Phe-Phe-OMe is necessary to produce an uniquely favorable complex indicates that the hydrophobic pocket of Cy3R is large. A large guest maximizes the contact surface area with the hydrophobic groups of Cy3R, resulting in a greater number of released water molecules. The observed drop in the K_A's for the HRs bound to Ac-Trp-Phe-Phe-OMe in DMSO and MeCN demonstrates the importance of the hydrophobic effect for the large K_A's observed in buffered water. In the three solvent systems, both wheels of Cy3R contribute to the binding event.

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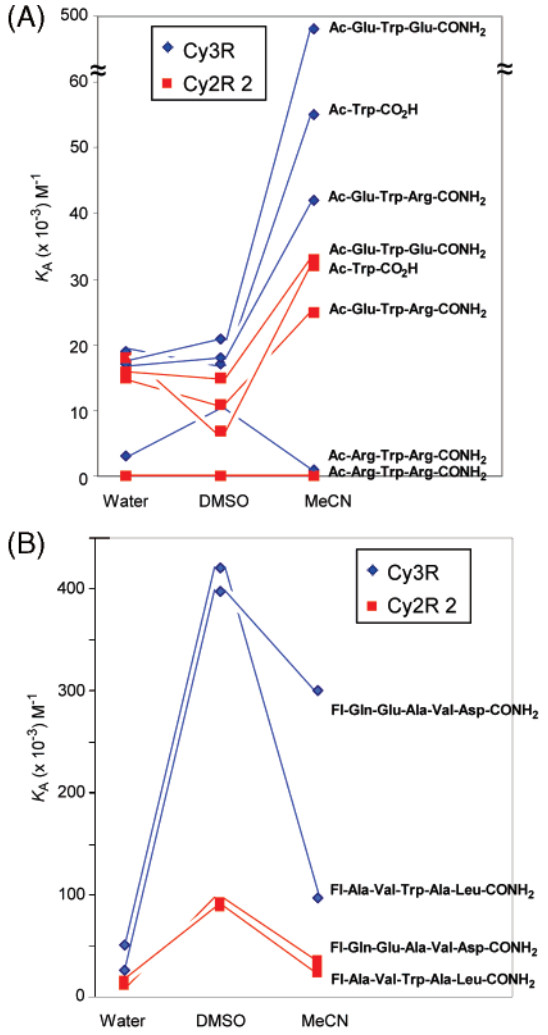

FIGURE 2. A comparison of the association constants (K_A's) shows that Cy3R forms more stable complexes than Cy2R. Cy3R is selective for (A) Ac-Glu-Trp-Glu-$CONH_2$ in MeCN and (B) the Fl-pentapeptides in DMSO. A large K_A is also observed for Fl-Gln-Glu-Ala-Val-Asp-$CONH_2$ bound to Cy3R in MeCN.

Two interesting trends are observed for the binding of the aromatic peptides in DMSO and MeCN. First, equivalently stable or more stable complexes are formed in DMSO than MeCN. Because greater energy is required to remove DMSO from the guanidinium moieties than MeCN, the arginines of the HRs appear not be involved in the complexes. This would leave aromatic−aromatic interactions as the driving force for association. DMSO and MeCN, however, should produce similar desolvation energies with aromatic surfaces since they have similar ET(30) values.[56] The ET(30) parameters are derived from the absorbance wavelength of the Reichard's dye in various solvents and have been shown to linearly correlate with the free energy of complexes formed between pyrene and an aromatic rich cyclophane in various solvents.[57] Furthermore, the second interesting trend is that as the number of aromatic rings within the guests increases the corresponding K_A's decrease. An opposite trend would be expected if complexation is driven by aromatic−aromatic interactions. The unexpected K_A's can be explained by a better binding geometry existing for the HRs in DMSO than in MeCN (see Discussion).

To further explore aromatic−aromatic interaction energies in HR complexes, pyrene was chosen as a guest. It has an extensive aromatic surface without the ability to form salt bridges and H-bonds, which are possible with the peptidic guests. K_A's for the HRs bound to pyrene are large in the aqueous solution (2.7×10^5 M^{-1}). The octamethoxy cyclophane performed as expected by providing tighter complexes of aromatic compounds in water than Cy2R **1**[34] (K_A for the Cy2R **1**·pyrene complex is 8×10^4 M^{-1}). Because the K_A's of the HRs bound to pyrene are approximately equal, a binding advantage is not provided by the second wheel of Cy3R. Desolvation of water molecules provides for the driving force for the association of pyrene since the complexes in DMSO and MeCN are substantially weaker. The second ring of Cy3R appears to be involved in the complex of pyrene in DMSO and MeCN, giving the observed 2-fold larger K_A than observed for Cy2R **2** binding pyrene.

The Complexation of Fluoresceinated Peptides and Large Peptides. The fluoresceinated peptides Fl-AVWAL and Fl-QEAVD were used as guests to determine the abilities of the HRs to bind larger peptides with aliphatic and aromatic side chains and with negatively charged side chains, respectively. Combined with fluorescein, these peptides were also used to determine the abilities of the HRs to transfer materials into cells. Fluorescein has an extensive aromatic surface like pyrene, but it also has two negatively charged groups in pH 7.0 water. The strong interactions between water molecules and the charged groups significantly weaken the attraction for fluorescein by either HR as compared to pyrene (Table 1). Fl-AVWAL is bound by the HRs in water with K_A values that are similar to those observed for the complexes with Ac-Trp-Phe-Phe-OMe. This is expected since both guests have multiple apolar side chains. Cy3R shows a slight selectivity for Fl-AVWAL over fluorescein. Apparently, the larger Fl-AVWAL and Ac-Trp-Phe-Phe-OMe make more favorable contact with the second wheel of Cy3R than smaller guests. Fl-QEAVD is more weakly bound than Fl-AVWAL by either HR in buffered water. Because of its negatively charged side chains, Fl-QEAVD has a large desolvation penalty for host−guest association.

Even though fluorescein forms essentially the same stable complex in the three solvent systems, the complex strength of the Fl-peptides shows a dramatic solvent dependency (Figure 2B). Cy2R **2** shows a 4-fold greater selectivity for the binding of the Fl-peptides in DMSO over the other solvents. Cy3R has an impressive 10-fold preference for the Fl-peptides in DMSO as compared to the same complex in buffered water. The complex of Cy3R·Fl-QEAVD in MeCN shows a 10-fold larger K_A than the Cy2R **2**·Fl-QEAVD complex. This binding selectivity is also seen for the association of Cy3R with the other highly negatively charged guest Ac-Glu-Trp-Glu-$CONH_2$. A greater preference is observed for the binding of Cy3R with the larger Fl-peptides (2-fold to 10-fold) than fluorescein (1.5 fold), as compared to Cy2R **2**. Thus, the larger Fl-peptides make greater contact with the second wheel of Cy3R than the smaller fluorescein. The same phenomenon was observed in the aqueous solution.

To more fully explore the size requirement for strong complexes, commercially available hormone fragments (hormone **1** and hormone **2**, Table 1) were investigated as guests.

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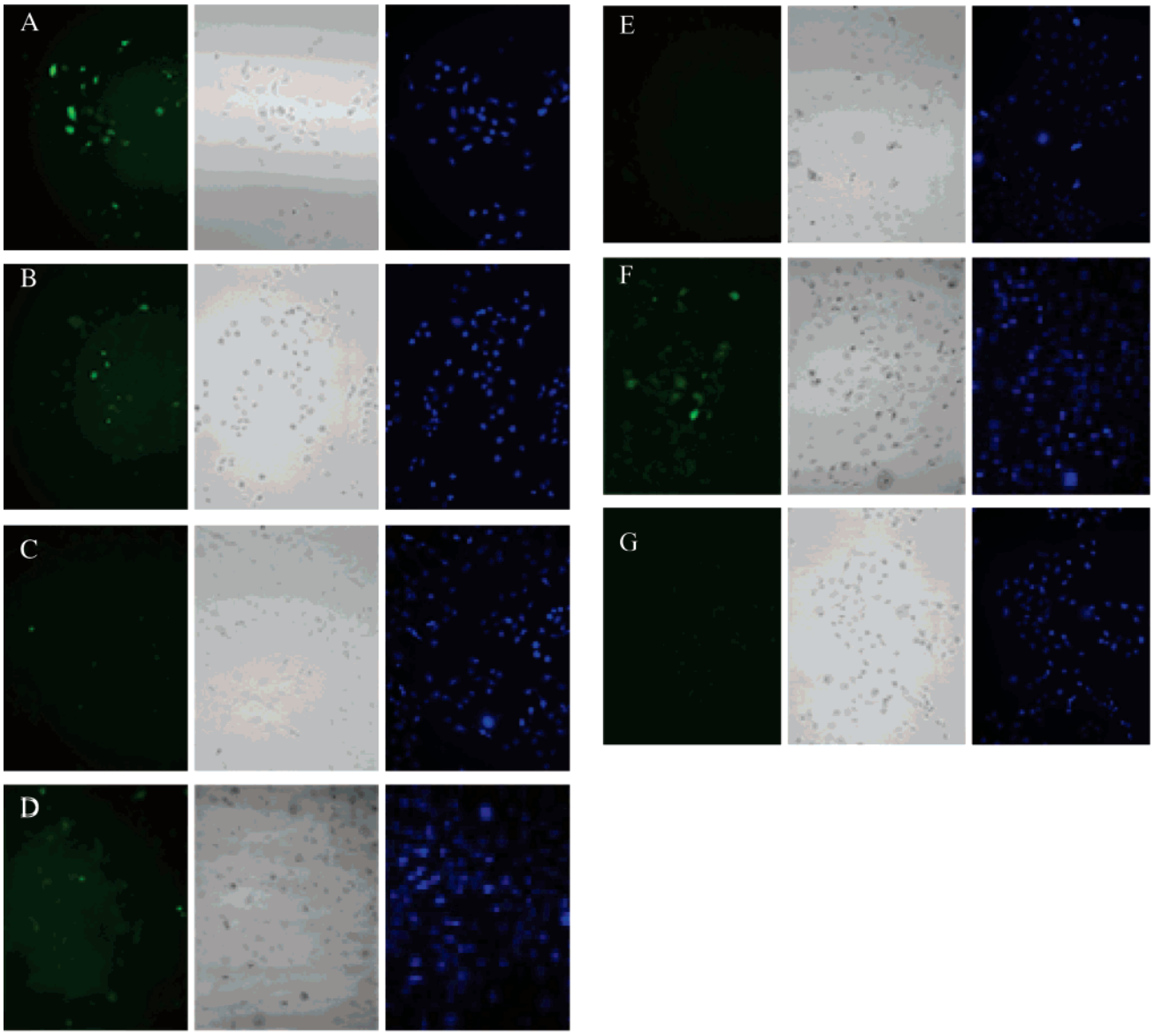

FIGURE 3. Fluorescence microscopy of COS 7 cells incubated with host-rotaxanes. Cells were viewed for Fl-peptide fluorescence (Fl-AVWAL or Fl-QEAVD uptake; left panels), white light (middle panels), and calcein blue fluorescence (viability; right panels). (A) Cy3R (10 μM) and Fl-AVWAL (10 μM), (B) Cy3R (10 μM) and Fl-QEAVD (10 μM), (C) Cy2R **2** (10 μM) and Fl-AVWAL (10 μM), (D) Cy2R **2** (50 μM) and Fl-AVWAL (20 μM), (E) Cy2R **2** (10 μM) and Fl-QEAVD (10 μM), (F) Cy2R **2** (50 μM) and Fl-QEAVD (20 μM), and (G) a typical example of a Fl-peptide (10 μM) only. Cell viability is similar for each combination of an HR and Fl-peptide (original magnification: 100×).

These 10-mer peptides contain the same amino acids as Ac-Arg-Trp-Glu-$CONH_2$, but the amino acids are not sequentially linked together. Hormone **1** and hormone **2** have different sequences, and three of the amino acids are different (Ser, Met, and Phe for hormone **1** and Gly, Pro, and Leu for hormone **2**). Considering these differences, we did not expect that the K_A's for the hormone fragments and for Ac-Arg-Trp-Glu-$CONH_2$ bound to the HRs would be very similar in buffered water and DMSO. Cy3R binds the hormones better than Cy2R **2**, except for hormone **2** in buffered water, and prefers hormone **1**. In MeCN, the strengths of the complexes are dramatically greater. The K_A's of the Cy3R·hormone **1** and Cy3R·Ac-Glu-Trp-Glu-$CONH_2$ complexes are similar in MeCN. This suggests that multiple salt bridges are formed with hormone **1**. Interestingly, a 15-fold larger K_A exists for the Cy2R **2**·hormone **1** complex than the Cy2R **2**·Ac-Glu-Trp-Glu complex. Most likely, the 10-mer peptide is large enough to interact with both guanidinium moieties of the single wheel of Cy2R **2**. The poor solubility of hormone **2** precluded K_A determination in MeCN.

Cellular Transport Efficiencies of the Host-Rotaxanes. We recently showed that HRs aid in the delivery of fluorescein and fluoresceinated peptides into COS 7 cells.[34,36,37] Of the Fl-peptides used previously,[37] Fl-AVWAL and Fl-QEAVD were chosen to test Cy3R and Cy2R **2** as cellular delivery agents. A comparison of the delivery levels of these guests will demonstrate any advantage provided by the aromatic and arginine moieties of the second wheel of Cy3R for transport. Cy3R displayed a high affinity for Fl-AVWAL (buffer $K_A = 5.1 \times 10^4$ M^{-1}, DMSO $K_A = 4.1 \times 10^5$ M^{-1}) and for Fl-QEAVD (buffer $K_A = 2.6 \times 10^4$ M^{-1}, DMSO $K_A = 4.0 \times 10^5$ M^{-1}). The magnitudes of these K_A's are similar to the values obtained for the association of Cy2R **1** with the same Fl-peptides. Cy2R

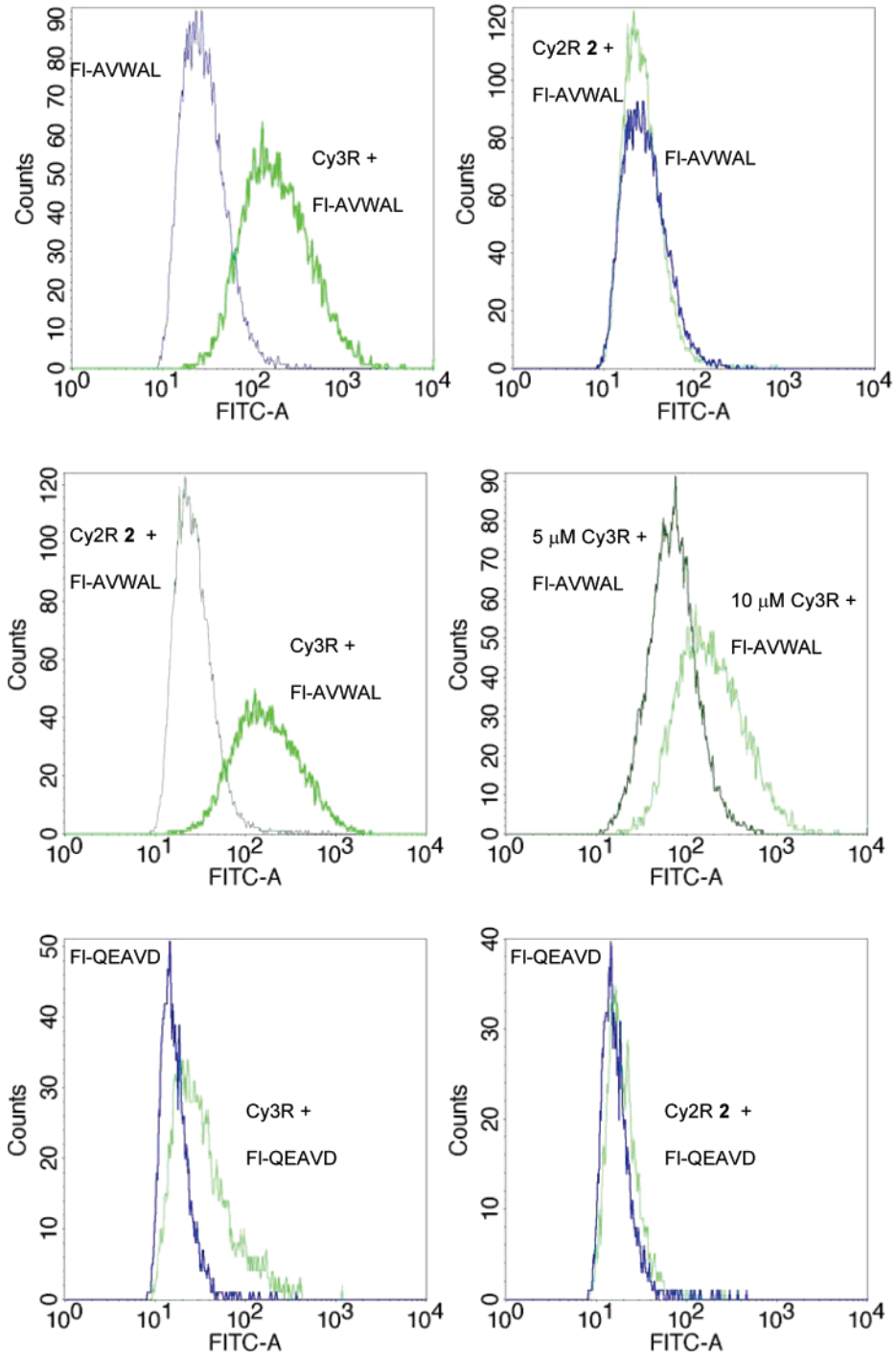

FIGURE 4. Relative fluorescence (FITC) of COS 7 cells exposed to Fl-AVWAL (10 μM) or Fl-QEAVD (10 μM) alone, with Cy3R (10 or 5 μM), or with Cy2R **2** (10 μM) as determined by flow cytometry.

2, on the other hand, forms weaker complexes for these guests, with generally a 2-fold to 4-fold reduction in K_A. If complex strength in these solvent systems provides a measure of the delivery levels, we would expect that Cy3R would deliver the peptides to a similar level as Cy2R **1**. Cy2R **2**, at the same concentration, would show a lower level of transport than the other HRs. Furthermore, raising the concentration of Cy2R **2** to compensate for the weaker complexes formed with the guests should result in intracellular delivery if host−guest complexation is necessary for membrane passage.

We used fluorescence microscopy analysis to determine whether CyR3 and Cy2R **2** delivered the fluoresceinated peptides at a concentration range consistent with the K_A values for the corresponding complexes. The assay procedure has previously been described in detail.[36] Briefly, COS 7 cells were incubated with an HR in a phosphate-buffered saline solution (PBS), pH 7.4, for 30 min before the addition of a Fl-peptide. After an additional hour, the cells were washed thoroughly, and calcein blue AM was added to each well to demonstrate cell viability or potential toxicity after incubation with rotaxanes

TABLE 2. Quantification of Fl-Peptide Uptake in COS 7 Cells by Flow Cytometry[a]

condition	compound(s)[b]	% high Fl-peptide cells	% low Fl-peptide cells	% total Fl-peptide cells	% cbAM[c] cells
rt	Cy3R (10 μM) + Fl-AVWAL	15	60	75	80
	Cy3R (5 μM) + Fl-AVWAL	5	53	58	76
	Cy3R (0.5 μM) + Fl-AVWAL	1	10	11	96
	Cy2R 2 (50 μM) + Fl-AVWAL (20 μM)	3	36	39	97
	Cy2R 2 (10 μM) + Fl-AVWAL	–	8.2	8	84
	Fl-AVWAL	0.3	4.9	5	86
	Cy3R (10 μM) + Fl-QEAVD	1	25	26	91
	Cy3R (5 μM) + Fl-QEAVD	–	16	16	77
	Cy3R (0.5 μM) + Fl-QEAVD	0.2	8.3	8	99
	Cy2R 2 (50 μM) + Fl-QEAVD (20 μM)	1	24	25	98
	Cy2R 2 (10 μM) + Fl-QEAVD	1	5.0	6	86
	Fl-QEAVD	–	4.6	5	89
	none	–	1.0	1	94
4 °C	Cy3R (10 μM) + Fl-AVWAL	5	29	34	76
	Cy3R (5 μM) + Fl-AVWAL	2	17	19	71
	Cy2R 2 (10 μM) + Fl-AVWAL	3	14	17	76
	Cy3R (10 μM) + Fl-QEAVD	5	35	40	84
	Cy3R (5 μM) + Fl-QEAVD	6	30	36	80
	Cy2R 2 (10 μM) + Fl-QEAVD	1	10	11	77
depleted ATP[d]	Cy3R (10 μM) + Fl-AVWAL	2	62	64	81
	Cy3R (5 μM) + Fl-AVWAL	–	24	24	83
	Cy3R (10 μM) + Fl-QEAVD	–	21	21	85
	Cy3R (5 μM) + Fl-QEAVD	1	20	21	68

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

and peptides. Cells were examined via inverted fluorescence microscopy at 100× magnification (Zeiss Axiovert 200M). High to moderate fluorescence was observed in cells that were exposed to Cy3R (10 μM) and Fl-AVWAL (10 μM) (Figure 3A). The majority of cells exposed to Fl-QEAVD (10 μM) and Cy3R (10 μM) were moderately to weakly fluorescent (Figure 3B). Cells exposed to Cy2R **2** (10 μM) and Fl-FAVWAL (10 μM) or Fl-QEAVD (10 μM) (Figure 3C,E) were only weakly fluorescent, similar to the background fluorescence obtained from exposure to the Fl-peptides alone (Figure 3G). Raising the concentration of Cy2R **2** to 20 μM did not increase the observed fluorescence (data not shown). Raising the concentration of Cy2R **2** to 50 μM and Fl-AVWAL or Fl-QEAVD to 20 μM resulted in moderately and weakly fluorescent cells (Figure 3D,F). Strong calcein blue fluorescence was observed in the majority of cells regardless of treatment, which demonstrates cell viability.

Flow cytometry was used to quantify the relative levels of Fl-AVWAL, Fl-QEAVD, and calcein blue AM within the cells. After incubating cells with an assay solution, trypsin digestion was used to lift the cells from the wells and to remove any residual Fl-peptide that may be nonspecifically attached to the outer cellular membrane.[58] The relative fluorescence intensity of these cells was quantified by multicolor flow cytometry (BD FacsAria). Thresholds and gates were set as previously described[36,37] to enable a direct comparison between the host-rotaxanes. Cy3R efficiently delivered Fl-AVWAL into a large percentage of the cells (75% above background; Figure 4 and Table 2). A small percentage of cells (15%) were highly fluorescent. A lower total percentage of fluorescent cells existed when the concentration of Cy3R was lowered to 5 μM (58%) and to 0.5 μM (11%). Lowering the concentrations of Cy2R **2** and Fl-AVWAL from 50 and 20 μM to 10 and 10 μM, respectively, also resulted in a lowering of the number of fluorescent cells (39 to 8%). The same concentration dependency is observed for the transport of Fl-QEAVD. The total fluorescence of cells exposed to Fl-QEAVD and an HR, however, is less than that for cells exposed to Fl-AVWAL and an HR. This reduction is reasonable considering that QEAVD has charged side chains and AVWAL does not. The observed concentration dependency for cellular fluorescence suggests that a noncovalent complex is formed between a Fl-peptide and HRs during the penetration process. Cells exposed to Fl-peptides alone resulted in a small percentage of weakly fluorescent cells, which was set at 5%. A high proportion of calcein blue positive cells was observed in most assays (see also Supporting Information), demonstrating that the HRs and Fl-peptides are only minimally toxic at these concentrations. Calcein blue and peptide fluorescence were independent variables.

Fl-peptide transfer depends on the concentration of an HR and a guest (Table 2 and Figure 5). The minimal concentration necessary to observe fluorescent cells matches the minimal concentration needed to form complexes in the solvents used in this study. These observations suggest that a host−guest complex forms during delivery. Cy3R forms tighter complexes with the Fl-peptides than Cy2R **2** in the solvents used to mimic the cellular environments. To determine which environment (as represented by a solvent) is crucial for complex formation, the ability of Cy3R and Cy2R **1** to deliver fluorescein into cells was measured. Cy2R **1** binds fluorescein strongly in DMSO ($K_A = 8.6 \times 10^5$ M^{-1})[34] and delivers it into cells. On the other hand, Cy3R binds fluorescein less favorably in DMSO by a factor of 20 ($K_A = 4.0 \times 10^4$ M^{-1}). The K_A's for these complexes in water only differ by a factor of 1.5. The same K_A's (within experimental error) are seen for these complexes in MeCN (4.1×10^4 and 4.3×10^4 M^{-1}). Flow cytometric results show that fluorescein at 0.5 μM is delivered into a small

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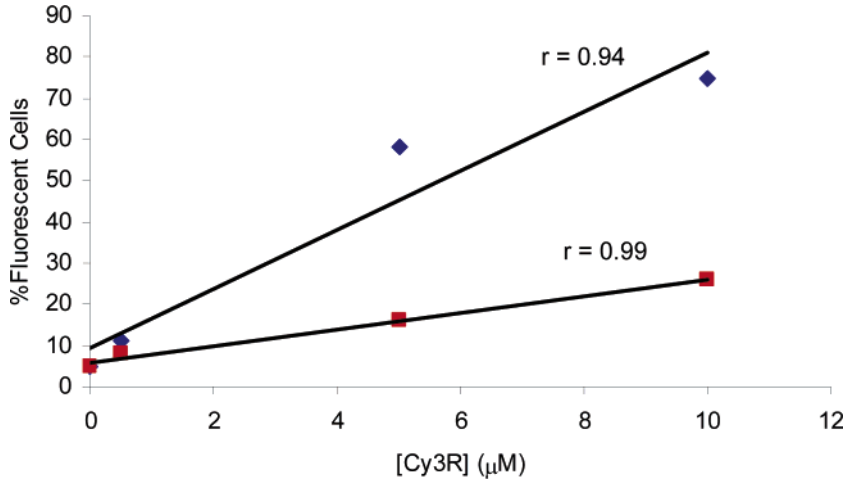

FIGURE 5. Plot of the total fluorescence of cells against the concentration of Cy3R that was used in the assay. The concentration of the Fl-peptides was 10 μM (Fl-FAVWAL diamonds and Fl-QEAVD squares).

percentage of cells (19%) when combined with Cy2R **1**, but not when combined with Cy3R (Table 3). These results are consistent with complexation being required for intracellular delivery and show that strong complexation in DMSO is required for these HRs to be delivery agents.

Determining the Cellular Delivery Mechanism. Because some highly argininated peptides enter cells through endocytosis,[58] we needed to determine whether Cy3R, which contains four arginine moieties, uses this pathway to deliver materials. Energy-dependent pathways were restricted by performing the delivery assays at 4 °C or by using the classic energy-depleting cocktail of 2-deoxyglucose and NaN_3.[58−69] Since some cell death is 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. For the energy-poisoned assay, cells were preincubated with the cocktail for 1 h prior to the addition of transporter/peptides to this solution. Cells with a depleted level of ATP showed a similar level of transport as seen for cells in PBS. For example, a minor reduction from 75 to 64% fluorescent cells was observed for cells exposed to 10 μM Cy3R and Fl-AVWAL after ATP depletion (Table 2). Similarly, 26 and 21% fluorescent cells were found for cells exposed to 10 μM Cy2R and Fl-QEAVD after ATP depletion.

Performing the assay at 4 °C resulted in a significant reduction in the number of fluorescent cells from 75 to 34% for cells exposed to Cy3R and Fl-AVWAL. Examination of the cells, however, showed that precipitation of Fl-AVWAL occurs at some of the cells (Figure 6A). This precipitation was not observed for cells exposed to Cy3R and Fl-QEAVD or for cells exposed to Fl-AVWAL alone. For cyclophanes, lowering the temperature generally increases the stability of their complexes[44] and reduces their water solubility. The precipitated material is most likely caused by the presence of Cy3R since precipitated material is not observed for Fl-AVWAL alone at 4 °C. Cy3R covers some of the hydrophilic moieties of Fl-AVWAL at the cells surface, which could reduce its solubility and reduce the level of delivered material. Other cells at 4 °C, however, fluoresced brightly with well-defined nuclei (Figure 6B). Fl-QEAVD shows an enhancement in the fluorescent intensity of cells at 4 °C (Figure 6C) as compared to rt (40 to 26%, respectively, for 10 μM Cy3r, Table 2). A slightly greater number of fluorescent cells is also observed for cells exposed to Cy2R **2** (10 μM) and Fl-QEAVD (10 μM) or Fl-AVWAL (10 μM) at 4 °C. The greater amount of material present within live cells at 4 °C is expected for a delivery mechanism that relies upon complex formation, which can be promoted with a lowering of the temperature. The similar delivery level observed at rt and at 4 °C suggests that holes are not formed in the membrane. Membrane integrity for cells exposed to the HRs at a concentration to obtain delivery was also verified by the low percentage of cells containing propidium iodide (8 and 12% of cells exposed to Cy3R (10 μM) and Cy2r2 (50 μM), respectively, untreated cells: 5%). Similarly, the level of lactate dehydrogenase released from the cells was not different than that of untreated cells (Supporting Information).

The energy-depleted cells were examined under 400× magnification to further explore the possibility that endocytosis

TABLE 3. Quantification of Fluorescein Uptake in COS 7 Cells by Flow Cytometry

compound(s)[a]	total % Fl-peptide cells	% cbAM cells
Cy2R **1** + fluorescein	19	99
Cy3R + fluorescein	5.2	94
fluorescein	4.6	86

[a] [HR] = 5 μM, [fluorescein] = 0.5 μM.

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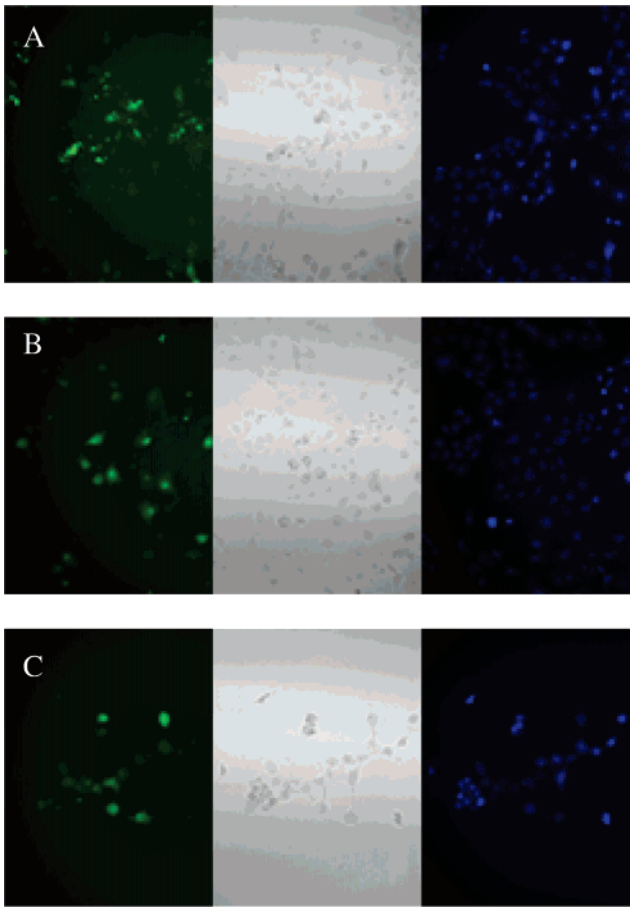

FIGURE 6. Fluorescence microscopy of COS 7 cells incubated with Cy3R and Fl-peptides at 4 °C. Cells were viewed for FITC fluorescence (Fl-AVWAL or Fl-QEAVD uptake; left panels), white light (middle panels), and calcein blue fluorescence (viability; right panels). Precipitated material appears to exist for some cells exposed to Cy3R and Fl-AVWAL (10 μM) (A), whereas other cells are highly fluorescent (B). Fl-QEAVD (10 μM) shows highly fluorescent cells. Cell viability is similar for each combination of a Cy3R and Fl-peptide (original magnification: 100×).

is the entry mechanism for the complexes. COS 7 cells were grown on microscope slides. After exposing cells to the various reagents, the unfixed cells were examined by fluorescence microscopy (Zeiss Axioplan 2). Fl-AVWAL was observed strongly in the nucleus and less in the cytoplasm (Figure 7A−C). Fl-QEAVD was more equally distributed throughout the cellular compartments (Figure 7D−F). The distribution and the fluorescence intensity of the Fl-peptides are not changed for cells lacking energy-dependent pathways. This holds for cells exposed to Cy3R and Fl-AVWAL at 4 °C. These observations are consistent with the flow cytometric results that suggest that endocytosis is not the major pathway for the delivery of materials into cells. Similar results were obtained for other HRs.[36,37]

Discussion

Host-rotaxanes adjust their binding domains with a change in environment to maintain stable complexes with a guest. Two dominant conformations exist (open and closed), depending on the environment. This feature is necessary for the ability of a cleft-rotaxane to transfer materials into cells[36] and most likely

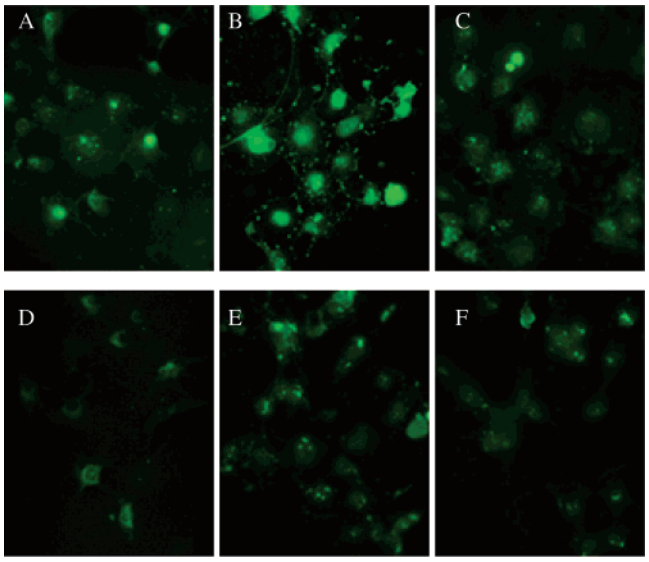

FIGURE 7. Fluorescence photomicrographs showing representative examples of COS 7 cells exposed to Cy3R (10 μM) and Fl-AVWAL (10 μM) (A−C) and Fl-QEAVD (10 μM) (D−F) at rt (A and D), depleted ATP (B and E), and 4 °C (C and F) (original magnification: 400×).

is necessary for the other HRs, as well, since they are built from similar components (pocket, wheel, axle, and blocking group). In apolar environments, such as lipids, the open conformation can exist in Cy3R and Cy2R **2** with the wheel(s) residing at the axle's ammonium ion. The geometry of the closed conformation was determined by molecular modeling to be at the two-carbon chain between the amide moieties of the axle. The distances between the wheel and the pocket for both conformations, however, are larger in CyR2 **2** and CyR3 as compared to CyR2 **1** because piperidinyl rings separate the wheel(s) from the pocket (Figure 1). Thus, the closed−closed conformation of Cy3R (Figure 8) and the closed conformation of Cy2R **2** do not form a tight aromatic pocket for small guests.

The separation of the wheel from the pocket is most likely largely responsible for the poor performance of Cy2R **2** to transfer the pentapeptides and diminishes the ability of Cy3R, as well. Although Ac-Trp-Phe-Phe-CO_2Me and the larger peptides are large enough to make simultaneous contact with the cyclophane pocket and aromatic rings of both wheels of Cy3R, the guanidinium moieties are forced away from the guests because of steric crowding. The steric clash between the guanidiniums over the pocket also keeps the smaller guests from simultaneously interacting with the pocket and both guanidiniums simultaneously. Thus, the closed−closed conformation of Cy3R most likely is not the dominant binding conformation in aqueous solutions. The optimum conformation for most guests is the closed−open conformation. It enables a guest to be embedded with the pocket and interact with both wheels. For Cy2R **2**, the preferred conformation for most guests is the closed conformation. Because of the piperidinyl ring, the binding of the guanidinium moiety to the guest forces the wheel near the pocket.

The additional arginine—provided by its second wheel—gives Cy3R a binding advantage over Cy2R **2**. For the series Ac-Trp-CO_2Me, Ac-Trp-Phe-CO_2Me, and Ac-Trp-Phe-Phe-CO_2Me bound to Cy3R, the indole ring resides in the pocket and one guanidinium can interact with the carbonyl oxygen atom of the ester and the other can form a cation−π interaction with a

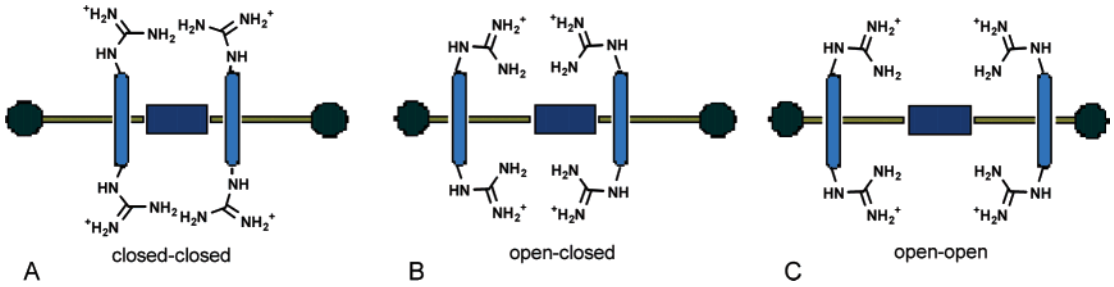

FIGURE 8. Conformations of Cy3R that are available in the host−guest complexes. (A) The closed−closed conformation brings the aliphatic and aromatic surfaces together, but the arginine moieties are moved away from the combining site because of steric crowding. (B) The open−closed conformation appears to be the optimal conformation for the binding of most guests in this study and forms readily in DMSO. (C) The open−open conformation is dominant in MeCN, and it is best suited for the binding of large guests.

guest's aromatic ring. Representative modeling pictures for the various complexes are available in Supporting Information. For the larger di- and tripeptides, both guanidiniums can form cation−π interactions. Similarly aligned complexes can exist with Ac-Trp-CO_2H, Ac-Glu-Trp-Glu-$CONH_2$, and Ac-Glu-Trp-Arg-$CONH_2$ except that one guanidinium makes a salt bridge with a guest's carboxylate. For Ac-Glu-Trp-Glu-$CONH_2$ as a guest, it can stretch across the pocket to form two salt bridges with a guanidinium from each wheel. Ac-Arg-Trp-Arg-$CONH_2$ also stretches across the pocket, but in this complex, it is the guest's guanidinium groups that make contact with the aromatic rings of opposing wheels. For Fl-AVWAL and Fl-QEAVD, all four guanidinium ions of Cy3R can form noncovalent bonds, whereas Cy2R **2** can use a maximum of two guanidinium ions. The additional guanidinium ions available for Cy3R readily account for its stable complexes with the Fl-peptides and the 10-mer peptides.

The modeling results (Supporting Information) and previous studies[36] indicate that complex strength depends on the conformation of the host-rotaxane. We also know that the dominant conformation of the HRs depends on the solvent. These facts combined provide an explanation for the unexpected discoveries that some complexes are more stable in DMSO than MeCN even though there should be a greater desolvation penalty for DMSO. DMSO interacts favorably with the ammonium ion of the axle. This enables the wheel to slide more freely along the axle to adopt stable conformations, such as the open−closed conformation of Cy3R. In MeCN, a strong noncovalent bond exists between the axle's ammonium ion and the wheel with a bond energy on the order of 1−2 kcal/mol.[48] Therefore in MeCN, the dominant conformation of Cy3R is the open−open conformation. To form the open−closed conformation in MeCN, one bond between the ammonium ion and the wheel needs to be broken. Breaking this bond detracts from the overall binding free energy.

Conformation selectivity appears to occur for the complexes of Ac-Arg-Trp-Arg-$CONH_2$ and the Fl-peptides in DMSO and hormone **1** in MeCN. Ac-Arg-Trp-Arg-$CONH_2$ fits best in the open−closed conformation of Cy3R. Its indole ring can reside in the pocket with both arginines in contact with each wheel. In the open−open conformation, only a single wheel can interact with Ac-Arg-Trp-Arg-$CONH_2$ if its indole ring resides in the pocket. Since the strength of the cation−π interaction is essentially solvent independent, a more stable complex is formed in DMSO because the open−closed conformation can more readily form in this solvent than in MeCN. In a similar manner, the open−closed conformation enables all four guanidiniums of Cy3R to interact with the Fl-peptides. Only a single wheel is close enough to make contact with a Fl-peptide in the open−open conformation. The large, 10-mer hormone **1** shows a significantly larger K_A in MeCN, as compared to that in DMSO for both HRs. The open−open conformation for Cy3R and the open conformation for Cy2R are dominant in MeCN. These larger binding domains of the HRs are a better fit for the large peptide. These studies show that guest selectivity for host-rotaxanes requires a matching of a host-rotaxane with a guest in the environment in which the complex would be formed.

These selectivity rules are also reflected in the transferring abilities of the HRs. At the same concentration of 10 μM, Cy3R transfers Fl-AVWAL and Fl-QEAVD, whereas Cy2R **2** does not. A comparison of the K_A's shows that Cy3R forms more stable complexes with the Fl-peptides than Cy2R **2**. The weakest complexes occur between Cy2R **2** and Fl-peptides in buffered water. This would suggest that it is the lack of complex formation in the extracellular domain that keeps Cy2R **2** from being a transporter. On the other hand, Cy3R is a better binding agent than Cy2R **2** in all three solvents. Therefore, we cannot determine whether it is the weaker association at the extracellular domain, the cell surface, the lipid portion of the cells, or all environments that reduce the delivery efficiency of Cy2R **2**.

The importance of complex formation at the cell surface, as represented by DMSO, is seen by comparing the properties of Cy2R **1** and Cy3R. Both HRs transfer the Fl-peptides into cells, and similarly large K_A's exist for the complexes in the three solvent systems. Fluorescein, however, is observed within cells for cells exposed to fluorescein and Cy2R **1**, but not for cells exposed to fluorescein and Cy3R (both HRs at 5 μM). Fluorescein is bound by Cy2R **1** and Cy3R with approximately equivalent stabilities in buffered water (pH 7.0) and MeCN. In DMSO, however, Cy2R **1** binds fluorescein with a very large K_A on the order of the Fl-peptides. Cy3R forms a 22-fold less stable complex with fluorescein in DMSO. The stability of the Cy3R−fluorescein complex in DMSO ($K_A = 4 \times 10^4$ M^{-1}) is on the same order of magnitude as that for complexes with other HRs, which transfer material into cells, in buffered water, MeCN, and chloroform (K_A 2.5×10^4 to 1×10^5 M^{-1}).[34,36,37] These results suggest that the tightest complexes need to form at the cell surface, and future delivery devices should be designed to bind guests strongly in DMSO.

Conclusion

To create more efficient delivery devices that operate at lower concentrations, a host-[3]rotaxane was constructed with a larger

more rigid cyclophane pocket and two wheels that contain two arginine moieties per wheel. A host-[2]rotaxane, which contains the same cyclophane pocket but only a single arginine-derivatized wheel, was constructed to determine whether the presence of a second wheel on a host-rotaxane would enhance guest association and intracellular delivery. A comparison between Cy2R **2** and the transporter Cy2R **1** shows that a greater separation of the wheel from the pocket results in a poorer binding agent for some guests and, more importantly, hampers the transport of materials into cells. The second wheel available in Cy3R can partially overcome these deficiencies. Its four guanidinium moieties can strengthen guest association and, apparently, enhances cellular delivery. Adding more arginines to an HR, however, did not result in greater Fl-peptide transport into cells or in a more selective delivery device for negatively charged peptides. At 5 μM, Cy2R **1** delivers Fl-AVWAL and Fl-QEAVD into 82 and 37% of the cells, respectively, whereas Cy3R delivers Fl-AVWAL and Fl-QEAVD into 58 to 16% of the cells, respectively. The size of K_A for complexes in DMSO appears to be a key indicator as to whether an HR will transfer the corresponding guest into cells. We are currently investigating the permeabilities of the HRs and guests using solid supported membranes. A better understanding of the properties of host-rotaxanes will facilitate the development of highly specific transporters for delivering therapeutic agents across cell membranes.

Experimental Section

Determination of Association Constants. Fluorescence quenching assays were performed to obtain the association constants. DMSO and MeCN were 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 phosphate (1 mM) at pH 7.0. A quantity of 2.7 mL of these solutions was placed into a 3.5 mL cuvette. Guests were added to these solutions from a DMSO stock solution to give final concentrations for most guests as 3.0×10^{-6} M. For complexes with larger K_A values, a 1×10^{-7} M solution of guest was used. For complexes with smaller K_A values, a 1×10^{-5} M solution of guest was used. Multiple aliquots of an HR stock solution in DMSO were added to the cuvette to give HR concentrations starting at approximately 0.3-fold lower than that of a guest's concentration and ending at approximately 40-fold higher concentration than that of a guest's concentration. The total change in volume caused by addition of the guest and a rotaxane was less than 2%. The fluorescence spectrum was recorded and analyzed after each addition of a rotaxane. 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.[70] The assays were duplicated, giving a standard deviation of less than 10% of the value obtained for the association constant. Benesi−Hildebrand analysis[71] was used to obtain the K_{A2} values for the HR_2·guest complexes.

Intracellular Transport Assays. We followed the procedures described in detail previously.[36] Briefly, cells were grown in 6-well culture plates in DME with 10% fetal calf serum until they had reached 50−70% confluency (approximately 1×10^5 cells per well). After 24 h, the growth medium 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. HRs and Fl-peptides were added from DMSO stock solutions to keep the amount of DMSO at less than 0.4% (v/v) of the total volume. A baseline level of fluorescence was obtained from wells containing Fl-peptide or fluorescein, and other wells contained no reagents except DMSO (referred to as untreated cells). An HR was added to a well followed by the addition of a Fl-peptide or fluorescein. After rocking the wells at rt for 1 h in the dark, the assay solutions were removed, and the cells were thoroughly washed. A calcein blue AM solution (1 μM in PBS) was added to each well. For cells exposed to the HRs, 2 μL of a 50 μg/mL propidium iodide solution was added to the wells. After 10 min, cells were observed by inverted fluorescence microscopy (Zeiss Axiovert 200M). 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 2 color flow cytometry (BD FACSAria). The thresholds for Fl-peptides alone were set at 5%, which was used as in the previous studies.[36,37] To determine the level of PI, all events were analyzed.

For cells assayed at 4 °C, the plates were stored at 4 °C for 15 min before the addition of an HR, 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 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.[57] HRs and guests 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 6-well culture plate with one coverslip per well. Cells were exposed to HRs and guest in the culture plate, as described above. The coverslips were then inverted and mounted onto microscope slides with Permafluor aqueous mounting medium (ThermoShandon). The unfixed cells were examined at 400× magnification by fluorescence microscopy (Zeiss Axioplan 2).

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Supporting Information Available: General experimental procedures, synthetic procedure for Cy3R and Cy2R **2**, ^{1}H NMR spectra, molecular modeling of Cy3R, photographs of cells exposed to Cy3R and Cy2R **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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