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Host-rotaxanes with oligomeric axles are intracellular transport agents

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ABSTRACT

Polymeric macromolecules are promising drug delivery devices with endocytotic properties that need to be resolved. Host-rotaxanes (HRs) also deliver materials into cells but require improved in vivo targeting capacity. Combining the targeting properties of nanoparticles with the transport function of HRs may improve drug efficacy. Our prototype HR (HR 1) has a short axle and is an efficient transporter. Here, we have constructed HRs that contain an oligo(ethylene glycol) (HR 2) or an oligoalkyl (HR 3) axle with the future goal of combining them with nanoparticles. HR 2 more efficiently delivers Fl-peptides into ovarian cancer cells than HR 3 and, in most cases, than HR 1. HR 2 appears to possess the appropriate balance between water solubility and lipophilicity to be an efficient transporter along with a suitable structure for incorporation into a larger nanoparticle.

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The cellular membrane blocks the passage of most materials into cells. Although necessary, this barrier severely limits the development of drugs and therapeutic methods. The challenge is to develop materials that selectively enter targeted cells without compromising their membrane and inducing cell death. We have developed a new class of intracellular transport agents, for example, host-rotaxane 1 (HR 1), that have low toxicities in the concentration range used for delivery and apparently pass through cell membranes unaided.[1] Host-rotaxanes are compounds with an interlocked wheel and axle with a blocking group and a synthetic host on the ends of the axle to keep the wheel threaded (Fig. 1).[2,3] Even though HRs are promising delivery devices, they lack a targeting mechanism for a particular cell type and so far have not been tested extensively in animals.

To provide a targeting mechanism and promote biostability, we proposed that the HRs can be combined with polymeric nanoparticles. Nanosized polymers can traverse many of the barriers imposed in living systems while protecting and improving the pharmacokinetic profile of an encapsulated drug. Although nanoparticles perform well in delivering drugs to cells, endolysosomal trafficking of the materials can degrade their payload. Cell-penetrating peptides (CPPs) have been combined with drug delivery materials to provide an alternative route for cellular entry.[4–8] Unfortunately, CPPs appear to enter cells via endocytosis.[9–12] HRs combined with polymeric nanoparticles may result in a more efficient delivery method.

We created a host-rotaxane with an oligo(ethylene glycol) chain (HR 2) to better equip the host-rotaxanes for noncovalent or covalent incorporation with polymers. This chain was chosen since polymeric ethers tend to be water-soluble, nontoxic, and nonimmunogenic.[13] Oligo(ethylene glycol) segments have been grafted onto long alkyl chains[14,15] or attached to the surface of a nanoparticle[16] and can be designed to release their payload at targeted sites.[17–20] The rest of the rotaxane was not changed to maintain efficient transport properties, including a cleft containing phenolic rings, which is used to house an aromatic guest, and a wheel containing arginine groups to promote cell surface binding.

The presence of an oligo(ethylene glycol) chain could greatly affect intracellular delivery. HR 2 when fully extended (4 nm) would span across half of a membrane (membrane thickness 6–9 nm). The full length of transporter HR 1 is approximately 2.5 nm. Long rotaxanes may undergo drastic conformational changes or become

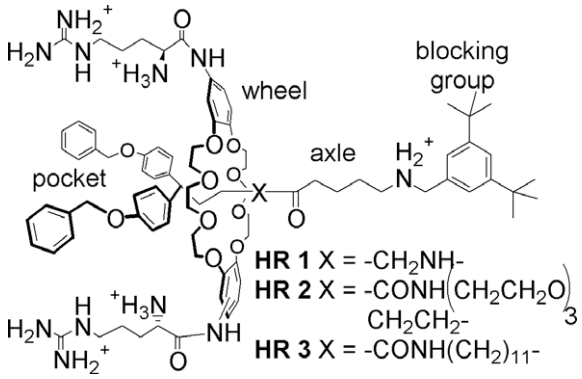

Figure 1. Host-rotaxanes used in this study and their components.

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permanently embedded within a membrane and halt transport. Additionally, changes made to the structure of the axle may affect properties such as permeability, guest recognition, or solubility of a rotaxane.

We were most concerned with the increase in the hydrophilicity of the rotaxane, which could decrease the ability of the HR to penetrate cellular membranes. A more lipophilic variant of HR 2 was created (HR 3) to improve the lipophilicity of long axle rotaxanes if needed. Its axle is made of repeating $-CH_2-$ groups, which increase the membrane permeability of HR 3, but decrease its water solubility. In this study, we show that HRs 2 and 3 bind and transport fluoresceinated guests into ovarian cancer cells. Surprisingly, HR 2 outperformed HR 1 and HR 3 for most guests in transport function; a process that was independent of endocytosis. Combining HRs with nanoparticles could prove to be a way to overcome the problem of endocytosis associated with the delivery of drugs by nanoparticles.

The synthesis of HR 2 and 3 started with the aromatic cleft component that was used in the synthesis of HR 1 (Scheme 1). It was oxidized to an acid to provide an attachment site for the oligoalkyl or oligo(ethylene glycol) strand. Synthesis of the oligo(ethylene glycol) strand followed the procedure of Martell.[21] The oligoalkyl strand was made from undecanedioic acid, which was amidated and then reduced. Once one amine of a strand was coupled to the cleft, the other end was exposed to a DCC–rotaxane.[22] DMSO was needed in the coupling reaction of the oligo(ethylene glycol) strand to improve its solubility. The amount of rotaxane formed with the oligoalkyl strand is lower (42%) than the yield usually obtained for rotaxane formation (around 70%). The wheels were deprotected and attached to Boc protected arginines, which were subsequently deprotected to produce the rotaxanes.

We have previously shown that efficient transport requires movement of the wheel along the axis. 2D NMR analysis demonstrated that dominant conformations exist for HR 1 bound to fluorescein in polar and apolar environments with the wheel residing near and far from the cleft pocket, respectively.[1] Additionally, we found that the likelihood of an HR to deliver a compound into cells is proportional to the strength of the complex that is formed between them, especially in DMSO.[23] Changing the length or the constituents of an axle could alter the spatial relationship between the wheel and pocket. This could result in restricted motion of the wheel or weak host–guest complexes. In either case, delivery could be diminished. We determined the binding enthalpy and entropy to explore the relationship between the wheel and pocket that exists in the host–guest complexes.[24]

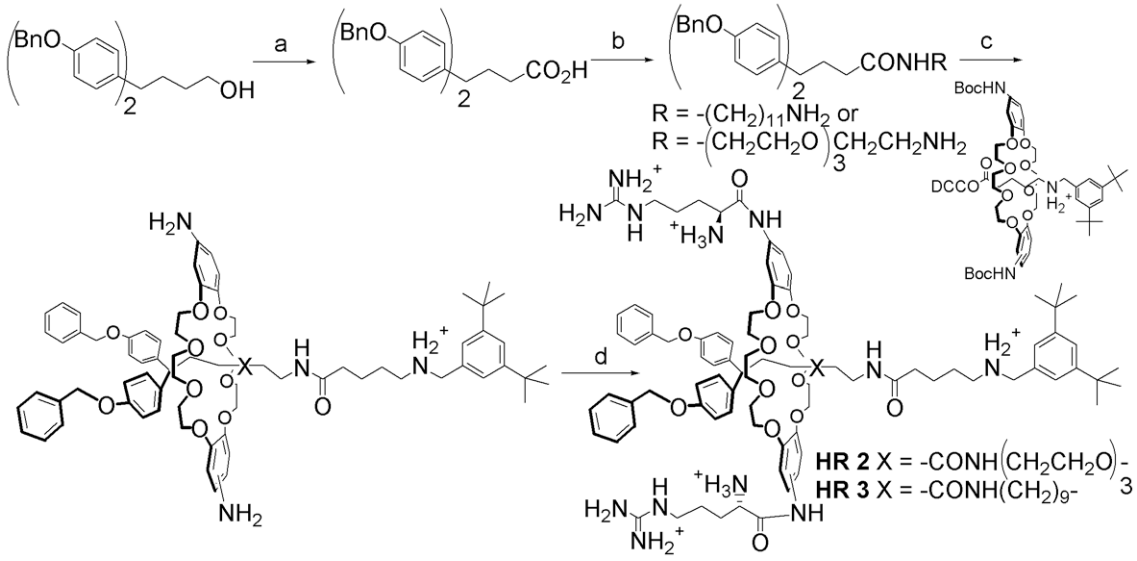
The thermodynamic parameters (see Supporting Information) for the HRs bound to fluorescein and to a fluoresceinated peptide, Fl-AVWAL, in buffered water (PBS, pH 7.3) and DMSO were determined (Table 1). These guests were chosen on the basis of their commonality with potential drugs and their extensive characterization in previous rotaxane studies.[23,24] The temperature dependence on the free energy was used to derive the binding enthalpies, according to the van't Hoff relationship. Knowing ΔG^0s and ΔH^0s, the binding entropies were obtained by solving the Gibbs free energy equation. A similar magnitude is seen in the binding free energies for the complexes formed between HRs 1–3 and the guests. The highly stable complexes in DMSO indicated that HRs 2 and 3 would be transporters. The results also show that the strength of a complex is insensitive to length and nature of the axle, at least for these axles.

On the other hand, the length and constituents of an axle greatly affects the magnitude of the enthalpy and entropy of binding. A wide range of values is seen for ΔH^0 and ΔS^0 in water ($\delta\Delta H^0 = 12$ kJ mol^{-1}and $\delta\Delta S^0 = 56$ J mol^{-1} K^{-1}) and in DMSO ($\delta\Delta H^0 = 23$ kJ mol^{-1} and $\delta\Delta S^0 = 82$ J mol^{-1} K^{-1}). The long axle rotaxanes HR 2 and 3 bind fluorescein in water with greater favor-

Table 1
Thermodynamic energies for HR complexes[a]

HR	Guest	Water[b]			DMSO		
		ΔG^0	ΔH^0	ΔS^0	ΔG^0	ΔH^0	ΔS^0
1[c]	Fl	−27	−13	47	−33	−11	74
	Fl-AVWAL	−30	−6	81	−33	−11	74
2	Fl	−28	−5	80	−31	−33	−8
	Fl-AVWAL	−22	−12	31	−32	−26	18
3	Fl	−26	−4	76	−31	−10	71
	Fl-AVWAL	−24	−16[d]	25	−31	−16	51

[a] $\Delta G^0 = -RT\ln K$, K from fluorescence quenching assays, uncertainty in K's $\leqslant 5\%$, ΔG^0 calculated for 25 °C, kJ mol^{-1}; ΔH^0 kJ mol^{-1}, from van't Hoff's analysis, uncertainty in $\Delta H^0 < 10\%$; $\Delta S^0 = (\Delta G^0 - \Delta H^0)/T$, calculated for 25 °C, J mol^{-1} K^{-1}, uncertainty in $\Delta S^0 < 10\%$.
[b] 1 mM phosphate pH 7.4.
[c] Ref. 24.
[d] From the integrated van't Hoff equation.



Scheme 1. Reagents and conditions: (a) $Na_2Cr_2O_7$, H_2SO_4, H_2O (88%); (b) i—ClCOCOCl, Et_3N, CH_2Cl_2; ii—$H_2N(CH_2)_{11}NH_2$, DMSO or $H_2N(CH_2CH_2O)_3CH_2CH_2NH_2$, CH_2Cl_2 (33%, 73%); (c) i—$CHCl_3$ (65%, 42%); ii—TFA, CH_2Cl_2 (96%, 84%); (d) i—$(Boc)_3$ArgOH, HOBt, DCC (68%, 56%); ii—TFA, CH_2Cl_2 (96%, 90%).

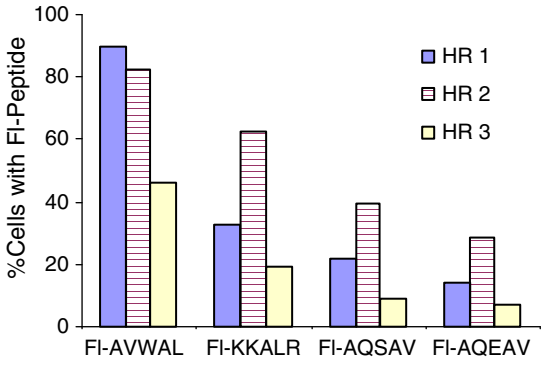


Figure 2. Delivery of Fl-peptides into CaOV3 cells by the HRs (standard deviations less than 10%).

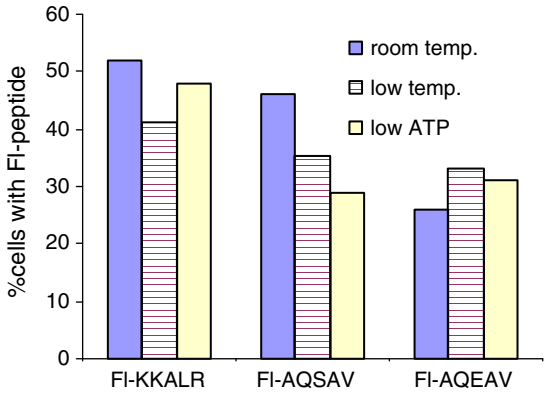


Figure 3. The delivery of Fl-peptides by HR 2 at different conditions to explore the transport mechanism (standard deviations less than 10%).

able binding entropy than HR 1. This could be the result of a greater number of water molecules being released from the axle as the wheel slides a greater distance. On the other hand, the binding of the larger Fl-AVWAL by HR 2 and 3 is more enthalpically driven than for its complex with HR 1 in water. In DMSO, HR 1 and HR 3, which have axles containing alkyl groups, bind the guests with similar energies and ΔH^0 and ΔS^0 both contribute to complex stability. The HR 2 complexes are driven through a favorable change in enthalpy. Thus, the source of the free energy does depend on the length or nature of the axle. A $\Delta H^0 - \Delta S^0$ compensation exists for these complexes, which lessens the differences in the free energies.

The ability of the HRs to transport materials into CaOV3 cells was investigated (see Supporting Information). Fluorescein was not used as a guest due to a high background level of fluorescence at the concentration needed for efficient delivery. Flow cytometry was used to quantify the relative level of Fl-AVWAL within the cells. The background level of fluorescence was set at 5%. Fl-AVWAL (10 μM) was delivered to a similarly high degree by HR 1 and HR 2 and approximately 50% less by HR 3 (Fig. 2). Since HR 3 is the least soluble in water of the HRs studied, the poorer performance could be caused by HR 3 or the HR 3·Fl-AVWAL complex precipitating during the assay. To enhance the water solubility of the HR complexes, the highly water-soluble Fl-KKALR, Fl-AQSAV, and Fl-AQEAV were tested as guests at 15 μM. The observed trend in delivery is consistent with previous studies of HRs.[25] HR 2 outperformed HR 1 in the delivery of these Fl-peptides, whereas HR 3 still delivered them less efficiently (Fig. 2). Since the multiple $-CH_2-$ groups of the axle should enhance the membrane permeability of HR 3, its poor water solubility most likely causes its less efficient transport.

The cellular assay solutions also contained calcein blue AM and propidium iodide (PI) to determine the number of viable and dead cells, respectively. A high proportion of calcein blue positive cells (90–98%) was observed in the assays, and less than 3% of the cells were dead, according to the level of PI within the cells. Calcein blue/PI and Fl-peptide fluorescence were independent variables. Membrane integrity was verified by measuring the amount of enzyme released from the cells during the assay. The level of lactate dehydrogenase (CytoTox-One Integrity Assay, Promega) released into the solution was 7–13% for cells exposed to the various reagents and 6% for untreated cells. These results demonstrate that the HRs and the Fl-peptides are minimally or not toxic at these concentrations.

Polymeric nanoparticles, as discussed above, and some highly argininated peptides enter cells through endocytosis. We previously observed that HR 1 delivers materials into cells in an energy independent process.[1] To determine whether endocytosis is the major pathway for cellular entry for HRs 2 and 3, the assays were repeated at 4 °C or by using an established ATP-depleting cocktail of 2-deoxyglucose and NaN_3 to deplete the cellular energy.[12] Fl-AVWAL was omitted as we have previously observed apparent precipitation in the assay solutions at 4 °C, most likely caused by the low solubility of the HR–Fl-AVWAL complex. Similar levels of transport were observed for each Fl-peptide under the energy-depleted conditions and in the physiologically relevant solution (Fig. 3). High levels of viable cells were measured in the assay performed at 4 °C and with depleted ATP (90–98% live cells, as indicated by calcein blue AM, and less than 7% dead cells, as indicated by PI). A larger number of dead cells were observed with a depleted level of ATP than at 4 °C. The flow cytometric results show that endocytosis is not the major pathway for the delivery of materials into cells. A cell-passive, rotaxane-dependent mechanism is more likely followed.

In summary, we found that the length or nature of an HR axle can increase or diminish the amount of a Fl-peptide that is delivered into cells. The results emphasize the importance of water solubility and lipophilicity, which are both needed by a host-rotaxane to be a transporter. HR 2, which has a oligo(ethylene glycol) as the axle, performed the best overall. It is highly soluble in water, forms strong complexes with Fl-peptides, and efficiently transports highly charged Fl-peptides into cells. A wide range of ΔH^0 and ΔS^0 values is seen for the HR–guest complexes. Notwithstanding the limited data set, these values do not correlate with transport efficiency, leaving ΔG^0 as the best indicator of transport.[23] More importantly, we found that HRs can be modified to enable nanoparticle attachment without interfering with efficient intracellular transport. Our ongoing studies focus on the mechanism of transport incorporation of long axle HRs with nanoparticles to improve the delivery of materials into cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.11.053.

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