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A Fine Line Between Molecular Umbrella Transport and Ionophoric Activity

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Abstract

A persulfated molecular umbrella derived from one spermine, four lysine and eight deoxycholic acid molecules was found to exhibit ionophoric activity, as shown by pH discharge, and Na⁺ and Cl[−] transport experiments. In sharp contrast, a moderately more hydrophilic analog derived from cholic acid showed no such ionophoric activity. Both molecular umbrellas crossed liposomal membranes by passive transport with experimental rates that were similar. These findings show how the interactions between such amphomorphic molecules and phospholipid bilayers are a sensitive function of the umbrella's hydrophilic/lipophilic balance (HLB). They also raise the possibility of exploiting molecular umbrellas in fundamentally new ways.

Molecular umbrellas are a unique class of conjugates made from two or more facial amphiphiles that have been covalently attached to a central scaffold (1). Such molecules are “amphomorphic” in the sense that they can produce a hydrophobic or a hydrophilic exterior when exposed to hydrophobic or hydrophilic environments, respectively. Because molecular umbrellas are capable of crossing lipid bilayers, even when strongly hydrophilic agents are coupled to them (e.g., oligonucleotides), they hold promise as drug carriers (2–4).

In previous work, we showed that highly sulfated molecular umbrellas, themselves, have interesting biological properties. Specifically, we showed that a bioconjugate derived from one spermine, four lysine and eight cholic acid groups, having all of its hydroxyl groups sulfated (i.e., **1**), exhibits significant anti-HIV and anti-HSV activity (Figure 1) (5,6). In a related investigation, we showed that a fluorescently-labelled analog of **1** readily enters live HeLa cells, and provided evidence that passive transport may be playing a significant role in this internalization process (7).

In the present study, we sought to test the feasibility of enhancing the membrane transport activity of a persulfated molecular umbrella by increasing its lipophilicity. Our motivation for this work was based on the presumption that if molecules of this type could cross the blood-brain barrier, they could have therapeutic value as anti-HIV and anti-HSV agents (8). With this aim in mind, we investigated more lipophilic analogs of **1**. According to the classic solution-diffusion model for bilayer transport, the permeability coefficient (P) of a permeant is directly proportional to its water-membrane partition coefficient (K) and its diffusion coefficient (D), but inversely proportional to the thickness (x) of the bilayer (1,9,10). Since $P=(K \times D)/x$, any

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Supporting Information Available

Experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

increase in lipophilicity is expected to increase P by increasing K , although exceptions to this model have already been noted for certain molecular umbrella-fluorophore conjugates (11).

As documented in this paper, we have discovered that a moderate increase in the lipophilicity of a persulfated molecular umbrella does not enhance its transport activity but, instead, leads to *ionophoric activity*; that is, a “fine line” exists between umbrella transport and ionophoric activity based on the umbrella’s hydrophilic/lipophilic balance.

Two specific molecular umbrellas that were chosen as synthetic targets for this work were **2** and **3** (Figure 2). Thus, replacement of all of the secondary amide groups with *N*-methylated amide units (i.e., **2**) was expected to result in a modest increase in lipophilicity. In contrast, removal of one sulfate group per sterol (i.e., **3**) was expected to lead to a more moderate increase in lipophilicity. As a control for umbrella-mediated transport, we chose a sulfonated polymer that was similar in size and charge to **1**, **2** and **3**, but devoid of facial amphiphilicity; that is, a β -naphthalene sulfonate/formaldehyde condensation polymer (PRO 2000) (12). This synthetic polymer, which is currently in clinical trials as an anti-HIV microbicide, was a gift from Indevus Pharmaceuticals (Lexington, MA).

Umbrella **3** was synthesized using methods analogous to those previously described for the preparation of **1**. In this case, deoxycholic acid was used in place of cholic acid. Umbrella **2** was prepared in a similar way except that *N*-methylated derivatives of *L*-lysine-dicholamide (i.e., **8**) and spermine (i.e., **9**) were employed (Figures 3) (5,13,14). Relative lipophilicities were determined by measuring the affinity of each agent towards liposomes (200 nm, extrusion) derived from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) (95/5, mol/mol). Partition coefficients, K , that are reported in Table 1 were calculated using the nonsaturable partitioning model (10). Thus, if C_0 is the equilibrium concentration of the conjugate in solution as measured in the absence of liposomes, and C is the concentration in the presence of liposomes, then the partition coefficient is calculated as $K = [(C_0 - C) \times W] / [C(L/2)]$. Here, $L/2$ is the concentration of the lipids in the external half of the bilayer with a weighted average molecular weight of 649 and W is the concentration of water, 55.5 M. If the inner leaflet contributes to binding, the true K values could be lower by a factor of two. The efflux rate for each molecular umbrella obeyed first-order kinetics; experimental rate constants, k_{obsd} , are also reported in Table 1.

The increase in lipophilicity on going from **1** to **2** proved to be so modest that it could not be detected by binding measurements. In contrast, the increase in lipophilicity of **3** was substantial. For PRO 2000, even stronger affinity to these lipid membranes was observed. Despite this variation in lipophilicities, the observed efflux rates for all three molecular umbrellas were similar. With PRO 2000, no efflux could be detected after 250 h.

An *in vitro* examination of **1** and **2** revealed that both molecular umbrellas had similar anti-HIV activity (Figure 4). In addition, neither of these agents showed any evidence of cytotoxicity at 1000 $\mu\text{g/mL}$. In sharp contrast, **3** was found to have significant cytotoxicity at 10 $\mu\text{g/mL}$. Because of its high toxicity, no attempt was made to determine the anti-HIV activity of **3**. Based on its relatively high lipophilicity, we hypothesized that the cytotoxicity associated with **3** was due to membrane-disrupting properties, possibly by acting as an ionophore (9).

To test for membrane permeabilization, we carried out a series of pH discharge experiments using liposomes made from POPC/POPG (95/5, mol/mol) containing 0.1 mM of entrapped pyranine in 25 mM HEPES buffer (pH 7.0, 50 mM NaCl) (15,16). After raising the pH of the external aqueous phase to 8.0, subsequent addition of 0.006 mol% of **3** led to a rapid increase in fluorescence intensity (Figure 5). A control experiment confirmed that exposure to **3** did not release any (i.e., <2%) of the dye from the aqueous interior of the liposomes within 4.5 h. Retention of this dye was quantified using an equilibrium dialysis cell, where the concentration

of dye in the source (i.e., liposome) and receiving sides were measured after 4.5 h. Taken together, these results show that **3** discharges the pH gradient across these liposomal membranes. Under similar conditions, **1**, **2** and PRO 2000 showed no permeabilization activity; that is, their fluorescence profiles were the same as that found in the absence of an added umbrella. Increasing the concentration of **1** by two orders of magnitude (i.e., using 0.6 mol%) still showed no evidence of permeabilization activity.

To determine whether **3** might be creating pores or channels in these lipid bilayers, we examined its ability to promote the transport of Na^+ and Cl^- ions across liposomal membranes. Using established procedures, a paramagnetic shift reagent [$\text{Dy}(\text{P}_3\text{O}_{10})_2\text{Na}_7$] plus NaCl were added to liposomal dispersions that were prepared from POPC (200 nm, extrusion) and 150 mM KCl (17). Subsequent addition of **3** resulted in an internalization of Na^+ , which could be monitored by $^{23}\text{Na}^+$ NMR (Figure 6). The biphasic nature of this kinetic profile is a likely consequence of the use of substoichiometric amounts of umbrella, which are expected to be distributed according to binomial statistics. Thus, the fast phase is presumed to be due to those liposomes that contain one or more umbrella molecules, and the slow phase due to a redistribution of the molecular umbrellas among the liposomes. Under similar conditions, **1** had no detectable Na^+ transport activity (not shown).

To test for Cl^- transport, we first attempted monitoring the kinetics of chloride ion influx using a liposome-entrapped chloride-sensitive dye (i.e., lucigenin) as a sensor. Such methods have previously been employed by other researchers (18). For our system, however, we found that the addition of **3** led to the complete release of the dye, as determined by equilibrium dialysis measurements. Because of this release, we chose an alternate approach, where we measured the efflux of chloride ion upon addition of **3**. Thus, a similar liposomal dispersion was first prepared from POPC and 100 mM NaCl in 10 mM sodium phosphate (pH 6.7). Subsequent dialysis against 100 mM NaNO_3 in 10 mM sodium phosphate (pH 6.7), followed by addition of 0.006 mol% of **3** resulted in $61 \pm 2\%$ release of the entrapped chloride ion after a 12 h at 23°C (see Supporting Information). In the absence of **3**, only $18 \pm 2\%$ of the entrapped chloride ion was released over this same time period. The fact that **3** promotes the transport of Cl^- ions rules out a simple carrier mechanism since chloride ion and the molecular umbrella are both negatively charged. Rather, it lends support for **3** acting as a pore- or channel-forming agent. The fact that two distinct resonances are observed for Na^+ further indicates that these openings are too small to permit the passage of the paramagnetic shift reagent but large enough to allow for a flow of Na^+ ions.

The difference between **1** and **3**, in terms of their interactions with phospholipid bilayers, is striking. One possible explanation for this difference is that **3**, being more lipophilic, prefers to reside within and across the bilayer, which leads to pore formation. In contrast, the more hydrophilic analog, **1**, may favor binding to the inner and outer surface of the membrane, and its presence in the hydrocarbon interior is only transient as it crosses from one leaflet to the adjoining one.

The strong dependency of molecular umbrella—lipid bilayer interactions on an umbrella's hydrophilic/lipophilic balance that we have discovered shows that a “fine line” exists between umbrella transport and ionophoric activity—a line that must not be crossed if such conjugates are to be used for drug delivery. At the same time, this finding raises the possibility of exploiting such molecules in fundamentally new ways; e.g., as biogenic ionophores and as cytotoxic drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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1, X=OSO₃Na

Figure 1.
Molecular structure of a persulfated molecular umbrella having significant anti-HIV and anti-HSV activity.

Figure 2.
 Molecular structures of **2** and **3** having a modest and moderate increase in lipophilicity, respectively, relative to **1**.

Figure 3.
Synthetic approach used for the synthesis of **2**.

Figure 4.

Dose-dependent inhibition of HIV-1 gene expression in U87 cells by different drugs. **(A)** U87 CD4⁺ CCR5⁺ cells pretreated with indicated concentrations of different drugs for 1hr followed by infection with JRFL envelope pseudotyped replication-defective HIV-1. Luciferase quantitation was performed on cell lysates 48 h post infection and is reported as Relative Light Units (RLUs). **(B)** Cytotoxicity of compounds **1**, **2** and **3** was done on U87 cells treated with different concentrations of the compounds. Cells were treated for 1 h with different drugs, then compounds were aspirated and replaced with control medium and cultured for additional 3 days. This was followed by addition of 20 uL of MTS/PMS solution and incubation for 2 to 4 h. Optical densities (OD) were read at 450 nm.

Figure 5.

Fluorescence intensity as a function of time (23°C) in liposomal dispersions made from POPC/POPG (95/5, mol/mol) in the absence (lower trace) and presence of 0.006 mol% of **3** (upper trace). An excess of Triton X-100 was used to destroy the liposomes by forming mixed micelles.

Figure 6. Entry of Na^+ into liposomes made from POPC as a function of time at 35°C after addition of (●) 0.002 mol% and (○) 0.004 mol% of **3**. In the absence of **3**, there was no detectable entry of Na^+ after 12 h (not shown).

Table 1

Binding and efflux data

Entrapped Permeant	Binding (%)	K^b	$10^3 k_{\text{obsd}}^c$ (h ⁻¹)
1	3.4	590	12.1
2	2.8	490	7.7
3	19	4000	9.8
PRO 2000	74	41000	0.0

^aMeasurements were at 24°C using POPC/POPG (95/5, mol/mol).

^bIn all cases, equilibrium was reached within 24 h.

^cExperimental first-order rate constant for efflux.