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Selective Transport of ATP across a Phospholipid Bilayer by a Molecular Umbrella

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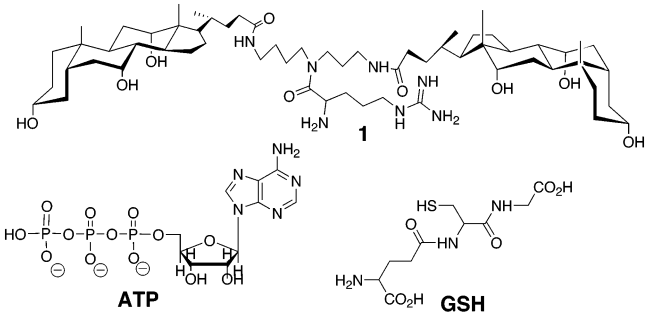
This paper reports the design and synthesis of a molecular umbrella (**1**), derived from three biogenic precursors (cholic acid, spermidine, and arginine), which readily transports adenosine 5-triphosphate (ATP), but not glutathione (GSH), across phospholipid bilayers made from 1-palmitoyl-2-oleyol-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG). The selectivity of such transport in a model system is without precedent, suggesting that umbrellas of this type may find use as drug delivery devices.



Devising methods for enhancing the passive transport of polar agents across biological membranes represents one of the greatest challenges currently facing medicinal chemists.[1] Our own approach to this problem has focused on the creation of "molecular umbrellas" as membrane transporters.[2] In essence, a molecular umbrella is composed of two or more facially amphiphilic units that are attached to a central scaffold. When drawn into a hydrocarbon environment (e.g., the interior of a lipid bilayer), the molecular umbrella can adopt a shielded conformation, thereby masking the hydrophilicity of an attached polar agent.

Previous studies from our laboratories have demonstrated the feasibility of using molecular umbrellas to transport covalently attached glutathione, and also adenosine 5′-*O*-(3-thiotriphosphate, across phospholipid bilayers.[2a,b] Recently, we have obtained kinetic evidence for a fundamentally new mechanism of transport in which the active species appears to involve a shielded conformer.[2c]

The primary aim of the work that is reported herein was to establish that *a suitably designed molecular umbrella can promote bilayer transport, even when the polar agent is not covalently attached to it*. For this purpose, **1** was chosen as a prototype due to the known affinity of the guanidinium moiety toward organic phosphates. Such affinity is presumed to result from hydrogen bonding and/or electrostatic association.[3] Specifically, we hypothesized that **1** would form a complex with phosphorylated agents of interest and that the resulting complex would readily cross lipid bilayers (Figure 1).

To test this hypothesis, ATP was selected as a model permeant.[4] Experimentally, we chose to entrap ATP within the aqueous interior of large unilamellar vesicles and to measure its release into the external aqueous phase.

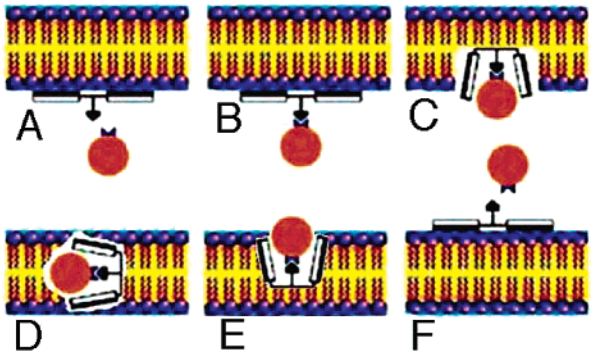
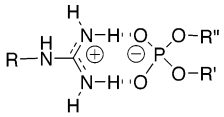


Figure 1. Hypothetical umbrella mechanism of bilayer transport, involving complex formation (**A** to **B**), monolayer insertion (**B** to **C**), bilayer permeation (**C** to **D** to **E**), and release of the polar permeant (**E** to **F**). This stylized illustration does not include the possibility that such transport may occur via an antiport or a symport process.



To distinguish among three different mechanisms of ATP release (i.e., umbrella-assisted transport, leakage, and membrane rupture), GSH was co-entrapped within the vesicles.[5] Here, our presumption was that guanidinium−phosphate interactions would be stronger than guanidinium−carboxylate interactions and that an umbrella-mediated transport should favor ATP.[6] Given the fact that GSH is smaller in mass and less polar than ATP, a release that occurs by a leakage mechanism should result in a greater percentage of GSH being released relative to ATP. In contrast, a release that is due to a rupture mechanism, whereby the vesicles "spill" their contents, should be reflected by the release of a similar percentage of GSH and ATP.[7]

The synthesis of umbrella **1** proved to be straightforward. Thus, condensation of N^1,N^3-spermidinebis[cholic acid amide] with N_α,N_δ,N_ω-tri-CBZ-L-arginine (Fluka), which had been activated with 3-hydroxyl-1,2,3-benzotriazin-4(3H)-one, and subsequent deprotection via hydrogenolysis afforded the requisite conjugate (Scheme 1). Examination of the surface tension of aqueous solutions of **1** in buffer (2 mM PIPES, 10 mM NaCl, 1 mM EDTA, pH 6.8) as a function of umbrella concentration yielded a critical micelle concentration of 330 μM.

Large unilamellar vesicles (150 nm diameter, extrusion) were prepared from POPC/POPG (95/5, mol/mol) in buffer containing 5.0 mM ATP and 3.0 mM of GSH. Removal of nonentrapped ATP and GSH via dialysis and subsequent dilution in buffer afforded a dispersion that was 4.0 mg/mL (5.26 mM) in phospholipid. In a typical transport experiment, 0.25 mL of the dispersion was mixed with 0.60 mL of a 100 μM solution of **1** in buffer and incubated at 35 °C with mild agitation for 48 h. The extent of ATP release was

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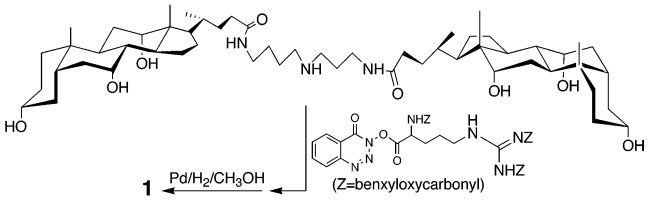
Scheme 1



then determined by withdrawing an aliquot, separating external ATP from vesicle-entrapped ATP via dialysis, and measuring the nucleotide concentrations by UV. In selected cases, the quantity of ATP that was released was confirmed by use of a Luciferase assay.[8] Under the experimental conditions used, spontaneous hydrolysis of ATP was negligible. The extent of GSH release was quantified by use of 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent).[2a]

Analysis for ATP in the external aqueous phase showed ca. 75% of that which had been entrapped.[9] In sharp contrast, the quantity of GSH that was present in this phase was negligible. In the absence of **1**, less than 1% of ATP was detected in the external aqueous phase. No difference in the extent of ATP release by **1** was found in vesicles that were made from 100% POPC or in vesicles that were devoid of GSH.

Control experiments that were carried out in which the same quantities of molecular umbrella, ATP, and GSH were added *externally* to empty vesicles showed significant (ca. 15%) adsorption of ATP, but not GSH, to the vesicles.[10] In the absence of the molecular umbrella, the extent of binding of ATP to the vesicles was negligible. These results are consistent with the selective uptake and transport of ATP by vesicle-bound umbrella molecules.

In an effort to drive the release of ATP into the external aqueous phase to completion, a combination of enzymes (Apyrase plus 3-Nucleotidase) was added, externally, to ATP-loaded vesicles. This combination converts ATP to adenosine. Under experimental conditions that led to an ca. 75% ATP release after 48 h (absence of enzymes), inclusion of the enzymes resulted in 100% ATP release with 97% retention of GSH. A control experiment that was carried out in which only the enzymes were added to the vesicle dispersion showed less than 6% ATP in the external aqueous phase after 48 h.

Based on the experimental conditions described above, the molar ratio of **1**/ATP was ca. 1.5. In an effort to demonstrate that **1** can act in a catalytic sense (i.e., there can be turnover), a similar experiment was carried out using 50 μM **1**, 10 mM entrapped ATP (corresponding to 148 μM ATP in the entire volume of the dispersion), a lipid/umbrella ratio of 132, and 0.25 units of each enzyme.[11] This corresponds to a molar ratio of **1**/ATP equalling 0.34. In this case, a turnover of >2.4 was observed after 48 h at 35 °C; that is, 81% of the ATP was present in the external aqueous phase. In this experiment, less than 4% of co-entrapped GSH (3 mM) was released. Taken together, these results provide compelling evidence for an umbrella-assisted bilayer transport favoring ATP over GSH.

The present findings suggest that conjugates such as **1** may find use in the area of drug delivery. For example, one can envision applying this approach to the transport of 3′-azido-3′-deoxythymidine AZT 5′-triphosphate, 2′,3′-dideoxyuridine-triphosphate, phosphorylated peptides, antisense oligonucleotides, and DNAs across phospholipid bilayers.[12−17] Studies aimed at exploring such possibilities are currently in progress.

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Supporting Information Available: Procedures for the synthesis of **1** and for the measurement of ATP and GSH release (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Juliano, R. L.; Alahari, S.; Yoo, H.; Kole, R.; Cho, M. *Pharm. Res.* **1999**, *16*, 494. (b) Ramaswami, V.; Zhu, Z.; Romanowski, M.; Haaseth, R. C.; Misicka, A.; Lipkowski, A. W.; Hruby, V. J.; O'Brien, D. F. *Int. J. Pept. Protein Res.* **1996**, *48*, 87. (c) De Mesmaeker, A.; Haner, R.; Martin, P.; Moser, H. E. *Acc. Chem. Res.* **1995**, *28*, 366.

(2) (a) Janout, V.; Zhang, L. H.; Staina, I. V.; DiGiorgio, C.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 5401. (b) Janout, V.; Jing, B.; Regen, S. L. *Bioconjugate Chem.* **2002**, *12*, 351. (c) Janout, V.; Staina, I. V.; Bandyopadhyay, P.; Regen, S. L. *J. Am. Chem. Soc.* **2001**, *123*, 9926.

(3) (a) Onda, M.; Yoshihara, K.; Koyano, H.; Ariga, K.; Kunitake, T. *J. Am. Chem. Soc.* **1996**, *118*, 8524. (b) Kato, Y.; Conn, M. M.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1994**, *116*, 3279. (c) Schiessl, P.; Schmidtchen, F. P. *J. Org. Chem.* **1994**, *59*, 509. (d) Sasaki, D. Y.; Kurihara, K.; Kunitake, T. *J. Am. Chem. Soc.* **1991**, *113*, 9685. (e) Andreu, C.; Galan, A.; Kobiro, K.; de Mendoza, J.; Park, T. K.; Rebek, J., Jr.; Salmeron, A.; Usman, N. *J. Am. Chem. Soc.* **1994**, *116*, 5501.

(4) Cholesteryloxycarbonyl-ATP has been found to release ATP into phospholipid vesicles with rates approaching ca. 10% in 9 days: Kreimeyer, A.; Andre, F.; Gouyette, C.; Huynh-Dinh, T. *Angew. Chem., Int. Ed.* **1998**, *37*, 2853.

(5) Sodium cholate has been shown to enhance the permeability of phospholipid bilayers toward nucleotides: Treyer, M.; Walde, P.; Oberholzer, T. *Langmuir* **2002**, *18*, 1043.

(6) (a) Schmuck, C. *Tetrahedron* **2001**, *57*, 3063. (b) Zafar, A.; Melendez, R.; Geib, S. J.; Hamilton, A. D. *Tetrahedron* **2002**, *58*, 683. (c) Linton, B.; Hamilton, A. *Tetrahedron* **1999**, *55*, 6027.

(7) Liu, Y.; Regen, S. L. *J. Am. Chem. Soc.* **1993**, *115*, 708.

(8) Admiraal, S. J.; Herschlag, D. *J. Am. Chem. Soc.* **1999**, *121*, 5837.

(9) As an alternative to an umbrella mechanism of transport (suggested by a referee), one can imagine a process in which the umbrella induces budding of the membrane, leading to the release of smaller vesicles that contain preferentially adsorbed ATP. Three lines of evidence argue against such a possibility: (i) budding or fusion processes should result in some release of entrapped GSH, which does not occur; (ii) ATP entrapped within budded vesicles would not be accessible to Apyrase plus 3-Nucleotidase; and (iii) dynamic light scattering shows a negligible change (±15%) in the average diameter of the vesicles after 48 h.

(10) Under the experimental conditions used, the captured volume represents ca. 0.85% of the total volume of the dispersion. Because of this low percentage, our estimate of ATP adsorption has little dependency on whether any of the ATP has entered the vesicles from the exterior phase. Thus, if it is assumed that ATP is fully equilibrated between the vesicular compartment and the bulk external phase, then the extent of adsorption is 14.2%; if one assumes negligible ATP entry into the vesicles, the extent of adsorption is 15%.

(11) In this experiment, the molar ratio of POPG/umbrella was 6.6. The fact that ATP transport proceeds, even when there is a signficant excesss of negative charge in the membrane, argues against an alternate transport mechanism (suggested by a referee), which is based on membrane/substrate electrostatics. Specifically, if the membrane maintained a net postive charge (e.g., by adsorption of large amounts of **1**), one could imagine a mechanism by which the trianionic ATP selectively adsorbs to the membrane, followed by a bilayer reorganization to achieve charge balance.

(12) Sastry, J. K.; Nehete, N.; Khan, S.; Nowak, B. J.; Plunkett, W.; Arlinghaus, R. B.; Farquhar, D. *Mol. Pharmacol.* **1992**, *41*, 441.

(13) van Wijk, G. M. T.; Hostetler, K. Y.; Kroneman, E.; Richman, D. D.; Sridhar, C. N.; Kumar, R.; van den Bosch, H. *Chem. Phys. Lipids* **1994**, *70*, 213.

(14) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003.

(15) Futaki, S.; Nakase, I.; Suzuki, T.; Zhang, Y.; Sugiura, Y. *Biochemistry* **2002**, *41*, 7925.

(16) Futaki, S.; Ohashi, W.; Suzuki, T.; Niwa, M.; Tanaka, S.; Ueda, K.; Harashima, H.; Sugiura, Y. *Bioconjugate Chem.* **2001**, *12*, 1005.

(17) Vigneron, J. P.; Oudrhiri, N.; Fauquet, M.; Vergely, L.; Bradley, J. C.; Basseville, M.; Lehn, P.; Lehn, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 9682.

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