The Agglutination of Erythrocytes by Influenza Virus is Strongly Inhibited by Liposomes Incorporating an Analog of Sialyl Gangliosides[1]

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Received April 23, 1992

Infection of a mammalian cell by influenza virus begins with the recognition of sialic acid (SA) groups on the cell surface by a viral surface protein, hemagglutinin (HA). Although virus binds tightly to cells,[3] solubilized HA binds only weakly ($K_d \sim 2.5 \times 10^{-3}$ M) to methyl α-sialoside.[4] This qualitative difference in the strength of binding is the basis for the hypothesis that the binding of virus to cell is controlled by polyvalent interactions.[5]

(1) This work is part of a collaboration with J. J. Skehel, J. R. Knowles, M. Karplus, and D. C. Wiley. Support provided by the NIH Grants GM 39589 and GM 30367.
(2) NSF Postdoctoral Fellow 1990–1992 (CHE-9002635).
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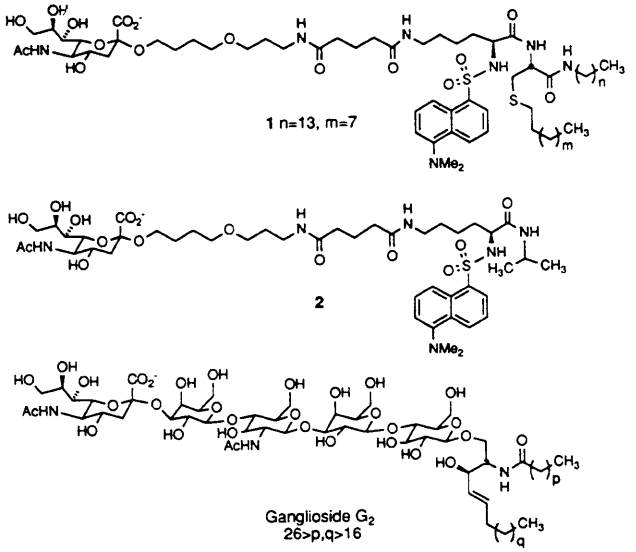
0002-7863/92/1514-7303$03.00/0

We are exploring the efficiency of polyvalent species presenting multiple SA groups as inhibitors of the binding of virus to cell and have reported that soluble polymers having a polyacrylamide backbone and pendant SA moieties inhibit virus-induced hemagglutination $\sim 10^5$ better than monomeric sialosides, when compared on the basis of SA residues.[6]

Here we compare synthetic and naturally occurring polymeric inhibitors with fluid bilayer liposomes presenting SA groups at their surface. Liposomes have been used as models for the fusion of virus to cell membranes[7] and as drug delivery vehicles.[8] Gangliosides[9] and antibodies[10] have been incorporated into liposome bilayers to probe ligand–receptor interactions. For our purposes, polyvalent, liposome-based inhibitors have three interesting features. First, we expect these systems to interact effectively with HA molecules on the surface of the virus. Second, in a fluid bilayer, the SA moieties can, in principle, move by lateral diffusion to optimize their binding to the virus. Third, the SA groups on the surface of a liposome are more localized in space than are the SA groups on a soluble polymer; they may, therefore, be more susceptible to detailed theoretical analysis.

To anchor the SA residue to the lipid bilayer, we designed and synthesized functionalized lipids that are intended to resemble sphingosine in their properties but are easier to synthesize. Compound **1** is a representative structure. A strategy for attachment of sugar moieties to liposomes using an analog of the naturally occurring glycosphingolipids has several advantages over one derived from natural materials. First, **1** is synthesized from



commonplace, inexpensive starting materials. Second, a convergent synthetic strategy readily accommodates structural variations and minimizes the quantity of saccharide needed. Third, a handle (in the form of an amine group in the dipeptide core) is included to which a fluorescent tag (or a tag with other useful properties) may be attached; this capability to modify and detect the glycolipid analog directly is useful in tracking it in the biological system. For comparison G_2, the major ganglioside on erythrocyte membranes, is shown.[11]

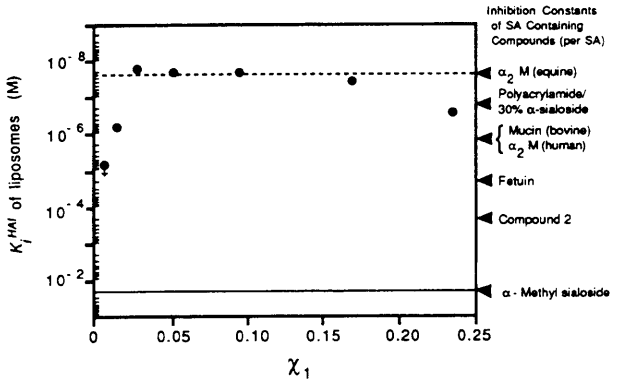


Figure 1. Inhibition of influenza virus-induced hemagglutination of chicken erythrocytes by liposomes incorporating **1**. The inhibition constant K_i^{HAI} is based on the content of SA groups in the system; values for SA groups on liposomes and polymers and the monovalent compounds are therefore directly comparable. The horizontal axis is the mole fraction of **1** used in preparing the liposomes, $\chi_1 = 1/(1 + PC + Chol)$. The data represent the averages of three independent trials in which independent preparations of liposomes were used. Inhibition constants of reference systems are taken from the following: Pritchett, T. J.; Paulson, J. C. *J. Biol. Chem.* **1989**, *264*, 9850 and ref 6.

Synthesis of **1** began with sequential amide formation between Boc-cystine and tetradecylamine, reduction of the disulfide group, and alkylation on the sulfur with bromodecane.[12] We used shorter alkyl chains than those found in gangliosides in the synthesis of **1** to improve solubility and simplify purification. The marker tag, here the fluorescent 5-(dimethylamino)naphthalene-1-sulfonyl (Dans) group, was incorporated by attachment to N_α of the lysine group. Coupling of the cysteine and lysine components by an amide group completed the anchor portion of **1**. The sugar component SA-2-O$(CH_2)_4$O$(CH_2)_3NH_2$[6] was joined to the dipeptide unit with a bridging glutaryl diamide unit. Compound **1** was prepared in six steps and 25% overall yield from Boc-cystine.

Liposomes were prepared using **1** and a lipid mixture comprised of egg phosphatidylcholine (PC) and cholesterol (Chol, 7:2 molar ratio). The appropriate amounts of **1** were added to buffered lipid, and the resulting mixture was sonicated until the turbidity dissipated to form small (25–100 nm) unilamellar liposomes.[13] The biochemical activity of the functionalized liposomes was evaluated using the hemagglutination inhibition (HAI) assay.[14] Briefly, the HAI assay consists of serial dilutions of inhibitors on 96-well microtitre plates followed by the addition of influenza virus X-31 and chicken red blood cells (RBCs). After 1–2 h, the wells are checked for agglutination of the RBCs. We define the hemagglutination inhibition constant K_i^{HAI} as the lowest concentration of SA residues that inhibits agglutination of erythrocytes by influenza virus at 4 °C under these assay conditions.

The HAI assay of the liposomes containing **1** revealed that they are extremely good inhibitors of hemagglutination, calculated on the basis of total SA groups contained in the system: $K_i^{HAI} \sim$ 20 nM (Figure 1). Independent liposome preparations give reproducible results within a factor of 4. The SA concentrations of liposome preparations were determined by taking half of the initial solution concentration of **1** to account for the material that is inaccessible on the inner bilayer surface of the liposome. The optimum mole fraction of SA on the surface of the liposome for inhibition is in the range 2.5–18%. This number is consistent with Haywood's observation that 3% of bovine ganglioside on liposomes is necessary to inhibit Sendai virus using the HAI assay.[10]

When **1** alone was assayed for inhibition, lysis of the RBCs occurred at concentrations above 10 μM. This result was expected for a surfactant such as **1**. Compound **2** was used to provide a

(6) This number reflects the increase in binding obtained when a monomeric SA moiety was incorporated into a polymer. Spaltenstein, A.; Whitesides, G. M. *J. Am. Chem. Soc.* **1991**, *113*, 686. Similar results have been obtained: Matrosovich, M. N.; Mochalova, L. V.; Marinina, V. P.; Bryamova, N. E.; Bovin, N. V. *FEBS Lett.* **1990**, *272*, 209. Roy, R.; Laferrière, C. A. *Carbohydr. Res.* **1988**, *177*, C1.

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(12) Experimental details of syntheses and characterization of these compounds are provided as supplementary material.

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soluble molecule against which to compare **1**.[15] A $K_i^{HAI} \sim 200$ μM (average of three trials) was obtained for **2**, a factor of 10^4 higher than for the polyvalent liposome system. Liposomes containing no **1** and liposomes containing the methyl ester of **1** were examined by the HAI assay as controls. Neither system inhibited hemagglutination.

These results establish that arrays of SA groups at the surface of liposomes are moderately more effective in inhibiting agglutination of RBCs by influenza virus than are SA groups linked to soluble polymers. More significantly, these SA functionalized liposomes are as good as or better than the best-known natural inhibitors of hemagglutination, the mucins and macroglobulins. We emphasize that the effective inhibition observed with **1** involves only a monosaccharide rather than a complex polysaccharide (i.e., a glycoprotein or ganglioside): this observation makes it unnecessary to synthesize the complex sialyl polysaccharides found in nature. It remains to be established whether this inhibitory activity is due to enhanced binding of SA to viral HA originating in polyvalency and entropic factors or to steric occlusion of the surface of the virus by bound liposome.[16] We will describe studies of the ability of these liposomes to inhibit infectivity of influenza virus in vivo later.

Supplementary Material Available: Experimental data for compounds **1**, **3–7**, and **9–12** (9 pages). Ordering information is given on any current masthead page.

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(15) Compound **1** (in a liposome) and **2** (in solution) have substantially different steric constraints. In the liposome, the Dans group is predicted to be constrained to an area near the lipid bilayer surface and unavailable to interact with HA; in **2**, the Dans group is sterically unconstrained. We have not yet investigated whether the Dans group in either case plays a role in binding. See: Toogood, P. L.; Galliker, P. K.; Glick, G. D.; Knowles, J. R. *J. Med. Chem.* **1991**, *34*, 3138.

(16) *The Effect of Polymers on Dispersion Properties*; Tadros, T. F., Ed.; Academic: London, 1982. Sato, T.; Ruch, R. *Stabilization of Colloidal Dispersions by Polymer Adsorption*; Marcel Dekker: New York, 1980.