Carbohydrate Materials Bearing Neuraminidase-Resistant C-Glycosides of Sialic Acid Strongly Inhibit the in Vitro Infectivity of Influenza Virus

Jon O. Nagy,*,‡ Peng Wang,†,‡ James H. Gilbert,§ Mary E. Schaefer,§ Tara G. Hill,∥ Matthew R. Callstrom,∥ and Mark D. Bednarski†,‡

Department of Chemistry, University of California at Berkeley, Berkeley, California 94720

The Center for Advanced Materials, Lawrence Berkeley Laboratory, Berkeley, California 94720

Department of Microbiology and Immunology, Glycomed, Inc., Alameda, California 94501

Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

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Sialic acids, derivatives of *N*-acetylneuraminic acid (NeuAc), are carbohydrate molecules found terminating cell-surface glycoproteins and glycolipids.[1-3] The α-glycosides of NeuAc are utilized by pathogens to attach to cells prior to infection using a class of glycoprotein molecules called lectins.[4-6] One of the most well-studied proteins in this class is the influenza hemagglutinin (HA).[7-9] A number of papers have recently demonstrated the remarkable cooperativity effect of polyvalent derivatives of O-linked sialic acids to inhibit the attachment of the virus to erythrocytes.[10-15] Of the materials tested, none were effective at inhibiting infectivity in vitro. The use of materials containing O-sialosides is also severely limited because enzymes that hydrolyse the saccharide linkage (neuraminidases (NA)) are present on the surface of the virus.[7] Materials to treat influenza must, therefore, be both resistant to viral NA and be able to inhibit viral plaque formation. We report the first example of a multivalent material that consists of the carbon glycoside (C-glycoside) of sialic acid that is resistant to hydrolysis by the viral NA and strongly inhibits viral binding and viral plaque formation.

The synthesis of the α-C-glycoside analog of sialic acid 1 has recently been reported by both our group and Paulsen et al. utilizing radical coupling reactions,[16,17] and by Vasella et al. using alkylation chemistry.[18] In a competitive viral binding assay,[19] we found that only the α-isomer binds to the virus.[20] Compound 2 was prepared from the ethyl ester of 1 by using standard chemistry (see supplementary material). (The β-epimer of compound 2 was similarly synthesized to show that no epimerization of the α isomer occurred over the course of the reaction sequence.) Compound 2 was then reductively aminated onto a 2-amidoglucose polymer 3 to give 4 (Scheme I). Carbohydrate-based materials such as 3 were chosen for conjugation of the C-sialosides because of their stability, high solubility in aqueous solutions, and the availability of latent aldehyde groups for ligand attachment.[21] Polymer 3 (concentration 30 mg/mL) was treated with compound 2 in a solution buffered at pH 8 (0.1 M borate buffer) and excess sodium cyanoborohydride. After incubation for 2 weeks at 37 °C, polymer 4 was recovered by extensive dialysis (Spectra/Por 25 000 MW cutoff) in water to remove all unbound monomer and borate salts. The material was lyophilized to give a fluffy white solid and the percentage of attached sialosides was estimated on the basis of the increase in weight of the polymer. Two materials were synthesized with different percentages of sialic acid by varying the ratios of compounds 2 and 3. Material 4a

† University of California at Berkeley.
‡ Lawrence Berkeley Laboratory.
§ Glycomed, Inc.
∥ The Ohio State University.

(1) Cornfield, A. P.; Schauer, R. Occurrence of Sialic Acids. In *Sialic Acids; Chemistry, Metabolism and Function*; Schauer, R., Ed.; Springer-Verlag Publishing Co.: New York, 1982; Vol. 10, pp 5–39.

(2) McGuire, E. J. *Biological Roles of Sialic Acid*; Rosenberg, A., Schengrund, C.-L., Eds.; Plenum Publishing Co.: New York, 1976.

(3) Sharon, N. The Sialic Acids. In *Complex Carbohydrates*; Addison-Wesley Publishing Co.: London, 1975; pp 142–154.

(4) Sharon, N.; Lis, H. Lectins as Cell Recognition Molecules. *Science* 1989, *246*, 227–234.

(5) Ruigrok, R. W. H.; André, P. J.; Hoof Van Huysduynen, R. A. M.; Mellema, J. E. Characterization of Three Highly Purified Influenza Virus Strains by Electron Microscopy. *J. Gen. Virol.* 1985, *65*, 799–802.

(6) Markwell, M. A. K. Viruses as Hemagglutinins and Lectins. In *Microbial Lectins and Agglutinins: Properties and Biological Activity*; Mirelman, D., Ed.; Wiley Series in Ecological and Applied Microbiology; Wiley-Interscience Publication, John Wiley and Sons Publishing Co.: New York, 1986; pp 21–53.

(7) For a review on the mechanism of virus infection, see: Wiley, D. C.; Skehel, J. J. The Structure and Function of the Hemagglutinin Membrane Glycoprotein of Influenze Virus. *Annu. Rev. Biochem.* 1987, *56*, 365–394.

(8) Weis, W.; Brown, J. H.; Cusack, S.; Paulson, J. C.; Skehel, J. J.; Wiley, D. C. Structure of the Influenza Virus Hemagglutinin Complexed with its Receptor Sialic Acid. *Nature* 1988, *333*, 426–431.

(9) Sauter, N. K.; Bednarski, M. D.; Wurzburg, B. A.; Hanson, J. E.; Whitesides, G. M.; Skehel, J. J.; Wiley, D. C. Hemagglutinins from Two Influenza Variants Bind to Sialic Acid Derivatives with Millimolar Dissociation Constants: A 500-MHz Proton Nuclear Magnetic Resonance Study. *Biochemistry* 1989, *28*, 8388–8395.

(10) Spaltenstein, A.; Whitesides, G. M. Polyacrylamides Bearing Pendant α-Sialoside Groups Strongly Inhibit Agglutination of Erythrocytes by Influenza A Virus. *J. Am. Chem. Soc.* 1991, *113*, 686–687.

(11) Glick, G. D.; Knowles, J. R. Molecular Recognition of Bivalent Sialosides by Influenza Virus. *J. Am. Chem. Soc.* 1991, *113*, 4701–4703.

(12) Sabesan, S.; Duus, J. O.; Domaille, P.; Kelm, S.; Paulson, J. C. Synthesis of Cluster Sialoside Inhibitors for Influenza Virus. *J. Am. Chem. Soc.* 1991, *113*, 5865–5866.

(13) Gamian, A.; Chomik, M.; Laferriere, C. A.; Roy, R. Inhibition of Influenza A Virus Hemagglutinin and Induction of Interferon by Synthetic Sialylated Glycoconjugates. *Can. J. Microbiol.* 1991, *37*, 233–237.

(14) Byramova, N. E.; Mochalova, L. V.; Belyanchikov, I. M.; Matrosovich, M. N.; Bovin, N. V. Synthesis of Sialic Acid Pseudopolysaccharides by Coupling of Spacer-Connected Neu5Ac with Activated Polymer. *J. Carbohydr. Chem.* 1991, *10*, 691–700.

(15) Matrosovich, M. N.; Mochalova, L. V.; Marinina, V. P.; Byramova, N. E.; Bovin, M. V. Synthetic Polymeric Sialoside Inhibitors of Influenza Virus Receptor-Binding Activity. *FEBS Lett.* 1990, *272*, 209–212.

(16) Nagy, J. O.; Bednarski, M. D. The Chemical-Enzymatic Synthesis of a Carbon Glycoside of N-Acetyl Neuraminic Acid. *Tetrahedron Lett.* 1991, *32*, 3953–3955.

(17) Paulsen, H.; Matschulat, P. Synthesis of the C-Glycoside of N-Acetyl Neuraminic Acid and Other Derivatives. *Liebigs Ann. Chem.* 1991, 487–495. The reported spectral data for the α-*C*-allylsialoside (1) is in error (Paulsen, H., private communication). The corrected values are as follows: $[\alpha]^{22}_D = +2.0°$ (c = 0.5, H_2O); ^1H NMR (D_2O, 500 MHz, ref 4.63 ppm HOD peak) δ (ppm) 1.46 (t, 1 H, J = 12.4 Hz, $H_{ax}C(3)$), 1.86 (s, 3 H, $NCOCH_3$), 2.36 (dd, 2 H, J = 3.2, 7.2 Hz, $CH_2CH{=}CH_2$), 2.44 (dd, 1 H, J = 4.6, 13.2 Hz, H_{eq}-C(3)), 3.39 (dd, 1 H, J = 1.2, 8.4 Hz), 3.43–3.55 (m, 7 H), 4.97 (s, 1 H, $CH_2CH{=}CH_2$), 4.99 (d, 1 H, J = 7.9 Hz, CH_2-$CH{=}CH_2$), 5.65 (m, 1 H, J = 2.7, 7.26, 10.1 Hz, $CH_2CH{=}CH_2$); ^{13}C NMR (125 MHz, CD_3OD, ref 49.0 ppm center CD_3OD peak) δ (ppm) 22.57, 42.12, 45.97, 54.52, 64.60, 69.81, 70.39, 73.07, 75.45, 82.25, 117.77, 134.77, 175.52, 178.74; HRMS FAB calcd m/z for $C_{14}H_{23}NO_8$ 334.1502 [(M + H)$^+$], found 334.1502.

(18) Wallimann, K.; Vasella, A. 147. C-Glycoside of N-Acetyl Neuraminic Acid. Synthesis and Investigation of its Effect on Vibrio cholerae Sialidase *Helv. Chim. Acta* 1991, *74*, 1520–1532.

(19) The cell-binding studies were performed following a method described by Pritchett et al.: Pritchett, T. J.; Brossmer, R.; Rose, U.; Paulson, J. C. Recognition of Monovalent Sialosides by Influenza Virus H_3 Hemagglutinin. *Virology* 1987, *160*, 502–506.

(20) Initial studies using X-ray diffraction of HA soaked with compound 1 showed that it bound to the same site as silylactose (Wiley, D.; Wang, P.; Nagy, J. O.; Sauter, N.; Bednarski, M. D., unpublished results).

(21) (a) Klein, J.; Herzog, D. Synthesis of Some Poly(Vinylsaccharide)s of the Amide Type and Investigation of their Solution Properties. *Makromol. Chem.* 1987, *188*, 1217–1232. (b) Callstrom, M. R.; Bednarski, M. D.; Hill, T. G.; Oehler, L. M.; Gruber, P. New Carbohydrate Materials. *Mater. Res. Soc. Proc.* 1990, *174*, 259–266.

0022-2623/92/1835-4501$04.00/0

Scheme I

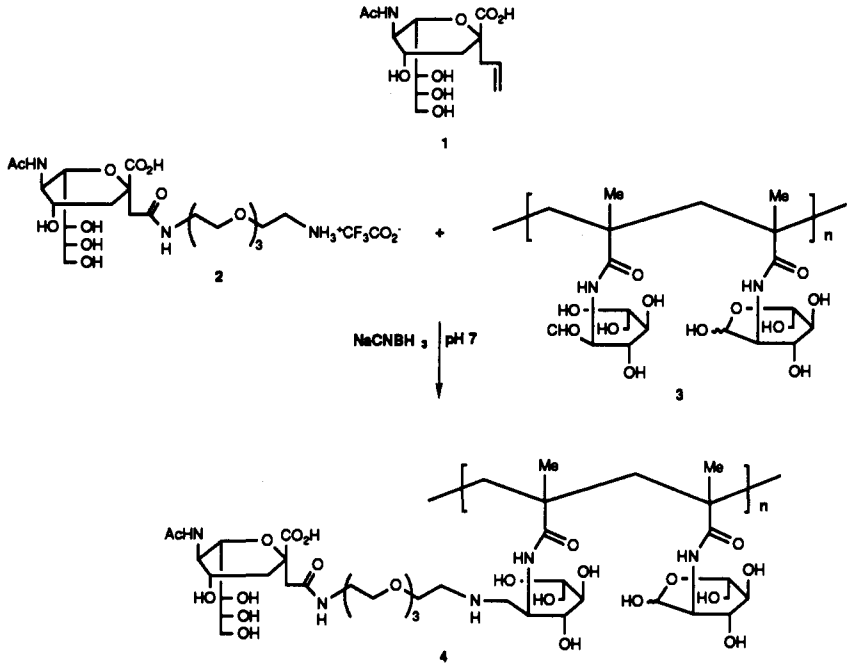

Table I. Comparison of α- and β-C-Sialoside Monomers and Multivalent C-Sialoside Polymers **4a** and **4b** in Their Ability To Prevent Viral Binding to Erythrocytes by Influenza Virus[a]

entry	compound	concn for 50% inhibn	rel potency
1	β-*C*-allyl sialic acid	>50 mM	<0.02
2	α-*C*-allyl sialic acid 1	10 mM	1
3	α-*O*-methyl sialic acid	4 mM	2.5
4	5% α-*C*-sialic acid polymer **4a**	1 mM	10
5	30% α-*C*-sialic acid polymer **4b**	0.2 μM	50000

[a] Material **4a** contains 5% sialic acid by weight and material **4b** contains 30%.[22] The inhibition of influenza virus (X-31) to resialylated erythrocytes was examined as previosly described.[19]

contained approximately 5% of the α-C-glycoside analog of sialic acid and material **4b** contained 30%.[22]

The monomers and polymers were tested in a standard viral binding assay (Table I).[19] To achieve 50% inhibition of viral binding, compound 1 required a concentration of 10 mM, the C-sialoside conjugate **4a** required 1 mM, and material **4b** only 0.2 μM. The enhancement of binding inhibition observed for material **4b** (approximately 50 000 times the value for monovalent sialosides) is in agreement with the values others have reported for multivalent O-sialoside complexes of similar sialic acid percent composition.[10,13-15] Carbohydrate polymer **3** alone showed no inhibition of viral binding.

We next turned our attention to assess the efficacy of polyvalent materials **4a** and **4b** to stop viral infectivity using a viral plaque assay.[23] It was found that at a concentration of 100 μM of **4b** the number of plaques was reduced by approximately 50% and at a concentration of 500 μM there was approximately 80% inhibition of plaque formation. The plaque size was also greatly reduced and plaque morphology was altered at these concentrations of **4b** in a dose-dependent manner. The polymer with 5% sialic acid **4a** showed only slight plaque reduction and the underivatized polymer **3**, C-sialoside **1**, and the α-*O*-methyl sialoside showed no inhibition of influenza virus plaque formation.[23] Compound 1 exhibited only weak inhibition of viral neuraminidase (approximately 40% inhibition at a concentration of 12 mM) but neither polymer **4a** nor **4b** inhibited the NA. These materials were also not substrates for the enzyme.[24]

In summary, we have synthesized a C-glycoside sialic acid containing material that is resistant to the viral NA and prevents viral binding to erythrocytes and viral plaque formation in vitro. We are in the process of testing these new materials in vivo.

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Supplementary Material Available: Experimental procedures and biological assay methods are given (5 pages). Ordering information is given on any current masthead page.

(22) The percentages of C-sialosides incorporated in the polymers were estimated by subtracting the weight of the polymer from the weight of the sialic acid polymer conjugate then subtracting the weight due to the linker from this difference and dividing this resulting amount by the weight of the sialic acid polymer conjugate.

(23) Tobita, K.; Sugiura, A.; Enomoto, C.; Furuyama, M. Plaque Assay and Primary Isolation of Influenza A Viruses in an Established Line of Canine Kidney Cells (MDCK) in the Presence of Trypsin. *Med. Microbiol. Immunol.* **1975**, *165*, 9-14.

(24) Isolated neuraminidase (H_1N_2 viron) was used in the assay of compounds **4a** and **4b** as per the procedure in the following: Baum, L. G.; Paulson, J. C. The N_2 Neuraminidase of Human Influenza Virus has Acquired a Substrate Specificity Complimentary to the Hemagglutinin Receptor Specificity. *Virology* **1991**, *180*, 10-15.