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Synthetic Mucus Nanobarriers for Identification of Glycan-Dependent Primary Influenza A Infection Inhibitors

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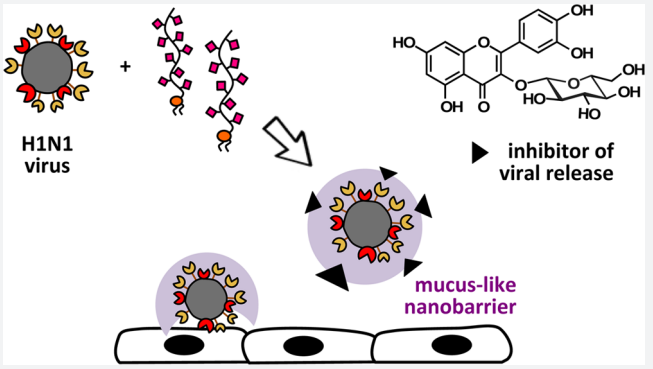
Supporting Information

ABSTRACT: Current drugs against the influenza A virus (IAV) act by inhibiting viral neuraminidase (NA) enzymes responsible for the release of budding virions from sialoglycans on infected cells. Here, we describe an approach focused on a search for inhibitors that reinforce the protective functions of mucosal barriers that trap viruses en route to the target cells. We have generated mimetics of sialo-glycoproteins that insert into the viral envelope to provide a well-defined mucus-like environment encapsulating the virus. By introducing this barrier, which the virus must breach using its NA enzymes to infect a host cell, into a screening platform, we have been able to identify compounds that provide significant protection against IAV infection. This approach may facilitate the discovery of potent new IAV prophylactics among compounds with NA activities too weak to emerge from traditional drug screens.



The influenza A virus (IAV) causes periodic pandemic outbreaks worldwide with substantial mortality and economic cost.[1,2] While powerful in combating IAV, vaccines rely heavily on the correct prediction of candidate pandemic strains. Small molecule antivirals offer a more general alternative for rapid deployment during outbreaks.[3−5] Currently approved IAV drugs (i.e., oseltamivir,[6] zanamavir,[7] peramivir,[8] and laninamivir[9]) target viral neuraminidases (NAs),[10,11] which are sialic acid-cleaving enzymes required for releasing budding virions from infected host cells and for preventing virion aggregation.[12]

Despite their usefulness, these anti-IAV drugs suffer from emerging viral resistance[13] and cross-reactivity with human neuraminidases (Neu2 and Neu3).[14,15] While the former can be addressed with new classes of structurally distinct NA inhibitors, current medicinal chemistry approaches focus on generating potent inhibitors, whose off-target activity against closely related human enzymes can be difficult to manage. Here, we describe an alternate strategy for the identification of IAV prophylactics that bolster the protective functions of the pulmonary mucosa.

IAV infection begins with the binding of viral hemagglutinin (HA) proteins to sialic acid-carrying glycans on host cells. However, the target tissues for IAV infection are covered with a layer of secreted mucus that contains highly sialylated mucin glycoproteins, which can act as viral receptor decoys that restrict viral entry (Figure 1A). The virus relies on its NA enzymes to destroy sialic acid receptors on secreted mucins that engage its HA and obstruct its path to the target cells.[12,16−18] Considering the key role of NA in facilitating the diffusion of IAV through the mucus, compounds with only a mild inhibitory effect on NA may effectively cause virus trapping in the mucus and its clearance with the natural turnover of the mucosal barrier.[19,20]

While conceptually intriguing, introduction of the mucosal component into screening assays to identify new NA inhibitors capable of suppressing IAV infection poses considerable challenges. Purified porcine mucins can offer broad-spectrum protection against some viruses, including IAV.[21,22] However, the effectiveness of viral inhibition is strongly dependent on the mucin source, with commercial products varying both in potency and cellular toxicity.[16,21]

Synthetic glycopolymers, which mimic the basic architecture of mucin glycoproteins, have a rich history as probes to evaluate the mechanism of HA binding to multivalent sialoglycan ligands[23,24] and as IAV inhibitors.[25,26] Recently, we reported the preparation of azide-functionalized sialoglycan polymers for immobilization in microarrays to analyze the effects of glycan presentation on recognition by IAVs.[27] Although soluble glycopolymers are known to inhibit viral entry,[25] they may not adequately recreate the densely sialylated microenvironment of the natural mucus. Inspired by reports of noninvasive labeling of influenza virions utilizing the IAV's lipid membrane

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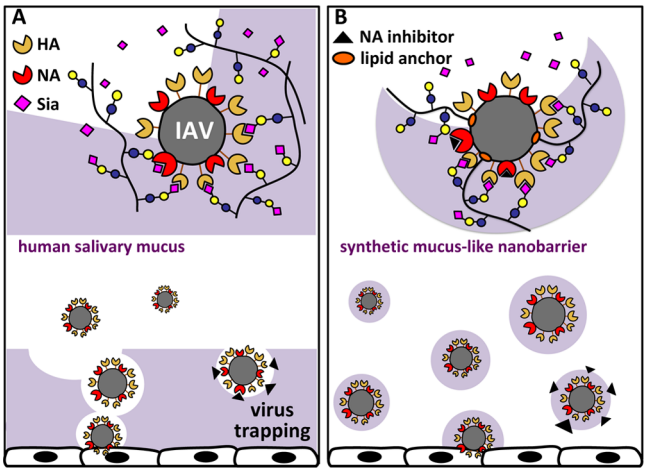
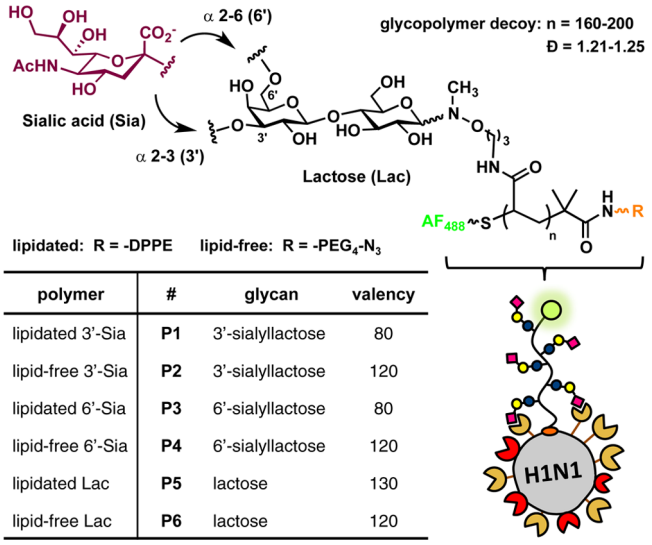


Figure 1. Secreted mucus engages influenza A viruses (IAV) en route to a host cell by presenting sialic acid glycan receptors to their hemagglutinin proteins (A). To avoid being trapped in the mucus, IAV employs neuraminidase (NA) enzymes to cleave sialic acids from the underlying mucin glycoproteins. Compositionally defined synthetic mucus-like nanoenvironments constructed around individual viruses allow for the identification of NA inhibitors that prevent infection by reinforcing the protective function of the mucosal barrier (B).



polymer	#	glycan	valency
lipidated 3'-Sia	**P1**	3'-sialyllactose	80
lipid-free 3'-Sia	**P2**	3'-sialyllactose	120
lipidated 6'-Sia	**P3**	6'-sialyllactose	80
lipid-free 6'-Sia	**P4**	6'-sialyllactose	120
lipidated Lac	**P5**	lactose	130
lipid-free Lac	**P6**	lactose	120

Figure 2. Mucin mimetic decoys comprise RAFT-derived glycopolymers carrying sialyllactose or lactose glycans armed with a 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethylamide (DPPE) lipid anchoring unit or a hydrophilic, lipid free, azido-tetraethylene glycol (PEG_4-N_3) group. The mimetics were tagged with AlexaFluor488 (AF488) for characterization and imaging.

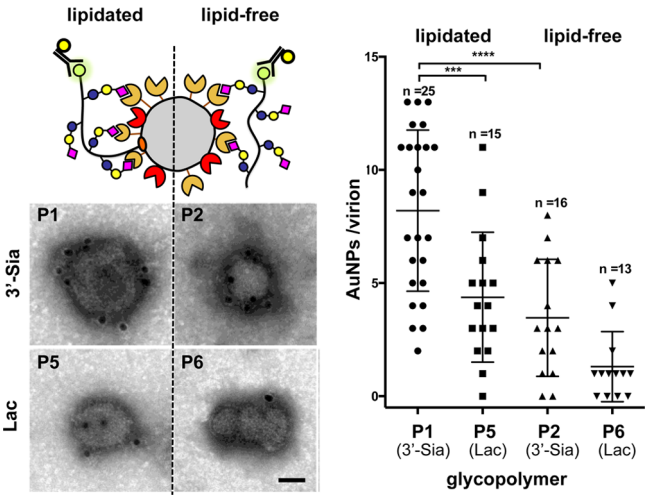


Figure 3. Incorporation of AF488-labeled decoys into the membranes of H1N1 virions was visualized by TEM after immunostaining with an anti-AF488 antibody conjugated with gold nanoparticles (Ab-AuNP, micrograph). The lipid anchor in polymers **P1** and **P5** promoted virion encapsulation, with precoordination of the sialoglycans in polymers **P1** and **P2** to the viral HA proteins providing additional enhancement (schematic and graph, ***$p \leq 0.001$, ****$p \leq 0.0001$ from Tukey's multiple comparison test).

envelope,[28,29] we have generated glycopolymers with a membrane-anchoring unit to facilitate the formation of a discrete nanoscale mucus-like environment proximal to individual virions. Such "nanobarriers" can be used to evaluate low-activity NA inhibitors for their potential to restrict the ability of IAV to escape from mucus and initiate infection (Figure 1B).

The passive insertion of lipidated glycoconjugates into membranes has emerged as a powerful tool to engineer new components into the cellular glycocalyx.[30−32] We have now extended this strategy to introduce mucin-mimetic glycopolymers terminated with a phospholipid tail to the surfaces of H1N1 (A/PR/8/34) virions (Figure 2). Using RAFT polymerization, we have generated polyacrylamide backbones with narrow chain length distributions and initiating with the lipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethylamide (DPPE; **P1**/**3**/**5**) as well as with a nonlipophilic azido-tetraethylene glycol moiety (PEG_4-N_3, **P2**/**4**/**6**). The polymers were decorated with *N*-methylaminooxypropyl side chains, which served as reactive sites for the attachment of 3′- and 6′-sialyllactose ligands for HA (**P1**/**2** and **P3**/**4**, respectively, in Figure 2).[27] Control polymers (**P5**/**6**) were functionalized with the nonbinding glycan lactose. To facilitate tracking of the polymers, their thiol termini were capped with an AlexaFluor 488 (AF488) maleimide dye.

To generate a discrete mucus-like environment, H1N1 (A/PR/8/34) viruses were incubated with lipidated 3′-sialyllactose (**P1**) or lactose (**P5**) glycopolymers, and membrane incorporation was examined with transmission electron microscopy (TEM) after immunostaining with an anti-AF488 antibody conjugated to gold nanoparticles (AuNPs, Figure 3). We observed higher levels of membrane incorporation for the sialylated polymers over those containing lactose glycans (8 ± 4 AuNPs/virion for **P1** vs 4 ± 3 AuNPs/virion for **P5**). This enhancement is likely due to precoordination of **P1** through binding to the viral HA and NA proteins. The lesser incorporation of the lipid-free polymers **P2** and **P6** (3 ± 3 vs 1 ± 2 AuNPs/virion, respectively) indicates the contributions from the membrane anchors to the surface remodeling process.

In the upper airways, the secreted mucus shields underlying epithelial cells from IAV infection by trapping the virus with sialylated mucin decoys, which are constantly cleared by mucociliary motion.[33] Inhibition of viral NA with oseltamivir can enhance this protective effect by impeding virus release from the mucus.[16] To replicate this mechanism in an *in vitro* assay, we treated Madin−Darby canine kidney (MDCK) cells adhered on a 96-well plate with purified sialylated human salivary mucins (HSM) followed by inoculation with H1N1

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(A/PR/8/34). After washing of excess HSM and virus, the cells were cultured, and H1N1 propagation was quantified after 24 h by measuring NA activity in the culture media.[34,35] We observed successful IAV infection with 1.5−3.0 nmol of HSM sialic acid per well (Figure S11). In contrast, when the virus is first preincubated with oseltamivir (1 μM), significantly less virus activity is detected in the cell media (Figure S11). Thus, inhibition of NA activity impairs the ability of IAV to escape from the HSM layer and to initiate infection.

In analogy to the HSM, the mucin mimetics also provided protection against IAV infection in the presence of oseltamivir in our assay (Figure 4). The MDCK cells were similarly

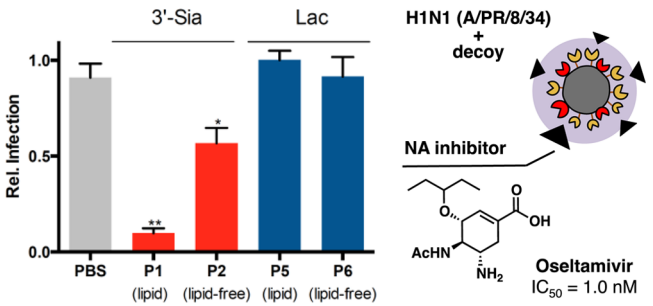

Figure 4. Sialoglycan decoys provide protection against IAV infection in the presence of the NA inhibitor oseltamivir. Relative infection was determined as a ratio of viral activity measured 24 h after inoculation with or without oseltamivir. Anchoring of decoy **P1** into the viral membrane via its lipid tail provides a robust mucus-like barrier difficult for the virus to breach in the presence of the inhibitor (*$p \leq 0.05$, **$p \leq 0.01$).

inoculated with the virus in the presence of the mucin mimetic decoys (17.5 nmol sialic acid/well) with or without oseltamivir (1 μM). Both 3′-sialyllactose polymers **P1** and **P2** significantly reduced IAV infection with oseltamivir. However, **P1** reduced infection to a much greater extent relative to **P2** (~80% vs ~35%, respectively), indicating the benefits of physically confining the virus within a mucus-like nanoenvironment. As expected, lactose polymers **P5** and **P6**, lacking sialic acid binding sites for either HA or NA, had no effect on infection (Figure 4). Polymers **P3** and **P4** carrying 6′-sialyllactose glycans, which engage HA proteins of H1N1 A/PR/8/34[27] but are not cleaved by its NA enzymes that preferentially hydrolyze α2−3 sialic acid glycosidic linkages,[36,37] provided no protection against infection in the presence of oseltamivir (Figure S12). The protective effect of the mucus-like nanoenvironment was also borne out in IAV hemagglutination experiments showing that the addition of oseltamivir (1 μM) improves the inhibitory capacity of the lipidated 3′-sialyllactose glycopolymer **P1** by ~16-fold (Tables S6−8 and S11). The lipidated mucin mimetics cannot differentiate between viral and cellular membranes, and, as expected, some membrane incorporation was observed in cells treated with the lipidated glycopolymers (Figure S13). However, the presence of these polymers on the cell surface does not significantly affect IAV infection (Figure S14).

Having established that the mucin-mimetic decoys can prevent infection, we next sought to introduce them into a screening platform to identify new NA inhibitors that prevent IAV infection by reinforcing the protective function of the mucosal barrier. We focused on a small panel of flavonoids known to inhibit NA (Figure 5). We hypothesized that,

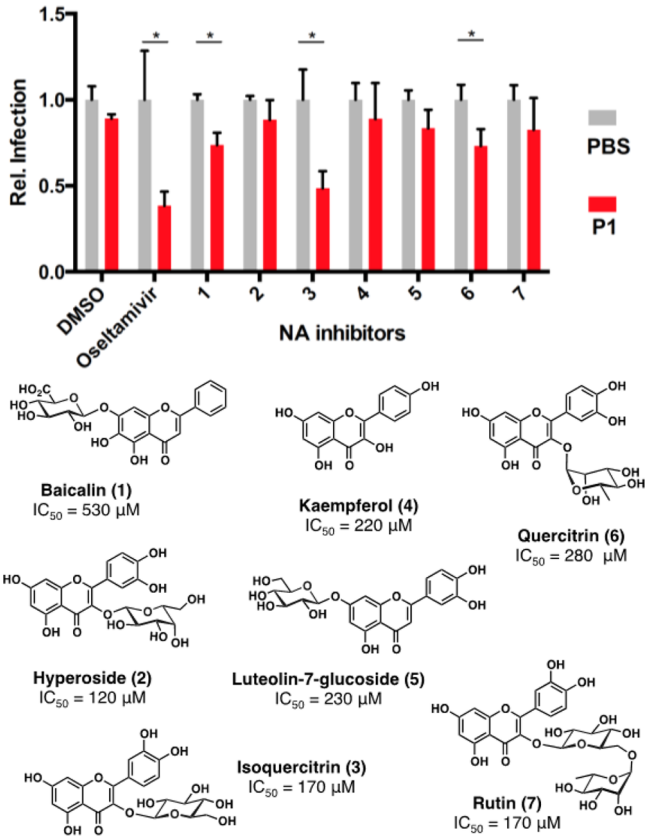

Figure 5. Flavonoids with known NA activity were tested for their ability to inhibit infection of MDCK cells by H1N1 (A/PR/8/34) remodelled with lipidated 3′-sialyllactose polymer decoy **P1**. Relative infection refers to a ratio of viral activity measured 24 h after inoculation with or without inhibitor. Isoquercitrin, a weak NA inhibitor, provided a similar extent of protection against IAV infection compared to the much more potent inhibitor oseltamivir (*$p \leq 0.05$).

although their low inhibitory activities ($IC_{50} \approx 10^2$ μM compared to ~1 nM for oseltamivir)[38,39] would likely disqualify them as hits in traditional screens, flavonoids may provide sufficient protection against IAV by enhancing virus trapping in the clearable mucus.

To test their ability to inhibit IAV, flavonoids **1**−**7** (0.1 mg/mL) as well as oseltamivir (1 μM) were incubated with the virus. MDCK cells were inoculated with the virus-inhibitor mixture with the lipidated glycopolymers, and washed. Fresh media was added and virus propagation was quantified after 24 h. As expected, oseltamivir reduced IAV infection by ~60−70% in the presence of **P1** compared to PBS (Figure 5). Among the flavonoids, several compounds exhibited some protection against IAV with isoquercitrin (**3**) inhibiting infection by ~50%. Isoquercitrin also enhanced glycopolymer-mediated inhibition in an IAV agglutination assay (Tables S9−S11). This indicates that despite its poor NA activity, isoquercitrin can provide protection against IAV at levels approaching those of oseltamivir, a much more potent inhibitor.

In summary, we report a conceptually novel drug-screening platform to identify inhibitors of viral NA to prevent the release of virions from sialylated mucins in mucosal barriers and, thereby, reduce infection of underlying cells. Using synthetic, membrane-anchored glycopolymer mimetics of mucin glycoproteins, we assembled a mucus-like environment at the virus surface with tunable size and glycan composition. These

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discrete nanobarriers can be used to identify small molecules that block viral escape from mucus and prevent infection. This technique is general and can be broadened beyond the small group of test compounds employed in this proof-of-concept study to identify potential new prophylactic antivirals among weak NA inhibitors that otherwise might have been overlooked in traditional drug screening assays.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.6b00191.

Synthesis and characterization of the mucin-mimetic glycopolymers, virus remodeling, TEM imaging, IAV infection, and agglutination assays (PDF)

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Notes

The authors declare no competing financial interest.

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