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Targeting Organic Anion Transporter 3 with Probenecid as a Novel Anti-Influenza A Virus Strategy

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Influenza A virus infection is a major global health concern causing significant mortality, morbidity, and economic loss. Antiviral chemotherapeutics that target influenza A virus are available; however, rapid emergence of drug-resistant strains has been reported. Consequently, there is a burgeoning need to identify novel anti-influenza A drugs, particularly those that target host gene products required for virus replication, to reduce the likelihood of drug resistance. In this study, a small interfering RNA (siRNA) screen was performed to identify host druggable gene targets for anti-influenza A virus therapy. The host organic anion transporter-3 gene (OAT3), a member of the SLC22 family of transporters, was validated as being required to support influenza A virus replication. Probenecid, a prototypical uricosuric agent and chemical inhibitor of organic anion transporters known to target OAT3, was shown to be effective in limiting influenza A virus infection *in vitro* (50% inhibitory concentration [IC_{50}] of 5.0×10^{-5} to 5.0×10^{-4} μM; $P < 0.005$) and *in vivo* ($P < 0.05$). Probenecid is widely used for treatment of gout and related hyperuricemic disorders, has been extensively studied for pharmacokinetics and safety, and represents an excellent candidate for drug repositioning as a novel anti-influenza A chemotherapeutic.

Influenza A virus is a global public health concern, causing morbidity and substantial mortality (1), with increasing trends in disease severity in the members of the population who are age 65 or older (2). Influenza vaccines are available; however, due to high virus mutation rates and constant antigenic drift, vaccines need to be developed annually (3). Currently, several antiviral drugs, including the neuraminidase (NA) inhibitors zanamivir and oseltamivir and the M2 ion channel inhibitors (amantadine and rimantidine), are available to treat influenza A virus infection (3–6). These antiviral drugs target viral components, a feature which provides selective pressure for development of drug resistance. The rapid emergence of drug-resistant influenza A virus strains has been increasingly reported, with a limited number of new antiviral drugs in the pipeline (7), highlighting the imminent need to identify novel drug targets and for the subsequent development of a new class of antiviral drugs.

High cost and lengthy approval processes are associated with development of new drugs for clinical use. Due to these factors, along with the limited number of new antiviral drugs currently in the pipeline, there is an increasing need for repositioning available currently approved drugs for treatment of other diseases. Drug repositioning allows faster availability of new treatments for a fraction of the cost of developing new drugs. Furthermore, the safety and pharmacokinetics of these drugs have already been assessed. Examples of such repositioning include the use of anticancer drugs zidovudine (AZT), decitabine, and gemcitabine for HIV (human immunodeficiency virus) treatment (8) and of the erectile dysfunction drug sildenafil, which was originally developed for antihypertension treatment (9).

Recently, numerous studies have utilized an RNA interference (RNAi) screen to identify cellular factors involved in virus replication. This strategy takes advantage of the fact that viruses lack their own machinery to replicate independently; thus, most use or co-opt host-derived gene products to facilitate entry, replication, and release. Therefore, targeting host factors involved in virus replication may serve as a therapeutic and/or prophylactic disease intervention strategy. In this study, RNAi was used to identify novel host drug targets for influenza A antiviral therapies. A small interfering RNA (siRNA) screen targeting 4,795 druggable genes in human lung type II epithelial (A549) cells was performed with influenza A/WSN/33 virus (WSN). This gene library was chosen for the druggability properties of the host gene products previously shown to favor interaction with drug compounds, thereby increasing the likelihood of identification of pharmacological inhibitors (10). Various druggable genes were identified in the siRNA screen, but only a few host genes were validated to substantially affect influenza A virus replication. One of these host genes was the organic anion transporter-3 gene, OAT3, a member of the SLC22 gene family. Transfection of A549 cells with siRNA targeting the SLC22A8 gene, also known as OAT3, completely blocked influenza A/WSN/33(H1N1) virus replication. The solute carrier (SLC) superfamily comprises 298 members grouped into 43 families, including SLC22 (11). The SLC22 family is further subdivided into three subfamilies: organic cation transporters (OCT), zwitterion/cation transporters (OCTN), and organic anion transporters (OAT) (12). The role of OAT transporters in the lung is not well characterized, but their substrates in intestine, liver, and kidney are targeted by several drugs that include diuretics, nonsteroidal anti-inflammatory compounds, β-lactam antibiotics, several antiviral drugs, xenobiotics, and endogenous compounds, e.g., cyclic nucleotide endogenous metabolites (11–13). Elsewhere, OAT family members have been implicated in homeostasis

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and sensing in brain, heart, eye, muscle, and olfactory epithelium (13). OAT3 is mainly expressed in the basolateral membrane of the kidney proximal tubule; however, its expression also occurs elsewhere, including the luminal surface of choroid plexus in brain, skeletal muscle, developing bone, and adrenal glands (12). In this report, we show that OAT3 is also expressed in both human and mouse lung epithelial cells and that siRNA silencing of other closely related transporters, i.e., OAT1, OAT2, OAT4, OAT7, and URAT1, did not affect influenza A/WSN/33 virus replication, indicating a specific role of OAT3 to support influenza A virus replication.

Probenecid {4-[(dipropyl-amino)sulfonyl] benzoic acid} is a classical inhibitor of OAT and is widely prescribed for therapeutic treatment of gout and other hyperuricemic disorders (14, 15). Probenecid usage for treatment of other OAT-mediated disorders such as hypertension has also been explored, along with its use to extend the plasma level of drugs identified as OAT substrates (such as β-lactam antibiotics and several antiviral drugs) (16–19). In this study, probenecid was shown to reduce OAT3 mRNA and protein levels *in vitro* and *in vivo*. Administration of probenecid alone reduced influenza A virus titer in agreement with the finding that OAT3 is important for influenza A virus replication. Additionally, probenecid has been previously reported to elevate plasma concentrations of an active oseltamivir metabolite, oseltamivir carboxylate; thus, its coadministration with oseltamivir has been suggested (19–21). This report shows that probenecid, the classical OAT3 inhibitor, can potentially be repositioned for a new anti-influenza A therapy.

MATERIALS AND METHODS

Cell cultures, influenza A virus stocks, and mice used. Human type II respiratory epithelial (A549) cells (ATCC; CCL-185) and Madin-Darby canine kidney (MDCK) cells (ATCC; CCL-34) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) in a 37°C incubator with 5% CO_2. Influenza virus strains A/WSN/33(H1N1), A/New Caledonia/20/99(H1N1) (New Caledonia), A/California/04/09(H1N1), A/California/07/09(H1N1) (CA), and A/Philippines/2/82/X-79(H3N2) (X-79) were propagated in 9-day-old embryonic chicken eggs, and titers in MDCK cells were determined as previously described (22, 23).

BALB/c female mice (6 to 8 weeks old) were obtained from the NCI (National Cancer Institute). All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia. All experiments were performed with 10 mice per group and repeated independently at least twice.

Human drug target library screen. A primary RNAi screen using four pooled siRNAs to target each of the 4,795 genes in the human drug target library (SMARTpool; Dharmacon ThermoFisher, Lafayette, CO) was performed using A549 cells infected with influenza A/WSN/33(H1N1) virus at a multiplicity of infection (MOI) of 0.001 as previously described (24, 25). A siRNA pool targeting MEK (also known as mitogen-activated protein kinase kinase [MAPKK]) was used as a positive control due to the known role of MEK in influenza A virus replication (26). A nontargeting siRNA was also used as negative control. A549 cells were reverse transfected with siRNA using DharmaFECT-1 reagent (Dharmacon, Lafayette, CO) as previously described (25). Briefly, siRNAs resuspended in Hanks' balanced salt solution (HBSS) were incubated with DharmaFECT 1 to form transfection complexes in 96-well tissue culture dishes. Following formation of complexes, 1.5×10^4 A549 cells suspended in DMEM with serum were added into each well containing a siRNA transfection complex. The final concentration of pooled siRNA oligonucleotides was 50 nM per well, with a final DharmaFECT amount of 0.4 μl per well in a 100-μl culture volume. Transfections were carried out for 48 h to allow maximal expression knockdown before cells were infected with influenza A/WSN/33 virus at an MOI of 0.001. The level of infectious virus was measured 48 h postinfection by titration of A549 cell supernatants on MDCK cells (22), and the results were normalized to nontargeting siRNA-transfected cell levels. In addition, adherent A549 cells on culture plates were fixed and analyzed for the presence of influenza virus nucleoprotein (NP) by immunofluorescence staining as described below. All assays were run in duplicate, and the entire screening assay was repeated twice.

***In vitro* and *in vivo* inhibition assays.** Probenecid (Invitrogen, Carlsbad, CA), the OAT3 pharmacological inhibitor, was resuspended in phosphate-buffered saline (PBS), and its cellular toxicity on A549 cells was determined by a ToxiLight BioAssay (Lonza, Walkerville, MD). For *in vitro* analyses, A549 cells were pretreated with increasing concentrations of probenecid for 24 h. Cells were subsequently infected with influenza virus A/WSN/33(H1N1), A/New Caledonia/20/99(H1N1), A/California/07/09(H1N1), or A/Philippines/2/82/X-79(H3N2) at the indicated MOI. At 24 or 48 h postinfection, cells were fixed with cold methanol:acetone for subsequent anti-NP immunostaining as described below or collected for total RNA isolation using a Qiagen RNeasy kit (Qiagen) to assess the OAT3 expression level and influenza A virus copy number using quantitative reverse transcription-PCR (qRT-PCR).

For *in vivo* studies to evaluate lung virus burden, probenecid was administered intraperitoneally (i.p.) at doses and time points pre- or post-influenza A virus infection as indicated in Results. At each indicated time point, mice were intranasally inoculated with a mouse-adapted strain of influenza A/WSN/33(H1N1) virus, nonadapted influenza A/New Caledonia/20/99(H1N1) virus, or influenza A/California/04/09(H1N1) virus at their respective 50% lethal dose (LD_{50}) of 70 PFU, 22 PFU, or 35 PFU. Lungs were collected 48 h postinfection, which corresponds to peak lung virus replication, and longitudinally sectioned for qRT-PCR and 50% tissue culture infective dose ($TCID_{50}$) analyses. Left lobes of lungs were homogenized in serum-free DMEM to assess virus titer, and right lobes were homogenized in TRizol solution (Invitrogen, Carlsbad, CA) for total RNA isolation. For virus titration analyses, lung homogenates were serially diluted and the titer was determined on MDCK cells for 72 h. Hemagglutination (HA) assays were performed using turkey red blood cells (RBCs) and virus-infected MDCK cell supernatant as described previously (22, 27). The HA titer was determined from the highest dilution factor that produced a positive HA reading, and virus titers were calculated as the $TCID_{50}$ using the Spearman-Karber formula (22, 27). For the *in vivo* survival study, mice were i.p. treated with PBS only or probenecid, as indicated in Results, pre- or postinfection with $10\times$ LD_{50} of influenza A/WSN/33(H1N1) virus (2.2×10^3 PFU per mouse). Mice were monitored for 14 days postinfection for survival and weight loss under the University of Georgia IACUC guideline.

Gene expression analyses. For measurement of the influenza A viral copy number, total RNA collected from infected A549 cells, or lungs of infected mice, was used for a quantitative RT-PCR assay using a OneStep RT-PCR kit (Qiagen). A 5-ng volume of total RNA was used per reaction. A universal influenza A virus primer-probe set was used for amplification and detection of influenza A virus RNA (InfA forward [SS118272-45], InfA reverse [SS118272-46], and InfA probe [SS118273-01]; Bioresearch Technologies, Inc., Novato, CA; provided by the CDC). RT-PCR was performed using an MX3005P thermocycler (Strategene; Agilent Technologies, Santa Clara, CA) under the following conditions: 30 min at 50°C (reverse transcription); 15 min at 95°C (*Taq* inhibitor activation); and 45 cycles of PCR amplifications (95°C for 15 s, 55°C for 30 s).

To assess OAT3 gene expression, cDNAs were synthesized from total RNA using a SuperScript Vilo cDNA synthesis kit (Invitrogen, Carlsbad, CA). cDNAs were subsequently used for quantitative PCR amplifications using OAT3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene-specific primers and RT^2 SYBR Green qPCR Master Mix (SABioscience) in an MX3005P thermocycler. The PCR conditions used for amplification were as follows: 10 min at 95°C followed by 40 cycles of 95°C for 30 s, 60°C

for 1 min, and 72°C for 30 s. OAT3 expression was normalized to GAPDH expression, and its expression relative to mock-treated samples was calculated using the $2^{(-\Delta\Delta Ct)}$ formula.

Immunoblot analyses. To evaluate OAT protein expression following siRNA transfection or probenecid treatment, A549 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, and 0.1% sodium dodecyl sulfate) supplemented with a protease inhibitor cocktail tablet (Roche, Germany), followed by 4°C centrifugation at 16,000 × *g* for 10 min to clarify lysates. Equivalent protein amounts were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting (Bio-Rad, Hercules, CA). Rabbit anti-OAT3 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-GAPDH polyclonal antibodies were used as primary antibodies for immunoblot analyses. Allophycocyanin (AP)-conjugated goat anti-rabbit IgG was used as a secondary antibody. Protein bands were visualized following addition of ECF substrate (GE Healthcare, Piscataway, NJ) and scanned using an Amersham Typhoon 9210 fluorescence scanner (GE Healthcare, Piscataway, NJ). Protein bands were quantified using ImageQuant software, and expression of OAT3 was normalized to expression of GAPDH.

Immunofluorescence staining. A549 cells were fixed with cold methanol:acetone (80:20) for 15 min and incubated with primary antibodies (mouse anti-NP monoclonal antibody [ATCC; H16-L10-4R5] [5 μg/ml] and/or goat anti-OAT3 polyclonal antibody [Santa Cruz Biotechnology, Santa Cruz, CA] [4 μg/ml]) followed by incubation with appropriate secondary antibodies (Alexa 488-conjugated goat anti-mouse, Alexa 488-conjugated donkey anti-goat, and/or Alexa 546-conjugated goat anti-rabbit [Invitrogen, Carlsbad, CA] [1 μg/ml]) and 4′,6-diamidino-2-phenylindole (DAPI) counterstain (Invitrogen, Carlsbad, CA) (2 μg/ml). Cells were visualized and counted using a Cellomics ArrayScan system (Thermo Fisher Scientific), an automated fluorescence microscope coupled with image and analytical software. Where indicated, percentages of infected cells (positive for NP staining) were calculated for probenecid 50% inhibitory concentration (IC_{50}) analysis.

Statistical analyses. Statistical analyses were done using Student's *t* test or one-way analysis of variance (ANOVA), as indicated. Results were calculated as means ± standard errors. Values of $P < 0.05$ were considered significant.

RESULTS

siRNA screen of host drug target genes. To identify novel host druggable targets, a screen was performed using a drug target siRNA library comprised of siRNA pools targeting 4,795 genes. The screen was performed in A549 cells infected with influenza A/WSN/33(H1N1) (WSN) virus at an MOI = 0.001 (Fig. 1A). As assay endpoints, infected cells were fixed and immunostained for influenza virus NP and culture supernatant was collected to measure virus titer by a hemagglutination (HA) assay 48 h following infection. Z-scores were calculated from HA assay results and plotted to display their distributions (Fig. 1B). Genes with negative Z-score values reduced influenza A virus titers when silenced with specific siRNA pools, indicating their importance for influenza A virus replication. Conversely, genes with positive Z-score values increased virus titer upon siRNA silencing, indicating their role in limiting influenza A virus replication. Transfection with MEK siRNA was used as a positive control due to its known importance in cellular signaling during influenza A virus infection. Genes with known pharmacological inhibitors available were chosen for further examinations in which the effects of these inhibitors on influenza A virus infection were assessed.

In the siRNA screen, the OAT3 (SLC22A8) gene was identified and subsequently validated as a druggable target gene for influenza A virus infection. Transfection of A549 cells with four pooled siRNAs targeting different seed regions on the OAT3 gene reduced virus titer in culture supernatant (Z-score = −0.854) compared to transfection of cells with nontargeting siRNA (Fig. 1B). Additionally, no influenza virus NP-positive cells were detectable following OAT3 siRNA transfection (Fig. 1C), supporting the idea of OAT3 as important for influenza A virus replication. siRNA transfection of other OAT family members in the library displayed weakly moderate or no reduction in virus titers or in the number of detectable NP-positive cells (Fig. 1B and C; see also Fig. S1 in the supplemental material). Curiously, transfection of siRNA targeting a closely related molecule, OAT1 (SLC22A6), which was previously reported to display tissue distributions and functions similar to those of OAT3 (12), resulted in increased virus titer (Z-score = 1.043) and numbers of NP-positive cells compared to nontargeting siRNA-transfected cell results (Fig. 1B and C), suggesting opposing roles of OAT1 and OAT3 during influenza A virus replication. Immunoblot analyses were performed to verify OAT3 protein expression following nontargeting (NT), OAT3, or OAT1 siRNA transfections (Fig. 1D and E). Transfection of OAT3 siRNA, but not nontargeting or OAT1 siRNA, resulted in a 61% reduction of OAT3 protein expression, indicating efficiency and specificity of OAT3 protein expression silencing by siRNA transfection. Together, these results demonstrate the importance of OAT3 during influenza A virus replication. Using gene pathway analysis, it was hypothesized that targeting OAT3 using its classical pharmacological inhibitor, probenecid, may limit influenza A virus replication.

Probenecid reduces OAT3 mRNA and protein levels in a dose-dependent manner. To examine the mechanism by which probenecid may affect OAT3 function, A549 cells were treated with increasing doses of probenecid, and OAT3 mRNA and protein levels were determined at 24 and 48 h following treatment. Twenty-four hours of probenecid treatment at a concentration of 1 μM or higher resulted in a >40% reduction in the OAT3 mRNA level as measured by qRT-PCR (Fig. 2A). A similar reduction was observed in cells treated with 0.1 μM probenecid for 48 h. Likewise, a 41% reduction of OAT3 protein expression was observed in cells treated with 1 μM probenecid for 24 h as assessed by immunoblotting (Fig. 2B) and a reduction of OAT3 staining was observed by immunofluorescence microscopy (Fig. 2C). Cytotoxicity assays were performed using A549 cell culture supernatant treated with increasing doses of probenecid for 24 or 48 h. Probenecid had a low level of cytotoxicity even when used at higher (1 mM) concentrations (Fig. 2D). These results show that probenecid acts to reduce OAT3 mRNA and protein expression and has minimal cytotoxicity and show for the first time that human lung epithelial (A549) cells express both OAT3 mRNA and protein.

Probenecid reduces influenza A virus replication *in vitro*. The effect of probenecid treatment on influenza A virus replication was evaluated in A549 cells pretreated with increasing doses of probenecid prior to infection with WSN at two different MOIs for 24 h. Pretreatment of cells with 10^{-1} μM probenecid significantly ($P < 0.005$) reduced virus copy numbers at both a low and a higher MOI of 0.05 and 0.5, respectively (Fig. 3A). A substantial reduction of virus copy numbers was also observed in cells infected with influenza A/New Caledonia/20/99 virus (H1N1; New Caledonia) (data not shown). As in Fig. 2A, probenecid treatment also reduced the OAT3 mRNA level in cells infected with WSN or New Caledonia virus (see Fig. S2 in the supplemental material). The percentage of NP-positive cells in probenecid-pretreated cells

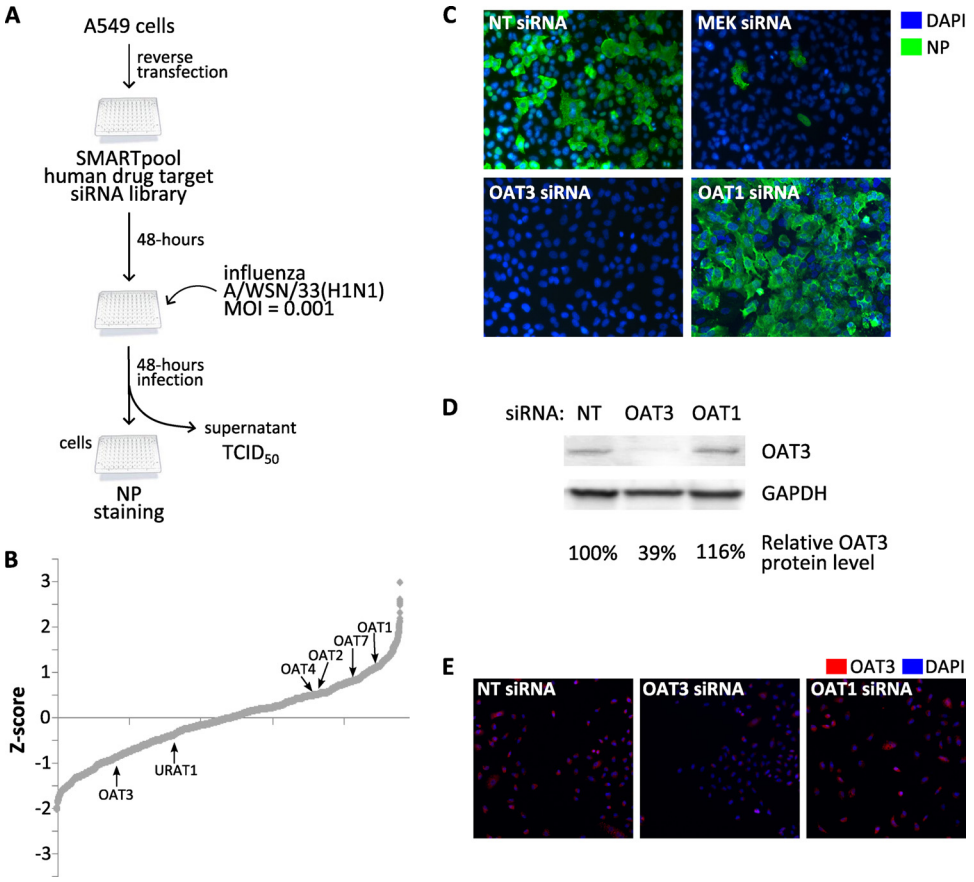


FIG 1 siRNA screen of host drug target genes important for influenza A virus replication. (A) Schematic outline of siRNA screen performed. (B) Z-score values were calculated based on virus titers for siRNAs targeting host drug target genes. Their distributions were plotted based on Z-score rank order, from siRNA with the lowest to that with the highest virus titer. Labels denote OAT gene members within the drug target gene library. (C) A549 cells were transfected with nontargeting (NT) siRNA and pooled siRNA to MEK, OAT3, or OAT1 for 48 h prior to infection with influenza A/WSN/33(H1N1) virus at an MOI = 0.001. Cells were fixed 48 h postinfection, stained for viral NP (green) and nuclei (DAPI; blue), and visualized using fluorescence microscopy. (D and E) OAT3 protein expression was evaluated by immunoblot (D) and immunofluorescence microscopy (E) following transfection of nontargeting (NT) siRNA and pooled siRNA to OAT3 or OAT1. For immunoblot analysis, OAT3 protein bands were quantified and normalized to GAPDH, and percent normalized OAT3 protein levels relative to nontargeting siRNA control were calculated. For immunofluorescence analysis, cells were stained for OAT3 (red) and nuclei (DAPI; blue) and visualized using fluorescence microscopy.

infected with WSN or New Caledonia virus for 24, 36, or 48 h was determined using a Cellomics ArrayScan immunofluorescence system and normalized to mock-treated cells to calculate the probenecid IC_{50} under each infection condition. It was determined that probenecid is effective in limiting influenza A virus replication *in vitro* at a IC_{50} ranging between 5.0×10^{-5} and 5.0×10^{-4} μM, depending on the influenza A virus strains and length of infection (Fig. 3C). To evaluate the effectiveness of probenecid in limiting replication of other influenza A virus strains, A549 cells were pretreated with increasing doses of probenecid and infected with influenza A/California/07/09(H1N1) (CA) (Fig. 3D) or A/Philippines/2/82/X-79(H3N2) (X-79) at a low and a higher MOI. In both cases, probenecid treatment reduced the percentage of NP-positive cells, although these reductions plateaued at 50% or higher versus mock-treated cells.

Probenecid reduces lung OAT3 expression and limits influenza A virus replication *in vivo*. As probenecid was effective in limiting replication of several strains of influenza A virus *in vitro*, its effects were evaluated in mice. Two probenecid doses were tested in BALB/c mice prior to infection with WSN, New Caledonia, or CA virus at their respective LD_{50}s (Fig. 4A). The levels of OAT3 mRNA were assessed in the lungs of mock- or influenza A virus-infected mice. Mice treated with 200 mg probenecid/kg of body weight at 24 h prior to infection with WSN (Fig. 4B), New Caledonia, or CA (data not shown) had a reduced lung OAT3 mRNA level as measured by qRT-PCR, with moderate decreases associated with lower doses of probenecid treatment. Importantly, there was a statistically significant reduction of lung virus load in mice pretreated with probenecid and infected with WSN, New Caledonia, or CA compared to PBS-treated mice as measured by qRT-PCR (Fig. 4C) and by evaluation of lung virus titers ($TCID_{50}$) (Fig. 4D; $P < 0.0001$). Maximum reductions of lung virus load were evident when mice were treated with 200 mg probenecid/kg at 24 h prior to infection ($P < 0.0001$), although significant ($P < 0.05$) reductions were also observed with a lower dose of probenecid (10 mg/kg).

Therapeutic treatment with probenecid effectively reduces influenza A lung virus burden and provides partial protection against lethal influenza A virus infection *in vivo*. To compare different probenecid treatment regimens, mice were prophylacti-

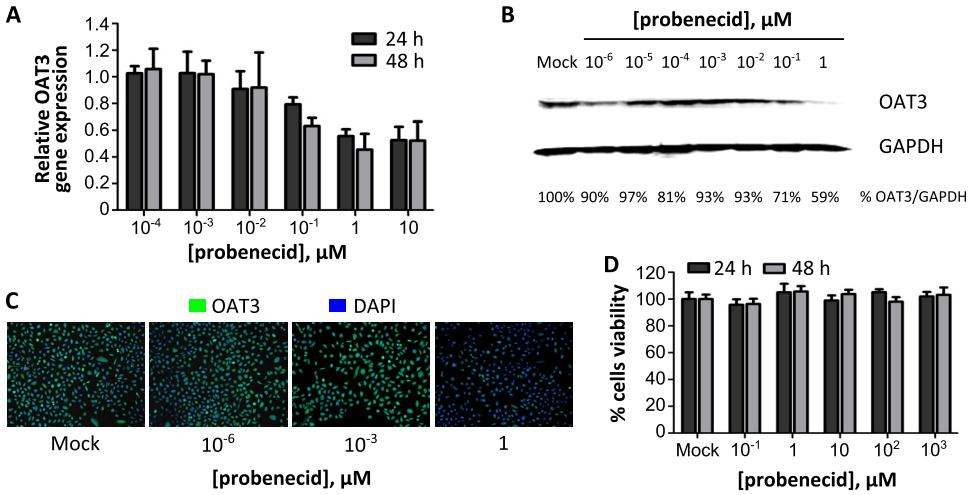


FIG 2 The OAT inhibitor probenecid reduces OAT3 mRNA and protein levels *in vitro* in a dose-dependent manner. (A to C) A549 cells were treated with increasing doses of probenecid. At 24 and 48 h posttreatment, cells were fixed for immunostaining or harvested for total RNA and protein. (A) OAT3 mRNA levels were assessed using qRT-PCR and normalized to GAPDH. Fold changes of OAT3 expression were calculated relative to mock-treated cell results. (B) OAT3 and GAPDH protein levels following 24 h of probenecid treatment were evaluated by immunoblot analysis, and protein bands were quantified. OAT3 protein levels were normalized to GAPDH, and percent OAT3 protein levels were calculated relative to mock-treated cell results. (C) Cells were fixed at 24 h following probenecid treatment, stained for OAT3 (green) and nuclei (DAPI; blue), and visualized using fluorescence microscopy. (D) Probenecid demonstrated low cellular cytotoxicity as evaluated by a ToxiLight BioAssay kit.

cally (24 h preinfection) or therapeutically (24 h postinfection) administered probenecid at 25 mg/kg and challenged with mouse-adapted WSN. Mice treated pre- or post-WSN infection had significantly ($P < 0.05$) reduced lung virus titers compared to mice subjected to mock treatment (Fig. 5A). The ability of probenecid to protect mice against infection with WSN at a lethal dose (i.e., 10× LD_{50}, or 2.2 × 10^3 PFU/mouse) was evaluated. Mice were treated prophylactically with 200 mg/kg of probenecid (24 h preinfection) or therapeutically with 200 mg/kg probenecid (24 h postinfection) or were administered 25 mg/kg probenecid daily for 3 days following infection (days 1, 2, and 3) and monitored for mortality or morbidity for the duration of the 14-day study. Mice treated daily over 3 days with 25 mg/kg probenecid following lethal infection were partially protected (60% survival; $P < 0.05$), whereas single-dose probenecid treatment given either prophylactically or therapeutically resulted in less protection, although the data were statistically nonsignificant (Fig. 5B). The reduced efficacy may relate to pharmacokinetics and drug decay over that time. These results indicate that probenecid can be utilized as a chemoprophylactic or chemotherapeutic treatment; however, multiple administrations of probenecid are required for ideal protection.

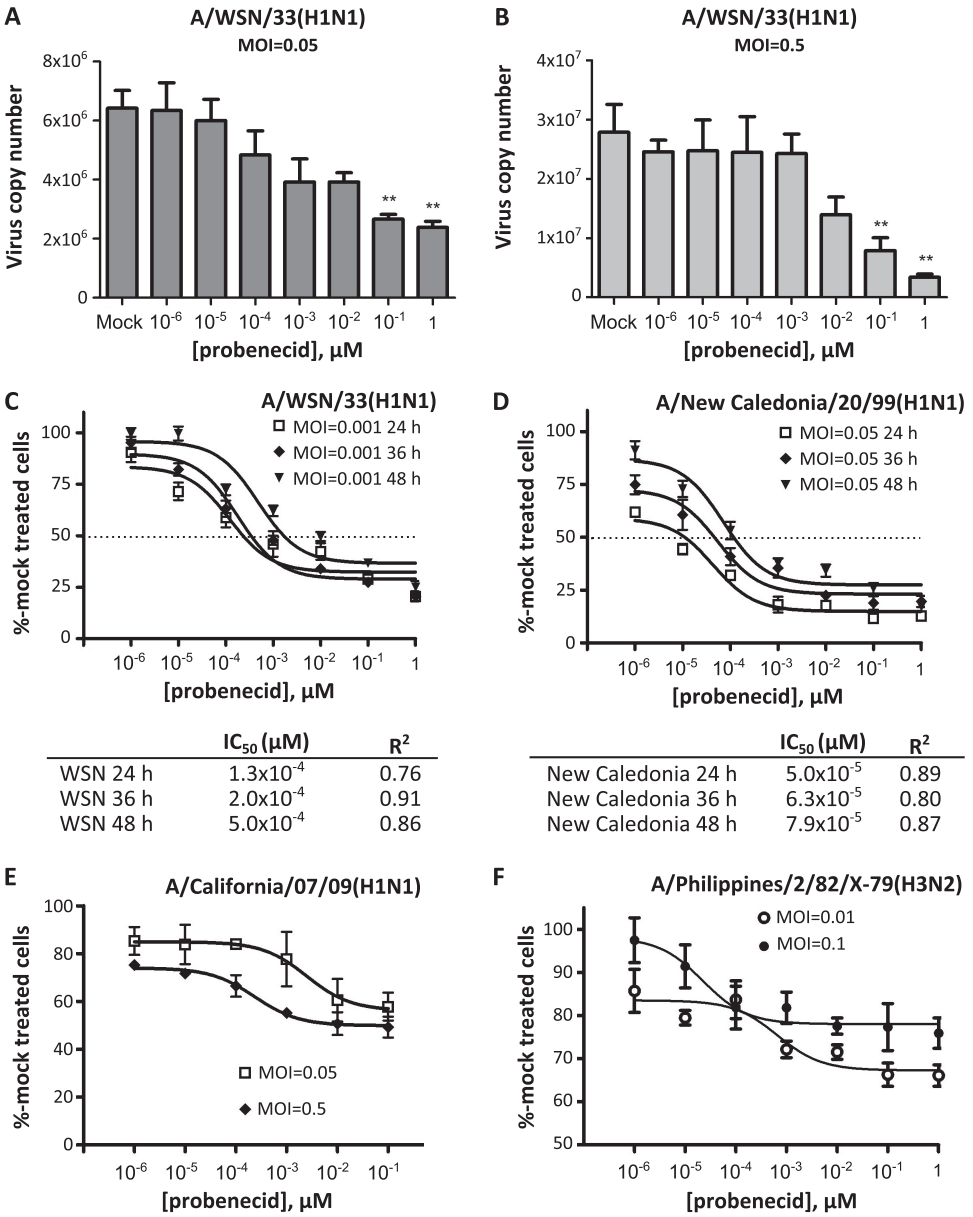
Since probenecid coadministration with oseltamivir has been reported to result in elevated oseltamivir carboxylate serum levels due to perturbation of OAT (19), a combinational probenecid/oseltamivir treatment regimen was evaluated *in vivo*. Mice infected with WSN or New Caledonia virus were treated 24 h later with different single or combinational probenecid and/or oseltamivir regimens once or twice daily for 72 h (see Fig. S3 in the supplemental material). All treatment conditions evaluated resulted in a significant ($P < 0.0001$) reduction of lung virus load. Treatment with a probenecid/oseltamivir combination twice daily better reduced New Caledonia lung virus titers than the recommended oseltamivir treatment for humans, which is a twice-daily regimen (see Fig. S3C in the supplemental material; $P = 0.0003$) (6). This regimen also appeared to better to limit WSN infection, although the findings were not statistically significant (see Fig. S3B in the supplemental material; $P = 0.115$). Collectively, these data feature probenecid as a new candidate for drug positioning for a novel class of anti-influenza A therapeutics.

DISCUSSION

Despite the availability of two different classes of approved anti-influenza drugs and the availability of vaccines, influenza A virus infection remains a major worldwide concern due to significant morbidity, mortality, and pandemic and epidemic potential. The occurrence of influenza A virus strains resistant to approved anti-influenza chemotherapeutics is a significant concern. Furthermore, there are limited numbers of novel anti-influenza A drugs under development, highlighting the need for anti-influenza drug discovery.

In this study, a siRNA screen was performed using a host drug target siRNA library in A549 cells infected with influenza A/WSN/33 virus. The screen and subsequent validation confirmed that a member of the organic anion transporter (OAT) family, OAT3, was important for influenza A virus replication. While it remains unknown how silencing OAT3 expression specifically decreases influenza A virus replication, it is hypothesized that transport of a particular host or virus factor through OAT3 is required during the course of infection. As siRNA silencing of OAT3 results in a complete block of influenza virus NP expression, it is likely that that this event occurs early during infection, i.e., before viral protein synthesis takes place. Further studies are needed to identify the OAT3 substrate that is required for influenza A virus infection.

Interestingly, this study showed that OAT3 functions to specifically facilitate influenza A virus replication among members of the OAT family, as siRNA transfection targeting other OAT members such as OAT1, −2, -4, and -7 and URAT1 did not result in substantial reduction of influenza A virus replication. All genes,



	IC_{50} (μM)	R^2
WSN 24 h	1.3×10^{-4}	0.76
WSN 36 h	2.0×10^{-4}	0.91
WSN 48 h	5.0×10^{-4}	0.86

	IC_{50} (μM)	R^2
New Caledonia 24 h	5.0×10^{-5}	0.89
New Caledonia 36 h	6.3×10^{-5}	0.80
New Caledonia 48 h	7.9×10^{-5}	0.87

FIG 3 The OAT inhibitor probenecid reduces influenza A virus replication *in vitro* in a dose-dependent manner. A549 cells were treated with increasing doses of probenecid for 24 h prior to infection with various strains of influenza A virus. (A and B) At 24 h following infection with influenza A/WSN/33(H1N1) virus at an MOI of 0.05 (A) or 0.5 (B), total RNA was collected and influenza A virus copy numbers were assessed using qRT-PCR. Graphs represent results from triplicate experiments, and error bars denote standard errors of the means. Statistical analyses were performed using one-way ANOVA. **, $P < 0.005$. (C to F) At 24, 36, or 48 h postinfection with A/WSN/33(H1N1) (C) or A/New Caledonia/20/99(H1N1) (D) or 24 h postinfection with A/California/07/09(H1N1) (E) or A/Philippines/2/82/X-79(H3N2) (F) at the indicated MOI, cells were fixed and stained for viral NP (green) and nuclei (DAPI; blue). Percent values of NP-positive cells were quantified using the Cellomics array scan system, normalized to mock treated cells, and plotted. Dose curves and IC_{50}s (when indicated) were determined using nonlinear regression. Graphs represent results from six replicate experiments, and error bars denote standard errors of the means.

with the exception of URAT1, appear to have roles opposite that of OAT3 during influenza A virus replication. Transporters of the OAT family are polyspecific such that they can transport multiple substrates and can translocate anions in either direction (12). Therefore, it is possible that OAT3 specificity may be explained in part by the directionality of a particular substrate or protein involved during influenza A replication, particularly during transport across cellular membranes. A known example of this directionality of transport is the urate reabsorption carried out by OAT1, OAT3, OAT4, and URAT1. OAT4 and URAT1 located at the luminal side of human kidney proximal tubule mediate reabsorption of urate into the proximal tubule cells. Conversely, OAT1 and OAT3 which are located on the basolateral surface mediate urate transport from the proximal tubule cells into plasma (12, 15, 28). Modulation of urate transport by OATs has been utilized for treatment of gout, a condition that is caused by accumulation of uric acid. Probenecid, a chemical inhibitor for OAT transport, is currently used to promote uricosuria, or the release of urate

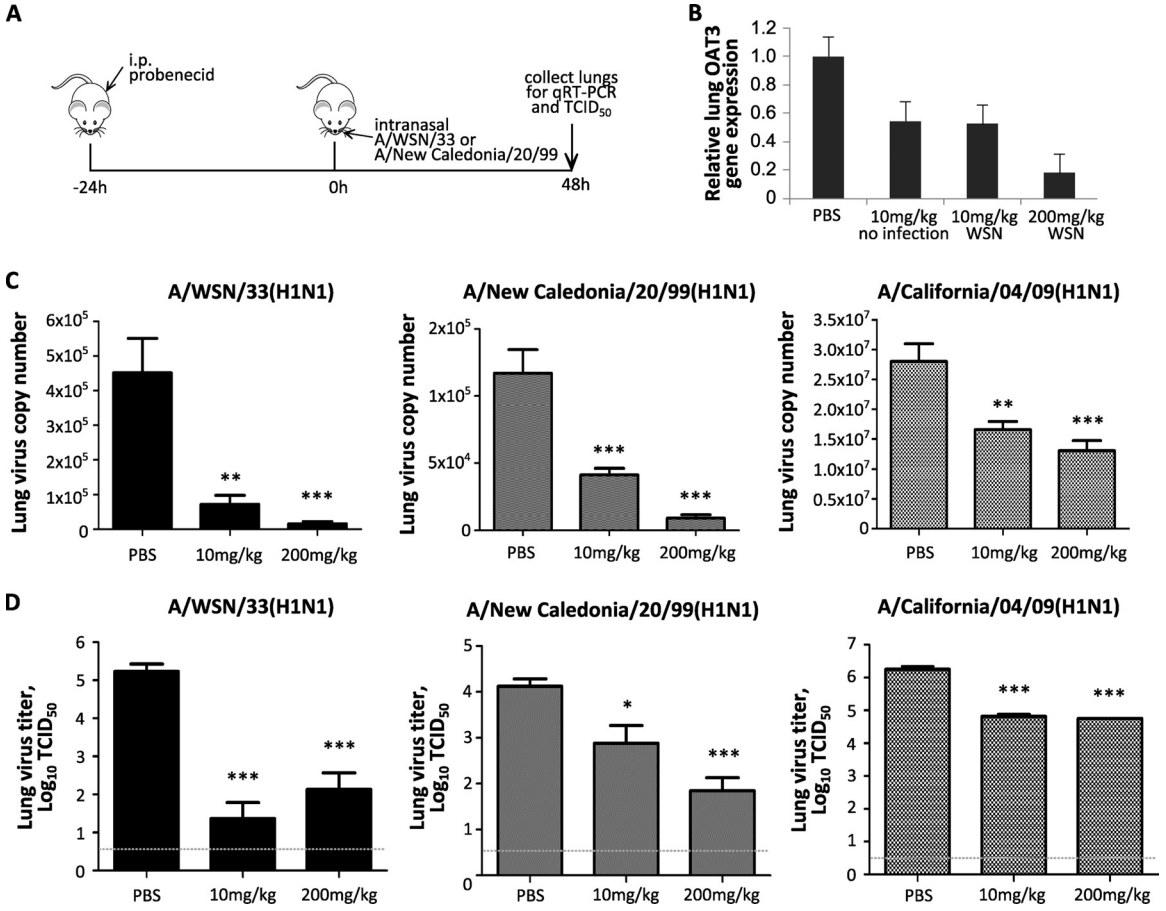


FIG 4 Probenecid treatment reduces OAT3 expression and influenza A virus load in lungs of infected mice. (A to D) BALB/c mice were intraperitoneally (i.p.) mock treated or treated with probenecid at 10 or 200 mg/kg of body weight 24 h prior to intranasal inoculation with 70 PFU of influenza A/WSN/33(H1N1) virus, 22 PFU of influenza A/New Caledonia/20/99(H1N1) virus, or 35 PFU of influenza A/California/04/09(H1N1) virus. At 48 h postinfection, animals were sacrificed and their lungs were collected. (A) Experimental outline; 0 h on the *x* axis denotes the start of infection. (B) OAT3 gene expression in lungs of PBS- or probenecid-treated mice infected with influenza A/WSN/33(H1N1) virus was assessed using qRT-PCR and normalized to GAPDH. Fold gene expression levels were calculated relative to PBS-treated mouse levels. (C) Lung influenza A virus copy numbers per 5 ng of total RNA were evaluated using qRT-PCR and graphed. (D) Lung influenza A virus titers are represented as $TCID_{50}$. Graphs represent results from 10 animals per experimental group, and error bars denote standard errors of the means. Dotted lines represent the limit of assay detection. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

through urinal excretion. Note, however, that probenecid is a nonspecific inhibitor of OATs, as it blocks transport by OAT1, OAT3, and URAT1, although it yields net excretion of urate. To understand the role of OAT3 during influenza A virus infection, future studies are planned to examine localization of OAT3 on the surface of polarized lung epithelial cells, in addition to identification of the substrate required during infection.

In this study, the anti-influenza A virus properties of probenecid were shown. *In vitro*, probenecid pretreatment of A549 cells resulted in a dose-dependent reduction of virus titer upon infections with several influenza A virus strains, A/WSN/33(H1N1), A/New Caledonia/20/99, and, to a lesser extent, A/California/07/09(H1N1) and A/Philippines/2/82/X-79 (Fig. 3). The IC_{50} of probenecid against influenza A infection *in vitro* for WSN and New Caledonia ranged from 5×10^{-5} to 5×10^{-4} μM. Additionally, it was shown that probenecid reduced mRNA and protein levels of OAT3, which suggests that probenecid blocks OAT-mediated transport by regulating the expression of transporter molecules itself (Fig. 2; see also Fig. S2 in the supplemental material). Probenecid administered prophylactically prior to influenza A virus infection resulted in reduced lung virus titers *in vivo* (Fig. 4 and 5). Likewise, probenecid given therapeutically at 24 h following influenza A virus infection also resulted in reduced lung virus titers, demonstrating the versatility of probenecid as an influenza A virus chemotherapeutic (Fig. 5; see also Fig. S3 in the supplemental material). Future studies should evaluate the efficacy of probenecid against other influenza A virus strains and determine optimal regimens for treatment of infections by a broad range of influenza A virus strains *in vivo*.

In addition to its direct anti-influenza A activity, probenecid has also been reported to maintain the plasma level of oseltamivir over a longer period of time (19, 21, 29, 30). Oseltamivir has been reported in several studies as a substrate for OAT1 and OAT3 (20, 31); therefore, blocking of OAT1/OAT3 transport with probenecid is thought to prevent oseltamivir renal excretion. For this reason, probenecid administration in conjunction with oseltamivir has previously been suggested (29, 32, 33). However, these studies were done in healthy individuals and are based on measurements of oseltamivir active as a metabolite in the plasma; thus, the efficacy of the oseltamivir-

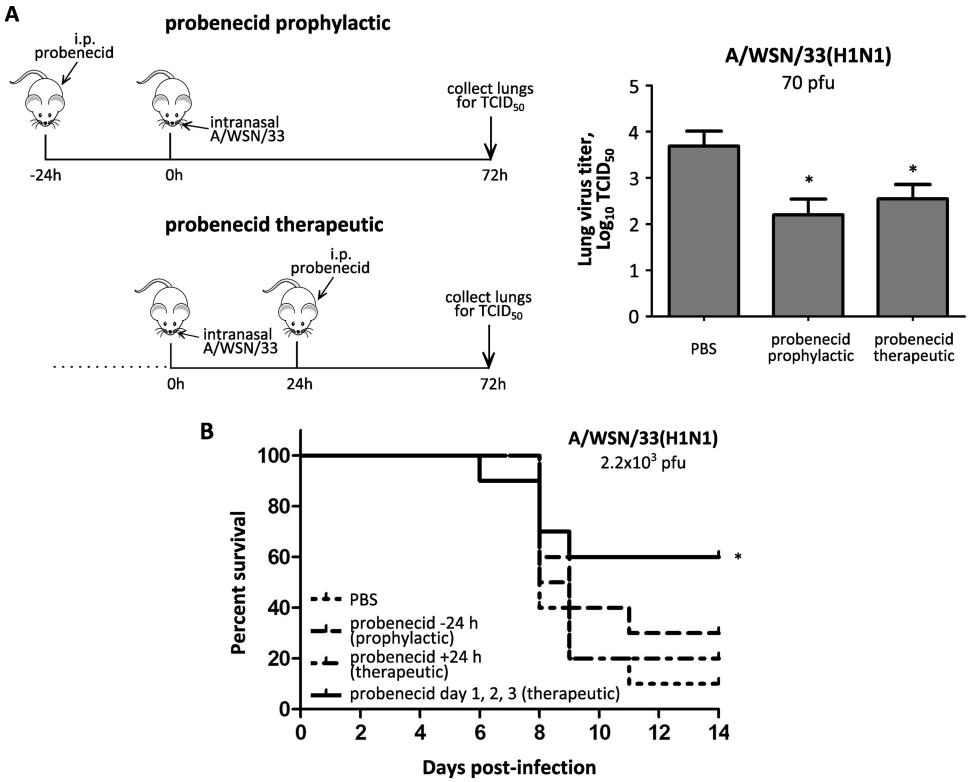


FIG 5 Probenecid administered either prophylactically or therapeutically reduces influenza A virus load in lung of infected animals. (A) BALB/c mice were i.p. treated with probenecid at 25 mg/kg body weight at 24 h prior to (−24 h; prophylactic) or following (24 h; therapeutic) infection with 70 PFU of influenza A/WSN/33 virus. In the experimental outline, 0 h on the *x* axis denotes the start of infection. At 72 h postinfection, animals were sacrificed and lungs were collected to evaluate viral load by $TCID_{50}$. Graphs represent results from 10 animals per experimental group, and error bars denote standard errors of the means. (B) BALB/c mice were subjected to a single-dose treatment with probenecid (at 200 mg/kg) i.p. 24 h prior to infection (prophylactic) or 24 h following infection (therapeutic) or to a three-dose treatment regimen (at 25 mg/kg each dose) on days 1, 2, and 3 postinfection with 2.2×10^3 PFU of influenza A/WSN/33 virus. Mice were monitored daily for 14 days for morbidity and mortality, and their survival was recorded and graphed. *, $P < 0.05$.

probenecid combinational therapy for treatment of influenza A virus infection has not been previously evaluated. In this study, combinational oseltamivir/probenecid therapy produced an anti-influenza A virus effect that was slightly higher than that seen with oseltamivir treatment alone using a mouse model.

Lastly, this study also identified host drug target genes, including many members of the OAT family such as OAT1, OAT2, OAT4, and OAT7, whose silencing led to increased influenza A virus replication. This suggests that they have a role in limiting influenza A virus replication; thus, silencing their expression or preventing their function with small-molecule chemical inhibitors could be useful in applications where amplification of virus replication is beneficial such as in the propagation of viruses during vaccine production. It also highlighted the breadth of new information obtained from siRNA screens performed during influenza A virus infection, both in the study reported here and in those done by others (24, 25, 34–39).

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