Influenza hemagglutinin (HA) glycoprotein is the primary surface antigen targeted by the host immune response and is a focus for development of novel vaccines, broadly neutralizing antibodies (bnAbs), and therapeutics. HA enables viral entry into host cells via receptor binding and membrane fusion and is a validated target for drug discovery. However, to date, only a very few bona fide small molecules have been reported against the HA. To identity new antiviral lead candidates against the highly conserved fusion machinery in the HA stem, we synthesized a fluorescence-polarization probe based on a recently described neutralizing cyclic peptide P7 derived from the complementarity-determining region loops of human bnAbs Fl6v3 and CR9114 against the HA stem. We then designed a robust binding assay compatible with high-throughput screening to identify molecules with low micromolar to nanomolar affinity to influenza A group 1 HAs. Our simple, low-cost, and efficient in vitro assay was used to screen H1/Puerto Rico/8/1934 (H1/PR8) HA trimers against ~72,000 compounds. The crystal structure of H1/PR8 HA in complex with our best hit compound F0045(S) confirmed that it binds to pockets in the HA stem similar to bnAbs Fl6v3 and CR9114, cyclic peptide P7, and small-molecule inhibitor JNA4796. F0045 is enantioselective against a panel of group 1 HAs and F0045(S) exhibits in vitro neutralization activity against multiple H1N1 and H5N1 strains. Our assay, compound characterization, and small-molecule candidate should further stimulate the discovery and development of new compounds with unique chemical scaffolds and enhanced influenza antiviral capabilities.

Influenza, together with subsequent complications by bacterial pneumonia (i.e., Streptococcus pneumoniae, Staphylococcus aureus), is among the top 10 leading causes of death in the United States with over 50,000 people succumbing to infection each year. According to the Centers for Disease Control and Prevention, the estimated number of US hospitalizations and deaths directly due to influenza during the 2017–2018 season was 808,000 and 61,000, respectively (1). More devastating are the unpredictable pandemic strains that can result in the mortality of one million (1957 Asian flu) to over 50 million (1918 Spanish flu) individuals (2). Thus, there is a critical need for readily available therapeutics to combat the global spread of influenza. Currently, three types of FDA-approved anti-influenza drugs are available and include: 1) neuraminidase inhibitors, such as oseltamivir and zanamivir, that prevent release of nascent virions postinfection (3); 2) M2 ion channel inhibitors amantadine and rimantadine that act by preventing viral uncoating during early stages of replication (4); and 3) the cap-dependent endonuclease inhibitor baloxavir marboxil, which is the most recent US FDA approved drug for influenza A and B viruses (5). Unfortunately, all of these molecules are subject to rapid resistance by influenza viruses, and resistant clinical isolates have been reported (6–8). Thus, there is a pressing and unmet need for new broad-spectrum influenza antivirals to combat pandemics and seasonal epidemics that can work alone or synergistically with current therapeutics and/or the host adaptive immune system.

The HA glycoprotein is the most abundant transmembrane protein on the surface of influenza and is necessary for initiating viral infection. HA is a trimeric class I viral fusion protein with a globular membrane-distal head containing the receptor binding site and a membrane-proximal stem housing the fusion machinery (9). These two regions of HA have two essential functions of facilitating entry into host cells through binding of HA to host sialosides (10) and membrane fusion upon entry into endosomes (11), respectively. Endosomal uptake is necessary for infection where the low pH of the endosome triggers conformational rearrangements in the metastable prefusion HA that lead to a postfusion state and subsequent membrane fusion (12, 13). Importantly, a number of bnAbs have now been characterized, including CR9114 and Fl6v3 (14, 15), that bind and stabilize the HA stem and prevent these conformational rearrangements in low pH conditions. Stabilization of the prefusion state HA by small molecules would then mimic the stem bnAbs and impede membrane fusion to effectively prevent infection (16).

Despite HA now being an established drug target, no FDA-approved therapeutics specifically block receptor binding or the...
fusion machinery. Umifenovir (Arbidol) is an antiviral small molecule used to treat influenza and other respiratory diseases in Russia and China (17, 18); however, large doses are required to achieve therapeutic efficacy (19). While Arbidol also has broad-spectrum antiviral capabilities against Ebola, hepatitis B, and hepatitis C (17, 18), we recently demonstrated that it binds to a specific hydrophobic cavity in the upper region of the HA stem, and this binding site likely accounts for its influenza antiviral effects (20). Notwithstanding, Arbidol needs extensive optimization to improve HA affinity and pharmacokinetic stability, and additional molecules that target HA at other surface epitopes are urgently needed.

We and colleagues recently reported a small cyclic peptide P7 that neutralizes group 1 HA influenza (21). P7 is based on the heavy-chain complementarity-determining region (HCDR) loop of bnAb FI6v3 and framework region 3 of CR9114. Similar to bnAb FI6v3, crystal structures and cellular assays demonstrate P7 binds to the highly conserved HA stem epitope and blocks the low pH-induced conformational rearrangements associated with membrane fusion. However, unlike stem-targeted bnAbs FI6v3 and CR9114, P7 is specific to influenza A group 1 HAs. The P7 specificity for group 1 is due, in part, to glycosylation of Asn38 in group 2 HAs as well as substitution of Thr49 (group 1) for a larger Asn (group 2) that interferes with binding. In another collaboration with Janssen, we also disclosed the small-molecule JNJ4796, a HA stem-targeted inhibitor that is orally active in mice (22). JNJ4796 can neutralize a broad spectrum of a human pandemic, seasonal, and emerging group 1 influenza A viruses and has promise as a therapeutic option with a complementary mechanism of action to existing antiviral drugs for the treatment of influenza.

The primary issue with the identification of HA-targeted small molecules is the lack of robust, rapid, and cost-effective high-throughput screening (HTS) assays (22–24). Identification of new antiviral small molecules has been attempted with both phenotypic and enzyme-linked immunosorbent assay formats, including a dual myxovirus reporter assay (23) and an AlphaLISA assay (amplified luminescent proximity homogeneous assay) (22). The AlphaLISA screen was used to identify the precursor hit molecule of JNJ4796 whereby a diverse library of ~500,000 compounds was screened for displacement of the decoy-designed small protein HB80.4 (25) from the group 1 HA stem epitope. While these assays helped to identify new lead antiviral candidates, AlphaLISA and phenotypic HTS formats can be expensive, convoluted, and difficult to develop counter-screens to eliminate false positives.

We present, here, the design, synthesis, and application of a P7 peptide-based fluorescence polarization (FP) probe that is selective for the screening of the stem epitope of influenza A group 1 HAs. As a proof of concept, we performed a HTS against 72,000 compounds to identify molecules with affinities to the H1/PR8 HA stem. We identified a novel small-molecule F0045(S) and biophysically characterized its binding to a panel of group 1 HAs with surface plasmon resonance, X-ray crystallography, and differential scanning fluorimetry. Importantly, F0045(S) neutralizes influenza A infection, and our assay and hit molecule, thus, represent key advancements in the ability to interrogate HA for additional molecular scaffolds that can be optimized for development of antiviral influenza drugs.

**Methods**

**HA Stem-Directed FP Probe and Assay.** FP is a powerful approach by which alterations in the apparent molecular weight of a fluororescent probe in solution are indicated by changes in the polarization of emitted light from the sample (26). A robust FP probe should ideally result in low and high FPs when incubated alone or in the presence of a protein target, respectively (Fig. 1A). Our FP probe was designed based on functional and structural considerations of the cyclic peptide P7 that targets the highly conserved stem epitope of influenza A group 1 HA with low nanomolar-binding affinity (21). Based on the crystal structure of H1 HA in complex with P7 (21), we attached a TAMRA fluorophore to a free amine located on the P7 aminopropanamide moiety as this side chain is located in a solvent channel with limited interactions to HA. We posited that modification of the cyclic peptide at this position would have minimal interference on P7 binding to HA (Fig. 1B). The P7-TAMRA probe was synthesized by a direct amine-carboxylic acid coupling between the pure P7 peptide (synthesized by standard Fmoc-based solid-phase peptide synthesis procedures) and TAMRA. The P7-TAMRA probe was purified by reverse-phase high performance liquid chromatography (HPLC) purification to yield >95% product (see Materials and Methods for additional details).

**Results and Discussion**

**HA Stem-Directed FP Probe and Assay.** FP is a powerful approach by which alterations in the apparent molecular weight of a fluorescent probe in solution are indicated by changes in the polarization of emitted light from the sample (26). A robust FP probe should ideally result in low and high FPs when incubated alone or in the presence of a protein target, respectively (Fig. 1A). Our FP probe was designed based on functional and structural considerations of the cyclic peptide P7 that targets the highly conserved stem epitope of influenza A group 1 HA with low nanomolar-binding affinity (21). Based on the crystal structure of H1 HA in complex with P7 (21), we attached a TAMRA fluorophore to a free amine located on the P7 aminopropanamide moiety as this side chain is located in a solvent channel with limited interactions to HA. We posited that modification of the cyclic peptide at this position would have minimal interference on P7 binding to HA (Fig. 1B). The P7-TAMRA probe was synthesized by a direct amine-carboxylic acid coupling between the pure P7 peptide (synthesized by standard Fmoc-based solid-phase peptide synthesis procedures) and TAMRA. The P7-TAMRA probe was purified by reverse-phase high performance liquid chromatography (HPLC) purification to yield >95% product (see Materials and Methods for additional details).

![](image1.png)

**Fig. 1.** Design and characterization of the P7-based FP probe. (A) Schematic of the FP assay to identify molecules with affinity to the HA stem. The polarization signal of the P7-carboxytemethylrhodamine (TAMRA) probe produces high and low FPs when bound or unbound to the HA protein, respectively. (B) Chemical structure of the P7-TAMRA probe. (C) P7-TAMRA probe (75-nM) and H1/PR8 (30-nM) competition assay against a dose-dependent increase in P7 peptide or stem-targeting bnAb S9-3–37 millipolarization (mP). (D) P7-TAMRA probe (75 nM) and other HA (A/California/04/2009 [H1/Cal04] [30-nM] or A/ Michigan/45/2015 [H1/Mich15] [100-nM]) competition assays against the P7 peptide.
FP-Based Competition Assay Validation. We first optimized the P7-TAMRA probe and H1/PR8 HA concentrations to yield the best separation between high and low FP signals in a 96-well plate format in the presence and absence of a protein. Importantly, FP of the P7-TAMRA probe increased only in the presence of H1/PR8 HA, while no observable change in polarization was detected when the P7-TAMRA probe was incubated with the group 2 A/Hong Kong/1/1968 (H3/HK68) HA (SI Appendix, Fig. S1). We next assessed the ability of P7 to compete with the P7-TAMRA probe for HA stem binding. Increasing concentrations of P7 were introduced to preincubated solutions of 30-nM H1/PR8 HA and 75-nM P7-TAMRA in a buffer consisting of phosphate-buffered saline (PBS), pH 7.4 and 0.01% Triton X-100 in a final volume of 60 μL. After a 1-min incubation at room temperature, FP was measured on a PerkinElmer EnVision plate reader, and we calculated a half maximal effective concentration (EC50) of 51 ± 5 nM (Fig. 1C). This value is comparable to that for P7 (EC50 = 30–70 nM) that was previously measured by an AlphaLISA assay using the small protein HB804 as a competitor for HA binding (21). In addition, we also performed the competition assay with a group 1-specific bnAb S9-3 and measured an EC50 of 36 ± 1 nM (Fig. 1C). The FP assay was next assessed for adaptability and sensitivity to measure FP of the P7-TAMRA probe in the presence of other group 1 HAs, including H1/Cal04 and H1/Mich15. A repeat of the P7 vs. P7-TAMRA competition assay with these HAs yielded relative EC50 values of 83 ± 4 and 31 ± 3 nM, respectively (Fig. 1D).

High-Throughput Screen to Identify HA Stem Binders. We next sought to optimize our cost-effective and throughput assay to identify new small molecules with affinity to the HA stem epitope. Similar to the proof-of-concept FP assays that used 30-nM H1/PR8 HA and 75-nM P7-TAMRA probes, we miniaturized the volume to 10 μL to perform HTS against commercially available small-molecule libraries. The assay was optimized for maximum differential FP between negative and positive controls consisting of dimethyl sulfoxide (DMSO) and 300-nM P7 peptide, respectively (Fig. 2A). A simplified two-step HTS process was performed whereby 100 nL of 2-mM DMSO stock solutions of small molecules were introduced into a premixture of HA and P7-TAMRA distributed into 384-well low-volume plates (final compound concentration was 20 μM). Plates were incubated for 30 min at room temperature and, subsequently, analyzed for FP as described in Materials and Methods. We screened H1/PR8 HA against 72,000 compounds consisting of the commercially available Maybridge HitFinder, ChemDiv, and Life Chemical libraries (Fig. 2A). The average Z’ for the HTS was 0.81 with an overall hit rate of 0.01% where molecules with FP < 3σ and the CV were considered hits (28, 29) (Fig. 2A). Importantly, our HTS assay has significant advantages over previous screening attempts as the FP can be rapidly measured upon reagent mixing, is cost effective due to the limited amount of reagents required (i.e., 7.5-nmol P7-TAMRA probe and 3-nmol HA per 10,000 compounds), and it is relatively easy to remove false-positive hits that are typically due to inherent fluorescence.

All library wells consisting of compounds with significantly reduced FP were submitted for LC–mass spectrometry (MS)/MS verification, and molecules were purchased from vendors for further in vitro characterization. One small molecule, termed F0045, was subjected to eight-point dose-response assays with H1/PR8 HA, and the resulting EC50 of 8.8 ± 2.4 μM provided confidence that this molecule was an authentic hit worth further analysis (Fig. 2B and SI Appendix, Fig. S2). We next employed an established trypsin-susceptibility (TS) assay to assess if F0045 protected H1/PR8 from low pH-induced conformational changes, akin to the endosomal membrane fusion event (20–22). Briefly, H1/PR8 HA is susceptible to trypsin digestion in the low pH postfusion state, but stem-binding small molecules (i.e., Arbidol, JNJ4796) or bnAbs (e.g., FI6v3, CR9114) prevent the HA from undergoing these conformation rearrangements on lowering of the environmental pH (Fig. 2C). Similar to the P7 peptide and P7-TAMRA probe, 50-μM F0045 prevented 5-μM H1/PR8 HA from trypsin digestion suggesting that the small molecule prevents HA from transitioning to the postfusion state at pH 5.0. Thus, F0045 has reasonable in vitro HA affinity in the...
low micromolar range and effectively prevents the biologically relevant low-pH HA conformational change.

**F0045 Targets HA Stem and Is Enantioselective.** Our best hit F0045 has a stereocenter, and the compound within the commercial library likely consisted of a racemic mixture (Fig. 2B). We further investigated if the spatial orientation of the phenyl group plays a role in HA affinity by synthesizing both F0045 enantiomers (Fig. 3 and see SI Appendix, Supplementary Methods for synthesis). The S enantiomer (i.e., F0045[S], EC$_{50}$ = 1.9 ± 0.3 μM) has a significantly reduced relative EC$_{50}$ than the R enantiomer (i.e., F0045[R], EC$_{50}$ = 43 ± 8 μM) when measured by our FP competition assay using the P7-TAMRA probe and H1/PR8 HA (Fig. 3B). A similar trend of improved affinity by the S-enantiomer F0045(S) was also observed for the HAs of other H1 strains, including H1/Cal04 (EC$_{50}$ = 1.4 ± 0.9 μM) and H1/Mich15 (EC$_{50}$ = 0.50 ± 0.16 μM). The spatial orientation of the phenyl group then has a significant effect on the compound’s ability to bind HA (Fig. 3B).

We then subjected these F0045 enantiomers and H1 HAs to additional biophysical methods to further validate and compare stem binding and affinity. For example, differential scanning fluorimetry analysis confirmed the preference of H1/PR8, H1/Cal04, and H1/Mich15 HAs for F0045(S) over F0045(R) as F0045(S) stabilizes the HA protein from temperature-induced unfolding at much lower concentrations than F0045(R) (SI Appendix, Fig. S3 and S4). Additionally, incubation of either F0045 enantiomer with group 2 H3/HK68 HA did not result in any observable increase in melting temperature (SI Appendix, Fig. S4). We anticipated group 2 HAs would have limited to no affinity for F0045 because the binding epitope has a few important differences that include an additional glycosylation site at Asn38 and some other amino acid substitutions that, when combined, would hinder F0045 binding. Steady state and kinetic binding of F0045(S) with H1/PR8 HA demonstrated a series of intermolecular polar and nonpolar interactions (Fig. 4). A cocrystal structure of F0045(S) in complex with H1/PR8 was determined at 2.69-Å resolution (Fig. 4D, Table S1). F0045(S) exhibited well-defined electron density and recognizes the hydrophobic cavity at the interface of the HA1–HA2 in the HA stem region (Fig. 4D and SI Appendix, Fig. S6). This region consists of residues His$_{18}$, His$_{38}$, Leu$_{42}$, and Thr$_{318}$ from HA1 and Asp$_{19}$, Trp$_{21}$, and Glu$_{38}$ from helix A of HA2. This HA1–HA2 interface consists of a number of small hydrophobic pockets, a few of which are occupied by the A to C rings of F0045(S) (Fig. 4D). Analysis of molecular interactions of F0045(S) with H1/PR8 HA demonstrated a series of intermolecular polar and nonpolar interactions (Fig. 4B and SI Appendix, Fig. S7). The amide carbonyl of F0045(S) makes a direct hydrogen bond interaction with the sidechain hydroxyl of Thr$_{318}$ from HA1, whereas C$_2$ CH from Thr$_{318}$ makes a CH–π interaction with the A ring. Similarly, the C ring of F0045(S) makes CH–π

![Fig. 3.](image-url) Characterization of F0045 enantiomers. (A) Chemical structures of F0045(S) and F0045(R). (B) Dose-response comparison (200 nM to 100 μM) of F0045(S) and F0045(R) binding a panel of H1 HAs as measured by the FP P7-TAMRA probe competition assay. The assay consisted of a solution of PBS, pH 7.4, 0.01% Triton X-100 and a 75-nM P7-TAMRA probe and H1 HA (30-nM H1/PR8, 30-nM H1/Cal04, and 100-nM H1/Mich15) mixed for several seconds at room temperature prior to measuring FP. DMSO and 300-nM P7 peptide represent the negative and positive controls, respectively. FP was measured in triplicate. (C) Steady state kinetic dose responses of F0045(S) (200 nM to 250 μM) against H1 HAs (H1/PR8, H1/Cal04, and H1/Mich15) as measured by SPR. (D) Dose-response comparison of F0045(S) binding a panel of 1 HAs (50 nM for H2 A/Adachi/2/1957 and H5 A/Vietnam/1203/2004 and 55 nM for H6 A/Taiwan/2/2013) measured as in A.
interactions with His$^{18}$ and Trp$^{21}$ from HA1 and HA2, respectively (Fig. 4B). These C-ring interactions of F0045(S) are strikingly similar to the interactions made by the D ring of small-molecule JNJ4796 in the JNJ4796-H1 HA complex (PDB ID 6CF7) (22). Nonpolar interactions of F0045(S) include contacts with the sidechains of Thr$^{318}$ and Val$^{40}$ from HA1 and Trp$^{21}$, Ile$^{45}$, Ile$^{48}$, and Val$^{52}$ from HA2. This network of polar and nonpolar interactions of F0045(S) with residues in the stem-binding site appears, then, to stabilize the HA1/HA2 interface in its prefusion conformation and prevent pH-induced conformational changes to the postfusion form (Fig. 2C).

Overall, F0045(S) buries $\sim$130 and 171 Å$^2$ on HA1 and HA2, respectively (Fig. 4C). F0045(S) occupies the same region as targeted by HCDR2 and HCDR3 loops of bnAbs.
CR9114 and Flv63, respectively, and the antibody-inspired peptide P7. The binding mode of F0045(S) is also strikingly similar to the binding modes of B, C, and D rings of small-molecule JNJ4796 (Fig. 4C). Thus, with a minimal footprint as compared to the currently known inhibitors and the presence of surrounding additional unoccupied pockets in the binding site, F0045(S) presents an excellent opportunity to elaborate the small molecule into nearby regions to improve the binding and neutralization activity against influenza viruses (Fig. 4 A–C).

To elucidate the structural basis for the stereospecific binding of F0045 toward group 1 influenza A HAs, we compared the two different configurations of F0045 in the binding site of H1/PR8. Based on the crystal structure of H1/PR8 with F0045(S) (Fig. 5A), we inverted the S stereocenter in the B ring of F0045(S) to the R form and compared the binding mode of these two different configurations that affect the relative disposition of the C ring. Compared to the C ring of F0045(S) in the F0045(S)-H1/PR8 complex, the modeled F0045(R) shows that the C ring would now be located inward with increased proximity to helix A and further steric clash with HA Ile45 and Trp7 (Fig. 5B). These steric considerations could explain the reduced binding and neutralization activity of F0045(R) against group 1 HAs as compared to F0045(S) (Fig. 5A).

Furthermore, to understand group 1 HA-binding specificity of F0045(S), the structure of F0045(S) bound to H1/PR8 was superimposed with the group 2 apo H3/HK68 HA structure (PDB ID 4FNK) (Fig. 5 C and D). The key differences in the F0045(S)-binding site on H1/PR8 vs. apo H3/HK68 HAs are the presence of a glycosylation site at HA1 Asn38 in group 2 HA, the orientation of HA2 Trp31, and the presence of Asn49 and Leu52 in helix A of group 2 H3/HK68 HA that could all lead to steric hindrance of F0045(S) with group 2 HAs and render it group 1 HA specific. This observation is consistent with the group 1 specificity of antibody CR6261 (30), cyclic peptide P7 (21), and small-molecule JNJ4796 (22).

F0045(S) Protects Human Cells from Influenza Infection. The most stringent in vitro assay used to assess the potential antiviral activity of chemical and/or biological interventions is with cell-based infection assays. MDCK-SIAT1 cells die after 72 h of incubation with influenza virus expressing the HA from H1N1 H1/PR8 (22) (CC50 values are toxic to MDCK-SIAT1 cells with cytotoxic concentration (R) are described previously (37). Please see SI Appendix, Supplementary Methods for full synthetic procedures.

Expression and Purification of the HA. The HAs used for binding and crystalization studies were expressed using the baculovirus expression system as described previously (37). Please see SI Appendix, Supplementary Methods for details regarding procedures.

Polarization Assay. A P7-TAMRA probe was incubated at a final concentration of 75 nM in the presence of one group of HA trimer (30-nM final concentration for H1/PR8 and H1/Cal04; 100 nM for H1/Mich15; 50 nM for H2 A/Adachi/2/1957 and H5 A/Vietnam/1203/2004; 55 nM for H6 A/Taiwan/21/2013) in an assay buffer containing PBS, pH 7.4, and 0.01% Triton X-100. A 100-μL volume of a P7-TAMRA probe and HA were dispensed into a black 96-well Costar flat-bottom polystyrene plate prior to FP measurement. Dose-dependent competition assays to determine relative EC50 values of P7, bNab S9-3-7, F0045(S) and (R), DMSO, or aqueous stock solutions were added to the premixed P7-TAMRA probe and HA, vortexed for 10 s at 1,000 rpm with FP immediately read on a PerkinElmer EnVision plate reader. All assay
conditions required n > 3 replicates. Data were analyzed using GraphPad Prism to determine EC50.

**High-Throughput Screen.** A 10 μL solution containing 30-nM H1/PR8 HA and 75-nM P7-TAMRA probe in assay buffer (PBS, pH 7.4 and 0.01% Triton X-100) was added into each well of a black 384-well Greiner low-volume plate with a Thermo Multidrop 384 dispenser. Next, 100-nL library compounds (2-mM stock) were added into each well using a Biomek FXP Laboratory Automation Workstation, and each plate was incubated at room temperature for 30 min. Fluorescence polarization was then measured on a PerkinElmer EnVision plate reader (ex. filter: 531 nm; em. filter: 595p and 595s; mirror: BODIPY TMR dual). Vehicle DMSO and 300-nM P7 peptide served as the negative and positive controls, respectively, and represented the upper and lower FP values for normalization of amP.

**Trypsin Susceptibility Assay.** The assay was performed as previously described (20). Some 50μM H1/PR8 HA were preincubated with 50 μM of P7 peptide, P7-TAMRA probe, or F0045 for 30 min at room temperature (control reactions consisted of a 2% DMSO vehicle). The pH of each reaction was lowered using 1-M sodium acetate buffer (pH 5.0). One reaction was retained at pH 7.4 to assess digestion at neutral pH. The reaction solutions were, then, thoroughly mixed and incubated for 20 min at 37 °C. The solutions were subsequently equilibrated to room temperature, and the pH was neutralized by addition of 200-nM Tris buffer, pH 8.5. Trypsin-ultra (NEB, Inc.) was added to all samples at a final ratio of 1:50 by mass, and the samples were digested for 30 min at 37 °C. After incubation with trypsin, the reactions were equilibrated to room temperature and quenched by addition of non-reducing SDS buffer and boiled for ~2 min at 100 °C. All samples were analyzed by 4–20% SDS-PAGE gel and imaged using a BioRad ChemDoc imaging system.

**Crystallization and Structure Determination of F0045(S)-H1/PR8 HA Complex.** Gel filtration fractions containing H1/PR8 HA were concentrated to ~10 mg/mL in 20-mM Tris, pH 8.0 and 150-mM NaCl. Before setting up crystallization trials, F0045(S) at ~5 molar excess was incubated with H1/PR8 HA for ~30 min at room temperature and centrifuged at 10,000 g for ~4 to 5 min. Crystallization screens were set up using the sitting drop vapor diffusion method using our automated CrystalMation robotic system (Rigaku) at The Scripps Research Institute. Within 3–7 d, diffraction-quality crystals were obtained using 0.2-M magnesium nitrate and 20% wt/vol PEG3350 as precipitant at 4 °C. Crystals were cryoprotected with 5–15% ethylene glycol and then flash cooled and stored in liquid nitrogen until data collection. Diffraction data were processed with HKL-2000 (38). Initial phases were determined by molecular replacement using Phaser (39) with an HA model from H1/PR8 (PDB ID 5W5S). Refinement was carried out in Phenix (40), alternating with manual rebuilding and adjustment in COOT (41). Detailed data collection and refinement statistics are summarized in SI Appendix, Table S1.

**Structural Analyses.** Two-dimensional depiction of the F0045(S)-binding sites on HA were rendered using the Flatland Ligand Environment View mode of Lidia module in COOT (41). Surface areas buried on the H1 PR8 HA upon binding of F0045(S) were calculated with the Protein Interfaces, Surfaces and Assemblies server at the European Bioinformatics Institute (42). Fab Fli6v3 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-molecule JNJ4796 (PDB ID 6CF7) bound to a H1N1 HA, and Fab CR9114 (PDB ID 3ZTN), designed peptide P7 (PDB ID 5W6T), and small-

**Data Availability.** The coordinates and structure factors for F0045(S) with H1/PR8 HA have been deposited in the Protein Data Bank (PDB), http://www.wwpdb.org (PDB ID code 6WCR).

**Viruses Neutralization Assay.** Some 25,000 MDCK-SIAT cells (Madin-Darby canine kidney cells overexpressing the α2,6-linked sialic acid receptor) were plated into each well of a 96-well plate in a total volume of 100-μL Dulbecco’s modified Eagle medium supplemented with 1× penicillin-streptomycin, 10% fetal bovine serum, and 1× nonessential amino acids. Plates were permitted to incubate overnight at 37 °C in a 5% CO2 incubator.
for MDCK-SIAT1 cells to attach to the plate. Cells were washed twice with Dulbecco’s PBS, and the medium was replaced with 100-μL OptiMEM diluent containing 0.8-μg/mL N-tosyl-l-phenylalaneine chloromethyl ketone-treated trypsin and 0.5% DMSO. Four H1N1 influenza A viruses (H1/PR8, H1/Beijing, H1/SI06, and H1/Cal04) and one H5N1 influenza A pseudovirus (H5 AVI-Vietnam/1203/2004) were used in this assay (44, 45). Some 500 μM of F0045(S)/(R) in 0.5% DMSO were twofold serially diluted in 50 TCID50 virus diluent in triplicates and incubated with cells at 37 °C for 72 h in a 5% CO2 incubator. CellTiter-Glo luminescent cell viability reagent was then added in each well according the manufacturer's instructions. Luminescence was measured on a PerkinElmer EnVision plate reader, and EC50 values were calculated with GraphPad Prism (n = 3 for each condition).

**ACKNOWLEDGMENTS.** We thank H. Rosen, R. L. Wiseman, and L. L. Lairson for access to instrumentation. This work was supported by The Scripps Research Institute (D.W.W.), The Skaggs Institute for Chemical Biology, TSRI (I.A.W.), NIH Grant R56 AI127371 (I.A.W. and D.W.W.), NIH Grant K99 AI139445 (N.C.W.), and the Bill and Melinda Gates Foundation Grant OPP1170236 (I.A.W.). C.-D.D.L. was sponsored by an Academia Sinica Fellowship, Academia Sinica, Taiwan.