Cross-Protection of Influenza A Virus Infection by a DNA Aptamer Targeting the PA Endonuclease Domain

Shuofeng Yuan,^a Naru Zhang,^a Kailash Singh,^b Huiping Shuai,^a Hin Chu,^a Jie Zhou,^a Billy K. C. Chow,^b Bo-Jian Zheng^a

Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR, China^a; School of Biological Sciences, The University of Hong Kong, Hong Kong SAR, China^b

Amino acid residues in the N-terminal of the PA subunit (PA_N) of the influenza A virus polymerase play critical roles in endonuclease activity, protein stability, and viral RNA (vRNA) promoter binding. In addition, PA_N is highly conserved among different subtypes of influenza virus, which suggests PA_N to be a desired target in the development of anti-influenza agents. We selected DNA aptamers targeting the intact PA protein or the PA_N domain of an H5N1 virus strain using systematic evolution of ligands by exponential enrichment (SELEX). The binding affinities of selected aptamers were measured, followed by an evaluation of *in vitro* endonuclease inhibitory activity. Next, the antiviral effects of enriched aptamers against influenza A virus infections were examined. A total of three aptamers targeting PA and six aptamers targeting PA_N were selected. Our data demonstrated that all three PA-selected aptamers neither inhibited endonuclease activity nor exhibited antiviral efficacy, whereas four of the six PA_N-selected aptamers inhibited both endonuclease activity and H5N1 virus infection. Among the four effective aptamers, one exhibited cross-protection against infections of H1N1, H5N1, H7N7, and H7N9 influenza viruses, with a 50% inhibitory concentration (IC₅₀) of around 10 nM. Notably, this aptamer was identified at the 5th round but disappeared after the 10th round of selection, suggesting that the identification and evaluation of aptamers at early rounds of selection may be highly helpful for screening effective aptamers. Overall, our study provides novel insights for screening and developing effective aptamers for use as anti-influenza drugs.

The threat from influenza A virus remains a serious global health issue. Even though extensive studies have been focused on anti-influenza research, fatalities due to H7N9 and H5N1 influenza viruses are continuously reported worldwide (1–4). Inhibitors targeting the matrix protein 2 (M2) or neuraminidase (NA) protein of influenza virus have been applied in clinical treatments (5). However, the emergence of drug-resistant mutants remains a substantial concern. M2 inhibitors block the acid activation of the M2 ion channel and render influenza viruses unable to complete the uncoating step after receptor-mediated endocytosis. However, this group of inhibitors is not effective against influenza B virus and exhibits poor protection against the H5N1 subtype of influenza A virus (6). Furthermore, due to the high frequency of drug resistance (7, 8), the U.S. CDC has recommended stopping the use of M2 inhibitors in the chemoprophylaxis or treatment of influenza virus infection (9). NA inhibitors act by destroying the viral neuraminidase cleavage activity and inhibit the newly assembled progeny virions releasing from an infected cell (10). This group of anti-influenza agents is attractive, as they are effective against both influenza A and B viruses by binding to the highly conserved region of the NA protein (11). However, viruses that have developed resistance to NA inhibitors (especially oseltamivir) have been reported globally, including influenza B virus and H5N1, H7N9, and pandemic H1N1 subtypes of influenza A viruses (12–15). A recent surveillance report reveals that the frequency of such cases is increasing (16). In this regard, new antivirals with cross-subtype protection are highly desired in response to the threats of the forthcoming influenza pandemics.

Recently, efforts to develop new antivirals have focused on targeting either influenza virus replication or host factors that are crucial to viral replication. These are the preferred means of targeting viruses in order to minimize the emergence of drug-resistant mutants. The polymerase complex of influenza virus serves as

the center of viral replication and transcription, which consists of the polymerase basic 1 (PB1), PB2, and polymerase acidic (PA) subunits (17). PA plays the role of an endonuclease, cleaving host mRNAs downstream of their mRNA cap structures, which are recognized and bound by PB2 (18).

The N-terminal domain of the PA protein (PA_N) holds the endonuclease activity site (19, 20); before the unveiling of this, inhibitors that block polymerase endonuclease reactions had been reported (21–23). Thus far, however, few studies have been carried out to target the functional PA_N domain directly. We conceive that blocking the endonuclease activity of the PA_N domain can suppress viral replication. Furthermore, since substitutions in the enzyme active site, particularly those located at the conserved catalytic residues, are expected to significantly reduce viral fitness (24), the emergence of escape mutants that are induced by PA_N antivirals will be significantly delayed. Therefore, we aimed to screen for an aptamer-based antiviral, which might inhibit PA endonuclease activity and thereby inhibit viral propagation. Aptamers are single-stranded DNA or RNA molecules that show high binding affinity and specificity toward a particular target af-

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Address correspondence to Bo-Jian Zheng, bzheng@hkucc.hku.hk.

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ter iterative rounds of selection (25). In 2005, pegaptanib became the first FDA-approved aptamer for the clinical treatment of age-related macular degeneration (26). Apart from detection, diagnosis, drug delivery, and other applications, aptamers have been widely applied in antiviral studies targeting viral proteins, including the hepatitis C virus (HCV) envelope (27) or replicase (28), HIV reverse transcriptase (29), and influenza virus nonstructural 1 (NS1) (30) or hemagglutinin (HA) (31).

In this study, a single-stranded DNA (ssDNA) library targeting the PA or PA_N domain of an H5N1 influenza virus was screened by systematic evolution of ligands by exponential enrichment (SELEX). We selected a DNA and not an RNA library for screening antiviral aptamers, based on several rationales. First, DNA aptamers are more stable than RNA molecules, due to the absence of the hydroxyl group at its 2' end, whereas RNA aptamers are inclined to be digested, owing to the generation of cyclic 2',3'-phosphate (32, 33). Second, the selection of RNA aptamers is more complicated and requires a longer time than that for DNA aptamers (34). Third, the price of RNA synthesis is higher than that for DNA, particularly on an industrial manufacturing scale. Our data suggest that four PA_N-selected aptamers showed antiviral efficacy, one of which exhibited cross-protection against infections of H1N1, H5N1, H7N7, and H7N9 viruses. Our results demonstrated that these aptamers exert antiviral effects through the inhibition of viral endonuclease activity. In addition, our data highlight the potential of the PA_N functional domain as an optimal target for antiviral aptamer screening. By monitoring the entire selection process, we illustrated that identification and evaluation of aptamers at early stages are potentially necessary to obtain the most effective aptamers. Overall, our study provides useful information for the future screening and development of effective aptamers for the therapies of viral infectious diseases.

MATERIALS AND METHODS

Cells and viruses. Sf9 insect cells were maintained in Sf-900 II serum-free medium (Gibco) at 28°C by suspension culture. Madin-Darby canine kidney (MDCK) cells and human embryonic kidney 293T (293T) cells were cultured in minimum essential medium (MEM) (Gibco) or Dulbecco's modified Eagle medium (DMEM) (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 50 units/ml penicillin, and 50 µg/ml streptomycin at 37°C with 5% CO₂. Four influenza A virus strains, A/HK/415742/09 (H1N1), A/Vietnam/1194/2004 (H5N1), A/Netherlands/219/2003 (H7N7), and A/Anhui/1/2013 (H7N9), were propagated in MDCK cells. Except for the H5N1 virus, all viruses were cultured in the presence of 1 µg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma). The cultured viruses were titrated by plaque assay and stored at -80°C in an aliquot. All experiments with live viruses were conducted using biosafety level 3 facilities, as described previously (35, 36).

Expression and purification of the full-length PA and PA_N domain. Full-length PA was expressed using the baculovirus expression system (Invitrogen). The coding region of strain A/Vietnam/1194/2004 (GenBank accession no. AY651610) was cloned into pFastBac HT B vector and transfected into Sf9 insect cells to generate recombinant baculoviruses. The PA protein was expressed by infecting the insect cells with recombinant baculoviruses at a multiplicity of infection (MOI) of 5. Infected insect cells were harvested 72 h postinfection, and cell pellets were lysed in I-PER insect cell protein extraction reagent (Pierce), according to the manufacturer's protocol. The PA_N domain was expressed in an *Escherichia coli* system. The cDNA fragment of the PA endonuclease domain of the same virus strain (residues 1 to 196) was amplified by PCR. To better expose the enzyme active site for downstream aptamer screening, a trun-

cated construct of PA_N with a 3-residue linker (Gly-Gly-Ser) to replace the loop between residues 50 and 73 (37) was cloned into the pET-32a(+) expression vector (Novagen) between the KpnI and NcoI sites. The plasmid was transformed into *E. coli* strain BL21(DE3) and overexpressed in LB medium, in accordance with a previous report (19). Both PA and PA_N were purified by His tag affinity chromatography from soluble lysate and dialyzed overnight in 50 mM HEPES (pH 7.4). They were further purified by Q ion exchange (GE Healthcare) and concentrated through a Vivaspin 20 centrifugal concentrator (GE Healthcare). The purified proteins were detected by Western blot analysis using anti-PA rabbit polyclonal antibody (for PA) and anti-His mouse monoclonal antibody (for PA_N), as described previously (38). A blank pET32a(+) vector (pET-blank) was expressed and purified using the same procedures as those described for PA_N as a negative control. The concentration of each protein was determined by the use of a Bradford protein assay kit (Bio-Rad), using bovine serum albumin as a standard.

ssDNA library and primers. A randomized ssDNA library was chemically synthesized by Integrated DNA Technologies and was composed of 30 random nucleotides that were flanked by two conserved sequences for primer binding and PCR amplification (5'-CCGTAATACGACTCACTA TAGGGGAGCTCGGTACCGAATTC-N₃₀-AAGCTTTGAGAGAGGA TCCTT-3') (39). Forward primer 5'-CCGTAATACGACTCACTATAGG GGAGCTCGGTACCGAATTC-3' and reverse primer 5'-AAGGATCCT CTCTGCAAAGCTT-3' in both 5'-biotinylated (for single-strand DNA isolation) and nonbiotinylated (for cloning) formats were ordered from the same supplier.

SELEX procedure. The selection of aptamer relied on a nickel nitrilotriacetic acid (Ni-NTA) magnetic agarose bead (Qiagen), as described previously (40). Purified PA or PA_N (30 µg) was first immobilized on the magnetic bead to form a protein-bead matrix. The ssDNA library was heated at 95°C for 5 min and cooled on ice for 10 min to form stable secondary structures. The denatured oligonucleotides were then incubated with the matrix for 1 h at room temperature with 0.1 µg/ml poly(dI-dC) (Sigma) as a nonspecific competitor. Following intensive washes, the aptamer-bound protein was eluted from magnetic beads with 50 mM NaH₂PO₄, 300 mM NaCl, and 200 mM imidazole. The bound aptamers were then dissociated from the target protein and amplified by Platinum Pfx polymerase (Invitrogen) using forward and 5'-biotinylated reverse primers. M-280 streptavidin beads (Invitrogen) were applied to separate ssDNA from the amplified PCR product, according to the manufacturer's protocol. During iterative rounds of selection, the incubation lengths of matrix and the ssDNA library from the last round were gradually reduced from 60 min to 30 min and the protein amount from 30 µg to 5 µg. Negative selections were introduced to eliminate nonspecific aptamer binding to magnetic beads every three rounds. For PA_N selection, an additional counterselection in the last round was performed to remove aptamers binding to the fusion regions other than the PA_N domain (e.g., His tag and Trx tag) by immobilizing the pET-blank protein instead of PA_N to the beads. At rounds 5, 10, and 15, the ssDNA pool was cloned into pCR-Blunt II TOPO vector (Invitrogen) for sequencing. To identify consensus sequences, those with mismatches in the primer binding regions or with random regions that differ in length from 30 nucleotides (nt) were discarded, and the remaining sequences were aligned.

Enzyme-linked oligonucleotide assay. To test the binding affinities of the selected aptamers to either PA protein or PA_N domain, an enzyme-linked oligonucleotide assay (ELONA) was performed, according to previous reports (41, 42). Briefly, an enzyme-linked immunosorbent assay (ELISA) plate (Greiner Bio-One) was coated with 500 ng of PA or PA_N in 100 µl of coating buffer (50 mM NaH₂CO₃ [pH 9.6]) and incubating at 4°C overnight. The antigen-coated plates were blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA). Individual 5'-biotinylated aptamer was denatured, and 100 µl of serial dilutions was added and incubated at 37°C for 2 h, followed by washing six times with PBS containing 0.05% (vol/vol) Tween 20 (PBST). After being diluted to 1:2,000, 100 µl of streptavidin horseradish peroxidase conju-

gate (Invitrogen) was applied to each well and incubated for another 30 min at 37°C. After washing, 100 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Pierce) was added to each well and incubated for 15 min at room temperature. The reaction was stopped by adding 50 μ l of 1 M H₂SO₄, followed by absorbance determination at a wavelength of 450 nm using an ELISA reader (Thermo Electron Corporation, Beverly, MA).

Isothermal titration calorimetry. To obtain a quantitative evaluation of binding affinity, the dissociation constant (K_d) of each aptamer was determined by isothermal titration calorimetry (ITC) (MicroCal, Inc., Studio City, CA). The MicroCal Origin software was utilized for data analysis by fitting to a single-site binding model. PA or PA_N (10 μ M) and each aptamer (200 μ M) were loaded into the cell and titration syringe, respectively, in the buffer of 50 mM HEPES (pH 7.4). The other parameters were set as described in a published protocol (42).

Endonuclease inhibitory assay. The endonuclease inhibitory activities of selected aptamers were tested against either PA or PA_N endonuclease by a DNA-gel-based assay (19, 43), with some modifications. Briefly, in a 10- μ l reaction volume, 100 nM or 10 nM denatured aptamers were incubated with 1 μ M enzyme for 1 h, followed by 3 h of incubation with 0.2 μ g of substrate M13mp18 (NEB) at 37°C. The reaction was quenched by adding 20 mM EGTA (Sigma), and the final products were loaded for agarose electrophoresis and ethidium bromide staining.

Antiviral evaluation of selected aptamers. Aptamers with both binding and endonuclease inhibitory abilities were further tested for antiviral effect. In 96-well plates (TPP), MDCK cells (4×10^4 cells/well) were transfected with aptamers at concentrations of 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0 nM by the use of X-tremeGENE HP DNA transfection reagent (Roche). At 6 h after transfection, the cells were washed and inoculated with the viruses at an MOI of 2 for 1 h. After the inoculum was removed, cells were washed and cultured in fresh MEM with TPCK-treated trypsin. At 7 h after virus inoculation, the supernatants were collected and subsequently applied to virus titration using a plaque assay.

MTT assay. A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to assess the potential cytotoxicity of the selected aptamers using an MTT kit (Invitrogen). MDCK cells (4×10^4 cells/well) were cultured in fresh MEM and incubated with individual aptamer (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15, and 0 μ M) for 24 h at 37°C. After that, 10 μ l of freshly prepared 5 mg/ml MTT solution was added to each well and incubated for another 4 h. Next, 100 μ l of 10% SDS with 0.01 M HCl was added to each well, and the mixture was incubated overnight. The final reading was obtained by an ELISA reader and represented by the optical density at 570 nm (OD₅₇₀).

DNase I footprinting assay. A DNase I footprinting assay was used to identify the aptamer-protein binding site (40, 44). In a 50- μ l reaction mixture, 2 μ M PA_N was incubated with 20 nM 5' 6-carboxyfluorescein (FAM)-labeled aptamer for 30 min at room temperature in the binding buffer (20 mM HEPES, 40 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol [DTT], 1 mM EDTA, and 10% glycerol). A 0.2-U aliquot of DNase I (Roche) diluted in the 20 μ l of digestion buffer (5 mM CaCl₂ and 10 mM MgCl₂) was incubated with the protein-bound aptamer for 5 min at 37°C. The reaction was stopped by adding 30 μ l of solution of 200 mM NaCl, 30 mM EDTA (pH 8.0), and 1% SDS, followed by denaturing at 70°C for 10 min. The digested products were then desalted by a PCR product purification kit (Thermo Scientific), according to the provided protocol. Purified DNA samples were submitted for GeneScan and fragment analysis (Center of Genome Sciences, the University of Hong Kong, Hong Kong).

Secondary structure prediction and molecular docking. Secondary structures of selected aptamers were predicted by Mathews Lab Web Servers (45). To predict the interaction between the selected aptamer PAN-2 and the PA_N domain, simulated flexible docking of aptamer binding nucleotides (CCGG) was carried out using the Molecular Operating Environment (MOE) (Chemical Computing Group, Quebec, Canada) software, Schrödinger software (46), and the patch dock server (47). The structure of PA_N was retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank with entry code 3EBJ. The

FIG 1 Expression and purification of PA and PA_N. PA (A) and PA_N (B) were expressed in insect cells and *E. coli*, respectively. (A) Lane 1, insect cell lysate before recombinant baculovirus infection; lane 2, insect cell lysate after infection; lane 3, purified full-length PA protein. (B) Lane 1, cell lysate before IPTG induction; lane 2, cell lysate after IPTG induction; lane 3, purified PA_N.

parameters used were as follows: 15 cycles total; iteration limit, 10,000; and no possible binding grid preselected. Other parameters were set to default.

Minireplicon assay. Polymerase activity analysis upon PAN-2 aptamer transfection was carried out by a previously described minireplicon assay (48), with some modifications. Briefly, 293T cells were seeded into a 24-well plate at the density of 2×10^5 cells per well. After incubation overnight, various concentrations (25, 12.5, and 6.25 nM) of PAN-2, library control (25 nM), and mock treatment control were transfected into the cells and incubated for 5 h at 37°C, followed by the secondary transfection of minireplicon plasmids. To prepare the secondary transfection mixture, 50 ng each of the pHW2K-PB1, pHW2K-PB2, pHW2K-PA, pHW2K-NP, firefly luciferase reporter plasmid pPoll-fluc, and pIRES-enhanced green fluorescent protein (eGFP) plasmid (Clontech) was diluted to a total volume of 25 μ l in Opti-MEM (Invitrogen). The mixture was subsequently added to 25 μ l of Opti-MEM containing 1 μ l of Lipofectamine 3000 (Invitrogen). Ten minutes later, the transfection complex was added directly to the 293T cells. The cells were lysed at 24 h post-secondary transfection, and eGFP fluorescence was first determined by the use of a Victor X3 multilabel plate reader (PerkinElmer) as an internal standard for transfection efficiency normalization. Afterwards, the luminescence was measured upon adding substrate (Promega). To explore whether there were changes in PA transcription upon PAN-2 treatment, similar procedures were performed to those described for the minireplicon assay. At 12 or 24 h post-secondary transfection, total RNAs of each sample were extracted from the cell lysate using the RNeasy minikit (Qiagen). The cDNA was synthesized using influenza universal primer Uni12 (5'-AGCAAAAGCAGG-3') and PrimeScript II reverse transcriptase (TaKaRa). The transcript expression level was determined using the LightCycler 96 real-time PCR system (Roche) using 480 SYBR green I master mix with specific primers of the PA gene.

RESULTS

Purification of target proteins. The expression and purification of the full-length PA protein and PA_N domain were confirmed by Western blot analysis. As shown in Fig. 1, cell lysates before infection of recombinant baculoviruses (Fig. 1A, lane 1) or before isopropyl- β -D-thiogalactopyranoside (IPTG) induction (Fig. 1B, lane 1) did not show any target protein, while cell lysates after infection and induction exhibited PA (Fig. 1A, lane 2) and PA_N (Fig. 1B, lane 2), respectively. After purification (Fig. 1A and B,

TABLE 1 Distribution of PA-selected and PA_N-selected aptamers with multiple copies at rounds 5, 10, and 15

Aptamer	Sequence of core region (5' to 3')	Copy no. (%) in round:		
		5	10	15
PA-1	CTTGGACCATTAAACACGTGTCTGCATCC		16 (40)	15 (37.5)
PA-2	GTCAACTTTTTTTTTTTTTTTTCATGCAT		12 (30)	23 (60.5)
PA-3	ATTCCTTTTTTTTTTTTTTTTCCG		8 (20)	
PAN-1	TTTAACTTTTTTTTTTTTTTCAATGAT	6 (15)	15 (37.5)	27 (67.5)
PAN-2	GCAAGCGTCTGCATCCCGTGGGACCATT	5 (12.5)		
PAN-3	TTCATTTTTTTTTTTTTTCTTCAGGAT	5 (12.5)	19 (47.5)	12 (30)
PAN-4	AAAGTTCCAATTAACGTAGAGTCTTCAG	5 (12.5)	4 (10)	
PAN-5	GAAGCGTCTGCACAATTATAATTGCCATTA	4 (10)		
PAN-6	GTTGTTTTTTTTTTTTTCCGTGGATC	3 (7.5)		

lane 3), recombinant PA (83 kDa) or PA_N (41 kDa) was detected at the expected molecular masses.

In vitro selection of DNA aptamers targeting PA or PA_N. To trace the enrichment of aptamers during the selection process, 40 randomly picked aptamer-containing transformants were sequenced and aligned to identify the consensual sequences every five rounds of selection. Sequences with multiple copies (more than three identical copies) are listed in Table 1. Analysis of sequences revealed that both PA and PA_N were in favor of binding AT-rich aptamers. Nevertheless, a single GC-rich aptamer (PAN-2) targeting PA_N was identified at the 5th round of selection. Three aptamers targeting PA were selected at the 10th round and accounted for 40%, 30%, and 20%. However, only two aptamers remained at the 15th round of selection, which accounted for 37.5% and 60.5%. Similarly, six aptamers targeting PA_N were identified at the 5th round of selection, but the numbers of the selected aptamers reduced to three at the 10th round and two at the 15th round of selection, which accounted for 67.5% and 30%, respectively. The results suggested that aptamers were indeed enriched during the selection process.

Binding affinity and specificity of selected aptamers. The binding affinity and specificity of selected aptamers were first detected by ELONA. The results showed that all PA_N-selected aptamers (PAN-1 to PAN-6) bound to PA_N (Fig. 2A) and PA (Fig. 2B), specifically in a dose-dependent manner, while the PA-selected aptamers (PA-1 to PA-3) showed relatively poor binding ability compared with that of PA_N-selected aptamers (Fig. 2B). Intriguingly, we noticed that the binding affinity of each candidate did not necessarily correlate with its enrichment efficiency, as indicated in Table 1. For example, PAN-1 (67.5%) and PAN-3 (30%) displayed higher prevalence than PAN-2 and PAN-4, which disappeared at the 10th and 15th rounds of selection, respectively. However, PAN-1, PAN-2, PAN-3, and PAN-4 exhibited similar levels of binding affinity, as determined by ELONA (Fig. 2A). We speculated that ELONA might be a rough reflection of the quantity of protein-bound aptamer. Therefore, ITC, a more precise tool, was carried out to measure the binding affinity of the same samples that were examined in ELONA. As shown in Table 2, the ITC results confirmed that the binding affinities of the PA_N-selected aptamers were higher than those of the PA-selected aptamers. At the same time, PAN-1, PAN-2, PAN-3, and PAN-4 showed similar binding affinities that range from 150 to 300 nM. Compared with that of 2,4-dioxo-4-phenylbutanoic acid (DPBA) (37), a metal-binding compound that also inhibits PA endonuclease, the binding affinities of these four PAN-selected aptamers were 15- to 30-fold higher.

Inhibition of endonuclease activity *in vitro*. Based on the binding affinity results, PA-2 and all PA_N-targeted aptamers (PAN-1 to PAN-6) were selected for an investigation of their inhibitory effects against PA_N and PA endonuclease activity. Our data demonstrate that both PA_N and PA possessed endonuclease activity (Fig. 3, lanes 3), as the substrate DNA was largely diminished. In contrast, the substrate was rarely affected under incubation with the control proteins (Fig. 3, lanes

FIG 2 Detection of binding affinity of selected aptamers by ELONA. (A) PA_N-selected aptamers were tested against PA_N protein. Serial dilutions of biotin-labeled PA_N-selected aptamer (1, 0.2, and 0.04 μM) were interacted with 500 ng of coated PA_N individually, with pET-blank protein included as a negative control. (B) Both PA-selected and PA_N-selected aptamers were tested against full-length PA protein. Each aptamer was serially diluted and incubated with 500 ng of coated PA protein. BSA was included as a negative control. The relative binding strength of each aptamer was measured by the absorbance at 450 nm. The experiments were carried out in triplicate and repeated twice. The results are presented as mean values + standard deviations (SD).

TABLE 2 Detection of binding affinity of selected aptamers by ITC^a

Aptamer	<i>K_d</i> (mean ± SD) (nM)
PA-1	— ^b
PA-2	1,075 ± 7
PA-3	—
PAN-1	137 ± 21
PAN-2	247 ± 11
PAN-3	147 ± 23
PAN-4	306 ± 11
PAN-5	1,300 ± 28
PAN-6	1,950 ± 14
PAN-2-M ^c	665 ± 3

^a The data correspond to the mean ± standard deviation (SD) values from two independent experiments.

^b —, >1 mM.

^c Mutated PAN-2 aptamer, as described in the legend to Fig. 6A.

2). Furthermore, the aptamers PAN-1, PAN-2, PAN-3, and PAN-4, which exhibited higher binding affinities than PAN-5, PAN-6, and PA-2, inhibited PA_N endonuclease activity at a concentration of 100 nM (Fig. 3A). To confirm this result, 100 nM

concentrations of each of the selected aptamers were tested for endonuclease inhibition against full-length PA (Fig. 3B). The result was consistent with that against PA_N (Fig. 3A).

Suppression of viral propagation by selected aptamers. The aptamers PAN-1, PAN-2, PAN-3, and PAN-4, which exhibited an inhibitory effect on endonuclease activity, were selected for further investigation for antiviral efficacy. In parallel, PA-2 showed binding affinity but no endonuclease inhibitory effect, and the original ssDNA library that was used for the screening was included in the experiments as a negative control. The results showed that PAN-1, PAN-2, PAN-3, and PAN-4 exhibited an antiviral effect against H5N1 virus infection (Fig. 4A). In contrast, an antiviral effect was not detected in PA-1, PA-2, PA-3 (Fig. 4A), or the aptamer library control (data not shown). The effective aptamers, with a 50% inhibitory concentration (IC₅₀) of <10 nM, reduced the virus titer in the supernatant by approximately 75% at the highest measured concentration of 12.5 nM. The results suggest that the effective aptamers might inhibit viral replication via suppression of the endonuclease activity of the virus. Because of the high sequence conservation of the PA_N domain among differ-

FIG 3 Detection of endonuclease inhibitory effect of the selected aptamers. (A) Inhibitory effects of selected aptamers on PA_N endonuclease activity were examined. Each aptamer (from lanes 6 to 19) at a final concentration (Concn) of 100 nM and 10 nM was denatured and mixed with 1 μM PA_N protein and then incubated with 0.2 μg of substrate M13mp18. A substrate control (lane 1), pET-blank protein control (lane 2), no-aptamer control (lane 3), and ssDNA library (Lib) (lane 5) were applied as negative controls. DPBA (10 μM) was taken as a positive control (lane 4). (B) Inhibitory effects of selected aptamers on full-length PA endonuclease activity were investigated. Each aptamer (lanes 6 to 12) at a concentration of 100 nM was interacted with the PA endonuclease using the same method as that described for panel A, except that BSA (lane 2) instead of pET-blank was used as one of the negative controls. The images were based on DNA agarose gels after ethidium bromide staining.

FIG 4 Antiviral evaluation of influenza virus infections by the selected aptamers. The antiviral effects of the selected aptamers were determined by detection of influenza virus replication. (A) Antiviral effect of the selected aptamers against H5N1 virus infection. MDCK cells were transfected with the indicated aptamers and subsequently infected with H5N1 virus at an MOI of 2. The supernatants were collected 7 h postinfection, and viral titers were detected by plaque assay. (B to E) Cross-subtype antiviral evaluation of the selected aptamers (PAN-1 to PAN-4) against infections with H1N1, H7N7, and H7N9 viruses. MDCK cells were transfected with indicated concentrations of the selected aptamers that exhibited anti-H5N1 effects and then infected with the indicated subtypes of influenza virus. The supernatants were collected 7 h postinfection, and viral titers were tested by plaque assay. The viral titers are expressed as 10^4 PFU/ml + SD from two independent experiments.

FIG 5 Detection of cytotoxicity of the selected aptamers by MTT assay. After 24 h of incubation of the selected aptamers at indicated concentrations with MDCK cells, cell viabilities were measured by an MTT kit. The data were calculated as the ratio between aptamer-treated and mock-treated cells. The experiments were carried out in triplicate and repeated twice. The results are presented as mean values + SD.

ent subtypes of influenza A virus, we next examined the antiviral efficacy of PAN-1, PAN-2, PAN-3, and PAN-4 against infections of H1N1, H7N7, and H7N9 viruses. Our results showed that PAN-2 inhibited viral replication of these three subtypes with similar IC_{50} to that of H5N1, whereas PAN-1, PAN-3, and PAN-4 were not effective in inhibiting H1N1, H7N7, and H7N9 virus replication (Fig. 4B to E).

Low cytotoxicity of effective antiviral aptamers. The potential cytotoxicity of the effective aptamers PAN-1, PAN-2, PAN-3, and PAN-4 was tested by MTT assay. Importantly, we detected extremely low toxicities upon a 24-h incubation of the aptamers with MDCK cells (Fig. 5). Notably, >90% of the MDCK cells survived the incubation period, even at an extremely high aptamer concentration of 10 μ M. Our results demonstrate that the 50% cytotoxicity concentration (CC_{50}) was >10 μ M and was >1,000-fold higher than their corresponding IC_{50} s, i.e., the selective index (CC_{50}/IC_{50}) of these effective aptamers is >1,000.

Binding site investigation and secondary structure prediction. Due to the cross-protection ability, the aptamer PAN-2 was chosen for further study. Using a DNase I footprinting assay, the binding site of PAN-2 in the PA_N endonuclease domain was mapped to residues 16 to 19 (CCGG) within its random region (Fig. 6A), in which the signal of FAM was rarely detectable (purple peaks) due to protection from the PA_N domain. To confirm this result, a PAN-2 mutant (PAN-2-M) with a mutated binding site from CCGG to TTTT was synthesized and subjected to ITC for determination of its binding affinity. The data showed an approximately 1.7-fold decrease in binding strength compared to that of the wild-type PAN-2 (Table 2). This result suggests that residues 16 to 19 (CCGG) of PAN-2 were essential for the binding of the PA_N domain. To explore the potential mechanism of cross-protection demonstrated by PAN-2, secondary structures of PAN-1 to PAN-4 were predicted. The results predicted that the binding site of PAN-2 was well exposed on the surface of the stem-loop structure formed by base pairs T14-A24, C15-G23, C16-G22, and C17-G21 (Fig. 6B), while the other three aptamers exhibited similar structures, with a small bulge outside the main circle (Fig. 6C

to E). Overall, our results illustrate the structural differences between PAN-2 and the other aptamers, which might in part explain the cross-protection capacity of PAN-2.

Potential interaction site on PA_N domain. The potential interaction site on the PA_N domain was predicted by molecular docking tools. Docking analysis showed that strong hydrogen bonds were formed between Tyr130 (PA_N) and C16 (PAN-2) or between Arg84 (PA_N) and G19 (PAN-2) (Fig. 7A). Furthermore, the aptamer PAN-2 also engaged the magnesium ions and water molecules through the backbone phosphate position (between C17 and G18) and the cytosine nucleotide base (C17), respectively. PA_N harbored an enzyme active cavity coordinated by the conserved amino acids His41, Glu80, Asp108, Glu119, and Lys134 (49); our data demonstrate that the identified binding site (CCGG) of PAN-2 directly occupied this enzymatic pocket by forming hydrogen bonds with the surrounding amino acids (Fig. 7).

Inhibition of polymerase activity. To explore whether the selected aptamer PAN-2 could impair influenza A virus polymerase activity, a minireplicon assay was performed. In the mock-treatment wells, the polymerase and NP proteins transcribed the virus-like RNA that was expressed by the reporter plasmid (pPolI-fluc) into mRNA, resulting in luciferase expression. A decrease in luciferase activity was observed in the PAN-2-transfected cells (at 25 and 12.5 nM), while transfection of the aptamer library (25 nM) had no effect (Fig. 8A). This result demonstrated that PAN-2, which inhibited PA endonuclease activity *in vitro*, also interfered with the catalytic activity of viral polymerase. To exclude the possibility that PAN-2 was acting on the transcriptional level, levels of mRNA transcription of the PA gene were compared between PAN-2-transfected and mock-transfected cells at 12 or 24 h post-transfection. No significant difference ($P > 0.1$) was detected at both time points (Fig. 8B), indicating that PAN-2 indeed inhibited the endonuclease activity of PA instead of suppressing the transcription of the PA gene.

DISCUSSION

In this study, we verified our hypothesis that the selected aptamers could inhibit viral replication by suppressing viral PA endonuclease activity. Among the selected aptamers, four (PAN-1, PAN-2, PAN-3, and PAN-4) that exhibited both binding affinity (Fig. 2 and Table 2) and influenza virus endonuclease inhibition (Fig. 3) were demonstrated to inhibit H5N1 virus replication (Fig. 4A). In contrast, the rest of the selected aptamers (PA-2, PAN-5, and PAN-6), which showed binding affinity but no endonuclease inhibitory effect, failed to suppress H5N1 virus replication. In this regard, the endonuclease activity assay is a valuable tool in the secondary screening of candidates with high binding affinities and in the exploration of the inhibitory mechanism.

Two parallel selection approaches utilizing either full-length PA or the PA_N endonuclease domain as screening targets were compared. Our results showed that none of the aptamers selected by PA exhibited an antiviral effect, while 4 of the 6 aptamers selected by PA_N showed antiviral activity against H5N1 virus infection (Fig. 4A). In addition, PA_N but not PA captured an aptamer with broad protection against infections of multiple subtypes of influenza A virus (Fig. 4C). The data indicate that PA_N was more capable than the intact PA in obtaining aptamers with antiviral ability. Multiple reasons might explain why PA_N is a superior screening target. First, PA_N is only one-third the length of the

FIG 6 Investigation of binding site and prediction of secondary structure. (A) Binding site of PAN-2 to PA_N domain was identified by DNase I footprinting assay. The 6-FAM-labeled PAN-2 was treated with PA_N protein (purple) or negative-control protein pET-blank (pink). The protected region was shown within the bracket. The GeneScan size standard is shown by the red line. (B) Secondary structure of aptamer PAN-2 was predicted and showed a stem-loop structure. The putative binding site identified in panel A is located on the top of the loop (red). (C to E) Secondary structures of aptamers PAN-1, PAN-3, and PAN-4 were predicted and showed a bulge outside the main circle.

A

B

FIG 7 Docking simulation of aptamer PAN-2 with influenza PA_N . (A) Two-dimensional analysis of the interaction between ssDNA sequence and PA_N . The chemical structure of PAN-2 binding nucleotides (CCGG) is shown in the center of the active pocket of the PA_N endonuclease, with the key interacting amino acids around it. Nucleotide C16 of PAN-2 interacted with the residue Tyr130, while G19 bound with Arg84. The phosphate group between C17 and G18 engaged the magnesium ion (Mg999), and C17 interacted with the water molecule. (B) Fitting of PAN-2 binding nucleotides to the enzyme pocket of PA endonuclease. PA_N is depicted as a ribbon structure, with the metal ion Mg^{2+} as a pink ball in the center. The binding nucleotides CCGG are shown as a stick structure (green). Key amino acids that are responsible for PA_N endonuclease activity (e.g., His41, Glu80, Asp108, Glu119, and Lys134) and residues that were predicted to interact with PAN-2 (Arg 84) are labeled.

full-length PA . As a result of the shorter length, the key amino acid sites of PA_N are more concentrated and are easier for aptamer enrichment. Second, PA_N is more conserved than full-length PA among influenza A viruses. Therefore, the selected aptamer is more likely to possess universal antiviral activity against influenza A viruses. Third, the PA_N used in this study was modified with the loop structure deleted for better exposure in drug screening. In this study, PA_N was purified from *E. coli*; however, the nuclease

contamination is an issue that deserves attention (50). We excluded this potential problem by setting the pET-blank construct protein as a negative control and demonstrated that the purified PA_N indeed has endonuclease activity without nuclease contamination.

Normally, SELEX will take 15 to 20 rounds for the selection of aptamers (51). In the present study, we sequenced the enriched ssDNA pool every five rounds in order to have a full view of the

FIG 8 Activities of the aptamer PAN-2 in minireplicon assays. (A) 293T cells were transfected with aptamer PAN-2 (25, 12.5, and 6.25 nM) or library control (25 nM). After 5 h of incubation at 37°C, the cells were further transfected with plasmids carrying genes encoding PB1, PB2, PA, nucleoprotein (NP), a firefly luciferase reporter gene flanked by the noncoding sequences of FluA NP, and a plasmid constitutively expressing eGFP that serves to normalize variations in transfection efficiency. Luciferase activity was determined at 24 h posttransfection. (B) mRNA transcription of the PA gene of each treatment was quantified by reverse transcription-qualitative PCR (RT-qPCR) at the indicated concentrations and time points. The data are represented as the means $\pm$ standard errors of the means (SEM) of triplicate data from two independent experiments. *, $P < 0.05$, and **, $P < 0.01$, versus the library (lib) control.

evolutionary history of the candidates. Surprisingly, aptamers were enriched as early as the 5th or 10th round (Table 1). Notably, the most potent aptamer, PAN-2, which exhibited a cross-subtype antiviral effect, disappeared after the 10th round of selection. In line with a previous report (52), these data highlighted the necessity of sequencing the enriched pool in the early rounds of selection, especially for the purpose of searching for candidates that possess designated biological functions instead of merely binding capacities. Otherwise, aptamers with high binding affinity that target undesired residues are likely to be obtained in the later stage of SELEX. There might be a number of reasons to account for the loss of an effective aptamer during continuous selection rounds. First, some aptamers that are enriched in the early stage (e.g., PAN aptamers at round 5) may have a similar binding site on the PA_N domain, while only the one possessing the highest binding competitiveness to the site lasts until the end of the selection procedure. Second, nonuniform amplification during the PCR procedure may happen, which depends on the features of template DNA. For example, GC-enriched regions of template usually cause the formation of stem-loop secondary structures that might promote polymerase jumping during PCR amplification. Thus, the whole template is not amplified completely (53). This is consistent with our result that AT-enriched sequences were dominant after rounds of selection, while a GC-enriched sequence was missing (Table 1). Similar cases of loss of sequence in subsequent selection were reported elsewhere (54, 55). Apparently, aptamers that survived until the last round of selection should have the highest binding affinity. However, for those aptamers that might act as inhibitors or blockers, binding to the appropriate residues on the target protein may be more critical than the binding affinity itself.

Among the 9 selected aptamers, only one (PAN-2) was GC enriched, while the other eight aptamers were AT enriched (Table 1). The GC-enriched aptamer in this study was demonstrated to exhibit a cross-subtype inhibitory effect against virus replication. It has been reported that effective aptamers are more likely to be GC enriched; this may be because sequences with GC-enriched regions more easily form hairpin structures, which results in bet-

ter conformational stability and stronger binding affinity than those of AT-enriched sequences (56, 57). In line with their findings, our results showed that the binding affinity of the PAN-2 mutant reduced when the binding site (CCGG) was replaced by TTTT (Table 2). Docking analysis on the PA_N and PAN-2 interaction predicted that the PA_N endonuclease cavity was fully occupied by the hairpin structure formed by nucleotides CCGG (Fig. 7). Furthermore, magnesium ions and water molecules, which are essential elements for endonuclease activity (49), were competitively engaged by a backbone phosphate group and the C17 nucleobase. These data illustrated that accommodation of the PA_N endonuclease pocket and interactions with metal ions and water molecules may impair the enzyme activity. Moreover, our results also demonstrate that PAN-2 interfered with the catalytic activity of influenza viral polymerase (Fig. 8A).

Among the 4 effective anti-H5N1 aptamers, only one (PAN-2) showed broad-spectrum protection (Fig. 4C). A comparison of the secondary structures between the 4 aptamers predicted that only PAN-2 formed a stable stem-loop structure, while the other three did not. We further identified that this hairpin structure of PAN-2 was responsible for its interaction with the PA_N domain (Fig. 6A and 7). Therefore, we speculated that the unique secondary structure of PAN-2 contributed to its stronger binding affinity across PA_N from different subtypes, which rendered PAN-2 capable of inhibiting not only H5N1, but also H1N1, H7N7, and H7N9 influenza viruses. Furthermore, alignment on the amino acid sequences by H5N1 with the other 3 subtypes revealed two substitutions (S58G and T126I) within the PA_N region (data not shown). It is possible that Ser58 and Thr126 were critical for coordinating the binding of PAN-1, PAN-3, and PAN-4 to H5N1 PA_N; when S58G and T126I substitutions occurred in H7N9, H1N1, and H7N7, the interactions of their PA_N and PAN-1, PAN-3, and PAN-4 were affected. To verify this, however, an experimental structural determination of PA_N aptamer crystals deserves further study.

Considering the high selective index ($>1,000$), the anti-influenza A aptamers discovered in this study may have high clinical potential. The fact that aptamers are liable to nuclease degradation

and renal filtration *in vivo*, however, limits their application in clinical therapy. Continuous efforts have been made in the modification of aptamers to enhance their pharmacokinetics and pharmacodynamics. Successful examples are available by conjugation of polyethylene glycol (PEG) polymers to aptamer termini (58) or by introducing oligonucleotide terminal caps, such as inverted nucleotides (34). Although it may be a long time before the discovered aptamers could be applied for clinical therapy, with a proper delivery system(s) and the extended stability *in vivo*, the application of antiviral-effective aptamers in the therapy of influenza diseases will be increasingly feasible.

In summary, we identified an aptamer with cross-protection against different subtypes of influenza A virus using the SELEX technology. By comparing the screening efficacy of the PA_N domain with that of the intact full-length PA, we demonstrated that PA_N had a higher chance of capturing effective aptamers and thus would be a good target for screening. Through tracing the evolution of enriched aptamers, we discovered that the ideal candidates might not be the ones that were most enriched at the final stage of selection. Therefore, analysis of the enriched pool from early rounds and at regular intervals was highly recommended, especially in the search for aptamers with specific biological functions.

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