Discovery of a novel specific inhibitor targeting influenza A nucleoprotein with pleiotropic inhibitory effects on various steps of viral life cycle

Running title: Characterization of a novel anti-influenza NP inhibitor

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Abstract

Influenza viruses (IAV) continue to pose an imminent threat to human due to annual influenza epidemics outbreak and episodic pandemics with high mortality. In this context, the suboptimal vaccine coverage and efficacy, coupled with recurrent events of viral resistance against a very limited antiviral portfolio, emphasize an urgent need for new additional prophylactic and therapeutic options, including new antiviral targets and drugs with new mechanisms of action to prevent and treat influenza infection. Here we characterized a novel influenza A nucleoprotein (NP) inhibitor FA-6005 that inhibited a broad spectrum of human pandemic, seasonal influenza A and B viruses in vitro and protects mice against lethal influenza A virus challenge. The small molecule FA-6005 targeted a conserved NP I41 domain and acted as a potential broad, multi-mechanistic anti-influenza virus therapeutic since FA-6005 suppressed influenza virus replication and perturbed intracellular trafficking of viral ribonucleoproteins (vRNP) from early to late stage. Cocrystal structures of the NP/FA-6005 complex reconciled well with concurrent mutational studies. This study provides the first line of direct evidence suggesting that the newly-identified NP I41 pocket as an attractive target for drug development that inhibit the multiple functions of NP. Our results also highlighted FA-6005 as a promising candidate for further development as an antiviral drug for the treatment of IAV infection and provide chemical-level details for inhibitor optimization.
Importance

Current influenza antivirals have limitations with regard to their effectiveness and the potential emergence of resistance. Therefore, there is an urgent need for broad-spectrum inhibitors to address the considerable challenges posed by the rapid evolution of influenza viruses that limit the effectiveness of vaccines and the emergence of antiviral drug resistance. Herein we identified a novel influenza A virus NP antagonist FA-6005 with broad-spectrum efficacy against influenza viruses and our study presented a comprehensive study of mode of action of FA-6005 with the crystal structure of the compound in complex with NP. The influenza inhibitor holds promise as an urgently sought-after therapeutic option offering a complementary mechanism of action to existing antiviral drugs for the treatment of influenza virus infection, and that should further aid development of universal therapeutics.

Introduction

Influenza A virus (IAVs), which is the major cause of morbidity and mortality through both epidemic and pandemic influenza, remains a significant threat to public health and the global economy(1-4). Although vaccination still remains the best strategy to prevent infection, but available vaccines are effective against a limited number of viral strains due to occurrence of the mutation on vRNA segments, the reassortment of vRNA segments between human and avian influenza virus strains, or the incorporation of new HA subtypes. Thus, approved anti-influenza drugs, including M2 ion channel inhibitors (amantadine, rimantadine), neuraminidase inhibitors (NAIs)(oseltamivir, zanamivir) and a cap-dependent endonuclease inhibitor targeting the viral PA polymerase(Baloxavir marboxil), are the stand-alone treatment for infected individuals especially
during influenza pandemics without effective vaccine (5-9). However, the CDC no longer recommends the M2 inhibitors for clinical use owing to drug resistance (6). Moreover, during the 2007-2008 influenza season, the circulating H1N1 strain gained nearly complete oseltamivir resistance (10). The quick spread of new influenza strains, cross-species transmission, and drug resistance (11), highlights an urgent need for new antiviral drugs (12) and combination therapies (13), with different mechanisms of action against alternative viral targets for treatment of broad spectrum of influenza viruses particularly the recurring resistant strains and unexpected influenza pandemics.

IAVs have a segmented genome consists of eight RNA molecules that are individually encapsidated into vRNP, which represents the minimum set of proteins for viral transcription and replication (14, 15). Following increased insights on the importance of vRNP, antiviral drugs that target on NP is an attractive strategy as NP is a highly conserved multifunctional protein that plays an essential role in infection by all subtypes of IAVs during the virus infection life cycle (16, 17). NP was reported to interact with RNA and oligomerize by self-interaction to form an oligomer to maintain the structure of RNP (18). In addition, NP is essential for viral transcription and replication, the major role of NP is encapsidation of RNA into RNP complex and maintaining conformation of the RNA template in a suitable order for transcription, replication, and assembling into virions (19). Another potential function of NP in transcription and replication is to switch the template mRNA transcription into vRNA replication (20). Furthermore, NP or RNP s participate in translocation between the cytoplasm and nucleus with the help of other viral proteins and host factors (21-24). Accumulating evidences suggested that NP was an ideal target for antiviral drugs due to its multi-functional role and the high sequence conservation (25-28). Recent
studies showed that viral replication is inhibited by small-molecule compounds that target NP, including nuclear localization signal (NLS)-binding compounds mycalamide analogues(25) and NP salt bridge inhibitors compound 3(26). Furthermore, our previous identification of nucleozin and its derivatives reported by others which have been identified as NP inhibitors that trigger aggregation of NP (28-30) promoted us to characterize other new potential drugs, with the potential for elucidating their mode of action.

Previously, a chemical library of 50,240 compounds were screened using a high-throughput system(28). Here we present one compound, FA-6005, out of 39 potential influenza inhibitors, as another NP inhibitor that impeded IAVs infection with a low micromolar median effective concentration (EC_{50}) and protected mice from lethal dose challenge of influenza H1N1 infection.

We demonstrated that FA-6005 inhibits IAVs replication via a mechanism that is different from that of existing antiviral drugs. The mode of action of FA-6005 was found to be interfering with the transcription and intracellular trafficking processes via interaction with NP. The crystal structure of small molecule FA-6005 in complex with a soluble mimic of NP trimer showed that FA-6005 is coating on the surface of NP, thereby inhibiting the multi-functions of vRNPs during virus infection and result in inhibitory effects on the entire viral life cycle. Taken together, the results of current study showed that the newly-identified NP pocket is a promising target for antiviral drugs that inhibit the multiple functions of NP. This detailed investigation also provides FA-6005 as a promising candidate for further development as an antiviral drug for the treatment of IAVs infection and provide chemical-level details for inhibitor optimization.
Results

Identification of a novel small-molecule Inhibitor against influenza in vitro and in vivo.

Based on the concept of chemical genetics, we have screened 50,240 structurally diverse compounds and selected and validated 39 potent antiviral compounds in our earlier publication in detail(28). Upon evaluation of the antiviral activities and toxicities of the selected 39 hits, a novel compound which we termed FA-6005 with potent antiviral activity on MDCK cells infected with influenza A/WSN/33 (H1N1) virus with desirable selective index was selected for further characterization in this study (Fig 1A). To assess its potential to inhibit virus replication, antiviral activities of FA-6005 against different strains of influenza A were determined by plaque reduction assays (PRA). The results revealed that FA-6005 decreased replication of all tested subtypes in a dose-dependent manner. FA-6005 impeded virus infection in MDCK cells by influenza A/WSN/33[H1N1], A/Puerto Rico/8/34[H1N1], A/Hong Kong/HKU38/2004[H3N2], A/Houston/21OS/2009[H1N1] and A/Shanghai/02/2013[H7N9] with an EC_{50} (half maximal effective concentration) of 2.82±0.33 μM, 2.88±1.68 μM, 1.64±0.31 μM, 6.22±0.63 μM, and 1.61±0.03 μM, respectively (Fig 1B). Moreover, FA-6005 significantly suppressed viral growth at 2 μM and offered 100% protection of MDCK cells in the presence of 20 μM FA-6005 in viral multicycle growth assay (Fig 1C). The CC_{50} (50% cytotoxicity concentration) for FA-6005 was estimated to be 125.69±0.61 μM as illustrated in cell viability assay, resulting in a selective index (SI = CC_{50}/EC_{50}) as high as 43.6 against A/WSN/33[H1N1](Fig S1). To demonstrate whether FA-6005 has broad-spectrum anti-influenza property, the antiviral activity of FA-6005 against B/Wisconsin/01/2010 was evaluated. Most interestingly, we found FA-6005 had similar antiviral
activity against B/Wisconsin/01/2010 with an EC\textsubscript{50} of 8.02±0.81 μM (Fig 1B). Thus, the above data demonstrated that FA-6005 had the capacity to inhibit the replication of both different subtypes of influenza A and influenza B viruses.

Since the free drug concentration in the tissue interstitial fluid is generally similar to the free plasma concentration under equilibrium, the doses of FA-6005 were selected according to the potential drug toxicity evaluated in mock-infected animals, maximum solubility in medium as well as the existing data regarding to EC\textsubscript{99} of FA-6005 in vitro. Therefore, BALB/c mice were inoculated with 10 LD\textsubscript{50} of A/PR/8/34 (H1N1) and intraperitoneally injected with FA-6005 (20mg/kg, final concentration = 100 μM per mouse) twice per day for 7 days, along with DMSO and zanamivir(100mg/kg) as controls. A sudden weight loss of all non-treated PR8-infected mice was observed during the first 5-day post-infection (p.i.) while the administration of FA-6005 or the potent NAI zanamivir resulted in a lower degree of weight loss and the body weight restored gradually when compared to control group (Fig 1D). Moreover, DMSO-treated control mice showed severe morbidity and 100% mortality at day 7 post-infection. By contrast, zanamivir treatment apparently protected the mice from influenza induced morbidity and lethality while FA-6005 treatment resulted in a delayed time-to-death with 80% surviving more than 14 days. The survival rates of PR8-infected mice treated with DMSO, FA-6005 and zanamivir were 0%, 80%, and 100%, respectively (Fig 1E). In parallel, five mice from each group were euthanized on day 6 post infection and lungs were harvested to determine viral titres. The virus load in lungs of both FA-6005 treated mice were significantly lower than that of control group while was no significant differences when compared to that of zanamivir treated mice (Fig 1F). Thus, these results collectively demonstrated that FA-6005 effectively inhibited against both laboratory and clinical
strains of both influenza A and B viruses in vitro and protected 80% of mice from death, suggesting that FA-6005 may be a promising drug against influenza viruses.

Characterization of NP as the antiviral target of FA-6005.

To explore the target of FA-6005, we generated resistant mutant virus from A/WSN/33 [H1N1] by passing the virus with increasing concentrations of FA-6005. The escaped mutant viruses resulting from 5 and 10 sequential passages were not susceptible to FA-6005 at concentration higher than 100 μM (Fig 2A) and the highly resistant mutants were used to identify the molecular targets of FA-6005. The whole genome of both escape mutants and the wild type virus were sequenced and the corresponding amino acid changes in the mutants summarized (Table S1). The EC₅₀s of FA-6005 against the corresponding escape mutant viruses were higher than 50 μM (Fig 2A). To further confirm that the resistance phenotype of mutant clones was attributable to these mutations, corresponding recombinant viruses were produced using reverse genetics(31). As demonstrated in the PRA assay, the recombinant NP I41T mutant virus showed resistance to high concentrations of FA-6005 and displayed a similar resistance profile to that of the originally isolated escape virus while the other substitution mutations showed no resistance to FA-6005 (Fig 2A and Table S2).

The resistance-bearing mutation sites indicate the target of FA-6005 to be NP protein. Moreover, no significant differences were observed in viral replication kinetics of NP I41T mutant virus in the absence or presence of 100μM FA-6005 throughout the assay course, further supporting that FA-6005 may interact with NP (Fig 2B). Furthermore, the growth kinetics of the NP I41T mutant virus was slightly lower than that of the wild-type virus prior to 45 h post-infection, though eventually reached viral yields that were comparable to the wild-type virus (Fig S2), indicating
that the mutation in NP did not critically affect the fitness and infectivity of the recombinant virus.

To obtain structural insights into the interaction between NP and FA-6005, FA-6005 was co-crystallized with NP and the complex structure was determined. Data collection and refinement statistics are summarized in Table 1. The NP/FA-6005 complex crystal belongs to space group C222₁ with only one NP trimer in each asymmetric unit. Three FA-6005 molecules were found in each trimer: one was in the I41 pocket, the major interactions involved in the I41 pocket including (1) hydrophobic interaction of the t-butyl group with G288, V285 and A286; Ring C with I41; Ring D with G54 and R55; (2) hydrogen bonding between amide O2 with T45 and (3) dipole-dipole interactions between Ring D with D51 and S283 (Fig. 2C); a second one was in the Y289 pocket and the interactions involved in Y289 pocket including (1) hydrophobic interactions between t-butyl group and Ring A with Y289, R305 and L306; and Rings A and B with N309 (Fig. 2D) and the third one around N224. However, N224 might not be a real pocket since the compound is seated between two symmetry-related NP molecules.

To better characterize the binding of FA-6005 to NP, residues in the I41T and Y289 pockets were systematically mutated, and the effect of these point mutations on the inhibition of FA-6005 was measured by PRA assay. We found that in the presence of FA-6005, mutation of residue in the I41 pocket displayed high resistance while that of residues in the Y289 pocket exhibited more tolerance to the compound (Table 2). These mutation studies provided further evidence of two independent ligand-binding sites on NP. Since the resistance-bearing mutation sites of FA-6005 were mainly mapped to the I41 pocket, the scope of this project primarily focuses on an investigation of residue 41. Importantly, protein alignments indicated that residue 41 on NP are
highly conserved among influenza A virus strains from different species, implying that the 
influenza virus is less prone to develop resistance to FA-6005 (Fig S3) and FA-6005 has a 
broad-range anti-influenza activity (Fig 1).

Although Y289 was previously identified to be binding site of nucleozin, which sit on either side 
of the putative I41 binding pocket, the binding sites of FA-6005 and nucleozin appear to be 
distinct with a small amount of overlap. FA-6005 would bind on the external surface of the NP and 
thus it may not interfere with the interaction between NPs during oligomerization (Fig 2E).

Moreover, as illustrated in Fig 2F, which shows the positions of FA-6005 and RNA, the binding 
of FA-6005 to NP molecules may not interfere with the interaction between NPs and RNA. Given 
the important role of the molecular target of FA-6005 in virus life cycle and FA-6005 is coating on 
the surface of the vRNPs, we therefore postulate that the compound-bound RNP may lose its 
capability to carry out proper biological functions, leading to inhibitory effects on the entire viral 
infection cycle.

FA-6005 inhibits vRNP transcription and replication.

NP is the most abundantly expressed protein during influenza infection with multiple functions. 
NP plays a very important role in the viral life cycle, such as nuclear import of incoming vRNPs, 
replication, transcription, and nuclear export of newly produced viral genome for packaging (16, 
19, 32). Given that NP is the molecular target of FA-6005, it is speculated that FA-6005 exerts its 
inhibitory effect on stages that NP or vRNP is involved in, including virus replication and 
trafficking. To verify which stage of viral infection the compound was inhibiting, we conducted a 
modified PRA assay by adding the compound at different time point. Influenza virus was firstly
inoculated with or without addition of FA-6005 and incubated for 2h for virus entry, the unbound virus was then removed. Agarose overlay containing the corresponding concentration of compound was added to the infected cells without FA-6005 treatment, while agarose with DMSO control was overlaid to the FA-6005 treated infected cells. Results showed that FA-6005 was able to maximally inhibit virus replication when it was added at 0h p.i or post-entry stage of the viral life cycle (2-72h p.i) when it was added 2h after infection. However, it is also suggested FA-6005 interfered with virus entry and subsequently inhibiting virus replication when it existed during virus entry stage (0-2h p.i), though with an higher EC50 at 7.68±0.50 μM (Fig S4). To elucidate the mode of action of FA-6005, we therefore further determined the effects of varying the timing of the compound addition on the viral infection cycle by conducting a time-of-addition (TOA) experiment at an MOI of 1 (A/WSN/33) with FA-6005 treatments at −1, 0, 2, 4, 6, or 8 h relative to infection. Strong inhibition was indeed observed when FA-6005 was added 1 h before infection and up to 6h after infection. However, FA-6005 did not inhibit virus replication when it was added 8 to 10 h after infection (Fig 3A). Thus, these results indicated that FA-6005 interferes with various stages of the influenza virus life cycle, including the adsorption, entry, replication and transcription, export processes.

Given the inhibitory effects exerted by FA-6005 during various stages of virus life cycle and our characterization of crystal complex FA-6005-NP, we then postulated that functions of NP or vRNPs during virus infection was restrained via FA-6005-NP interaction and thereby FA-6005 was sufficiently responsible for conferring antiviral activities. To examine this further, we first explored the possibility that FA-6005 affects viral genome replication and/or transcription, both of which occur during the 2-6h post infection stages during viral life cycle. In agreement with our
results from TOA assay, FA-6005 effectively abolished the replication of the virus in a
dose-dependent manner in the modified luciferase reporter-based mini-genome assay (33) which
measures the polymerase activity of RNP in the context of a reconstituted viral ribonucleoprotein.
However, FA-6005 had little effect on the NP I41T variant mutant, indicating that the I41T
mutation overcame the observed antagonistic effects of FA-6005 against RNP polymerase
activities in vitro (Fig 3B). Thus, these results indicated that FA-6005 inhibits the activity of
vRNP complex.
To further validate the inhibitory effect of FA-6005 on replication and/or transcription of IAVs
infection, the macromolecular synthesis of viral proteins and RNAs were explored by qPCR and
western blotting. Expression level of viral proteins increased over time in the absence of the
compound (Fig 3C). Amounts of HA, NP and M1 were hardly detected at 8 h post infection when
FA-6005 was added at the beginning of infection, indicating that FA-6005 had effectively
abolished viral proteins synthesis (Fig 3C). By using strand specific primers, expression of vRNA,
mRNA, and cRNA of NP and NA were examined (34). Increasing amounts of different types of
viral RNA of both NP and NA (Fig 3D and Fig S5) were detectable from 4 h post infection in the
absence of FA-6005. Notably, viral RNA synthesis of both NP and NA were markedly reduced
when FA-6005 was added, suggesting that the function of influenza virus polymerase activity was
perturbed by FA-6005. The inability of FA-6005 to inhibit I41 variant NP in mini-genome assay
(Fig 3B), expression of vRNA (Fig 3D) indicates that I41T residue on NP is responsible for the
resistance to FA-6005. Taken together, these results suggested that FA-6005 restrained the function
of vRNP complexes during virus transcription and replication. We can also conclude that NP is the
molecular target for FA-6005, with amino acid residue 41 as a critical binding partner.
FA-6005 inhibits nuclear export of NP or vRNP complex.

NP is the major component of the vRNP complex. Recent studies have demonstrated that blocking vRNP nuclear transport impairs the activity of vRNP complex (19). Therefore, to further investigate the effects of FA-6005 on the translocation of vRNP complex, FISH combined immuno-imaging was employed to visualize the migration of viral nucleoprotein and the distribution of vRNA and mRNA of the segment 5 (NP) under the influence of inhibitors. NP/vRNPs in the cells treated with DMSO (negative control) was predominantly located in the cytoplasm at 10 h p.i. while FA-6005 treatment induced most of NP/vRNPs retention in the nucleus (Fig 4A), indicating that the nucleocytoplasmic transportation of influenza vRNP complex was inhibited by FA-6005 during infection. Accordingly, a reduction in viral vRNA and mRNA of segment NP was also detected, in agreement with our qPCR assays (Fig 4A). However, nuclear export of the NP I41T mutant virus was not inhibited by FA-6005, further indicating that the NP-compound interaction results in the nuclear retention of NP/vRNPs (Fig 4B). The influenza virus NP is a nuclear shuttle protein that contains both nuclear localization signals (NLSs) and nuclear export signals (NESs) (35). I41T mutation located in the putative nuclear export signal (NES) area of NP (35), which raises the question whether FA-6005 exerts effects on the localization of NP and thereby inhibiting vRNP complex export. To test our hypothesis, we next examined the effects of FA-6005 on translocation of NP in MDCK cells transiently expressing NP. Importantly, we found that NP was mainly detected in the cytoplasm at 24 h post-transfection (p.t.) in the control group while WT NP export but not I41T NP was retained in the presence of FA-6005 (Fig 4C). Taken together, these results suggested that FA-6005 depressed the NP/vRNPs export via interacting with residue I41 (located on putative NES) of NP protein and thereby...
exerting an inhibitory effect on virus infection.

FA-6005 perturbs virus uncoating process and vRNP import at early stage.

The results from TOA assay have indicated that FA-6005 impeded virus infection at the early stage of infection. To validate the entry process perturbed by FA-6005 was not HA and NA-specific, we explored the inhibitory activity of FA-6005 through the pseudotype virus containing the luciferase gene and encoding either the VSV envelope protein (pseudo-VSV) or H5 HA and N1 NA (pseudo-H5N1) in the presence of DMSO or FA-6005. The EC_{50}s of FA-6005 against both pseudo-VSV and pseudo-H5N1 were found to be higher than 100 μM, further indicating that FA-6005 inhibits NP-involved entry steps (Table S3). pH-induced fusion of influenza envelops with the limiting membrane of endocytic vacuoles provides the virus genome (that is, viral ribonucleoproteins (vRNPs)) with a portal of access to the cytoplasm(32). Dissociation of vRNP from M1 is a pre-requisite for the vRNP translocation into the nucleus as it exposes the NLS (36). We therefore examined whether FA-6005 would affect the uncoating process that NP is involved in. As M1 disperses into the cytosol when undergoing pH-triggered disassociation from the vRNP complexes, M1 can be detected in the cytoplasm following the uncoating process(21). We then visualized M1 proteins to evaluate the dispersal level of M1 in the cytoplasm by immunofluorescence assay. To prevent the synthesis of new viral proteins, 1 mM cycloheximide was added to the infection medium. Strong inhibition on M1 signal was observed after the addition of FA-6005, indicating that FA-6005 inhibited uncoating of virions and restricted disassociation of vRNP-M1 complexes (Fig 5A). Followed by uncoating, vRNPs are imported into nucleus for viral transcription and replication. We next probed the action of FA-6005 during virus nuclear import. The nuclear accumulation of vRNPs was detected by IF assay. Accordingly,
the vRNPs failed to enter into the nucleus in FA-6005 treated group (Fig 5B). Finally, trafficking of vRNPs at the early stage was perturbed by FA-6005. Therefore, these data collectively suggested that the inhibitory effect of FA-6005 at early stage during virus infection resulted from NP or vRNPs, which further supports the conclusion that the molecular target of FA-6005 is NP.

FA-6005 impairs the trafficking of circulating RNPs in the cytoplasm and leads to marked defects in virions budding.

Our findings have indicated two mechanisms of action for FA-6005: the direct inhibition of viral transcription, and the blocking of nuclear trafficking of NP. However, it could not explain why FA-6005 displayed a late-acting effect, as shown in the TOA assay (Fig 3A). As FA-6005 has the same Y289 binding pocket as nucleozin, we thus postulated that FA-6005 might affect vRNP cytoplasmic trafficking to the apical PM region, a hallmark for late-stage infection, by trapping the vRNPs in the cytoplasm. We therefore infected MDCK cells with WT virus or the NP I41T mutant virus in order to follow the localization of vRNPs by FISH assay. Rab11 was also stained since Rab11-positive vesicles transport vRNPs to the PM region at the apical membrane(37). The majority of Rab11 was distributed throughout the cytoplasm in both treated and untreated cells. In the untreated cells, vRNA was exported from the nucleus 6 h post infection and accumulated in the cytoplasm around 8 h post infection with a large portion of the vRNA and Rab11 being co-localized in the cytoplasm (Fig 6A). In contrast, large RNP complexes co-localized with Rab11 were seen in the perinuclear region in FA-6005-treated group (Fig 6A). Therefore, the timing of the effect we observed, as well as the inclusion of Rab11 in the complexes (Rab11 interacts with RNP s but not non-RNP NP), suggested that the drug blocked the trafficking of RNP s. Importantly,
the localization of vRNP and Rab11 exhibited no differences in the NP I41T mutant, with or without FA-6005 (Fig 6B). Our results suggested that the interaction of FA-6005 with vRNP-Rab11 complex has perturbed the cytoplasmic transport during the late stage of the infection cycle, and since the proper trafficking of the vRNPs for virion budding was halted.

The prominent cytoplasmic aggregates of Rab11 and vRNA clearly reflected the disruption of normal vRNP trafficking, hence we next tested if this resulted from the drug “dismantling” assembled RNPs. A system in which cells are transfected with FLAG-NP and superinfected with virus to produce FLAG-tagged RNPs that can be affinity purified was enacted. A WSN/33 H1N1 virus was inoculated into MDCK cells 12 h after FLAG-NP transfection, and cells were treated with compound 6 h later and lysed at 8 h p.i. Western blotting for aliquots of the cell lysates and affinity fractionation over flag-magnetic beads was also examined. The results indicated that all of the viral proteins tested were present in the supernatant of infected cells in equal quantity, as was the cellular protein GAPDH. The NPs in RNP and M1 were co-precipitated equally in the absence and presence of FA-6005 after affinity purification of FLAG-NP. Little effect was evident in the disruption of vRNP assembly by FA-6005 (Fig S6). The system successfully detected RNP-M1 complex formation, which was not substantially changed by FA-6005 treatment. We also examined whether the FA-6005 would influence the recruitment of RNA to NP by EMSA assay. An RNA shift pattern was visualized by staining with ethidium bromide, while the protein display pattern was stained by Coomassie Brilliant Blue G-250. From the gel staining, it was discovered that NP-RNA binding activity, as well as NP-RNA complex formation, did not change with or without FA-6005 (Fig S7), which is consistent with our NP/FA-6005 co-crystal structure. Therefore, it can be concluded that the FA-6005 induced-aggregation of vRNPs in the cytoplasm...
did not result from the disruption of RNP assembly and NP-vRNA interaction. These results are consistent with our speculation for NP-FA-6005 crystal complex.

The vRNP cytoplasmic trafficking are interconnected in an intricate network whereby disruption of one pathway can have secondary effects on other vesicular pathways. To eliminate the possibility that FA-6005 may exert a general effect on the exocytic pathway and, as a consequence, induces disruption of the trafficking of viral membrane proteins, we then examined the localization of α-tubulin which participated in RNP-Rab11 transport pathway and the distribution of HA was also investigated by FISH assay. The localizations of both α-tubulin and HA was found to be identical between the untreated and treated groups while compound-induced aggregation of vRNP was observed (Fig S8). Thus, the normal trafficking of cellular and additional viral proteins suggested that FA-6005 did not affect the function of the microtubule network and the effects of FA-6005 on viral and cellular trafficking pathways are apparently specific to the RNP-Rab11 complex in infected cells.

As a consequence of impaired RNP cytoplasmic trafficking, the viral genome would be impeded reaching the plasma membrane (PM) in the late stage of virus life cycle, leading to marked defective budding of virions. To investigate this relationship, we analysed virions budding from WT and variant mutant virus in the absence or presence of FA-6005 by TEM. As illustrated in Fig 6C, mature viruses budded out from PM by 8 h pi in the control group while little mature virions were detected after treatment with FA-6005 6 h pi onward, and the budding event exhibited obvious defects compared to the untreated group. Importantly, no significant difference was observed regarding the budding process in NP I41T mutant virus, with or without FA-6005. Thus,
FA-6005 clearly impeded vRNP cytoplasmic trafficking, and resulted in virion budding defects, which can match the antiviral effects of FA-6005 in the late stage of viral infection (Fig 3A). Collectively, we have successfully identified another novel NP inhibitor FA-6005 with distinct mode of actions from previously reported inhibitors. The co-crystal structure of NP/FA-6005 complex clearly showed the binding pocket of FA-6005 and suggested the potential mechanisms. We have also elucidated the inhibitory mechanism of FA-6005, and showed that it exerts pleiotropic inhibitory effects on various steps of viral infection cycle. FA-6005 inhibited viral RNA and protein synthesis and post-entry process including uncoating, nuclear import and export process when FA-6005 is present at the start of infection. Moreover, when added at later time points, FA-6005 potently blocked virus infection without affecting viral macromolecular synthesis. Instead, it induced large perinuclear aggregates of RNPs with cellular Rab11 that had undergone nuclear export, thereby leading to much reduced production of virus progeny. Furthermore, the amino acid residue found in the binding pocket at FA-6005 are highly conserved among strains of influenza virus, suggesting that the small pocket within NP may be a promising target for antiviral drugs, particularly a multifunctional NP inhibitor. This work also provides proof of FA-6005 to be a new lead of anti-influenza compounds, and highlight a novel strategy for developing new drugs that target the NP pocket.

**Discussion**

The influenza virus poses a global public health concern and remains a human menace that has not
been fully addressed yet. Currently we are limited in the countermeasures to prevent and treat influenza virus infection. Influenza virus claims 250,000–500,000 lives annually, even with the availability and use of vaccine and limited antiviral drugs (38, 39). While M2 ion channel inhibitors are not recommended for clinical use due to their reduced effectiveness, NAIs and a recent approved cap-dependent endonuclease inhibitor Baloxavir are the mainstay of antiviral treatment for influenza infections (9). However, with regard to the segmented reassortment feature, coupled with the vast range of natural hosts for influenza A virus, and the emergence of reduced susceptible viruses to NAIs and Baloxavir, novel antiviral drugs with different mechanisms of action are therefore urgently needed.

Influenza nucleoprotein stands out as one of the most promising drug targets since it is highly conserved among influenza strains isolated from different species (16). The multi-functional role of NP provides a number of opportunities for therapeutic intervention. This study has successfully illustrated that chemical genetics is an effective approach and a powerful tool in identification of novel small-molecular inhibitors against influenza virus. Following the high-throughput screening of 50,240 compounds, we identified a novel compound FA-6005 as potential anti-influenza NP inhibitor with a defined mechanism of action. FA-6005 was shown to extend its breadth to multiple influenza A virus subtypes and even to influenza B viruses. Furthermore, in vivo data indicated that FA-6005 prevented body weight loss, increased the survival rate and reduces the viral titers of influenza-infected mice, thereby rendering it as a promising lead compound for the treatment of severe influenza (Fig 1). Though FA-6005 showed less efficiency than zanamivir for treatment of influenza A virus-infected mice, the inhibitory effect of this compound could be further improved by development of its congeners. Moreover, it has been reported that emergent
amino acid substitutions in the polymerase acidic (PA) protein occurred at a frequency of 2-20% and was found to confer reduced susceptibility to Baloxavir(40). The rise in resistance to currently approved influenza antivirals such as amantadine has emphasized the potential benefits of antiviral cocktails of small molecules for future therapeutical strategy(13). Given the different target of FA-6005, this compound would be a good candidate to include in a potential anti-influenza drug cocktail by providing synergistic effect combined with current clinical use of zanamivir or Baloxavir for reduced susceptibility virus treatment and lowering the chances of developing resistance.

Substitution mutation I41 was discovered in the viral nucleoprotein for resistant mutant raised with FA-6005, as resistant phenotypes were confirmed by reverse genetics. X-ray crystallography incorporating FA-6005 and monomeric NP identified two potential binding sites for FA-6005 (Fig 2). Consistent with our mutational study, FA-6005 interacts with several amino acid residues around I41 binding pocket: I41, D51, and A286, etc. Furthermore, the configuration of FA-6005 at NP I41 pocket also revealed strong binding energy in our molecular docking model (data not shown). Moreover, the residues around Y289 binding pocket are less resistant to FA-6005, indicating Y289 pocket is not the major binding domain for FA-6005. Taken together, FA-6005 may exert inhibitory effects against IAVs through interaction with residue at I41 pocket and the crystal structures were also very helpful in rationalizing the mechanisms of action for FA-6005.

The NP protein is highly conserved at the FA-6005 binding pocket across seasonal, pandemic, and highly pathogenic avian influenza A viruses. NP I41 is highly conserved in currently circulating influenza viruses as the most common amino acid at position 41 in the NP is isoleucine (99.8%).
and the frequency of polymorphic natural mutation NP I41V in 2223 of human influenza A virus strains was only 0.2%. Therefore, the resistant substitution mutation NP I41T generated by FA-6005 perhaps occurs only in the presence of our compound. Thus, it is not yet obvious that the resistance of viruses to FA-6005 would be problematic during clinical use.

The broad-spectrum feature of FA-6005 against various influenza A strains could be explained as amino acids are well-conserved among influenza A species though their functional importance for viral replication remains to be further studied. However, it is surprising that FA-6005 also has its inhibitory property on influenza B virus with an EC_{50} of 8.02±0.81 μM, whose structure is quite different from influenza A virus. The NP proteins of influenza A and B do not share identical amino acid at the identified resistant mutation site I41 and R55 but show identical amino acids at position D51. By mapping the corresponding resistant mutations onto the crystal structure of the influenza B NP protein, it was found that I41 was located on the surface and will not form a pocket for FA-6005 to access. These results suggest that FA-6005 may interact with influenza B NP protein at a different position which is worthy for further study.

FA-6005 was found to perturb virus transcription and replication at post-entry stage while it was also found to inhibit virus entry and subsequently inhibiting virus infection though with a higher EC_{50} in a modified PRA assay (Fig S4). Such results indicated that FA-6005 interferes with various stages of the influenza virus life cycle, including the adsorption, entry, replication and transcription, export processes. Moreover, FA-6005 showed distinct inhibitory effects on influenza virus infection in TOA assay (Fig 3), indicating that FA-6005 inhibited viral replication via a pathway different from that of approved antiviral drugs. The mode of action of FA-6005 in
infected cells was further demonstrated by the isolation and characterization of NP I41T variants with reduced sensitivity to the compound. When added early until the end of one cycle during virus infection, FA-6005 was found to inhibit RNP activities, viral RNA and protein synthesis. However, this cannot explain the early-acting drug effect when FA-6005 exists at 0-2 h p.i. during virus life cycle. Further analyses showed that FA-6005 interfered with virus entry involving NP proteins, including the uncoating process and the nuclear import process. Furthermore, we found FA-6005 also blocked the nuclear export of vRNPs, which may be explained as residue I41 is predicted to locate in a putative NES(41). The above result indicating the NP-FA-6005 binding pocket may play a role in M1-RNP disassociation and vRNPs shuttling between nucleus and cytoplasm.

Regarding the undoubted ability of FA-6005 to inhibit earlier steps in the virus life cycle, given that no effect on viral RNA and viral protein synthesis but induced rapid aggregation of cytoplasmic RNP with cellular Rab11 was observed when adding the compound during the late stage of virus replication and such aggregation of RNP-Rab11 complex also correlated with a marked reduction in the amount of virus budding. More importantly, we saw little effect of FA-6005 treatment on other viral proteins and microtubule network during cytoplasmic trafficking stage. We therefore conclude that the late-acting effect of FA-6005 on virus replication is directly attributable to effects on RNP cytoplasmic trafficking and the proper trafficking of the viral genome may be specifically required to allow efficient virus assembly and/or budding. Taken together, the results above could be due to the protein aggregation capacity of FA-6005 as the location of the Y289 binding sites of FA-6005 overlaps with those conferring resistance to nucleozin, whose mechanism is defined to cause aggregation. These results expand the role of the
interacting binding pocket in NP nuclear transport and virus replication and strongly support a distinct antiviral mechanism of FA-6005 in prohibiting virus replication and trafficking.

Recently, several compounds targeting NP have been identified as novel antiviral drug candidates (26, 27, 42-44). However, they showed weak antiviral effects since they disrupt only one of the NP functions such as NP export or self-oligomerization and RNA-binding capacities. Developing a drug that inhibits multiple NP functions will be extremely effective because NP is the most abundant multifunctional viral protein in infected cells(16). While the mechanism of nucleozin is well-defined(28, 30), the rapid appearance of resistant viruses and the limited antiviral spectrum of nucleozin promoted us to characterize other NP inhibitors that perturbed several NP functions.

Our current study reveals an important antiviral mechanism of FA-6005 by targeting influenza A nucleoprotein with pleiotropic inhibitory effects on various steps of viral life cycle. We also provide evidence that binding pocket of FA-6005 are effective druggable targets by structural analysis and identify its multi-functional role during virus infection.

Given the different target between current approved anti-influenza therapeutics and our compound, it is expected that NP I41T variant mutant virus is highly susceptible. It has also been reported that single use of baloxavir is associated with the frequent emergence of variants with reduced susceptibility(40), However, the use of a combination of antiviral compounds that could rapidly reduce viral replication and limit the emergence of antiviral resistant strains remains an attractive approach. Thus, it is highly promising that the current generation of this NP inhibitor FA-6005, particularly when used in combination with current approved NAIs or baloxavir, has the potential to be added to our therapeutic armamentarium in fighting against the fast-evolving influenza virus.
and providing our repertoire of antiviral options including more effective treatment for severely ill
or hospitalised patients. Moreover, it has been reported that single-dose baloxavir showed
significant postexposure prophylactic efficacy in preventing influenza in household contacts of
patients with influenza(45), thus, considering FA-6005 as another broad-spectrum inhibitor with
pleiotropic inhibitory effects on functions of vRNPs of influenza virus, it is highly anticipated that
our current compound can be considered as both the prophylaxis and therapy of influenza A and B
virus infections.

502  Materials and Methods

503  Cell lines and viruses. 293T and Madin-Darby canine kidney (MDCK) cell lines were purchased
from ATCC and shown to be mycoplasma-free using the MycoAlert mycoplasma detection (Lonza;
LT07-318). Cells were maintained in MEM or DMEM with 10% heat inactivated fetal bovine
serum (HI-FBS). Influenza A virus subtypes A/WSN/33 [H1N1], and A/PR/8/34 [H1N1] were
propagated in MDCK cell culture in either plain MEM supplemented with 0.2% FBS. Other virus
strains, including A/Hong Kong/415742/2009 [H1N1], A/California/NHRC0007/2005 [H3N2],
clinical isolate [H7N9] and B/Wisconsin/01/2010 were propagated in either MEM or DMEM
without FBS. All experiments involving live clinical isolate [H7N9] followed the standard
operating procedures of the approved Biosafety Level 3 facility according to a previous report
(46).

513  Antibodies. Influenza A virus nucleoprotein antibody (MAB8257, EMD Millipore); GAPDH
antibody (G9545, Sigma-Aldrich Inc); Influenza A virus M1 antibody (GTX125928, Genetex);
Anti-Influenza A Antibody (ab1074, EMD Millipore); Influenza A virus H1N1 HA
(Hemagglutinin) antibody (GTX127357, Genetex); alpha Tubulin Monoclonal Antibody (A11126,
Thermo Fisher Scientific); RAB11 Polyclonal Antibody (71-5300, Thermo Fisher Scientific);
The Donkey anti-Mouse IgG (H+L) Secondary Antibody AF488 (A32723, Thermo Fisher Scientific);
The Donkey anti-Mouse IgG (H+L) Secondary Antibody AF594 (A-11032, Thermo Fisher
Scientific); horseradish peroxide (HRP) linked goat anti-rabbit antibody (31460, Thermo Fisher
Scientific).

Plaque reduction assay (PRA). The PRA assay was performed in triplicate as described in details
previously (28). In brief, confluent MDCK cells were seeded in 24-well tissue culture plates (TPP,
Switzerland) using MEM (Thermo Fisher Scientific, USA) in 10% FBS one prior to conducting
the assay. After 16 to 24 h, cells were infected with either 50 plaque forming unit (pfu) of
influenza virus for 24 well plates with or without the addition of serial diluted compounds.
Infected cells were allowed to incubate at 37 °C with 5% CO₂ for 1.5 h before removing unbound
viral particles by aspiration. At the end of incubation, the MEM was mixed with 1 % FBS, 1
μg/mL TPCK-treated trypsin (Thermo Fisher Scientific, USA) containing the corresponding
concentration of compounds and 0.75% low melting agarose (LMA) (Thermo Fisher Scientific,
USA), and overlaid the infected cells. Agarose plugs were then removed and monolayers were
stained with 0.7% crystal violet (BDH, Poole, England) and the plaques were counted. The
percentage inhibition of plaque formation at each compound concentration relative to the control
(without compound) was determined, and the median effective concentration, EC₅₀, representing
the concentration of a compound that is required for 50% inhibition in vitro, was calculated by
Sigma plot (SPSS, USA). No FBS was included in the overlying medium for strains: A/Hong Kong/415742/2009 [H1N1], A/California/NHRC0007/2005 [H3N2], the clinical isolate [H7N9] and influenza B viruses.

**Cell viability assay.** CellTiterGlo kit (Promega) was used to test the cell viability of selected compounds by detection of ATP levels as a function of cell viability according to manufacturer’s instructions. The assay was performed by seeding MDCK cells at 20000 cells/well in a total volume of 100 μL MEM with 10% FBS to 96-well cell culture plates one day prior to conducting the assay. After 24 h, cells were washed once with 1×PBS and replenished with MEM before addition of the compound. The Compound was serially diluted in 96-well plates using MEM with 1% FBS, starting from 1 mM by two-fold dilution. The monolayer MDCK cells were treated with serially diluted FA-6005 or dimethyl sulfoxide (DMSO) as the control in triplicates. After 72 h of incubation at 37°C with 5% CO₂, a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium present in each well was added. The luminescence signal was read using a DTX880 multimode detector followed by incubation of the reagent and cells for 10 min at room temperature. The relative metabolic activity of ATP was calculated by normalization the mean of raw signals for each concentration of the compound to the mean signal for the negative control. This calculation normalizes negative control to 100% ATP activity.

**Animal experiments.** 5 to 7-week-old BALB/c female mice were kept in biosafety level 2 housing and were provided access to standard pellet feed and water ad libitum. All experimental protocols followed the standard operating procedures of approved biosafety level 2 animal facilities and were approved by the Committee on the Use of Live Animals in Teaching and
Research (CULATR). Experiments were performed in triplicate as reported previously(28). As the maximum solubility threshold of FA-6005 is 100 μM in 2% DMSO/MEM using a generic UPLC method, we therefore firstly dissolved FA-6005 in DMSO to prepare a 250 mM stock solution. 30 μL of stock solutions containing FA-6005 or DMSO were further dissolved in 3ml 50% v/v methyl cellulose/PBS to make 2.5mM solutions. The doses of candidate molecule FA-6005 were selected according to the potential drug toxicity evaluated in mock-infected animals. Moreover, since the free drug concentration in the tissue interstitial fluid is generally similar to the free plasma concentration under equilibrium and thus, we can link EC₉⁹ of FA-6005 in vitro to its in vivo tissue interstitial fluid concentration. Therefore, the mice were divided into three groups: One group (10 mice) was injected intraperitoneally (i.p.) with zanamivir(100mg/kg) (GlaxoSmithKline), a second group (10 mice) was injected i.p. with the compound FA-6005(10mg/kg, which is equal to a final concentration of 100 μM in 5ml body fluid) and the untreated group (10 mice) was injected with 200μl of DMSO 1 h prior to inoculating the mice intranasally with 30 LD₅₀ (6000 PFU) of the influenza A/PR/8/34 H1N1 virus in zanamivir, FA-6005 or DMSO. Two doses per day of i.p. zanamivir, FA-6005 or DMSO solutions were given for 7 days. The mice were observed for illness and survival rates for 14 days until death. Five mice from each group were sacrificed 6 days post-infection to determine viral titre and pathological changes in lungs by plaque assay (half lung).

Generation of escape mutant influenza A virus resistance to FA-6005. The method of generating a mutant virus followed the protocol in our previous study(28). The development of viruses resistant to selected compound, FA-6005, was generated by serial passage of A/WSN/33 [H1N1] in MDCK cells in the presence of increasing concentrations of inhibitors. The A/WSN/33
[H1N1] parental strain was also passaged in the MDCK cell line without inhibitors as a control. Influenza A/WSN/33 virus at MOI 1 were inoculated into MDCK cells with the addition of two concentrations for the compound FA-6005. The lower concentration of compounds used in the first passage was 10 μM, 5 x higher than its initial EC50, while the higher one was 20 μM. At 72 h post-infection, if CPE was observed in both concentrations of the compound, virus supernatant in the presence of the higher compound concentration would be harvested; otherwise, the one with the lower compound concentration would be harvested. The strategy and the condition of the compound to generate escape mutants were shown in table 3:

As CPE was observed in the condition of higher concentration of the compound in the first four passages, the concentration of the compound subsequently increased until 80 μM. The desired resistant viral clones were purified by plaque isolation on MDCK cell monolayers in the presence of FA-6005. Three individual purified clones were propagated into MDCK cells in the presence of the compounds followed by viral RNA extraction, complementary DNA (cDNA) of all eight segments was obtained by reverse transcription using Superscript III reverse transcriptase (Invitrogen) and cDNA was amplified by PCR and subjected to whole genome sequencing.

was used to transfect the co-cultured 293T and MDCK cells according to manufacturer’s instructions. The infectious particles from the supernatants were harvested at 72 h post-transfection, and the recombinant virus titer was determined by plaque assay.

Growth kinetics of recombinant influenza virus. MDCK cells were infected by resistant mutant viruses, recombinant virus or A/WSN/33 [H1N1] virus at MOI of 0.001. Virus supernatant was collected at 16 h, 24 h, 40 h, 48 h and 71 h post-infection by speed centrifugation to remove cell debris. The collected supernatant was immediately stored at -80 °C. Finally, viral titer of each virus at various time points was measured by plaque assay to determine their growth rate during viral life cycle. Each time point was performed in triplicates as described previously (47).

Protein expression and purification. Full-length genomic segments of influenza A/WSN/33 NP sequence was cloned into a pET28 vector and the recombinant plasmid pET28-NP was transformed into competent *E. coli* strain Rosetta 2. The bacteria were cultured at 37 °C until the OD600 of the cell culture reached 0.6. And then isopropyl-β-thiogalactoside (IPTG) was added to induce protein expression at 18 °C for about 16 h. The cell pellet was harvested and lysed by sonication in 50 mL of lysis buffer (20 mM HEPES, pH 7.5, 500 mM NaCl). The cell debris was removed by centrifugation and the cleared lysate was loaded into HisTrap HP column (GE Healthcare) mounted on an AKTA purifier (GE Healthcare). The column was washed with 50 mL of lysis buffer and then the protein was eluted with a linear gradient of 20-200 mM imidazole. The peak fractions were collected, concentrated and loaded onto Superdex-200 HiLoad 16/60 gel filtration column (GE Healthcare) pre-equilibrated with buffer containing 20 mM HEPES at pH
7.5, and 150 mM NaCl. The purified protein was concentrated to 20 mg/ml for crystallization.

**Crystallization of FA-6005 with NP.** NP was mixed with FA-6005 (final concentration of 0.5 mM) before crystallization. Crystals were obtained in 0.1 M sodium acetate, 0.05 M magnesium acetate, 0.1 M MES, pH 6.0, 7% PEG8000 and 5% glycerol, and then the crystals were frozen by quick immersion in liquid nitrogen. X-ray diffraction data were collected at beamline BL17U, Shanghai Synchrotron Radiation Facility and processed with HKL2000 package. Molecular replacement was applied to solve the phase problem of NP/FA-6005 complex, using the program Phaser in the CCP4 program suite. The apo H1N1 NP monomer structure (PDB code 2IQH, Chain A) was used as a search model to determine the NP structure, and its tail loop was not included in the initial calculations. The unliganded structural model was refined using program REFMAC5 in the CCP4 suite, and program COOT was used for manual model building and adjustment after each refinement cycle of REFMAC5. The space group is C222₁, with one trimer and three FA-6005 molecules contained in each asymmetric unit, and these three FA-6005 molecules were located in three different sites of NP molecules. The quality of the current structural model was evaluated by PROCHECK in the CCP4 program suite. Data collection and refinement statistics are summarized in Table 1. Coordinates and structure factors have been deposited in the protein data bank, with PDB ID code 6J1U.

**Time of addition assay (TOA).** The protocol of TOA assay followed the literature described by Furuta et al. (48). 5 × 10⁴ cells per well MDCK cells were cultured one day prior to the assay.

After 16 to 24 h, cells were washed once with PBS and subjected to A/WSN/33 [H1N1] virus infection at MOI 1 on ice (approximately 4°C) in fresh DMEM containing 0.2 % FBS and 1
μg/mL TPCK-treated trypsin. After adsorption for 60 min (-1 h), cells were washed three times with PBS and replaced with fresh DMEM containing 0.2 % FBS and 1 μg/mL TPCK-treated trypsin, then incubated at 37°C with 5% CO₂ (time zero). 20 μM FA-6005 or DMSO were added at the indicated time points(-1-0,0-2,2-4,4-6,6-8,8-10h). After each incubation period, the monolayers with compounds or control were washed three times with PBS and incubated with fresh medium until 10 h post-infection. Samples were collected at 10 h post-infection and subjected to titer determination using plaque assays after two freeze and thaw cycles. Each time point in the TOA was conducted in triplicates in 24-well tissue culture plates.

**Luciferase reporter assay for polymerase complex activity.** The luciferase reporter assay was performed as described previously(28). 2 x 10⁴ HEK293T cells per well were seeded in a 96-well tissue culture plate one day before assay. Components of the RNP complex, comprising of pHW2000-NP, plus pHW2000-PA, pHW2000-PB1, and pHW2000-PB2, or respective mutant plasmids, combined with a luciferase reporter plasmid, pHY-Luci, which contains noncoding sequences from the M segment of influenza A virus driven by PolI were co-transfected into cultured HEK293T cells using Trans-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer’s instructions. A phRL-TK (Promega Co., Madison, WI, USA), which expresses renilla luciferase, was also co-transfected as an internal control for data normalization. After 2h post transfection, serial-diluted compound solutions in 2-fold dilution were added into the transfected cells. At 24 h post transfection, cell lysates were prepared for the Dual-Luciferase® Reporter Assay System (Promega Co., Madison, WI, USA), and luciferase activity was measured using a DTX880 multimode detector (Beckman, USA).
**Western blotting.** Cells were infected with influenza A/WSN/33 [H1N1] virus at MOI 10 in fresh MEM containing 0.2% FBS and 1 μg/mL TPCK-treated trypsin in the presence of 20 μM of FA-6005 or DMSO. At 2, 4, 6, and 8 h post infection, virus was removed and cells were washed with PBS once and lysed. Supernatants were collected after centrifugation at 12000 rpm for 10 min. Equal amounts of proteins dissolved in the cell lysate were loaded into wells of the SDS-PAGE gel, along with a molecular weight marker and ran for 1–2 h at 100 V. Gels were transferred to a nitrocellulose membrane at a constant current of 400 mA for 2.5 h. Then, the membrane was blocked with 10% w/v not-fat milk in PBST for 1h at room temperature. Membranes were incubated with appropriate dilutions of primary antibodies of α-H1N1 and GAPDH in a blocking buffer overnight, then washed by PBST three times at 15 min each. The membranes were incubated with the recommended dilution of conjugated secondary antibodies in a blocking buffer at room temperature for 1 h. The membrane was washed in three washes of TBST at 15 min each, dried and processed for image taking by the Odyssey Imaging System (LI-COR Biosciences, USA).

**Quantitative RT-PCR assays for viral RNA synthesis:** MDCK cells were infected with influenza A/WSN/33 virus at MOI 10 in fresh MEM containing 0.2% FBS and 1 μg/mL TPCK-treated trypsin in the presence of 20 μM of FA-6005 or DMSO. At 2, 4, 6, and 8 h post infection, cells were harvested followed by extraction of viral RNAs using the RNeasy Mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer’s instructions. Each time point was conducted in triplicates. The expression levels of viral mRNA, vRNA, and cRNA of the NP and NA segment were quantified as described(34).
Fluorescence in situ hybridization (FISH) and Immunofluorescence microscopy (IF). MDCK 684 cells were grown to 70–80% confluency on coverslips. Cells were propagated with influenza A/WSN/33 wild-type virus or mutant viruses at MOI 10 in fresh MEM containing 0.2% FBS and 1 μg/mL TPCK-treated trypsin. 20 μM of FA-6005 or DMSO was added at the indicated time point. Infections were stopped at indicated time points by fixation with 500 μL of 4% paraformaldehyde (Electron Microscopy Sciences) at room temperature for 15 min. After fixation, the cells were washed with PBS for three times and permeabilized with 1ml 70% ethanol at 4°C overnight. Custom Stellaris® FISH Probes for detection vRNA and mRNA of segment 5 of influenza were designed by utilizing the Stellaris® FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA). The assay for simultaneous IF and FISH was performed following the manufacturer’s instructions available online at www.biosearchtech.com/stellarisprotocols. The slides were stored at 4 °C overnight before being sealed with nail polish to prevent drying. The slides were observed and photographed using a Leica DMIL inverted microscope equipped with a DC300F digital imaging system (Leica Microsystems, Germany) or LSM710 confocal microscopy (Carl Zeiss AG, Oberkochen, Germany).

Pseudotype virus platform. For the preparation of pseudotype virus, avian pseudotype viruses expressing H5 HA and N1 neuraminidase (NA) were produced by cotransfecting 18 μg of pNL4-RE Lac+ plasmid and 3 μg each of two plasmids (pPolII-HA and pPolII-NA) into 293FT cells with polyethylenimine (PEI) transfection solution (Mirrus, Madison, WI). The virus supernatant was collected at 48 h post-transfection by centrifugation to remove the floating cells and cell debris. The supernatant was immediately divided into aliquots and stored at −80°C. Pseudotype virus bearing vesicular stomatitis virus (VSV) envelope protein was also prepared.
similarly by cotransfecting pNL4-R-E-Luc$^+$ and pVSV plasmids. For the antiviral assay for pseudotype virus, compound was 2-fold serially diluted in DMEM supplemented with 10% FBS and then incubated with a $100 \times 50\%$ tissue culture infective dose (TCID$_{50}$) of pseudotype virus at room temperature for 1 h. MDCK cell monolayers were infected with the compound-virus mixture at 37°C for 72 h. Finally, cells were lysed and the luciferase reading was recorded using a luciferase assay system (Promega) according to the manufacturer's manual. The luciferase reading of cells treated with the compound FA-6005 only was regarded as the background, while the samples incubated with the virus and DMSO were regarded as representing the maximum transduction. The EC$_{50}$ of FA-6005 against the pseudotype virus was estimated by determining the percentage of inhibition.

**Uncoating assay and RNP import assay.** Confluent MDCK cells were infected with influenza A WSN/33 virus or a corresponding mutant virus in an infection medium (MEM with 0.2% FBS and 1μg TPCK-treated trypsin) at MOI 100 at 4°C for 1 h. After virus adsorption, the cells were washed with ice-cold PBS to remove the unbound virus particles. Fresh infection medium, with or without compounds, was added to the bound particles, and cells were allowed to internalize at 37°C with 5% CO$_2$ incubator at indicated time. 1 mM cycloheximide was added into the infection medium to prevent the synthesis of new viral proteins. Following 2 h of infection, the infected cells were fixed and permeabilized with 0.1% Triton-X100 in PBS for 3-5 min at RT and incubated with purified mouse monoclonal M1 antibody (Abcam, USA) in PBS (1:100) for 1 h to stain the viral M1. Cells were washed with PBS, followed by incubation with secondary anti-mouse IgG-AF488 (1:150). Nuclei were stained with ProLong® Gold anti-fade reagent with DAPI (Thermo Fisher Scientific, USA). Coverslips were then imaged. While RNP import was
detected at 3 h infection, the infected cells were stained with purified mouse monoclonal NP antibodies in PBS (1:150) for 1 h to stain the viral RNP followed by incubation with a secondary anti-mouse IgG-AF488 (1:150). Nuclei were stained with ProLong® Gold anti-fade reagent with DAPI (Thermo Fisher Scientific, USA). Coverslips were then imaged.

**Pulldown assays.** The protocol for Pulldowns of flag-tagged proteins were modified and performed using Anti-FLAG® M2 Magnetic Beads (Sigma-Aldrich Inc., St. Louis, USA) as previously described M. J. Amorim et al. (24). Confluent 6-well dishes of MDCK cells were transfected with 500 ng of pHW2000-FLAG-GFP-NP. After 24 to 48 h, cells were infected with influenza A WSN/33 virus at a MOI equal to 10. At 6 h post infection, 20 μM FA-6005 was added into the infected cells. DMSO was added as negative control. All samples were collected and lysed at 8 h post infection (hpi) in 500 μL of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Roche Life Science, Switzerland) on ice for 30 min. Cell lysates were pre-cleared with mouse immunoglobulin G (IgG) agarose and the supernatant was bound to mouse Anti-FLAG® M2 Magnetic Beads on ice for 2 h. Beads were washed before and after sample binding with a wash buffer containing 50 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 0.5% NP40, and 10% glycerol (Sigma-Aldrich Inc., St. Louis, USA). Bound proteins were eluted by being boiled in SDS-PAGE sample buffer. Western blotting was performed and imaged as above described.

**Electrophoretic mobility shift assay (EMSA).** NP proteins were incubated with compounds at RT for 30 min, followed by the addition of 24 nt small RNA and incubation for another 30 min, and addition of up to 10 μL of nuclease-free water. The final concentration of small RNA was 2
μM, and the molar ratio of NP: RNA was kept at 4:1 (18). After incubation, the samples were mixed with 3 μL 6 X DNA loading dye (0.25% bromophenol blue, 0.25% xylene xyanol, 40% sucrose) and loaded into sample well of non-denatured 4-12% gradient Bis-Tris NuPAGE gel (Thermo Fisher Scientific, USA). The gel was equilibrated by pre-electrophoresis at 50 V in 1 X TBE, and electrophoresis was performed at a constant voltage of 150 V for 35 min at room temperature in 1 X TBE. Gel was visualized by EtBr stain for small RNA shift patterns. The same gel was also stained with Comassie brilliant blue G-250 to display NP protein shift patterns.

Transmission electron microscopy (TEM). To understand the effects of compound for virion formation, TEM was performed as our previous study (17). 5 × 10⁶ MDCK cells were seeded into a 10 mm tissue culture dish (TPP, Switzerland) in MEM with 10% FBS. On the following day, cells were washed once with PBS and infected with influenza A/WSN/33 or corresponding mutant viruses at MOI 10 in MEM with 0.2% FBS and 1μg/mL TPCK-treated Trypsin. Budding virions of either strain were not detected at 4 hpi as we described before, thus 20 μM of compound was added into the cells after 6h pi, and DMSO was added as a control. At 8 h post infection, cells were washed with a 0.1 M phosphate buffer at pH 7.4 three times and incubated at 4°C in 2.5% glutaraldehyde (EM grade) in 0.1M phosphate buffer (pH 7.4) overnight. The monolayer was washed with phosphate buffer the following day, and cells were scraped into phosphate buffer and harvested by centrifugation at 14,000 × g for 10 min. The supernatant was replaced with fresh phosphate buffer and processed by the Electron Microscope Unit at HKU. Images were acquired using a FEI Philips CM100 transmission electron microscope equipped with a Deben AMT digital camera and an EDAX Genesis XM4 EDX system.
Statistical analysis: Statistical analysis was performed using GraphPad Prism. Statistical significance was determined with student \( t \)-test and two-Way ANOVA. \( p \) value <0.05 were considered significant. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) and ****\( p < 0.0001 \). NS, no statistical significance.

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Figure legends

**Fig 1. Chemical structure and antiviral activities of FA-6005.** (A) Chemical structure of FA-6005. (B) Broad spectrum antiviral activities of FA-6005 against influenza A subtypes and Flu B viruses. (C) Antiviral activity of FA-6005 in multicycle growth assays. (D-E) Efficacies of FA-6005 and zanamivir in a mice influenza A PR/8/34 H1N1 infection model on (D) body weight; (E) survival rate; (F) viral load in the lungs. Error bars represent the s.d. of the mean from three biological replicates (n = 3) in an individual experiment. Three independent experiments were performed for PRA assay, viral growth assay and animal study, and representative data are shown. *Results significantly different from that of each control by t test (p< 0.05). ****, results significantly different from that of each control by t test (p < 0.0001).

**Fig 2. FA-6005 targets on Influenza A NP.** (A) Escape mutant virus and recombinant virus carrying the I41T substitution in influenza A NP confer resistance to high concentrations of FA-6005. (B) Growth kinetics of NP I41 mutant virus in the presence of FA-6005. (C) Crystal structure of NP-FA-6005 complex showing the I41 binding pocket. Left panel) the interacting residues of FA-6005 are determined by LigPlot+ software(49) . The compound exhibits hydrophobic interactions with I41, D51, G54, R55, S283, V285, A286 and G288. Right panel) the
binding pocket of FA-6005 on NP involves the I41 residue. The NP protein is colored in green while the side chains of the interacting residues are shown and colored in purple. (D) Crystal structure of NP-FA-6005 complex showing the Y289 binding pocket. Left panel) the interacting residues of FA-6005 are determined by LigPlot+ software (1). The compound shows hydrophobic interactions with Y289, R305, L306 and N309. Right panel) the binding pocket of FA-6005 on NP involves the Y289 residue. The NP protein is colored in green while the side chains of the interacting residues are shown and colored in purple. (E) RNP models and the possible FA-6005 positions on its central regions. Reconstruction of the central region of RNP using PDB models 4BBL and 2YMN, respectively, and ligands are fitted in the I41 and Y289 pockets. (F) Detail view of modeled FA-6005 and RNA (represented by brown spheres) in one NP molecule.

Fig 3. FA-6005 inhibits virus transcription and replication. (A) Time-of-addition experiments examining the effect of FA-6005 on various stages of IAVS life cycle. (B) FA-6005 exhibits inhibition of the parental virus NP activity, but not the resistant I41T variant virus NP in a luciferase reporter assay. (C) FA-6005 abolishes viral protein synthesis. MDCK cells were infected with IAVS at MOI 10 in the presence of 20 μM FA-6005. DMSO was added as a negative control. (D) FA-6005 inhibits viral NP vRNA synthesis. **, results significantly different from that of each control by Dunnett’s t test (p < 0.005), ***, results significantly different from that of each control by Dunnett’s t test (p < 0.0005). ****, results significantly different from that of each control by t test (p < 0.0001).

Fig 4. FA-6005 blocks vRNP and NP export. (A) FA-6005 kept wt vRNP retained in nucleus
while (B) did not block I41T variant vRNP export. MDCK cells were firstly infected with A/WSN/33 [H1N1] virus or I41 mutant NP virus at MOI 10, DMSO or 20 μM FA-6005 was added to each set at 2h p.i. Samples were fixed at 10h p.i and stained for NP (shown in green) and processed for FISH for the NP vRNAs (shown in red) and NP mRNAs (shown in purple). Merged images include a DAPI channel shown in blue. (C)FA-6005 has inhibitory effect on free NP export. MDCK cells were transfected with phW2000-NP plasmid 24 h prior to the addition of compounds. Samples were fixed 8 h after DMSO or compound treatment and stained for NP (shown in green). Merged images include a DAPI channel, shown in blue. Each experiment was performed three independent times.

Fig 5. FA-6005 perturbs entry step that NP is involved. MDCK cells was infected with influenza A virus at an MOI of 100 in the presence of 1mM cycloheximide. Infection was carried out on ice for 60 minutes to synchronize virus entry before incubation at 37 °C for the times indicated. (A)Uncoating process was interfered in the presence of FA-6005. Disassociated M1 were visualized by M1 staining and cell nuclei by DAPI staining. (B)Import of NP (green) was blocked in cells treated with FA-6005 while in the DMSO treated cells, virus particles were allowed to enter into the nucleus at 37°C for 3 h. Incoming NP proteins (green) were detected within the nucleus (blue) by staining with mouse monoclonal antibody. Each experiment was performed three times.

Figure 6. FA-6005 impairs vRNP cytoplasmic trafficking, leading to defects on virus budding. (A)FA-6005 induces wt vRNP and Rab11 aggregates in the cytoplasm. (B)I41T vRNP and Rab11
fail to aggregate in the presence of FA-6005. Samples were fixed at 8 hpi and stained for NP (shown in green) and processed for FISH for the NP vRNAs (shown in red) and NP mRNAs (shown in purple). Merged images include a DAPI channel, shown in blue. (C) TEM visualization of budding virions in infected MDCK cells with or without drugs. MDCK cells were infected with WT or NP variant mutant viruses and treated with DMSO or FA-6005 at 6 hpi, then fixed at the 8 hpi and processed for TEM. Images were acquired using a FEI Philips CM100 transmission electron microscope. Arrows indicate defective budding events. Each experiment was performed three independent times.
Table 1. Crystallographic data collection and refinement statistics

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<td>α, β, γ (°)</td>
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*Values in parentheses are for highest resolution shells. Table Footnote.
Table 2. Effect of selected NP point mutations on antiviral activities for FA-6005

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<tr>
<th>Sensitive residues</th>
<th>EC₅₀ (µM) of FA-6005</th>
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<tr>
<td>NP I41T</td>
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<td>NP A286T</td>
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<th>Tolerant residues</th>
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<tr>
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Table 3. Strategy used for generating resistant mutant viruses for FA-6005.

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**Fig 4**

A

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B

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C

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