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Discovery of Novel Antiviral Agents Directed Against the Influenza A Virus Nucleoprotein

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1. Introduction

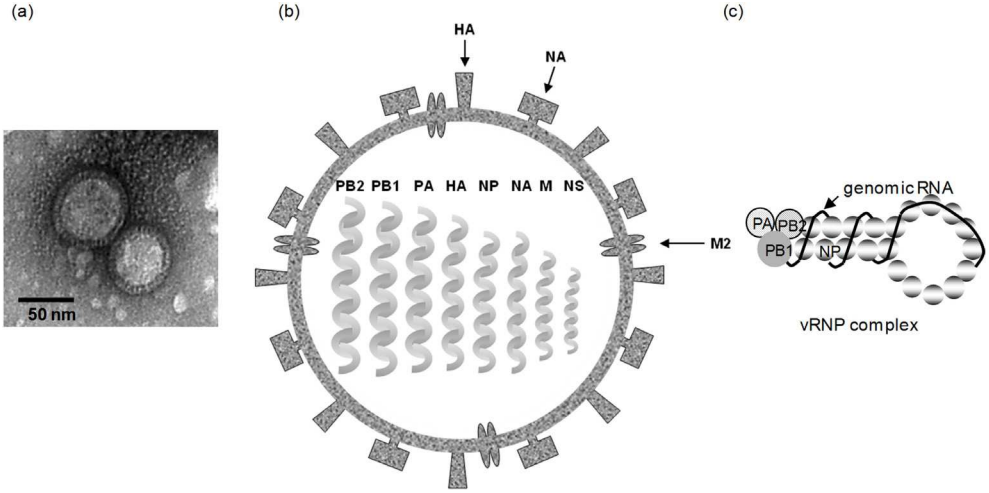
The influenza virus types A, B, and C belong to the family *Orthomyxoviridae*. Influenza B and C viruses are predominantly human pathogens, whereas influenza A virus spreads not only in humans but also in many animals, including birds and pigs. This characteristic of influenza A viruses is a major cause of influenza pandemics in humans. Influenza A viruses are responsible for periodic widespread epidemics, or pandemics, which have taken the form of respiratory diseases with cold-like symptoms, but also sometimes serious disease with high mortality rates (Webster et al., 1992). Four outbreaks of influenza occurred in the last and current centuries: Spanish influenza (H1N1) in 1918, Asian influenza (H2N2) in 1957, Hong Kong influenza (H3N2) in 1968, and H1N1 influenza in 2009. Recently, an influenza pandemic was caused by swine influenza (H1N1) in 2009, and according to the European Centre for Disease Prevention and Control, so far this global pandemic brought on 1.2 million infections and took the lives of more than fourteen thousand people. A strong sense of fear pervaded, but many lessons were learned from this pandemic. For example, this pandemic illustrated once again that the available vaccines against influenza virus were not completely effective for the prevention of influenza outbreaks, due to the extraordinarily rapid mutation rate that influenza viruses possess (Steinhauer & Holland, 1987). In addition, we did not have enough efficient antiviral drugs to cover all of the people infected (Stiver, 2004). On the other hand, there had been avian influenza (H5N1) infections in humans several years ago in Southeast Asia (Tran et al., 2004), and, importantly, that virus caused a high mortality rate. Putting it all together, in the face of the persistent threat of human influenza A infections, and moreover, the possibility of outbreaks of an avian influenza (H5N1) pandemic (Ungchusak et al., 2005; Wang et al., 2008), there is much concern about the shortage of effective anti-influenza virus agents, and for this reason, the development of novel anti-influenza virus agents is being strongly demanded.

Influenza A virus is a negative-stranded RNA virus with an eight-segmented genome that encodes 12 different proteins, including polymerase basic (PB)1, PB1-F2, an N-terminally truncated version of the polypeptide (N40), the translation of which is directed by PB1 codon 40, PB2, polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein (M)1, M2, nonstructural protein (NS)1 and NS2 (Wise

et al., 2009) (Fig. 1b). Nine of these proteins are incorporated into the virion. The influenza virus particles, which are ~100 nm in diameter, form by budding from the plasma membrane of infected cells (Fig. 1a). On the surface of the virion, there are two main antigenic determinants, the spike glycoproteins HA and NA. There are considerable antigenic variations among influenza viruses consisting of 16 different types of HA (H1-H16) and 9 different types of NA (N1-N9) (Fouchier et al., 2005; Hinshaw et al., 1982; Kawaoka et al., 1990; Rohm et al., 1996; World Health Organization, 1980). In addition, another viral protein is inserted in the viral membrane: M2, a low-abundance ion channel involved in uncoating and HA maturation (Fig. 1b). Underlying the membrane is the matrix or M1 protein, the major structural component of the virion, which is thought to act as an adaptor between the lipid envelope and the ribonucleoprotein (RNP) complexes and is probably the mediator of virus budding (Gómez-Puertas et al., 2000). Inside the shell of M1 lie the viral RNP complexes (vRNPs), which comprise the genomic RNA segments in association with a trimeric RNA polymerase (PB1, PB2 and PA subunits) and the stoichiometric quantities of NP (Fig. 1c). Also found in the virion are small quantities of the NEP and NS2 proteins.

The surface proteins HA, NA, and M2 are the targets of vaccines and anti-viral drugs. Two classes of drugs including adamantanes (amantadine and rimantadine) and NA inhibitors (oseltamivir, zanamivir, peramivir, and laninamivir) are available for the treatment of the influenza infection (Kubo et al., 2010; Vanvoris et al., 1981; Wingfiel et al., 1969; Yamashita, 2011). Because the adamantanes exert several toxic effects on the central nervous system (Bryson et al., 1980; Keyser et al., 2000) and also because of the emergence of resistant variants (Bright et al., 2005, 2006), the use of these drugs is limited. Currently, the NA inhibitors are used widely for drug therapies to treat influenza patients because of high inhibitory effects and little toxicity. However, resistant strains, and especially oseltamivir-resistant strains, have also been reported in recent years (Besselaar et al., 2008; Dharan et al., 2009; Hauge et al., 2009; Hurt et al., 2009). The fact that available drugs target only two steps of the viral life cycle, along with the appearance of oseltamivir-resistant influenza strains, strongly highlights the need for treatment alternatives or novel antiviral drugs targeting other proteins besides M2 or NA.

In contrast to the surface proteins, after entry into the cytoplasm, the vRNPs are imported into the nucleus for the production of viral messenger RNAs. RNA polymerases, such as PB1, PB2, and PA, transcribe and replicate the virus genome, while NP encapsidates the virus genome to form an RNP complex for the purposes of transcription and packaging. Thus, influenza virus transcription and replication are initiated after transport to the nucleus of vRNPs. A promising target for blocking influenza A viruses is the NP, which is expressed in the early stage of infection and plays important roles in numerous steps of viral replication. NP preserves viral genomic RNA (vRNA) stability and contains many functional domains in its sequence, such as a nuclear localization signal (NLS), an RNA binding site, an NP-NP binding site, and a PB2 binding domain. In addition, NP is relatively well conserved compared with viral surface spike protein. Here, we summarize current knowledge about influenza therapy, the functions of NP involved in the nuclear-import step of influenza virus replication, and how this could facilitate the discovery of a new small molecule involved in influenza replication.

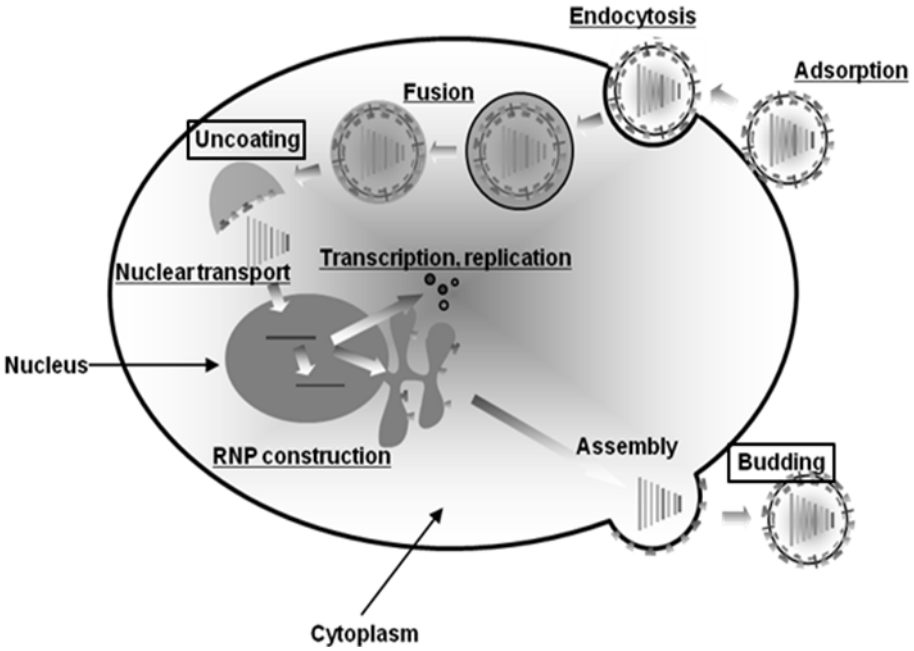


(a) Influenza virus particles (A/WSN/33) were isolated by sucrose density gradient ultracentrifugation, and purified particles were observed by electron microscope with uranyl acetate-staining. (b) Model of influenza virus particle. The particle contains eight segments that make up the RNA genome. There were three major proteins (HA, NA, and M2) on the surface of the viron. (c) Diagram of the viral ribonucleoprotein (vRNP). The vRNA is associated with NP, PB2, PB1, and PA, which are composed of vRNPs.

Fig. 1. Electron microscopy and model of influenza virus particles.

2. Viral life cycle

Influenza A virus is one of the rare RNA viruses that replicate in the nucleus. The design of effective anti-influenza virus therapeutics is based on detailed knowledge of the biology of the virus. Fig. 2 shows the viral life cycle of the influenza virus. Generally, the influenza virus is adsorbed to the host cell through the binding of HA glycoproteins with sialic acid groups as receptors on the host cell, which are distributed on membrane-bound proteins and lipids. Then, the influenza virus is taken up into host cells through receptor-mediated endocytosis (Matlin et al., 1981). In the acidic environment of the endosome, HA changes to the active form and promotes fusion of the viral envelope with the endosomal membrane (Stegmann et al., 1987; White et al., 1982). The acidification of the endosome is necessary not only for the membrane fusion of HA but also the activation of the M2 ion channel. The activation of the M2 ion channel leads to proton entry into the virions, which causes uncoating of the vRNPs (Bui et al., 1996; Pinto et al., 1992). All vRNA are associated with the NP, which is bound at a distance of 24 nucleotides (Compans et al., 1972; Ortega et al., 2000). It is also suggested that vRNA is associated with trimeric RNA polymerase, which are composed of vRNPs (Klumpp et al., 1997). Influenza virus transcription and replication are initiated after the transport of vRNPs into the nucleus. After uncoating, the vRNPs are transported into the nucleus through importin α/β transport systems, where they undergo transcription and replication (Herz et al., 1981; Martin & Helenius, 1991). Transcription of vRNA requires capped RNA primers snartched from cellular pre-mRNAs and premature poly(A) termination of transcripts (Bouloy et al., 1978; Plotch et al., 1979; Robertson et al.,



The crucial steps in Influenza virus multiplication are: 1) attachment of the virus to its target cell; 2) entry of the virus via receptor-mediated endocytosis; 3) fusion of endosome and viral membranes; 4) the RNPs release into the cytoplasm and 5) transport into nucleus; 6) virus transcription and replication in nucleus; 7) the RNPs construction; 8) virion assembly and 9) viral budding. Anti-influenza drugs target two different steps in the viral life-cycle; these steps are shown in boxes.

Fig. 2. Model of the life cycle of the influenza virus.

1981). In contrast, the replication of vRNA is performed in a primer-independent manner (Nagata et al., 2008). The newly synthesized vRNPs are exported from the nucleus to the cytoplasm in association with the viral proteins M1 and NS2 and the cellular protein chromosome region maintenance 1 (CRM1) (Cros & Palese, 2003; Neumann et al., 2004). These vRNPs are incorporated into budding virions. The vRNA is specifically packaged in preference to other cellular RNAs and the different vRNAs are present in an equimolar ratio within a population of virions (Palese, 1977). A mechanism for the specific packaging of vRNA is mediated by *cis*-acting packaging signals in the vRNAs. Specific packaging signals exist in the UTRs and coding regions at both the 5′ and 3′ ends of the vRNAs (de Wit et al., 2006; Fujii et al., 2003; Liang et al., 2008; Muramoto et al., 2006; Noda et al., 2006; Ozawa et al., 2007). The structure of eight separate segments is associated with inter-segment interactions (Muramoto et al., 2006). However, it remains uncertain whether there are specific interactions among the eight RNPs within the virions. The M1 and M2 proteins play central roles in the assembly and budding process. The M1 protein is associated with the cytoplasmic tail of HA and NA. This binding allows for M1 to associate with lipid raft membrane domains, triggering a conformational change that enables M1 polymerization at the site of virus budding (Barman et al., 2004; Gómez-Puertas et al., 2000; Ruigrok et al., 2001). The M2 protein is required for the membrane scission of the budding virions. M2

binds to cholesterol and this allows M2 to alter membrane curvature at the site of virus budding (Rossman et al., 2010a, 2010b). Bud formation and bud release are the last steps of the viral life cycle. NA is responsible for cleaving terminal sialic acid residues from the ends of glycoconjugates on both the virus particle and the host cell in order to facilitate virus release (Air & Laver, 1989).

3. Influenza therapy

The life-cycle of influenza virus is a major target for drug development. Accordingly, significant efforts have been made recently to identify molecules that inhibit the different stages of the influenza virus life cycle. As shown in Fig. 2, the current treatments for influenza infections target two steps of the replication cycle: uncoating and budding. Six drugs are currently available (Table 1): the adamantanes and neuraminidase inhibitors, including amantadine, rimantadine, zanamivir, oseltamivir, peramivir, and laninamivir (Kubo et al., 2010; Vanvoris et al., 1981; Wingfiel et al., 1969, Yamashita, 2011). The adamantanes block the function of the M2 ion channel, preventing acidification-triggered uncoating. The adamantanes were the first effective drugs licensed for influenza treatment (Davies et al., 1964; Dolin et al., 1982; Wang et al., 1993). Despite a degree of treatment effectiveness, however, both drugs induced significant adverse effects in the central nervous system, as well as the emergence of drug-resistant mutants (Bright et al., 2005, 2006; Bryson et al., 1980; Keyser et al., 2000). Recently, the vast majority of circulating seasonal influenza strains has been adamantanes-resistant (Bright et al., 2005, 2006). Neuraminidase inhibitors inhibit the release of virions by competitively inhibiting viral NA. Currently, zanamivir and oseltamivir are widely used to treat acute uncomplicated illness due to influenza A and B. Zanamivir mimics the natural substrate, which fits into the active site pocket of NA (Varghese et al., 1992, 1995; von Itzstein et al., 1993). Oseltamivir was developed through the modification of the sialic acid analogue framework (Kim et al., 1997). Many reports have shown that both drugs are highly efficient in the treatment of influenza (Cooper et al., 2003; Hayden et al., 1997; Monto et al., 1999; Nicholson et al., 2000). In recent years, peramivir and laninamivir, which also target NA, have been licensed as anti-influenza drugs (Kubo et al., 2010; Yamashita, 2011). For oseltamivir, the appearance of drug-resistant mutants has significantly increased in many countries (Besselaar et al., 2008; Dharan et al., 2009; Hauge et al., 2009; Hurt et al., 2009). The oseltamivir-resistant H275Y virus also displays reduced susceptibility to peramivir *in vitro* (Nguyen et al., 2010). On the other hand, no zanamivir-resistant virus has emerged at present. However, because zanamivir requires treatment by the intravenous route, it is not commonly used in clinical treatment.

As mentioned above, in addition to the fact that available drugs target only two steps of the viral life cycle, this appearance of oseltamivir-resistant influenza strains strongly highlights the need for treatment alternatives or novel antiviral drugs targeting other proteins besides M2 or NA. Potential targets for blocking influenza A virus replication are influenza virus RNA polymerases and NP, which is required to form the RNPs. Recently, favipiravir, a novel therapeutic drug targeting viral replication and translation, has been identified (Furuta et al., 2005). Favipiravir, developed by Furuta *et al*. at Toyama Chemical Co., Ltd., inhibits the replication and translation of influenza viruses in a GTP-competitive manner. In addition to these drugs, a few novel antiviral compounds, mycalamide analogs (Hagiwara et al., 2010), nucleozin (Kao et al., 2010) and nucleozin analog FA-2 (Su et al., 2010), were

recently reported as influenza inhibitors targeting NP. Moreover, another anti-influenza compound, 367, has been identified that targets the PB1 protein and influenza RNA-dependent RNA polymerase activity (Su et al., 2010). These observations suggest that the drug targeting of components of the RNPs, such as PA, PB1, PB2, and NP, may provide a new strategy for influenza therapy.

Classification	Generic Name	Commercial Name
M2 Channel Inhibitor	Amantadine	Symmetrel
	Rimantadine	Flumadine
Neuraminidase Inhibitor	Oseltamivir	Tamiflu
	Zanamivir	Relenza
	Peramivir	Rapiacta
	Laninamivir	Inamivir

Table 1. Available drugs against influenza virus infection.

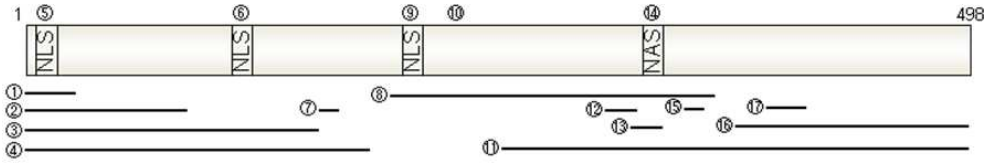
4. NP of influenza A virus

4.1. Function of NP

The influenza virus NP, which is encoded by the fifth genome segment, is expressed in the early stage of infection. It is the major component of the RNP and contains many functional domains in its sequence, such as a NLS, an RNA binding site, an NP-NP binding site, and a PB2 binding domain (Albo et al., 1995; Biswas et al., 1998; Cros et al., 2005; Davey et al., 1985; Elton et al., 1999; Ketha & Atreya, 2008; Kobayashi et al., 1994; Neumann et al., 1997; Ozawa et al., 2007; Wang et al., 1997; Weber et al., 1998; Ye et al., 2006). The major functional domains of NP are summarized in Fig. 3. Thus, NP plays important roles in numerous stages of viral replication, such as in the nuclear transport of NP and RNPs, replication and transcription of genomic RNA, and nuclear export and packaging of RNPs. In addition to these, some domains that are important for the maintenance of three-dimensional structure, such as the tail-loop structure, pocket structure, and regulation of particle formation, as well as domains that are important for interactions with host proteins, have been reported (Fig. 3) (Digard et al., 1999; Momose et al., 2001; Ng et al., 2008, 2009; Noton et al., 2009; Wang et al., 1997; Ye et al., 2006). Moreover, phylogenetic analysis of viral strains isolated from different hosts revealed that the NP gene is relatively well conserved (Shu et al., 1993), especially in the functional domains (Heiny et al., 2007; Li et al., 2009; Ng et al., 2009). Thus, all of these functional domains could be considered potential targets for antiviral agents.

4.2 Structure of NP

The crystal structure of NP of influenza A virus H1N1 (Ye et al., 2006) is shown in Fig. 4. The N-terminal truncated NP derived from H1N1 forms trimers through NP-NP interactions using a tail-loop structure constructed from the segment at amino acid positions 402-428 (Fig. 3 & 4) and pocket structures constructed from segments at amino acid positions 160-167, 321-334, and 340-349 (Fig. 3, Ye et al., 2006).



Position	Function	Reference
①1-20:	RAF-2p48 interaction	Momose et al., 2001
②1-73:	NPI-1/NPI-3 interaction	Wang et al., 1997
③1-160:	PB2 interaction	Biswas et al., 1998
④1-180:	RNA interaction	Albo et al., 1995; Kobayashi et al., 1994
⑤3-13:	unconventional nuclear localization signal	Cros et al., 2005; Neumann et al., 1997; Ozawa et al., 2007; Wang et al., 1997
⑥90-121:	overlapping bipartite nuclear localization signal	Ketha & Atreya, 2008
⑦160-167:	pocket for tail loop	Ng et al., 2008, 2009; Ye et al., 2006
⑧189-354:	NP-NP interaction	Elton et al., 1999; Ye et al., 2006
⑨198-216:	bipartite nuclear localization signal	Ozawa et al., 2007; Weber et al., 1998
⑩239:	regulation site of particle formation	Sarah et al., 2009
⑪256-498:	PB2 interaction	Biswas et al., 1998
⑫321-334:	pocket for tail loop	Ng et al., 2008, 2009; Ye et al., 2006
⑬327-345:	F-actin interaction	Digard et al., 1999
⑭339-345:	nuclear accumulation signal	Davey et al., 1985
⑮340-349:	pocket for tail loop	Ng et al., 2008, 2009; Ye et al., 2006
⑯371-498:	NP-NP interaction	Elton et al., 1999; Ye et al., 2006
⑰402-428:	tail loop	Ng et al., 2008, 2009; Ye et al., 2006

NLS, nuclear localization signal; NAS, nuclear accumulation signal

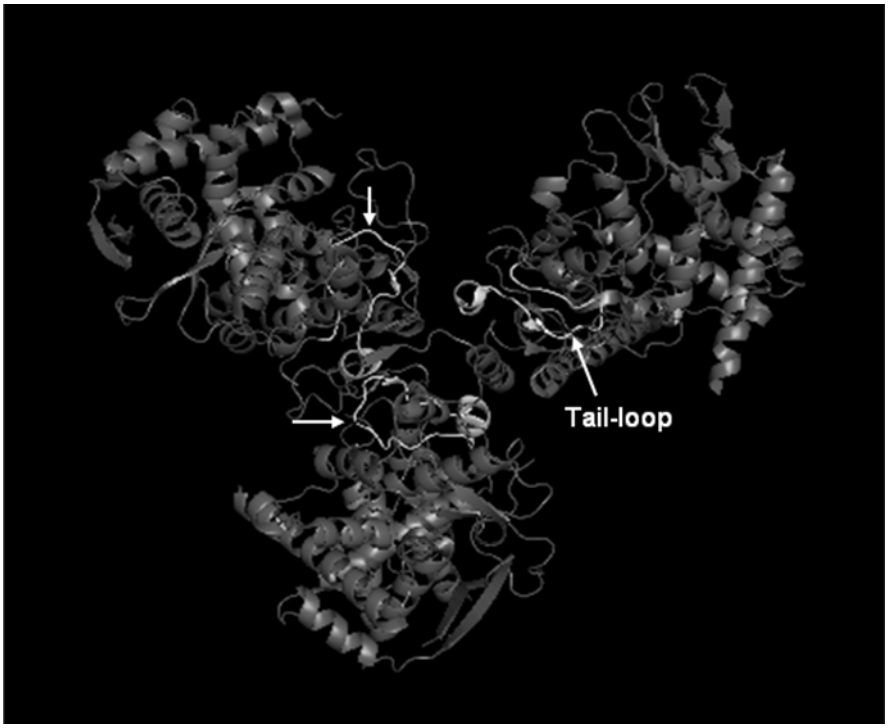
Fig. 3. Summary of NP functions.

The nucleoprotein has been proven to bind non-specifically to RNA at one in every 24 nucleotides (Compans et al., 1972; Ortega et al., 2000), and indeed, the protein has been shown to have an RNA-binding groove between the head and body domains. This groove is located exterior to the nucleoprotein oligomers. The surface of the groove is occupied by several basic residues, such as R65, R150, R152, R156, R174, R175, R195, R199, R213, R214, R221, R236, R355, K357, R361, and R391 (Ye et al., 2006), which can interact with nucleotides. These distributed basic residues are highly conserved, and therefore it is likely that the compounds targeting these regions can inhibit viral multiplication.

The crystal structure of NP derived from H5N1 has also been reported. It forms a different trimer compared with H1N1; however, the tail-loop interactions were identical (Ng et al., 2008). Both papers suggest that the tail-loop binding pocket is a good target for the development of anti-influenza virus drugs.

5. Nuclear transport of NP

Most RNA viruses that lack a DNA phase replicate in the cytoplasm (Cros & Palese, 2003). However, several negative-stranded RNA viruses, such as influenza, Thogoto, and Borna disease viruses, replicate their RNAs in the nucleus, taking advantage of the host cell's



The NP trimer was constructed according to NP-NP interactions using pocket structure (amino acid positions 160-167, 327-334 and 340-349) and tail-loop structure (amino acid positions 402-428). The positions of the three tail loops are highlighted in white and indicated by arrows. This structure is based on the available structural information for NP (PDB code: 2IQH).

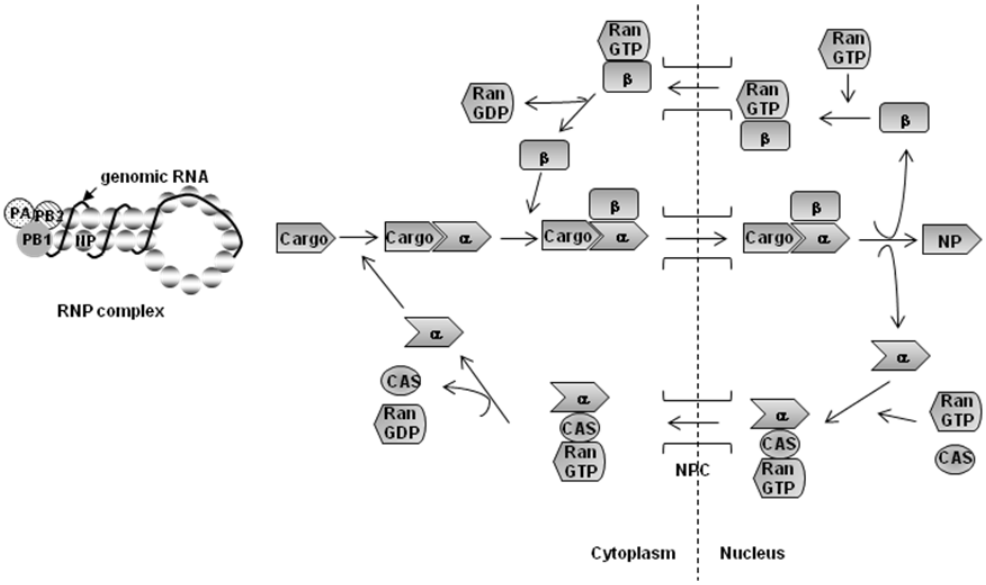
Fig. 4. Crystal structure of influenza virus NP trimer analyzed by X-ray.

nuclear machinery. The cell nucleus is separated from the cytoplasm by a double-layer membrane contiguous with the ER called the nuclear envelope (Terry et al., 2007). This nuclear envelope is composed of two lipid bilayers, the outer and inner nuclear membranes (Gruenbaum et al., 2005). Viruses that replicate their genome in the host cell nucleus have evolved strategies for moving viral components across this membrane barrier. These membranes are separated by a lumen and joined at nuclear pore complexes (NPCs) (Fahrenkrog & Aebi, 2003) that serve as gates for traffic crossing the nuclear envelope. Entrance into and exit from the nucleus occurs via these NPCs, which are > 60 MDa macromolecular structures that form channels spanning the nuclear envelope (Cronshaw et al., 2002; Rout et al., 2000). Each NPC is equipped to facilitate both the import and export of proteins and RNAs (Dworetzky & Feldherr, 1988).

The movement of ions, metabolites and other small molecules through the NPC occurs via passive diffusion, but the translocation of cargos larger than ~40 kDa generally requires specific signals known as NLSs. The nuclear import of basic NLS-bearing proteins is mediated by specific soluble factors, including importin-α (Impα) (Goldfarb et al., 2004), importin-β (Impβ) (Harel & Forbes, 2004), small GTPase Ran/TC4 (Quimby & Dasso, 2003),

and NTF2 (Stewart, 2000). Impα functions as an adaptor molecule, binding Impβ via its amino-terminally located Impβ-binding (IBB) domain and binding an NLS-bearing protein via its two central region-located NLS-binding sites (Herold et al., 1998; Kobe, 1999) (Fig. 5). Impβ is the transport receptor that carries the Impα-NLS complex from the cytoplasm to the nuclear side of the NPC. Once the heterotrimer consisting of Impα, Impβ, and the NLS-bearing protein reaches the nuclear face of the NPC, the GTP-bound form of Ran binds directly to Impβ, releasing Impα and the NLS-bearing protein into the nucleoplasm. Ran, which is found in its GDP-bound form in the cytoplasm and in its GTP-bound form in the nucleus, is a major determinant of the directionality of transport across the nuclear membrane.

A challenge faced by influenza virus is that of the trafficking of viral components into the nucleus through the NPC (Fig. 5). The genomic RNAs of influenza virus associate with proteins to form large complexes called vRNPs, which exceed the size limit for passive diffusion through the NPCs. The vRNPs is believed to be 10–20 nm wide (Compans et al., 1972). The vRNA, coated by NPs (1NP for each 24 nucleotides) (Compans et al., 1972; Ortega et al., 2000), forms a loop (Fig. 1C & 6). The trimeric polymerase complex, PB1, PB2, and PA, binds to the partially complementary ends of the vRNA, giving rise to a complex panhandle structure (Martin-Benito et al., 2001).



NPC, nuclear pore complex

Fig. 5. Model of the nuclear transport through classical importin α/β import pathway and influenza virus.

To ensure efficient transport across the nuclear membrane, influenza virus uses NLSs exposed on PB2, PB1, PA and NP. These signals recruit cellular import complexes, which are responsible for the translocation of the vRNPs through the NPC. Although all of the

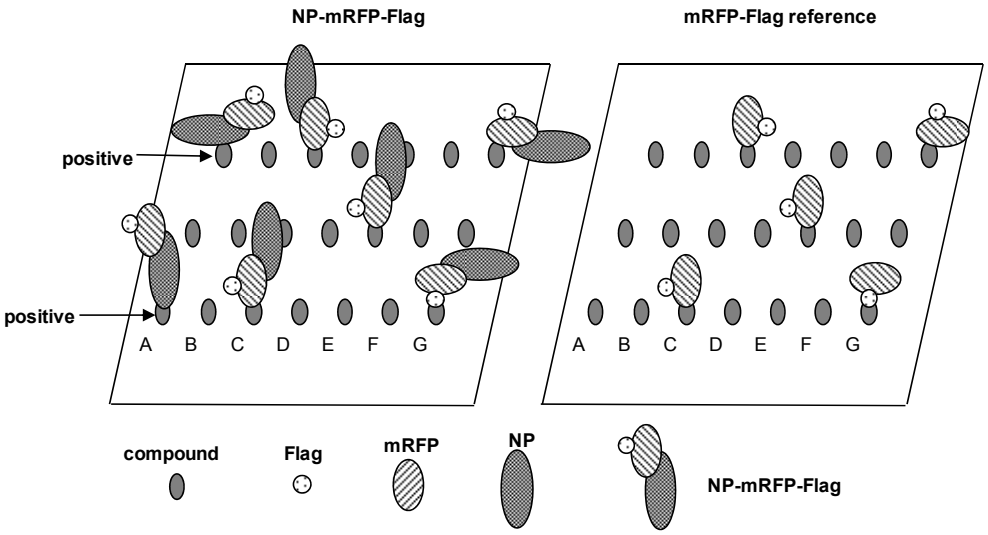
proteins of the vRNPs carry NLSs (Akkina et al., 1987; Jones et al., 1986; Nieto et al., 1994), the NP was shown to be sufficient to mediate the nuclear import of viral RNAs (O'Neill et al., 1995). As shown in Fig. 3, a more detailed analysis revealed the presence of three NLSs on the NP (Neumann et al., 1997; Wang et al., 1997), including an unconventional NLS at the very N-terminus, located between amino acids 3 and 13. A second NLS resides in the central part of the NP, located between amino acids 198 and 216. This bipartite signal appears to be weaker than the unconventional N-terminal NLS (Weber et al., 1998). Of these NLSs, the unconventional, N-terminal NLS of the NP is indispensable for the nuclear transport of NP and vRNPs (Cross et al., 2005; O'Neill et al., 1995). Alanine-substituted mutants of this unconventional NLS of the NP have shown that the amino acids at position 7 and 8 are critical for nuclear localization (Neumann et al., 1997). Furthermore, the mutation of amino acids at position 7 and 8 of NP leads to a reduction of viral growth compared with wild-type virus (Ozawa et al., 2007).

The transport of vRNPs of influenza A virus into the nucleus is performed through the classical nuclear import pathway, the impα/β transport system shown in Fig. 5. As an adaptor, impα binds with the NLSs of viral proteins and then NLS-impα binds to the receptor on impβ, through the IBB domain on impα (Cross et al., 2005; O'Neill et al., 1995). This NLS-bearing protein/receptor complex is imported into the nucleus. The impα family comprises six members in humans. Based on structural similarity, the impα family is grouped into three subfamilies, impα1/Rch1 (Rch1), impα3/Qip1 (Qip1), and impα5/NPI-1 (NPI-1) (Goldfarb et al., 2004). Interestingly, NP binds to several types of human impα, including Rch1, Qip1, and NPI-1, and regulates not only the nuclear transport of vRNPs but also host cell tropism and the growth of influenza virus (Gabriel et al., 2011). Therefore, NP functions as the main regulator of vRNPs trafficking and is a potentially useful target for the development of novel compounds that inhibit influenza A virus replication.

6. Screening of anti-viral drugs for NP

Recently, we demonstrated that NP is a novel target for the development of new antiviral drugs against the influenza virus using screening of NP-binding compounds by photo-cross-linked chemical arrays. Chemical arrays represent one of the most promising and high-throughput approaches for screening ligands against proteins of interest (Kanoh et al., 2006), and several successful results from chemical arrays have been reported (Koehler et al., 2003; Kuruvilla et al., 2002; Miyazaki et al., 2008). The screening protocol using chemical array was shown in Fig. 6. Approximately 25,000 small-molecules have been developed at RIKEN were fixed on a glass plate by photo-cross linker. To firstly identify inhibitors of NP, a large-scale chemical array approach of 6,800 compounds from an RIKEN NPDepo chemical library was used that detected specific interactions of small molecules with NP.

Using purified, recombinant influenza virus (A/WSN/33) NP, which was fused to monomeric red fluorescent protein (mRFP), we succeeded in detecting 72 compounds as positive. Next, plaque assay was used to investigate whether the 72 compounds inhibited multiplication of the influenza virus (A/WSN/33). Among them, 9 compounds showed inhibitory activity against influenza virus multiplication (Table 2). Furthermore, to obtain the compound which shows high inhibition activity, we searched for the derivatives of compounds from RIKEN NPDepo and found three derivatives of compound 1, which is



Approximately 2,000 compounds are spotted, with duplicates, on a glass plate using a photo-cross linker.

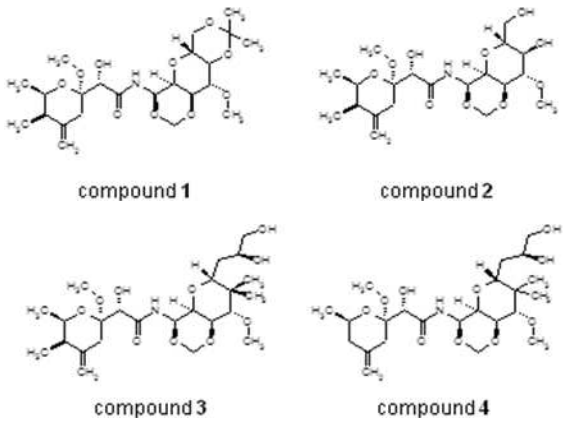
Fig. 6. Summary of compound screening using chemical array.

Methods	Targeting	Positive	Negative
Photo-crosslinked chemical array assay	Binding to NP	72	6,800
Plaque assay	Inhibition of viral replication	9	63
Plaque assay using derivatives	Inhibition of viral replication	1	8 (not determined)
Surface plasmon resonance	Binding of four derivatives to NP	4	0

Table 2. Screening for NP inhibitors from the NPDepo RIKEN Natural Products Depository.

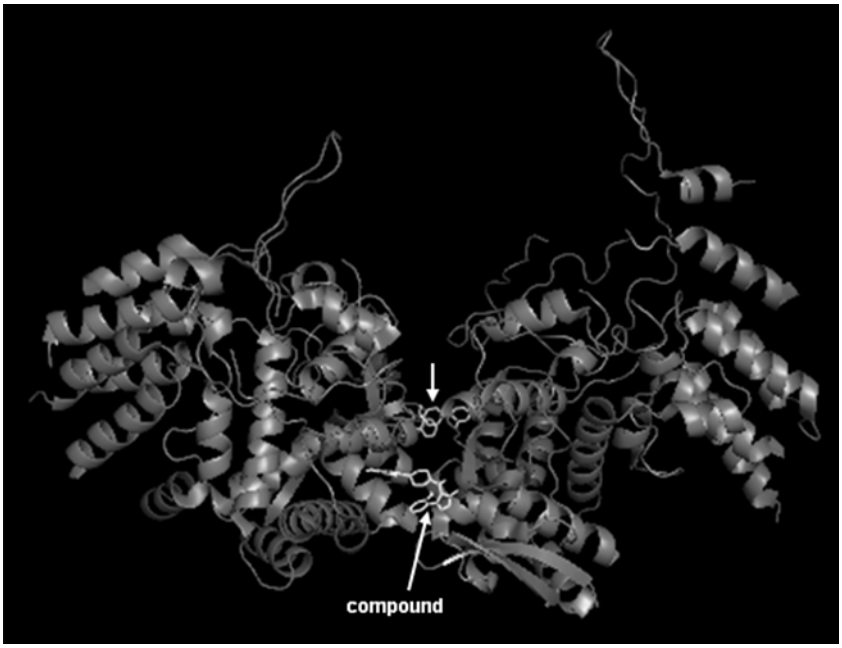
artificial analog of mycalamide. Among them, two derivatives showed lower inhibitory effect compared with compound 1, whereas compound 4 (Fig. 7, Table 2) showed strong activity reaching inhibition level up to 97% (Hagiwara et al., 2010).

Furthermore, surface plasmon resonance imaging experiments demonstrated that the binding activity of each compound to NP correlated with its antiviral activity. Finally, it was shown that these compounds bound NP within the N-terminal 110-amino acid region but their binding abilities were dramatically reduced when the N-terminal 13-amino acid tail was deleted, suggesting that the compounds might bind to this region, which mediates the nuclear transport of NP and its binding to viral RNA. These data suggest that compound binding to the N-terminal 13-amino acid tail region corresponding to an unconventional NLS may inhibit viral replication by inhibiting the nuclear transport of NP.



Compound 1, which is artificial analog of mycalamide was finally selected as the anti-influenza virus agent. Compound 4 was the derivative of compound 1 that showed the strongest activity in inhibiting influenza virus multiplication.

Fig. 7. Chemical structure of three derivatives of compound 1.



The positions of the compounds are highlighted in white and indicated by arrows.
The compounds induce the aggregation of NP. This structure is based on the available structural information for NP (PDB code: 3RO5).

Fig. 8. Structure of influenza virus NP bound to compounds.

7. Alternative anti-influenza virus compounds targeting NP

As described earlier, we first experimentally identified NP as a valuable drug target for inhibiting the influenza virus (Hagiwara et al., 2010). Interestingly, two other compounds that inhibit the function of NP have been reported. One compound, called nucleozin, was randomly screened from a commercial chemical library. Nucleozin inhibits nuclear localization of NP by inducing aggregation of NP (Kao et al., 2010). Furthermore, some nucleozin derivatives have been developed (Gerritz et al., 2011; Su et al., 2010) and the co-crystal structures of NP and these nucleozin derivatives were also reported (Gerritz et al., 2011). The crystal structures indicated that two derivatives bound to the NP dimer (Fig. 8) and might induce the aggregation of NP, thereby inhibiting nuclear transport of NP and thereby influenza virus multiplication.

Another compound was screened *in silico* and found to inhibit construction of the NP trimer. The compound interacts with E339-R416 salt bridge in the tail-loop binding pocket and thereby inhibits functional oligomerization of NP, which would in turn inhibit the multiplication of the influenza virus (Shen et al., 2011).

Using three different methods (direct binding, random screening, and *in silico* screening), compounds that inhibit the function of NP have been obtained. The results strongly suggest that NP is a good target for the development of an anti-influenza virus drug.

8. Conclusions

Influenza A viruses are responsible for seasonal epidemics and high mortality pandemics. Influenza A virus has a segmented genome of eight negative-strand RNA segments, which are packaged into virions as RNPs. In addition to RNA, RNP contains the viral NP and the three subunits of the RNA-dependent RNA polymerase, PB1, PB2, and PA. The NP is expressed in the early stage of infection and plays important roles in numerous steps of viral replication. NP also is relatively well conserved compared with viral surface spike proteins. Using three different methods, direct binding, random screening, and *in silico* screening, several small molecules that interact with NP and inhibit virus multiplication were discovered. Since there are currently only two types of drugs available for the treatment of influenza virus infection, M2 inhibitors and NA inhibitors, the discovery of a novel mechanism of inhibition of influenza virus replication may supply the field of drug development with an effective new strategy.

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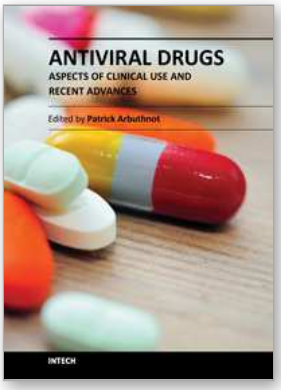

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The articles that appear in Antiviral Drugs - Aspects of Clinical Use and Recent Advances cover several topics that reflect the varied mechanisms of viral disease pathogenesis and treatment. Clinical management and new developments in the treatment of virus-related diseases are the two main sections of the book. The first part reviews the treatment of hepatitis C virus infection, the management of virus-related acute retinal necrosis, the use of leflunomide therapy in renal transplant patients, and mathematical modeling of HIV-1 treatment responses. Basic research topics are dealt with in the second half of the book. New developments in the treatment of the influenza virus, the use of animal models for HIV-1 drug development, the use of single chain camelid antibodies against negative strand RNA viruses, countering norovirus infection, and the use of plant extracts to treat herpes simplex virus infection are described. The content of the book is not intended to be comprehensive, but aims to provide the reader with insights into selected aspects of established and new viral therapies.

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