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REVIEW

A new role of neuraminidase (NA) in the influenza virus life cycle: implication for developing NA inhibitors with novel mechanism of action

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SUMMARY

The entire life cycle of influenza virus involves viral attachment, entry, replication, and release. Previous studies have demonstrated that neuraminidase (NA) is an essential glycoprotein on the surface of influenza virus and that it is responsible for release of progeny virions from the host cell to infect new cells. However, recent studies have also suggested that NA may play other roles in the early stages of the viral life cycle, that is, viral attachment and entry. This review focuses on the new role of NA in the early stages of influenza life cycle and the corresponding development of novel NA inhibitors. Copyright © 2016 John Wiley & Sons, Ltd.

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INTRODUCTION

Aside from its impact on health and well-being, influenza poses a serious threat to world economies. Seasonal flu outbreaks are commonplace; in particular, the 1918 pandemic killed millions of people [1]. In 2009, a new influenza pandemic caused by a novel S-OIV H1N1 has resulted in millions of infections in more than 214 countries [2,3]. H5N1 virus was also classified as a highly pathogenic avian influenza A virus because it can cause severe illness and high mortality. Recent studies have identified just five mutations (known as N1581D/N2241K/Q2261L/T3181I) in the hemagglutinin (HA) protein that allow H5N1 virus to spread through the air between ferrets [4–6]. Furthermore, new strains of influenza viruses were recently detected, including H7N9, H5N8, and H5N5 [7–9].

Genetic variation of influenza A virus is a key problem that results from genetic drift and genetic shift, making it almost impossible to produce a timely and sufficiently effective vaccine to prevent epidemic outbreaks. For this reason, antiviral drugs have offered an important option to combat avian flu, including H7N9 and the highly pathogenic avian influenza virus H5N1 [10–12]. Currently, two classes of anti-influenza drugs are available. These target the M2 ion channel and neuraminidase (NA) expressed on the virus envelope, respectively. Since 2003, M2 ion channel inhibitors have had extremely limited clinical application as a result of neurological side effects and widespread drug resistance [13,14]. NA inhibitors (NAIs) are currently the most effective therapy against all strains of influenza A and B viruses [12]. Oseltamivir and zanamivir were licensed and recommended for treatment and prevention of acute uncomplicated flu caused by influenza [12,15–18]. Two other NAIs, peramivir

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Abbreviations
S-OIV, swine-origin influenza A virus; HA, hemagglutinin; NA, neuraminidase; NAIs, NA inhibitors; Neu5Ac, N-acetyl neuraminic acid; MDCK, Madin Darby canine kidney; MERS-CoV, Middle East respiratory syndrome coronavirus; RBC, red blood cell.

and laninamivir, have been approved for seasonal use in Japan and are under clinical evaluations in other countries [19,20]. Peramivir is also approved for use in Korea and China, as well as the USA. According to surveillance data between 2013 and 2014, 98.2 and 100% of 2009 H1N1 viruses tested for surveillance were susceptible to oseltamivir and zanamivir, respectively (http://www.cdc.gov/flu/professionals/antivirals/antiviral-drug-resistance.htm). These drugs have offered new prospects for influenza management. However, virus strains resistant to NAIs are constantly emerging. In addition, dual resistance to both oseltamivir and amantadine has been detected [21–23]. Thus, it is urgent and important to investigate new antiviral drugs against influenza virus.

THE LIFE CYCLE OF INFLUENZA VIRUS

The three types of influenza viruses are A, B, and C. Influenza A belongs to the family of *Orthomyxoviridae*. It is an enveloped virus with a genome made up of negative sense, single-stranded, segmented RNA. Only influenza A viruses are further classified by subtype based on the two main surface glycoproteins, HA and NA.

The influenza A virus life cycle can be divided into four stages (Figure 1). First, HA binds to the host cell's sialic acid residues. Second, receptor-mediated endocytosis occurs, and the virus enters the host cell in an endosome. The endosome has a low pH of around 5 to 6, which triggers the fusion of the viral and endosomal membranes. Following release into the cytoplasm, the vRNPs enter the nuclei of infected cells. Third, when completing the transcription and replication of the viral genome, vRNPs are exported from the nucleus, followed by assembly and budding at the host cell membrane. Fourth, viral particles are released from the cell [24–26]. One of the most important steps that must occur before the newly made viral particle can leave the plasma membrane is the cleavage of sialic acid residue from glycoproteins and glycolipids. Without this process, the viral particle could not be released from the plasma membrane and go on to infect neighboring cells. Recently, the receptor-binding function of NA has been reported [27–34].

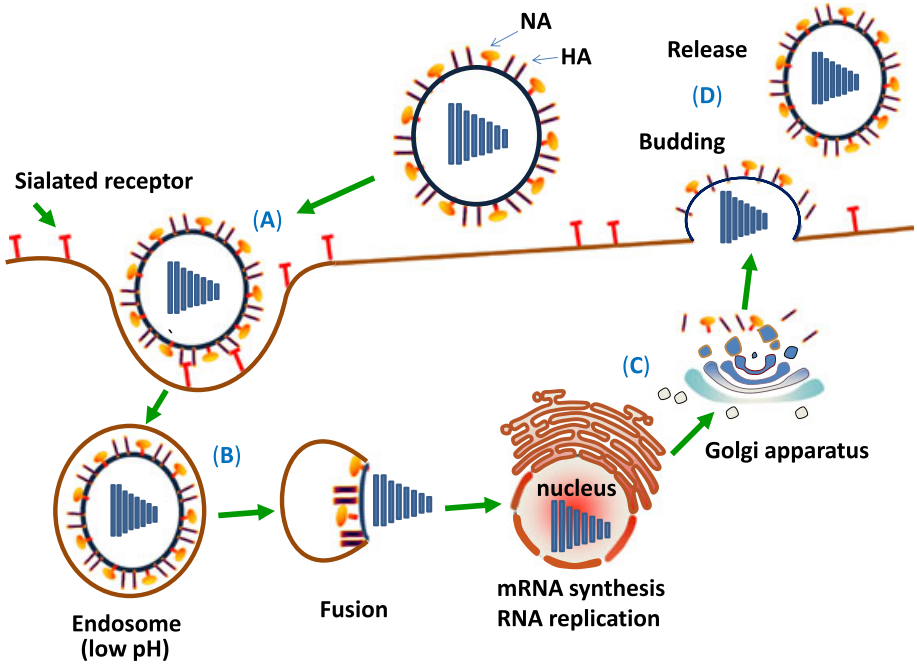

Figure 1. The life cycle of an influenza A virus (IAV). (A) IAV binds to target cells through the interaction between sialylated receptors and HA or NA. (B) IAV envelope fuses with endosomal membrane under low pH, releasing viral genetic materials into cytosol. (C) Viral replication occurs in nucleus and Golgi apparatus. (D) Progeny virions are assembled, budded, and released from the infected host cell to infect neighbor cells

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THE ROLE OF NA

Generally, the major function of viral NA occurs during the final stage of infection. First, NA displays sialidase activity, which cleaves off the terminal *N*-acetyl neuraminic acid on 2-3 or 2-6 sialic acid moieties of host cells. Then, NA facilitates the release of progeny virions from the host cell, aiding virus transmission. In addition, NA is able to prevent newly assembled viruses from aggregating [15,35]. Several other attributes of NA have been reported in recent years, as summarized in Table 1, indicating that NA may play multiple roles in the viral life cycle, including viral attachment and entry.

The new role of NA in the initial stage of influenza infection

Recently, it has been proposed that NA may facilitate virus entry into target cells in the initial stage of viral infection, and some attempts have been made to analyze this new role. It is well known that NA promotes virus access to target cells in airways by mucous degradation. Consistent with this hypothesis, some studies showed that NA facilitates the spread of virus through mucous that covers the human airway epithelium. The most likely mechanism is related to removal of decoy receptors on mucins, cilia, and cellular glycocalyx [36,37]. However, lung cancer A549 and MDCK cells, which are widely used as influenza models, do not secrete mucin. It is therefore possible that NA has adopted a novel way to aid in virus entry, and recent studies have demonstrated that NA does, indeed, play a direct and early role in influenza A virus entry. Ohuchi *et al.*, for example, found that NA facilitates virus transfer from an endocytosis-inactive site to an endocytosis-active site, thus facilitating virus entry into target cells, noting that such activity can be blocked by NAIs [38]. Other authors have suggested that NA expression can directly impact HA-dependent influenza virus fusion and virus infection efficiency based on the enzymatic activity of NA [39]. With the unchanged HA, NA replacement in the background of PR8 virus had significant effect on the efficiency of infection initiation, virus release and fusion of infected cells [40]. Such results call for more research to confirm the role of NA in facilitating influenza virus entry to susceptible cells.

Surprisingly, several research groups have reported that NA has acquired the capacity to bind receptors. These reports have challenged the generally accepted concept of NA as an effector during viral fusion and release (Figure 2). Under this new concept, NA can bind receptors on the host cell surface and complement the process of HA receptor binding. A recent report revealed a D151G NA mutant that promoted NA-dependent and NAI-sensitive hemagglutination and caused the attachment of recently isolated human H3N2 viruses via their NA to sialic acid receptors refractory to catalytic cleavage. Although HA is still required for viral entry, the specificity of NA

Table 1. The function of NA in the early and late stages of influenza infection

Virus (subtype)	The function of NA	References
Influenza A and B viruses	NA cleaves sialic acids from cell surface and releases progeny virus from the infected cells, promoting the spread of the infection.	[25,26]
Influenza A viruses (H3N2 and H1N1)	NA can bind to receptor on target cell surface and to complement the process of HA receptor binding.	[27–29]
Influenza A virus (H3N2, H1N1, and SOIV)	NA sialidase activity is required to free virions from sialylated host mucins decoy during initial stages of infection.	[36]
Influenza A viruses (H3N2, H1N1, H7N7, H7N1)	NA plays an early role in infection, providing a further rationale for the prophylactic use of NA inhibitors.	[37]
Influenza A viruses (H3N2 and H1N1)	NA promotes virus entry into target cells during the initial stage of viral infection.	[37–39]

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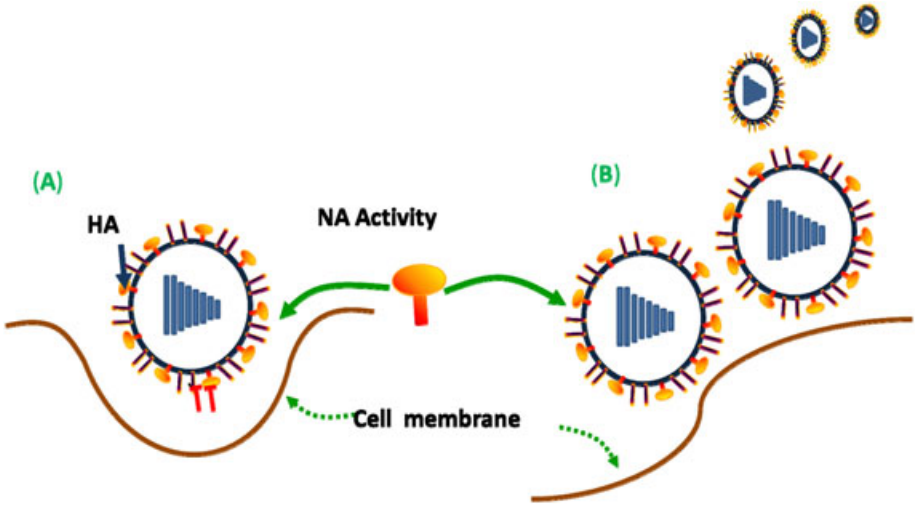

Figure 2. The roles of NA in the early and late stages of influenza A virus life cycle. (A) NA mediates attachment of the virus to receptors on host cells to facilitate viral entry. (B) NA mediates cleavage of sialic acid from receptors on host cells to allow release of progeny virions to infect neighbor cells

appears to complement a deficiency in HA binding. Thus, NA displays two biological activities, including the complementary binding specificities of HA and the cleavage of sialic acid at the early and late stages, respectively, of viral life cycle. It was subsequently shown that D151G NA mutant has sialic acid binding activity for avian α2-3 and human α2-6 sialic acid glycans in their active sites and that oseltamivir blocked the binding to α2-3-linked sialic acids of human H3N2 isolates with D151G [27,29–32], again highlighting that D151G NA mutant plays a role in viral entry.

Another laboratory-generated mutant, G147R, enables NA subtype N1 to completely co-opt the receptor-binding function. This new mutation enables some recent human H3N2 isolates to grow to high titers, even in the absence of the HA receptor-binding function. Specifically, G147R NA allows NA to mediate viral infection and RBC agglutination in a manner that can be reversibly blocked by NAIs and anti-NA antibodies [33]. Subsequent studies have shown that the NAs of some human influenza A (H3N2) viruses isolated as early as 1994 had gained such receptor-binding function [34]. However, some researchers still challenge the theory that NA can facilitate virus entry at all [41]. Therefore, the receptor-binding properties of NA must involve some still unknown mechanisms, which therefore call for the accumulation of more relevant *in vitro* and *in vivo* data.

Indeed, many factors must be taken into consideration when speculating about the possible receptor-binding mechanisms of NA, including influenza subtype, species, and cell lines. Also, binding site, stalk length, variation in other functional domains, substrate specificity of NA, and potential glycosylation sites [42] might also affect NA activity.

Development of NAIs with novel mechanism of action by targeting the entry stage of influenza virus

To date, there are four licensed NAIs available for the treatment and prophylaxis of influenza virus infections (Figure 3). NAIs are the only class of antiviral drugs recommended by WHO for the treatment and prophylaxis of influenza A and B infections, including currently emerging avian H5N1 and H7N9 strains [43]. Unfortunately, development and spread of existing NAI-resistant strains significantly limit the effectiveness of these inhibitors [21,22,44,45]. Because NA remains a promising target for anti-influenza drug discovery, NAI resistance gives added urgency to the search for novel NAIs.

Blockade of viral entry would certainly be a promising therapeutic strategy in the fight against virus infection, including HIV-1, MERS-CoV, and influenza virus [46–49]. Notably, applications of viral entry/fusion inhibitors, such as anti-HIV

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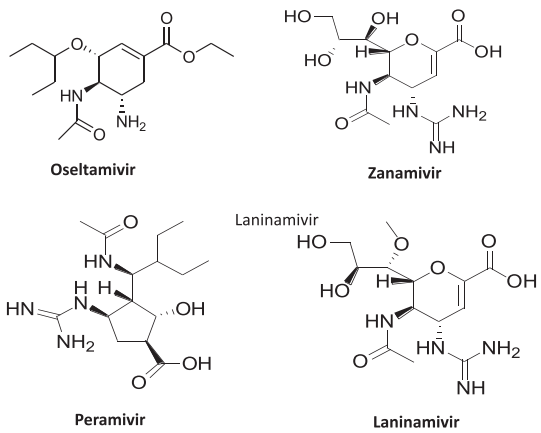

Figure 3. Chemical structures of four NA inhibitors currently used in clinics to treat influenza virus infection

peptide T20 (enfuvirtide), either alone or in combination with other antivirus agents, have become an attractive antiviral strategy [50]. Moreover, small molecules as potential entry inhibitors have been the object of research for many years [51–54]. To date, however, no drug able to block viral entry is available for the treatment and prevention of influenza. On the other hand, based on the speculated receptor-binding characteristics of NA and studies thus far showing that NA plays a role at the early stage of the virus life cycle, we asked whether NAIs could be developed as entry inhibitors against influenza infection.

NA contains a mushroom-shaped homotetrameric glycoprotein with a stalk domain anchored to the viral membrane and a globular head that contains a catalytic site. The active site of NA is composed of 8 functional residues (R118, D151, R152, R224, E276, R292, R371, and Y406), which are surrounded by 11 framework residues (E119, R156, W178, S179, D198, I222, E227, H274, E277, N294, and E425) (N2 numbering system). These residues are highly conserved in all NA subtypes [55–58]. Based on the conserved catalytic site, intensive research has focused on structural modification of existing NAIs, as well as the design of novel NAIs, some of which have been previously reviewed [59–62]. For example, new derivatives of oseltamivir were prepared by modifying the amino group with glycyl, acetyl, benzyl, and prolyl moieties in order to develop novel influenza virus inhibitors [63,64].

The location of residue 151 in the 150 loop at the edge of the active site of NA and the sensitivity of its binding to oseltamivir suggest that D151 is a critical residue involving in the catalytic activity of the wild-type NA [27,28]. The principal role of D151 is believed to stabilize the transition state intermediate in the cleavage reaction [65–67]. The key hydrogen bond between glycosidic oxygen of sialic acid and the residue D151 is a critical interaction for the enzymatic activity of NA (Figure 4A). The residue D151 in wild type of NA may function as a "scissor" to cleave the sialic acid receptor. Therefore, it is understandable that wild-type NA exhibits no detectable binding to sialosides. However, once the aspartic acid 151 of NA is replaced by glycine, NA is able to bind to the receptor, resulting in the attachment of virus via its NA to sialic acid receptors on red blood cells. The entry of virus carrying NA with D151G mutation could be blocked by NAIs, indicating that G151 locates in the binding site of NA to its receptor [27]. As shown in Figure 4B, the pivotal

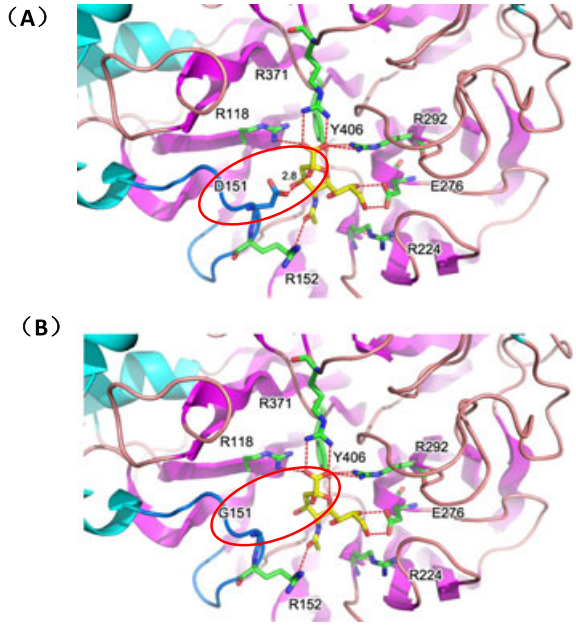

Figure 4. Schematic representation of the structure of the complex between NA of influenza virus and sialic acid receptor (PDB code 2BAT). (A) Wild-type NA protein in complex with sialic acid. The side chain of conserved catalytic sites of NA (R118, D151, R152, R224, E276, R292, R371, and Y406) was shown as green sticks. Location of residue 151 and the 150 loop was shown in marine. Sialic acid was shown as yellow sticks. The hydrogen bond was represented as red dot line (highlighted in red circle). (B) NA protein with D151G mutation in complex with sialic acid. All colors remain the same as shown in panel (A)

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hydrogen bond of residue D151 involving the cleavage activity of NA is eliminated because of the insufficient length of residue G151 side chain. Therefore, unlike the wild-type NA, binding of the NA with D151G mutation to the α2-3-linked sialic acid receptor does not effectively cleave the receptor but rather mediates the entry of the virus into the target cell.

The receptor-binding characteristics of NA, as described in the preceding texts, alter the specificity of NA mainly through amino acid substitutions in residues 151 or 147 in the NA. It is worth noting that NA with the D151G or G147R mutation not only readily bind sialic acid receptors but they are also sensitive to the NAIs. This activity would represent a nonclassical role for the NA. If receptor-binding NA variants turn out to be common, perhaps it could provide valuable insight into NA as a potential emerging target for the development of influenza virus entry inhibitors and provide data for expanding avenues of research, such as small molecules able to block NA activity, leading, in turn, to a new class of NAIs targeting viral entry.

COMBINATION THERAPY

As novel NAIs become identified against influenza virus entry, they may prove to be most effective when used in combination with other antiviral agents. Combining drugs with different modes of action is a strategy that has been successful in antiviral therapy and one that may be particularly useful for additive antiviral effects, as well as avoiding the selection of resistant viruses [68]. For instance, the combination of oseltamivir with other antiviral agents is under investigation, making it possible to optimally suppress influenza infection [69,70]. Similarly, novel NAIs could be designed and synthesized such that their combinatorial use will become the best option for prophylaxis and control under pandemic conditions.

CONCLUSION

Much remains to be learned about the influenza A viral life cycle, including the molecular mechanisms underlying viral entry. However, the new role of NA as a potential target during the early stage of viral entry has led to renewed interest in NAIs and highlights the development of new NAIs against virus entry, with particular emphasis on their use in combination therapies. Therefore, it is necessary to continue studying the roles of NA in the virus life cycle in anticipation of identifying more novel targets for the development of new antiviral agents for treatment and prevention of influenza virus infection.

CONFLICT OF INTEREST

The authors have no competing interest.

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