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Antivirals Targeting the Neuraminidase

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The neuraminidase (NA) of influenza A and B viruses plays a distinct role in viral replication and has a highly conserved catalytic site. Numerous sialic (neuraminic) acid analogs that competitively bind to the NA active site and potently inhibit enzyme activity have been synthesized and tested. Four NA inhibitors are now licensed in various parts of the world (zanamivir, oseltamivir, peramivir, and laninamivir) to treat influenza A and B infections. NA changes, naturally occurring or acquired under selective pressure, have been shown to reduce drug binding, thereby affecting the effectiveness of NA inhibitors. Drug resistance and other drawbacks have prompted the search for the next-generation NA-targeting therapeutics. One of the promising approaches is the identification of monoclonal antibodies (mAbs) targeting the conserved NA epitopes. Anti-NA mAbs demonstrate Fab-based antiviral activity supplemented with Fc-mediated immune effector functions. Antiviral Fc-conjugates offer another cutting-edge strategy that is based on a multimodal mechanism of action. These novel antiviral agents are composed of a small-molecule NA inhibitor and an Fc-region that simultaneously engages the immune system. The significant advancements made in recent years further support the value of NA as an attractive target for the antiviral development.

NEURAMINIDASE AS A TARGET

Neuraminidase (NA) is an appealing target for the development of therapeutics against influenza A and B viruses (Air and Laver 1989). This viral enzyme is expressed on the surface of type A and B virus particles and infected cells, which makes it readily accessible to antiviral agents and antibodies. NA, a glycoside hydrolase (EC 3.2.1.18), cleaves the terminal sialic (N-acetylneuraminic) acids from glycoproteins and glycolipids. NA is a type II integral membrane glycoprotein and is formed by four identical monomers. Each monomer consists of a globular head that carries the enzyme active

site (Fig. 1A), a stalk of variable length, a membrane-anchoring hydrophobic transmembrane domain, and a cytoplasmic tail (Varghese et al. 1983; Colman et al. 1987; Air and Laver 1989).

The NA active site has been directly identified by soaking neuraminic acid substrate into NA (N2 subtype) crystals (Colman et al. 1983). It is a shallow pocket formed by highly conserved amino acid residues, some of which bind directly to the substrate and participate in catalysis, whereas others provide the framework (Colman et al. 1993). A remarkable feature of the NA active site is the large number of charged residues. The residues R118, D151, R152, R224, E276, R292, R371, and Y406 are defined as

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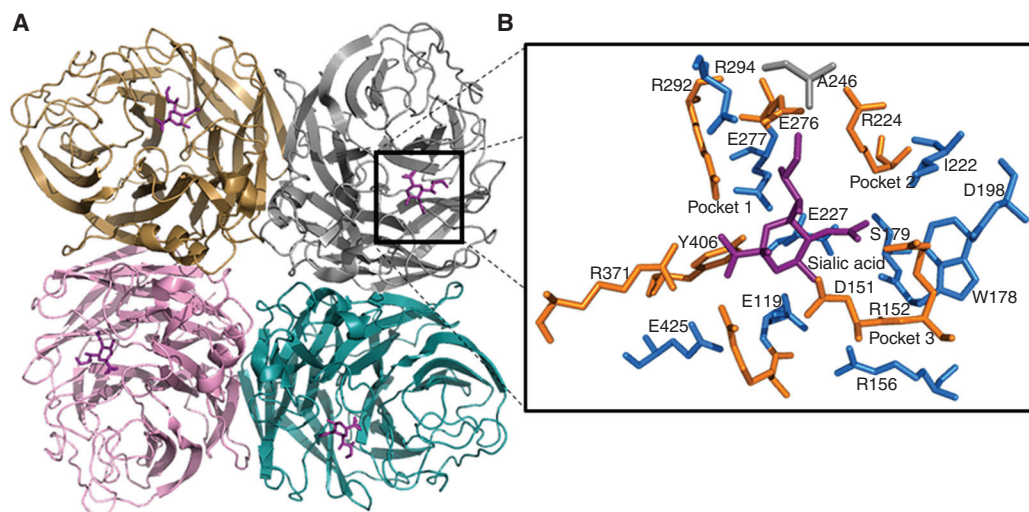


Figure 1. Crystal structure of neuraminidase (NA) catalytic head with sialic acid. The structure was visualized in PyMOL using information from Protein Data Bank code 4GZX and 4GZQ (A/Tanzania/205/2010 N2 NA). (A) A ribbon representation of an NA tetramer, in which each monomer is individually colored and sialic acid is shown in purple. (B) Black-boxed region is magnified and shows the catalytic (brown sticks) and the framework (blue sticks) residues in the three major binding pockets.

catalytic (or functional), whereas E119, R156, W178, S179, D198, I222, E227, N294, and E425 belong to the framework (N2 amino acid numbering is used throughout the text) (Fig. 1B; Colman et al. 1993). Of note, aspartic acid (D) is present at residue 198 in all type A NAs, except subtypes N7 and N9, which have asparagine (N) at this position. Based on the crystal structure, the NA active site and adjacent residues can be divided into three major binding pockets, which could be explored when designing inhibitors. Pocket 1 is formed by E276, E277, R292, and N294 residues and interacts with the glycerol moiety of sialic acid. Pocket 2 is formed by A246, I222, and R224 residues and is not utilized by sialic acid for binding. Pocket 3 is surrounded by E119, D151, R152, W178, S179, I222, and E227 residues and participates in sialic acid binding (Fig. 1B; Kim et al. 1997). The role of the individual residues has been investigated to elucidate the underlying enzymatic mechanism (Lentz et al. 1987; Ghate and Air 1998; Watson et al. 2003; Chan et al. 2012). Notably, during catalysis, amino acid residues of the active site bind more tightly to the transition state of the substrate (oxocarbenium ion) than to its

ground state form (neuraminic acid). This feature has been exploited extensively in designing the structure-based NA inhibitors representing chemical analogs of the substrate's transition state.

INHIBITORS OF NA ENZYME ACTIVITY

NA inhibitors compete with neuraminic acid (substrate) by reversibly binding to the NA active site. The earliest transition state analog of the substrate, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA) (Fig. 2A), binds rapidly to NA and is a weak inhibitor of NA enzyme activity. Modifications that conferred a time-dependent or slow binding can improve potency of this inhibitor (Smith et al. 2001). Its trifluoroacetyl derivative (FANA) (Fig. 2B) was shown to be a potent inhibitor in vitro; however, it failed to protect animals against influenza challenge (Palese et al. 1974; Schulman and Palese 1975; Palese and Compans 1976).

Years later, the first generation of potent influenza NA inhibitors was rationally designed using information gained from extensive NA sequence analysis, detailed enzyme crystal struc-

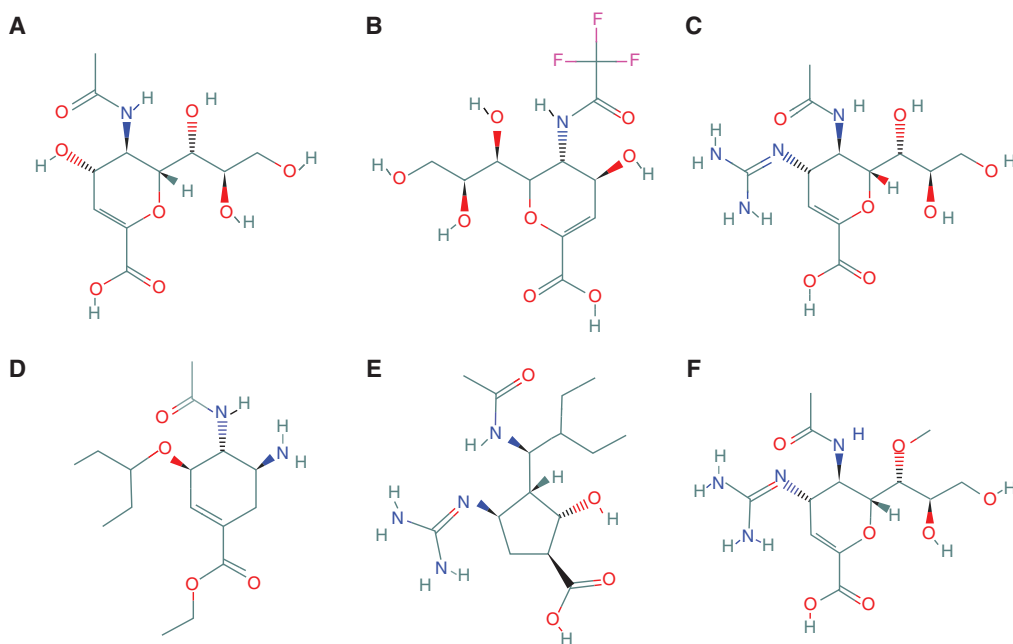


Figure 2. Chemical structure of neuraminidase (NA) inhibitors. Structures were drawn using the PubChem compound database. (A) DANA (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid), (B) FANA (2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid), (C) zanamivir (4-guanidino-Neu5Ac2en), (D) oseltamivir carboxylate (GS-4071), (E) peramivir, and (F) laninamivir (R-125489).

tures, and computational chemistry tools (Colman and Ward 1985; Burmeister et al. 1992; von Itzstein et al. 1993; Kim et al. 1997; Kobasa et al. 1999; Moscona 2005).

LICENSED NA INHIBITORS

Four NA inhibitors are currently licensed in various parts of the world for the treatment and prevention of influenza A and B infections: zanamivir, oseltamivir, peramivir, and laninamivir. The first two NA inhibitors, zanamivir and oseltamivir, were approved in 1999. Peramivir and laninamivir were approved in 2014 and 2010, respectively; the latter is available only in Japan. The four NA inhibitors share a certain degree of similarity in chemical structure, but differ by the route of administration, treatment regimen, and other attributes.

Zanamivir (4-guanidino-Neu5Ac2en) (Fig. 2C) was designed using a minimalistic approach (Colman 1999). It is an analog of DANA, where a hydroxyl group linked to C-4 was replaced with

a positively charged guanidine group. This modification resulted in a significant increase in binding affinity. It has been demonstrated that guanidinium formed a hydrogen bond to E119 (Taylor et al. 1998); however, some researchers suggested that it formed an ionic contact with this framework residue through a stacking interaction (Varghese et al. 1983, 1995). Zanamivir has low bioavailability (2%) and therefore requires topical administration. Zanamivir is approved as an inhaled drug that needs to be taken twice a day for 5 d. Over the years, an intravenous formulation of zanamivir has been developed and provided for compassionate use. In 2019, it received a marketing authorization in the European Union to be used under exceptional circumstances (e.g., resistance to other anti-influenza medications). The transdermal delivery system of zanamivir in the treatment of influenza using microneedle patch has also been investigated (NIH-R43AI129122).

Oseltamivir carboxylate (GS-4071) (Fig. 2D) was designed with the aim to simplify synthesis

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and improve bioavailability (Lew et al. 1999). This compound utilizes a carbocyclic scaffold instead of the pyran of zanamivir, where the 3-pentyl ether side chain replaced the hydrophilic glycerol side chain and the amino group replaced the guanidine group (von Itzstein and Thomson 2009). Unlike zanamivir, oseltamivir relies on strong hydrophobic interactions rather than polar interactions. The oseltamivir–NA complex crystal structure indicates that one branch of the 3-pentyl side chain of oseltamivir contacts with A246, I222, and R224, resulting in a significant increase in binding affinity. In contrast, the other branch of the 3-pentyl side chain of oseltamivir binds to the highly polar pocket 1. Such binding becomes possible because the E276 side chain moves away from the middle of NA pocket 1 and adopts an alternative conformation. This conformational change effectively makes pocket 1 larger and less polar to accommodate the hydrophobic moiety of oseltamivir. Oseltamivir is a potent inhibitor of NA activity; however, its bioavailability was lower than desired. Therefore, oseltamivir phosphate (GS-4104) was developed as a prodrug of oseltamivir carboxylate. Oseltamivir phosphate is prescribed as an oral medication, to be taken twice a day for five days. It is converted to the active metabolite (GS-4071) by host hepatic esterases (Kim et al. 1997; He et al. 1999).

Peramivir (Fig. 2E) is a cyclopentane (five-membered ring) derivative, making it structurally the most different among the other approved NA inhibitors. However, it shares similar functional groups—namely, a guanidino moiety of zanamivir and a hydrophobic side chain of oseltamivir (Babu et al. 2000). Its performance as an oral medication was not very effective, and this facilitated the development of an intravenous formulation. Because of a prolonged half-life, peramivir was approved as a single-dose treatment. It may provide a substitute for patients unable to take medication via the oral or inhalation routes.

Laninamivir (R-125489) (Fig. 2F) was derived by methylation of the C-7 hydroxyl group of zanamivir. It is administered as an octanoyl ester prodrug, laninamivir octanoate (CS-8958). After absorption by the respiratory tract epithe-

lial cells, laninamivir octanoate is rapidly hydrolyzed to an active form (R-125489) (Kiso et al. 2010). Laninamivir acts as a long-lasting NA inhibitor and showed therapeutic efficacy after a single nasally inhaled dose (Yamashita et al. 2009; Kubo et al. 2010a,b; Sugaya and Ohashi 2010). Different mechanisms leading to the retention of the drug in target organs have been proposed, but not yet fully elucidated (Koyama et al. 2009; Yamashita et al. 2009; Ikematsu and Kawai 2011).

OTHER NA INHIBITORS

The licensure of the first-generation NA inhibitors has inspired a search for compounds with improved properties (Laborda et al. 2016). Among those, multimeric derivatives and diversely decorated analogs of zanamivir and oseltamivir have been reported (Macdonald et al. 2004, 2005; Watson et al. 2004).

Dimeric derivatives of zanamivir conjugates with various linking groups have been shown to be more potent than zanamivir both in vitro and in vivo (Macdonald et al. 2005). Trimeric and tetrameric compounds of zanamivir were significantly more active than the monomeric ones and, more importantly, showed long-lasting protective activity in a mouse model (Watson et al. 2004). Zanamivir derivatives, such as zanamivir-amino acid prodrugs and zanamivir phosphate congeners, have also been patented (Han and Amidon 2000; Shie et al. 2011; Dahan et al. 2014). Among other modifications, the additions of the biodegradable polymer and poly-L-glutamine were also explored (Watson et al. 2004; Weight et al. 2011; Lee et al. 2012).

A series of oseltamivir derivatives carrying a phosphonate and alkyl substituents at carboxylic acid and 4-amido and/or 5-amino groups, respectively, were reported but did not show better efficacies than oseltamivir (Rungtongmongkol et al. 2009; Hussain Basha and Prasad 2012; Venkatramani et al. 2012). Oseltamivir derivatives bearing a pyridyl group were explored and some of the compounds exhibited more potent inhibitory activity against A(H5N1) NA than oseltamivir (Wang et al. 2019). A panel of other oseltamivir derivatives

with modifications at carboxyl-modified or C1 displayed robust anti-influenza potencies in vitro against A(H5N1) or A(H5N6) and both viruses, respectively (Ju et al. 2018; Wang et al. 2018). The latter is potentially propitious because of the improved membrane permeability and oral drug absorption (Wang et al. 2018). Many other oseltamivir derivatives designed through structure–activity relationship studies were also investigated (Gong and Xu 2008; Schade et al. 2014; Li et al. 2017; Zhang et al. 2018; Ye et al. 2019).

Among NA inhibitors with a different scaffold, cyclopentane amide and pyrrolidine derivatives named A-192558 and A-315675, respectively, were synthesized and tested (Wang et al. 2001; Kati et al. 2002). Both compounds effectively inhibited NA activity of influenza A and B viruses (Abed et al. 2008). A-315675 exhibited comparable potencies against a panel of A(H1N1) and A(H3N2) influenza viruses and was found to be significantly more potent than oseltamivir against type B isolates (Kati et al. 2002). Prodrug of A-315675 (A-322278) was used for oral administration and showed promising anti-influenza activity in animal models (Mishin et al. 2005a). An aromatic compound with an oxopyridine scaffold was discovered to inhibit NA activity of A(H1N1) influenza virus in the micromolar range (Karpf and Trussardi 2001).

Instead of focusing on compounds that bind reversibly, a research team applied a different approach to develop NA inhibitors that bind covalently to NA (Kim et al. 2013). In this study, 2,3-difluorosialic acid (DFSA) and its derivatives were used as a substrate exhibiting a slow turnover. These compounds function through the formation of a stabilized covalent intermediate with NA, which can permanently inactivate this viral enzyme. DFSA inhibitors demonstrated comparable efficacies to that of zanamivir and showed broad-spectrum activity against drug-resistant strains. They provide valuable insight into the design of next-generation covalent mechanism-based influenza therapeutics (Kim et al. 2013). Notably, to prove the possibility of covalent inhibition of NA, the role of conserved Y406 residue in the NA active site

was highlighted (Vavricka et al. 2013). They demonstrated that Y406 acts as a catalytic nucleophile and plays an integral role in the catalytic mechanism. The drug-resistant mutations conferred by substitution of Y406 were enormously unfavorable for the virus (Lentz et al. 1987; Ghate and Air 1998; Vavricka et al. 2013). Therefore, targeting conserved Y406 with covalent inhibitors may be a promising strategy for NA inhibitor development (Vavricka et al. 2013).

The advancements made in the analysis of crystal structures of NA from various subtypes also stimulated the search for improved antiviral compounds. With the exception of recently identified N10 and N11 in bats (Tong et al. 2012, 2013; Mehle 2014), NAs of type A viruses are divided into two major groups: group 1 (N1, N4, N5, and N8) and group 2 (N2, N3, N6, N7, and N9) (Sui et al. 2009; Nabel and Fauci 2010). The NAs of group 1 possess a flexible loop (known as the 150-loop) and a cavity (the 150-cavity formed by amino acids 147–152 of the 150-loop), which is adjacent to the NA active site. After binding with inhibitors, NAs of group 1 can render the conformational change from the “open” to the “closed” form, whereas group 2 NAs assume a “closed” form at all time (Russell et al. 2006). Different oseltamivir derivatives targeting the 150-cavity (e.g., modifications at C-5 NH₂ position, addition of N/O/C fragment/linker, and lipophilic side chains at C-5) were synthesized. These derivatives had significantly improved potency (Wang et al. 2010; Xie et al. 2014; Lin et al. 2018; Zhang et al. 2018). As these inhibitors target the 150-cavity and can specifically inhibit only the group 1 NAs, including the A(H1N1)pdm09, they may provide a prospective way to design selective NA inhibitors.

NA CHANGES THAT AFFECT INHIBITOR BINDING

All four approved NA inhibitors potently reduce influenza NA activity and display low IC₅₀ values (subnanomolar–nanomolar range) in NA inhibition assays (Gubareva et al. 2001b; Ike-matsu et al. 2015; Lackenby et al. 2018). Overall,

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peramivir shows the lowest IC_{50} values across influenza types and subtypes and thus is the most potent NA inhibitor in vitro (Okomo-Adhiambo et al. 2010; Lackenby et al. 2018). Despite the high conservation of amino acid residues of the NA active site, the potency of NA inhibitors can be influenced by a variance in NA sequence and structure. For example, all three NA inhibitors that are approved in the United States (zanamivir, oseltamivir, and peramivir) more potently inhibit the NA activity of type A viruses compared to type B. Moreover, certain NA changes, naturally occurring or acquired during virus replication in the presence of NA inhibitor, can reduce the potency of a NA inhibitor. Notably, these NA changes interrupt the slow binding or faster dissociation of NA inhibitors (McKimm-Breschkin and Barrett 2015).

The common change associated with zanamivir resistance in N2-subtype viruses is E119G substitution, although A and D substitutions were also reported (Gubareva et al. 1997; Zurcher et al. 2006; L'Huillier et al. 2015; Tamura et al. 2015). E119 is critical for interactions with the C4-guanidino of zanamivir and the C4-amino group of oseltamivir. E119G confers high-level resistance only to zanamivir, whereas E119D affects interaction with all NA inhibitors (McKimm-Breschkin et al. 1996; Colacino et al. 1997; Tamura et al. 2015). A recent study implicated the 150-loop in oseltamivir resistance caused by the E119D substitution (Hanpaibool et al. 2019). Noteworthy, a different substitution at the same residue, E119V, confers oseltamivir resistance in viruses carrying the N2 subtype; however, it does not affect binding of zanamivir (Mishin et al. 2005a; Pizzorno et al. 2011; Eshaghi et al. 2014; Gaymard et al. 2016a).

For oseltamivir, the most common changes conferring resistance are H274Y (H275Y in N1 amino acid numbering) in the N1 subtype and R292K in the N2 subtype (Whitley et al. 2013; Roosenhoff et al. 2019).

In the N1 background, H274Y reduces binding of oseltamivir to NA by several 100-fold. Tyrosine at 274 prevents the conformational change needed to accommodate the bulky pentyl ether side chain of oseltamivir, whereas zanamivir and sialic acid can still bind to the

mutant NA. It was proposed that tyrosine at 274 stabilizes the E276, so that there is a high-energy penalty for the movement of E276 and this results in weak oseltamivir binding and high resistance. Although most of the published studies on H274Y focused only on the drug-bound system, the direct effects of this substitution on NA itself prior to drug binding remain unclear. The structural changes in the NA binding site caused by H274Y substitution were examined (Yusuf et al. 2016). They observed that the H274Y substitution disturbed the NA binding site because of the repulsive effect of Y274 in the 250-loop (a loop formed in the region of residue 250 in NA). The result may be an adverse effect in the flexibility and positioning of this loop and its contribution in the hydrogen bonding network involved in binding of NA inhibitors (Yusuf et al. 2016; Wan et al. 2018). H274Y was shown to cause a slower association and faster dissociation rate of oseltamivir, whereas it does not affect the kinetics for binding of zanamivir (Barrett et al. 2011). Of note, the H274Y substitution has also been found to affect the binding of peramivir (Mishin et al. 2005a).

Interestingly, H274Y oseltamivir-resistant A(H1N1) viruses first broadly circulated in Europe (prior to the 2009 pandemic) (Lackenby et al. 2008), where oseltamivir was not commonly used, have subsequently become prevalent worldwide. The NA of these resistant A(H1N1) viruses has shown a slightly higher activity and affinity for the substrate than that of previously circulating sensitive viruses (Rameix-Welti et al. 2008). Furthermore, in addition to the H274Y substitution, some possibly permissive changes in the NA and hemagglutinin (HA) proteins were also implicated (Bloom et al. 2010; Ginting et al. 2012). Thus, these two causes may have permitted oseltamivir-resistant A(H1N1) viruses to acquire the capacity for efficient human-to-human transmission. These oseltamivir-resistant viruses exhibited cross-resistance to peramivir but remained sensitive to zanamivir and laninamivir (McKimm-Breschkin 2000; Baum et al. 2003; Baz et al. 2007; Collins et al. 2009; Hurt et al. 2009; Takashita et al. 2013). Notably, the transmission of oseltamivir-resistant H274Y A(H1N1)pdm09 strains to date

has been limited. The clusters of oseltamivir-resistant A(H1N1)pdm09 viruses in Australia (2011) (Hurt et al. 2012b) and in Japan (2013–2014) (Takashita et al. 2015a) were reported. Other studies also confirmed that transmission most commonly occurred in closed and near-contact settings (Le et al. 2010; Wolfe et al. 2010; Chen et al. 2011; Graitcer et al. 2011; Storms et al. 2012).

In the N2 background, R292K confers oseltamivir resistance by introducing an obstacle to the structural transition through which E276 engages R224 to create the novel hydrophobic binding pocket. It was suggested that a hydrogen bond between E276 and K292 is responsible for this failure (Varghese et al. 1998). R292K has a greater effect on binding of oseltamivir and peramivir compared to other NA inhibitors (Varghese et al. 1998; Gubareva et al. 2017). This substitution also affects the NA enzyme activity (Sleeman et al. 2013; Yen et al. 2013). This is not surprising as R292 is a catalytic residue and is a part of the highly conserved arginine triad (R118, R292, R371) shared by all sialidases (Colman et al. 1983; Roggentin et al. 1989; Chavas et al. 2005). The R292K substitution confers the same profound effect among all group 2 NAs, including N6 and N9, as per the random mutagenesis and reverse genetic approaches (Song et al. 2015; Gaymard et al. 2016a).

Over the years, numerous NA changes have been implicated in the decreased potency of one or more NA inhibitors, at least in vitro (Thorlund et al. 2011; Samson et al. 2013; WHO 2018). Among those, N294S (N295S in N1 amino acid numbering) confers resistance to oseltamivir by virtue of hydrogen bonding to E276 and restraining the necessary conformational change associated with oseltamivir binding (Collins et al. 2008). The D198E (D197E in B amino acid numbering) in type B affected the interaction of R152 with the N-acetyl group, thus reducing binding of oseltamivir and peramivir (Oakley et al. 2010). A combination of NA changes has been shown to enhance drug resistance (Nguyen et al. 2010). For example, a dual H274Y and I222K/R change (H275Y and I223K/R in N1 amino acid numbering) has been reported to greatly reduce the drug binding

(Singh and Soliman 2015; Hsu et al. 2017). Noteworthy, the long-timescale dynamics simulations demonstrated that the mechanism of oseltamivir resistance of dual I222R/H274Y mutations was due to the loss of key hydrogen bonds between the inhibitor and residues in the 150-loop (Woods et al. 2012).

Furthermore, changes affecting the 250-loop have been reported to reduce the binding of zanamivir, laninamivir, and oseltamivir. One such NA substitution, S247P, was detected in the influenza A(H3N2) variant virus (also known as “A(H3N2)v” subtype, according to World Health Organization [WHO] nomenclature) (Sleeman et al. 2014). It was speculated that having proline at position 247 increases rigidity of the 250-loop by imposing strict torsion angles on this NA portion. This disturbance may in turn alter the hydrogen-bonding network involved in drug binding (Sleeman et al. 2014). Notably, not only substitutions, but also deletions, can affect drug binding. It was shown that a 4-amino acid deletion, such as 245–248 or 247–250 in the N2 subtype, adversely affect binding of oseltamivir and zanamivir (Abed et al. 2009; Tamura et al. 2015). Moreover, substitutions at remote residues that are neither conserved nor having direct interaction with the substrate can also confer resistance. For example, the Y155H substitution conferred reduced susceptibility to both oseltamivir and zanamivir in NA inhibition assay (McKimm-Breschkin et al. 2013).

Notably, influenza A viruses of animal origin can cross the interspecies barrier causing sporadic infections and large outbreaks in humans or even trigger pandemics (Vincent et al. 2014; Nelson and Vincent 2015; Subbarao 2019). A recent example is the emergence of avian A(H7N9) viruses in China since 2013 (Gao et al. 2013). Avian influenza A viruses of the H5N1, H5N6, H6N1, and H10N8 subtypes are also known to cause human infections (WHO 2014a, 2016b, 2019). Therefore, analysis of the identified substitutions has been expanded to NAs of different subtypes. These studies were conducted using recombinant NA proteins and reverse genetically engineered viruses (Pizzorno et al. 2011, 2013; Baek et al. 2015; Gubar-

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eva et al. 2017; Choi et al. 2018; Kwon et al. 2018; Tewawong et al. 2018). It was observed that NA proteins from the same group share certain similarities in their drug resistance profiles. For example, R292K caused a drastic reduction in oseltamivir potency among all group 2 NAs, including N9 (R289K in H7N9 amino acid numbering) and N6 (Song et al. 2015; Gaymard et al. 2016a). There were also apparent differences in drug resistance profiles within the group. Notably, H274Y in N2 did not affect oseltamivir binding, whereas in N9, it did reduce the drug potency by 100-fold in NA inhibition assay (Wang et al. 2002; Gubareva et al. 2017).

HA AND NA FUNCTIONAL BALANCE

Although the approved antiviral agents potently reduce NA enzyme activity, numerous studies indicate that their effect on virus replication can be less consistent. Influenza NA is not vital for virus entry, replication, assembly, or budding (Liu et al. 1995). Furthermore, some NA-lacking viruses can replicate in cell culture to appreciable titers. Such viruses were selected in cell culture, generated by reverse genetics, and even detected in clinical specimens (Gubareva et al. 2002; Mishin et al. 2005c; Moules et al. 2010). Taken together, these findings seem to challenge the appropriateness of NA as the target for drug design. On the other hand, a large body of evidence highlights the importance of NA during virus replication in vivo. Indeed, reduced virus replication in the respiratory tract of animals and humans treated with NA inhibitors was observed (von Itzstein et al. 1993; Hayden et al. 2000; Nishikawa et al. 2012). Moreover, the NA-lacking virus displayed severe attenuation in a ferret model (Mishin et al. 2005b).

To better understand the antiviral effect of NA inhibitors, it is essential to consider the roles played by both NA and HA (Wagner et al. 2002; Du et al. 2019; McAuley et al. 2019). Both surface glycoproteins bind to neuraminic acid-containing receptors that are used by influenza A and B viruses to enter cells. It was postulated that HA enables virus attachment, whereas NA facilitates release of nascent virus particles by destroying receptors (Gaymard et al. 2016b).

However, the HA–NA interplay is a more complex matter. To optimize virus replication, the opposing HA and NA activities must be balanced at all stages. NA's role in preventing the virus entrapment by mucin-rich secretions and in virus entry was recognized in several studies (Matrosovich et al. 2004; Cohen et al. 2013; Zanin et al. 2016). By using R18-labeled viruses and a fetuin-coated surface, it was discovered that influenza viruses can move via the HA-receptor exchange mechanism (Sakai et al. 2017). Moreover, NA contributes to the initiation of crawling and gliding and elongation of gliding. Together HA and NA act as novel motile machinery, and this movement accelerates virus entry into cells. Blocking this viral movement resulted in the suppression of virus endocytosis by cells (Sakai et al. 2017). The contribution of NA to virus-receptor binding was revealed using the biolayer interferometric analysis (Guo et al. 2018). They also observed that NA activity strongly stimulates virus rolling on surfaces covered with receptors. It was concluded that after attaching to the mucus layer in vivo, virus particles reside in a receptor-bound state while rolling through the mucus layer and over epithelial cells driven by the HA–NA-receptor balance. The receptor gradient is likely to serve as the driving force for virus rolling. Dynamic and motile interactions between HA and NA are crucial for penetration of the mucus layer and subsequent infection of cells (Guo et al. 2018). Further insights into how HA and NA balance works to meet both needs—to penetrate mucus and to attach to cells—were also provided (Vahey and Fletcher 2019). They demonstrated that the asymmetrical distribution of receptor-binding and receptor-destroying activities on the surface of the viral particle facilitates its persistent (directional) motion while maintaining stable attachment (Vahey and Fletcher 2019).

The HA affinity has a profound effect on NA enzyme activity and specificity toward sialic acid-containing receptors and receptor analogs (Lai et al. 2019). The results of this study have direct implications for numerous laboratory methods used for virus characterization, including the assessment of NA inhibitor susceptibility (Lai et al. 2019). The influence of



HA-receptor specificity may explain why NA inhibition results (small substrate-based assays) better correlate with the outcomes of studies in ferrets than with the traditional cell culture-based assays. Although traditional cell cultures (e.g., Madin–Darby canine kidney [MDCK]) predominantly express receptors linked to the adjacent carbohydrate moiety via α -(2,3) linkage, the HA of human viruses prefers receptors linked with α -(2,6) linkage. The ferret upper respiratory tract epithelium predominantly expresses α -(2,6) receptors, which makes ferrets a suitable model for assessing the NA inhibitor sensitivity of human viruses (Gubareva et al. 1998; Yen et al. 2005). Conversely, the HA of avian and egg-adapted human viruses preferably binds to the receptors with α -(2,3) linkage, and this manifests in an apparent higher sensitivity of avian viruses versus human viruses in traditional cell culture-based assays. Viruses exhibiting a drug-resistant phenotype in humans and ferrets can display drug sensitivity in cell culture and vice versa (Blick et al. 1998; Gubareva et al. 1998, 2001a; Abed et al. 2002a). Moreover, the role of HA glycosylation in virus susceptibility to NA inhibitors was evidenced and thus provides a further insight into the functional balance of HA and NA (Mishin et al. 2005c). Based on many observations made over the years since the approval of the first NA inhibitor, cell culture-based assays are not recommended for the assessment of virus susceptibility to NA inhibitors.

For the purpose of virological surveillance, sensitivity to NA inhibitors is assessed using biochemical NA inhibition assays that utilize small synthetic substrates (i.e., 4-methylumbelliferyl *N*-acetyl- α -*D*-neuraminic acid [MUNANA] and NA-Star) (Potier et al. 1979; Buxton et al. 2000; Takashita et al. 2015b). However, there are also NA assays that utilize a large substrate (e.g., fetuin) in enzyme-linked lectin assay (ELLA) (Lambré et al. 1990; Gao et al. 2016). Despite the clear advantages, there are also limitations to this approach. Namely, these assays cannot accurately predict the drug's effect on virus replication, and it has been challenging to establish laboratory correlates of clinically relevant NA inhibitor resistance.

Another manifestation of the HA and NA interplay is the emergence of NA mutants during virus culturing. The HA of human A(H3N2) viruses has evolved in such a way that it displays a low affinity for receptors expressed on the surface of MDCK cells (Asaoka et al. 2006; Yang et al. 2015). As a result, culturing of these viruses often leads to the emergence of NA mutants with substitution at D151 in the NA active site (Lin et al. 2010; Tamura et al. 2013). The substitution at D151 allows switching from the receptor-destroying to the receptor-binding activity (Xue et al. 2018). A virus isolate may contain two NA subpopulations: One (D151) acts as an enzyme, whereas the other (N151) acts as the receptor-binding protein. This cell culture artifact interferes with drug susceptibility monitoring (Mishin et al. 2014). To delay the selection of NA mutants, it is recommended to use cell cultures that were genetically modified to overexpress α -(2,6)-containing receptors, such as MDCK SIAT1 (Matrosovich et al. 2003) and AX-4 cells (Hatakeyama et al. 2005). Moreover, the recently developed hCK cell line may further delay the selection of NA mutants as it additionally expresses extremely low levels of α -(2,3) sialoglycans (Takada et al. 2019). Interestingly, the wild-type NA genotype can be restored by passage of mutated viruses in human primary respiratory cell cultures (Brown et al. 2019).

ANTI-NA ANTIBODIES

Since the 1980s (Webster et al. 1988), there has been growing experimental evidence supporting the role of antibodies raised against NA as an important correlate of protection against influenza (Memoli et al. 2016; Krammer et al. 2018). Because of a lower rate of evolution compared to HA and its highly conserved active site, NA is viewed as an attractive target for the development of therapeutic monoclonal antibodies (mAbs) (Abed et al. 2002b; Doyle et al. 2013c; Clark et al. 2017; Tewawong et al. 2017). Unlike antibodies targeting the HA, anti-NA antibodies do not seem to directly neutralize the infectivity of the virus, although they can impede virus spread. They may also help to clear virus by engaging the Fc region, thereby medi-

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ating antibody-dependent cellular cytotoxicity (ADCC), antigen-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (Hashimoto et al. 1983; Co et al. 2012, 2014; He et al. 2016; Wohlbold et al. 2017). Anti-NA antibodies can also act like NA inhibitors by sterically interfering with NA's access to sialic acid moieties on receptors. In recent years, there have been numerous reports describing the generation and characterization of mAbs that broadly and potently inhibit NA activity.

Human infections with A(H1N1)pdm09 and the highly pathogenic avian influenza (HPAI) A(H5N1) viruses have stimulated research toward the antigenic properties of N1 NA. The murine mAb 2B9, generated against a plant-derived recombinant N1 NA, was shown to broadly inhibit NA activity in the MUNANA-based assay (Shoji et al. 2011). Repeated treatment with mAb 2B9 significantly decreased morbidity and mortality of mice and ferrets infected with HPAI A(H5N1) viruses. Additionally, a panel of murine mAbs was generated to map strain-specific and conserved N1 antigenic domains (Wan et al. 2013). Given prophylactically, the cross-reactive mAb fully protected mice against a lethal challenge with A(H1N1) and A(H1N1)pdm09 viruses and provided partial protection against A(H5N1) virus. In another study, a unique 30-amino acid epitope that spans the lateral side of a NA dimer was identified with the use of CD6 mAb. Because this lateral CD6 epitope is conserved among circulating A(H1N1)pdm09 viruses, it may be used as an effective therapeutic target against this subtype (Wan et al. 2015). Furthermore, two mouse mAbs, N1-7D3 and N1-C4, were identified to bind to NAs of multiple N1 subtype viruses (Job et al. 2018). MAb N1-7D3, which recognizes a linear epitope, cross-reacted with NAs of N1 subtype but failed to inhibit NA activity and protect mice against lethal challenge. Conversely, N1-C4 mAb, which recognizes a conformational epitope, displayed NA inhibitory activity by ELLA. However, this was not seen in the MUNANA-based assay, indicating that this mAb did not block access of a small substrate to the enzyme active site. mAb N1-C4 showed

partial protective potential without cross protection in mice.

A research team developed two mAbs (IgG2a isotype) against A(H1N1)pdm09 virus; 3C02 was strain-specific, whereas 3C05 cross-reacted with multiple N1 strains (DiLillo et al. 2016). The former was 100-fold more potent in vitro (DiLillo et al. 2016). Using a mouse model, they demonstrated that 3C05 relied on Fc-FcγR interactions to mediate protection, whereas 3C02 did not (DiLillo et al. 2016). Furthermore, the authors emphasized that for broadly acting mAbs with in vitro antiviral activity (e.g., prevention of egress), the FcγR requirement for protection can be circumvented by increasing the dose of mAb.

The emergence of avian A(H7N9) viruses in China represents a significant public health concern and has been a focus of intense research since 2013 (WHO 2017a, 2017b). A murine mAb 3c10-3 directed against A(H7N9) NA showed prophylactic and therapeutic efficacy in mice; it inhibited N9 NA activity in MUNANA-based assay by binding across the enzyme active site. Notably, affinity analysis revealed that mAb 3c10-3 binds equally well to wild-type NA and NA carrying the R292K substitution that confers cross resistance to NA inhibitors. This mAb also restricted the cell-to-cell spread of the virus. As mAb 3c10-3 belongs to IgG1/κ isotype, the question was posed whether a version with the IgG2a Fc would be more protective (Wilson et al. 2016, 2017).

Influenza A viruses of N8 subtype also present a concern because of evidence suggesting their circulation in humans in the nineteenth century (Worobey et al. 2014), recent zoonotic infections in China by avian A(H10N8) viruses (Chen et al. 2014; WHO 2014b), and the spread of HPAI A(H5N8) viruses in birds (WHO 2016a). A panel of murine N8-reactive mAbs that displayed broad binding, encompassing both North American and Eurasian N8 lineages, was generated and characterized (Wohlbold et al. 2016). The 2E11 mAb showed strong antiviral activity in vitro and protection in mice.

Studies also included the NA of influenza B viruses, in which broadly reactive epitopes were elucidated. A panel of mAbs (1F2, 1F4, 3G1,

4B2, and 4F11) that can bind to NAs of viruses from both lineages was generated (Wohlbold et al. 2017). The conserved epitopes targeted by these mAbs are located on the NA head and are distant from the active site. These mAbs demonstrated broad binding to NAs spanning more than 70 years of type B evolution. They broadly inhibited NA activity in ELLA, albeit with relatively high IC_{50} values. When administered prophylactically at high dose (5 mg/kg), mAbs protected mice against lethal challenge with viruses from both the lineages (Wohlbold et al. 2017).

Influenza A and B NAs share a unique universally conserved sequence spanning amino acids 222 to 230, ILRTQESEC (Doyle et al. 2013a,b,c). Notably, it encompasses three amino acid residues (222, 224, and 227) of the NA active site. A rabbit mAb, HCA-2, was raised by immunization with this short peptide. In ELLA, this mAb caused a significant decrease in the enzyme activity of the N1–N9 subtypes and influenza B viruses from both lineages. Although the inhibitory effect was broad, it was not very potent ($IC_{50} > 10 \mu\text{g/mL}$). MAb HCA-2 provided heterosubtypic protection in mice lethally challenged with A(H1N1) and A(H3N2) viruses; however, high doses were required (Doyle et al. 2013a,b).

Recently, a panel of human NA (N1 and N2)-reactive antibodies was isolated from NA-reactive B cells of infected patients (Chen et al. 2018). Among anti-N2 mAbs, several displayed broad reactivity in both ELLA and NA-Star assay, spanning five decades of A(H3N2) antigenic drift. They also identified anti-N1 mAbs that were active in ELLA and NA-Star assays against distant strains of N1 subtype. There was an overlap between the mAb binding footprints and the site occupied by oseltamivir. These mAbs protected mice from lethal challenge, even against distant strains within the NA subtype (Chen et al. 2018).

A study in which broadly reactive NA epitopes for type A and B viruses were identified represents an important milestone (Stadlbauer et al. 2019). Human mAbs derived from plasma-blasts of an individual infected with a seasonal A (H3N2) virus were characterized. One of them,

mAb 1G01, showed the broadest binding activity toward NAs of type A (N1–N9 subtypes) and type B viruses. In ELLA, this mAb broadly inhibited NA activity of type A viruses (except for one N5 subtype virus) and type B/Victoria lineage viruses. In NA-Star assay, mAb 1G01 displayed a high potency in inhibiting NA activity of N2 viruses ($IC_{50} \sim 1 \text{ nM}$), indicating that this antibody directly targets the enzyme active site. Remarkably, mAb 1G01 fully protected animals against lethal challenge with different influenza A and B viruses (Stadlbauer et al. 2019).

It has been challenging to identify anti-NA mAbs displaying broad inhibitory activity, especially when their activity was measured using small substrates. This is not surprising considering that the NA active site presents a surface smaller than the footprint of a typical antibody-binding site (Colman et al. 1987). An innovative approach that utilizes novel molecules known as antiviral Fc-conjugates (AVCs) may overcome this challenge. The lead conjugate, CB-012, combines an antiviral molecule with the Fc-fragment of human IgG1. CB-012 was designed to target the virus and simultaneously engage the immune system (www.cidara.com/cloudbreak). In cell culture, this conjugate was 98- and 20-fold more potent than oseltamivir against influenza A and B viruses, respectively (Locke et al. 2019). Its efficacy was assessed in a lethal mouse model using oseltamivir-sensitive and -resistant influenza A(H1N1) and A(H3N2) viruses. A single intravenous dose administered 4 h prior to infection provided full protection in mice (Levin et al. 2019). Moreover, it was shown that the conjugate engages the immune system to accelerate elimination of the pathogen (Döhrmann et al. 2019). These findings validate the utility of multimodal mechanism of action—direct antiviral activity and immune-mediated clearance—for the prevention and treatment of influenza infections.

CONCLUDING REMARKS

The studies conducted around the world have contributed substantially to improve knowledge of NA structure, function, and interplay with

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HA (Colman et al. 1993). For two decades, NA inhibitors, the sialic acid analogs, have been the mainstay of antiviral therapy against influenza. Four NA inhibitors, oseltamivir, zanamivir, peramivir, and laninamivir, are currently licensed in different parts of the world to control influenza A and B infections (Ison 2017). Influenza viruses that display resistance to NA inhibitors typically contain amino acid substitutions in the NA active site. Because of the difference in NA inhibitors' chemistry and subtle variance in NA structures, resistance can be NA inhibitor-specific and NA type/subtype-specific. Changes outside of the NA active site have also been shown to affect drug binding (Hurt et al. 2012a; Li et al. 2015). Resistance to NA inhibitors, especially to oseltamivir and peramivir, is a public health concern. Over the years, extensive studies have been conducted with the goal to identify NA inhibitors with improved characteristics (multimeric derivatives and diversely decorated analogs, covalent inhibitors, selective NA inhibitors, etc.).

A new and vibrant area of research is the identification of mAbs that can be used as broadly acting anti-influenza therapeutics. Anti-NA mAbs can prevent virus transmission and protect against lethal virus challenge in animal models (Doyle et al. 2013b,c; Wan et al. 2013, 2015; Liu et al. 2015; Jiang et al. 2016; Memoli et al. 2016; Wohlbold et al. 2016, 2017; Chen et al. 2018). The mAbs that display potent NA inhibitory activity in a small substrate-based NI assay seem to be most promising. Another innovative approach utilizes an AVC, a molecule that directly inhibits viral replication while simultaneously engaging the immune system. Although reports describing the outcomes of influenza AVC studies are not yet published, the data presented on AVC high potency and low clearance raise hopes of protection after a single dose, regardless of a patient's immune status.

Efforts made to improve the understanding of NA-based immunity and protection may pave the way for the development of new influenza therapeutics of greater cross protection and efficacy, while reducing the threat of drug resistance.

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