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Review Article

Neuraminidase as a target for drugs for the treatment of influenza

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Abstract

Neuraminidase, or sialidase, is a surface glycoprotein that possesses enzymatic activity essential for viral replication in both influenza A and B viruses. To date, 9 neuraminidase subtypes (N1-N9) have been identified from influenza A viruses and only 1 subtype has been identified from influenza B viruses. Because of the importance of this enzyme in the pathogenesis of influenza virus infection and the close correspondence of the conserved residues of the active sites from all influenza neuraminidases, the enzyme structure has been successfully used for the design of neuraminidase inhibitors, which would be effective for various influenza viruses. The neuraminidase inhibitors zanamivir and oseltamivir are now commonly used for the therapy of influenza virus infections with minimal adverse effects, and they can also be used for the prophylaxis of influenza, especially when vaccination is unsuitable or ineffective. Up to now, the data on neuraminidase inhibitors suggest that the enzyme is an attractive target for the discovery of new antiinfluenza drugs.

Introduction

Influenza virus is the main cause of respiratory disease and is associated with significant morbidity and mortality (1, 2). During infection, the highly contagious virus is easily transmitted by air and can spread very quickly, producing characteristic epidemics. The first approved drugs for influenza were the adamantanes amantadine and rimantadine, which act specifically against influenza A virus by blocking the ion channel of the M2 protein (3). However, these compounds are not widely used owing to their limited spectrum of activity and adverse effects, and also because of the rapid emergence of resistant virus during treatment. Thus, there is an obvious need for new drugs to treat and control the spread of influenza virus infection.

Because neuraminidase, or sialidase, one of two glycoproteins on the surface of the influenza virus, plays a key role in virus replication and entry and release from the host cell, it has become a target for screening antiinfluenza drugs. Two neuraminidase inhibitors, zanamivir and oseltamivir, which are effective against both influenza A and B, have been approved for human influenza infection in many countries (4) and can also be used for the prevention of influenza (5-9). New neuraminidase inhibitors are also in the research or development stage (10, 11).

Neuraminidase

Chemical and biological features

Neuraminidase (E.C.3.2.1.18), an enzyme hydrolyzing sialic acid linkages in other molecules, exists widely in mucus virus, mycoplasma, bacteria and animal tissues (12, 13). Human neuraminidase occurs in a high-molecular-mass complex with several other proteins, including cathepsin A and β-galactosidase, which makes it difficult to purify and characterize the human enzyme. The bacterial enzyme is a monomer. Although the monomers are of similar size (about 380 residues) to human neuraminidase, the sequence similarity is low (about 15%) (14). The neuraminidase from influenza virus is located on the surface of the virus and is a tetramer. Influenza virus neuraminidase is composed of about 450 residues coded for by RNA6 and its molecular weight is 50 kD (15).

The biological features of neuraminidases from influenza viruses A and B are described in detail here since these are the only types that cause widespread influenza outbreaks among the three types of influenza viruses (A, B and C). Neuraminidase and hemagglutinin, two surface glycoproteins of influenza viruses, show great diversity in their sequences. Based on the differences between their two surface glycoproteins, the viruses are classified into different subtypes. Currently, 9 neuraminidase subtypes (N1-N9) and 15 hemagglutinin subtypes (H1-H15) have been identified from influenza A viruses. Viruses with all neuraminidase and hemagglutinin subtypes have been recovered from aquatic birds, but only 2 neuraminidase subtypes (N1 and N2) and 3 hemagglutinin subtypes (H1, H2 and H3) have established stable lineages in the human population since 1918. Only 1 subtype each of neuraminidase and hemagglutinin has been identified from influenza B viruses (16).

For influenza A viruses, 3 viral proteins (hemagglutinin, neuraminidase and M2 protein) are incorporated into the lipid bilayer, whereas there is no M2 protein in influenza B viruses. Neuraminidase tetramers and hemagglutinin trimers form spikes on the surface of the virion (17) (Fig. 1). Neuraminidase is composed of a cytoplasmic tail, a transmembrane domain, a stalk region and a mushroom-shaped head. There are about 100 molecules of this tetramer present in each influenza virion (18-20).

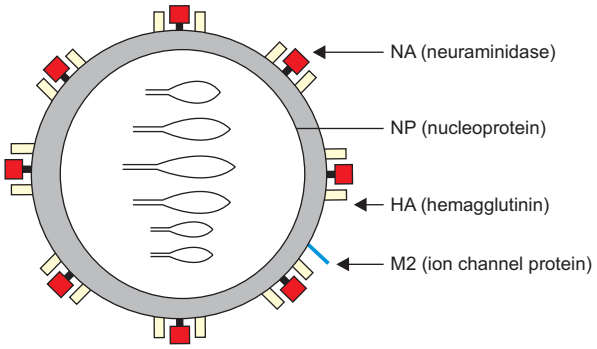

Fig. 1. Simple schematic representation of an influenza A virion.

Biological functions

In the replication cycle of influenza virus, hemagglutinin is responsible for recognizing the receptors to initiate virus penetration and promotes fusion of viral and cellular membranes, while neuraminidase is in charge of destroying these receptors by catalyzing the cleavage of the α-ketoside bond linking a terminal neuraminic acid residue to the adjacent oligosaccharide moiety. This breaking of the bond has several important effects that facilitate the movement of the virus to and from sites of infection in the respiratory tract. First, it allows the release of virus from infected cells. Second, it prevents the formation of viral aggregates after release from host cells. Third, this

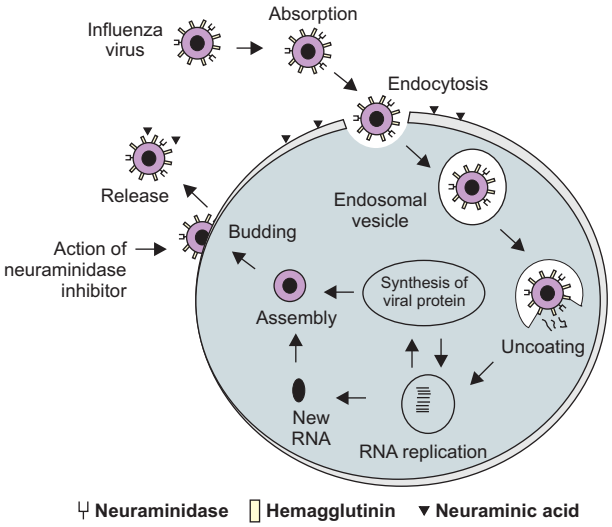

Fig. 2. Schematic diagram showing the replication cycle of influenza virus.

enzyme, by cleaving the sialic acids found in respiratory tract mucus, may prevent viral inactivation and promote viral penetration into respiratory epithelial cells (21).

Virion release requires neuraminidase to destroy the activity of the receptor on both viral surface glycoproteins and the cellular membrane. In the presence of neuraminidase inhibitors, virions stay attached to the membrane of infected cells and cause self-aggregation, and virus spread is inhibited (Fig. 2). In addition, neuraminidases hydrolyze the terminal, nonreducing sialic acid linkage in glycoproteins, glycolipids, gangliosides, polysaccharides and synthetic molecules, and a variety of assays are available to measure neuraminidase activity, making it an attractive target for new antiinfluenza drugs (22).

Neuraminidase as a drug target

As previously mentioned, 9 subtypes of neuraminidase have been identified from type A influenza and 1 subtype from type B influenza. The first crystallographic study on the enzyme was performed with subtype N2, which was from virus A/Tokyo/3/67 and RI/5-/57 (23-25). Subsequently, the structures of neuraminidase from other strains of influenza virus were characterized (26-28). Although the sequence homology between neuraminidases from influenza A and B viruses is not high (about 30%), the conserved residues of the active sites from all influenza neuraminidases superimpose very closely, with both the main-chain and side-chain atom positions showing a close correspondence. The structural similarities between the active sites of influenza A and B neuraminidases are reflected in the activities of current neuraminidase inhibitors, such as zanamivir and oseltamivir.

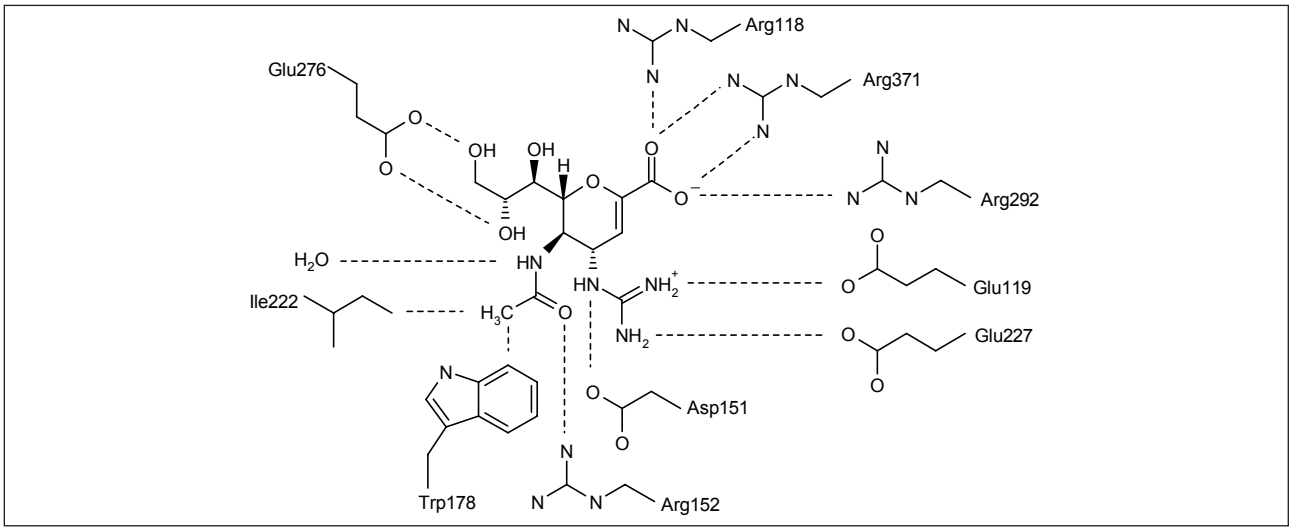

Fig. 3. Schematic drawing of the binding mode of zanamivir to neuraminidase showing the major protein-ligand hydrogen bonding and van der Waals interactions.

The active site of neuraminidase is located within a pocket on the surface of each glycoprotein subunit that is lined by amino acids which are conserved among all influenza A and B neuraminidases characterized so far. Some of the residues, such as Arg371, Arg118, Arg292, Glu227, Asp151, Arg152, Trp178, Ile222 and Glu276, are conserved residues, directly contacting the bound substrate neuraminic acid, and are regarded as the active binding residues (Fig. 3), whereas others provide a structural framework for the functional residues (29).

Neuraminidase inhibitors

Because of the importance of influenza neuraminidase in viral replication and pathogenesis, and also its highly conserved active site, interest has focused on the development of selective inhibitors of this enzyme, in particular sialic acid analogues.

The first sialic acid analogue, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) (Fig. 4), was developed in 1969 but exhibited low potency and specificity (30). Among a number of DANA derivatives examined for efficacy, FANA was reported as the most potent compound, with about 6-fold lower concentrations required compared to the parent compound. However, these compounds lacked specificity, *i.e*., they were at least as effective in inhibiting bacterial neuraminidase as the viral enzyme (31).

Following the determination of the crystal structure of the enzyme in 1983 and of the complex structure with the natural substrate sialic acid (23, 32), structural knowledge allowed molecular modeling to design more potent and selective inhibitors of the enzyme. 4-Amino-Neu5Ac2en (Fig. 4), was identified using structure-based design by simulating the geometry of the transition state during the enzymatic reaction. To increase the interaction between 4-amino-Neu5Ac2en and the amino acid residues forming the enzyme active site, the amino group was substituted by a guanidyl on carbon atom 4, resulting in zanamivir (Fig. 4) (33). Another approach, using a cyclohexene ring and replacement of a polar glycerol with lipophilic side chain, led to oseltamivir (Fig. 4). The bioavailable prodrug oseltamivir is an ethyl ester that is converted to the active carboxylate by hepatic esterases (34-36). Another neuraminidase inhibitor, peramivir (BCX-1812, RWJ-270201; Fig. 4), is a cyclopentane derivative with a guanidinyl group and lipophilic chains (37). Zanamivir and oseltamivir were approved for the treatment of human influenza infection in the 1990s by the FDA and peramivir is in phase III clinical trials.

These molecules interact differently in terms of hydrogen bonding and electrostatic and van der Waals interactions within the enzyme active site, and the differences may influence antiviral activity and the emergence of resistance, but all three are potent and selective influenza neuraminidase inhibitors. Recently, several new neuraminidase inhibitors have been reported. Although they are in the research stage, their structures can offer a basis for medicinal chemists to design new neuraminidase inhibitors with high activity (11, 38, 39).

In vitro activities of neuraminidase inhibitors

Zanamivir

Woods and colleagues have provided the most extensive data regarding the *in vitro* activity of zanamivir (40). It was reported that the 50% effective concentrations (EC_{50}) of zanamivir were consistently lower than those of 4-amino-Neu5Ac2en, DANA, amantadine, rimantadine and ribavirin, as determined by plaque formation inhibition assays in Madin-Darby canine kidney (MDCK) cells

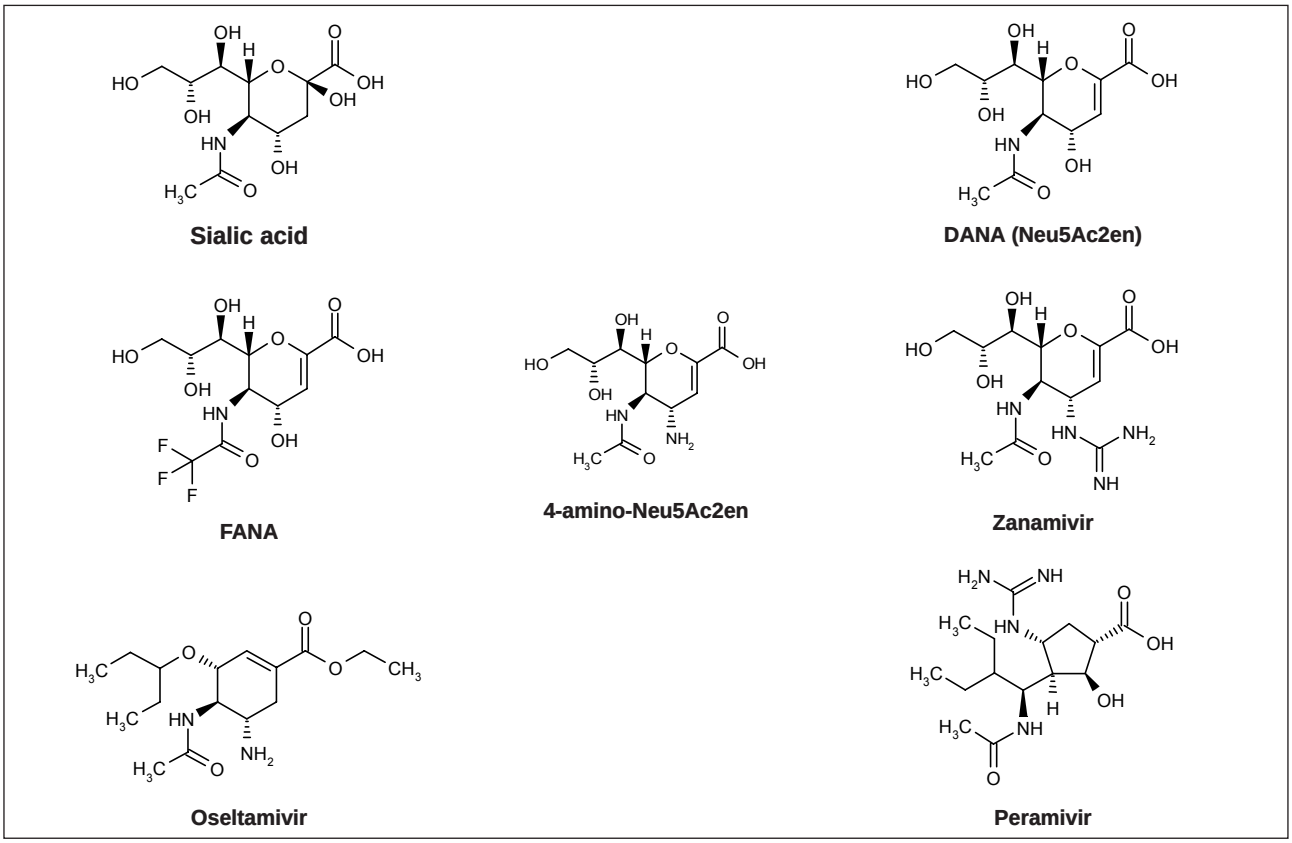

Fig. 4. Molecular structures of sialic acid neuraminidase inhibitors.

with both laboratory-passaged strains and clinical isolates of influenza A and B viruses, although the EC_{50} values had a much greater range for clinical isolates (0.002-16 mmol/l) than for laboratory-passaged isolates (0.004-0.014 mmol/l). However, when the EC_{50} was determined by inhibition of purified neuraminidase, zanamivir was shown to be a potent inhibitor of neuraminidases from all isolates. Plaque assay studies also showed that zanamivir was active at concentrations of 0.002-1.5 mmol/l against clinical isolates resistant to amantadine and rimantadine.

Studies have also evaluated the cytotoxicity and selectivity of zanamivir. As compared with earlier neuraminidase inhibitors, zanamivir exerts much less inhibition of human and other noninfluenza neuraminidases. These neuraminidases are inhibited only at > 10^6 higher concentrations compared with the influenza enzyme. Even at concentrations of up to 10 mmol/l, no cytotoxicity against human cells has been seen.

Oseltamivir

Generally, oseltamivir has an *in vitro* spectrum and potency comparable to zanamivir against neuraminidase activity and the replication of influenza A and B viruses (41). Oseltamivir appears to be several times more active against H3N2 subtype viruses in cell culture and in enzyme inhibition assays than zanamivir (42). No cytotoxicity or inhibition of neuraminidases from other sources was seen at concentrations as high as 1 mmol/l, the highest concentration tested (43).

Peramivir

The third inhibitor under development, peramivir, is a highly selective inhibitor of neuraminidases from influenza A and B viruses and a potent inhibitor of influenza A and B virus replication in cell culture. The *in vitro* potency appears to be greater than either zanamivir or oseltamivir carboxylate, based on the generally lower EC_{50} values (37).

Clinical therapy of influenza infection

The previously available antiinfluenza drugs amantadine and rimantadine are only effective against influenza A and are also associated with neurological and gastrointestinal adverse effects. In the absence of the ability to differentially diagnose influenza A from influenza B infections, the introduction of neuraminidase inhibitors which are effective against both influenza A and B should pro-

vide a significant benefit to those infected with any influenza virus. A further important consequence is that treatment with neuraminidase inhibitors should also limit the spread of influenza (44).

Zanamivir

Clinical therapy for the treatment of influenza A and B with zanamivir was studied in randomized, double-blind, placebo-controlled trials conducted in North America, Europe, Canada, Japan, Australia and other countries (44-48). Patients were recruited into trials when they experienced influenza-like symptoms, a fever of > 37.6 °C or feverishness and two symptoms from among headache, myalgia, sore throat and cough for < 36-48 h. The primary clinical endpoint was the length of time to reduction of all major symptoms for at least 24 h. Results demonstrated earlier relief of symptoms by a mean of 1-1.5 days when all patients were included in the analysis. The time for those presenting with fever or who had experienced symptoms for < 30 h varied from 2 to 4.5 days. Subjects in high-risk groups also experienced a substantial benefit (2.5 days). There was also a significant reduction in the time to resumption of normal activities and in the use of symptom relief medication and antibiotics (49-51). Similar efficacy was observed in pediatric trials, with a mean time to symptom relief of 1.25 days, reduced use of fever relief medications and a faster return to normal activities (52). Another study showed that zanamivir was also an effective treatment for influenza associated with asthma or chronic obstructive pulmonary disease (COPD), with a safety profile similar to placebo (53, 54). No adverse effects were noted in any group.

Oseltamivir

Treatment with oseltamivir was also studied in randomized, double-blind, placebo-controlled trials in patients challenged with influenza A virus or with naturally acquired infection (55-57). Another separate investigation was carried out in patients infected with influenza B (58). Patients recruited into the trial had to have fever of > 38 °C and two other symptoms (fever, myalgia, headache, sore throat, cough) and symptoms for < 36 h. Clinical efficacy was based on the duration and severity of symptoms and viral shedding. Relief of symptoms was seen a mean of 1-1.5 days earlier following treatment with oseltamavir when all patients were included, and the benefit for patients presenting symptoms for < 24 h was 2 days. In pediatric trials, the efficacy was similar to in adults (59). Oseltamivir was well tolerated, although a few patients experienced transient nausea (60, 61).

Zanamivir (Relenza®; GlaxoSmithKline) and oseltamivir (Tamiflu® ; Gilead/Roche) are now being used worldwide, although oseltamivir can cause vomiting. The drugs need to be given very soon after infection to be effective, only inhibit influenza virus and are very expensive (6, 7). Recently, a few cases of side effects from neuraminidase inhibitors were reported in patients with complications, but no side effects have been reported in otherwise healthy patients (62, 63).

Prophylaxis of influenza infection

While the prevention of influenza by vaccination plays a significant role, this approach still does not provide complete protection, due to either a poor match of the vaccine strain or a poor immune response (64, 65). Studies of neuraminidase inhibitors for the prevention of influenza showed that they may provide household prophylaxis or adjunctive prophylaxis in high-risk vaccinated patients during an outbreak of the disease, or for patients in whom vaccination is unsuitable or ineffective (6, 66, 67).

Zanamivir

To examine the efficacy of zanamivir in the prevention of influenza infection and disease, Monto *et al.* administered the drug by oral inhalation for 4 weeks in a randomized, double-blind, placebo-controlled trial (68). The results showed that zanamivir was 67% effective in preventing laboratory-confirmed clinical influenza and 84% effective in preventing laboratory-confirmed illnesses with fever. The nature and incidence of adverse events in the zanamivir group did not differ from placebo.

Hayden *et al.* performed a double-blind, placebo-controlled study of inhaled zanamivir for the prevention of influenza in families (69) in which contacts were treated for 10 days. Zanamivir was shown to be 79% effective in preventing influenza in contacts. It was effective against both influenza A and B and no drug resistance was detected.

Oseltamivir

Initial prophylaxis studies with oseltamivir were conducted in subjects experimentally infected with either influenza A or B (57, 58). In the influenza A trial, oseltemivir was 61% effective in preventing influenza infection based on serology and no patients shed virus, but in the influenza B trial, oseltamivir did not reduce the incidence of infection, although it did reduce the virus titer and duration of virus shedding. Overall, a protective efficacy of 74% was shown. The efficacy of oseltamivir in preventing influenza in household contacts was also studied (70). The overall protective efficacy against clinical influenza was 89% for individuals and 84% for households. An investigation on the protective efficacy of oseltamivir in a frail elderly population showed that prophylaxis was associated with a 92% reduction in the incidence of laboratory-confirmed clinical influenza compared with placebo (71).

Conclusions

Because of the critical role of neuraminidase in the pathogenesis of influenza virus infections and the conserved binding residues, neuraminidase inhibitors are expected to be effective for the treatment/prophylaxis of influenza virus infections. The neuraminidase inhibitors zanamivir, oseltamivir and peramivir have been shown to provide significant antiviral effects. These drugs appear to offer the benefit of activity against both influenza A and B viruses with minimal adverse effects. Up to now, the data on neuraminidase suggest that it is an attractive target for new drug discovery in the field of influenza viruses and neuraminidase inhibitors may soon fill a large void in the current antiinfluenza armamentarium, despite the fact that zanamivir suffers from problems of administration and oseltamivir is less potent against influenza B (9).

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