Chapter 31
Influenza M2 Ion-Channel and Neuraminidase Inhibitors

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1 Introduction

1.1 Influenza Viruses

There are two main types of influenza viruses, influenza A and B, that cause mild to serious respiratory disease in humans, which is associated with increased deaths every year, and referred to as seasonal influenza. In addition, influenza A viruses which infect several different animal species, are able to undergo genetic reassortment and mutate to produce new antigenic sub-types which are capable of causing pandemics of serious influenza infections in humans, associated with high mortality. Influenza A viruses are divided into sub-types based on the surface glycoproteins that project through the lipid membrane of the virus, the haemagglutinin (HA) or virus receptor and neuraminidase (NA), an enzyme that cleaves terminal sialic acid from glycoproteins/glycolipids. There are a total of 16 HAs (H1–16) and 9 NAs (N1–9) which together form the antigenic sub-types. Within the lipid envelope of the virus are also found virus M2 ion channels. Lining the inside of the lipid membrane is the M1 matrix protein which encloses the ribonucleoprotein complexes. Influenza viruses have a segmented negative-strand RNA genome, consisting of eight RNA segments which produce a total of 10 (A viruses) and 11 (B viruses) viral proteins.

1.2 Influenza Virus Replication

Knowledge of how the virus replicates in cells is important for understanding the mechanism of action of antiviral agents (see Fig. 1). First, the virus has to enter the cell, and this occurs through binding of the surface haemagglutinin to the terminal sialic (neuraminic) acid containing cell receptors. Once binding has occurred the virus is endocytosed into the cell. The haemagglutinin undergoes an acid-induced conformational change within the endosome which triggers fusion of the virus lipid membrane at low pH with the cellular lipid membrane of the endosome. The M2 ion channels facilitates entry of hydrogen ions into the virus from the acidified cellular endosome and the low pH inside the virus particle triggers M1 protein uncoating and release of the ribonucleoprotein complexes into the cytoplasm. Transport of the ribonucleoprotein complexes to the cell nucleus occurs followed by primary virus transcription of the vRNA, by the polymerase present in the virus, into positive sense mRNA ready for production of virus proteins within the cytoplasm. Later a switch to synthesis of full-length complementary RNA occurs from which vRNA will be produced for the progeny virus. New virus products are transported to the cell membrane where assembly and packaging of the ribonucleoprotein complexes occurs. Insertion of the viral glycoproteins into the cell membrane is followed by budding through the cellular membrane to form new virus particles. Finally, to allow release of virus from the cell and to aid spread of virus, the neuraminidase enzyme, which functions extracellularly, removes terminal sialic acid from the surface of the virus and surrounding glycoproteins and glycolipids.

2 M2 Ion-Channel Inhibitors: Amantadine and Rimantadine

Amantadine (1-adamantanamine hydrochloride, Symmetrel™, Lysovir™, Symadine™) and its close analogue rimantadine (α-methyl-1-adamantanemethylamine hydrochloride, Flumadine™, Roflual™) (see Fig. 2) were first shown to possess potent anti-influenza A activity in 1964/1965 in cell culture and in ferret and mouse animal models (1, 2). Later amantadine was approved for the prophylaxis and treatment

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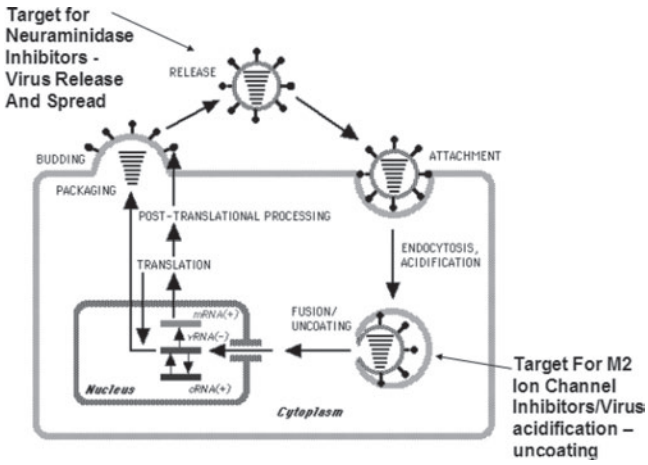

Fig. 1 Influenza virus replication cycle taken from: www.tulane.edu/~dmsander/WWW/335/Orthomyxoviruses.html, showing targets for inhibition by the M2 inhibitors, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir

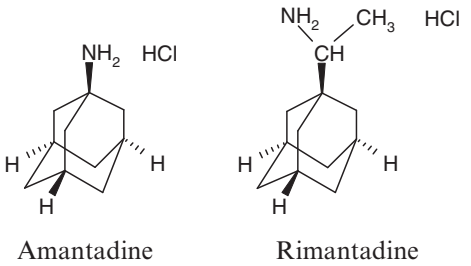

Fig. 2 Chemical structures of the M2 ion-channel inhibitors: amantadine and rimantadine

of influenza A (H2N2, Asian) virus infections in humans in the USA in 1966, and after further clinical evaluation for all influenza A infections in 1976. Rimantadine, which was first widely used for influenza prophylaxis in the USSR, was only later approved for use against influenza A infections in the USA in 1994 after demonstrating similar potency but reduced side effects to amantadine (3, 4).

2.1 Antimicrobial Mechanisms of Action

The anti-influenza A activity of amantadine and rimantadine was discovered empirically during random large-scale screening of molecules for activity against influenza either in cell culture or in ovo (4, 5). In vitro, rimantadine is up to eightfold more active than amantadine with activity (50% inhibitory concentrations by plaque reduction) at <5 µM or <1 µg/mL against the most susceptible strains in cell culture (6). Both amantadine and rimantadine were used in the clinic long before the target protein, the M2 protein, was identified and consequentially before the more detailed molecular mechanism of inhibition could be elucidated. These inhibitors became valuable tools using resistance studies to help identify the target protein and to later help understand the function of this protein as the first viral ion channel to be discovered. Today viral ion channels have been discovered in many viruses including HIV, HCV, influenza B, rhinoviruses (7, 8) and are a target for antiviral chemotherapy, although still a relatively difficult target to discover inhibitors using specific ion-channel assays.

2.1.1 Direct Studies on the Mechanism of Action in Cells

After the fortuitous discovery of these clinically potent influenza A inhibitors, amantadine and rimantadine, work was rapidly initiated to try to understand further the mechanism of action in cell culture. The majority of this work has been undertaken with amantadine but appears to apply equally to rimantadine. Early studies were rather limited by the technology available but it was shown that an early stage in the replication of human influenza viruses, during virus entry into the cell, was blocked. Under single cycle conditions, with influenza A/WSN/33(H1N1) and A/Japan/305/57(H2N2) the inhibitor was only active when administered within 10 min of infection (9). Adsorption of the virus to the cell surface was not affected, since a considerable drop in the virus titre of the supernatant fluid occurred within 1 h of infection in the presence of amantadine hydrochloride. The above workers suggested that penetration of the virus through the cell membrane into the cell was inhibited by amantadine, by demonstrating that infectious virus remained sensitive to neutralisation by antisera. Later it was shown using fowl plague virus that virus penetration was not affected but that amantadine inhibited uncoating of virus as demonstrated by a block in the loss of photosensitivity of virus labelled by neutral red (10). More detailed understanding of the mechanism of inhibition of uncoating by amantadine came several years later with detailed studies of the structure and function of the M2 protein.

2.1.2 General Structure and Function of the M2 Protein

The M2 protein consists of just 97 amino acids, which is an integral membrane protein present as a homotetramer channel in the virus membrane (23–36 copies per virion) and virus-infected cell membranes (11). The M2 channel includes a 24-residue N-terminal extracellular domain, a 19-residue highly conserved hydrophobic transmembrane domain and a 19-residue cytoplasmic tail (12).

The M2 channel was later demonstrated to be an acid pH-activated ion channel and the passage of ions could be blocked by amantadine and rimantadine (13–15). The channel is closed at physiological pH and is activated at pH

≤6.2. The M2 protein is therefore involved with uncoating of the virus during endocytosis by mediating the passage of protons from the acidic medium of the endosome into the virion to induce low pH mediated dissociation of the RNP/M1 complex and release of the RNP complex into the cytoplasm for transport to the nucleus. Based on viral resistance studies and the observation that inhibition of virus uncoating mediate by M2 occurs at relatively low inhibitor concentrations (<5 μM or <0.75 μg/mL), this is considered the clinically relevant mechanism of inhibition.

The M2 ion channel plays a second role in virus replication in that the ion-channel activity of M2 can increase the pH within vesicles of the trans-Golgi network and protect the structure and function of the acid sensitive HA of some avian A/H7 viruses during transport to the cell surface. Thus, the virus was able to bud from the cell surface with intact HA ready to infect further cells. In the presence of amantadine the pH of the trans-Golgi network decreased and cleavage of HA into HA1/HA2, the low pH form, occurred, and release of infectious virus was inhibited (16, 17). Again, studies with amantadine helped to determine the second role of the M2 ion channel in replication of some influenza viruses.

2.1.3 Structure/Function/Inhibitor Binding to the M2 Protein

Further studies have attempted to understand the detailed mechanism of the block in M2 function. Kinetics of inhibition suggest irreversible binding of one molecule of inhibitor per channel (15, 18). It was postulated that amantadine/rimantadine binds to the M2 protein at an allosteric site which triggers a conformational change in the pore region which interferes with proton transfer through the ion channel across the membrane of the virus or endosome (14). However, neutron studies and resistance studies suggested interaction of the inhibitor with the region between residues 22 and 46 of M2 which would have a direct effect on the pore region (19, 20). Molecular modelling of the ion channel using molecular dynamics calculations (21) or based on mathematical analysis of the functional properties of a series of mutants (22) produced similar three-dimensional structures of the trans-membrane region. The predicted structure consists of four parallel trans-membrane α-helices around a central channel. It was proposed that amantadine binds to hydrophobic groups lining the pore which form a widening near the centre of the bilayer (22).

Analysis of the structure and function of the M2 protein have been undertaken using site-directed mutants. Residues 25–44 in the transmembrane domain were individually replaced by cysteine and it was shown that A30, G34, H37 and W41 line the pore (23). Further, H37 is important in the conduction mechanism of the channel and is believed to form a hydrogen-bonded interaction with the ammonium group of amantadine (22). The indole side chain on tryptophan, W41 in the transmembrane domain acts as a gate that opens and shuts the pore and H37 acts selectively on transport of protons (24). This reveals the simplicity of the mechanism of the M2 ion channel in that only two residues are responsible for the functions of selectivity and activation. Transient exposure to low pH outside the membrane will result in lasting acidification of the virus because the protons are retained by the tryptophan gate. The channel is believed to transport protons by way of a proton wire using a continuous water molecule file (25, 26).

Further structural studies of the M2 channel using solid-state NMR and site-specific Fourier transform infra-red dichroism analysis have identified a helix tilt and determined the rotational pitch angles of the helices within the functional channel, and refined the backbone structure of M2 (27–31).

2.1.4 Other Effects of Amantadine/Rimantadine on Virus Replication

At high inhibitor concentrations (>100 μM, >15 μg/mL), amantadine and rimantadine block cell fusion by directly raising the pH of the cellular endosome and preventing the acid-induced conformational changes in the cleaved HA which is required for fusion of virus with the endosomal cell membrane. This non-specific effect is also observed with other weak bases (32), but is not considered to be clinically relevant due to the high concentrations of inhibitor required.

2.2 Mechanism of Drug Resistance

2.2.1 Genetics–Mutations Associated with Resistance

Understanding the mechanism of drug resistance and the mutations responsible for resistance has been key to unravelling the clinically relevant mechanism of action of amantadine and rimantadine. Immediately after the discovery of amantadine it was shown that resistant virus could be readily isolated after one or two passages of virus with amantadine in cell culture (2, 33). Similarly, it was possible to isolate resistant virus from virus stocks at an estimated frequency of 1 in 10^3/10^4 (34), and some early isolates such as A/WSN/33(H1N1) and A/PR8/8/34(H1N1) were naturally resistant to amantadine before ever being exposed to the drug (35). Later drug-resistant strains were also isolated in vivo in the mouse model after one pass in lung tissue (36), and may be readily isolated within 6 days in the ferret model for

human influenza (37). In humans naturally resistant isolates have been observed sporadically before exposure to the drug (38, 39), and during treatment or prophylaxis with amantadine and rimantadine (6, 40–47). More recently, the avian H5N1 viruses circulating in South East Asia in 2004/2005 have been reported to be resistant to amantadine/rimantadine (48), and there has been a substantial increase in the number of adamantine-resistant viruses circulating world-wide (49).

Initial studies with genetic reassortments, using dual infections with an early resistant strain A/Bel/42 (H1N1) and an amantadine sensitive strain A/Japan/57(H2N2) demonstrated that resistance was linked to RNA segment 7 (34). Influenza segment 7 codes for the M gene which produces two products, a co-linear truncated product, the M1 matrix protein and a spliced product, the M2 protein. Other reassortment studies with avian strains implicated that the neuraminidase (NA), the nucleoprotein (NP) (50) and HA genes (51) may contribute to drug resistance. The later observation may be explained by the effect of M2 on transport of avian HA to the cell surface.

Further studies with resistant viruses, selected after passage in cell culture or by plaque selection in the presence of amantadine or rimantadine, and then analysed by NA sequencing, confirmed that the M gene was linked with resistance. These studies further defined that the M2 protein was the drug target due to the presence of mutations in the M2 region downstream of the M1 termination site (52). Analysis of in vitro derived resistant virus revealed that single amino acid substitutions were sufficient to produce total resistance to amantadine or rimantadine. This conflicts with some of the early passage studies which implied varying degrees of resistance to amantadine developed in cell culture (2, 33). However, this probably reflects that virus mixtures of sensitive and resistant virus were present in early studies, whereas later studies generally used cloned viruses.

Initially, from in vitro studies, mutations at four amino acid residues, and later a mutation at a fifth amino acid residue were identified in the M gene, at L26H, V27A/G/D or I27S/T/A/N, A30T/P/S, S31N or G34E all within the transmembrane domain of the M2 protein (51, 53). Based on these findings, analysis of amantadine/rimantadine resistance in humans included NA sequencing of the M gene and susceptibility analysis using ELISA assays (40). The first study in humans revealed the presence of mutations at residues V27A, A30V and S31N consistent with observations made in cell culture (40). Later studies have confirmed that these mutations may arise after treatment or prophylaxsis with amantadine or rimantadine, (41, 43–47) and identified a fourth mutation, at residue L26F (42). Similar studies in chickens identified mutations at residues I27S/T, A30S/T and S31N, (54) and in ferrets at residues L26F, V27A, A30S/T/V, S31I/N (37). Further details of the changes observed are shown in Table 1.

In all these studies changes in the M2 have consistently been linked to abolition of susceptibility to amantadine and/or rimantadine, showing that this is the specific mechanism of inhibition of these inhibitors in vivo for both human and avian strains of influenza virus. The amantadine-induced changes in HA of avian influenza viruses could be abrogated by M2 mutations alone which would affect the pH of the trans-Golgi network (16). Thus, HA mutations which change pH stability of avian strains have relatively minor effects on amantadine/rimantadine susceptibility compared to the total loss of drug susceptibility with M2 mutations, and do not appear to be clinically important (55). Analysis and mutagenesis of the M2 sequences of the Rostock and Weybridge avian strains revealed that residue 44 alone mapped to the trans-Golgi pH modulation whereas changes in residues 27, 38 and 44 were required to switch the activation characteristics of the Weybridge M2 to those of the Rostock M2 (56).

2.2.2 Effect of Mutations on Function and Structure of the M2 Ion Channel

Functional studies examining effects of amantadine mutations in the M2 protein on ion-channel activity have confirmed that this is the specific mechanism of virus inhibition by amantadine. When the influenza virus M2 protein was expressed in Xenopus oocytes it was shown to possess ion-channel activity with selectivity for monovalent ions, the transport of which could be blocked by amantadine (13). Further when amantadine-resistant mutants were expressed in Xenopus oocytes it was shown that amantadine no longer blocked the ion-channel activity of these mutant M2 proteins. Similarly, studies using deuterium-labelled amantadine and influenza M2 peptides suggested that amantadine bound 0.5 nm from the centre of the bilayer in an area between V27 and S31, a location consistent with the formation of a steric block within the ion channel (20). Similar studies with an amantadine-resistant mutant peptide revealed no interaction with amantadine. Based on solid-state NMR structural studies the amantadine mutations at residues 27, 30 and 34 were positioned facing the pore of the channel, and residue 31 was positioned partially in the protein-protein interface and partially in the pore (57). Fig. 3 shows amantadine bound within the ion channel of wild-type influenza virus.

Binding analysis of amantadine to M2 peptides from different viral strains showed that the virus developed two processes of overcoming the amantadine block (57).

1. The channels mutate so that amantadine can no longer bind.
2. A novel mechanism which retains binding of amantadine but the mutation maintains the function of the pore.

In this second process the pore size is increased thus allowing protons to move through the channel in the presence of the inhibitor. It was shown that mutations that introduced a

Table 1 M2 Mutations observed from in vitro, in vivo and clinical studies with amantadine and rimantadine

Inhibitor	Virus/sub-type/source	Mutation (no. of isolates)	Selected (reference)			Transmission in humans
			In vitro	In vivo	In clinic	
Amantadine	H3N2 (human)	L26F (2)			Yes (42)	NR
Amantadine	H7N1 (avian)	L26H (1)	Yes (53)			
Amantadine	H3N2 (human)	L26F (1)			Yes (68)	NR
Amantadine	H3N2 (human)	L26F (1)		Yes (37)		
Amantadine	H2N2 (human)	V27A (2)	Yes (51)			
Amantadine	H7N7 (avian)	V27A (3)	Yes (51)			
Amantadine	H7N1 (avian)	V27A (1)	Yes (51)			
Rimantadine	H3N2 (human)	V27A (1)			Yes (40)	NR
Rimantadine	H3N2 (human)	V27A (1)			Yes (41)	NR
Amantadine	H3N2 (human)	V27A (3)			Yes (43)	Yes
Rimantadine	H3N2 (human)	V27A (1)			Yes (45)	NR
Amantadine	H3N2 (human)	V27A (1)		Yes (37)		
Amantadine	H3N2 (human)	V27A (1)	Yes (46)			
Amantadine	H3N2 (human)	V27A (8)			Yes (47)	Yes
Amantadine	H7N7 (avian)	V27G (2)	Yes (51)			
Amantadine	H7N7 (avian)	V27D (1)	Yes (51)			
Amantadine	H5N2 (avian)	I27S (1)		Yes (54)		Yes
Amantadine	H7N1 (avian)	I27S (17)	Yes (51)			
Amantadine	H7N1 (avian)	I27S (10)	Yes (53)			
Amantadine	H5N2 (avian)	I27T (3)		Yes (54)		Yes
Amantadine	H7N1 (avian)	I27T (8)	Yes (51)			
Amantadine	H7N1 (avian)	I27T (6)	Yes (53)			
Amantadine	H7N1 (avian)	I27T (1)	Yes (53)			
Amantadine	H7N1 (avian)	I27A (1)	Yes (51)			
Amantadine	H7N1 (avian)	I27N (2)	Yes (53)			
Rimantadine	H3N2 (human)	A30V (2)			Yes (40)	NR
Rimantadine	H3N2 (human)	A30V (1)			Yes (41)	NR
Amantadine	H3N2 (human)	A30V (1)			Yes (43)	NR
Amantadine	H3N2 (human)	A30V(2)			Yes (44b)	NR
Rimantadine	H3N2 (human)	A30V (1)			Yes (45)	NR
Amantadine	H3N2 (human)	A30V (3)			Yes (47)	Yes
Amantadine	H3N2 (human)	A30V (4)		Yes (37)		
Amantadine	H3N2 (human)	A30V (1)	Yes (46)			
Rimantadine	H3N2 (human)	A30T (1)			Yes (40)	NR
Rimantadine	H3N2 (human)	A30T (1)			Yes (41)	NR
Amantadine	H5N2 (avian)	A30T (1)		Yes (54)		Yes
Amantadine	H3N2 (human)[a]	A30T (1)			Yes (46)	No
Amantadine	H2N2 (human)	A30T (6)	Yes (51)			
Amantadine	H7N7 (avian)	A30T (7)	Yes (51)			
Amantadine	H3N2 (human)	A30T (1)			Yes (41)	
Amantadine	H7N7 (avian)	A30T (11)	Yes (53)			
Amantadine	H3N2 (human)	A30T (1)			Yes (67b)	
Amantadine	H3N2 (human)	A30T (3)		Yes (37)		
Amantadine	H7N7 (avian)	A30P (2)	Yes (51)			
Amantadine	H5N2 (avian)	A30S (1)		Yes (54)		Yes
Amantadine	H7N1 (avian)	A30S (1)	Yes (53)			
Rimantadine	H3N2 (human)	S31N (10)	Yes (5)[a] (40)		Yes (5)[a] (40)	NR
Rimantadine	H3N2 (human)	S31N (14)			Yes (41)	Yes
Amantadine	H3N2 (human)	S31N (1)			Yes (43)	NR
Amantadine	H3N2 (human)	S31N (2)			Yes (42)	NR
Amantadine	H5N2 (avian)	S31N (1)		Yes (54)		Yes
Amantadine	H2N2 (human)	S31N (8)	Yes (51)			
Amantadine	H7N1 (avian)	S31N (4)	Yes (51)			
Rimantadine	H3N2 (human)	S31N (5)			Yes (6)	Yes
Amantadine	H7N1 (avian)	S31N (5)	Yes (53)			
Amantadine	H3N2 (human)	S31N (5)			Yes (39)	Possible
Amantadine	H3N2 (human)	S31N (2)			Yes (67b)	
Amantadine	H3N2 (human)	S31N (4)			Yes (44b)	
Rimantadine	H3N2 (human)	S31N (6)			Yes (45)	Yes
Amantadine	H3N2 (human)	S31N (16)	Yes (9)[a] (46)		Yes (7[a]) (46)	Yes
Amantadine	H3N2 (human)	S31N (5)		Yes (37)		
Amantadine	H3N2 (human)	S31I (1)		Yes (37)		
Amantadine	H7N7 (avian)	G34E (29)	Yes (51)			
Amantadine	H7N1 (avian)	G34E (4)	Yes (53)			

[a] = number of isolates, NR - not recorded. [b] = immunocompromised

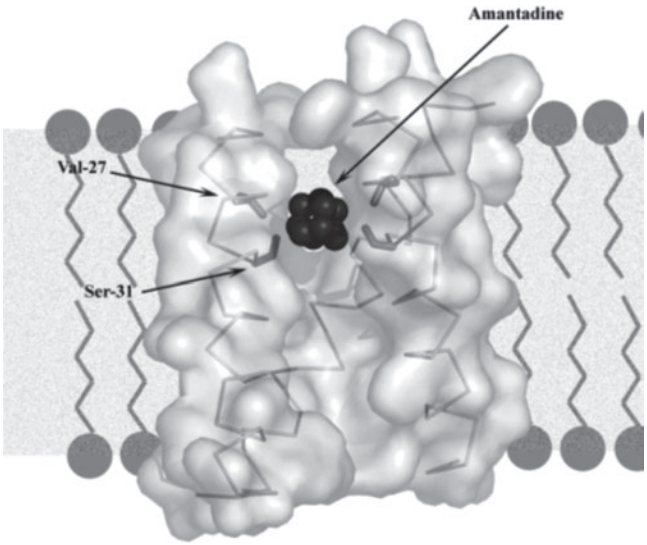

Fig. 3 Cross-section of the tetrameric A/M2 ion (proton) channel within the viral lipid membrane showing binding of amantadine. The hydrophobic adamantane moiety is associated with the pore-lining residues of the amantadine-sensitive proton channel. The two most common amino acid residues associated with amantadine resistance, S31 and V27, are highlighted. Kindly prepared and provided by Dr Alan Hay, MRC National Institute for Medical Research, Mill Hill, London, UK

larger amino acid either S31N or A30T blocked binding of amantadine possibly by reductions in pore size (S31N), or changes in chemical nature (A30T), i.e. steric hindrance or chemical incompatibility due to changes in hydrophobicity. Mutations that introduce a smaller amino acid either V27G/S/T/A retain amantadine binding but the pore is larger in size so that the drug does not block the pore. Based on these studies amantadine appears to be located near residues 30 and 31, whereas the water molecule file required for the H^+ wire is located in the vicinity of residue 27 (57). Mutants that lose binding of amantadine due to reductions in pore size also have reduced proton transfer, whereas mutations that increase pore size have increased proton transfer.

2.3 Cross-Resistance

Amantadine and rimantadine are structurally very similar (see Fig. 2) and it is assumed that binding within the M2 ion-channel pore would be identical for both molecules. Data from cross-resistance analysis would confirm this because selection of resistant variants and the total resistance observed for mutations at each of the five different residues selected appear the same. Cross-resistance has been reported for other related molecules such as cyclo-octylamine (58), cyclononane (59) and BL-1743 (60) (see Sect. 2.5).

2.4 Mechanism of Spread of Resistance

The majority of studies on emergence of resistance to amantadine or rimantadine whether in vitro or in vivo, including clinical studies, demonstrate the relative ease with which resistance can develop to the M2 ion-channel blockers. In vitro or in vivo resistant isolates are stable during replication in the absence of inhibitor. This fits with the data obtained with functional studies on amantadine-resistant M2 ion channels. Selective proton ion-channel activity is retained, although mutations may result in some impairment or enhancement of activity, but sufficient activity must be retained to result in acidification of the interior of the virus and in uncoating of the RNP complexes. Comparison of amantadine-resistant and parent virus from studies with avian viruses showed no differences in replication capacity, on transmissibility or in pathogenicity (54, 61). Similarly, for human influenza viruses no differences were observed for amantadine or rimantadine selected drug-resistant influenza A viruses compared with the drug-sensitive progenitor strains in replication in cell culture, or in ovo (36, 40). When isolates from a rimantadine clinical study, containing single mutations at either V27A, A30V or S31N, were compared with parent virus in the ferret model, no differences in replicative capacity or virulence were observed between matched isolates, although the parent isolates differed in pathogenicity (62). Clinical studies in paediatrics and in family studies or nursing homes have shown that resistant isolates appear to have the same ability to cause illness and may be readily transmitted (6, 41, 43, 45, 63, 64). There is no evidence either that human infections caused by resistant viruses are any more severe. Overall the data show that mutations have no apparent deleterious effect on the virus, but also do not confer any advantages on the virus in the absence of drug.

Prevalence of the different mutants selected during prophylaxis and therapy does appear to vary (see Table 1). The S31N is the most frequent mutant isolated from the clinical setting and has been shown to be transmitted the most during therapy (6, 41, 45). Similarly, surveillance studies, although reporting that isolation of amantadine/rimantadine resistance was rare, the mutation most frequently detected was the S31N virus (65, 66). Analysis of H1N1 virus isolates from the 1930s also detected S31N mutants (35), and the Avian H5N1 strains circulating in South East Asia in 2004/2005 have the S31N mutation. Switches from one resistance mutation to another have also been found suggesting that the S31N may have some advantage over other mutants (67, 45). In vitro studies with human H2N2 virus also showed increased frequency of selection of the S31N mutant over the V27A and A30T (51). In contrast, with avian strains other mutations may be dominant including I27S or G34E suggesting there may be some variation with sub-type (see Table 1).

From clinical studies of H3N2 influenza, amantadine- and rimantidine-resistant isolates have been observed in up to 30% of patients including children and adults (68), and more recently in up to 80% of patients (69). Children have been shown to secrete resistant virus for longer periods of time than wild-type although this did not appear to affect resolution of symptoms but would potentially increase the risk of spread (63). This high potential for resistance development and transmission of resistant virus has led to discussion of how best to use these M2 inhibitors to limit development of resistance. Interestingly, early clinical studies in families with amantadine indicated that prophylaxis of contact cases was 100% protective whereas in a later study treatment of both index and contact cases lead to only 20% protection (70, 71). A later study with H1N1 influenza showed 69% protection with prophylaxis without treatment of index cases (72). Although no clinical studies have been run comparing these different treatment strategies directly, the clinical data indicate that the increased problem of resistance development when both index and contact cases are treated seriously compromises efficacy of the M2 inhibitors (73). Studies with the newer neuraminidase inhibitors indicate this may not be an issue with inhibitors with lower thresholds of resistance development (74, 75). Despite the high potential for resistance development with amantadine/rimantadine, surveillance studies had not reported high levels of circulating resistant virus from year to year. This may have been due to relatively low rates of use of these inhibitors in the community. However, it was suggested that this may be linked with the seasonal epidemiology of influenza where new strains under antigenic pressure tend to arise each year (6, 76). Since 2003 the number of resistant isolates has increased substantially initially in China (77) and the Far East where it is believed there was increased use of amantadine for respiratory conditions as a result of increased awareness of influenza due to the Avian H5N1 outbreaks. Similarly in other parts of the world with increased use of these inhibitors high levels of resistance have been observed circulating worldwide (49). In the USA the high levels of resistance observed has prompted the CDC to issue warnings not to use the M2 inhibitors for treatment of influenza infections (78, 79).

2.5 Alternative Agents

Over the years since the discovery of amantadine there has been tremendous effort and resource put into the synthesis and evaluation of diverse chemical series, many with cage-like structures which resemble amantadine (5, 80–84). Several active series have been identified but only three compounds have progressed into clinical trials. One of the best of these was spiroadamantadine (1′-methyl-spiro (adamantine-2,3′-pyrrolidine) maleate) which in clinical studies at 70 and 120 mg/day had only modest prophylactic (85) and therapeutic efficacy (86) and was not developed further. Similarly a cyclononane was developed at ICI in the UK and after initial promise at 100 or 200 mg/day in experimental prophylaxis studies showed minimal efficacy in experimental therapeutic studies (87). Finally, cyclo-octylamine hydrochloride, a cyclic amine which was administered intranasally as a 0.4% solution every 2 h had only marginal efficacy (88). Despite all this effort amantadine and rimantadine still remain the only two M2 ion-channel inhibitors available in the clinic.

The more detailed structural studies of amantadine binding to the M2 ion channel have renewed the interest in developing further novel M2 ion-channel inhibitors. More recently an inhibitor developed using an M2 expression yeast system (89) was shown to have specific M2 ion-channel inhibitory activity in Xenopus oocytes (60). The inhibitor BL-1743[2-(3-azaspiro (5,5)undecanol]-2-imidazoline] is a reversible inhibitor of the M2 ion-channel activity in the M2 oocyte model, compared to amantadine which is an irreversible inhibitor in the assay system used. This molecule must have similarities in binding the M2 channel to amantadine since all the amantadine-resistant isolates were also resistant to BL-1743. However, one mutant selected by BL-1743 had >70-fold resistance to BL-1743 and only tenfold resistance to amantadine indicating some differences in binding in the ion channel. Although BL-1743 was not developed these studies do show that new approaches may yield M2 ion-channel inhibitors with different characteristics and possibly increased potency over amantadine/rimantadine.

Further discovery and development of inhibitors to block the M2 ion channel would greatly benefit from improved assay design to measure ion-channel activity with higher throughput. Detailed structural information is also required to help design new inhibitors to this proven anti-influenza target. Suggestions made to exploit other areas of the pore include targeting the key amino acid residues H37 and W41 involved with ion-channel activity which appear conserved in an HXXXW motif in both influenza A and influenza B ion channels (90). There may be the potential to design inhibitors to this target in the future which are active against both influenza A and B and to amantadine-resistant isolates and where there may be more constraints on resistance development.

3 Neuraminidase Inhibitors: Zanamivir and Oseltamivir

Zanamivir [4-guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetyneuraminic acid, or 4-guanidino-Neu5Ac2en, Relenza™] (see Fig. 4) was first described in 1993 as a potent and selective influenza A and B inhibitor designed to inhibit the influenza virus neuraminidase enzyme, and which inhibited virus

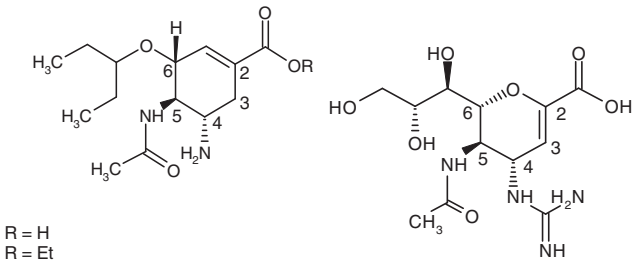

Fig. 4 Chemical structure of the neuraminidase inhibitors: zanamivir and oseltamivir, R = H parent active molecule, R = Et pro-drug converted by liver esterases to active parent

replication in both cell culture and in animal models (91). After clinical evaluation, zanamivir, given by oral inhalation, was approved for therapeutic use against influenza A and B infections in the USA and in Europe in 1999, for prophylaxis use in 2006, and for both prophylactic and therapeutic use in many countries from 1999 to 2007. In addition, an IV formulation of zanamivir, at 600 mg BID, was evaluated in experimental infection in humans and was shown to be highly efficacious in preventing infection with A/Texas/36/91(H1N1)(92).

A further series of carbocylic sialic (or *N*-acetylneuraminic) acid analogues were reported in 1997 and an ethyl ester derivative, oseltamivir phosphate [(3*R*, 4*R*, 5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid, GS4104, Ro 64-0796, Tamiflu™] (see Fig. 4) was developed as an orally available prodrug which is converted by liver esterases to the active form oseltamivir carboxylate [GS4071, Ro 64-0802] which is a potent inhibitor of influenza A and B viruses. Like zanamivir, oseltamivir was active in cell culture and the prodrug active in vivo in different animal models (93–95). Oseltamivir was approved for prophylactic and therapeutic use against influenza A and B infections in the USA, Europe and in many other countries in 1999/2000. Both oseltamivir and zanamivir have been shown to be active, in vitro and in the murine model, against avian strains of virus isolated from the Far East, including the H5N1 strain, A/HongKong/156/97, which caused lethal infections in humans (96–98). Further evaluation of oseltamivir against a highly pathogenic A/Vietnam/1203/04 (H5N1) strain in mice showed that more prolonged dosing (8 days) and higher doses of oseltamivir produced a more beneficial antiviral effect (99). Similar studies in the ferret model examined post-exposure prophylaxis and treatment started 4 or 24 h after infection, using low dose 10–10^2 EID_{50} of either highly pathogenic A/Vietnam/1203/04 (H5N1) or low pathogenic A/Turkey/ 15/06 (H5N1). Again higher doses of oseltamivir were required to protect the ferrets from lethal infection with the more pathogenic virus and if treatment was delayed for 24 h with the low pathogenic strain (100).

3.1 Antimicrobial Mechanisms of Action

3.1.1 Function of the Neuraminidase in Viral Replication

The neuraminidase of influenza A and B functions by cleaving terminal sialic acid residues from glycoproteins, glycolipids and oligosaccharides. Specifically, the viral enzyme catalyses the hydrolysis of α-(2, 3) or α-(2, 6) glycosidic linkages between the terminal sialic acid and the adjacent carbohydrate moiety. It has also been called receptor-destroying enzyme because it cleaves the sialic acid residues that are bound to the viral receptor HA, which is a sialic acid specific receptor, and thus causes release of virus from the cell surface. The functions of the NA and HA appeared to be in opposition, and from studies with neuraminidase inhibitors it has become clear that there is a fine balance between the affinity of binding of the HA to the sialic acid-containing cell-receptor and entry into the cell and release of virus from the cell surface through receptor cleavage by the NA.

The function of the NA is, therefore, in the release of virus particles from the cell surface, in preventing aggregation of virus particles which occur through the virus/virus HA sialic acid interactions after budding from the cell, and in vivo, in aiding spread of virus through the mucus layer in the respiratory tract by removing sialic acid from mucin. Blocking of neuraminidase function using neuraminidase inhibitors in vitro has shown aggregation of virus particles at the cell surface (101). In cell culture neuraminidase function is not essential for replication of virus (102), however, in vivo the function appears critical for spread of virus within the respiratory tract and to aid release and transmission of virus to the next host.

3.1.2 Structure of the Neuraminidase and Enzyme Active Site

The development of the clinically active neuraminidase inhibitors, which occurred roughly 30 years later than the M2 inhibitors amantadine and rimantadine, benefited from all the advances that had been made in molecular virology over this time. However, screening to look for neuraminidase inhibitors was also initiated in the 1960s (103) and later the transition-state analogue Neu5Ac2en (DANA) and the more potent trifluoro-acetyl analogue (FANA) were shown to be potent inhibitors of neuraminidase in vitro but not in vivo (104, 105). In this 30-year period extensive studies were undertaken to try to understand the structure and function of the surface glycoproteins of influenza, the haemagglutinin and the neuraminidase, and their high antigenic variability.

Large quantities of proteins were produced for structural studies and in 1981 the crystallographic structure of the H3 haemagglutinin was published (106), and 2 years later in1983 the structure of the N2 neuraminidase was presented (107) together with a detailed description of the invariant catalytic pocket (108). Later, further NA structures followed with the avian N9 in 1987 (109) and influenza B in 1992 (110). Comparison of these three NA structures further supported the high conservation of the NA active site and the suitability of this as a target for drug design. In 2006 further crystal structures of avian N1, N4 and N8 were solved by molecular replacement (111). Comparison of these structures further confirmed overall similarities of the active site but also revealed significant differences in the form of a cavity close to the active site in these three NAs. Based on phylogenetic analysis the neuraminidases of influenza A have been divided into two groups, Group 1 contains N1, N4, N5 and N8 and Group 2 N2, N3, N6, N7 and N9 (111).

Influenza virus neuraminidase is a tetrameric glycoprotein with a total molecular weight of 240 kDa. It characteristically has a mushroom shaped morphology with a hydrophobic stalk peptide which anchors the molecule in the cell membrane, and a globular head in which each monomer contains a deep pocket, the conserved enzyme active site, surrounded by the highly variable antigenic sites. Each enzyme monomer consists of six β-sheets of four anti-parallel strands arranged like the blades of a propeller (107). Accumulation of sequence data from different neuraminidase sub-types plus studies on binding of inhibitors to the enzyme identified 24 key active site residues which have been numbered here based on the original N2 structure (107, 108, 112). The 24 conserved residues identified contain a large number of potentially charged amino acids including six basic arginine residues, R118, R152, R156, R224, R292, R371, and a basic histidine H274 and asparagine N294, six glutamic acid residues E119, E226, E227, E276, E277, E425 and five aspartic acids D151, D198 (N198 in N7, N9 sub-types), D243, D293, D330, plus four hydrophobic residues, tryptophane W178, tyrosine Y406, leucine L134 and isoleucine I222, plus a hydrophilic residue, serine S179.

3.1.3 Binding of Substrate and Inhibitors to the Active Site

The detailed structural data on the Group 2 (N2 and N9) neuraminidase enzyme, particularly the highly conserved active site, allowed the development of rational drug design based on the understanding of the structure of the target molecule and its interaction with the substrate, sialic acid. Sialic acid (Neu5Ac) is bound within this pocket in the α-anomer in a half-chair configuration. The carboxylate moiety of the sugar lies between R118, R292 and R371 residues and the glycerol side-chain is H-bonded to G276, the 4-hydroxy interacts with E119, and the glycosidic oxygen interacts with the carboxylate oxygen of R151. The 5N-acetyl side chain is in a hydrophobic pocket formed by W178 and L134, with the N–H group interacting with a bound water molecule in the floor of the active site, and the oxygen is hydrogen bonded with R152.

Using computer modelling and based on this detailed understanding of the molecular binding of sialic acid to the neuraminidase enzyme, two potent inhibitors, were designed (91). These inhibitors were substrate analogues with modifications at the 4-hydroxy group to produce, 4-amino Neu5Ac2en and 4-guanidino-Neu5Ac2en (zanamivir). These substitutions were sufficient to increase the dynamics of binding and stability of the molecules to result in inhibition of virus in both cell culture and intranasally in vivo in the mouse and ferrets models, which was a major advance in developing neuraminidase inhibitors to be active in the clinic. Substitution of the 4-hydroxyl group by an amino group produced a significant increase in the overall binding interaction due to a salt bridge formation with the side chain carboxylic acid group of E119. The replacement of the 4-hydroxyl group with the more basic guanidino group produced a tighter affinity due to lateral binding through the terminal nitrogens of the guanidino group with E119. Binding of zanamivir to the NA of different influenza sub-types including A (Group 1 and 2) and B strains is considered to be similar (91, 111, 113). In neuraminidase enzyme assays both inhibitors were potent competitive inhibitors with inhibition constants of 5×10^{-8} M for the 4-amino derivative and 2×10^{-10} M for the 4-guanidino derivative (91). Further enzyme kinetics studies showed the 4-guanidino-derivative, zanamivir to be a slow-binding inhibitor of both influenza A (A/Aichi/ 2/68(H3N2)) and B (B/Beijing/1/87) viruses, with association and dissociation constant almost identical for both enzymes (114). This slow binding is suggested to occur due to slow release of a tightly bound water molecule by the guanidinium group (115). Further studies showed zanamivir to possess similar potency to all nine NA sub-types including different human sub-types (116) and against different sub-types of avian origin (117, 118). Later surveillance studies conducted by the Neuraminidase Inhibitor Susceptibility Network (NISN) against large numbers of virus isolates have recorded mean enzyme susceptibility for zanamvivir against the H3N2 isolates of 2.17 nM ($n=664$), for H1N1 isolates of 0.61 nM ($n=139$) and for influenza B isolates of 2.57 nM ($n=148$) (119). Activity against the neuraminidase of avian strains isolated in 1997 and 2005 with the potential to infect humans have been recorded. The IC_{50} values for these H5N1 viruses were 0.5–5 nM (96, 97, 120), and for H9N2 isolates

7–10 nM (97). This broad-spectrum activity is believed to derive from the structural design of the inhibitor to bind to only highly conserved residues within the active site.

The rational chemistry/drug design approach lead to the development of a second series of potent inhibitors of the neuraminidase enzyme, the carbocylic sialic acid inhibitors which culminated in the development of the orally active drug, oseltamivir (121). Oseltamivir differs from zanamivir in having a cyclohexene ring structure, a hydrophobic substitution replaced the glycerol moiety at the 6-position, and the 4-guanidino group was replaced by a 4-amino group. When binding to the active site, due to the presence of the hydrophobic substitution at the 6-position, oseltamivir causes a small conformational change in the active site of the NA to accommodate binding of the inhibitor. The residue E276 forms a salt link with R224 (see Fig. 5) and this conformational change results in the formation of a hydrophobic pocket for the substituent at the 6-position (122). From enzyme kinetic studies oseltamivir is also reported to be a slow binder of influenza A (A/Tokyo/3/67(H3N2)) and B (B/Memphis/3/89) viruses due to slow rate of dissociation of the compound from the neuraminidase (123). From surveillance studies by NISN, the mean IC_{50} values for oseltamivir against H3N2 isolates was 0.62 nM ($n=767$), for H1N1 isolates 0.92 nM ($n=139$), and for influenza B was 5.21 nM ($n=148$) (119). Activity against the neuraminidase of avian strains isolated from 1997 to 2005, with a potential to infect humans, have been recorded. The IC_{50} values for these H5N1 viruses were 0.08–7.0 nM (98, 120, 124), and for H9N2 10–15 nM (98). Again broad spectrum activity was observed which is characteristic of the NAIs.

3.2 Mechanism of Drug Resistance

3.2.1 Development of Resistance to the NAIs

One rational for developing the neuraminidase inhibitors which bind only to highly conserved residue in the active site of the NA was that there would be major constraints on the development of resistance to these drugs (91, 107). Following on from the discovery of zanamivir, the first NA active in vivo, there was a flurry of activity to look at the potential for resistance development in cell culture with zanamivir. Overall it was found that passage of virus in increasing levels of zanamivir did result in reductions in susceptibility of the virus to zanamivir and drug resistance mutations were characterized (125–134). Fewer cell culture passage studies have been undertaken with oseltamivir, but the results also showed that it was possible to generate resistant variants to oseltamivir (135). However, unlike amantadine and rimantadine, it was found that many passages in cell culture at relatively high drug concentrations were required before resistant variants to the NAIs were selected. Only one study has been undertaken in vivo, in the ferret model to look at development of resistance to zanamivir in comparison with amantadine. Whereas resistance to amantadine developed rapidly within 6 days, similar to that observed in the clinic, no resistance to zanamivir was detected after two passages over 18 days' treatment (37). No comparative passage studies using in vivo models appear to have been done with oseltamivir and amantadine, although prophylaxis and treatment in the mouse model did not give rise to resistance to either drug (136). A later study in an immunocompromised murine

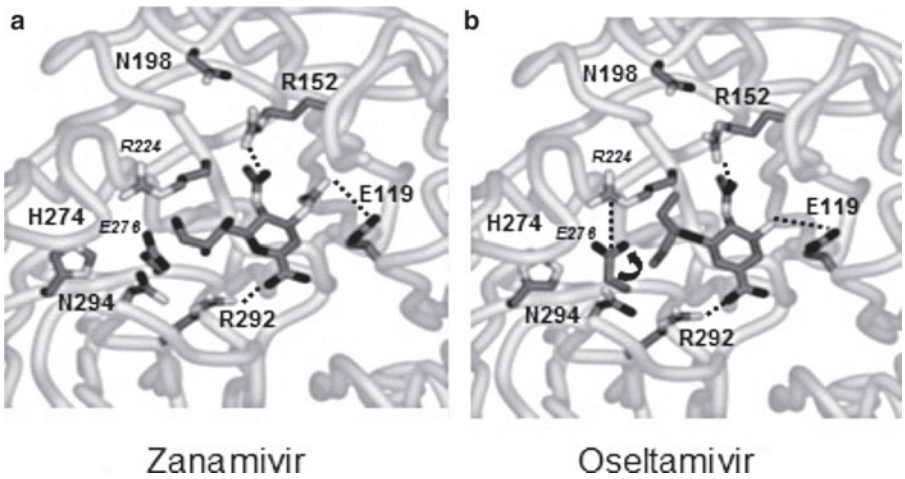

Fig. 5 (**a**) Binding of zanamivir within the active site of N9 NA without change in shape of the active site. (**b**) Binding of oseltamivir within the active site of N9 NA showing a conformational shift of the E276 residue to form a salt bridge with R224 to make a pocket to accommodate binding of oseltamivir. Some oseltamivir-resistance mutations (R292K, H274Y, N294S) prevent this conformational change blocking binding of oseltamivir but not zanamivir nor the natural sialic acid receptor. Residues associated with NAI resistance development E119, R152, N198, H274, R292, N294 are highlighted. Kindly prepared and provided by Dr Jennifer McKimm-Breschkin and Dr Mike Lawrence, CSIRO, Melbourne Australia

model infected with A/Japan/305/57(H2N2), oseltamivir was compared with another neuraminidase inhibitor A-32278. Resistance development was monitored by clonal analysis and one oseltamivir resistant virus but no resistance to A-32278 was detected (137).

Extensive surveillance studies on influenza viruses circulating worldwide have been undertaken by the NISN to look at NAI susceptibility using the NA enzyme assay. No naturally occurring resistant isolates were observed in more than 1,000 isolates circulating between 1996 and 1999 before the introduction of the NA inhibitors into the clinic (119). Similar studies have also been conducted by the CDC and WHO Australia, and no natural resistance detected at this time (138, 139). These data are consistent with these drugs targeting the highly conserved active site of the NA enzyme. Monitoring for resistance development during treatment and prophylaxis studies has been undertaken for the clinical development of both NAIs, using the NA enzyme assay and sequencing of the NA and HA genes. No emergence of zanamivir-resistant mutants has been detected during treatment of more than 5,000 immunocompetent patients with zanamivir (140). For immunocompetent patients treated with oseltamivir, resistance has been detected in viruses isolated from 1 to 2% of adults (140, 141), and from 5 to 6% of children (142), and more recently in Japan from 16 to 18% of children infected with H1N1 and H3N2 virus respectively (69, 143).

The potential for development of resistance in immunocompromised patients is expected to be higher than for immunocompetent subjects because of the higher levels of virus produced and the prolonged virus replication times. Although few immunocompromised subjects have been included in studies after treatment with NAIs, resistance has been observed to develop in at least six subjects, one (influenza B infection) after treatment with zanamivir, five (four A and one B infections) after treatment with oseltamivir (140, 144–147). Two of these subjects (both A infections) were later treated with zanamivir and only wild-type virus was isolated (141, 146, 147).

NISN has undertaken further worldwide monitoring of NAI susceptibility of 2,287 isolates of influenza A and B, circulating during the first 3 years (1999–2002) of NAI use (119, 148). These studies revealed eight isolates (two B, six A) with reduced susceptibility (>10-fold shift) to oseltamivir of which two (one A and one B) also had reduced susceptibility to zanamivir. Drug use at this time was relatively stable except for a tenfold increase in the use of oseltamivir in Japan in 2002. It was concluded that the frequency of isolation of variants did not increase significantly over this time (1, 0.22% in 1999/2000; 3, 0.46% in 2000/2001; and 4, 0.41% in 2001/2002). Later local surveillance studies within Japan have shown marked reduced susceptibility of influenza B isolates circulating within the community to oseltamivir (median IC_{50} values 55.8–85.1 nM) and slight reductions to zanamivir (median IC_{50} values 7–15.8 nM) (149). In addition, six pretreatment isolates had high level resistance to oseltamivir with IC_{50} values from 204.2 to 513.8 nM, and four of these with moderate reductions in susceptibility to zanamivir (IC50 values 29.5–61.7) and one other B isolate with an IC_{50} of 191.3 nM to zanamivir (149). This data has led to concerns that increased clinical treatment with NAIs in Japan, possibly may have selected for natural influenza B variants with reduced NAI susceptibility (150) but the magnitude of these changes may partly relate to differences within NA assays methods used (151). In the 2007/2008 season, the WHO/ECDC and CDC reported that worldwide drug susceptibility monitoring had identified widespread transmission of oseltamivir resistance in H1N1 viruses, which was not associated directly with drug treatment.

Susceptibility monitoring of highly pathogenic A(H5N1) avian influenza viruses circulating in poultry in various regions of South East Asia between 2004 and 2006 showed most strains to be highly sensitive to both zanamivir and oseltamivir. However, two isolates had reduced susceptibility to oseltamivir and one of these also showed a significant reduction in susceptibility to zanamivir (152). A second study of H5N1 isolates in poultry in the Far East revealed that while all isolates were sensitive to both oseltamivir and zanamivir there was up to 30-fold reduction in susceptibility to oseltamivir between some of the clade 1 isolates and the clade two isolates from Indonesia (153).

3.2.2 Genetic Analysis of Resistance to the NAIs

HA Variants (Mutations Based on H3 Sub-type Numbering)

Analysis of virus variants from some of the earliest passage studies with zanamivir in cell culture revealed mutations in the HA gene only, generally in residues close to those involved with receptor binding (see Table 2). However, the structure of the sialic acid binding site for HA is very different from that for NA and these inhibitors based on the design strategy should not bind to the HA site. The mechanism of drug resistance was considered to be due to decreases in affinity of binding of HA to the cell receptor such that the virus was less dependent on neuraminidase function for virus release. Mutations in the HA which resulted in weaker binding of the virus to the cell appeared not to compromise virus replication in vitro, but it was not known if this was relevant to the in vivo situation where neuraminidase function is essential. However, when one of these HA variants was used to infect ferrets and treated with zanamivir the virus was fully susceptible to zanamivir (133). Similar variants may occur naturally in the clinic, and although showing resistance in cell-based assays, are not resistant in the ferret model (154). This difference in susceptibility between in vitro and in vivo assays may reflect differences in receptor usage between cell culture (primarily α-(2–3)

Table 2 NA and HA mutations observed from in vitro, in vivo and clinical studies with NAIs

Inhibitor	Virus sub-type	NA mutations N2 numbering	HA mutations[b] H3 numbering	In vitro	In clinic
Zanamivir	A/H1N9 (human/avian recombinant)	N346S[a]	T155A	Yes (126, 127)	
		None	R229S		
		G90Q[a]	V223I/R229I		
Zanamivir	A/H1N9 (human/avian recombinant)	None	S165N,S186F	Yes (128, 129)	
		None	S186N,K222T		
Zanamivir	A/H2N2 (human)	None	E135A	Yes (133)	
		None	R137Q		
		None	A138T		
Zanamivir	B/(human)	None	L226Q,V93A	Yes (134)	
Zanamivir	A/H4N2	R249K[a]	G75E	Yes (130–132)	
		None	Y234L/A35T/K68R		
Oseltamivir	A/H3N2 (human)	None	A28T/R124M	Yes (135)	
Oseltamivir	B/ (human)	None	H103Q	Yes (216)	
Peramivir	A/H3N2 (human)	None	K189G	Yes (217)	
Zanamivir	A/H1N9 (human/avian recombinant)	E119G	None	Yes (128, 129)	
		E119G	S186F		
Zanamivir	A/H1N9 (human/avian recombinant)	E119G	None	Yes (125)	
Zanamivir	B/ (human)	E119G	N145S	Yes (125)	
		E119G	N150S		
Zanamivir	A/H4N2 (avian)	E119G	None	Yes (130–132)	
Zanamivir	A/H4N2 (avian)	E119A/R249K[a]	G75E	Yes (130–132)	
Zanamivir	A/H4N2 (avian)	E119D	None	Yes (130–132)	
Zanamivir	B/(human)	E119G	L226Q, V93A	Yes (134)	
Oseltamivir	A/H3N2 (human)	E119V(1)	None		Yes (161)
Oseltamivir	A/H3N2 (human)	E119V(3)	None		Yes, (141)
A-315675	A/H1N9 (human/avian recombinant)	E119D	None	Yes (162)	
		E119D	R233K,S339P		
Oseltamivir	A/H1/N9 (human/avian recombinant)	E119V/R305Q	H154Q	Yes (161)	
		E119V/R292K	None		
Oseltamivir	A/not defined (human)	E119V	None		Yes (142)
Zanamivir	B/(human)	E119A	Q218K	Yes (216)	
Oseltamivir	A/H3N2 (human)	E119V (2)	None		Yes (69)
Oseltamivir	A/H3N2 (human)^	E119V	A142G,Y195F,I239R		Yes (146)
Oseltamivir	A/H3N2 (human)[c]	E119V	V226I		Yes (146)
Oseltamivir	A/H3N2 (human)[c]	E119V,I222V			Yes (147)
Oseltamivir	A/H3N2 (human)	E119V			Yes (151)
Zanamivir	B (human)[c]	R152K (1)	T198I		Yes (144)
Oseltamivir	A (H1N1)	H274Y	None	Yes (165)	
Oseltamivir	A (H1N1)	H274Y (2)	None		Yes (164)
Oseltamivir	A (human) not defined	H274Y (1)	None		Yes (142)
Oseltamivir	A/H1N1 (human)	H274Y (1)	None		Yes (141)
Oseltamivir	AH1N1) (human)[c]	H274Y (7)			Yes (145)
Oseltamivir	A/H1N1 (human)	H274Y (7)			Yes (143)
Oseltamivir	A/H5N1 (avian)	H274Y (2)			Yes (166)
Oseltamivir	A/H5N1 (avian)	H274Y (1)			Yes (120)
Peramivir	B	H274Y		Yes (189)	
Peramivir	A/H1N1 (human recombinant)	H274Y	None	Yes (218)	
Oseltamivir	A/H1N1 (human)	H274Y			Yes (151)
6-Carboximide derivative of zanamivir	A/H1N9 (human/avian recombinant)	R292K	N199S	Yes (155)	
Zanamivir	A/H4N2 (avian)	R292K	Y234L/T267K/D304N/K68R	Yes (130–132)	
Oseltamivir	A/H3N2 (human)	R292K	A28T, R124M,	Yes (135)	
Oseltamivir	A/H3N2 (human)	R292K (8)	None		Yes (142)
Peramivir	A/H2N2 (human)	R292K	G130D	Yes (196)	
Oseltamivir	A/H3N2 (human)	R292K (10)	None		Yes (141)
Oseltamivir	A/H3N2 (human)	R292K (6)	S262N (1)		Yes (69)
Oseltamivir	A/H3N2 (human)	R292K			Yes d(137)
Oseltamivir	A/H3/N2 (human)	R292K			Yes (151)
Oseltamivir	B/ (human)[c]	D198N (1)	None		Yes (140, 146)
Oseltamivir	A/H3N2 (human)	N294S (1)	None		Yes (69)
Oseltamivir	B/ (human)	G402S			Yes (149)
Zanamivir	A/H1N1 (human recombinant)	Deletion 92–362	A200T	Yes (218)	
Oseltamivir	A/H3N2 (human)	Deletion SASG245–248			Yes (151)

[a] NA mutations recorded outside the NA active site, probably due to natural variation

[b] HA mutations are included when reported but for some clinical studies the HA may not have been sequenced

[c] Immunocompromised patients

[d] in vivo murine model

receptors) and in vivo (primarily α-(2–6) receptors). Variations in HA binding probably explain the characteristic larger variation in susceptibility to NAIs seen with different influenza strains in cell culture assays (95, 116).

NA Variants and Effects of HA Mutations (NA Mutations Numbered Based on the N2 Sub-type Numbering)

Later studies in cell culture revealed that mutations could arise within the active site of the NA, although generally also accompanied by changes in HA. The mutations observed in the NA active site are selected presumably due to direct effects on drug binding. A number of NA mutations have been observed after cell passage in vitro and in the clinic, and have been listed in Table 2, together with any accompanying HA mutations. The HA mutations observed in further cell culture studies with either zanamivir or oseltamivir have been scattered on the HA molecule, but some do appear to reduce receptor binding in cell culture (155) thus reducing virus dependence on NA. Some HA and NA mutations appear to work synergistically increasing the levels of resistance detected in cell culture (129, 156).

From the clinical studies with oseltamivir, NA-active site mutations have been linked with treatment, but HA mutations, although observed are probably natural variants not associated with drug treatment and have not shown altered drug susceptibility (157). However, with zanamivir the one clinical isolate with zanamivir resistance had both an NA-active site mutation, and an HA mutation with altered cell-culture binding properties. The HA mutation in cell-based assays totally masked the change in susceptibility resulting from the presence of the NA mutation as observed in enzyme-based assays (144). The cell-based assay results with this resistant variant plus the data from selection of HA variants in cell culture confirmed that cell-based assays were unreliable in monitoring susceptibility to NAIs (158). To overcome this problem, the MDCK cell line has been modified to overexpress human α-2,6-sialyltransferase(SIAT1), such that these cells have twofold increased expression of α–(2,6) receptors and twofold-lower α–(2,3) receptors (160). This improved the consistency of susceptibility recorded for NA mutations between the cell-based and enzyme-based assays (160). There must be constraints on reductions in affinity of HA binding that still allow efficient entry of virus into cells in vivo. This means that HA mutations alone will probably not compromise NAI therapy in the clinic. However, it may be possible that some HA variants may predispose the virus to the development of NA resistance mutations. Currently, NISN recommends the use of NA enzyme assays for monitoring susceptibility to NAIs in the clinic together with sequencing of the NA gene.

NA Variants Selected During In Vitro Passage or During Treatment in the Clinic

A total of seven NA residues within the enzyme active site region have been linked with selection of NAI resistance to date and are listed in Table 2. However, some mutations have only been observed in vitro, and some only in vivo. Interestingly some differences in the mutations observed have been seen between the different NA sub-types, and with the two inhibitors used, which relate to differences in binding within the active site.

Four different mutations have been observed at residue E119, three in vitro with zanamivir (E119G/A/D) in influenza A (H1N9), A(H4N2) and influenza B strains (125, 127, 129, 130–132, 134) and one (E119V) with oseltamivir in influenza A (H3N2) isolates from the clinic (69, 141, 142, 161) and in vitro (162). Despite the frequent isolation of E119 mutants by zanamivir in vitro, no E119 variants have been selected by zanamivir in the clinic. Based on studies with H3N2 viruses generated by reverse genetics, it has been suggested that these viruses may not readily arise in vivo due to their poor viability (163).

The most frequent mutation observed (see Table 2) in the clinic with oseltamivir treatment of influenza A(H3N2) viruses, is at the catalytic residue R292K (69, 141, 142, 157). This mutation was also selected by oseltamivir in vitro (135) and has also been selected in vitro by zanamivir in an avian influenza A strain (H4N2) (132).

A novel mutation within the NA active site was detected at residue H274Y in a volunteer study using experimental infection with influenza A/Texas/36/91(H1N1) to evaluate the efficacy of oseltamivir in humans (164). Subsequently, in vitro studies with an H1N1 strain were described where an H274Y variant was isolated suggesting that there were some differences structurally between the N1 and N2 enzymes which influenced selection of resistant variants to oseltamivir (165). This mutation has since been detected in H1N1 viruses isolated from two further subjects during clinical trials with oseltamivir in adults and children (141, 142), in 16% (7/43) children in an oseltamivir clinical study in Japan (143), and in H5N1 infections in the Far East (120, 166).

Limited studies have also been undertaken in immunocompromised subjects to evaluate the risk of development of resistance where virus clearance is more difficult and treatment courses are longer. The mutation R152K has been isolated once in an influenza B strain in an immunocompromised child after 12 days treatment with zanamivir (144) and similarly a mutation D198N in an influenza B isolate has been obtained from an immunocompromised subject treated with oseltamivir (140). For influenza A, resistant isolates have been detected in four immunocompromised patients treated with oseltamivir. Mutations included those already observed in

immunocompetent subjects, that is, the E119V (three patients) (140, 146, 147) and the H274Y (one patient) (140, 145). One patient with the E119V had a second mutation I222V (147) which is a highly conserved framework residue in all influenza A and B strains and has been observed previously from in vitro studies with oseltamivir together with the E119V or H274Y mutations (148). This I222V mutation enhanced the resistance observed with the E119V mutations. In addition, two patients treated with both oseltamivir and amantadine/rimantadine developed resistance to both inhibitors (M2 – S31N, plus NA H274Y or E119V) (140, 145, 147).

Finally, a new mutation was recorded in influenza A (H3N2) at residue N294S within the NA active site from one paediatric patient in Japan treated with oseltamivir (69). This mutation has since been observed in H5N1 virus isolated from one patient in Vietnam (120), and at least two patients in Egypt (151).

The degree of resistance that all these different mutations may confer against the selection drug, as evaluated in the enzyme assay, range from approximately 10- to >10,000-fold with the exception of the I222V which only produces low-level resistance (approximately twofold) to the selection drug. Susceptibility data will be discussed in detail in the following section on cross-resistance. Although susceptibility of some isolates, particularly in vitro isolates, have also been evaluated using cell culture assays, these data will be influenced by the presence of HA mutations, and cell receptor specificity, and therefore may be unreliable (158). Such data has been reviewed previously (155).

Variants Detected in Untreated Subjects During Surveillance Programmes

One important function of the NISN surveillance programme was to evaluate if resistance mutations selected during treatment may circulate within the community. Early studies did not detect any known NAI resistance mutations circulating (119) and during the first 3 years of NAI use only one H1N1 isolate contained an NA mutation previously found in clinical trials to be associated with oseltamivir resistance (H274Y) (148). However, later studies by NISN of annual surveillance of influenza viruses circulating in Japan reported further low level isolation of viruses with known oseltamivir resistant mutations, in 2003/2004, 1 × E119V (0.16%) and 2 × R292K (0.08%) in H3N2 viruses and in 2005/2006, 4 × H274Y (2.2%) in H1N1 viruses (159). This suggested that transmission of resistant virus may be possible. In other surveillance studies in Japan both the A198N and I222T oseltamivir resistance associated mutations were observed circulating in influenza B viruses in untreated subjects, with some reduced susceptibility to both oseltamivir and zanamivir (149). Surveillance in the 2007/2008 season revealed widespread circulation in man of H274Y variants in H1N1 virus in many countries (WHO/ECDC and CDC web-sites).

In addition to mutations observed during treatment with NAIs, some natural variants have been identified by the members of NISN surveillance programme that have given rise to some NAI resistance (148). Mutations observed include Y155H in A/Hokkaido/15/2002(H1N1) which is a natural variant with the Y155 conserved in all human N1 viruses, and H155 found in some swine and avian N1 viruses and some earlier N2 viruses which are susceptible to NAIs. Interestingly the A/Hokkaido variant gave resistance to both oseltamivir and zanamivir, indicating that the NA background in this isolate must be having secondary conformational effects on the active site which affect drug binding and influence susceptibility. One double natural variant, G248R/I266V, in an N1 background was also associated with resistance to both oseltamivir and zanamivir. Other mutations of unknown significance include E41G and Q226H in an N2 background which gave rise to low level oseltamivir resistance. In influenza B isolates, natural variants were observed at D198E and in I222T. Both of these residues, but with different amino acids, have previously been observed associated with development of oseltamivir resistance (140, 147, 148). In Australia, another natural variant N198E in an influenza B isolate was associated with low level resistance to oseltamivir and zanamivir (167). Finally, one isolate with a S250G mutation showed reduced susceptibility to zanamivir (149).

Susceptibility monitoring of highly pathogenic A(H5N1) avian influenza viruses circulating in poultry in various regions of South East Asia between 2002 and 2004 revealed two isolates with mutations associated with reduced susceptibility. Mutations identified included I117V plus I314V in a dual resistant virus and V116A in a variant with reduced susceptibility to oseltamivir. All three mutations are close to residues which are within the active site (152).

Other NA variants from in vitro studies

Finally, in some studies site-directed mutagenesis has been used to look at further mutations within the active site not seen by selection, either in vitro or in the clinic (168–170). Mutations at the E119 residue in the N9 enzyme background which were shown to produce reduced susceptibility to zanamivir but were not tested against oseltamivir included E119Q, E119T and E119L (168). Mutations at the H274 residue in the N1 enzyme background which produced reduced susceptibility to oseltamivir included the large substituent H274F, whereas some zanamivir resistance was observed with the mutants H274N, H274G, H274S, H274Q. In the third study mutations constructed in an N2 virus background (A/Wuhan/359/95(H3N2) and tested for susceptibility to zanamivir and oseltamivir included R118K, R371K, E227D, R224K, E276D, D151E. The R224K, E276D and R371K mutations conferred resistance to both zanamivir and oseltamivir, and the D151E mutation gave reduced

susceptibility to oseltamivir. However, based on genetic stability and replication efficiency data, they concluded that only the E276D variant might be viable in vivo (170).

3.2.3 Enzyme Functional Studies

Mutations observed in the vicinity of the neuraminidase active site may be divided into two types, those affecting the structural scaffold, the framework residues (E119, H274, N294, D198) and those affecting catalytic residues which interact directly with the substrate (R152, R292) (69, 119, 140, 146, 171). All the mutations studied have been reported to reduce enzyme activity and/or enzyme stability. Ideally, for accurate determinations of enzyme activity in comparison to wild-type, quantitation of native NA protein using conformational specific NA antibodies is important.

The framework mutation E119G affects stability of the enzyme but not the enzyme-specific activity (172), whereas E119A the most conservative mutation at residue 119 has a greater effect on reducing susceptibility and on specific activity of the enzyme (132). Kinetic analysis of zanamivir binding demonstrated that the E119G variant did not exhibit slow binding characteristic of that seen with the wild-type enzyme (128). All three 119 residue mutations in the avian (H4N2) virus have altered stability as determined using thermal and pH effects (132). In an H3/N2 virus plasmid rescue system the different 119 mutations were compared for enzyme activity, E119G had just 4.1% activity, E119A 5.2%, E119D 15% and E119V 45% NA activity compared to wild-type, allowing for different levels of native enzyme protein (163). This suggests that the 119V enzyme, the only 119 variant observed in the clinic has the greatest stability of all these 119 variants. This E119V variant has also been reported to have twofold greater binding affinity for the enzyme substrate than the E119 wild-type (161). Similarly, analysis of enzyme activity of the framework mutant H274Y in the N1 background (A/Texas/36/91) showed that this mutant had substrate affinity and enzymatic activity equivalent to wild-type (173). A later study using an in vitro derived mutant AWS/33(H274Y), enzymatic activity of the mutant (H274Y) was reported to have 30% activity of wild-type (169).

In contrast, the mutations in the catalytic residues (R152, R292) would be expected to have major effects on the enzyme activity with the natural substrate. The R292 residue is one of three arginines within the catalytic triad of the NA active site which are all highly conserved. Studies from in vitro-generated R292K mutants showed that all the variants in different N2 backgrounds had reduced specific activity from 2 to 44% activity, but high enzyme stability in contrast to the other variants described above (132, 135, 156). There was a reduction in affinity of the R292K enzyme for substrate of about fivefold and the pH optimum of the mutant enzyme had reduced to pH 5.0/pH 5.3 (132, 135). Further direct comparisons of the effect of the R292K mutation and the E119V mutation generated by reverse genetics in the same N2 virus background revealed that the R292K caused a greater reduction in enzyme activity and thermostability than the E119Vmutation (170).

Characterization of the NA enzyme activity of the R152K mutant virus isolated from an immunocompromised subject also showed substantial reductions in enzyme activity, 3–5% of parent virus (144).

3.2.4 Mutant Enzyme Structural Studies

Crystallographic analysis of the mutant G119 NA indicated that reduced binding of zanamivir was due partly to loss of stabilizing interactions between the guanidino moiety and the carboxylate at residue 119, and partly from alterations to the solvent structure of the active site (128). The carboxylate of wild-type E119 is involved with binding to the 4-guanidino group in zanamivir, but in the G119 mutant the carboxylate is replaced by a water molecule (128). Structural data on other 119 mutants has not been published. Although the E119 does not interact with the 4-amino-group of oseltamivir directly (91), increase in size of the amino acid substituents at the 119 residue, such as with 119V, may lead to increased displacement of oseltamivir resulting in greater resistance (155). Why the 119V retains susceptibility to zanamivir is not understood without structural data on this mutant.

Structural studies with the R292K mutant enzyme revealed that this mutation affects the binding of both substrate and NAI substrate analogues through the carboxylate group on the sugar (122). This correlates with reduced enzyme activity of the variant and would account for reduced binding to zanamivir. For oseltamivir this mutation has much greater effects because the lysine at 292 prevents the formation of a salt bridge with A224, and the more stable E276 residue does not move to form a hydrophobic pocket to accommodate the bulky pentyl ether group, resulting in a substantial decrease in the binding of oseltamivir. However, interactions with the 2-carboxylate, the 4-amino group and the 5-acetamido group of oseltamivir are retained.

Detailed structural analysis has not been published for either the R152K mutant or the H274Y mutant. The wild-type R152 has been shown to form a hydrogen bond to the acetamide of the substrate, sialic acid, bound in the active site (109), and would be expected to affect the binding of all NAI substrate analogues. The H274Y mutant in H1N1 viruses is reported to act similarly to the R292K in the H3N2 viruses in that it forms a salt bridge stabilising the E276 residue preventing re-orientation and formation of the hydrophobic pocket to accommodate the pentyl ether group (136). The D294E mutations in the N2 background also affects the formation of the salt bridge preventing the conformational change to allow oseltamivir binding (174).

3.3 Cross-Resistance

Zanamivir and oseltamivir target the same region, the active site of the NA molecule, and therefore it may be expected that both drugs would show high levels of cross-resistance. However, because the two molecules bind in different ways within the active site not all mutations show cross-resistance, or the levels of resistance observed are different. These differences could be important in the clinic for use of the two drugs if resistance should become a problem (174). Generally tenfold shifts in susceptibility from wild-type are classified as resistant, however since wild-type strains may vary in susceptibility, care must be taken when interpreting shifts in susceptibility which should relate to IC_{50} values and drug levels achieved in the clinic. To date neither in vitro shifts in susceptibility nor IC_{50} values have been related to clinical efficacy to produce meaningful guidelines on clinical cut-off levels. However, recent clinical efficacy data with oseltamivir against influenza A and B virus infections in Japanese adults and children has shown reduced efficacy and susceptibility of influenza B strains (175, 176). This was also linked to longer virus shedding in influenza B than influenza A patients treated with oseltamivir. In contrast, zanamivir has shown similar clinical efficacy against influenza A and B strains (177). Further comparison of the effectiveness of zanamivir and oseltamivir in the treatment of influenza A and B infections in Japan showed comparative efficacy of the two inhibitors against influenza A but increased efficacy of zanamivir over oseltamivir against influenza B. These efficacy findings may relate to slightly greater susceptibility of influenza B strains to zanamivir compared with oseltamivir and possibly to higher local respiratory levels of zanamivir (178, 179) (see Fig. 6). Use of higher doses of oseltamivir may be required to increase efficacy against influenza B (151).

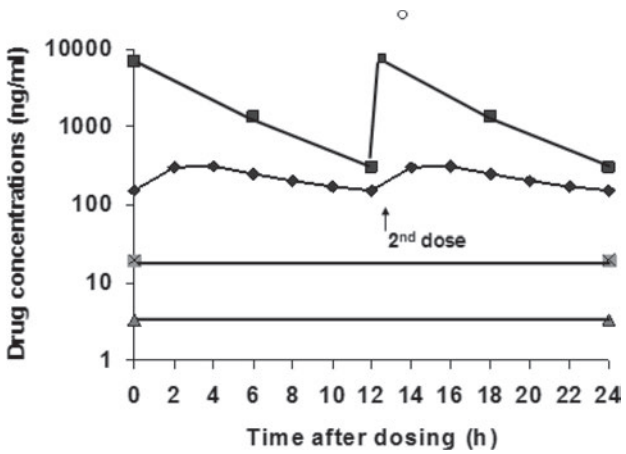

Fig. 6 Zanamivir (sputum) and oseltamivir carboxylate (plasma) q12h steady-state drug levels compared with median IC_{50} values for influenza B viruses isolated from Japanese subjects in 2004/2005 season. Information taken from publications by Hatakeyama S, Peng AW and He G (149, 178, 179). Zanamivir sputum drug levels (*filled square*); zanamivir median IC_{50} value (*filled triangle*); oseltamivir carboxylate plasma levels (*filled diamond*); oseltamivir carboxylate median IC_{50} value (*times symbol*)

3.3.1 Cross-Resistance Analysis with NA Variants Obtained from In Vitro Passage or Clinical Studies with NAIs

As stated earlier, the enzyme assay is the most suitable direct assay to study cross-resistance due to the NA mutations, since cell-based assays are influenced by receptor usage and changes in HA binding. Cross-resistance analysis has been undertaken with both in vitro derived and clinical isolates, with comparisons made using different assay conditions in the fluorescent NA assay (140, 180–182). Further comparisons of NAI cross-resistance have been carried out by the global NISN who have used the clinically derived NAI resistance isolates as controls in monitoring susceptibility of circulating influenza strains (183). Three NA assays have been compared in this study, two using the fluorescent substrate 2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUN or MUNANA), and one using a chemiluminescent substrate, 2′-(4 –NA Star)α-*D*-*N*-acetylneuraminic acid (NA-Star). Overall, the results obtained from all three assays were similar but the chemiluminescent assay was the more consistent. In Table 3, the published fold changes in NA susceptibility have been compared.

From this Table it may be seen that the most common NA mutant observed in the A/N2 sub-type in the clinic after treatment with oseltamivir, the 292K, shows high level resistance to oseltamivir >1,000- to 16,000-fold resistance, whereas cross-resistance to zanamivir is a relatively low 3.7- to 24.5-fold. The 274Y first seen in the A/N1 sub-type selected by oseltamivir in experimental infection and later in natural A/H1N1 infection in clinical trials and more recently in the highly pathogenic avian A/H5N1 infections in humans has lower resistance to oseltamivir than the 292K mutant with a shift of approximately 225- to 1,000-fold to oseltamivir but no apparent resistance to zanamivir. These differences in susceptibility for the two NAIs for these two mutants are consistent with the structural studies which indicate that both these mutations cause a block in the conformational change required for binding of oseltamivir. Zanamivir binds in a similar manor to natural substrate not requiring a conformational change.

The residue 119 group of mutations are interesting because differences in susceptibility are observed with the two inhibitors depending on which substituent is present. For the 119G selected in vitro by zanamivir but not seen in the clinic, high levels of resistance to zanamivir in the A/N2 sub-type are observed, from 40- to 333-fold shift in susceptibility but no significant or low level <10-fold resistance to oseltamivir (180–182), and in the B sub-type, 4,218- to 7,830-fold change to zanamivir and 35- to 119-fold change to oseltamivir (184). For the 119V in the A/N2 sub-type isolated from

Table 3 Cross-resistance analysis using NA enzyme assays, of neuraminidase variants obtained from in vitro passage studies and clinical studies with NAIs

NA mutations	Sub-type	Resistance to oseltamivir (fold-shift)[a]	Resistance to zanamivir (fold-shift)[a]	References
R292K	N2 avian	R (9,375)	I (8)	(180)
	N2 avian	R (>1,000–16,666)	I-R (8–12)	(181)
	N2 avian	R (15,000)	I (8)	(182)
	N2	R (>8,000–11,440)	I-R (3.7–24.5)	(183)
	N2	R (>1,600)	I (5)	(182)
R152K	B	R (187.5)	R (28.5)	(180)
		R (67–338)	R (20–94)	(184)
		R (>25–174)	R (30–3125)	(181)
		R (9.6–147.8)	R (12.5–36)	(183)
		R (100)	R (70)	(182)
H274Y	N1	R (1,000)	S (0.6)	(180)
		R (225 > 500)	S (1.1–1.3)	(181)
		R (353.5–634.8)	S (1.4–1.6)	(183)
		R (>700)	S (1)	(182)
	N1 avian	R (1,271–1,813)	S (1.4–3.4)	(120)
	B	I (2.4–6.6)	S (0.5–0.6)	(189)
	N1 recombinant	R (617)	S (1.3)	(218)
E119G	N2 avian	S (0.8)	R (40)	(180)
	N9	S (1–2)	R (249–984)	(184)
	B	R (35–119)	R (4,218–7,830)	(184)
	N2 avian	S (0.8)	R (40–333)	(181)
	N2 avian	S (2)	R (200)	(182)
E119A	N2 avian	S (2)	R (20)	(180)
		R (2.8–27)	R (20–417)	(181)
		I (9)	R (100)	(182)
E119D	N2 avian	S	R (60)	(180)
		R (1.3–9)	R (60–3,333)	(181)
		S (4.5)	R (323)	(182)
E119V	N2	R (52–335.4)	S	(183)
		R (130)	S (1)	(182)
		R (277)	S (3)	(182)
		R (276)	S (2.7)	(146)
D198N	B	I (9)	I (9)	(182)
		I (8.2)	R (10.7)	(146)
N294S	N2	R (300)	Not tested	(69)
	N1 (avian)	R (11.8–20.8)	S/I (3.2–6.2	(120)
G402S	B	R (3.9[b])	R (7)	(149)

[a] Fold shift = S < 5-fold difference from reference wild-type, I ≥ 5 < 10-fold difference from wild-type, R ≥ 10-fold difference from wild-type. In vitro fold-shifts have not been related to clinical efficacy

[b] Baseline virus high IC_{50}, resistant virus IC_{50} 281 nM, highly resistant – shift an underestimate

the clinic with use of oseltamivir, shifts in susceptibility for oseltamivir range from 50- to 335-fold, whereas zanamivir shows no shift in susceptibility (181, 182).

3.3.2 Cross-Resistance Analysis Using NA Variants Derived by Reverse Genetics or Recombinants Expressed in HeLa, 293T, or Insect Cells

Further cross-resistance analysis has been undertaken with neuraminidase variants prepared by reverse genetics or with recombinants expressed in insect cells, HeLa, or human kidney 293T cells (163, 168–170, 185–188). In this way the known resistance mutations have been studied and susceptibility to NAIs compared in different neuraminidase sub-types. The results of this cross-resistance analysis have been summarised in Table 4.

From this analysis it became apparent that the same mutations engineered into different NA sub-types may show marked differences between sub-types in the levels of susceptibility to oseltamivir and zanamivir. For the R292K mutation resistance to oseltamivir was highest in N2 but was also observed in the B background, but for zanamivir the resistance was much lower but variable in the N2 and B

Table 4 Cross-resistance analysis using NA enzyme assays of neuraminidase variants in different NA sub-types, derived by reverse genetics or from recombinants expressed in HeLa, insect cells or human kidney 293T cells

NA mutations	Sub-type	Resistance to oseltamivir (fold-shift)[a]	Resistance to zanamivir (fold-shift)[a]	References
R292K	N2	R (>10,000)	R (134)	(187)
	N2	R (>1,580)	S (2.5)	(163)
	N2	R (>60,000)	R (7)	(170)
	B	R (>300)	R (28.5)	(186)
R152K	N2	S (2.7)	I (5.5)	(163)
	N2	S (1)	S (1)	(170)
	N9	S (1.9)	R (9.6)	(163)
	B	R (252)	S (4.7)	(186)
H274Y	N1	R (200)	S (3)	(169)
	N1	R (427.8)	S (1)	(185)
	N1	R (754)	S (1)	(187)
	N1 (H5 avian)	R (292)	S (1)	(188)
	N1 (H5avian)	R (1,672)	S (2)	(188)
	N2	S (2.5)	ND	(169)
	N2	I (7)	I (5)	(187)
	N2	S (2.6)	S (3.7)	(163)
	N2	S (0.8)	S (1.2)	(163)
	N9	R (80)	S (2.7)	(163)
E119G	N1	I (8.74)	S (4)	(185)
	B	R (31.1)	R (>560)	(186)
E119A	B	R (>300)	R (>560)	(186)
E119D	N2	S (3.16)	R (32)	(163)
	B	R (>300)	R (>560)	(186)
E119V	N1	R (1,727)	R (2,144)	(187)
	N2	R (1,028)	I (7)	(187)
	N2	R (14–18)	S (0.8–1)	(163)
	N9	ND	R (145)	(168)
	B	R (>300)	S (1.9)	(186)
N294S	N1	R (197)	I (5)	(187)
	N1 (H5 avian)	R (83)	S (3)	(188)
	N1 (H5avian)	R (21)	S (3)	(188)
	N2	R (1,879)	I (8)	(187)

[a]Fold shift = S < 5-fold difference from reference wild-type, I ≥ 5 < 10-fold difference from wild-type, R ≥ 10-fold difference from wild-type. NAs used were B/Beijing/1/87 (186); A/WSN/33 (N1), A/Sydney/5/97(N2) (185, 187); A/Sydney/5/97 (N2), A/Tokyo/67 (N2), A/G70c N9 (163); A/G70c (N9) (168); A/WS/33 (N1) (169), A/Wuhan/359/95(N2) (170); A/Vietnam/1203/04(N1) (188).

variants. For the R152K selected during treatment with zanamivir in an influenza B infection, only a small shift in susceptibility to zanamivir (fivefold) was observed when introduced into B/Beijing1/87 background compared to a 250-fold change for oseltamivir. When introduced into N2 or N9 NA only small shifts in susceptibility were observed in both inhibitors (≤10) but were marginally greater for zanamivir. The H274Y only showed high level resistance to oseltamivir in the N1 and avian N9 backgrounds suggesting some similarity between these two sub-types in contrast to the N2 NA. The B NA was not constructed with the H274Y mutation, but previous data from in vitro passage with peramivir and influenza B virus has shown that this mutation also confers oseltamivir resistance in B virus (189).

Interesting results were observed with some of the 119 mutations, particularly in the N1 background. The in vitro mutation most frequently selected by zanamivir (E119G) and which gave high level resistance in the N2, N9 and B background was shown to remain sensitive to zanamivir in the N1 NA. Conversely, the E119V which was selected in the clinic by oseltamivir was sensitive to zanamivir in the N2 and B NAs but was highly resistant to zanamivir in the N1 background. These differences are not understood but may reflect structural differences between the Group 1 (N1) and Group 2 (N2, N9) NAs (111). The E119V mutation gave high level resistance to oseltamivir in all three NAs studied (N1, N2 and B).

For N294S resistance to oseltamivir was observed in both N1 and N2 constructs but the level or resistance was higher in N2 consistent with clinical data (69, 120), but for zanamivir only low level shifts were observed less than tenfold. As indicated in the structural studies these variations in

susceptibility suggest that there must be structural similarities and differences around the active site between the different sub-types (111).

3.4 Mechanism of Spread of Resistance

Based on functional studies, all the NA mutant enzymes show either substantial reductions in stability or enzyme activity, that is they all have compromised enzyme fitness. When replication kinetics were examined in cell culture, reductions in replication rates compared to wild-type strains have been detected for H274Y (173), and for R292K in two out of three studies (132, 135, 156), but not for E119G (125, 129), E119A (132) or E119V (161). However, kinetic studies in cell culture are not straightforward for NA mutants, because the presence of HA mutations, in addition to NA mutation, may result in increased growth of virus (156). In vivo studies using either the mouse or ferret models have also shown reductions in infectivity and pathogenicity, for the E119A (132) and E119V mutants (161, 190) but not the E119G mutant (129), and reductions for the R152K mutant (144), the R292K mutant (131, 132, 135) and the H274Y mutant (173). Again some of these studies were undertaken with HA and NA double mutants, where the HA background may have resulted in increased growth. Detailed studies in the mouse have indicated that HA mutations may play some role in resistance in vivo, although this has not been demonstrated in the ferret model (155). To overcome this problem the E119V and the R292K mutations were introduced into the N2 background and the same virus background using reverse genetics to allow direct comparisons of viral fitness in vitro and in vivo (191). This study confirmed the differences between these two mutations.

In addition, reverse genetics studies using two different H5N1 highly pathogenic virus backgrounds revealed that introduction of the H274Y or N294S mutations retained the high level pathogenicity in mammalian species (188).

Studies on fitness of NA variants gave rise to the theory that NAI variants would not be transmitted in humans. Although NA mutations may be detected during acute infection their presence has been reported to not affect resolution of symptoms (142), but virus shedding may be prolonged, particularly in children and the immunocompromised subjects increasing the risk of transmission (69, 140). Clinical trial studies and surveillance studies by the global NISN have revealed some potential for circulation of resistant viruses to date, with five H1N1 isolates with the H274Y mutation and two H3N2 isolates with the R292K mutation and one H3N2 isolate with the E119V mutation observed from untreated subjects (148, 159). Widespread surveillance studies are ongoing to determine how much of a problem transmission of resistant virus may be, in particular, transmission of the H274Y mutant in H1N1 in untreated subjects, observed in 2007/2008.

A model for influenza transmission in ferrets has been developed and used to study the potential for transmission of oseltamivir resistant clinical isolates from immunocompetent subjects (190, 192). This model which involves infecting four ferrets per group with mutant or wild-type virus and then housing these infected ferrets with three uninfected ferrets should help determine the potential for clinical transmission of the different influenza variants. The first studies with the R292K mutant showed no transmission for mutant virus but all the uninfected controls became infected showing transmission of wild-type virus. In addition, some contacts from the mutant virus group became infected with wild-type virus due to reversion to wild-type in the originally infected ferrets. Similar studies with the H274Y and E119V variants revealed under these experimental condition that these variants grew to high titre and could be transmitted, although the 274Y mutant had reduced infectivity and a 100-fold higher dose of virus was required to infect the ferrets (173). On transmission the virus variants remained stable and did not revert to wild-type. The E119V had similar infectivity to wild-type, and grew to similar titres as wild-type in both the donor and recipient ferrets (173, 191). Based on these studies the E119V variant in the H3N2 background appeared to have the highest potential for transmission (173, 191). However, the frequency of isolation of this N2 variant is very low compared to the more debilitating R292K mutant.

From all the clinical studies it appears that NA resistance arises due to single mutations within the highly conserved region of the active site of NA all of which have some effect on virus fitness. Interestingly there is one report from in vitro selection studies with oseltamivir of isolation of a double active site mutant (E119V/R292K), but at very low levels <10% of the virus population suggesting this virus was substantially compromised (162). Apart from the H274Y variants in man there is limited evidence that compensatory mutations in either the NA or HA could overcome the fitness deficit of the active site mutations. Further in a self-limiting disease where virus is normally cleared within 6–10 days there is a time limit during treatment on development of multiple mutations to produce fit virus. Even in immunocompromised subjects where virus shedding may be prolonged, and where both HA and NA mutations have been observed the virus fitness was still compromised (144). Currently it is not clear if different HA variants circulating in the community as described (154) may predispose virus to developing NA resistance and which may result in fitter virus with increased ability to transmit. From the NISN susceptibility surveillance studies and recent studies with H274Y transmission there are indications that differences in NA background may influence susceptibility and transmission of some mutations ((148), WHO/ECDC and CDC web-sites). However, limited cross-resistance between the NAIs may prove valuable. Compared

with amantadine/rimantadine, the potential for drug resistance to be a problem for the NAIs in treating influenza infections appears much reduced.

3.5 Alternative Agents

Zanamivir and oseltamivir are the only two neuraminidase inhibitors that are licenced for use against influenza infections in humans, with similar potency in both treatment and prophylaxis of seasonal influenza infections (193, 194), but with zanamivir showing greater potency against influenza B in vitro and in the clinic (177). Further substrate analogues have been designed and shown to be highly potent in vitro against influenza A and B viruses (195–198). These included a cyclopentane analogue [(4-acetylamino)-3-guanidinobenzoic acid, RWJ-270201, BCX-1812, peramivir] discovered at Biocryst and which showed oral efficacy in vivo in animal models (199, 200), including highly pathogenic H5N1 viruses (201), and was further evaluated in clinical studies by R.W Johnson Pharmaceuticals. In phase I studies, peramivir was shown to be effective in lowering virus titre but at relatively high drug concentration of 400–800 mg/kg/day (202). In phase 2 and 3 clinical trials, the primary end point of time to relief of symptoms did not reach statistical significance, probably due to the low oral bioavailability of peramivir (≤3%) (203). The clinical development of oral peramivir was halted in 2002. However, with the continued spread of the highly pathogenic avian H5N1 virus there is an increased need for influenza drugs to treat life-threatening influenza. Alternative formulations of peramivir, intramuscular and intravenous are being evaluated in clinical studies with seasonal influenza (204). In the mouse model it was shown that a single intramuscular injection of peramivir significantly reduced weight loss and mortality in mice infected with A/NWS/33(H1N1) or A/Victoria/3/75(H3N2) comparable to 5-day treatment with oral oseltamivir (203). The efficacy of a single intramuscular dose is explained by the tight binding of peramivir to the active site of neuraminidase. For N9 NA the peramivir $t_{1/2} > 24$ h compared with a $t_{1/2} = 1.25$ h for oseltamivir and zanamivir (203). In vitro passage studies of an A/H3N2 virus with peramivir selected for the mutation R292K in NA with a 10- to 20-fold reduction in NA susceptibility (196) and of H274Y in influenza B with a 16- to 31-fold reduction in susceptibility (189).

A second inhibitor, a novel pyrrolidine-based compound, [5-(1R,2S)-1-(acetylamino)-2-methoxy-2-methyl pentyl-4-[(1Z)-1-propenyl]-(4S,5R)-D-proline, A-135675, and isopropylester pro-drug A-322278] was discovered by Abbott Laboratories (197, 198), but despite showing good efficacy in vitro has not been developed further. In vitro passage studies of influenza A/N9 G70 virus in the presence of A-135675 selected E119D with a 162-fold reduction in NA susceptibility (162).

The development of resistance to oseltamivir, in the treatment of both seasonal influenza and avian H5N1 infections in humans, has renewed interest in drugs that will treat resistant virus. A detailed comparison between oseltamivir, zanamivir, peramivir and A-135675 susceptibility of resistant isolates obtained from both in vitro and clinical studies with oseltamivir and zanamivir has been undertaken (182). Interestingly A-135675 had the lowest cross-resistance profile, followed by zanamivir. This probably relates to some similarities of binding of these two inhibitors in that they do not cause a conformational change in binding to the NA active site whereas both oseltamivir and peramivir cause a conformational change due to the presence of the bulky hydrophobic substituent at the sixth position (162, 205). Against the H274Y variant, which has been isolated from some patients infected with H1N1 and H5N1 virus treated with oseltamivir, both A-315676 and zanamivir were active but peramivir showed a 100-fold reduction in susceptibility (182). In addition, the isopropylester pro-drug of A-135675 (A-322278), was shown to be effective against an oseltamivir selected B variant (D198N, N2-numbering) in the mouse (182). The additional structural studies undertaken with the N1, N4 and N8 of the Group 1 NAs have revealed a cavity close to the active site that closes on ligand binding which may be exploited in further drug design of NA inhibitors (111).

In addition to structural design, further approaches to development of more potent inhibitors has been that of the study of large hydrophobic pro-drugs of close analogues of zanamivir and multivalent zanamivir molecules by Sankyo in Japan, and Biota in Australia (206–208). These approaches were reported to prolong deposition of drug within the respiratory tract after oral inhalation with the potential for much reduced dosing frequencies, of possibly one prophylactic treatment per week, or once only treatment (209, 210). One inhibitor CS8958 in co-development by Daiichi-Sankyo and Biota is in phase II/III evaluation against seasonal influenza A and B.

The increased awareness of the potential for a new influenza A pandemic has stimulated research into the development of new influenza inhibitors. The rapid development of widespread clinical resistance to the M2 ion-channel inhibitors plus the emergence of some resistance to the NAI, oseltamivir, emphasises the need for new agents. These new influenza inhibitors may be developed to the same targets (NA and M2 ion channel) to cover resistant isolates as described above, or inhibitors to other targets within the influenza replication cycle should be developed.

Inhibitors in development to alternate targets include a sialidase fusion protein (DAS-81, Fludase) which is a recombinant fusion protein containing a sialidase catalytic domain derived from *Actinomyces viscosus* fused with a respiratory epithelium-anchoring domain. DAS-81 works by tethering to the respiratory epithelium and cleaving both α(2,6)-linked and α(2,3)-linked sialic acid receptors which are recognised by human and avian strains of influenza viruses,

and therefore blocks virus attachment (211). The molecule shows potent activity in vitro (IC_{50} values 0.04–0.9 nM) against both influenza A and B viruses and efficacy when given intranasally to mice before or after infection with H1N1 and H5N1 viruses (211, 212) DAS-81 is undergoing Phase 1 studies in humans.

A second inhibitor of interest is T-705 which is a substituted pyrazine 6-fluoro-3-hydroxy-2-pyrazinecarboximide which inhibits influenza virus RNA polymerase after conversion to the triphosphate (213). This inhibitor has activity against influenza A and B viruses in vitro with IC_{50} values in the range of 0.013–0.48 µg/mL (214), and in vivo efficacy against an A/Duck/MN/1525/81(H5N1) virus at 30–300 mg/kg/day, and was more effective than oseltamivir administered at 20 mg/kg/day (215). In 2007 T-705 entered clinical development in Japan to determine its efficacy and safety in humans.

Development of new inhibitors either to the proven targets, M2 ion channels and neuraminidase or to new targets and which may be active against resistant virus should improve treatment options for controlling influenza in the future. Some of these inhibitors may prove suitable for use in combination therapy which may limit the chances for resistance to develop.

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