

Erhard van der Vries and Michael G. Ison

## 1 Introduction

There are three classes of antiviral drugs approved for the treatment of influenza: the M2 ion channel inhibitors (amantadine, rimantadine), the neuraminidase (NA) inhibitors (laninamivir, oseltamivir, peramivir, zanamivir), and the protease inhibitor (favipiravir); some of the agents are only available in selected countries [1, 2]. These agents are effective at treating the signs and symptoms of influenza in patients infected with susceptible viruses. Clinical failure has been demonstrated in patients infected with viruses with primary resistance, i.e., antivirals can be present in the virus initially infecting the patient, or resistance may emerge during the course of therapy [3–5]. NA inhibitors are active against all nine NA subtypes recognized in nature [6], including highly pathogenic avian influenza A/H5N1 and recent low-pathogenic avian influenza A/H7N9 viruses [7]. Since seasonal influenza is usually an acute, self-limited illness in which viral clearance usually occurs rapidly due to innate and adaptive host immune responses, the emergence of drug-resistant variants would be anticipated to have limited effect on clinical recovery in otherwise healthy patients, as has been demonstrated clinically [3, 8, 9]. Unfortunately, immunocompromised or immunologically naïve hosts, such as young children and infants or those exposed to novel strains, are more likely to have mutations that confer resistance emergence during therapy; such resistant variants may also result in clinically significant adverse outcomes [10–13].

Factors that influence the clinical and epidemiologic importance of drug-resistant influenza viruses include the magnitude of phenotypic resistance, its frequency and rapidity of emergence, its stability and ability of resistant variants to compete with wild-type virus in the absence of selective drug pressure, and the effects of resistance mutations on viral replication competence, pathogenicity, and transmissibility in vivo. Prior to being replaced by the pandemic 2009 A/H1N1 virus, most circulating seasonal A/H1N1 viruses in the 2008–2009 season contained the His275Tyr mutation and were therefore highly resistant to oseltamivir while retaining susceptibility to zanamivir. All currently circulating A/H1N1 and A/H3N2 viruses have mutations conferring M2 inhibitor resistance. There is no data to date indicating that M2 inhibitor resistance is associated with worsened viral virulence, atypical influenza, or enhanced transmissibility. Sporadic viruses with primary resistance mutations resulting in neuraminidase inhibitor resistance have been described. Most but not all NA mutations conferring resistance in clinical isolates have been associated with reduced infectivity, replication, and pathogenicity in animal models of influenza. Such features are important not only in clinical management of individual patients but also are key factors that need to be considered by health authorities and governments when making decisions regarding the stockpiling of antivirals for response to pandemics or other influenza threats [14, 15]. Concerns about antiviral resistance, particularly to NA inhibitors, should not dissuade countries from developing adequate antiviral inventories for pandemic response [14, 16].

The frequency of resistance emergence during therapy is higher with M2 inhibitors than NA inhibitors. Development of resistance during the course of therapy was very common among initially M2 inhibitor-susceptible viruses in the past. Mutations in one of five amino acids in the M2 gene result in cross-resistance to both amantadine and rimantadine. Resistance emergence during therapy with neuraminidase inhibitors generally remains rare for circulating strains of A/H1N1, A/H3N2, and B viruses, with higher rates in children

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E. van der Vries, Ph.D.  
Research Center for Emerging Infections and Zoonoses, University  
of Veterinary Medicine, Hannover, Germany

M.G. Ison, M.D., M.S. (✉)  
Divisions of Infectious Diseases and Organ Transplantation,  
Northwestern University Feinberg School of Medicine,  
Chicago, IL, USA  
e-mail: [mgison@northwestern.edu](mailto:mgison@northwestern.edu)

and immunocompromised patients. A common feature of patients who develop resistance despite ongoing therapy is high-level replication and longer duration of replication in the presence of antiviral therapy. Resistance to neuraminidase inhibitors results from mutations in the neuraminidase gene, the hemagglutinin gene, or both. The specific mutation determines the degree of resistance and which neuraminidase inhibitor has reduced susceptibility. Further, the frequency and magnitude of NA inhibitor resistance vary with drug, virus, and neuraminidase type and subtype. Resistance has been demonstrated to develop during the course of therapy with avian viruses, particularly A/H7N9, with greater frequency than seasonal human viruses. Compensatory mutations may also occur that improve the fitness and transmissibility of resistant viruses and may play a role in establishing persistent transmission, as was demonstrated with the seasonal A/H1N1 during the 2007–2008 and 2008–2009 seasons.

Several new classes of antivirals, many with novel mechanisms of action, are currently undergoing development. Resistance mutations have been identified for many of these agents from in vitro passage experiments, but clinical evidence of resistance emergence is still in its infancy. The following sections review clinical and epidemiological data on antiviral resistance for the three classes of available anti-influenza agents. Information from experimental animal models of influenza is incorporated to supplement the limited data derived from clinical studies.

## 2 M2 Ion Channel Inhibitors (Amantadine, Rimantadine)

The M2 ion channel allows the influx of protons into the viral particle which, in turn, facilitates uncoating [17]. M2 inhibitors bind to the M2 ion channel and limit the influx of protons resulting in its antiviral effect. Since the M2 protein is present only on influenza A viruses, M2 inhibitors have no activity against influenza B [17]. There are currently two approved M2 ion channel inhibitors, amantadine and rimantadine. Early studies demonstrated that influenza variants with high-level resistance to amantadine and rimantadine could be selected in the laboratory though in vitro and in vivo passage in virus in the presence of the drug [3, 18]. Studies of resistance helped to determine the mechanism of antiviral action of the M2 inhibitors [19].

Mutations in the M2 inhibitor gene at one of five commonly recognized sites (position 26, 27, 30, 31, or 34 of the M2 protein) in human viruses result in reduced binding of the M2 inhibitors or in enlargement of the pore diameter. The function of the M2 pore of viruses with any of the mutations is preserved in the presence of the inhibitor [3, 20, 21].

Resistance mutations do not affect transmissibility or replication fitness as compared to wild-type viruses; documented transmission from person-to-person has been well established [22]. Resistance affects both drugs in the class equally and appears to be persistent over time [3].

During routine treatment with M2 inhibitors for documented influenza, resistant variants emerge frequently. The clinical implications of resistance became apparent in studies during the 1980s of treated children, in whom a high frequency of resistance emergence was documented, and subsequently of households and nursing homes, where transmission of drug-resistant variants was implicated in failures of drug prophylaxis [23–26]. About 30% of adults treated with M2 inhibitors will have resistant variants detected during the course of their illness with high frequency (up to 80%) of resistance emergence in immunocompromised patients, patients hospitalized for influenza, and children [8, 23, 25, 27, 28]. Until recently, the frequency of M2 inhibitor resistance among seasonal isolated was low (1–3%) [29]. Since 2002, though, the prevalence of resistance to M2 inhibitors among circulating influenza A/H3N2 increased globally, and now the majority of A/H3N2 globally is resistant to this class of drugs [29, 30]. Resistance has resulted from the S31N substitution of the M2 inhibitor. M2 inhibitor resistance has also been documented in several important novel strains of influenza: A/H5N1, A/H7N9, and 2009 pandemic A/H1N1 virus [31–36]. Most clade 1 A/H5N1 viruses and all swine-origin A/H1N1 are resistant to the M2 inhibitors as a result of the S31N substitution, while most (~80%) of clade 2.1 A/H5N1 are resistant secondary to S31N or V27A substitution [31, 32, 37]. Of note, most of the clade 2.2 and 2.3 A/H5N1 viruses remain susceptible to M2 inhibitors [37]. Since most circulating strains of influenza are currently resistant to the M2 inhibitors, this class is not recommended for the prevention or treatment of influenza currently [2].

### 2.1 Detection of Resistance

There are currently no rapid tests that can screen for and identify the presence of M2 inhibitor resistance. M2 resistance may be diagnosed using phenotypic assays or gene sequencing. Most phenotypic assays, including plaque reduction, yield reduction, and ELISA, utilize the growth of virus in cell culture exposed to a range of concentrations of the drug of interest; these assays are not widely available. Pyrosequencing methods for rapid analysis of mutations in the M2 gene associated with resistance have been described and are used in several reference laboratories [21, 38]. Neither assay is typically available in most clinical labs. As a result, most clinicians rely on data generated from groups

actively monitoring the resistance among circulating strain—in the United States this is actively done by the Centers for Diseases Control and Prevention (<http://www.cdc.gov/flu/professionals/antivirals/antiviral-drug-resistance.htm>).

Detection of M2 inhibitor resistance has usually relied on virus isolation from respiratory samples and susceptibility testing of virus in cell culture. Several assays have been described including plaque reduction, yield reduction, and ELISA [39]. Following phenotypic analysis, genotypic M2 inhibitor resistance has been confirmed by nucleotide sequence analysis of the M2 gene and detection of the characteristic mutations. Genotypic detection can be accomplished quickly by the use of PCR restriction length polymorphism (RFLP) analysis of RNA extracted from respiratory samples using commercially available endonucleases for discrimination of point mutations in the M2 gene [38, 40]. Greater sensitivity in detecting resistant clones has been described with reverse transcription-polymerase chain reaction amplification of RNA followed by sequencing of multiple clones [4, 38, 41]. Recently, the rapid pyrosequencing technique has been shown to be a reliable, high-throughput method for detecting genotypic resistance in large numbers of community isolates [4, 29, 38].

## 2.2 Susceptibility of Field Isolates

Historically, human isolates of influenza A/H1N1, A/H2N2, and A/H3N2 were initially susceptible to amantadine and rimantadine [19, 24, 39, 42]. Even after licensure of the M2 inhibitors, there were low levels of primary resistance in community isolates (see Table 71.1). This began to change when field isolates of A/H3N2 viruses from China were noted to have a significant increase in the resistance to the M2 inhibitors, possibly related to increased use of over-the-counter amantadine after the emergence of severe acute respiratory syndrome (SARS) [38]. During the 2004–2005 influenza season, approximately 70% of the A/H3N2 isolates from China and Hong Kong and nearly 15% of those from the United States and Europe showed resistance due to a Ser31Asn mutation, and this frequency increased to over 90% in the United States during the 2005–2006 season [29, 38]. Since then, most clinical isolates of A/H3N2 were noted to have Ser31Asn mutations in the M gene conferring resistance to M2 inhibitors (Table 71.1). This spread occurred despite the absence of sustained selective drug pressure, possibly because the resistant M gene was incorporated into efficiently spreading HA antigenic variants. Phylogenetic

**Table 71.1** Representative studies of M2 inhibitor susceptibility of influenza A field isolates from adults and children

	Site	Period	Method	No. tested by subtype	No. (%) resistant
Belshe et al. [24]	United States	1978–1988	EIA, S	65 H1N1	0
				181 H3N2	5 (2.0%) <sup>a</sup>
Valette et al. [202]	France	1988–1990	EIA	28 H1N1	0
				77 H3N2	0
Ziegler et al. [203]	43 countries	1991–1995	EIA, S, PCR-RFLP	2017	16 (0.8%) <sup>b</sup>
Dawson [204]	UK	1968–1999	EIA, plaque	1813	28 (1.5%)
Suzuki et al. [205]	Japan	1993–1998	Not stated	55	0
		1999–2000	Not stated	179	6 (3.4%)
Shih et al. [206]	Taiwan	1996–1998	Plaque, S	84	1 (1.2%)
Bright et al. [38]	Global	1994–2005	S	6525	392 (6.0%)
		1994–2002		H3N2	0.3–1.8%
		2003–2005			12.3–13.3% <sup>c</sup>
		1998–2004		589 H1N1	2 (0.3%)
Bright et al. [29]	United States	2005	S	205 H3N2	193 (92.3%)
				8 H1N1	2 (25%)
Saito et al. [207]	Japan	2005–2006	S	354 H3N2	231 (65.3%)
				61 H1N1	0
Barr et al. [43]	Australia, New Zealand, Asia, South Africa	2005	S	102 H3N2	43 (42%)
				37 H1N1	0

*Abbreviations:* S M2 gene sequence analysis, PCR-RFLP polymerase chain reaction-restriction length polymorphism, EIA enzyme immunoassay

<sup>a</sup>All resistant viruses from family members receiving rimantadine

<sup>b</sup>Over 80% of tested isolates were H3N2 subtype and all resistant ones were of this subtype. Separate analysis found that 9 (4.5%) of 198 strains from Australia, 1989–1995, were resistant

<sup>c</sup>In 2004–2005 the frequencies of resistance in H3N2 viruses were 73.8% in China, 69.6% in Hong Kong, 22.7% in Taiwan, 15.1% in South Korea, 4.3% in Japan, 30.0% in Canada, 19.2% in Mexico, 14.5% in United States, and 4.7% in Europe

analyses of the HA1 and M2 genes have suggested a common origin of these viruses [43]. This experience clearly indicates that this resistance mutation does not reduce transmissibility and is stable over time.

The frequency of resistance in seasonal A/H1N1 viruses increased from 2005 to 2007, primarily due to the Ser31Asn mutation [29, 30]. Fortunately, the incidence of primary resistance declined in 2008 and 2009 among seasonal A/H1N1 viruses as oseltamivir-resistant viruses predominated [44]. This seasonal A/H1N1 virus, which was replaced by the 2009 pandemic A/H1N1 virus, was primarily resistant to the M2 inhibitors generally due to the Ser31Asn mutation [44]. As a result, all currently circulating strains of influenza A are primarily resistant to the M2 inhibitors, and this class of drug is not recommended for the prevention or treatment of influenza [2].

M2 proteins show considerable evolution in human and swine viruses, and the H3 and H1 subtype viruses have phylogenetically different M2 proteins [45]. This may influence the mutations that are more advantageous for conferring M2 inhibitor resistance. A characteristic feature of A/H1N1, A/H1N2, and A/H3N2 swine viruses circulating in Europe since 1987 has been the presence of Ser31Asn mutation, as well as Lys27Ala in some isolates, that confers resistance to M2 inhibitors [46]. The postulated role of swine as intermediate hosts in the emergence of some novel human viruses and direct interspecies transmission from birds may be another mechanisms for a reassortment event leading to acquisition of an M gene encoding resistance in a human strain [47, 48].

Although the initial human isolates of highly pathogenic avian A/H5N1 viruses in Hong Kong in 1997 were M2 inhibitor susceptible, resistance to this class of drugs has become more prevalent [32, 37]. Most clade 1 A/H5N1 viruses are resistant to the M2 inhibitors as a result of the Ser31Asn substitution, while most (~80%) of clade 2.1 A/H5N1 are resistant secondary to Ser31Asn or Val27Ala substitution [32, 37]. Of note, most of the clade 2.2 and 2.3 A/H5N1 viruses remain susceptible to M2 inhibitors [37]. Isolates of A/H7N9 infected humans have also had the Ser31Asn mutation conferring resistance to the M2 inhibitors [49, 50].

## 2.3 Resistance in Posttreatment Isolates

Studies in experimentally infected animals and treated humans have documented the common emergence of resistant variants as the course of infection progresses over time. Following treatment, approximately 70–90% of amino acid substitutions in resistant viruses occur at position 31, and about 10% each are found at positions 27 and 30 [40]. The Ser31Asn mutation has been responsible for the resistant A/H3N2 and A/H1N1 variants recently identified globally [29, 38].

### 2.3.1 Animal Studies

The rapid emergence of resistant variants in M2 inhibitor-treated patients has been found also in studies of experimentally infected animals. In a study of a chicken A/H5N2 virus, resistant viruses are detectable by 2–3 days after starting drug administration and persisted thereafter [51]. A study in ferrets inoculated with a human influenza A/H3N2 virus detected M2 inhibitor resistance mutations in four of nine amantadine-treated animals by day 6 after inoculation; in each instance two or more M2 gene mutations were identified [52].

### 2.3.2 Immunocompetent Patients

Resistant variants arise commonly and rapidly in M2 inhibitor-treated children and adults with acute influenza (Table 71.2). One study of adults found that resistant virus could be detected in 50% of six rimantadine recipients by day 3 of treatment, although the nasal lavage titers were lower than in placebo recipients shedding susceptible virus [27]. Another study found that 33% of 24 adult and pediatric household members receiving rimantadine shed resistant virus on day 5 of treatment; none were positive when tested 5 days later [27]. A larger pediatric trial found emergence of resistant virus in 27% of 37 rimantadine recipients, including 45% of those still virus positive on day 7, compared to 6% of 32 acetaminophen recipients [23]. Resistant virus was detected as early as day 3 in one child but was usually present on days 5–7. A study of Japanese children treated with amantadine found that 30% of 81 in the 1999–2000 season and 23% of 30 during the following season had resistant virus detected on day 3–5 after a 3-day course [53]. Resistant

**Table 71.2** Recovery of resistant influenza A during M2 inhibitor treatment

Study	Seasons	Patient group	Treatment	No. treated	No. (%) shedding resistant viruses
Hall et al. [23]		Children	Rimantadine	37	10 (27%) H3N2
Hayden et al. [25]	1987–1989	Children	Rimantadine	21	6 (29%) H3N2
Hayden et al. [27]	1988–1989	Adults	Rimantadine	13	5 (38%) H3N2
Englund et al. [28]	1993–1994	Immunocompromised	Amantadine, rimantadine	15	5 (33%) H3N2
Saito et al. [53]	1999–2001	Children	Amantadine	111	22 (33%) H3N2 9 (20%) H1N1
Shirashi et al. [41]	1999–2001	Children (hospitalized)	Amantadine	15	8 (100%) H3N2 4 (57%) H1N1

variants were detected more frequently in A/H3N2-infected children (33%) than in A/H1N1-infected children (20%). Another study employing sensitive molecular cloning detection methods found mutations conferring resistance in 80% of 15 hospitalized children during or immediately after amantadine treatment [41]. Nine (75%) of 12 children had 2–4 resistance mutations detected in clones from a single sample, sometimes mixed with wild-type virus. In a randomized study patients hospitalized with influenza were to receive either rimantadine alone or rimantadine plus nebulized zanamivir [54]. Rimantadine-resistant virus was detected in 2/20 (10%) of rimantadine monotherapy patients, while non-resistant variants were detected in the 21 patients receiving combination therapy [54].

### 2.3.3 Immunocompromised Hosts

Resistant influenza A viruses may be shed for prolonged periods in immunocompromised hosts, who can serve as a reservoir for nosocomial transmission. One study of adult bone marrow transplant and acute leukemia patients recovered resistant virus in 5 (33%) of 15 M2 inhibitor-treated patients and in 5 (83%) of 6 patients with illness who shed virus for  $\geq 3$  days [28]. The median time between the first and last virus isolation was 7 days with range up to 44 days. Death associated with influenza occurred in 2 of 5 (40%) patients with resistant virus, compared to 5 of 24 (21%) without, and prolonged illness was noted in several with protracted shedding. Other reports have documented prolonged shedding of resistant variants in immunocompromised hosts with or without continued drug exposure, including one transplanted SCID child who shed for 5 weeks and one adult leukemia patient who shed resistant virus for  $\geq 1$  week of therapy [55]. Another case report documented recovery of resistant virus >1 month after cessation of a course of amantadine, as well as shedding of mixtures of wild-type virus and variants with different resistance genotypes [56]. Heterogeneous populations of resistant variants with sequential or dual mutations have been found in several immunocompromised hosts [28, 55].

One stem cell transplant recipient shed dually M2 inhibitor and oseltamivir-resistant virus for at least 5 months and probably over 1 year [57]. The prolonged shedding of resistant variants in immunocompromised hosts is consistent with the genetic stability of such variants observed in experimental animal models [51].

## 2.4 Transmissibility of Resistant Variants

The transmissibility of M2 inhibitor-resistant viruses has been demonstrated in animal models and in several clinical settings. Competition-transmission studies with an avian A/chicken/Pennsylvania/1370/83 A/H5N2 virus compared the transmissibility of wild-type virus with resistant variants possessing M2 substitutions at positions 27, 30, or 31 [51]. Contact birds shedding resistant virus due to earlier incorporation of amantadine in the drinking water of donors (4 days only) were caged with birds shedding susceptible virus, and the virus was allowed to transmit through three more sets of contact birds in the absence of selective drug pressure. Resistant virus was detected from the final set of contact birds in three of four experiments over four cumulative transmission cycles.

### 2.4.1 Households

Both amantadine and rimantadine are effective for postexposure prophylaxis of illness due to susceptible strains in household contacts, when ill index cases are not given concurrent treatment (Table 71.3). In contrast, two studies have found no significant reduction in secondary influenza illness in household contacts receiving either amantadine or rimantadine for postexposure prophylaxis, when the ill index cases received treatment with the same drug, and one of these documented failures of prophylaxis due to infection by drug-resistant variants, most likely transmitted from the treated index cases [25]. These findings indicate that the strategy of using M2 inhibitors for both index case treatment and postexposure prophylaxis in households should be avoided.

**Table 71.3** Influenza prevention in households with postexposure prophylaxis (PEP)

Study	Drug (age of contacts)	Season (virus)	Index case treated	Influenza A illness in contacts		PEP efficacy (%)
				No./total evaluable (%)		
				Active	Control	
Galbraith et al. [208]	Amantadine ( $\geq 2$ years)	1967–1968 (A/H2N2)	No	0/91 (0%)	12/90 (13%)	100
Bricaire et al. [209]	Rimantadine ( $\geq 1$ year)	1988–1989 (A/not stated)	No	8/151 <sup>a</sup> (5%)	26/150 <sup>a</sup> (17%)	70
Monto et al. [210]	Zanamivir ( $\geq 5$ years)	2000–2001 (A/H3N2, B)	No	12/661 (2%)	55/630 (9%)	82
Welliver et al. [211]	Oseltamivir ( $\geq 13$ years)	1998–1999 (A/H3N2, B)	No	4/493 (1%)	34/462 (72%)	89
Galbraith et al. [212]	Amantadine ( $\geq 2$ years)	1968–1969 (A/H3N2)	Yes	5/43 (12%)	6/42 (14%)	6
Hayden et al. [25]	Rimantadine ( $\geq 1$ year)	1987–1989 (A/H3N2, A/H1N1)	Yes	11/61 (18%)	10/54 (19%)	3
Hayden et al. [213]	Zanamivir ( $\geq 5$ years)	1998–1999 (A/H3N2, B)	Yes	7/414 (2%)	40/423 (9%)	82
Hayden et al. [214]	Oseltamivir ( $\geq 1$ year)	2000–2001 (A/H3N2, B)	Yes	11/400 (3%)	40/392 (10%)	73

<sup>a</sup>Clinical influenza

### 2.4.2 Chronic Care Facilities

Transmission of M2 inhibitor-resistant viruses is well documented in nursing home outbreaks of influenza A and may be manifested by a persistent or an increasing number of virus-positive patients despite amantadine prophylaxis. The recovery of the same genotype of resistant virus from multiple patients on prophylaxis or from patients or staff not receiving drug indicates ongoing transmission in this setting [26, 58]. This particularly true with multiple isolations of a less commonly observed resistant variant, as was found with nine isolates of a Leu26Phe variant in one nursing home outbreak [58]. The frequency of instances in which amantadine or rimantadine has failed to control outbreaks because of resistance emergence is not well defined, although existing studies demonstrate a range of protective efficacy from 59 to 76% [59]. Studies have demonstrated patients who developed infections with resistant viruses, typically with the Ser31Asn mutation, in 16–28% of residents' long-term care where M2 inhibitors were used during an outbreak [40, 60]. Such findings emphasize the importance of proper isolation of treated persons and of using NA inhibitors for treatment of ill persons.

### 2.5 Pathogenicity

M2 inhibitor-resistant influenza A viruses appear to cause typical influenza illness without obviously enhanced or attenuated symptoms [25, 27]. Illness occurs in both the presence and absence of the drug, a finding that indicates the loss of antiviral effectiveness *in vivo*. Although severe and progressive infection with resistant virus has been noted in immunocompromised and debilitated elderly patients, this is likely more of a marker of the patients underlying condition than virulence of the resistant virus [57, 61]. In most patients, M2 inhibitor-resistant virus has similar risks of pneumonia, hospitalization, or death compared to those with wild-type illness [39].

While the M gene mutations do not appear to attenuate or potentiate the virulence of human influenza viruses, more subtle effects on biologic fitness cannot be excluded by studies to date. In occasional patients wild-type virus replaces resistant variants after cessation of amantadine [41]. As noted for some avian A/H7 viruses, this reversion in the absence of selective drug pressure suggests diminished replication competence of some resistant genotypes. However, the most common resistant variant with Ser31Asn has no apparent loss of replication competence or transmissibility. In studies in birds and ferrets, influenza viruses with Val27Ala, Ala30Val, or Ser31Asn mutations had no impact of virulence, mortality, febrile responses, peak nasal viral titers, or nasal inflammatory cell counts [22, 51]. In general,

it appears that M2 inhibitor-resistant human influenza A viruses that emerge *in vivo* do not differ substantially in replication ability or pathogenicity from drug-susceptible wild-type viruses, and resistance phenotypes were typically retained in the absence of drug.

In treated patients the emergence of resistant virus may be associated with persistence of viral recovery and in some studies delays in resolution of illness in immunocompetent persons. Although patients who had resistance emergence during the course of therapy had a somewhat longer time to resolution of symptoms, fever, and possibly functional impairment, they still had a more rapid recovery than placebo-treated patients [23, 27]. Patients may have worsening symptoms or viral titers when resistance emerges as well [23].

### 2.6 Treatment Alternatives

Amantadine and rimantadine share susceptibility and resistance, so that resistance to one M2 inhibitor confers high-level cross-resistance to another one and the entire class of compounds targeting M2 protein. Because of their different mechanism of antiviral action, neuraminidase inhibitors and protease inhibitors (discussed below) retain full activity against M2 inhibitor-resistant viruses and are appropriate choices for both prophylaxis and treatment of suspected M2 inhibitor-resistant infections. Clinical studies suggest that both oseltamivir and zanamivir are successful in terminating institutional outbreaks where amantadine resistance is proven or highly probable [58, 62, 63]. *In vitro* testing suggests that the synthetic nucleosides ribavirin and favipiravir are also inhibitory for M2 inhibitor-resistant influenza A and B viruses and are a therapeutic consideration [64].

Combination therapy may also be an option. A small study randomized hospitalized adults to rimantadine monotherapy or rimantadine-nebulized zanamivir combination therapy. The combination arm had a trend to less cough and fewer patients with detection of M2 inhibitor resistance mutations over the course of therapy [54]. Recently, a triple combination of amantadine, oseltamivir, and ribavirin has been studied *in vitro*, *in vivo*, and in infected patient and appears effective at inhibiting viral replication *in vitro* and improved outcomes *in vivo* [65, 66].

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## 3 Neuraminidase Inhibitors

The initial design of the NA inhibitors was accelerated after solving X-ray structures of NA co-crystallized with the chemical compound *2,3-dehydro-2-deoxy-N-acetylneuraminic acid* (DANA) [67, 68]. This transition state

analogue of the NA substrate sialic acid has served as the scaffold for the NA inhibitor derivatives [69]. In vitro studies to determine the genetic antiviral resistance profiles of the NA inhibitors zanamivir and oseltamivir were initiated shortly after their development (reviewed in [70, 71]). Due to differences in drug binding interactions and structural differences in the enzyme active site, NA inhibitors show varying antiviral resistance profiles in NA that depend on virus type and subtype (reviewed in [72–74]). Sequential passage in cell culture to select resistant variants found that changes in HA could confer resistance in vitro also [75–77]. These HA changes are predominately found at the receptor binding site and thought to restore the functional balance between HA receptor-binding and NA receptor-destroying properties [78]. Mechanisms which influence the viral HA/NA functional balance may have played a role in the emergence of oseltamivir-resistant A/H1N1 viruses during the 2007–2008 season [79–82]. The frequency and possible importance of resistance emergence during drug administration have been studied largely in the context of controlled clinical trials conducted in the late 1990s that served as the basis for approval of zanamivir and oseltamivir in 1999 and, more recently, for approval of laninamivir and peramivir [83–87]. Although zanamivir and oseltamivir have been available in many countries since 1999, their use has been quite limited, except in Japan and during the 2009 influenza A/H1N1 pandemic [88]. Clinical importance of antiviral resistance emergence was assessed when comparing clinical outcome of oseltamivir-treated patients infected with a susceptible or oseltamivir-resistant A/H1N1 virus during the 2007–2008 influenza season [89–93]. A retrospective clinical study by Dharan et al. showed that patients infected with an oseltamivir-susceptible virus ( $n=182$ ) had significantly fewer days of fever if treated with oseltamivir ( $n=64$ ) as compared to non-treated patients ( $n=93$ ;  $P=0.02$ ). In contrast, patients infected with an oseltamivir-resistant viruses ( $n=44$ ) did not benefit from oseltamivir treatment ( $n=43$ ;  $P=0.5$ ) [91]. Similar finding was reported in a study by Saito et al. where a reduction of fever on days 3–6 was reported in treated oseltamivir-susceptible A/H1N1-infected patients ( $P<0.01$ ), but not in the patients infected with the oseltamivir-resistant A/H1N1 virus strain [93]. This oseltamivir-resistant A/H1N1 virus variant with an NA H275Y amino acid change emerged first in Norway during the 2007–2008 season and was able to spread rapidly in humans [94, 95]. As it became the dominant variant, it was suggested that the oseltamivir-resistant virus was able to spread more easily in the population than wild type [79, 96]. This was unexpected, as both in vitro and in vivo animal studies had claimed reduced virulence and transmissibility of NA inhibitor-resistant viruses before the beginning of 2007–2008 influenza season [97–99].

### 3.1 Detection of NA Inhibitor Resistance

Unlike the situation for M2 inhibitors, cell culture-based assays have not been validated for detecting phenotypic resistance in clinical isolates, partly because of the differences in cellular receptor specificity between human respiratory epithelium and available cell culture types (reviewed in [71]). In addition, these types of assays are labor intense and require an additional virus titration step, which make these assays unfavorable for high-throughput surveillance. Humanized Madin-Darby canine kidney (MDCK) cell lines that stably overexpress human 2,6-sialyltransferase (SIAT1) to increase alpha 2,6-linked sialic acids may overcome this first limitation. However, these cells have not been widely utilized to date [100, 101]. Other challenges are the broad variation in morphology of influenza plaques between different influenza types and subtypes and the reduced sensitivity of yield reduction assays. The NA enzyme inhibition phenotypic assays have, therefore, been the preferred assay to screen for clinically relevant NA inhibitor resistance mutations in influenza antiviral resistance surveillance [5, 102, 103]. Both fluorometric (MUNANA) and chemiluminescent (NA-star) type of phenotypic assays are available. Both assays have the same limitations, such as the necessity of a virus propagation step, and may therefore not reliably detect resistant subpopulations and do not detect HA-mediated NA inhibitor resistance [104]. To standardize interpretation and reporting of NA inhibitor susceptibility of influenza viruses, clear definitions were formulated in 2012 using 50% inhibitory concentration ( $IC_{50}$ ; the concentration of drug required to inhibit a standardized amount of NA activity by 50%) fold-change thresholds, compared to the median for viruses from the same type/subtype/lineage showing “normal inhibition” [105, 106].

Besides the phenotypic resistance assay, numerous genotypic PCR-based resistance assays have been developed for detection of previously identified antiviral resistance mutations in NA [107–110]. As compared to the phenotypic assays, these types of assays are rapid and easy to perform, and they allow minor variant detection (~1–5% of the quasi-species) with no requirement of an additional virus culture step. Unknown resistance patterns in newly emerging influenza subtypes or novel NA inhibitors cannot be identified using PCR-based resistance assays.

The typical NA mutations conferring resistance depends on the drug and NA subtype [72, 74, 111, 112]. For oseltamivir, His274Tyr (based on N2 numbering) confers resistance in N1 [113], whereas Arg292Lys and Glu119Val are the most common antiviral resistance mutations in N2-containing viruses (Table 71.4). Because of the differences in interaction among drugs with the active enzyme site, varying patterns of cross-resistance are found for particular NA mutations.

**Table 71.4** Effects of NA mutations that confer oseltamivir resistance on viral fitness measures in clinical isolates of influenza

Virus (ref)	Mutation	Enzyme activity or stability (% of parental virus)	Infectivity in mice/ferret	Replication in ferret	Transmissibility in ferret
A/H3N2 Yen et al. [138] Herlocher et al. [98]	Glu 119 Val	↓	↓ (>10–100-fold)/ <sup>a</sup>	– <sup>a</sup>	–
A/H3N2 Yen et al. [138] Herlocher et al. [165] Carr et al. [97]	Arg 292 Lys	↓↓ (2%)	↓ (>100-fold)/↓ (>100-fold)	↓↓ Reversion to wild type observed	0 or ↓↓
A/H1N1 Ives et al. [163] Herlocher et al. [98]	His 274 Tyr	–	↓ (>1000-fold)/↓ (≥100-fold)	– or ↓	– 1–2 days delay
A/H5N1 Le et al. [130]	His 274 Tyr	NR	NR/NR	↓	NR
B Mishin AAC [228]	Asp198Asn	NR	NR	–	NR
B Gubareva et al. [12] Jackson et al. [112]	Arg 152 Lys	↓↓ (3–5%)	NR/↓	↓	NR
2009 A/H1N1 [13, 166, 167]	His 275Tyr	–	–/↓	–/↓	/↓
2009 A/H1N1 [229]	Ile223Arg	↓ (50%)	–	–	–
A/H7N9 Yen et al. [230]	Arg292Lys	↓	–	–	– Reversion to wild type observed

Abbreviations: – no change compared to wild type, ↓ decreased, O absent, NR not reported

<sup>a</sup>Days of fever in ferrets exposed to the parental A/H3N2 virus was greater than in ferrets exposed to the E119V mutant virus (≥2 days vs. 1 day, respectively; P > .05).

Importantly, zanamivir and laninamivir retain full inhibitory activity against variants with either the His274Tyr or Glu119Val mutation and partial activity against the Arg292Lys variant [114]. Viruses with a His274Tyr are also cross-resistant to peramivir. Antiviral resistance may be caused by a single resistance mutation or a combination of additional mutations, which may enhance the level resistance and/or causes multidrug resistance [115–117].

HA binding efficiency and associated susceptibility to NA inhibitors are affected by amino acid changes in the receptor binding [112]. Consequently, HA mutations have been looked for in clinical isolates usually by comparing the sequence of pre- and post-therapy isolates and in some instances by examining changes in receptor affinity [10, 11]. HA variants that have reduced receptor affinity show cross-resistance in vitro to all NA inhibitors but in general retain susceptibility to NA inhibitors in vitro and in animal models [118–120].

### 3.2 Drug Susceptibility of Circulating Viruses

With the exception of the influenza seasons between 2007 and 2009 when the oseltamivir-resistant A/H1N1 viruses were circulating, the global incidence of circulating A and B viruses with de novo resistance to the NA inhibitors has

been very low since the approval of these drugs (Table 71.5) [9, 103, 106, 121]. A recent study in which 10,641 viruses were collected globally in 2013–2014 by collaborating National Influenza Centers to determine IC<sub>50</sub> data for NA inhibitors oseltamivir, zanamivir, peramivir, and laninamivir 172 viruses (1.6%) showed highly reduced inhibition (>100-fold) against at least one of the four drugs and 32 viruses (0.3%) with only reduced inhibition (between 10- and 100-fold reduction) [106]. Most of these highly resistant isolates were 2009 pandemic influenza A/H1N1 viruses with a His274Tyr amino acid change (*n* = 169). Only a single resistant A/H3N2 virus was detected, which carried a Glu119Val amino acid change. Two influenza B viruses with and Glu119Gly (B/Victoria) and His273Tyr (B/Yamagata) were detected. In a recent global observational multicenter clinical trial (IRIS) with follow-up sampling of influenza-infected patients after admission to a clinic (2009–2013; *n* = 1799), no genotypic resistance was detected at baseline in respiratory specimens of influenza A or B virus-infected patients apart from the A/H1N1 viruses with an inherited His275Tyr amino acid change [103]. In 19 of 1014 patients (1.9%) receiving an antiviral, emergence of resistance to oseltamivir could be detected during treatment, in most cases children below the age of 5 (*n* = 14; 74%). In 17 of these cases, a 2009 A/H1N1 His274Tyr amino acid change was detected. In two oseltamivir-treated children with



**Table 71.5** Representative studies of oseltamivir and zanamivir susceptibility of field isolates of influenza A and B viruses

Study	Location	Seasons	Assay	No. tested	No. (%) resistant	Mutations detected
McKimm-Breschkin et al. [215]	Worldwide	1999–2002	NAI-FA, NAI-CL, S	139 A/N1	0	
				767 A/N2	0	
				148 B	0	
Hurt et al. [216]	Australia, Southeast Asia	1998–2002	NAI-FA	235 A/N1	0	
				169 A/N2	0	
				128 B	0 <sup>a</sup>	
Bovin and Goyette [217]	Canada	1999–2000	NAI-CL	38 H3N2	0	
				40 H2N1	0	
				23 B	0	
Mungall et al. [218]	Worldwide	2000–2002	NAI-CL	567 A/N2	0	
				271 A/N1	0	
				712 B	0	
Monto et al. 2006 [143]	Worldwide	1999–2002	NAI-CL, S	922 A/N2	3 (0.3%)	Gln41Gly, Gln226His
				622 A/N1	3 (0.5%)	His274Tyr, Tyr155His, Gly248Arg
				743 B	2 (0.3%)	Asp198Glu, Ile222Thr
Ferraris et al. [219]	France	2002–2005	NAI-FA, S	788 H3N2	0 <sup>b</sup>	
NISN WER [220]	Japan	2003–2004	NAI-CL, S	1180 H3N2	3 (0.3%)	2 Glu119Val, 1 Arg292Lys
				171 B	0	
Hatakeyama et al. [221]	Japan	2004–2005	NAI-FA, S	422 B	7 (1.7%)	3 Asp198Asn, 3 Ile222Thr, 1 Ser250Gly
NISN WER [222]	Japan	2004–2005	NAI-CL, S	558 H3N2	0	4 His274Tyr
		2005–2006	S	60 H1N1	0	
				251 H3N2	0	
				178 H1N1	4	
Whitley et al. [103]	Worldwide	2009–2013	NAI-CL, S, PCR	335 H3N2	0	47 His274Tyr
				47 sH1N1	100	
				889 2009H1N1	0	
				518 B	0	
Meijer et al. [121]	Worldwide	2012–2013	NAI-FA, S	2343 H1N1	18 (<0.1%)	18 His274Tyr
				5109 H3N2	4 (<0.1%)	3 Glu119Val, 1 Arg292Lys
				3935 B	2 (<0.1%)	2 His273Tyr
Takashita et al. [106]	Worldwide	2013–2014	NAI-FA, S	5152 H1N1	169 (3.3%)	169 His274Tyr
				2574 H3N2	1 (<0.1%)	1 Glu119Val
				2915 B	1 (<0.1%)	1 His273Tyr

*Abbreviations:* NAI neuraminidase inhibition, CL chemiluminescence, FA fluorescence, S sequence analysis of neuraminidase gene, PCR polymerase chain reaction

<sup>a</sup>One B/Perth/211/2001 isolate had ninefold reduced susceptibility to zanamivir and 14-fold to oseltamivir compared to the mean inhibitory concentrations of influenza B strains and contained a mixed population including resistant variants with a Asp197Glu mutation [79]

<sup>b</sup>Four isolates (0.5%) with NA deficiency were found to be resistant to NA inhibitors in cell culture-based assays

an A/H3N2 virus infection, an Arg292Lys change emerged posttreatment. Although the incidence of NA inhibitor-resistant viruses is currently low, the occasional clusters of 2009 oseltamivir-resistant influenza A/H1N1 viruses with an His274Tyr are a reason for concern [122–124]. Resistance to zanamivir was reported due to an amino change Gln136Lys [125, 126]. The presence of this mutation, however, may be caused by an artifact propagation of the virus in Madin-Darby canine kidney (MDCK) cell cultures [127]. With regard to the highly pathogenic avian influenza A/H5N1 viruses and low-pathogenic avian influenza A/H7N9 viruses, these are susceptible to the NA inhibitors [128].

Like A/H1N1 influenza viruses, amino acid changes at 119, 274, and 294 were found in 2.4% of human and 0.8% of avian A/H5N1 virus sequences, which were deposited to GenBank [33]. Additionally, markers of reduced NA inhibitor susceptibility at amino acid positions 116, 117, 150, 222, and 246 were found in 0.8% of human and 2.9% of avian A/H5N1 isolates [129]. Although the His275Tyr change has been the major antiviral resistance pattern found in highly pathogenic avian influenza A/H5N1 viruses [130–133], a A/H5N1 isolate was reported [130], with an Asp295Ser amino acid change causing an 80-fold and sevenfold increase of the IC<sub>50</sub> for oseltamivir and zanamivir, respectively [134–

137]. This Asn295Ser change has also been observed in A/H5N1 virus isolates. The emergence of an Arg292Lys amino acid change in the low-pathogenic avian influenza A/H7N9 viruses circulating in China since 2013 causes high NA inhibitor resistance to oseltamivir and peramivir and reduced resistance to zanamivir [138, 139]. Unlike A/H3N2 viruses carrying the Arg292Lys amino acid change, A/H7N9 virus does not seem to be much attenuated by this change [128, 140]. Like the influenza A viruses, NA inhibitor resistance in influenza B viruses is currently low [141]. Nevertheless, several oseltamivir-resistant B viruses have been isolated from treated or untreated patients [142–144]. Antiviral resistance to neuraminidase may be caused by changes at residues Asp198 and Ser250. In addition, also influenza B viruses have been found with an Ile221 [144, 145]. These mutations cause only a two- to threefold increase in IC<sub>50</sub> to oseltamivir, zanamivir, and peramivir.

### 3.2.1 Immunocompetent Hosts

In natural infections, oseltamivir-resistant variants have been detected much more commonly in treated children than adults (Table 71.6). In the past, analysis of samples from over 2500 influenza patients treated with oseltamivir as outpatients indicated that the frequency of resistance detection is about 0.4% in adults and about 4.5% in children [146]. Similar observations were made more recently in the IRIS trial where 14 of 19 oseltamivir-treated outpatients with resistance development were children aged below 5. The higher level of replication with longer duration of virus shedding increases the chance of developing antiviral resistance as compared to adults. Two studies in Japanese children reported high frequencies of 16 and 18% oseltamivir resistance emergence during oseltamivir therapy [8, 147]. The use of weight-based dosing for children in Japan, as contrasted with unit dosing in most countries, is associated with lower drug exposure in young children. This has been postulated to be a major factor in the higher frequency of resistance detected in these studies. Among 54 volunteers experimentally infected with an A/H1N1 virus, oseltamivir-resistant variants with His274Tyr mutation were detected in two subjects in association with apparent rebounds in viral replication [148]. This study found that oseltamivir-treated subjects were less likely than placebo to have late viral isolates showing reversion of the egg-adapted inoculum virus to a human receptor HA genotype. The His274Tyr finding suggests that HA mutations with reduced affinity for human receptors might have a replication advantage over viruses with human receptor preference during oseltamivir use in humans. Interestingly, amino acid changes in the HA of the influenza A/H1N1 viruses prior to the emergence of the oseltamivir-resistant A/H1N1 virus in the 2007–2008 season have been predicted to have facilitated the emergence of the His274Tyr amino acid change [149, 150].

### 3.2.2 Immunocompromised Hosts

Immunocompromised individuals tend to suffer from influenza longer with more serious complications than otherwise healthy patients [151–154]. Since immunocompromised patients are more likely to acquire influenza [155], showing relatively high influenza-associated mortality [10, 11, 153], effective antiviral is crucial for these patients. Like with influenza and young children, the higher level of replication with longer duration of virus shedding in immunocompromised patients increases the chance of developing antiviral resistance [156]. Several recent clinical studies have reported that the emergence of antiviral resistance among treated immunocompromised patients is not uncommon [151, 157, 158]. Recently, a prospective clinical study aimed to study antiviral resistance in immunocompromised patients ( $n=24$ ); a resistance prevalence of 17% (4/24) was reported [158]. In all four cases the NA His275Tyr was detected by RT-PCR of 2009 influenza A/H1N1 virus-infected patients. In other retrospective studies, similar rates have been reported [151, 157]. The NA His275Tyr amino acid change has been described frequently during the 2009 influenza A/H1N1 virus pandemic in case reports of antiviral-treated immunocompromised patients [117, 159]. Amino acid changes at position 223 have also been reported to cause increased levels of resistance (48-fold) to oseltamivir. The impact on therapy is unclear for such moderate increase in oseltamivir resistance; however viruses with the combination of Ile223Arg and His275Tyr are highly resistant to oseltamivir in vitro (1750-fold) [160]. In the past, emergence of resistance in immunocompromised patients has been also described for influenza A/H3N2 and influenza B virus-infected patients treated with oseltamivir and zanamivir with mutations in both the viral HA and NA glycoproteins [8, 10, 12, 161]. Most fatal cases during influenza pandemics and seasonal epidemics are patients belonging to the traditional high-risk groups for developing severe disease, including the very young children, elderly, and immunocompromised patients [162]. Given the high mortality and morbidity, the moderate effectiveness of current antivirals, and the relatively high prevalence of resistance in immunocompromised patient, better treatment strategies are clearly needed for these patients.

### 3.3 Pathogenicity and Transmissibility of Resistant Variant

Before the 2007–2008 influenza season, it was thought that NA inhibitor resistance development was to go hand in hand with reduction of virus fitness [97, 163]. Mathematical modeling predicted a 10% relative transmissibility of oseltamivir-resistant variants would result in low levels of resistant viruses circulating in the community [164]. Based

**Table 71.6** Frequency of resistance emergence to oseltamivir or zanamivir during treatment

Drug/study	Population	Assay	Virus type	No. isolates tested	No. (%) resistant	Mutations detected
<i>Oseltamivir</i>						
Gubareva et al. [148]	Adults	NAI, S	A/H1N1	54	2 (4%)	2 His274 Tyr
Roberts [146]	Adults	NAI, S	A/H3N2	418	5 (1%)	4 Arg292Lys, 1 Glu119Val
Whitley et al. [223] <sup>a</sup>	Children—outpatient	NAI, S	A&B	150 A	10 (6.7%)	8 Arg292Lys, 1 Glu119Val, 1 His274Tyr
				66 B	0	
Kiso et al. [8] <sup>a</sup>	Children—outpatient + hospitalized	Cloning + S	A/H3N2	50	9 (18%)	6 Arg292Lys, 2 Glu119Val, 1 Asn294Ser
Ward et al. [224] <sup>a</sup>	Children—outpatient + hospitalized	NAI, S	B	74	7 (16%)	7 His274Tyr
	Children—outpatient	NAI, S			1 (1.4%)	Gly402ser
Whitley et al. [103]	Children + adults outpatient	NAI, S	A&B	759 A	19 (2.5%)	17 His275Tyr, 2 Arg292Lys
				256 B		
Hatekayama et al. [221]	Children—outpatient	NAI, S	B	77	0	1 Gly402Ser
Stephensen et al. [225]	Children—outpatient	NAI, S	A&B	43 A	1 (1.3%)	3 His275Tyr, 1 Arg292Lys
				19 B		
Harvala et al. [227]	Children—outpatient	NAI, S	A	32 A	4 (7.4%)	5 His275Tyr
					0	
Tramontana et al. [226]	Adults + children hospitalized	PCR	A	30 A	5 (15.6%)	4 His275Tyr
	Adults—hospitalized	NAI, S			4 (13.3%)	
<i>Zanamivir</i>						
Barnett et al. [88]	Adults	NAI, S	A + B	41	0	

<sup>a</sup>These pediatric studies used a 2 mg/kg dose of oseltamivir that has been shown to give reduced drug exposure because of more rapid clearance in children under the age of 5 years. Insufficient drug exposure may have contributed to resistance emergence in these studies

on animal experiments, the reduced fitness and replication competence of certain NA resistance mutations appeared to be depending on virus subtype and resistance mutation. For instance, an oseltamivir-resistant influenza A/H3N2 virus with an Arg292Lysine amino acid change did not transmit between infected and naïve ferrets and showed a 10–100-fold reduction in nasal virus titers [165]. For the Glu119Val oseltamivir-resistant mutant, however, it was found that the mutant was as transmissible as wild type with comparable nasal virus titers in both donor and recipient animals [98]. An influenza A/H1N1 virus with a His275Y Tyr mutation required 100-fold higher inoculum to infect the donor ferret, but once infected, they transmitted the virus to contact animals with a delay of 1–3 days compared to wild-type virus. Early after the outbreak of the 2009 pandemic, it was questioned whether a His275Tyr oseltamivir-resistant mutant would be attenuated [13, 166, 167]. In vitro replication and in vivo pathogenicity studies were performed using resistant isolates; however, the answers were conflicting. Some researchers found slight attenuation of the early His275Tyr mutant A/H1N1 viruses [166], while others did not find such differences [167]. At most, from these conflicting data, it can be concluded that the differences between a wild-type 2009 pandemic A/H1N1 virus and its

His275Tyr-resistant counterpart are too close to call by means of its pathogenicity and transmissibility [74]. Additional compensatory mutations may facilitate the emergence of NA inhibitor resistance mutations, which cause an initial loss of virus fitness [96]. For instance, for the His275Tyr amino acid change in 2007–2009 A/H1N1 viruses, several permissive amino acid changes have been suggested to have facilitated the emergence of this oseltamivir resistance change. The Asp344Asn amino acid change, which appeared before the 2007–2008 season, had increased the enzymatic properties of NA prior to the introduction of the His275Tyr amino acid change [113, 168]. Amino acid changes Val234Met and Arg222Gln maintained high NA expression in vitro, which was reduced if the single His275Tyr was expressed [80, 169]. In A/H3N2, compensatory roles for amino acid changes at position 222 have been assigned to compensate for the loss of fitness due to the Glu119Val oseltamivir resistance mutation [170, 171]. The observed community clusters of 2009 A/H1N1 viruses with a His275Tyr amino acid change do not seem to be attenuated by the His275Tyr amino acid change either [122]. These viruses contain, in addition to the His275Tyr change, changes at amino acid positions 241, 369, and 386. These mutations may also have permissive effects [122].

### 3.4 Treatment Alternatives

The patterns of NA inhibitor cross-resistance vary by virus type and subtype, such that zanamivir retains inhibitory activity for the most common resistant variants that emerge during the therapeutic use of oseltamivir or peramivir. Zanamivir is fully inhibitory for oseltamivir-resistant variants possessing the Glu119Val substitution in N2 or His275Tyr or Asn294Ser in N1 [102, 172]. Depending on the virus and assay, zanamivir is partially inhibitory for resistant variants with Arg292Lys substitution in N2, in that the loss of susceptibility is about 5–25-fold compared to the wild type [102, 172–174]. There is controversy about the role of peramivir in the management of variants that are resistant to oseltamivir as *in vitro* and *in vivo* models have given conflicting results [175–177]. Oseltamivir is not inhibitory for the Arg152Lys mutation in influenza B NA that confers reduced susceptibility to zanamivir [178].

Given these findings, most experts recommend using zanamivir for the treatment of patients who develop resistance or virologic failure to oseltamivir. Inhaled zanamivir has been utilized in a few patients with variable success but has not been studied systematically in oseltamivir-resistant infections; success is less likely in patients with influenza pneumonia [179–182]. Intravenous zanamivir has been utilized most frequently for patients with proven or suspected resistant influenza; while the therapy is effective for some patients, available data precludes assessing the optimal role of this intervention given the severity of illness of many patients at conversion to therapy and significant prior exposure to numerous interventions [183–186]. Other NA inhibitors and zanamivir dimers that have prolonged duration of antiviral effect after topical application are currently under development [187]. These may provide NA inhibitor prevention and perhaps treatment alternatives in the future.

Ribavirin would also be expected to be inhibitory for influenza A and B viruses resistant to the NA inhibitors, but there are no reports of its use in human influenza infections due to such variants. Ribavirin combined with a NA inhibitor exerts additive to synergistic antiviral activity *in vitro* [188]. In mice experimentally infected with influenza A, the combination of orally administered ribavirin and peramivir was associated with improved survival relative to ribavirin alone but not to peramivir alone [189]. A more recent study found that a combination of ribavirin and oseltamivir was no more effective than ribavirin alone against a lethal influenza A(H1N1) infection but superior to single agents against influenza B [189]. Further studies of such ribavirin-NA inhibitor or T-705-NA inhibitor combinations (see below) are warranted to determine whether this strategy offers the possibility of treating severe influenza, particularly that due to M2 inhibitor-resistant viruses. Recently, triple combinations of amantadine, ribavirin, and oseltamivir have been

studied *in vitro*, *in vivo*, and in humans with influenza infection [65, 66, 190]. Given the promise of this combination, a prospective phase 2 study is ongoing to assess the safety and clinical efficacy of this combination for the treatment of influenza. Combination therapy has been demonstrated to reduce the development of resistance in clinical studies and therefore may be of benefit in populations at increased risk of development of resistance emergences [54].

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## 4 Novel Agents

### 4.1 T705/Favipiravir

Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is an antiviral drug that is phosphoribosylated by cellular enzymes to its active form, favipiravir-ribofuranosyl-5'-triphosphate (RTP), and selectively inhibits the RNA-dependent RNA polymerase of influenza virus [191]. It is highly active against seasonal strains A/H1N1, A/H3N2, and influenza B; the 2009 pandemic A/H1N1 virus; highly pathogenic avian influenza virus A/H5N1 isolated from humans; A/H1N1 and A/H1N2 isolated from swine; and A/H2N2, A/H4N2, and A/H7N2. The antiviral is active against viruses that are resistant to amantadine, rimantadine, oseltamivir, and zanamivir, in addition to dually resistant viruses (M2 and NA inhibitor resistant) [191, 192]. In studies of serial passage of two seasonal (A/Brisbane/59/2007 and A/New Jersey/15/2007) and two 2009 pandemic (A/Denmark/524/2009 and A/Denmark/528/2009) A/H1N1 viruses in MDCK cell lines in the presence or absence of low concentrations of favipiravir, no favipiravir-resistant viruses were phenotypically or genotypically (PB1, PB2, PA, and NP sequencing) detected. Sequence analysis, though, did demonstrate an enrichment of G → A and C → T transversion mutations, increased mutation frequency, and a shift of the nucleotide profiles of individual NP gene clones under drug selection pressure [193]. Few clinical studies have been published with this novel compound, so the frequency of resistance emergence is not fully understood at this point. The drug is currently licensed in Japan for use selectively when approved by the Ministry of Health; studies of efficacy are ongoing in the rest of the world with the goal of seeking regulatory approval in the near future.

### 4.2 Antibodies

Recent studies have reported the development of neutralizing antibodies to specifically target conserved regions of the virus HA [194, 195]. HA binding of the antibodies was elegantly shown by X-ray crystal structures of HA-antibody protein complexes [196, 197]. These antibodies differ in

their recognition sites: Some are targeted to the sialic acid RBS and globular head, while others bind to the stalk region [195]. As the stalk region is more conserved between different HA subtypes, cross-reactive immunity against several influenza subtypes may be obtained with broadly neutralizing capacities. Although the antibodies are being developed against conserved regions of HA, mutations do arise at the antibody target sites, which may result in viral escape.

## 5 Implications and Future Research Directions

Currently, circulating strains of influenza are primarily resistant to the M2 inhibitors but are generally susceptible to the clinically available neuraminidase inhibitors. Sporadic cases of neuraminidase inhibitor resistance have been recognized, and limited regional transmission has been demonstrated [4, 44, 198]. Further, resistance in seasonal A/H1N1 became widespread during the 2008–2009 influenza season. Lastly, NA inhibitor resistance has been demonstrated to emerge during therapy in highly pathogenic avian influenza viruses that infect humans, with the highest frequency in A/H7N9 viruses [35, 36, 49]. As such, most regions of the world are currently limited to a single class of drug, the neuraminidase inhibitor, for the management of influenza infections. The risk that resistance could emerge and result in global spread poses a serious threat and requires the development of novel agents and combinations [128, 187].

Lessons learned from the 2009 pandemic suggest that there is a significantly higher frequency of antiviral resistance emergence in the pandemic virus compared to inter-pandemic influenza. Further, the clinical and epidemiologic implications of antiviral resistance in a future pandemic influenza virus cannot be predicted with confidence. As a result, the great progress made in developing global systems to rapidly monitor the susceptibility patterns of circulating strains needs to be maintained and potentially expanded to include regions with sparse surveillance [106]. Further, surveillance of resistance patterns in animals may give early warnings about future pandemic influenza viruses.

A number of unanswered questions remain regarding antiviral drug resistance in influenza viruses. With contemporary next-generation sequencing, it is possible to understand the kinetics of the emergence of resistance from minor variant populations to the predominant population in a given host. Such data can inform the optimal timing of screening and intervention. Specific risk factors beyond generic concepts, such as immunocompromised and young age, should be identified that predict the emergence of resistance. From a therapeutic perspective, the optimal approach, including the duration of therapy and the benefit of combination therapy in patients with severe illness or who are predicted to have

prolonged shedding, needs to be carefully studied. Currently, there is a significant gap in the capacity to test specimens for resistance, and as a result, many patients with potential resistance may be missed. As a result, there is a desperate need for susceptibility assays that can be utilized broadly in the clinical laboratory. Lastly, there is need for ongoing and expanded surveillance of antiviral susceptibility patterns in human and animal influenza viruses, especially community isolates in countries with higher antiviral use, and for resistance transmission in high-risk epidemiologic settings.

Given the current pattern of antiviral susceptibility in circulating strains, M2 inhibitors should not be utilized for the prevention or treatment of influenza, while any of the neuraminidase inhibitors should be considered whenever therapy is indicated. Such therapy should be started as early as possible to improve the benefit obtained from the use of the therapy. Given its slightly broader activity against most oseltamivir-resistant variants, zanamivir would be the preferred therapy for patients with proven or suspected oseltamivir-resistant influenza. Novel agents, optimally with novel mechanisms of action, need to be developed. Drugs in advance stages of development include the polymerase inhibitor favipiravir [191], the receptor-destroying sialidase DAS181 [199], and nitazoxanide [200]. Neutralizing antibodies and convalescent plasma need to be studied further to optimize the treatment of patients, particularly with novel or highly resistant viruses [201]. Lastly, combinations of antivirals should be studied to understand their ability to prevent and overcome resistance clinically [128].

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