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Review

Resistance of influenza viruses to neuraminidase inhibitors — a review

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1. Introduction

Influenza virus is a negative stranded RNA virus. It contains two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA exists as a trimer and is responsible for binding to the terminal sialic acid bound to receptors on the surface of the target cell, leading to attachment and subsequent penetration by the virus into the cell. Influenza virus isolates from different animals appear to have a preference for specific receptor linkages. Equine and avian isolates bind preferentially to the α2,3 galactose structure, while human isolates bind preferentially to the α2,6 galactose structure (Leigh et al., 1995). A single amino acid mutation is sufficient to change receptor specificity (Rogers and Paulson, 1983; Nobusawa and Nakajima, 1988; Martin et al., 1998). Sequence analysis and alignment has identified key residues conserved across all HA subtypes, which are involved in receptor binding (Nobusawa et al., 1991). The sialic acid binding site forms a groove across the top of the HA surrounded by antibody binding sites (Weis et al., 1988). Residues 134–138 form the right side of the ligand binding site, and residues 224–228 form the left side. Other conserved residues appear to play a role in orienting several of the surface atoms for binding to the sialic acid, these include Tyr 98, Trp 153, His 183, Glu 190, Leu 194 and Tyr at 195 (Weis et al., 1988; Nobusawa et al., 1991).

After replication of the virus, progeny virions bud from the cell surface. NA is thought to be responsible for cleavage of terminal sialic acid moieties from receptors, to facilitate elution of progeny virions from the infected cell. Since they are also glycosylated, newly synthesized HA and NA on virions may also contain sialic acid residues on their oligosaccharide chains. Removal of these terminal sugars is therefore also necessary to prevent self-aggregation, due to the HA of one virion binding to the sialic acids on an adjacent

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virion. NA may also play a role in enabling the virus to penetrate the mucin layer in the respiratory tract.

Structural analysis of NA of influenza virus (Varghese et al., 1983) revealed it was a tetramer, and that there were several invariant residues in both influenza A and B which formed part of the active site. There are nine conserved residues which contact the sialic acid, and there are a further ten which provide substructure or a scaffold, on which amino acids contacting the bound sugar are supported (Burmeister et al., 1992; Varghese et al., 1992). The conserved residues include three arginines at positions 118, 292 and 371, which interact with the carboxylate of the sugar. Other important residues include the hydroxyl groups of the glycerol side chain which are hydrogen bonded to Glu 276 and the 4-hydroxyl group on the sugar is directed towards Glu 119 (Varghese et al., 1992). Based on the structure of NA in complex with sialic acid (Varghese et al., 1992) a program of inhibitor design was begun, based on the earlier work of Meindl and Tuppy who first synthesized the unsaturated sialic acid analog, Neu5Ac2en (2-deoxy-2,3-didehydro-D-*N*-acetylneuraminic acid or DANA) (Meindl and Tuppy, 1969). DANA is a weak inhibitor of NA enzyme activity and demonstrates weak antiviral activity in vitro, but not in vivo when administered intraperitoneally (Palese and Schulman, 1977). Two new inhibitors were designed, substituted at the 4 position, 4-amino-Neu5Ac2en, and 4-guanidino-Neu5Ac2en, zanamivir, (von Itzstein et al., 1993). Zanamivir has a 100-fold higher affinity than the 4-amino-Neu5Ac2en, which in turn is around 100-fold more effective than DANA. The 4-amino group is predicted to form a strong salt bridge with the acid group of Glu 119 and the 4-guanidino group is predicted to interact not only with Glu 119, but also with Glu 227 (von Itzstein et al., 1993) accounting for the further increase in its affinity. Zanamivir is not just an effective inhibitor of the viral enzyme, but is a potent inhibitor of virus replication in both cell culture and animals (von Itzstein et al., 1993; Woods et al., 1993). The inhibitor prevents release of progeny virions from the infected cells, and in vivo is only effective when applied at the site of infection, either by inhalation or intranasal instillation (von Itzstein et al., 1993; Hayden et al., 1996, 1997) since orally administered drug is rapidly cleared by the kidneys (Woods et al., 1993). Zanamivir has now been approved for general use in the USA, Europe, Australasia and Japan.

Based on the efficacy of zanamivir, other neuraminidase inhibitors are also being developed. GS4071, oseltamivir carboxylate, the active form of the ethyl ester prodrug GS4104, oseltamivir phosphate, is a potent carbocylic inhibitor with a cyclohexene scaffolding (Li et al., 1998). In addition, oseltamivir contains a bulky lipophilic side chain, a pentyl ether, at the 6-position, instead of the glycerol group in zanamivir, and is orally active when administered as the ethyl ester prodrug, oseltamivir phosphate, which undergoes activation by hepatic esterases. It has been approved for marketing in the USA and Sweden. Johnson and Johnson are developing an orally active cyclopentane derivative from Biocryst Pharmaceuticals (Bantia et al., 1999a,b) which has both the guanidinium group as well as a bulky hydrophobic side chain in a position sterically corresponding to the glycerol side chain in sialic acid.

However, as with any new anti-infective it is important to establish whether resistance to these new inhibitors arises readily. There have now been several publications in this field, predominantly on the characterization of laboratory generated mutants, unexpectedly the mechanisms of resistance are complex. As the drugs are now available for clinical use the aim of this paper is to review the current available data on resistance, to provide some insight into the challenge faced in trying to evaluate clinical isolates for possible resistance.

2. Generation of mutants

Mutants have been generated in vitro in Madin Darby Canine Kidney, MDCK cells by a combination of limit dilution passaging and plaque purification under increasing concentrations of inhibitor (Blick et al., 1995; Staschke et al., 1995; Gubareva et al., 1996a,b; McKimm-Breschkin et

al., 1996a,b; Penn et al., 1996; Gubareva et al., 1997; Tai et al., 1998; McKimm-Breschkin et al., 1998; Barnett et al., 1999; Carr et al., 1999). In each case, it has taken many passages before resistant variants have been selected, unlike amantadine in which resistant variants can be selected after a single passage in vitro or directly from amantadine-treated patients (Grambas et al., 1992; Houck et al., 1995; Englund et al., 1998).

Mutations have arisen in the laboratory in previously conserved residues in NA in both structural (Staschke et al., 1995; Blick et al., 1995; Gubareva et al., 1996a,b, 1997; Carr et al., 1999; Barnett et al., 1999) and catalytic residues (Gubareva et al., 1997; McKimm-Breschkin et al., 1998; Tai et al., 1998).

However, the predominant mutations generated in vitro map to the virus HA gene, in residues proximal to those involved in receptor binding (McKimm-Breschkin et al., 1996a,b; Blick et al., 1998; McKimm-Breschkin et al., 1998). The mechanism of resistance appears to be as a result of a decrease in the affinity of binding of HA to the cellular receptor, so that there is less dependence on the activity of NA for elution of virus from cells (McKimm-Breschkin et al., 1996a,b). These results have highlighted the importance to the virus of maintaining a balance between the strength of HA binding to receptors, and the efficiency of NA in removing them.

As yet there have been no resistant mutants isolated from normal patients treated with zanamivir. However, a drug resistant influenza B virus was isolated from an immunocompromised patient treated with zanamivir (Gubareva et al., 1998). This virus initially acquired an HA mutation near residues known to be involved in receptor binding. After further treatment a double mutant was isolated, with a mutation in NA, Arg 152 Lys. The Arg 152 is thought to play a structural role, but the mechanism of resistance is not yet clear.

There have been three different mutants isolated from patients treated with oseltamivir phosphate (oseltamivir pack insert). The catalytic mutation, Arg 292 Lys, seen in vitro (Gubareva et al., 1997; McKimm-Breschkin et al., 1998; Tai et al., 1998) has been isolated from four patients (Covington et al., 1999). A second mutation of Glu 119 Val isolated after in vitro selection with oseltamivir, has also been isolated from one patient treated with oseltamivir phosphate (Covington et al., 1999). Information is not yet available on the third mutant.

Mutants have been tested for altered drug sensitivity in either an enzyme inhibition assay or in a plaque reduction assay (von Itzstein et al., 1993; Bethell et al., 1999; McKimm-Breschkin et al., 1999c). As the inhibitor prevents release of progeny virions, the effect in a plaque assay is initially to decrease the size of plaques, therefore, some laboratories have used a decrease in plaque size to determine drug sensitivity, (Blick et al., 1995; McKimm-Breschkin et al., 1996a,b; Sahasrabudhe et al., 1998) whilst others have used a decrease in plaque number (Staschke et al., 1995; Gubareva et al., 1996a; Penn et al., 1996). The latter generally requires a significantly higher concentration of drug to achieve an IC_{50}. While this assay has been used for characterization of laboratory generated mutants of influenza, many clinical isolates do not plaque well, hence the plaque reduction assay is not universally applicable for monitoring resistance of clinical specimens (Tisdale, 2000). Sensitivity in a $TCID_{50}$ assay may be used for viruses which plaque poorly (McKimm-Breschkin et al., 1999c). Data has now accumulated from different laboratories using different methods of characterization, which are reviewed here to enable comparisons of properties.

3. Neuraminidase mutations

3.1. Mutations at Glutamic acid 119

Four different mutations at residue Glu 119 have been selected in several different strains of both influenza A and B in vitro after passaging in zanamivir (Blick et al., 1995; Staschke et al., 1995; Gubareva et al., 1996a,b, 1997; Barnett et al., 1999) and in vivo from a patient in an oseltamivir phosphate treatment study (Carr et al., 1999).

Glu 119 Gly was isolated in the strain NWS/G70C, a reassortant containing the HA of A/NWS/33 and NA of A/Tern/Australia/G70C/75

(H1N9), (Blick et al., 1995), A/Turkey/Minnesota/833/80 (A/Turkey/Minn, H4N2) (Gubareva et al., 1996a,b, 1997), the B/HK/Lee strain, a reassortant with the HA of B/HK/8/73 and NA of B/Lee/40 (Staschke et al., 1995), and B/Beijing/1/87 (B/Beijing) (Barnett et al., 1999). In enzyme inhibition assays there was a decrease in binding of zanamivir, but there was no significant effect on binding 4-amino-Neu5Ac2en (Table 1). The inhibitor no longer bound slowly, as seen in the wild type (Blick et al., 1995; Gubareva et al., 1996a,b; Bethell et al., 1999). Structural analysis shows that the loss in affinity of the mutant enzyme for zanamivir derives in part from the loss of stabilizing interactions between the guanidino moiety and the carboxylate residue at 119, and in part from alterations to the solvent structure at the active site. A water molecule occupies the position where the carboxylate was in the glutamate (Blick et al., 1995). While the carboxylate of Glu 119 is involved in binding to the 4-guanidino group in zanamivir (von Itzstein et al., 1993; Varghese et al., 1995), it does not directly interact with the amino group at the 4-position. Recently it was reported that under certain assay conditions the A/Turkey/Minn Glu 119 Gly was not resistant to the new Biocryst inhibitor, despite the latter having a guanidinium group in a position sterically corresponding to the 4-guanidino in zanamivir (Gubareva et al., 1999). The solvent structure may be different in this case, but determination of the structure of a Glu 119 Gly NA with the Biocryst compound will lead to a better understanding of the interactions involved.

A further two mutations Glu 119 Ala, and Glu 119 Asp were also described from in vitro passaging of A/Turkey/Minn (Gubareva et al., 1996a,b). In enzyme assays, these both exhibited resistance to 4-amino-Neu5Ac2en (Table 1), suggesting that they may also have altered binding to oseltamivir carboxylate, which has an amino group at the 4-position. Consistent with this observation is the recent report of a Glu 119 Val mutation leading to oseltamivir resistance in the human strain A/Wuhan/359/95 (H3N2). The structural consequences of the other 119 mutations have not been determined, but altered binding may be as a consequence of the water molecule occupying the position previously occupied by the carboxylate of Glu 119 (Blick et al., 1995). The Asp, Val and Ala

Table 1
Effect of neuraminidase mutations at Glutamic acid 119 on sensitivity to different inhibitors in an enzyme inhibition assay[a]

Virus	Fold decrease in sensitivity				
	Mutation	DANA	4-Amino-neu5Ac2en	Zanamivir	Oseltamivir carboxylate
NWS/G70C[b]	Glu 119 Gly	10	0	250	0
rec G70C[c]	Glu 119 Gly	–	–	1000	–
	Glu 119 Ala	–	–	340	–
A/Turkey/Minn[d]	Glu 119 Gly	10	3	700	–
	Glu 119 Ala	7	35	600	–
	Gly 119 Asp	20	200	2500	–
B/HK/Lee[e]	Glu 119 Gly	Resist		500	–
B/Beijing/87 [f]	–	–	–	33	–
A/Wuhan/95[g]	Glu 119 Val	–	–	–	20

[a] All assays based on 2′-(4-methylumbelliferyl)-α-D-*N*-acetyl neuraminic acid, (MUNANA) as substrate, except e, which used a fetuin based assay.
[b] (Blick et al., 1995).
[c] Recombinant G70C NA (Goto et al., 1997).
[d] Gubareva et al. (1996a,b, 1997).
[e] Staschke et al. (1995).
[f] Barnett et al. (1999).
[g] Carr et al. (1999).

are all larger amino acids than Gly, and they may not as readily accommodate the water molecule. This may result in displacement of the 4-amino substituted inhibitors, resulting in greater resistance with increasing size of the substituted amino acid.

In plaque assays (Table 2) the magnitude of resistance is at least one-tenth of that observed in an enzyme inhibition assay. The sensitivity in a plaque assay is also complicated as if there is a concomitant HA mutation, NA and HA mutations can act synergistically (Blick et al., 1998). To determine the role of Glu 119 Ala the A/Turkey/Minn virus containing both NA and HA mutations was reassorted into another wild type virus, the A/NWS/1/33 (H1N1) strain (Gubareva et al., 1997) to produce an NWS/Turkey (H1N2) reassortant. However, while the reassortant with only the NA mutation still appeared to be 1000-fold resistant, the combination of the wild type NWS HA and the Turkey NA gave 'natural' resistance of 50-fold compared to the A/Turkey/Minn wild type (Table 2). Hence the resistance due to the Glu 119 Ala compared to the reassortant wild type was only 20-fold. Thus, to determine the role of individual mutations it is critical to compare the reassortants to the homologous wild type pairing of HA and NA. Alternatively one can reassort back into the original parental wild type, so that HA and NA are still matched (Blick et al., 1998; McKimm-Breschkin et al., 1998).

Mutations at Glu 119 have an adverse effect on the stability of the enzyme (McKimm-Breschkin et al., 1996c; Colacino et al., 1997; Sahasrabudhe et al., 1998). While the specific activity for the Glu 119 Gly NA is the same as wild type NA (McKimm-Breschkin et al., 1996c) the protein is more unstable. This initially led to the wrong conclusion that the Glu 119 Gly enzyme had a lower specific activity based on activity per total protein which contained significant amounts of inactivated NA (Table 3) (Staschke et al., 1995). Use of a conformational specific monoclonal or polyclonal anti-NA antibody should be used to determine the amount of native protein for more accurate determinations of specific activity (McKimm-Breschkin et al., 1996c, 1999c. Also, care should be exercised in handling viruses which may have an unstable NA.

Table 2
Fold decrease in sensitivity of different Glutamic acid 119 neuraminidase mutants to zanamivir in plaque assay, based on reduction in plaque size

Virus	Mutation		Fold decrease in sensitivity to zanamivir
	NA	HA	
NWS/G70C[a]	Glu 119 Gly		10
A/Turkey/Minn[b]	Glu 119 Ala	HA1 Tyr 234 Leu HA2 Ala 35 Thr Lys 68 Arg	10 000[c]
NWS/Turkey	Wt.		50[d]
	Glu 119 Ala		1000[e]
	Glu 119 Ala		20[f]
B/HK/Lee[g]	Glu 119 Gly	Asn 145 Ser Asn 150 Ser	100[c]

[a] Blick et al. (1998).
[b] Gubareva et al. (1996a, 1997).
[c] Resistance due to both NA and HA mutations.
[d] Natural resistance due to reassorting of wild type Turkey NA and NWS HA, compared to the A/Turkey/Minn parent.
[e] Resistance compared to the A/Turkey/Minn parent.
[f] Resistance compared to homologous reassortant NWS/Turkey parent.
[g] Staschke et al. (1995).

Table 3
Properties of the neuraminidase of different Glutamic acid 119 mutants

Virus	Mutation	Reported specific activity	Stability
NWS/G70C[a]	Glu 119 Gly	Wt.	Unstable
NWS/G70C[b]	Glu 119 Gly	5%	Unstable
rec G70C[c]	Glu 119 Gly	30%	Low specific activity probably due to instability
A/Turkey/Minn[d]	Glu 119 Gly	46%	Unstable to high temp, and low pH
	Glu 119 Asp	3%	Unstable to high temp and low pH
	Glu 119 Ala	23%	Unstable to high temp and low pH
B/HK/Lee[b]	Glu 119 Gly	0.6%	Unstable

[a] McKimm-Breschkin et al. (1996c).
[b] Staschke et al. (1995), based on total protein, including inactive NA.
[c] Goto et al. (1997).
[d] Gubareva et al. (1997).

Whilst Glu 119 Asp would seem to be the most conservative mutation, it has the greatest effect on both drug resistance as discussed above, (Table 1) as well as on specific activity (Table 3) (Gubareva et al., 1997). All 119 mutations in the A/Turkey/Minn virus lead to altered thermal and pH stability. The effect of the Glu 119 Val mutation on stability has not been published, but it is reported to bind substrate with a two-fold greater affinity (Carr et al., 1999), which would be consistent with valine producing a more stable molecule than glycine.

Growth of the Glu 119 Gly NWS/G70C virus is compromised in cell culture, producing smaller plaques than wild type (Blick et al., 1998), and showing a slight delay in growth kinetics, although eventually reaching the same titers as wild type virus (Staschke et al., 1995). Instability of the NA may compromise growth, as mutant plaques can be rescued by the presence of exogenous *Clostridium* NA in the overlay (Blick et al., 1998). Although the A/Turkey/Minn and B/HK/Lee mutants also exhibited lower apparent enzyme activities their growth in cell culture was reported to be comparable to wild type virus (Staschke et al., 1995; Gubareva et al., 1997). However, each of these mutants had a concomitant HA mutation, which have been shown to rescue poor growth due to low NA activity (McKimm-Breschkin et al., 1998), hence no conclusions can be drawn regarding the sole effect of the NA mutation on growth in these viruses.

3.2. Mutation at Arginine 292 to Lysine

Sialic acid, zanamivir and 4-amino-Neu5Ac2en contain a glycerol side chain at the 6 position of the sugar ring, while other inhibitors contain a bulkier more hydrophobic group such as a carboxamide (Taylor et al., 1998; Smith et al., 1998, 1999), or the pentyl ether in oseltamivir carboxylate (Kim et al., 1998). A small change in the active site of the NA occurs to enable these inhibitors to be accommodated. Glu 276 changes its position to form a salt link with Arg 224, and thereby creates the necessary hydrophobic pocket for the binding of the bulkier substituents. Passaging of the NWS/G70C virus in the 6-carboxamide derivative of zanamivir generated a series of mutants with various HA mutations, but an Arg 292 Lys NA mutation was also selected (McKimm-Breschkin et al., 1998). The same NA mutation was generated after passaging A/Victoria/3/75 (A/Vic/75 H3N2) in oseltamivir carboxylate in vitro Tai et al. 1998) and from patients treated with oseltamivir phosphate (Covington et al., 1999). Passaging of the A/Turkey/Minn virus in zanamivir also generated the same mutant (Gubareva et al., 1997).

Arg 292 is one of three highly conserved arginines that form part of the catalytic triad of the NA active site (Varghese et al., 1992). Not surprisingly the mutant enzymes all exhibited lower specific activity, $\sim 20\%$ for NWS/G70C (McKimm-Breschkin et al., 1998), less than 10%

Table 4
Effect of Arginine 292 Lysine mutation on neuraminidase sensitivity in a MUNANA based enzyme inhibition assay

Virus	Fold-reduction in enzyme sensitivity			
	DANA	4-Amino-Neu5Ac2en	Zanamivir	Oseltamivir carboxylate
NWS/G70C[a]	20	33	55	6500
A/Turkey/Minn[b]	2	4	10	
A/Vic/75[c]		30	24	30 000

[a] McKimm-Breschkin et al. (1998).
[b] Gubareva et al. (1997).
[c] Tai et al. (1998).

Table 5
Drug sensitivity of Arginine 292 Lysine neuraminidase mutants in plaque reduction assay[a] and effect of concomitant hemagglutinin mutations[a]

Virus	Mutation		Fold decrease in drug sensitivity	
	HA	NA	Zanamivir	Oseltamivir carboxylate
NWS/G70C[b]	Asn 199 Ser	–	30	100
	Asn 199 Ser	Arg 292 Lys	100	>30 000
		Arg 292 Lys[c]	3[c]	>300[c]
A/Turkey/Minn[d,e]	Tyr 234 Leu	Arg 292 Lys	1000	–
	Thr 267 Lys	–	–	–
	Asp 304 Asn	–	–	–
	Lys 68 Arg^{HA2}	–		
		Arg 292 Lys	2	–
A/Vic/75[f]	Ala 28 Thr		7	7
	Arg 124 Met^{HA2}		–	–
	Ala 28 Thr	Arg 292 Lys	60	3000
	Arg 124 Met^{HA2}	–	–	–
		Arg 292 Lys[c]	8[c]	400[c]

[a] Resistance based on reduction in plaque size for NWS/G70C and A/Turkey/Minn, plaque number for A/Vic/75.
[b] McKimm-Breschkin et al. (1998).
[c] Relative resistance for NA Arg 292 Lys mutants has been inferred from the difference between the HA mutants and the HA/Arg 292 Lys double mutants.
[d] Gubareva et al. (1997).
[e] Gubareva et al. (1996a), Gubareva et al. 1996b).
[f] Tai et al. (1998).

for A/Vic/75 (Tai et al., 1998), but somewhat higher at 44% for A/Turkey/Minn (Gubareva et al., 1998). In contrast to the Glu119 mutations, the NA itself was stable, but the A/Turkey/Minn virus exhibited instability at low pH (Gubareva et al., 1997). It also demonstrated a shift in pH optimum from pH 5.9 to pH 5.3 (Gubareva et al., 1997).

This mutation had a differential effect on the relative resistance to each of the inhibitors, both in enzyme assays (Table 4), and in cell culture (Table 5), demonstrating minimal resistance to zanamivir and greater resistance to the compounds with the hydrophobic side chains (McKimm-Breschkin et al., 1998; Tai et al., 1998).

The A/Turkey/Minn HA*Arg 292 Lys (three mutations in HA1 and 1 in HA2)(Gubareva et al., 1996b) virus was 1000-fold less sensitive to zanamivir in cell culture, and when it was reassorted with the A/NWS/33 wild type, an NWS HA/Arg 292 Lys NA reassortant was generated, which was 100-fold less sensitive to zanamivir (Gubareva et al., 1997). However reassorting of the two parental viruses also generated a virus which was 50-fold less sensitive to zanamivir. Hence the Arg 292 Lys mutation actually only gave a two-fold resistance, when compared to the homologous reassortant background. In light of the minimal resistance to zanamivir it is interesting that the Arg 292 Lys mutant arose in the A/Turkey/Minn virus passaged in zanamivir.

Virus carrying only the Arg 292 Lys NA mutation produced both smaller plaques and lower yields of virus when kinetics of replication was analyzed (McKimm-Breschkin et al., 1998). Plaques were too small to determine drug sensitivity either by reduction in plaque size or number, hence drug sensitivity had to be determined by a yield reduction assay. This approach may also be necessary for poorly plaquing human isolates. The growth defect could be rescued by the presence of exogenous *Clostridium* NA, and also by concomitant HA mutations (McKimm-Breschkin et al., 1998). Decreasing the affinity for the receptor would compensate for the low NA activity, and thus allow the virus to elute.

There was no decrease observed in replication of the A/Turkey/Minn mutants in cell culture or embryonated eggs. However, the virus used had concomitant HA mutations, which could have rescued the poor growth.

Detailed structural analysis revealed three main mechanisms of resistance (Varghese et al., 1998). There is altered binding of the triol group and the carboxylate group on the sugar, which would affect substrate binding as well as all sialic acid analogs, correlating with reduced enzyme activity, as well as altered drug sensitivity. This would correlate with a similar decrease in binding to DANA, 4-amino-Neu5Ac2en and zanamivir. The lysine at 292 stabilizes the Glu 276 from moving to accommodate the bulkier hydrophobic groups, resulting in decreased binding of both the 6-carboxamide, and oseltamivir carboxylate. When the 6-carboxamide inhibitor binds the movement does occur, but at a significant energy penalty, resulting in a greater decrease in binding compared to the compounds with the triol group. The energy penalty is too great to accommodate the pentyl ether in oseltamivir carboxylate, hence a further order of magnitude decrease in binding is observed (Varghese et al., 1998).

Based on these results it has been suggested that the more removed the inhibitor is from the structure of the natural substrate the more likely the virus is able to mutate to maintain substrate binding, but decrease inhibitor binding (Varghese et al., 1998). Hence a minimalist approach to drug design would be less likely to generate viable mutants.

3.3. Mutation at Arg152 to Lys

An immunocompromised child suffering from and influenza B infection with a B/Beijing/184/93-like virus, was treated with zanamivir, after unsuccessful ribavirin therapy. After 8 days of zanamivir treatment a virus with a mutation in HA, Thr 198 Ile was obtained (Thr 189 H3 numbering) (Gubareva et al., 1998). This mutation abolished glycosylation on Asn 187, near the receptor binding site (Nobusawa et al., 1991). The virus also exhibited altered reactivity to polyclonal monospecific serum, presumably due to the loss of the carbohydrate side chain. While this mutation decreased the affinity for the terminally linked α2,6 sialic acids found on human cells, which would produce resistance in vivo, it increased the binding for terminal α2,3 sialic acids, the predominant linkage on MDCK cells. This was demonstrated by binding to MDCK cell membranes, erythrocytes from different species with different linkage specificities and sialyloligosaccharides (Gubareva et al., 1998). Due to the increase in affinity for α2,3 linked sialic acids, in a plaque assay in MDCK cells the virus actually appeared more sensitive to zanamivir (Gubareva et al., 1998).

By 12 days post-treatment a virus with an additional mutation, Arg 152 Lys in the NA was isolated. Arg 152 is conserved in all influenza A

and B viruses and forms a hydrogen bond to the acetamide of sialic acid bound in the active site (Burmeister et al., 1992). Expression of Arg 152 Lys in an SV40 recombinant system had previously shown that protein was expressed, but had no detectable activity (Lentz et al., 1987). These observations correlate with the very low enzyme activity in the mutant, 3–5% of the parent. The enzyme was about 1000-fold less sensitive to zanamivir. However, in MDCK, Vero and primary rhesus monkey kidney cells, the combination of a stronger HA and weak NA would make it difficult for the virus to elute, hence the isolate appeared more sensitive. Such a mutant would obviously be missed if the current cell culture assays were the sole means of screening potential isolates. Thus although resistance arising from both HA and NA mutations may be clinically important they could be missed in cell culture based assays.

3.4. Hemagglutinin mutations

Unexpectedly, the majority of mutants isolated after in vitro passaging in any of NA inhibitors have mutations in HA (Staschke et al., 1995; Penn et al., 1996; Gubareva et al., 1996a,b; McKimm-Breschkin et al., 1996a,b; Gubareva et al., 1997; Blick et al., 1998; McKimm-Breschkin et al., 1998; Barnett et al., 1999). Furthermore, it was subsequently demonstrated that in cell culture based assays the effects of two HA or an HA and NA mutation could be synergistic (Blick et al., 1998; McKimm-Breschkin et al., 1998). To date, one clinical isolate from a zanamivir treated immunocompromised child has also demonstrated an HA mutation with altered binding properties (Gubareva et al., 1998). Although HA mutations have also been found in isolates from patients treated with oseltamivir phosphate, none as yet have been shown to be related to drug treatment, or altered drug sensitivity (Covington et al., 1999).

Sequence analysis revealed changes in residues in the vicinity of the receptor binding site (Nobusawa et al., 1991) which could alter the affinity of HA for the receptor. A decrease in affinity would allow release of virus from infected cells, without the need for significant NA activity. Electron micrographs of infected MDCK cells show the wild type A/Turkey/Minn virus aggregated at the cell surface in the presence of zanamivir (Gubareva et al., 1996a). The double HA/NA mutant was not aggregated. Surprisingly the HA mutant was also aggregated, which did not support the hypothesis of the low affinity HA allowing virus release. However, while the concentration of zanamivir used to treat the infected cells was the IC_{50} for the double mutant, it was 100-fold higher than the IC_{50} for the HA mutant. Hence as it was inhibited in the plaque assay, one would also expect to see inhibition of virus release by electron microscopy. However, at lower drug concentrations, which would still cause aggregation of the wild type, one would expect to see normal budding of the HA mutant virus.

However, if the decrease in HA affinity is too great, this has a deleterious effect on the fitness of the virus. Such viruses have difficulty in adsorbing to cells, before the NA cleaves off the target receptors, and therefore infect cells more efficiently if the NA is inhibited by drug. These mutants exhibit a drug dependent phenotype (Gubareva et al., 1996a; McKimm-Breschkin et al., 1996a,b; Barnett et al., 1999). Levels of resistance vary from ten to greater than 10 000-fold. The mutants showing the greatest resistance tend to be drug dependent. HA mutants are cross-resistant to all classes of neuraminidase inhibitors, since the effect of the lower affinity is independent of how the NA is inhibited (McKimm-Breschkin et al., 1996a,b). Some mutations can also introduce carbohydrate side chains (Staschke et al., 1995; Blick et al., 1998), others result in the loss of carbohydrate residues (Staschke et al., 1995; McKimm-Breschkin et al., 1998), both of which can alter receptor binding through steric effects. As observed with the Glu 119 NA mutations, mutations in HA can also be deleterious, rendering it more unstable (McKimm-Breschkin et al., 1996c).

There do not seem to be any particular hot spots for mutations, many different regions can apparently have an effect on receptor binding, including the stalk region, HA2, and the second potential ligand binding site (Table 6). It is not

known yet whether the decrease in binding is due to an alteration in affinity and/or specificity of the target receptor. Assays for demonstrating a decrease in affinity for cellular receptors include HA elution, where the virus elutes more readily from red blood cells due to the weakly binding HA (McKimm-Breschkin et al., 1996a,b; Bantia et al., 1998; Bethell et al., 1999; McKimm-Breschkin et al., 1999c), binding to erythrocytes modified to contain predominantly α2,3, or α2,6 sialic acid linkages (Gubareva et al., 1998), kinetics of adsorption to MDCK cells, measured by immunofluorescence of infected cells by nucleoprotein staining 5–6 h post-infection (Sahasrabudhe et al., 1996; Blick et al., 1998; McKimm-Breschkin et al., 1999c), numbers of plaques adsorbed at various time points (McKimm-Breschkin et al., 1998), or binding to MDCK membrane extracts (Gubareva et al., 1998). Binding to sialyloligosaccharides has also been used, however due to different steric effects they may not always reflect cell binding experiments (Gubareva et al., 1998).

HA mutations can rescue the poor growth of a mutant virus with low NA activity (Blick et al., 1998; McKimm-Breschkin et al., 1998), thus masking any growth defect, but increasing resistance. However, an HA mutation can also mask resistance due to an NA mutation (Gubareva et al., 1998) when assayed in a heterologous system. A human isolate with a mutation in HA had decreased affinity for α2,6 linked sialic acid receptors, which would confer resistance in humans, but when assayed in MDCK cells, the predominant receptor is an α2,3 linked sialic acid, the HA mutation actually increased the affinity of binding to these receptors. When there was a concomitant NA mutation, generating resistance in the enzyme assay, resistance in MDCK cells was masked by the stronger HA binding. Thus, due to the lack of an appropriate cell line expressing α2,6 sialic acid receptors a dilemma exists for screening clinical isolates for drug sensitivity in cell culture, whether by plaque assay or yield reduction assay.

As some of the mutants isolated have had both HA and NA mutations, reassortants have been generated in order to determine the role of the individual mutations. However, wild type reassortants with HA from one parent and the NA from the other parent can demonstrate decreased drug sensitivity, due to an alteration in the balance of HA binding and NA eluting as shown by reassorting the wild type viruses of A/Turkey/Minn and A/NWS (Gubareva et al., 1997).

These observations have highlighted how natural resistance can be demonstrated, i.e. without any mutations, simply by an altered HA:NA balance. Some avian viruses have been shown to be inherently more resistant to zanamivir due to lower affinities of their HAs and a decreased dependence on their NA for elution (Baigent et al., 1999). We have also recently demonstrated that the converse is true. An NWSm/G70C reassortant generated by co-infecting the NWSm/Tokyo (H1N2) virus and the NWSc/G70C (H1N9) virus, was approximately 30-fold less sensitive to zanamivir. While both have NWS HAs there are two amino acids different between them, one being in the receptor binding site, (McKimm-Breschkin et al., 1996a). (The reassortants were raised in laboratories in Canberra and Memphis, NWSc and NWSm have been previously used to distinguish their HA (Yang et al., 1997)). The reverse reassortant was as sensitive as both parents. Treating cells with purified N9 or N2 NA which had the same activity in an enzyme assay, showed that the N9 was much more efficient than the N2 NA at removing the receptors to which the NWSm HA bound, based on the efficiency of adsorption of 100 pfu of virus (McKimm-Breschkin et al., 1999a). Thus pairing of the efficient N9 NA with the NWSm HA allowed the NA to be inhibited by zanamivir, but still retained sufficient activity to elute the virus. Furthermore, this demonstrates that enzyme activity in vitro does not necessarily correlate with the efficiency of receptor removal on the target cell. Thus when the balance in any virus is such that HA < NA, or NA > HA, then resistance can occur.

4. In vivo studies

Both the mouse and ferret model have been used for evaluating pathogenicity of influenza isolates for many years. More recently they both have also been used for determining sensitivity of

Table 6
Hemagglutinin mutations leading to altered sensitivity to neuraminidase inhibitors

A/Singapore/1/57[b] Virus strain	Ala 28 Thr^{HA2} Mutation H3 numbering	≥1000-fold, drug dept. Resistance to NA inhibitors	Reported in conjunction with above mutations, Comments
A/Turkey/Minn[a]	Glu 114 Lys^{HA2}	10-fold	Egg to MDCK adaptation
	Gly 75 Glu^{HA2}	100–1000-fold	Near second ligand binding site
	Tyr 234 Leu	1000-fold	Lys 68 near second ligand binding site
	Ala 35 Thr^{HA2}		
	Lys 68 Arg^{HA2}		
	Thr 267 Lys	ND	Reported in conjunction with above mutations, role in resistance not known
	Asp 304 Asn		
A/Singapore/1/57[b]	Gly 135 Asp	1000-fold	O of COOH interacts with *N*-5 on sialic acid
[c]	Arg 137 Gln	>20-fold	NH interacts with COOH of sialic acid
[c]	Ala 138 Thr	ND	Adjacent to sialic acid contacts
A/Vic/75[d]	Ala 28 Thr^{HA2}	10-fold double mutant	Both stalk–effect on cleavage or fusion?
	MArg 124 Met^{HA2}		
NWS/G70C[e]			
ju2	Gly 143 Glu	5–10-fold	Close to right edge of receptor pocket, 134–138
Variant 7[f]	Thr 155 Ala	1000-fold, drug dept.	Sialic acid interacts with Trp 153. 155 mutation altered affinity for *N*-glycolyl neuraminic acid
Variant B[g]	Thr 155 Ile	1000-fold, drug dept.	Sialic acid interacts with Trp 153. 155 mutation altered affinity for *N*-glycolyl neuraminic acid
Variant C[g]	Thr 155 Met	10 000-fold,drug dept.	Sialic acid interacts with Trp 153. 155 mutation altered affinity for *N*-glycolyl neuraminic acid
tg2[e]	Ser 165 Asn	30-fold	Addition of CHO on N165 impairs access of adjacent trimer to receptor
P1-1[h]	Ser 186 Phe	10-fold	S186I role in egg adaptation, avian versus human receptor
P3-1[h]	Ser 165 Asn	≥1000-fold, drug dept.	See above 2 mutants. Synergistic effect of 2 HA mutations
	Ser 186 Phe		
P3-2[h]	Ser 186 Phe	≥1000-fold, drug dept.	222 near left edge of receptor pocket. Synergistic effect of 2 HA mutations
	Lys 222 Thr		
xy22/ty4[e]	Asn 199 Ser	30-fold	Addition of CHO on N197 masks binding of 190, 194, 195 to receptor. Rescues poor NA.
Variant 1[f]	Val 223 Ile	100-fold	229 left edge of receptor pocket, thermolabile
	Arg 229 Il[e]		
Variant 5[f]	Arg 229 Ser	100–1000 fold, drug dept.	229 left edge of receptor pocket, thermolabile
B/Beijing/184/93-like[i]	Thr 189 Ile	Sensitive in cell culture, masked NA resistance	Loss of CHO on N187, near receptor binding site. Decrease in α2,6 linked sialic acid binding, increase in α2,3, binding. Altered HA antigenicity.
B/Hong Kong/	Asn 145 Ser	~10-fold double mutant	Loss of CHO on 145
-Lee[j]	Asn 150 Ser		Addition of CHO on 148
B/Beijing/1/87[k]	Leu 226 Gln	>1000-fold double mutant, drug dept.	226 in A strains role in specificity of receptor binding
	Val 93 Ala		93 in A strains, near second sialic acid binding site

[a] Gubareva et al. (1996a,b).
[b] Penn et al. (1996).
[c] Bantia et al. (1998).
[d] Tai et al. (1998).
[e] McKimm-Breschkin et al. (1998).
[f] McKimm-Breschkin et al. (1996a,b).
[g] McDonald et al. (1999).
[h] Blick et al. (1998).
[i] Gubareva et al. (1998).
[j] Staschke et al. (1995).
[k] Barnett et al. (1999).

influenza viruses to neuraminidase inhibitors, either by inhalation (von Itzstein et al., 1993) or oral administration (Mendel et al., 1998) of drug. In mice influenza infects cells in the lower respiratory tract, and infectivity and drug sensitivity are determined using either LD_{50} or lung virus titer. Replication in ferrets is more analogous to the human situation, primarily an upper respiratory tract infection, with a pyrexic response 3–5 days post-infection. Virus replication is monitored by assaying virus titers in the nasal washes or lung virus titer. Drug sensitivity is determined by a decrease in virus titer, or abrogation of the pyrexic response. However, the limitation in vitro of a cell line expressing appropriate receptors for evaluating human isolates also applies to in vivo evaluation of potential resistant variants. Ferret tracheal epithelial cells are thought to express primarily α2,6 linked terminal sialic acid receptors, and few if any α2,3. linkages (Leigh et al., 1995) similar to those in humans. Viruses with preference for α2,3 linkages can still replicate in ferret lungs, but not as well those with a preference for α2,6 linkages. In the nasal passages they replicate equally well, suggesting both linkages may be present. Therefore different results may be obtained testing mutants with altered receptor specificity, depending on whether one uses the virus titer in ferret nasal washes, or lung virus titer. The receptor specificity of mouse epithelium is not known. Variants generated in vitro in MDCK cells, may have altered affinity for either α2,3 or α2,6 terminally linked sialic acids, whereas resistant isolates from humans would be expected to have altered affinity for α2,6 linked sialic acids. Whether resistance is manifested in an animal model will therefore depend on the specificity of the target receptors for both HA and NA.

Several variants with altered sensitivity to neuraminidase inhibitors in vitro have been tested for infectivity and drug sensitivity in both mice and ferrets. Based on mouse lung virus titers, infectivity of the NWS/G70C Glu 119 Gly mutant was comparable to wild type virus, although the virus used had a concomitant HA mutation of Ser 186 Phe (Blick et al., 1998). The mutant virus was slightly less sensitive to zanamivir in mice. There was no difference in infectivity or drug sensitivity of the double mutant in ferrets (Blick et al., 1998). The Glu 119 Ala mutant demonstrated slightly lower infectivity than wild type virus in mice, but drug sensitivity was not tested (Gubareva et al., 1997). However, this was also a double HA and NA mutant, hence the role of each mutation cannot be ascertained.

All Arg 292 Lys mutants have exhibited very poor infectivity in mice and ferrets (Gubareva et al., 1997; McDonald et al., 1998; Tai et al., 1998; Covington et al., 1999; McKimm-Breschkin et al., 1999b). For the NWS/G70C Arg 292 Lys the resistance profile corresponded to the in vitro resistance of the NA. The single Arg 292 Lys mutant had lower resistance to zanamivir, but increased resistance to the 6-carboxamide, and the greatest resistance to oseltamivir carboxylate (McDonald et al., 1998; McKimm-Breschkin et al., 1999b). Although the weak HA rescued the poor growth of the Arg 292 mutant in cell culture, infectivity of the double HA and NA mutant was extremely compromised in mice, indicating that both the weaker HA affinity and the poor NA activity had an adverse effect. The double mutant was also more resistant to all drugs than the single NA mutant.

The B/Beijing/93-like mutant, HA Thr 189 Ile, NA Arg 152 Lys, isolated from an immunocompromised child had significantly reduced infectivity in mice, and although drug sensitivity was not determined, in the presence of drug it outgrew the wild type virus, although the input ratio was 60:1 of mutant to wild type (Gubareva et al., 1998).

Infectivity and drug sensitivity of HA mutants in vivo has varied. Both the A/Stockholm, naturally resistant virus (Woods et al., 1993), and the A/Singapore Gly 135 Asp mutant were sensitive in mice (Penn et al., 1996). We have tested several HA mutants in mice and found that the infectivity of many is compromised presumably due to their weak binding HAs, but some do exhibit drug resistance (Table 7). Drug depen-

dence is also demonstrated by some mutants in vivo.

We have also recently shown that altering the balance of HA binding and NA eluting by reassorting wild type viruses can lead to resistance in vitro, if the NA is more efficient at cleaving the HA receptor (McKimm-Breschkin et al., 1999a). In mice, this wild type reassorting was also shown to have altered drug sensitivity (Table 7).

Therefore, while the mouse model may not be the optimal model for resistance studies, due to receptor specificities, it has demonstrated that in principle both HA and NA mutations can play a role in resistance to neuraminidase inhibitors in vivo. Resistance in ferrets has not yet been demonstrated. While the model may be more analogous to the human infection, they may not have the appropriate receptors for MDCK generated mutants.

The difficulty of in vitro selection of mutants resistant to NA inhibitors has so far been predictive of the difficulty of generating resistance in vivo. The mechanisms of resistance in vitro

Table 7
Infectivity and drug sensitivity of NWS/G70C mutants in mice

Virus	HA mutation	NA mutation	Fold resistance and dependence plaque assay	Lung virus titer for 10^5 pfu + PBS mock treated ($TCID_{50} \times 10^4$)	Lung virus titer for 10^5 pfu treated with 1 mg kg^{-1} zanamivir ($TCID_{50} \times 10^4$)
NWSc/G70C	Wild type			14.5	2.3
NWSm/G70C[a]	NWSc/G70C		30	740	321
Wild type	NWSm/Tokyo				
Reassortant					
G70C4-G[b]	Ser 186 Phe	Glu 119 Gly	100	1.47	1.11
Variant 1[c]	Val 223 Ile Arg 229 Ile		100	1.97	2.26
Variant 5[c]	Arg 229 Ser		100–1000 drug dept.	0.01	0.94
Variant 7[c]	Thr 155 Ala		1000 drug dept	0.93	0.12
Variant B[d]	Thr 155 Ile		1000 drug dept	0.003	1.
Variant C[d]	Thr 155 Met		10 000 drug dept	33	134
P3-1[c]	Ser 165 Asn Ser 186 Phe		$\geq$1000 drug dept.	$<$0.001	$<$0.001
P3-2[c]	Ser 186 Phe Lys 222 Thr		$\geq$1000 drug dept	$<$0.001	0.088
tg2[e]	Ser 165 Asn		30	25	0.5
xy22[e]	Asn 199 Ser		30	4.5	0.7
ir2[e]		Arg 292 Lys			
Zanamivir			3	0.39	0.1
Oseltamivir Carboxylate			$>$300	0.06	0.15
ty4[d]	Asn 199 Ser	Arg 292 Lys			
Zanamivir			100	0.19	0.16
Oseltamivir carboxylate			$>$30 000	0.18	0.20

[a] McKimm-Breschkin et al. (1999a).
[b] Blick et al. (1998).
[c] McKimm-Breschkin et al. (1999b)
[d] McDonald et al. (1999).
[e] McDonald et al. (1998).

are however complex, being through mutations in both the NA and HA. These results highlight the fact that the roles of HA and NA cannot be viewed in isolation. The virus must maintain a balance between HA binding and NA eluting for optimal survival. Although exhibiting a drug resistant phenotype in vitro, a virus with such combinations of compensating mutations may not be sufficiently fit to survive in the clinical situation. Although the NA mutations have been more difficult to isolate in vitro, resistant NAs have been more readily identified from clinical samples because the enzyme inhibition assay is not subject to any of the complications of receptor specificity.

Various phenotypic properties of HA mutants have been described, however, all assays have potential limitations due to receptor differences between humans and the laboratory systems available. Although one HA mutant has been isolated from a patient, the general significance of HA mutations in humans is yet to be determined. Until more suitable screening assays are developed, isolation of HA mutants from clinical specimens will remain a challenge.

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