

Review

Position statement: global neuraminidase inhibitor susceptibility network

Maria Zambon ^{a,1}, Frederick G. Hayden ^{b,*,1}

(On behalf of the Global Neuraminidase Inhibitor Susceptibility Network)

^a Public Health Laboratory Service, London, UK

^b University of Virginia Health Sciences Center, Box 800473, Department of Internal Medicine, Charlottesville, VA 22908, USA

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1. Introduction

The global Neuraminidase Inhibitor Susceptibility Network (NISN) was established in 1999 to address public health and regulatory concerns regarding the potential emergence and consequences

of drug resistance in influenza viruses following the introduction of the influenza neuraminidase inhibitor (NI) class of antiviral agents. The first meeting was held on December 13, 1999, and subsequent meetings were conducted on June 30, 2000 and September 23, 2000. The Network initially included investigators and public health persons with demonstrated interest in neuraminidase inhibitors or anti-viral resistance and has expanded to include representatives of each of the four WHO global influenza reference laboratories and scientists from regions of the world where increasing use of these drugs is anticipated. The Network's activities are funded currently by two corporate sponsors, GlaxoSmithKline and Hoffman-LaRoche, and company representatives are invited to attend meetings as observers. However, the deliberations and actions of the Network itself are intended to be independent of any company, and the core working group of the Network is composed of scientists drawn either from academic or public health sectors. The broad objectives of the Network are to: (1) provide a coherent approach to global NI resistance monitoring from

* Corresponding author. Tel.: +1-804-9245059; fax: +1-804-9249065.

E-mail address: fgh@virginia.edu (F. G. Hayden).

¹ Other current Network members: Michele Aymard, Université Claude Bernard, Lyon, France; Alan Hay, National Institute for Medical Research, Mill Hill, London, UK; Alan Hampson, WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia; Alexander Klimov, Centers for Disease Control, Atlanta, GA; Jenny McKimm-Breschkin, Biomolecular Research Institute, Melbourne, Australia; Arnold Monto, University of Michigan, Ann Arbor, MI; Masato Tashiro, WHO Collaborating Center for Influenza, Tokyo, Japan; Robert Webster, St. Jude Children's Research Hospital, Memphis, TN. Daniel Lavanchy, Coordinator, Epidemic Disease Control, Department of Communicable Disease Surveillance and Response, WHO, Geneva, Switzerland is an advisor to the Network. Pharmaceutical sponsor observers include Margaret Tisdale, Rob Fenton, and Michael Elliot (GlaxoWellcome) and Noel Roberts, Emma Covington, and Penelope Ward (Hoffman-LaRoche).

both public health and research perspectives; (2) examine data from the scientific literature and from specific monitoring programs to make recommendations for appropriate general strategies and specific assays for monitoring resistance; (3) conduct longitudinal prospective surveillance for resistance emergence through a link with the existing WHO Global Influenza Surveillance Network; and (4) communicate this information to the scientific community. In particular, the Network will select appropriate monitoring assays, determine the NI susceptibility of representative clinical isolates (> 1000) collected before introduction of these drugs, and continue surveillance for resistance emergence on a sustained basis (> 5 years). The four WHO Collaborating Centers for Reference and Research on Influenza will continue to provide the Network with viruses isolated in the post-licensing period. The current position paper of the Network covers the rationale, mechanisms of NI resistance including phenotypic and genotypic characterization, currently recommended approaches and assays, and future directions for neuraminidase inhibitor resistance monitoring in influenza viruses. This consensus statement derives from deliberations of the group over the past 18 months and is intended to provide the foundation for subsequent communications which will deal with assay selection and validation, statistical considerations, baseline susceptibility results, and surveillance data.

2. Antimicrobial resistance

The emergence and societal implications of antimicrobial resistance has received increasing attention in recent years. The development of effective antiviral drugs is an important scientific achievement and has led to the licensing and use of over two dozen specific antiviral drugs in the developed world in the last two decades. The most recently licensed class of antiviral compounds, the neuraminidase inhibitors of influenza viruses (zanamivir [Relenza™, GlaxoSmithKline] and oseltamivir [Tamiflu™, Roche]) has sparked optimism but also controversy. Inevitably, the licensing of new antiviral drugs provokes concerns about the development of antiviral resistance. Such concerns

are rooted in the accumulated experiences with the first class of anti-influenza agents, the M2 protein inhibitors amantadine and rimantadine, resistance to drugs for HIV infection, and on the significant problems associated with the emergence of antimicrobial resistance in non-viral pathogens (pyogenic bacteria, tuberculosis, malaria). Indeed, the naivety of the mid 20th century towards the conquering of infectious diseases has been replaced by mature realism and respect for the mutability of microbial pathogens.

The experience with the M2 inhibitors, amantadine and rimantadine, illustrates the potential of influenza A viruses to rapidly develop drug resistance in the clinical setting (reviewed in Hayden, 1996). The basis of resistance is point mutations in the M gene with corresponding single amino acid changes in the M2 protein that confer high level cross-resistance between the drugs in vitro (Hay, 1992; Belshe et al., 1988). Rarely, amantadine-resistant variants predominate in clinical isolates. However, resistant variants are present in low concentration in virus samples and emerge within 2 to 5 days of initiating drug therapy in $\sim 30\%$ of treated immunocompetent adults and children (Belshe et al., 1988; Hayden et al., 1991; Hall et al., 1987). More prolonged virus replication, as seen in immunocompromised hosts, is associated with high frequencies of resistance emergence (Englund et al., 1998). Amantadine-resistant variants are genetically stable, are not reduced in infectivity or virulence in animal models, cause typical influenza illness in humans, and are transmissible from person to person under conditions of close contact. Spread of these resistant variants has caused failures of drug prophylaxis in households and nursing homes (Hayden et al., 1989; Degelau et al., 1992; Mast et al., 1991). Thus, amantadine/rimantadine-resistant variants possess the biological properties associated with clinically, and possibly epidemiologically, important drug resistance. To date the use of amantadine and rimantadine has been relatively limited and has been associated uncommonly ($< 1\%$) with recovery of resistant variants in the general population (Ziegler et al., 1999).

In part because of this experience, the introduction of the neuraminidase inhibitors (NI) requires the establishment of longitudinal surveillance to determine the possible emergence, clinical impor-

tance, and epidemiological consequences of resistant strains, particularly in patient populations under-represented in clinical trials. This necessity may be translated into regulatory requirements following the introduction of new antiviral drugs. Although there has been limitation on public sector prescription of NIs in several European countries because of reimbursement issues (NICE, 1999), there was extensive prescribing in primary care in the USA and to a lesser extent in western Europe during the 1999–2000 influenza season. Thus the possibility of emergence of antiviral resistance is realised for the first time as the drugs are used outside of the clinical trials setting. With the anticipated extension of NI availability to other countries and increased awareness of their value, it is expected that use will continue to increase, and with it the potential for drug pressure to select resistant variants.

3. Requirements for surveillance of NI resistance

Influenza is a global disease with seasonal variability and geographic unpredictability. The emergence and spread of new variants is rapid and relentless. Comprehensive NI resistance surveillance therefore needs to extend globally. One important advantage in monitoring the emergence of NI resistance is that the WHO has a well-established surveillance network for monitoring the impact of influenza worldwide and the associated antigenic and genetic changes of the responsible viruses (WHO, 1996) (www.who.int/health-topics/influenza.html). Monitoring for antiviral susceptibility is important not only in its own right, but also for the potential impact antiviral drugs may have on the generation of antigenic diversity. Comprehensive surveillance of NI resistance requires co-operation of national/regional governmental agencies and other public health authorities responsible for influenza surveillance, as well as liaisons with the pharmaceutical groups responsible for antiviral drug development and marketing. In addition, the generation of meaningful data on NI resistance, wherever it is carried out, requires robust, reproducible assays of drug susceptibility and an understanding of the specific

technical problems which limit the usefulness of many current assays (e.g. cell culture-based phenotypic assays).

Clearly, there is a need to determine the potential for development of resistance to NIs during wide spread use for the treatment of acute influenza, and the possible consequences should resistance develop. Key questions include the frequency and rapidity of resistance development; the genetic stability, virulence and transmissibility of any resistant strains; and whether alterations in drug susceptibility result in antigenic changes in circulating strains. Answers to these questions may be technically challenging to achieve. Assessment of NI resistance should therefore include:

- Use of suitable, validated assays for resistance testing with appropriate controls. Currently, measuring inhibition of NA enzymatic activity *in vitro* is the most sensitive and specific phenotypic means of detecting NA variants, due to the lack of predictive cell culture-based assays.
- A suitable range of viral isolates to establish baseline susceptibility prior to the introduction of NI drugs.
- Analysis of post treatment isolates, particularly from populations at higher risk for protracted virus replication (e.g. infants and young children, immunocompromised hosts, elderly adults).
- A panel of well-characterized resistant viruses to incorporate into the screening assays.
- All marketed NIs.

4. Mechanism of action of NIs and basis of resistance

NA and haemagglutinin (HA) work in concert during viral entry and release from the cell. At cell entry, HA binds to the cell via receptors bearing terminal sialic acid residues. Following budding, progeny viruses remain attached to the host cell and to each other through HA binding to sialic acid-bearing receptors on the cell surface and on the HA and NA of progeny virus. The viral neuraminidase (NA), which is required to complete the viral replication cycle, cleaves the sialic acid residues from these receptors and facilitates

release of new virions from infected cells and spread of virus within the respiratory tract (reviewed in Gubareva et al., 2000). The structure of the active site is highly conserved across all nine influenza A NA subtypes and influenza B (reviewed in Colman, 1994). The essential role of NA in viral replication and the conservation of the active site thus make the NA an attractive target for drug action. The NIs bind to the catalytic site of the NA and competitively inhibit this key viral function.

Resistance to NIs has been shown to arise in vitro and in vivo by two mechanisms to date (reviewed in McKimm-Breschkin, 2000 and in Mendel and Sidwell, 1998):

- Mutations in HA which decrease virus receptor binding affinity. This facilitates progeny virus release and reduces the requirement for NA enzyme activity, hence reducing viral sensitivity to any NIs. However, such mutations in HA can also decrease the infectivity of the virus by reducing binding at viral entry. It is not known currently if HA mutations alone can produce clinically important NI resistance in vivo.
- Mutations in NA which decrease inhibitor binding affinity. Such mutations have also caused reduced enzyme catalytic activity or stability and have often resulted in reduced replication and decreased virulence in vivo (Table 1).

5. Resistance in vivo

Neither NA nor HA mutations have been selected readily in vitro. Sequential passage in vitro has typically led to appearance of HA variants first and NA mutants only later (Tisdale, 2000). The NA mutants selected in vitro usually but not necessarily predict those observed in vivo. Pre-clinical resistance studies in animals (Mendel and Sidwell, 1998; Sidwell et al., 1998) and monitoring of influenza isolates during clinical trials with zanamivir (Boivin et al., 2000; Barnett et al., 2000) and oseltamivir (Treanor et al., 2000), although limited in number, suggest that resistance will not develop rapidly. To date the frequency of recovery of resistant virus with NA mutations was $\sim 1\text{--}2\%$ in immunocompetent adults receiving oral oseltamivir for acute treatment of influenza and has not been observed in immunocompetent persons receiving inhaled zanamivir. Furthermore, resistant isolates studied have been compromised in their NA activity or stability, and in the majority of cases this has translated into a reduction in virus infectivity/replicative ability (Table 1) (reviewed in Tisdale, 2000; McKimm-Breschkin, 2000). Most of the oseltamivir-resistant clinical isolates possess a mutation at Arg292Lys. Moreover, it will be necessary to consider resistance to each neuraminidase subtype separately, even though the active site is highly conserved across all subtypes. For example, oseltamivir has been

Table 1
NA mutations observed in preclinical and clinical studies and their effects on enzyme function^a

Inhibitor	NA Type/subtype	NA mutations	Selected		Enzyme function
			In vitro	In clinic	
Zanamivir	A/N2, B	E119G/A/D	Yes	No	Reduced stability
	A/N9	R292K	Yes	No	Reduced catalytic activity (<20% wild-type)
	B	R152K	No	Yes	Reduced catalytic activity (3–5% wild-type)
Oseltamivir	A/N2	R292K	Yes	Yes	Reduced catalytic activity (<20% wild-type)
	A/N2,N9	E119V	No	Yes	Reduced catalytic activity
	A/N1	H274Y	Yes	Yes	Reduced catalytic activity

^a N2 numbering is used for all types and subtypes (Colman, 1994).

shown to select for Arg292Lys in N2 and for His274Tyr in N1 containing influenza A viruses both in vitro and in treated persons. Large-scale studies are required to monitor this further during widespread use of drugs in different countries.

6. In vitro assessment of resistance

There is no clear relationship between the phenotypes and genotypes of viruses that emerge during exposure to NI drugs in vitro, and this reflects the complexity of resistance to NI and the need to consider both the NA and HA virus components (Blick et al., 1998; Barnett et al., 1999). Although useful for evaluating resistant isolates selected in cell culture, the standard methods for detecting viral drug resistance based on changes in antiviral phenotype in cell culture (plaque reduction assay, yield reduction, EIA) have, so far, not proved reliable for screening virus isolates from clinical trials of NIs. Both false positive and false negative resistance results have been recognized (Gubareva et al., 1998; Penn et al., 1996; Gubareva et al., 2001). For example, zanamivir susceptibility in vivo in experimental animal models of influenza correlates well with in vitro susceptibility determined by NA inhibition assay but not with plaque assay in MDCK cells (Tisdale, 2000).

Limited passage clinical isolates tend to give higher antiviral IC_{50} values in MDCK cells (i.e. they appear much less sensitive to NI) compared with laboratory strains of virus. Such viruses usually have a fully susceptible NA by enzyme inhibition assay and are inhibited in growth in human cells (explant cultures) and in animals (Penn et al., 1996; Woods et al., 1993). For example, the MDCK plaque assay IC_{50} values of representative sets of clinical isolates ranged over 700-fold, whereas variation in an NA inhibition assay values was generally within a 10-fold range (Woods et al., 1993). Such results probably reflect the sub-optimal binding of clinical isolates to the $\alpha(2,3)$ -linked sialic receptors of MDCK cells. Human influenza viruses bind preferentially to sialic acid linked to the penultimate galactose by an $\alpha(2,6)$ linkage, whereas MDCK and many other cell types have predomi-

nately $\alpha(2,3)$ linkages. If HA binds with lower affinity to receptors, then the virus is less dependent on NA activity for release and appears less sensitive to NIs in vitro. Furthermore, many low passage clinical isolates plaque so poorly that such assays are not feasible. In addition, at least one influenza B isolate recovered from an immunocompromised child receiving nebulised zanamivir was fully sensitive in MDCK cells but resistant in a NA enzyme inhibition assay (Gubareva et al., 1998). This variant has a NA catalytic site mutation that confers resistance to NIs but also an HA mutation that results in enhanced binding to MDCK cell receptors and apparently increased susceptibility to zanamivir in this cell type.

6.1. HA-related resistance

If HA binds less tightly to host receptors, the virus elutes more easily from receptors and is less dependent upon NA activity for release of progeny virus. Hence the virus will appear less sensitive to NIs in cell culture-based assays. Mutations in HA which change receptor binding may mask resistance due to NA mutations in cell culture. Weak HA binding and/or disproportionately high NA activity may result in failure of virus to infect cells due to release of attached virus before viral cell penetration occurs. Such a phenomenon has been described in in vitro-selected, drug dependent HA mutants (Barnett et al., 1999). Conversely, a strongly binding HA could mask a resistant NA.

HA mutations resulting in altered sialic acid receptor binding would be expected to occur in the sialic acid receptor region of HA1, although other sites have been reported from in vitro studies (reviewed in McKimm-Breschkin, 2000). However, mutations which result in decreased affinity of HA to one cell receptor type may not confer decreased affinity for receptors on other cell types. Other carbohydrate residues on the cellular receptor and glycosylation of the HA may also affect receptor binding. Hence, mutations observed in HA during in vitro studies to select NI-resistant virus may be (relatively) specific for the cell type used in the experiment (usually MDCK) and may not be predictive of HA mutations required to reduce binding affinity to receptors in the human

respiratory tract (Gubareva et al., 2000). Conversely, mutations in HA which decrease affinity for the receptors in the human respiratory tract may not have decreased affinity for receptors in laboratory cell lines. A cell line carrying $\alpha(2,6)$ linked sialic acid receptors reflective of the human respiratory tract and which supports growth of fresh clinical isolates is not currently available. Phenotypic assays for HA mutations (e.g. RBC elution, binding to artificial receptors) are not yet standardized and require further development.

These observations present a major, and as yet unresolved problem for phenotypic assay of HA-mediated resistance to NI drugs. Further, expansion of virus from clinical samples may allow variants in HA to arise as the virus adapts to the cell type used for culture. Currently, comparison of the sequence of HA from the pre- and post-treatment samples, looking specifically for mutations at or near residues involved in sialic acid binding, may be the best option to evaluate emergence of mutations in HA. The occurrence of natural (i.e. not drug induced) variants in HA on passage through a patient must be expected and factored into the outcomes analysis.

6.2. NA-related resistance

Since NA functions extracellularly and NIs are active without entering the host cell, direct NA inhibition assays are likely to be more predictive of *in vivo* resistance than cell culture-based assays. Decreased sensitivity to NIs due to mutations in NA may be assayed using an *in vitro* enzyme assay using an artificial substrate. Although assays have been developed to detect influenza NA enzymatic activity directly in clinical samples (i.e. Zstat[®] influenza test), usually virus must be expanded in cell culture prior to susceptibility assay. Decrease in sensitivity of the NA to NI between pre and post treatment samples may indicate resistance. However, the high level potency of NIs means that clinical isolates may have a wide range of sensitivity in an NA assay but remain inhibited by clinically achievable concentrations of the drug. Shifts in sensitivity which reliably predict clinical resistance need to be defined, but will probably be substantially higher

(> 10-fold) than the limit of variability noted in pre treatment isolates. The absolute concentration above which resistance may be predictive of clinical failure (i.e. lack of antiviral effects *in vivo*) also has still to be determined, but will probably be greater than 50 nM. An assessment of the potential to lose mutants during cell culture or to fail to detect the activity of mutant NAs is required. To confirm resistance, the virus NA should also be sequenced to identify the genotypic change(s) responsible for the change in phenotype. Optimally such sequencing studies should be performed on original clinical samples to exclude *ex vivo* selection of resistance mutations during growth of virus in cell culture systems containing residual drug.

6.3. Determination of endpoints

For the NI Susceptibility Network there is a need to determine as accurately as possible the susceptibility of a large number of clinical isolates throughout the world. This should allow detection of significant changes in susceptibility occurring in the circulating viruses from year to year. Highly accurate statistical analysis may prove useful in identifying resistant variants should they arise and will be dependent on precise *in vitro* measures to define the usual ranges of susceptibility.

The 50% inhibitory concentration (IC₅₀) is the most precise value for this purpose (Richman, 1996). The relationship between the inhibition of NA activity and the log of the drug concentration is usually a sigmoid curve and the IC₅₀ is derived from the center of the linear portion of the curve. Other endpoints (e.g. IC₉₀ or IC₉₉) may permit the detection of a heterogeneous mixture with resistant sub-populations, but their calculation is much less precise and subject to artifactual errors. The IC₅₀ determination of susceptibility is not a therapeutic target concentration, which must be determined independently for each drug (Richman, 1996), and may not detect low level resistance or resistant subpopulations. Inspection of the inhibition curves is useful for the latter, particularly when there is failure to inhibit NA enzyme activity fully at high NI concentrations.

While it may be valuable to relate achievable drug concentrations to antiviral activity, this is not necessarily an accurate predictor of clinical efficacy and is dependent on various pharmacokinetic parameters that will be unique to each drug. Also the relationship of the IC_{50} to the IC_{90} will vary with different inhibitors and will be dependent on the slope of the linear part of the curve. To understand the level of susceptibility that is clinically relevant for each inhibitor will require clinical efficacy data and/or additional analysis in a suitable *in vivo* model.

The observed IC_{50} value is also influenced heavily by the type and concentration of the substrate, as reflected by the following equation: $IC_{50} = K_i \times (1 + [S]/K_m)$ where K_i , binding constant for inhibitor, K_m , binding constant for the substrate, and $[S]$, substrate concentration. Therefore, if the substrate concentration is fixed in a particular assay, then the IC_{50} is proportional to the K_i for any particular NA of fixed K_m . If the substrate concentration is much greater than K_m , then the observed IC_{50} value is substantially increased. The IC_{50} values for different assays and viruses will differ considerably depending on the K_m values for the particular substrates used. Currently the most widely used substrate is the fluorogenic reagent 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA), although a chemiluminescent reagent, the 1,2-diotetane derivative of sialic acid (NA-STAR) may offer greater sensitivity (Buxton et al., 2000). K_m values will also vary by NA type (A versus B), subtype, and from isolate to isolate. Increasing substrate concentrations can magnify shifts in susceptibility determined by NA inhibition assays but also increase the background noise and variance of the system. Before screening of large numbers of clinical isolates, it is essential to validate assay conditions, including optimal substrate concentrations and buffer systems.

7. Sampling and interpretation of resistance data

Influenza virus infection is an acute event in the immunocompetent. Virus is cleared completely by the host's immune system following each infec-

tion. When sequential respiratory samples are taken and shown to progress from culture positive to culture negative, it may reasonably be assumed that a person has cleared the virus. Provided the last culture positive sample is not resistant, it is highly unlikely that subsequent culture negative samples from the same patient will contain clinically important levels of resistant virus. It therefore follows that patients whose samples are culture positive pre-treatment but culture negative at all post-treatment sample times do not carry clinically important levels of drug resistant virus. In calculating the incidence of resistance, patients whose post-treatment virus samples were resistance assay negative or culture negative can reasonably be included in the denominators used to evaluate the proportions of treated patients shedding resistant virus. Of note, immunocompromised hosts can have protracted shedding of influenza viruses for weeks and sometimes months (Klimov et al., 1995). Careful scrutiny of viral isolates from such patients is important. Furthermore, different routes of drug administration (i.e. inhaled versus oral) provide different drug concentrations and associated selective pressures for resistance emergence within the respiratory tract. These differences should be considered in collecting samples for recovery of potentially resistant viruses. For example, orally inhaled zanamivir is predominately deposited in the pharynx and tracheobronchial tree. Consequently, throat or lower respiratory samples (sputum, tracheal aspirates) would be more appropriate than nasal ones in searching for resistant variants.

8. Characterization of resistant variants and potential for transmission

The probability of transmitting NI-resistant influenza virus person-to-person relative to wild-type influenza could be very low, particularly if resistant variants have compromised viral fitness. Thus, broadly based surveillance may underestimate the frequency of resistance emergence in individual patients. Therefore, it would be useful, although more difficult, to include sampling of patients at higher risk of prolonged replication

and resistance emergence (e.g. children, immunocompromised, hospitalized) during treatment. Whenever possible, last day isolates collected on or after day 3 of therapy should be submitted for analysis. Here, the chances of observing resistance should be higher, particularly if resistant strains are compromised and not easily transmitted. In the absence of a reliable cell-based assay, it would also be useful to sequence the HA from matched pairs of isolates during treatment to observe if any consistent patterns of mutations in HA emerges. The antigenic characterization of viruses pre and post drug exposure, particularly those in which HA mutations affecting the receptor binding sites are recognized, is also important.

The HA/NA balance required to infect MDCK (or other laboratory cell lines) may not reflect that for human respiratory tract epithelium. A suitable human respiratory cell system for phenotypic characterization of NI-resistant variants remains to be established. Thus growth characteristics in MDCK cells of any mutant virus compared to wild-type may not reflect potential growth (infectivity and replication) characteristics in the human respiratory tract. HA mutations can mask potential defects in growth due to NA instability or reduced activity *in vitro*, so that growth properties in cell cultures or eggs may not reflect loss of viral replication fitness *in vivo*. Infectivity/replicative ability of mutant viruses is currently best assessed *in vivo* in the ferret. This species is potentially the best model for human influenza, given the similarity between the two species with regard to receptor type and the consequent ability to infect ferrets without virus adaptation. Furthermore, correlation has been established between the *in vivo* inhibitory effect of NIs in the ferret and virus susceptibility determined by NA inhibition assay, but not in MDCK plaque assays (Tisdale, 2000).

9. Initial strategy for NI susceptibility determination

No cell culture-based assay (e.g. plaque reduction, yield reduction, EIA) can currently be recommended for reliable assessment of NI susceptibility (reviewed in Tisdale, 2000; see be-

low). However, representative isolates and original samples should be stored for the time that reliable cell-based assays are available. Preliminary data on the variation in susceptibility of over 1000 natural influenza isolates collected from many regions of the world prior to the introduction of NIs is being generated currently with the co-operation of the WHO Global Influenza Surveillance Network. Susceptibility is being assessed by NA inhibition assay for both approved NIs. NA gene sequencing will be determined for samples showing reduced or outlying susceptibility. Results will be scrutinised by the Network members, and this process will provide information of the baseline of NI susceptibility prior to the introduction of drugs. This assessment will provide data regarding the magnitude of natural variation in susceptibility in clinical isolates and provide one point of reference for subsequent prospective monitoring of resistance emergence. It should also provide informed comment about the different technical approaches to determination of NI resistance to ensure that there are accurate estimates of resistance. If clinically significant resistance emerges, it will be important to rapidly disseminate this information to assist planning in different regions.

10. Conclusions

1. There are a number of technical difficulties associated with determining NI resistance in influenza viruses. Previous cell culture methodologies used for other viral systems may not be suitable, and novel assays need to be established. The plaque reduction assay is not suitable as the sole assay for testing NI resistance and currently an NA inhibition assay is the most predictive assay for susceptibility monitoring.
2. Mutations in viral NA and HA can both contribute to the resistance phenotype, although the relative significance of these remains *in vivo* to be established. Sequence analysis of key regions of the HA gene and assessment of antigenic changes in isolates

with apparent drug-related changes are necessary at present.

3. The relationship between phenotype and genotype of NI-resistant viruses remains to be fully clarified, although certain NA mutations have been shown to confer decreased enzyme inhibition by NI.
4. Surveillance for NI resistance should be established globally to reflect the global impact of influenza and to reflect the increasing use of different NA inhibitors.
5. Because of the limited information regarding resistance emergence during clinical use, targeted surveillance in risk populations, including young children, immunocompromised hosts, and elderly institutionalized adults, and correlations of resistance detection with viral replication and clinical outcomes are needed.

In summary, NA inhibition phenotyping of initial (pre-treatment) and final positive virus isolates, supported by NA and HA sequencing, currently provides a reliable and reasonably comprehensive approach to identification of NI resistant clinical isolates.

References

- Barnett, J.M., Cadman, A., Burell, F.M., Madar, S.H., Lewis, A.P., Tisdale, M., et al., 1999. In vitro selection and characterisation of influenza B/Beijing/1/87 isolates with altered susceptibility to zanamivir. *Virology* 265, 286–295.
- Barnett, J., Cadman, A., Gor, D., Dempsey, M., Walters, M., Candlin, A., et al., 2000. Zanamivir susceptibility monitoring and characterization of influenza virus clinical isolates obtained during phase II clinical efficacy studies. *Antimicrob. Agents Chemother.* 44 (1), 78–87.
- Belshe, R.B., Smith, M.H., Hall, C.B., Betts, R., Hay, A.J., 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. *J. Virol.* 62, 1508–1512.
- Blick, T.J., Sahasrabudhe, A., McDonald, M., Owens, I.J., Morley, P.J., Fenton, R.J., et al., 1998. The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. *Virology* 246 (1), 95–103.
- Boivin, G., Goyette, N., Hardy, I., Aoki, F.Y., Wagner, A., Trottier, S., 2000. Rapid antiviral effect of inhaled zanamivir in the treatment of naturally occurring influenza in otherwise healthy adults. *J. Infect. Dis.* 181, 1471–1474.
- Buxton, R.C., Juo, R.R., Voyta, J.C., Tisdale, M., Bethell, R.C., 2000. Development of a sensitive chemiluminescent neuraminidase assay for the determination of influenza virus susceptibility to zanamivir. *Anal. Biochem.* 280, 291–300.
- Colman, P.M., 1994. Influenza virus neuraminidase: structure, antibodies, and inhibitors. *Protein Sci.* 3 (10), 1687–1696.
- Degelau, J., Somani, S.K., Cooper, S.L., Guay, D.R., Crossley, K.B., 1992. Amantadine-resistant influenza A in a nursing facility. *Arch. Intern. Med.* 152 (2), 390–392.
- Englund, J.A., Champlin, R.E., Wyde, P.R., Kantarjian, H., Atmar, R.L., Tarrand, J.J., et al., 1998. Common emergence of amantadine and rimantadine resistant influenza A viruses in symptomatic immunocompromised adults. *Clin. Infect. Dis.* 26 (6), 1418–1424.
- Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R., Webster, R.G., 1998. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J. Infect. Dis.* 178 (5), 1257–1262.
- Gubareva, L.V., Kaiser, L., Hayden, F.G., 2000. Influenza virus neuraminidase inhibitors. *Lancet* 355, 827–835.
- Gubareva, L.V., Kaiser, L., Matrosovich, M.N., Soo-Hoo, Y., Hayden, F.G., 2001. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J. Infect. Dis.* 183, 523–531.
- Hall, C.B., Dolin, R., Gala, C.L., Markovitz, D.M., Zhang, Y.Q., Madore, P.H., et al., 1987. Children with influenza A infection: treatment with rimantadine. *Pediatrics* 80 (2), 275–282.
- Hay, A.J., 1992. The action of adamantanes against influenza A viruses: inhibition of the M2 ion channel protein. *Semin. Virol.* 3, 21–30.
- Hayden, F.G., 1996. Amantadine and rimantadine-clinical aspects. In: Richman, D.D. (Ed.), *Antiviral Drug Resistance*. Wiley, New York, pp. 59–77.
- Hayden, F.G., Belshe, R.B., Clover, R.D., Hay, A.J., Oakes, M.G., Soo, W., 1989. Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. *N. Engl. J. Med.* 321 (25), 1696–1702.
- Hayden, F.G., Sperber, S.J., Belshe, R.B., Clover, R.D., Hay, A.J., Pyke, S., 1991. Recovery of drug-resistant influenza A virus during therapeutic use of rimantadine. *Antimicrob. Agents Chemother.* 35 (9), 1741–1747.
- Klimov, A.I., Rocha, E., Hayden, F.G., Shult, P.A., Roumilat, L.F., Cox, N.J., 1995. Prolonged shedding of amantadine-resistant influenza A viruses by immunodeficient patients: detection by polymerase chain reaction-restriction analysis. *J. Infect. Dis.* 172 (5), 1352–1355.
- Mast, E.E., Harmon, M.W., Gravenstein, S., Wu, S.P., Arden, N.H., Circo, R., et al., 1991. Emergence and possible transmission of amantadine-resistant viruses during nursing home outbreaks of influenza A (H3N2). *Am. J. Epidemiol.* 134 (9), 988–997.
- McKimm-Breschkin, J.L., 2000. Resistance of influenza viruses to neuraminidase inhibitors — a review. *Antivir. Res.* 47, 1–17.
- Mendel, D.B., Sidwell, R.W., 1998. Influenza virus resistance to neuraminidase inhibitors. *Drug Resist. Updates* 1, 184–189.

- NICE, 1999. National Institute for Clinical Excellence. Zanamivir (Relenza) in the management and treatment of influenza. Guidance 1999.
- Penn, C.R., Barnett, J., Bethell, R., Fenton, R.J., Gearing, L., Healy, N. et al., 1996. Selection of influenza virus with reduced sensitivity in vitro to the neuraminidase inhibitor GG167 (4-guanidino-Neu5Ac2en): changes in haemagglutinin may compensate for loss of neuraminidase activity. In: Brown, L.E., Hampson, A.W., Webster, R.G., editors. *Options For The Control of Influenza III*. Elsevier Science BV, Amsterdam, The Netherlands, pp. 735–740.
- Richman DD, 1996. Issues and Challenges. In: Richman DD, editor. *Antiviral Drug Resistance*. Chichester: Wiley, p. 6.
- Sidwell, R.W., Huffman, J.H., Barnard, D.L., Bailey, K.W., Wong, M.H., Morrison, A., et al., 1998. Inhibition of influenza virus infections in mice by GS4104, an orally effective influenza virus neuraminidase inhibitor. *Antiviral Res.* 37, 107–120.
- Treanor, J.J., Hayden, F.G., Vrooman, P.S., Barbarash, R.A., Bettis, R., Riff, D., et al., 2000. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza. *JAMA* 283 (8), 1016–1024.
- Tisdale, M., 2000. Monitoring of viral susceptibility: new challenges with the development of influenza NA inhibitors. *Rev. Med. Virol.* 10, 45–55.
- WHO, 1996. WHO influenza surveillance, *Weekly Epidemiological Record* 22; 71 (47): 353–357.
- Woods, J.M., Bethell, R.C., Coates, J.A., Healy, N., Hiscox, S.A., Pearson, B.A., et al., 1993. 4-Guanidino-2,4-dideoxy-2,3-dehydro-*N*-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses in vitro. *Antimicrob. Agents Chemother.* 37 (7), 1473–1479.
- Ziegler, T., Hemphill, M.L., Ziegler, M.L., Perez-Oronoz, G., Klimov, A.I., Hampson, A.W., et al., 1999. Low incidence of rimantadine resistance in field isolates of influenza A viruses. *J. Infect. Dis.* 180 (4), 935–939.