Influenza Antivirals: Efficacy and Resistance
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Contents

Introduction 03

Influenza therapeutics and antiviral policy 04
Current antivirals 04
Potential new drug targets 04
Clinical impact of resistance mutations 05
Clinical management of influenza 05
Public health importance of influenza antivirals 05

Surveillance 06

Detection and characterisation of resistance 07
Molecular Basis of Resistance 07
Assays of antiviral susceptibility 07
Fitness of Resistant Viruses 08

Outstanding issues and areas for further research 09
Introduction

The inaugural conference of the newly established Antiviral Group (AVG) of the International Society for Influenza and other Respiratory Virus Diseases (isirv) was organised in conjunction with Fiocruz (Fundacao Oswaldo Cruz) in Rio de Janeiro, Brazil. The objectives of the conference were to increase awareness of the available influenza antivirals and to promote understanding of their use and limitations. The meeting also focused on the development and consequences of antiviral resistance and the need for surveillance of the emergence of drug-resistant influenza viruses. The meeting’s location in Rio de Janeiro reflected the Antiviral Group’s ongoing commitment to supporting influenza surveillance, laboratory and policy development in South America, as elsewhere.

The approach was multidisciplinary, with 130 participants drawn from clinical, laboratory and policy backgrounds at national and international level. Organisations represented included national public health agencies and academic centres from the USA, Canada, Australia, Japan, Vietnam, and many South American, European and Asian countries, and the WHO. The three day conference included state of the art reviews by medical and scientific leaders in their fields, workshops facilitating expert discussion, and interactive demonstrations on technical aspects of resistance. The topics covered three main themes: Influenza therapeutics including antiviral policy, laboratory diagnosis and characterisation of antiviral resistance, and surveillance.
Influenza therapeutics and antiviral policy

**Current antivirals**
The amino-adamantanes, amantadine and rimantadine, are M2 ion channel blockers which were introduced in the 1960s and are now of little value due to widespread resistance among circulating seasonal and 2009 pandemic viruses. The main drugs of current clinical value are the neuraminidase inhibitors (NAIs) oseltamivir (oral delivery) and zanamivir (inhaled), licensed almost universally. Intravenous zanamivir is also in phase III trials. Two other neuraminidase inhibitors, peramivir (intravenous) and laninamivir (inhaled), currently have limited licence approval (Japan and Korea, and Japan, respectively), and both are under development for wider approval.

It is widely recognised that new classes of drugs are needed and a number of new developments were reviewed. Arbidol, a fusion inhibitor which binds to the HA stabilising it against the low pH-induced conformational change necessary for membrane fusion, is licensed in Russia and China. Another fusion inhibitor, which binds to HA2, is a 16-mer peptide, flufirvitide-3, which has been shown to be active against primary infection and to inhibit virus transmission in ferrets, and is currently in Phase I studies in man. The polymerase inhibitor favipiravir (T-705) is in Phase II/III clinical studies in Japan. Older drugs have also been explored for anti-influenza activity. For example, the antiparasitic agent nitazoxanide which inhibits the maturation of the influenza HA at high doses has shown some clinical benefit. A number of existing drugs have possible value as adjunctive treatments, such as statins, fibrates, glitazones, COX2 inhibitors, NAC and resveratrol, and merit further exploration.

Alternative treatment modalities are also being explored. A recent review of the 1918 pandemic suggests that convalescent blood products reduced mortality at that time and a small trial in Hong Kong has supported this conclusion. Broadly neutralising monoclonal antibodies against conserved epitopes of the HA are another promising line of investigation. New and existing drugs need to be evaluated not just singly but also in combination, as combinations of conventional antivirals with immunomodulators, convalescent blood products, or monoclonal antibodies may provide more effective therapies. Challenges in designing clinical trials for new therapies include the need for surrogate endpoints, the choice of study subjects, and in particular the difficulty in including severe disease, and the varying performance of point of care diagnostics leading to difficulty in selecting patients to enrol.

**Potential new drug targets**
The current heavy reliance on NAIs is clearly not ideal and several speakers indicated other targets being considered for potential new drug development. These include the polymerase PB1, PB2, PA complex, the NP protein which is essential for viral replication, the NS1A multifunctional protein, and monoclonal antibodies. Vertex VX-787, which was discovered by high throughput sequencing and has a novel mechanism, is currently in Phase I/II development. Also mentioned were potential host targets such as the cell proteases that cleave HA into HA1 and HA2, and other cell proteases involved in virus entry. The concept that targeting a host protein would avoid resistance was discussed, although there is evidence to the contrary from experience with the HIV treatment maraviroc. Note was made of the possibility of immune modulation, such as by a sphingosine analogue shown to suppress the cytokine storm in a murine model, and of alternative approaches such as antisense RNA products, one of which is currently in phase I development.
Clinical impact of resistance mutations
The clinical importance of both naturally occurring and drug-induced resistance was discussed. Studies in vitro and in vivo show that the interactions between antiviral resistance, virus fitness and transmissibility are complex. Resistance mutations correlate with poor clinical outcomes in immunocompromised patients, but this has not been consistently borne out in observational studies of healthy patients. Metanalysis of data from the pandemic shows an association between oseltamivir resistance and pneumonia, though no significant association with hospitalisation. The clinical importance of some of the newly recognised mutations such as Y155H and I223R are not yet clear. The same concerns apply to H5N1; in a series of 8 H5N1 cases, 2 of 3 deaths had resistant virus emerging at about 5 days into the illness. Further data are needed on treatment of drug-resistant influenza and the relationship between clinical and laboratory resistance. For example, H275Y influenza A viruses demonstrate a rise in peramivir IC50 in vitro, but retain therapeutic activity in a mouse model. It was also noted that with multiple circulating mutations, the potential for reassortment must be monitored.

Clinical management of influenza
The medical need for more effective therapy was recognised, particularly for severe disease and high risk groups. Many speakers referred to the challenges of treating special groups such as the immunocompromised and pregnant women. The immunocompromised have poorer responses to immunisation and a higher risk of developing antiviral resistance, due in part to prolonged viral replication. Antivirals are clearly of benefit in the immunocompromised, but the optimal choice of drug, dose and duration of treatment are undefined. There is also demonstrated reduction in adverse outcomes amongst pregnant women treated with oseltamivir and the potential benefit is considered to outweigh risk. The obese are a newly recognised risk group, and studies using a murine model support the increased susceptibility of the obese to influenza, as seen during the pandemic. Although current evidence has not supported increased antiviral dosing in the obese, the mouse model demonstrated greater protection by an increased dose of oseltamivir.

Public health importance of influenza antivirals
The 2009 pandemic has highlighted the contrast between the process and data for drug development and the data that is needed for public health policy. Most of the licensed uses of oseltamivir which have important public health impact are off-label. This puts public health agencies in the difficult position of advocating off-label use of drugs.

Another challenge is to gather the evidence that is required to support policy. Global data collated by WHO suggest that there was an inverse relationship between access to antivirals and influenza mortality during the pandemic. However data tend to come from well resourced countries. The limitations in the evidence base are recognised and it is essential to be explicit and transparent about this in making recommendations. Key aims are to reduce inappropriate use without losing appropriate use, to implement effective containment where appropriate to complement drug use, and to foster innovation and R&D of new tools. Resistance is recognised as a public health threat and there is continuing debate about the contributory role of prophylactic use to the development of resistance.

Individual countries reported on their antiviral policies and the challenges faced during the pandemic. Issues discussed included the use of stockpiles, with several countries preferring to buy antivirals during the pandemic rather than deplete their stockpile, inequitable access to
antivirals within individual countries as well as between different countries, and the difficulties of disseminating good diagnostic and therapeutic practice. Medical experience of antivirals was recognised as an important factor in successful use, and exemplified by Japanese experience and their extremely successful use of antivirals during the pandemic. Many countries revised their policies several times during the pandemic, identifying this flexibility as an important component of the pandemic response. It was agreed that it is important to extract data about antiviral use and outcomes during the pandemic at an international level and this work has been started by WHO.

Surveillance

Pre-2009 pandemic surveillance has shown that emergence of resistance is not always linked to drug use, that compensatory mutations can allow a resistant virus to overcome fitness deficits, and has emphasised the link between HA and NA activity. Conventional and innovative strategies during the pandemic were discussed, such as the UK’s use of community self-swabbing. Future priorities should include establishment of more community and risk-based surveillance programmes with good links to clinical use and guidance in prescribing. It is important to link this to virological, structural and animal studies to ensure an increasing understanding of the virus, and to inform new drug development.

Reports on the incidence of oseltamivir resistance, due to the H275Y substitution in NA, among influenza A(H1N1)pdm09 viruses were consistent with previous data, with a reported incidence of about 1% or less in countries represented at the meeting, including Argentina, Australia, Brazil, Chile, China, Mexico, the UK and the USA. Most of the resistant viruses detected in these studies, as previously, were from drug-treated, severely unwell patients, many of whom were immunocompromised.

However, there were some trends which might cause concern. Three larger studies all showed that although the overall resistance incidence was low, there was a gradual increasing trend throughout the pandemic. Thus, UK data showed incidences of approximately <0.3%, 1.2% and 1.8% for the three phases of the pandemic. In the USA incidence for 2009-2010 was 0.5% and for 2010-2011 was 0.9%. Similar data were recorded in Australia, where a localised cluster of viruses was also described in the region around Newcastle, with a resistance incidence of 14% (25/184) in July 2011. These viruses were genetically related and potential permissive co-mutations were identified. However, the incidence of resistant viruses collected from the same area the following month was slightly lower (9%) and resistance was not found in any virus samples in September or October. These observations of resistance were not all associated with treated patients; the incidence of resistance among viruses isolated in the community (i.e. from untreated patients) also increased with time (in the UK, 0% in first two waves, but 8/59 in the third wave; in the USA 4/35 for 2009-2010, but 25/33 for 2010-2011).

Several reports on seasonal virus drug sensitivity were presented. All confirmed the general situation that most influenza A viruses remained resistant to amantadine and rimantadine, that there had been a high level of oseltamivir resistance (but zanamivir sensitivity) in residual seasonal H1N1 viruses from the 2007-2009 outbreaks and that resistance to both oseltamivir and zanamivir in A(H3N2) and B viruses was essentially zero.
Detection and characterisation of resistance

Molecular Basis of Resistance
Antiviral drugs bind to a specific molecular target in the virus and mutations in that target molecule, which prevent drug binding, may result in resistance. Amantadine and rimantadine bind to the M2 ion channel of influenza A viruses. Several mutations in the ion channel (most commonly S31N) can prevent drug binding without serious effects on ion channel function, such that the viruses carrying resistance mutations are as fit as wild type virus. Increased use of amantadine may have been responsible for the progressive increase in resistance in circulating A(H3N2) viruses observed from about 2001 onwards. A(H1N1) pdm09 viruses acquired amantadine resistance as a property of the M gene of Eurasian swine viruses, which acquired amantadine resistance in the mid 1980s. Consequently almost all current human influenza A viruses are resistant to amantadine and rimantadine.

The NAIs bind to the active site of the neuraminidase enzyme. For effective binding to influenza A, but not influenza B, viruses oseltamivir requires a change in orientation of glutamic acid 276 in the enzyme active site, whereas zanamivir binds similarly to the natural substrate sialic acid. Hence mutations in the enzyme active site that block the movement of glutamic acid 276 cause resistance to oseltamivir but not to zanamivir. The mutations causing resistance to oseltamivir appear sub-type specific. The principal mutations in N1 viruses are H275Y and , much less commonly, N294S. In N2 viruses the resistance mutation preventing this re-orientation is R292K. Both drugs derive some binding affinity from an interaction to E119, and in vitro resistance to both drugs can be caused by mutations at this position, but in vivo so far only E119V, causing resistance to oseltamivir, has been found.

A novel NA resistance mutation Y155H found in a seasonal H1N1 isolate, A/Hokkaido/15/02, conferred resistance (~100 fold) to all neuraminidase inhibitors as a class. However, this residue is not conserved and 155H is found in other isolates which are not NAI resistant. Reverse genetics studies showed that the Y155H mutation resulted in reduced enzyme activity, reduced and slower substrate binding and a narrower pH profile. In NAI sensitive viruses the NA/HA balance was maintained by a D225 in the HA receptor binding site, while in the case of A/Hokkaido/15/02 the HA carried a D225G substitution which reduced binding, such that NA activity was less essential for virus detachment, resulting in lack of sensitivity to all NAIs. NAI-sensitive revertants were generated on passage in cell culture by selection of compensatory mutations V247I and L430Q in NA, which despite their distance from the active site enhanced NA activity, restoring the NA/HA balance while retaining 155H.

Assays of antiviral susceptibility
Several speakers described the currently used assays for monitoring resistance of influenza viruses to NAIs. Phenotypic cell culture assays are not suitable to detect NAI resistance, although this is the method of choice for the amino adamantanes and arbidol. Resistance to NAIs is determined functionally by inhibition of neuraminidase activity, and by NA, and sometimes HA, gene sequencing. The two commonly used substrates for inhibition assays are MUNANA (a fluorometric assay) and NA-Star (a chemoluminescent assay). IC50 values will differ between the two assays and will depend on local assay conditions for each assay. Panels of standard resistant and wild-type viruses to characterise newly established assays and to act as ongoing internal controls are available.
from the isirv-AVG through both Aeron Hurt (Melbourne) and the UK Health Protection Agency (HPA). The MUNANA assay may be better at detecting mixtures of resistant and wild-type viruses.

NAIs as a class exhibit slow binding kinetics which may be affected by resistance mutations. Two novel assays were described to assess these kinetic changes; a real-time kinetic assay using MUNANA to measure rates of binding, and a novel solid-phase assay which allows the simultaneous evaluation of dissociation of NAIs from multiple mutant and wild-type viruses.

Several genotypic assays are available to detect known resistance mutations. Novel resistance mutations can only be detected by a combination of phenotypic and genotypic analysis. Assays were described using conventional (Sanger) sequencing, various forms of RT-PCR or pyrosequencing, and the single nucleotide polymorphism assay (SNaPshot assay). All have different advantages and disadvantages, and the emergence of natural variants may necessitate the re-design of many published assays. A novel allele specific RT-PCR assay using locked nucleic acids in the primers was described, which is very sensitive (lower limit of detection approximately 100 vp/ml), linear over a large range (1.9-8.5 log10 vp/ml) and capable of detecting 1-5% of minority species (e.g. 1% of H275Y in 99% wild-type). This assay is being used to monitor resistance in the Roche IRIS study of oseltamivir and, perhaps not surprisingly, detects about 4 times more resistant virus than the phenotypic assay. Several databases are now available where genetic data can be compared with that of previous isolates to aid the interpretation of variation in sequence.

**Fitness of Resistant Viruses**

As mentioned above for the aminoadamantanes, if resistance mutations do not affect the fitness of the virus the resistant virus will readily compete with wild type virus. Comparative fitness can be assessed *in vitro* by growth competition experiments. Changes in infectivity and transmissibility can be assessed in ferrets by parallel experiments with resistant mutant and corresponding wild type virus. Such experiments with early clinical isolates resistant to oseltamivir showed that all three major mutations (H275Y, R292K and E119V) resulted in significantly less fit viruses. Modelling studies suggest that just a 1-2% difference in fitness will determine which virus prevails. However, since these studies, we have seen H275Y influenza A circulating as the predominant variants among the 2007-2009 H1N1 seasonal viruses. Modelling studies have shown the emergence of this virus could not have been driven by drug usage, and it is apparent that compensatory mutations in NA (and/or HA) transformed an unfit resistant virus into a fit resistant virus. Influenza A(H1N1)pdm09 viruses can accommodate H275Y with little change in fitness, as can some of the more pathogenic H5N1 viruses which have very high NA activity.

Virus fitness is complex to assess experimentally. A summation of data on weight loss, duration of illness, clinical score, pathological changes, and contact and aerosol transmissibility are required to accurately reflect the overall viral fitness in animal models. Understanding of how drug resistance mutations interact with transmission and fitness is critical to successful public health preparedness for seasonal and pandemic influenza.
Outstanding issues and areas for further research

The need for new therapeutic agents and treatment modalities was repeatedly stressed, as well as the importance of developing the evidence base for both new and existing agents, to support public health policy. Current policy should be transparent about the lack of supporting data. There is currently an opportunity to use the international data generated by the pandemic, which already points to the importance of equitable access to antivirals. Clinical evidence is needed in the areas of treating severe disease and at-risk groups such as the immunocompromised, but the challenges in clinical trial design are recognised.

Data from the UK HPA’s international laboratory proficiency assessment involving 16 countries exemplified many of the outstanding issues in interpretation of resistance data. There was considerable spread of IC50 values for the same samples and a mixed ability to detect and classify whether a virus was resistant or sensitive from genotypic assays. These and other data presented at the meeting highlight the following issues:

1. There are extremely limited data by which a correlation can be made between IC50 value and clinical resistance. 2. The impact of different resistance mutations on the IC50 are variable and this is poorly reflected in reporting. 3. Genotyping can detect and quantify mixed viral populations with increasing sensitivity and discrimination, but the clinical impact of mixed infections is unclear and the optimal reporting strategy has not been defined. 4. NA/HA imbalance could result in resistance to NAI as a class, but there is no simple validated assay for such a characteristic.

It is clear that some guidelines on interpretation are needed, even in the absence of clear clinical correlates, so that a standardised interpretation of resistance or not can be made from both phenotypic and genotypic data across all labs. For this to operate, the inclusion of standard viruses in each assay would seem imperative.

The value of conventional and innovative surveillance systems during the pandemic was recognised. Improvement of surveillance systems depends in some part on resolution of the laboratory concerns described. However, it is important to ensure good links of surveillance with the clinical and scientific communities where it can influence treatment guidance and new drug development.