

Overview of the conference

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Introduction

The ISIRV Antiviral Group conference on *Influenza Antivirals: Efficacy and Resistance* 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 importance of surveillance of the emergence of antiviral-resistant influenza viruses. The meeting's location in Rio de Janeiro reflected the Antiviral Group's commitment to supporting influenza surveillance, laboratory and policy development in South America.

The programme was multidisciplinary, with 130 participants 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 3-day conference consisted of plenary sessions with topical reviews by medical and scientific leaders in their fields, workshops facilitating expert discussion, and interactive demonstrations facilitating educational discussions and potential collaborations.

The topics covered related to three main themes: influenza therapeutics including antiviral policy, laboratory detection and characterisation of antiviral resistance, and surveillance. This overview summarises information presented on the main topics discussed.

Influenza therapeutics and antiviral policy

Current and pipeline anti-influenza drugs

The aminoadamantanes, amantadine and rimantadine, are inhibitors of the M2 ion channel which were introduced in

the 1960s, but are now of little value due to widespread resistance in circulating seasonal and pandemic 2009 viruses. The main drugs of current clinical value are the neuraminidase inhibitors 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). Both are under development for wider approval.

It is widely recognised that new classes of drugs are needed and new developments were reviewed. Arbidol, licensed in Russia and China, acts as a fusion inhibitor that binds to a hydrophobic site in HA2, stabilising the HA against the low pH-induced conformational change necessary for membrane fusion; however, whether this activity or its immunomodulatory properties are the basis of its *in vivo* efficacy has not been established. Flufirvitide-3, a 16-mer peptide, is another fusion inhibitor that also binds to HA2. It has been shown to be active against primary infection and to inhibit virus transmission in ferrets and is currently in Phase I studies in humans. 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, such as the antiparasitic agent nitazoxanide that inhibits the maturation of the influenza haemagglutinin and at high doses showed a reduction in time to alleviation of symptoms in a Phase III study. A number of other existing drugs have possible value as adjunctive treatments, such as statins, fibrates, glitazones, COX2 inhibitors, NAC and resveratrol, and merit further evaluation.

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. Broadly neutralising monoclonal antibodies against conserved

epitopes on the haemagglutinin are another promising line of investigation. New and existing drugs need to be evaluated not just singly but also in combinations, 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, in particular the difficulty in including those with severe disease, and the varying performance of point of care diagnostics causing difficulty in selecting patients to enrol.

Potential new drug targets

The current heavy reliance on neuraminidase inhibitors is clearly not ideal, and several speakers indicated other targets being considered for potential new drug development. These include activities of the polymerase PB1, PB2, PA complex; the nucleoprotein (NP) protein which is essential for virus replication; the NSA1 multifunctional protein; and conserved epitopes on the HA targeted by neutralising monoclonal antibodies. Vertex VX-787, which has a novel mechanism of action, 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 to activate infectivity, or 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.

Issues in clinical management of influenza

The medical need for more effective therapies 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 heterogenous immunocompromised group has poorer response to immunisation, prolonged viral replication and a higher risk of developing antiviral resistance. Antivirals are of clear benefit in this group, but the optimal choice of most suitable drug, dose and duration of treatment are undefined. Reduction in adverse outcomes has been demonstrated among pregnant women treated with oseltamivir, and the potential benefit is considered to outweigh risk. The obese are a newly recognised risk group, and studies in mice showing increased lung inflammation and decreased lung cell proliferation, independent of virus titre, in infected obese mice support the increased susceptibility of the obese to influenza seen during the pandemic. Although current evidence has not supported increased

antiviral dosing of obese patients, the mouse model demonstrated greater protection of obese mice by an increased dose of oseltamivir.

Clinical impact of resistance mutations

The clinical importance of both naturally occurring and drug-induced resistance mutations was discussed. Studies *in vitro* and *in vivo* show that the interactions between antiviral resistance, fitness and transmissibility of viruses 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. Meta-analysis of data from the pandemic shows an association between oseltamivir resistance and pneumonia, although no significant association with hospitalisation. The clinical importance of some of the newly recognised mutations such as Y155H and I223R in NA is not yet clear. The same concerns apply to H5N1; in a series of 8 H5N1 cases, 2 of 3 deaths had resistance emerging at about 5 days into the illness. Further data are needed on the treatment for drug-resistant influenza and the relationship between clinical and laboratory resistance. For example, the H275Y mutation causes a rise in the IC₅₀ for peramivir *in vitro*, but peramivir retains therapeutic activity in a mouse model. It was also noted that with multiple circulating mutations, the potential for reassortment must be monitored.

The public health importance of influenza antivirals

The 2009 pandemic has highlighted the contrast between the process and data required for drug development and the data that are 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 of 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 the use of drugs; and to foster innovation and R&D for new tools. Resistance is recognised as a public health threat, and there is continuing debate about the contributory role of prophylaxis to development of resistance.

Individual countries reported on their antiviral policy 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 antiviral use during the pandemic. There was acknowledgement that many countries had revised policy several times during the pandemic, and this flexibility is an important component of the pandemic response. It was agreed that it is important to derive data about antiviral use and outcomes during the pandemic at an international level, and this work has been started by WHO.

Surveillance

New data from surveillance studies

A summary of surveillance for influenza antiviral resistance described pre-pandemic findings that emergence of resistance is not always linked to drug use, that compensatory mutations can allow a resistant virus to overcome fitness deficits, and emphasised the importance of balance between HA and NA activity. These concepts also have implications for H5N1. Conventional and innovative strategies during the pandemic were discussed, such as the UK's use of community self-swabbing. Priorities going forward are to establish more community and risk-based surveillance programmes with good links to the clinical community and prescribing guidance and stewardship. Integration with structural and animal work is also important to ensure increasing understanding of the viruses and to inform new drug development.

Influenza A(H1N1)pdm09

New reports on the incidence of oseltamivir resistance, due to the H275Y substitution, in influenza A(H1N1)pdm09 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 slightly increasing trend with progression through the pandemic. Thus, UK data showed incidences of resistance among A(H1N1) pdm09 viruses of approximately <0.3%, 1.2%, and 1.8% for the three phases of the 2009 influenza pan-

demic. In the USA, incidence for 2009–2010 was 0.5%, and for 2010–2011, it was 0.9%. Similar data were recorded in Australia, where a localised cluster of resistant viruses occurred in a region around Newcastle, with 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 these viruses decreased (9% in August), and they were not detected in September or October. Resistance was not all associated with the treatment of patients; the incidence of resistance in viruses isolated in the community (i.e. from untreated patients) also increased with time (in the UK, 0% in the first two waves, 8/59 in the third wave; in the USA, 4/35 for 2009–2010, but 25/33 for 2010–2011), and only one of the 25 resistant viruses in the Australian cluster was associated with oseltamivir use.

Surveillance of seasonal viruses

Several reports on seasonal virus drug sensitivity were presented. All confirmed the general observations that most influenza A viruses remained resistant to amantadine and rimantadine, that there remained a high level of oseltamivir resistance (but zanamivir sensitivity) among the residual seasonal H1N1 viruses since the 2007 emergence of oseltamivir resistance and that resistance to both oseltamivir and zanamivir in H3N2 and B viruses was essentially zero.

Laboratory diagnosis 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, will result in resistance. The aminoadamantanes (amantadine and rimantadine) bind to the M2 ion channel of influenza A viruses. Several mutations in the channel (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. In contrast, A(H1N1) pdm09 viruses acquired amantadine resistance of as a property of the M gene of Eurasian swine viruses, which had acquired amantadine resistance in the mid-1980s. Consequently, almost all current influenza A viruses are resistant to the amantadine and rimantadine.

The NAIs bind to the active site of the neuraminidase enzyme. However, for effective binding to influenza A (but not influenza B), NA oseltamivir requires a change in orientation of glutamic acid 276 (E276) in the enzyme active site. Zanamivir does not require this re-orientation and binds much more like the natural substrate sialic acid.

Hence, mutations in the enzyme active site that block the movement of E276 will cause resistance to oseltamivir, but not to zanamivir. These mutations may be subtype specific; the principal mutation in N1 viruses is H275Y, and much less commonly, N294S, while in N2 viruses the principal mutation preventing re-orientation of E276 is R292K. Both drugs derive some binding affinity from an interaction with glutamic acid 119 (E119). Resistance selected *in vitro* to either drug can be generated by mutations at this position, but so far only the E119V mutation, causing resistance to oseltamivir, has been found *in vivo* and has been rarely observed.

Properties of resistant viruses; studies in ferrets

As mentioned above for the aminoadamantanes, if resistance mutations do not affect the fitness of the virus, then the resistant virus will readily compete with wild-type virus. Comparative fitness can be assessed *in vitro* by growth competition experiments, while *in vivo* effects on virus infectivity, pathogenicity and transmissibility are 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 viruses with the H275Y mutation emerge as the predominant seasonal A(H1N1) viruses circulating in 2007–2009. Modelling studies have shown that the emergence of this virus could not have been driven by drug usage. On the contrary, it is apparent that compensatory mutations in NA (and possibly HA) were responsible for the fitness of this oseltamivir-resistant virus. The influenza A(H1N1)pdm09 virus also appears to be able to accommodate the H275Y mutation with little change in fitness, as can some of the more pathogenic A(H5N1) viruses, in part due to their very high NA activity.

Virus fitness is complex to assess experimentally. A summation of data on weight loss, duration of illness, clinical score and pathological changes, as well as contact and aerosol transmissibility, is required to accurately reflect overall virus fitness in animal models. Understanding of how drug resistance mutations impact transmission and fitness is critical to successful public health preparedness for seasonal and pandemic influenza.

Studies of new mutations

A novel NA resistance mutation Y155H in a seasonal H1N1 isolate A/Hokkaido/15/02 caused resistance to all NAIs as a class (ca 100-fold to zanamivir and peramivir). However, this residue is not conserved and is remote from the active site, and H155 is found in other isolates that are not resistant to NAIs. The reduced activity of the Y155H

NA appears to be compensated by a D225G mutation in HA to maintain the HA/NA balance such that NA activity is less essential for virus detachment, resulting in lack of sensitivity to all NAIs. In NAI-sensitive revertants, which retained the Y155H mutation, the effects of the mutation were compensated by two mutations in the NA, V247I and L430Q, both remote from the location of residue 155, which enhanced NA activity, restoring NA/HA balance while retaining H155.

Viruses with an I223R mutation, alone or together with a H275Y mutation, have been isolated from immunocompromised children treated with oseltamivir or oseltamivir, then zanamivir (in the former case). The I223R mutation caused resistance to oseltamivir and to a lesser extent zanamivir and peramivir and did not compromise replicative ability or transmissibility of the virus in ferrets, although pathogenicity was reduced. The dual mutation resulted in marked enhancement of resistance to oseltamivir.

Assay development

Several speakers described the currently used assays for resistance to NAIs of influenza viruses. Phenotypic assays in cell culture are not applicable to detect NAI resistance, although this is the method of choice for the aminoadamantanes and arbidol. Resistance to NAIs is determined functionally by NA inhibition assays and by sequencing the NA (and sometimes HA) gene. The two commonly used substrates for inhibition assays are MUNANA (a fluorometric assay) and NA-Star (a chemiluminescence assay). IC₅₀ values differ between the two assays and depend on the particular conditions within each assay. The MUNANA assay may be better at detecting mixtures of resistant and wild-type viruses. NAIs as a class exhibit slow binding kinetics which are affected by resistance mutations. Two novel assays were described to assess these kinetic changes: a real-time kinetic assay using a modified MUNANA assay to measure rates of binding and a novel solid-phase assay that allows the simultaneous evaluation of dissociation of NAIs from multiple mutant and wild-type viruses.

Panels of standard oseltamivir-resistant and oseltamivir-sensitive wild-type viruses to characterise newly established assays and to act as internal controls are available from the isirv-AVG through the VIDRL, Melbourne, and the HPA, London.

Several genotypic assays are available to detect known resistance mutations; however, novel resistance mutations can only be detected by a combination of phenotypic and genotypic analysis. Methods include conventional (Sanger) sequencing, various forms of RT-PCR or pyrosequencing and the single nucleotide polymorphism assay (SNAPshot assay). All have different advantages and disadvantages. 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 has detected about four times more resistant viruses than the phenotypic assay. Several databases are now available where genetic data can be compared to aid the interpretation of mutations detected in new sequences. A novel molecular surveillance tool FluSurver is available online to facilitate screening for particular mutations, the co-occurrence of other mutations and associated phenotypes to determine the frequency of occurrence and potential significance.

Issues outstanding and areas for further research

Influenza therapeutics and antiviral policy

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

Laboratory detection of resistance

Data from an international laboratory proficiency assessment involving 16 countries, co-ordinated by the HPA, London, exemplified many of the outstanding issues in the 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 outstanding 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 is 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 imbalance.

It is clear that some guidelines on interpretation are needed, even in the absence of clear clinical correlates, so that a standardised nomenclature for resistance (or reduced susceptibility) could be agreed for both phenotypic and genotypic data. For this to operate, the inclusion of standard viruses in each assay would seem imperative.

Surveillance of antiviral resistance

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

Conflicts of interest

Professor N. Roberts has received fees for consultancy services during the last two years from Hoffmann-La Roche, Pfizer, Biota and Vertex..