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Meeting report

Meeting report: 4th ISIRV antiviral group conference: Novel antiviral therapies for influenza and other respiratory viruses

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ABSTRACT

The International Society for Influenza and other Respiratory Virus Diseases (isirv) held its 4th Antiviral Group Conference at the University of Texas on 2–4 June, 2015. With emerging resistance to the drugs currently licensed for treatment and prophylaxis of influenza viruses, primarily the neuraminidase inhibitor oseltamivir phosphate (Tamiflu) and the M2 inhibitors amantadine and rimantadine, and the lack of effective interventions against other respiratory viruses, the 3-day programme focused on the discovery and development of inhibitors of several virus targets and key host cell factors involved in virus replication or mediating the inflammatory response. Virus targets included the influenza haemagglutinin, neuraminidase and M2 proteins, and both the respiratory syncytial virus and influenza polymerases and nucleoproteins. Therapies for rhinoviruses and MERS and SARS coronaviruses were also discussed. With the emerging development of monoclonal antibodies as therapeutics, the potential implications of antibody-dependent enhancement of disease were also addressed. Topics covered all aspects from structural and molecular biology to preclinical and clinical studies. The importance of suitable clinical trial endpoints and regulatory issues were also discussed from the perspectives of both industry and government. This meeting summary provides an overview, not only for the conference participants, but also for those interested in the current status of antivirals for respiratory viruses.

Contents

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1. Background

The International Society for Influenza and other Respiratory Virus Diseases (isirv) is an independent, international scientific professional society promoting the prevention, detection, treatment, and control of influenza and other respiratory virus diseases. The isirv Antiviral Group (isirv-AVG) was established following the merger in April 2011 of the former Neuraminidase Inhibitor Susceptibility Network (NISN) with isirv. The Group aims to promote understanding of the means of prevention, treatment and control of influenza and other respiratory virus diseases, including the emergence of antiviral resistance. To communicate preclinical and clinical developments of potential novel/new antivirals three meetings have been held annually in different regions of the world.

The 4th isirv Antiviral Group Conference was held at the University of Texas on 2–4 June, 2015. With emerging resistance to the drugs currently licensed for treatment and prophylaxis of influenza (primarily the neuraminidase inhibitor (NAI) oseltamivir phosphate (Tamiflu) and the M2 inhibitors amantadine and rimantadine) and the lack of effective interventions against other respiratory viruses, the 3-day programme focused on the discovery and development of inhibitors of several virus targets and key host cell factors involved in virus replication or in mediating the inflammatory response. Virus targets included the influenza haemagglutinin, neuraminidase and M2 proteins, and both the respiratory syncytial virus (RSV) and influenza polymerases and nucleoproteins. Therapies for rhinoviruses and Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV). and severe acute respiratory syndrome coronaviruses (SARS-CoV) were also discussed. With the emerging development of monoclonal antibodies as therapeutics, the potential implications of antibody-dependent enhancement of disease were also addressed. Topics covered all aspects from structural and molecular biology to preclinical and clinical studies. The importance of suitable clinical trial endpoints and regulatory issues were also discussed from the perspectives of both industry and government. This meeting summary provides an overview, not only for the conference participants, but also for those interested in the current status of antivirals for respiratory viruses.

2. Keynote lectures

2.1. Public health impact of antiviral therapy for respiratory diseases

Nancy Cox, Centers for Disease Control and Prevention, Atlanta, USA.

Prior to licensing of the NAIs in 1999, the aminoadamantanes were the only drugs used for the treatment and prevention of influenza. These target the virus M2 ion channel protein, involved in virus uncoating in the endosome (see 9.3 below). However, due to central nervous system complications in the elderly and lack of efficacy against influenza B, they were not widely employed. Additionally, since 2000 many viruses have acquired mutations in the M2 gene conferring resistance, including the current human A(H3N2) and A(H1N1)pdm09 viruses, and avian influenza A(H5N1) and A(H7N9) viruses which have caused sporadic human infections. Resistance initially emerged in China, possibly related to the ready availability of the aminoadamantanes in over the counter medications, and use in poultry feed.

The NAIs Tamiflu (prodrug oseltamivir phosphate), taken orally as a capsule, and Relenza (zanamivir), an inhaled powder, were approved in 1999–2000. More recently, peramivir (Rapivab), administered intravenously, as well as a long-acting derivative of zanamivir, laninamivir (Inavir), have been approved in some countries. The US CDC recommends the use of NAIs for patients with suspected or confirmed influenza who are hospitalised or at high risk for complications due to influenza, including patients less than 2 and more than 65 years of age, those with underlying medical conditions or compromised immunity, and pregnant women (CDC, 2015b). Clinicians are advised to use clinical judgement to guide NAI treatment of outpatients who are not at high risk. One study showed that antivirals are under-prescribed for high-risk outpatients, with only 19% receiving a prescription when presenting at less than 48 h post symptom onset (Havers et al., 2014). Furthermore, only 7.5% of all laboratory-confirmed influenza outpatients were given an antiviral prescription, versus 30% who were prescribed antibiotics. With the recent emergence of the novel influenza A(H5N2) circulating in poultry in the USA, CDC recommends antiviral treatment as soon as possible for hospitalised patients with novel influenza A viruses associated with severe infection.

While influenza vaccines are still recommended as the means of primary protection (CDC, 2015a), in 2014 a new human H3N2 strain emerged which was antigenically distinct from the H3N2 vaccine component. Interim vaccine effectiveness against H3N2 virus infections was estimated at only 23% in January 2015 (Flannery et al., 2015). This new H3N2 virus resulted in the highest numbers of H3N2 hospitalizations in the USA since records began more than 10 years ago. Thus the CDC focused communications on reminding clinicians about recommendations for NAI use, regardless of vaccination status.

Recently there has been controversy over the efficacy of the NAIs. The Cochrane group carried out meta-analysis of clinical data from 20 oseltamivir and 26 zanamivir trials (all enrolled outpatients with uncomplicated influenza), concluding that oseltamivir provided a 17 h benefit for adults and a 29 h benefit for children, and zanamivir, a 0.6 day benefit among all enrolled participants, regardless of influenza infection (intention to treat (ITT) population) (Jefferson et al., 2014). They failed to find a significant decrease in the number of hospitalizations after treatment, and concluded that, with the increased risk of nausea from oseltamivir (4%) and possible psychosis, the harm from treatment outweighed the benefits. However, the PRIDE study (Muthuri et al., 2014), using meta-analysis of global hospitalizations of more than 29,000 individual patients in 78 observational studies from 2009 to 2010, showed that NAI treatment led to a 19% decrease in mortality in adults, and, with early treatment, to a 50% reduction in mortality. No significant benefit was observed in children.

More recently, the Multi Party Group for Advice on Science (MUGAS) repeated a meta-analysis of randomised, placebo-controlled clinical trials of oseltamivir enrolling outpatient adults using individual level patient data. They demonstrated a 1 day decrease in the time to alleviation of symptoms, a 44% reduction in antibiotic prescriptions and a 60% decrease in hospitalizations in the ITT influenza-infected population (Dobson et al., 2015).

New antivirals are needed, but they face higher benchmarks to prove efficacy, including demonstration of reduction of severe complications due to influenza, and a recent mistrust of studies funded by industry. This will lead to higher costs of clinical trials and ultimately of the drugs. The dilemma is who will pay?

2.2. Clinical development of antivirals for respiratory diseases

Fred Hayden, University of Virginia, USA.

Recent clinical trials were disappointing for both the inhaled long-acting derivative of zanamivir, laninamivir, in uncomplicated influenza and intravenous peramivir in hospitalised patients, both failing to meet the primary clinical endpoint of alleviation of symptoms or normalization of vital signs. Additionally, DAS181, a sialidase fusion protein, which acts by removing potential sialic acid receptors for influenza (Belser et al., 2007), showed some decrease in virus load in uncomplicated influenza, but no significant clinical benefit (Moss et al., 2012). Combination therapy, mostly of NAIs with other drugs against different targets (Hayden, 2013), are currently under evaluation, to try to increase potency and reduce the likelihood of oseltamivir resistance. DAS181 is also being tested against parainfluenza (PIV) viruses, which also use sialic acid receptors (Jones et al., 2013).

For rhinoviruses, pleconaril, a capsid binding inhibitor, resulted in some decrease in total symptom severity, but it was not broadly effective against the multiple different strains of the virus. A newer derivative, vapendivir, is being tested in adults with rhinovirus infections and asthma (ClinicalTrials.gov, 2015). Many other inhibitors were discussed in more detail in the subsequent program.

3. Inhibitors of virus polymerases, nucleoproteins and accessory proteins

3.1. Influenza

3.1.1. Keynote lecture: structure, mechanism and drug targeting of influenza polymerase

Stephen Cusack, EMBL, Grenoble, France.

Influenza RNA-dependent RNA polymerase is a good drug target since its active site is highly conserved. The polymerase complex, composed of three proteins, PA, PB1 and PB2, is essential for transcription, to generate capped viral mRNAs, replication, to generate the full length vRNA genome for incorporation into new virions, and antigenome cRNA, providing the template for full length vRNA. PB2 binds host cell pre-mRNA associated with the cell Pol II, then short 5′ capped RNA fragments are cleaved off by the PA endonuclease, in a process known as cap snatching. Transcription elongation is then initiated by PB1 using the capped primer. Early attempts at crystallization of soluble fragments had limited success. However more recently, expression of the complete trimeric polymerases, using self-cleavable polyproteins, of the novel bat influenza virus H17N10 and influenza B enabled solving the X-ray crystal structures in complex with the RNA promoter, comprising 16–18 nucleotides from the 3′ and 5′ ends of vRNA, to between 2.7 and 3.4 Å resolution (Pflug et al., 2014; Reich et al., 2014). The structures suggest that the cap-binding domain of PB2 rotates by 70°, first allowing the endonuclease to cleave the host pre-mRNA, to an alternative configuration in which the capped oligomer can enter the polymerase active site and prime transcription. All three subunits are thus involved in RNA binding and transcription. When the influenza B polymerase was complexed with 5′ vRNA only, it yielded a different structure to that of the complex with both the 3′ and 5′vRNAs, which may represent an alternative conformation active in cap snatching.

The La Crosse bunyavirus which has only 3 RNA segments has a single L polymerase protein. This is similar in total size to the influenza complex, and structural analysis showed it to have similar overall architecture and domain structure as the influenza enzyme, despite their divergence (Gerlach et al., 2015).

Structure-guided drug design has been used to develop inhibitors against the cap snatching PB2 subunit and the PA endonuclease (Kowalinski et al., 2012; Pautus et al., 2013). The PA inhibitor chelates the two critical manganese ions in the active site of the enzyme. Savira (an ESRF spin off) and Roche are undertaking further development of the polymerase inhibitors. Resistance studies *in vitro* have shown that after 10 passages of A/Aichi/2/68 (H3N2) virus in the presence of an endonuclease inhibitor there was no change in the IC_{50}.

3.1.2. Safety and efficacy of JNJ-63623872 (VX-787)

Lorant Leopold, Janssen Pharma, Titusville, USA.

JNJ-63623872 (VX-787) is a non-nucleoside inhibitor targeting PB2 (Byrn et al., 2015; Clark et al., 2014). It inhibits production of viral mRNA, preventing death of infected cells, unlike the NAIs which still allow cell death. It was effective against all influenza A strains tested in cell culture, including those which are resistant to NAIs, and was also effective in reducing mortality in both H1N1 and H5N1 lethal mouse models. At 3 mg/kg it resulted in up to 50% decrease in mortality due to A/Puerto Rico/8/34 (PR8, H1N1) infection in mice. With 10 mg/kg it could be delivered up to 120 h post-infection and still lead to an 80% decrease in mortality. Synergy was demonstrated in combination with oseltamivir, zanamivir or favipiravir *in vitro*. While 0.3 mg/kg alone was not protective in mice, there was a synergistic effect with oseltamivir leading to 100% survival.

In a human challenge study different doses were tested, including 100 or 400 mg once daily for 5 days, or a loading dose of 900 or 1200 mg on the first day followed by 600 mg once daily for 4 days. The primary endpoint was the area under the curve for virus shedding, determined by virus infectivity in cell culture and qRT-PCR. A statistically significant decrease in virus shedding was seen in the 1200/600 cohort, although the duration of virus shedding was not affected. There was also a dose-dependent decrease in symptom scores and duration of symptoms. Resistance screening revealed a mutation M431I in the PB2 gene in four of 72 patients, which resulted in a 57-fold reduction in sensitivity. However, mutant viruses had reduced fitness, estimated to be 8% of wild type. Cusack commented that resistance was seen within 2 passages *in vitro*. Mutation of phenylalanine 323, involved in tight binding of the inhibitor to influenza A, to serine leads to resistance. Lack of this phenylalanine in influenza B PB2 accounts in part for naturally poor binding and lack of activity against influenza B. A phase 2b trial is evaluating the dose range and once versus twice daily dosing in healthy patients.

3.1.3. The nucleoprotein of influenza virus, a target for new antivirals

Anny Slama-Schwok, INRA, Jouy en Josas, France.

Influenza ribonucleoprotein (RNP) complexes are composed of the three subunits of the polymerase associated with the viral RNA genome covered by multiple copies of the nucleoprotein (NP). The NP is a highly conserved protein and thus potentially a good target for a broadly-reactive antiviral. The recent structures of the polymerase complex and cryo-electron microscopy studies of the RNP have provided the basis for *in silico* screening of the Sigma catalogue for inhibitors of NP-polymerase and NP-RNA interactions. Naproxen, a known anti-inflammatory inhibiting cellular cylcooxygenase 2 (COX-2), was identified as a competitor of NP–RNA interaction, and protected the NP C-terminus against proteolysis. Naproxen reduced infection of both H3N2 and H1N1 viruses in cells with an EC_{50} of 50 μM, and at 2–8 mg/kg per day decreased virus titers in mice (Lejal et al., 2013). Two derivatives were synthesised to be more specific for viral RNP, naproxen A and CO (Tarus et al., 2015). Naproxen CO competes with RNA binding to NP and destabilizes NP-RNA oligomers while stabilizing monomeric NP. It was more potent with an IC_{50} of 2–3 μM, more soluble and less toxic than naproxen, but no longer inhibited COX-2 in human A549 cells.

Work on anti-inflammatory compounds targeting the NADPH

oxidase (NOX) are aimed at a dual antiviral and anti-inflammatory approach.

3.1.4. Identification and characterization of influenza variants resistant to a viral endonuclease inhibitor, L-742,001

Gyanendra Kumar, St Jude Children's Research Hospital, Memphis, USA.

L-742,001 is a diketo acid inhibitor of the influenza virus polymerase PA subunit (Stevaert et al., 2015). Stevaert et al. (2013) previously described mutations which confer reduced sensitivity (Stevaert et al., 2013). Kumar carried out random mutagenesis as natural mutants did not emerge after 10 passages in cell culture. They generated a plasmid library, then used reverse genetics to generate recombinant PR8 viruses. These were then passaged in the inhibitor for 3–4 passages, and then potentially resistant viruses were selected. Mutations included I79L, E119D, T20A (also seen by Stevaert et al., 2013), and F105S. Mutations marginally reduced polymerase activity, but viruses did not appear to lose fitness, with similar growth properties *in vitro* and similar weight loss, virus titers and MLD_{50} in mice.

3.1.5. Structure-based development of a new class of influenza endonuclease inhibitors

Joseph Baumann, Rutgers University, USA.

Crystals of an N-terminal domain of the endonuclease (PA) of an A(H1N1)pdm09 virus were used for fragment screening to identify new chemical entities binding to the enzyme active site. A 3-hydroxy-2-pyridone scaffold was shown to bind at 3 sites, identifying a new mode of chelation of the active site metal ions (Bauman et al., 2013). A third Mn^{++} ion, not previously identified, was also detected. SAR studies led to improved binding, reducing the IC_{50} from 16 μM to 11 nM in a fluorescence based enzyme assay, and 11 μM in a plaque reduction assay (PRA) against PR8 (H1N1). The IC_{50}s in enzyme assay varied depending on whether Mg^{++} or Mn^{++} was in the reaction. The most active compounds had an IC_{50} of 0.2 μM in the PRA.

3.1.6. Clinical and anti-influenza virus effects of favipiravir, a novel anti-RNA virus, anti-influenza agent

Carol Epstein, Medivector Inc, Boston, USA.

Favipiravir, T-705, is a selective inhibitor of the RNA-dependent RNA polymerase of influenza virus, and is also reported to inhibit a broad range of other RNA viruses (Furuta et al., 2013; Oestereich et al., 2014). Additionally, since it does not target the NA, it is effective against oseltamivir-resistant viruses (Tarbet et al., 2014). Over 1500 patients have been studied in clinical trials, evaluating symptom improvement and emergence of resistance. In the first phase 2 trial of 550 patients over 5 seasons, no significant benefit was observed. The second phase 2 trial evaluated twice (BID) versus three times (TID) daily administration with different dosing: 1200 mg TID for 1 day, then 600 mg TID for 4 days; a 2400 mg loading dose and two 600 mg doses on day 1, followed by 600 mg TID for 4 days; or 1800 BID on day 1 and 800 mg BID on days 2–5. The higher loading dose was needed to maintain the C_{min} levels of 20 μg/ml. The BID and TID groups both showed a more rapid decrease in virus titers compared to the placebo groups. Interestingly, the BID group showed a statistically significant decrease in the time to resolution of symptoms, but the TID did not. Among individual symptoms, a statistically significant decrease was demonstrated for 6 symptoms, but not for fever. No resistance has been detected in more than 700 clinical samples tested so far, with more than 1500 samples to test from a Phase 3 multi-country trial that recently concluded enrolment.

3.2. Respiratory syncytial virus

3.2.1. Discovery and development of ALS-8176, a nucleoside analogue inhibitor of the RSV RNA polymerase

Julian Symons, Alios Biopharma Inc, San Francisco, USA.

The RSV L protein is a viral RNA-dependent RNA polymerase that contains multiple enzyme activities required for RSV replication. Using a cell based screening assay, ALS-8112, a novel cytidine-based analogue, and its orally available prodrug, ALS-8176, were identified as potent and specific inhibitors of the replication of laboratory and clinical strains of RSV A and B (Deval et al., 2015), with EC_{50}s of approximately 100 nM and a K_i of 90 nM for the enzyme. Both a 2′F and the 4′$ClCH_2$ groups contributed to the selectivity of ALS-8112. *In vitro*, the inhibitor caused chain termination of viral RNA synthesis, but did not inhibit cellular or unrelated virus RNA polymerases. It also inhibited related paramyxoviruses, including human metapneumovirus and parainfluenza virus 3. High levels of the ALS-8112-triphosphate formed in A549 and primary epithelial cells. Oral administration to non-human primates resulted in high levels of the NTP in the lung, with a half-life of 29 h.

In human challenge studies in adult volunteers (DeVincenzo et al., 2015), ALS-8176 was administered either with a high loading dose of 750 mg followed by a maintenance dose of 150 or 500 mg, or as a constant dose of 375 mg. In the placebo group, by quantitative RT-PCR the peak RSV RNA titer was around 10^4 log_{10} at 3.5 days post challenge. In the 750/500 mg and 750/150 mg groups there was an immediate decrease in the RSV RNA levels, and a decrease in symptom scores and mucus weight. In the 375 mg group there was an initial increase in virus RNA followed by a decrease, thus indicating the importance of the high loading dose. ALS-8176 was well tolerated and no resistant virus was isolated, although *in vitro* after 35 passages mutations were found in the active site of the L protein. It is currently undergoing evaluation in hospitalised infants.

3.2.2. RSV polymerase and nucleoprotein inhibitors: mechanism of action and resistance

Qin Yu, AstraZeneca R&D Boston, Waltham, USA.

AZ-27, a polymerase inhibitor (Tiong-Yip et al., 2014), was active against both RSV A (average $EC_{50} = 24 \pm 9$ nM) and B (average $EC_{50} = 1.0 \pm 0.28$ μM) subtypes in many cell lines, and was effective when added up to 24 h post infection. There was no detectable cytotoxicity at 100 μM. AZ-27 inhibits an early stage in mRNA transcription, as well as genome replication, by inhibiting initiation of RNA synthesis from the promoter (Noton et al., 2015). Resistant viruses were isolated after 7 passages in culture; a dominant mutation, Y1631H, in the putative capping enzyme domain of L protein, resulted in a greater than 5000-fold increase in EC_{50} against AZ-27.

The RSV nucleoprotein (N) is essential for virus replication and assembly as part of the viral RNP complex. Previously, RSV604, a benzodiazepine, has been reported to inhibit both RSV RNA synthesis and the infectivity of released virus, with an EC_{50} of approximately 1 μM (Challa et al., 2015). Its potency is cell line dependent. Yu and colleagues reported here that they have identified a novel thienodiazepine as an N inhibitor with improved potency and broad spectrum activity against both RSV A and B strains, although it was also cell type dependent. The compound demonstrated direct binding to recombinant N fragments by surface plasmon resonance (SPR) with a K_d of 1.93 μM. An X-ray co–crystal structure showed the binding site overlapped the N–P interaction site. Mutations at residues N80 and N28 caused reduced inhibition, being resistant to 2x, but not to 10x, the EC_{50}. They did not, however, affect inhibitor binding to the N protein as assessed by SPR, suggesting that the mechanism of 'resistance' may not be

due to reduced inhibitor–N binding. The dual mechanism of action for these N inhibitors highlights the potential for this class of inhibitor in delivering efficacy and suppressing resistance.

3.3. Influenza NS1

3.3.1. Novel broad spectrum antiviral against influenza blocks dsRNA binding to NS1A protein and restores antiviral responses

Ji-Young Min, Institut Pasteur, Korea.

The influenza A virus non-structural 1 (NS1A) protein is a known antagonist of the host antiviral response. A small molecule, named BOC, inhibited human seasonal H1N1, H3N2, and B viruses and avian H5 and H7 subtype viruses. The dsRNA binding pocket of the NS1A protein was shown to be the putative target based on an *in vitro* dsRNA binding assay. Influenza A virus-infected cells showed increased NF-κB nuclear translocation and elevated levels of various cytokines related to interferon-stimulated genes. BOC administered to mice infected with an avian influenza virus reduced lung virus titers in the early stage of infection. Results thus suggest BOC may abrogate the antagonistic responses of the NS1A protein.

4. New inhibitors of influenza NA and M2 activities

4.1. Neuraminidase

4.1.1. The influenza neuraminidase - old target, new approaches

Jenny McKimm-Breschkin, CSIRO, Parkville, Australia.

While the current NAIs are based on the transition state analogue DANA, a new series of compounds, based on the natural substrate sialic acid, 2,3, difluoro sialic acids (DFSA), have recently been described (Kim et al., 2013). DFSAs require the catalytic activity of the NA to bind, hence are termed mechanism-based inhibitors. They form a covalent link to a tyrosine in the enzyme active site. Unlike the NAIs, they are not competitive inhibitors and their efficacy depends on a slow turnover of the covalently linked inhibitor. Substitution on the sugar ring, with either 4-amino or 4-guanidino groups, affected affinity and rates of binding and dissociation in an IC_{50} kinetics assay (Barrett et al., 2011). Even more striking was the effect of the orientation of the 3 fluoro group, with the equatorial being more potent than the axial. DFSAs have broad efficacy, with IC_{50}s in enzyme assays and in plaque reduction assays in the low nM to μM range, and can protect mice from a lethal influenza infection. They are also effective against viruses resistant to oseltamivir and zanamivir. Theoretically resistance to DFSAs should be more difficult to select, since mutations would compromise the NA function. No resistant viruses were selected after 12 passages of an A(H1N1) pdm09 and an influenza B virus *in vitro*, with virus reduced to almost undetectable levels. A further 3 passages in a lower drug concentration, however, led to isolation of a small plaque variant from the A(H1N1)pdm09 virus, which was highly resistant in the plaque reduction assay. The mutant virus had barely detectable enzyme activity, but replicated to comparable titers *in vitro* as the wild type virus. Preliminary results suggest a novel mutation/s in the NA, which remains to be confirmed. These DFSAs are also active against human PIV3 (Dirr et al., 2015; Streltsov et al., 2015). Since the NAs of human H3N2 (Mohr et al., 2015) and H1N1 (Hooper et al., 2015) viruses can demonstrate receptor binding as well as the catalytic function, with a recent focus on antivirals targeting the HA we need to understand this secondary role of the NA, which may compensate for inhibition of the HA receptor binding function. We may need to target both the HA and NA.

4.1.2. New inhibitors of influenza A neuraminidases

Mario Pinto, NSERC, Ottawa, Canada.

In addition to the catalytic site, in group 1 NAs amino acids 149–152 form a loop, which either projects into the active site in the closed conformation, or remains outside the active site in the open conformation, creating the 150 cavity (Adabala et al., 2013; Amaro et al., 2011; Wang et al., 2011). Pinto and colleagues have created a carbocyclic analogue of zanamivir, by replacing the hydrophilic glycerol side chain with the hydrophobic pentyloxy group of oseltamivir (Kerry et al., 2013; Mohan et al., 2014). This inhibitor targets only the catalytic site, and showed nM potency in the NAI assay (Ki = 0.46 nM; Ki (zanamivir) = 0.16 nM) and an EC_{50} of 80 nM in cell culture; activity was only 10-fold lower against the H275Y oseltamivir resistant mutant (100–1000 fold resistant). This appears to be due to increased flexibility of the pendant pentyloxy group and the ability to pivot about a strong hydrogen-bonding network. However, its properties suggest that it would still only be suitable for topical application.

Pinto also described a series of triazole-containing carbocyles, related to oseltamivir, which bound in both the catalytic site and 150 cavity (Adabala et al., 2013; Amaro et al., 2011; Mohan et al., 2010; Wang et al., 2011). Inhibition of virus replication *in vitro* with triazole derivatives containing either an amino or guanidino modification indicated that the guanidinium compound had higher efficacy against an N2 subtype, at a concentration of 20 μM, and did not inhibit replication of an N1 subtype virus even at a concentration of 100 μM. He also briefly described the serendipitous discovery of a potent spirolactam NA inhibitor (Mohan et al., 2014).

4.1.3. Delayed oseltamivir and T-705 combination therapy protects mice against lethal influenza A(H5N1) infection

Bindumadhav Marathe, St Jude Chidren's Research Hospital, Memphis, USA.

Due to the emergence of resistance to oseltamivir, several groups are investigating combinations of therapeutics directed against different virus targets. Oseltamivir (20 mg/kg), T-705 (50 mg/kg) or a combination of the two was administered to mice at 48, 72, 96 or 120 h post-infection. All mice were protected at 48 and 72 h, but at 96 h only the combination gave 100% protection. After 120 h the single drugs had a 25% survival rate, compared to 80% with the combination. There was a 2 $\log_{10}$ decrease in mouse lung virus titers for the monotherapy, and a 4 $\log_{10}$ decrease for the combination therapy. Analysis of lung tissue by immunohistochemistry and histomorphometry revealed that combination therapy restricted virus spread and reduced the extent of lesions by 30% compared with monotherapy. Combination therapy also reduced the pro-inflammatory cytokine response, which reduced disease severity and facilitated recovery.

4.1.4. Influenza viral load and peramivir kinetics after a single administration in children

Masaktoki Sato, Fukushima Medical University, Japan.

While peramivir (Rapivab) administered as a single dose (i.v.) has been licensed in Japan for treatment of adults, children and infants, it has only been approved in the USA for acute uncomplicated influenza in patients 18 years and older. This study investigated the pharmacokinetics of peramivir in children (Sato et al., 2015). While the mean serum peramivir concentration among 28 children at 0.5 h after a 10 mg/kg dose was 88.9 μM, after 1, 2, 3, 4 and 5 days it was reduced to 83.2, 25.1, 17.1, 6.2 and 4.6 nM, respectively. The mean concentration in the URT at 0.5 h after administration was 5.1 μM and 48.3 nM at 1 day, but the drug was undetectable after day 2. While viral RNA load in both H3N2 and H1N1 infections decreased from day 0 to day 3, it increased in 9/12 patients after day 3. The decrease in viral RNA load was much less for influenza B than influenza A. No resistant virus was detected. Hence a single peramivir dose appears to be inadequate virologically, and

readministration should be considered in severe cases.

4.2. Influenza M2

4.2.1. Is M2 a good target to combat drug resistance in the influenza A viruses?

Jun Wang, University of Arizona, Tucson, USA.

Once the influenza virus binds to sialic acid receptors, it is taken up into the endosome, where the low pH activates the formation of the M2 ion channel, causing an influx of protons, into the virion interior. This results in dissociation of the M1 matrix protein from the virus RNP, which is then released into the cell. The M2 protein is highly conserved, and can be inhibited by the adamantanes. However resistance arises rapidly, due to three major mutations, L26F, V27A or S31N, S31N being the most common. The S31N mutant has a more contracted channel compared with that of the wild type (WT). It is hypothesised that the pore undergoes a conformational change from open-closed to closed-open as the H^+ goes from outside to inside. Guided by molecular dynamics simulations, NMR and X-ray crystallography studies, the group developed amantadine derivatives which are effective against the resistant mutants (Wang et al., 2015, 2013). One compound which contains a charged ammonium group, M2WJ332, sits inside the channel, and in the plaque reduction assay has an EC_{50} of 153 nM against the S31N mutant in comparison to 328 nM for amantadine against the WT M2. Four passages in drug concentrations increasing from 300 to 600 nM did not select a resistant mutant. However, greater increase in drug concentrations (330, 376 nM, 1.6 and 35.9 μM) selected resistant mutants with an L26I mutation, and by passage 6 a reversion of the N31S occurred, which decreased binding. However, the limitation remains that new compounds need to bind both the wild type and mutant M2s, and the L26I mutation is found naturally in avian viruses isolated from wild birds. Additionally, the BM2 of influenza B viruses is not inhibited by aminoadamantanes.

5. Inhibitors of virion attachment/fusion proteins

5.1. Influenza

The influenza virus HA protein is a homotrimer, in which each monomer is composed of two disulfide-linked subunits, HA1 and HA2. The HA binds to sialic acid receptors on the cell surface, and the virus is then taken up into endosomes, where the low pH leads to a conformational change in the HA trimer to its fusogenic form. HA1 forms the head region of the protein, which is primarily involved in receptor binding, whereas HA2, referred to as the stem region, is involved in promoting virus–host membrane fusion. Various groups have targeted the HA to try to either stabilize the prefusion form or block the receptor binding site. Antibodies directed against the head region appear to be more potent, whereas those directed to the stalk region appear to be more broadly reactive.

5.1.1. The influenza HA as an antiviral target

George Gao, Chinese Academy of Sciences, Bejing, China.

Gao described the discovery of an anti-stem monoclonal antibody, CT149, which was isolated from convalescent plasma from patients infected with the A(H1N1)pdm09 virus in 2009 (Wu et al., 2015). CT149 neutralised all group 2 H3N2 viruses tested and two H7N9 viruses, but surprisingly only some group 1 influenza viruses, including 1/6 H1, two H5 and two H9N2, inhibiting low pH-induced HA-mediated membrane fusion. Both the heavy and light chains are involved in binding, and the antibody cross-links two monomers of the trimer. This antibody protects against H3N2 infection both *in vitro* and in mice.

A second antibody, MAb#14, derived from an H7N9 virus-infected patient binds near the receptor binding site. It sits above the 190 helix, interacting partly with the 130 and 150 loops in the head of the HA. However it is specific only for the H7 HA, since this region is not conserved.

5.1.2. Blocking influenza virus by stabilizing the pre-fusion conformation of HA

Megan Shaw, Mount Sinai Hospital, New York, USA.

High throughput screening used a recombinant influenza A/WSN/33 virus with the luciferase gene replacing the HA gene and cells expressing the HA. Luminescence was only detected if whole virus was produced. From almost 10^6 compounds, the hit rate was 0.5%, (4582), with 744 compounds showing a dose response effect (White et al., 2015). The top five were screened against wild type viruses, with three showing influenza A specific inhibition, but not broadly reactive. The other two were broadly reactive, M4 reacting against a subset of viruses and M53 showing reactivity against both RNA and DNA viruses, suggesting a host cell target. The three influenza-specific hits were S57, which had broader reactivity, interacting with the C-terminus of the NP, S119, reacting with the N-terminus of the NP protein, and S20, reacting with the HA of group 1 viruses, but not H3N2 or influenza B viruses. When tested against wild type virus, S20 had an IC_{50} of 80 nM and caused a maximum reduction in virus titers of >3 $\log_{10}$ with low toxicity (CC_{50} 40 μM). Time of addition assays and use of an HA/NA pseudotype HIV particle showed that S20 inhibited entry. Resistant viruses were selected after 4 passages *in vitro*, all with mutations affecting the HA, primarily around the "B" loop structure connecting the large and small helices of the HA2 subunit (White et al., 2015). In a red cell fusion assay, the pH of fusion (50% fusion at pH 5) was lowered below pH 5 in the presence of S20, indicating stabilisation of the prefusion form by S20. S20 also protected the HA from conversion to the trypsin sensitive form under low pH conditions. In contrast, the pH of fusion of a M59I mutant was unaffected by S20. In silico docking studies predicted that S20 binds both the HA1 and HA2 in the stalk region, corresponding to the area where resistance mutations occurred.

5.1.3. Prophylactic and therapeutic protection against influenza by a computationally engineered protein

Merika Treants, University of Washington, Seattle, USA.

A computationally designed and engineered small protein HB36.6 (90 amino acids) protected mice against weight loss and death when administered either as a single prophylactic dose 48 h prior to virus challenge, or from 24 h post-infection when administered daily at 3 mg/kg. A synergistic response providing full protection was seen with suboptimal doses of oseltamivir (5 mg/kg) and HB36.6. To determine whether an immune response developed to the protein mice were immunised with multiple doses and were then challenged with virus. No immune response to HB36.6 was seen as mice were fully protected. IL-6, IL-10, IFN-γ and IL-12 p7 all of which increased in control mice, were all lower in the HB36.6-treated mice, correlating with lower virus titers and more rapid recovery. HB36.6 also reduced clinical signs in influenza-infected ferrets. After intravenous administration HB36.6 could not be detected in blood, hence pharmacokinetic studies are using inhaled administration. Labelled compound appears to coat the respiratory tract.

5.1.4. Novel family of peptides with potent antiviral activity against influenza viruses

Seema Jasim, The Roslin Institute & Royal (Dick) School of Veterinary Studies, University of Edinburgh, UK.

Approaches to novel antiviral compounds include the

development of synthetic peptides that disrupt the entry of virus into cells. Two peptides, "FluPep" (a family of short, hydrophobic peptides related to suppressors of cytokine signalling-1, SOCS, proteins) and "Entry Blocker" (derived from the signal sequence of fibroblast growth factor-4) were shown to have antiviral activity against a panel of viruses *in vitro* and *in vivo* (Nicol et al., 2012). In plaque reduction assays the FluPep was more effective against H1 than H3 or H4, although PR8 had lower sensitivity compared to the A(H1N1)pdm09 virus. IC_{50}s ranged from the low nM to ~100 nM (Nicol et al., 2012). In PR8 recombinants with different HAs, sensitivity was dependent on the HA. The Entry Blocker did not show the same strain specificity. They acted upstream of the nuclear import of the RNPs. The peptides caused aggregation of fluorescently labelled virus particles on the surface of host cells, and appeared to slow internalisation. Given prophylactically in mice there was a decrease in virus titer, and some protection against weight loss.

5.2. Respiratory syncytial virus

5.2.1. Discovery and proof of concept of GS-5806 in RSV disease

Seth Toback and Mike Perron, Gilead, Foster City, USA.

GS-5806 is an orally available RSV inhibitor that targets the fusion protein, blocking virus envelope fusion with the host cell membrane (DeVincenzo et al., 2014; Mackman et al., 2015). It is highly effective against both RSV A and B with a mean EC_{50} of 0.43 nM against 75 clinical isolates, (RSV A 0.51 nM and B 0.35 nM), and low toxicity (SI > 23,000-fold). GS-5086 does not block RSV attachment to cells, but interferes with virus entry, and cell–cell fusion. In a cell-based fusion inhibition assay the EC_{50} was ~2 nM. Resistant viruses were isolated after 9 passages *in vitro*, with mutations in the F protein, including L138F or F140L/N517I in RSV A, conferring more than 2000-fold resistance, and F488L or F488S in RSV B. These were cross-resistant to another RSV fusion inhibitor VP-14637 (Douglas et al., 2005; McKimm-Breschkin, 2000), also undergoing clinical development as an inhaled product (MDT-637). In cotton rats a single IP dose of 0.3, 3 or 30 mg/kg GS-5806 was administered 1 h prior to intranasal challenge with RSV. A 1.8 $\log_{10}$ difference was seen in virus RNA load in nasal wash and lung lavage samples on day 4, in the 30 mg/kg group. In a small trial with 4 calves infected with 10^5 pfu of nebulised bovine RSV, and treated 1 h prior to infection and twice daily for 7 days with 2 mg/kg, there was a non-significant 3-fold decrease in virus titer, but a significant 3-fold decrease in the symptom score.

A double blind placebo-controlled trial of GS-5806 was carried out in healthy subjects experimentally infected with 10^4 pfu of the RSV Memphis-37b strain. Nasal wash samples from day 2 were tested for RSV by RT-PCR, and when positive or at day 5, whichever was sooner, subjects were randomised into 7 cohorts, with ~20 in each, and treated as follows: Cohorts 1–4 (day 1 50 mg; days 2–5 25 mg daily), Cohort 5 (day 1 50 mg; days 2–3 25 mg daily), Cohort 6 (day 1100 mg), Cohort 7 (day 1 10 mg; days 2–5 5 mg daily). All patients in treatment groups 1–4 showed a significant decrease in the area under the curve (AUC) for $\log_{10}$ virus load, (250.7 vs 757.7 $\log_{10}$ pfu equivalents), lower mucus weight (during 5 days after first dose 6.9 vs 15.1 g, $p = 0.03$), and lower symptom scores. Cohorts 5–7 had comparable results, although Cohort 7 did not show a decrease in mucus weight. Resistance frequency was 5%, with mutations the same as selected *in vitro*, but the viruses showed decreased fitness. Plans are to test a higher single dose, >100 mg, to try to avoid resistance.

5.2.2. RSV antivirals: fusion inhibitors and beyond

John DeVincenzo, University of Tennessee, Memphis, USA.

John gave an overview of clinical observations and correlates of protection. A higher virus load in the nose corresponds with more severe disease and faster virus clearance correlates with faster clinical improvement. Detection of virus mRNA by RT-PCR correlates with virus RNA and is more sensitive than virus culture, with RNA detected often to day 13, although generally cultures are negative after day 6 (IgA in the sample may inhibit virus growth). Lung pathology shows high virus staining, and low numbers of immune cells. Unlike influenza where virus peaks at days 2–3 and symptoms at days 3–4, for RSV virus load and symptoms peak at the same time. Children have higher virus titers and shed virus for longer compared to adults. Palivizumab/Motavizumab monoclonal anti-fusion antibodies, which are effective prophylactically (AAPCID, 2014) showed no clinical efficacy for treatment of children hospitalised with RSV (Ramilo et al., 2014).

A different approach to those already discussed is the use of RNAi, for example, ALN-RSV01 targeting the N gene (DeVincenzo et al., 2010). A trial using a nasal spray of ALN-RSV01, administered daily for 2 days before and 3 days after RSV inoculation, showed antiviral activity against RSV infection, with the proportion of culture-defined RSV infections 71.4 and 44.2% in placebo and in ALN-RSV01 recipients, respectively.

6. Inhibitors of seasonal and emerging threats

6.1. Antiviral strategies for prevention and treatment of rhinovirus infections

Ronald Turner, University of Virginia, USA.

There are currently no antiviral agents licensed for the treatment of rhinovirus infections. The common cold, generally a self-limiting disease, was an original target, but no broadly effective inhibitor has been demonstrated; additionally, it would need to be cheap with no side effects. Interferon, protease inhibitors, and capsid binding inhibitors all showed efficacy in laboratory models, but not in patients (Hayden, 2013). Although rhinovirus (RV) is an important cause of exacerbations of COPD and asthma, hence being alternative targets, there is no good clinical model for evaluation of therapies. Prophylaxis may be more feasible. In a family study there was a significant decrease in asthma scores with pleconaril prophylaxis. However, approaches to therapy have been further complicated by the discovery of a new species of rhinovirus, RV-C, in 2006, in addition to the known RV-A and RV-B viruses (Palmenberg and Gern, 2015). As with RV-B they are inherently resistant to capsid binders and have a different cellular receptor to both RV-A and RV-B (CDHR3 instead of ICAM-1). Host-directed therapies, such as TLR antagonists and probiotics, may be promising, and recent studies have reported some beneficial effect of probiotics (Lactobacillus spp.) on the occurrence of common cold illnesses in children (Ballengee and Turner, 2014).

6.2. Severe illness associated with EV-D68 infection in the United States and approaches to management

Sue Gerber, Centers for Disease Control and Prevention, Atlanta, USA.

Enterovirus EV-D68 was first identified in 1962. Unlike other enteroviruses, which can cause febrile rashes and neurological syndromes, EV-D68 is commonly associated with respiratory illness and may play a role in asthma exacerbation. In the summer and fall of 2014 there was a large increase in severe respiratory illness in the USA and the CDC and state health laboratories identified 1,529 people PCR positive for EV-D68 (Midgley et al., 2015); however rhinovirus, Coxsackie virus and echoviruses will also be detected by the rhinovirus/enterovirus multiplex PCR. Using VP1 sequencing to identify EV-D68, 46% of specimens were positive for EV-D68. Several cases of flaccid myelitis have been temporally related to EV-D68

infection but a causal role of EV-D68 has not been established. A new real-time RT-PCR test is available from the CDC (Zhuge et al., 2015). Three strains have been identified with 92% belonging to one strain, 7.4% to the second, and a single case of the third strain. The virus grows in MK cells and human lung A549 cells and binds to α2,6 linked sialic acids. It is sensitive to pleconaril and other capsid binding inhibitors *in vitro*, and a randomised double blind placebo controlled trial suggested a shorter time to culture and PCR negative status with oral pleconaril. DAS181 which removes sialic acid receptors is also being evaluated (Abzug et al., 2015).

6.3. New targets and approaches for coronavirus antiviral inhibitors

Mark Denison, Vanderbilt University, Nashville, USA.

Coronaviruses (CoVs) are endemic respiratory pathogens, causing mainly colds, bronchiolitis and pneumonia. Additionally, zoonotic transmissions have occurred, with the SARS-CoV and more recently MERS-CoV. A large number of endogenous bat CoVs have been identified. They have the largest genome of any RNA virus, 26–32 kB, with up to three proteases, and a proof reading exonuclease (nsp14-ExoN), endonuclease and helicase-ATPase. The SARS ExoN-minus virus appears to acquire more lethal mutations with 5-fluoro uracil mutagen treatment than the wild type (Smith et al., 2013). It is also attenuated in mice. Hence high replication fidelity is critical for CoVs. Treatment *in vitro* with ribonucleoside analogues EV524 and EV1081 (Gilead) caused a 5 $\log_{10}$ reduction in virus titer compared to the control. A combination of fidelity inhibitors and nucleoside analogues may provide broad activity against multiple CoVs.

6.4. Functional dipeptidyl peptidase (DPP4) in mink supports entry and replication of Middle Eastern Respiratory Syndrome Coronavirus: American mink (Neovision vision), a novel in vivo model of MERS-CoV infection

Thomas Voss, SRI International, Harrisonburg, Virginia, USA.

Dipeptidyl peptidase 4 (DPP4/CD26) is the receptor for MERS-CoV. It is a multifunctional protein and is a target for diabetic therapy, and apoptosis. A cell line derived from mink lung epithelium is susceptible to infection by MERS-CoV. Although ferret cells express DPP4, based on antibody reactivity, ferrets cannot be infected. Mink challenged with MERS-CoV showed no signs of illness or virus in any tissue. Different challenge routes were tried, intranasal, oral, intratracheal and intraocular, and challenge doses from 10^6 -10^9, and different challenge strains, including one adapted to mink lung cells. As an alternative animal model, while normal mice are not susceptible to MERS-CoV, expression of the human DPP4 (hDPP4) overcomes the lack of susceptibility. The transgenic hDPP4 mice develop severe and lethal respiratory disease upon inoculation with MERS-CoV and are potentially a suitable animal model for efficacy testing of therapeutic and prophylactic countermeasures (van Doremalen and Munster, 2015).

6.5. Phase III multi-center clinical trial of nitazoxanide in adult patients with uncomplicated influenza A and B and other influenza-like illness: results on 1876 subjects from the United States, Canada, Australia and New Zealand

Jean-Francois Rossignol, Romark Laboratories, Florida, USA.

Nitazoxanide has been approved for treatment of diarrhoea associated with *Giardia* and *Cryptosporidium* infections. It has been used in more than 75 million patients in the USA and Latin America alone, hence has a known safety profile. It is an orally available, small MW drug, with what appears to be broad antiviral activity, including against influenza, PIV and RSV. Nitazoxanide appears to block the maturation of the influenza virus HA (Rossignol, 2014). In a phase 2b/3 trial in 2010–11, comparing 300 mg–600 mg twice daily in 624 patients, only treatment with 600 mg twice daily for 5 days was associated with a reduction in the duration of symptoms, and a 1 $\log_{10}$ decrease in virus titer, in recipients with acute uncomplicated influenza (Haffizulla et al., 2014). Nitazoxanide has also shown synergistic effects *in vitro* with NAIs (Belardo et al., 2015). In a current Phase III trial, 600 mg of nitazoxanide is being compared to nitazoxanide plus oseltamivir, oseltamivir, or placebo.

7. Monoclonal antibodies as therapeutics

7.1. Monoclonal antibodies as therapeutics against respiratory viruses

Wayne Marasco, Harvard Medical School, Boston, USA.

In studies of respiratory viruses, this group has used non-immune human antibody phage display libraries as a source of human monoclonal antibodies (mAbs). As a complementary strategy, they have used single memory B cell cloning to isolate broadly neutralizing antibodies. To isolate antibodies against the SARS-CoV and MERS-CoV blood samples were obtained from China and Arabia. Binders were identified using pseudotyped viruses. An initial SARS-CoV mAb, 80R, which neutralised all 2002–3 isolates, interferes with the glycoprotein spike S, binding to its ACE2 receptor site. However, viruses isolated from patients in a small outbreak in 2004 were all resistant to neutralization due to a single Asp/Gly mutation. A new mAb was made to cover these escape mutants (Sui et al., 2008). However, recent work has shown that even two mAbs in combination do not prevent the emergence of escape mutants (Sui et al., 2014).

MERS-CoV emerged in the Arabian Peninsula in 2012, but appears to have been endemic for more than 20 years. Many camels are infected, including elite racing camels, although the primary host may be bats. To June 2015, there had been 1150 cases with 434 deaths. Using an ScFv phage library, 7 mAbs, encompassing three different epitopes of the S1 spike, were identified, all inhibiting binding to the DPPD4 receptor in an SPR assay. Escape mutants identified changes in five amino acids critical for neutralization escape. Loss of binding to one epitope did not have a major impact on binding of Abs directed to other epitopes, but mutants had reduced fitness, resulting in decreased virus amplification and reduced inflammation in the lungs of infected rhesus monkeys (Tang et al., 2014).

Screening for antibodies against influenza A(H5N1) identified 10 mAbs which bound a common epitope on the HA stem, preventing the post-attachment fusion process (Sui et al., 2009). All appeared to use the IGHV1-69 variable heavy chain region, with high conservation of CDR-2. These antibodies protected mice and decreased the virus load. The antibodies cross-reacted with other subgroup 1 influenza HAs, but not with subgroup 2. While generation of escape mutants was difficult, those selected had decreased fitness; mutations were outside the epitope, causing destabilization of the trimer. Another mAb, I14, was broadly active against both groups 1 and 2, stabilizing the pre-fusion form of the HA, as assessed by blocking trypsin cleavage of the HA0 to HA1 and HA2. Unexpectedly, despite being selected against a group 1H5N1 strain, its efficacy *in vitro* and *in vivo* is higher against group 2 viruses.

7.2. New antibody-based strategies against viral respiratory diseases

Qing Zhu, Medimmune, Gaithersburg, USA.

The humanised mAb Synagis (palivizumab), which is still the

only mAb against a viral disease approved for clinical use by the US FDA, has been widely used for prevention of RSV infections in neonates and immunocompromised individuals. RespiGam® is a human polyclonal antibody preparation also used for prevention of RSV, but there is significant batch variation. A new mAb, MEDI8897, which has 100-fold higher potency and neutralizes both RSV A and B strains, has an EC_{90} in the cotton rat model of 6.5 μg/ml, compared to 57 μg/ml for palivizumab. The half life was ~40 days in monkeys and ~80 days in a Phase 1a trial in adult humans. The aim is to increase the half life to enable a single intramuscular injection to be effective for the whole season, thus providing vaccine-like immunity.

Another mAb, MEDI8852, neutralizes all influenza A strains by binding a highly conserved epitope in the HA stalk, with a median IC_{50} of 0.76 μg/ml. It has multiple effects on virus-induced membrane fusion, HA maturation and cell–cell spread. It is claimed to be superior to oseltamivir when given late in infection in mice and ferrets. Given 3 days post-infection in mice there was 100% protection, versus 40% protection by oseltamivir. A combination of MEDI8852 and oseltamivir at a suboptimal dose demonstrated a synergistic effect. The aim is to generate a bispecific antibody against both influenza A and B.

7.3. VIS410 monoclonal antibody demonstrates potent efficacy against neuraminidase inhibitors-susceptible and –resistant influenza A(H7N9) viruses and protects mice from ARDS

Tatiana Baranovich, St Jude Children's Research Hospital, Memphis, USA.

VIS410 is a broadly neutralizing human IgG1 anti-HA antibody, which reacts with a region of the HA stalk conserved in group 1 and 2 influenza viruses (Tharakaraman et al., 2015). It appears to have lower affinity to H7 HA, hence its efficacy was tested against the oseltamivir-susceptible A/Anhui/1/2013 or -resistant A/Shanghai/1/2013 (R292K-NA) H7N9 influenza viruses in mice. VIS410 was administered via intraperitoneal injection of either a single 50 mg/kg dose 12 h before (prophylaxis), or a 2, 10, or 50 mg/kg dose 24 h after (treatment) H7N9 virus inoculation. Weight loss, morbidity and virus lung titers were monitored: Prophylactic administration of the antibody resulted in 100% protection of the mice; a single IP administration of 50 mg/kg 24 h post infection also provided 100% survival, whereas the 10 mg/kg dose protected 70% of the Anhui-infected and 90% of the Shanghai-infected mice. All doses of VIS410 tested protected mice from H7N9-induced acute respiratory distress syndrome (ARDS). There was a dose response in the numbers of cells positive for NP. Mice developed an anti-HA immune response, sufficient to provide protection against a 25 MLD_{50} challenge with the homologous virus.

8. Antibody-dependent enhancement (ADE) of disease: implications for therapeutic monoclonal antibody development

8.1. Influence of antibodies and T cells on dengue disease outcome

Sujan Shresta, La Jolla Institute for Allergy and Immunology, La Jolla, USA.

The four types of Dengue virus (DENV) cause up to 100 million cases, with 2.1 million severe cases and up to 21,000 deaths per year. Infections range from asymptomatic to a self-limited febrile illness, dengue fever (DF), to life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The roles of antibodies and T cells in mediating protection versus pathogenesis remains unclear. Antibody-dependent enhancement (ADE) was identified more than 40 years ago, and is hypothesised to be due to the presence of subneutralizing levels of antibody and the consequent binding of antibody-coated virus to the Fc receptors (FcR) which facilitates virus entry, leading to enhanced infection. In the case of secondary infections and of maternal antibody in newborns, there may be some cross-reactive antibodies, but insufficient to neutralize virus. A model of DHF/DSS-like disease has been developed in AG129 mice lacking the type I IFN receptor (IFNAR) and the IFN-γR (IFNAR−/−; IFN-γR−/−). This mouse model reproduces key pathophysiological features of DHF/DSS, including similar cellular and tissue tropism, lethal vascular leakage, cytokine storm, low platelet count, elevated hematocrit, and hemorrhage (Tang et al., 2015). In order to test the hypothesis, mice were pretreated with different levels of antibody IP, followed by DENV challenge: 0.5 μg of antibody was too low to have any effect; subneutralizing doses of 2–15 μg led to severe disease and death; while a fully neutralizing dose of 500 μg protected all mice. Virus load was significantly higher at 48 h in the liver of ADE mice compared with non-ADE mice. The importance of the interaction between the Fc in the DENV-Ab complex and Fcγ receptor was confirmed using an antibody with a mutant Fc region that cannot bind Fcγ receptors, or Fcγ receptor-blocking antibody or the Fab fragment of anti-DENV Ab. These interventions could decrease the enhanced virus titer in the livers of ADE mice to non-ADE levels. In another model using a VEE replicon and the DENV E2 protein (virus replicon particles), AG129 immunised mice had a higher survival rate and lower virus titers when challenged with DENV (Zellweger et al., 2013). However, passive transfer of 50–1500 μl of immune sera resulted in ADE in recipient mice.

To determine the role of T cells, CD4 or CD8 depletion was used to show that whereas CD4-depleted mice had low virus titers, CD8 depleted mice had high virus titers. Thus CD8 T cells conferred greater protection. When mice were primed with UV-irradiated dengue virus, CD8 T cells protected against ADE. Thus in terms of vaccine development, both cellular and humoral immune responses need to be induced, and the Fc–FcR interaction controlled.

8.2. Influenza vaccine-induced anti-HA2 antibodies promote virus entry and enhance lung pathology after influenza A infection

Surender Khurana, FDA, Bethesda, USA.

During the 2009 influenza A(H1N1) pandemic, several reports suggested prior vaccination with the 2008–2009 seasonal inactivated influenza vaccine increased severity of clinical disease following infection with the A(H1N1)pdm09 virus (Skowronski et al., 2010). The mechanism of vaccine-associated enhanced respiratory disease (VAERD) is not well understood. Using a swine model, Khurana et al. (2013) showed that vaccination of pigs with an H1N2 virus and challenge with a A(H1N1)pdm09 virus resulted in the pigs having enhanced pneumonia and disease. Using recombinant expression of HA1 and HA2 and SPR binding assays, they showed that the H1N2 sera reacted with the A(H1N1)pdm09 virus, but that antibodies bound only to the HA2 of the H1N1 virus, and competed with other stem-targeting antibodies. The H1N2 sera also enhanced A(H1N1)pdm09 infection of MDCK cells and promoted virus fusion activity. Only adsorption of the sera with HA2 had any effect in reducing the enhancement effect. Live vaccination of pigs caused no ADE; however administration of the same, but UV-inactivated, vaccine resulted in ADE. The findings of these studies have generated concerns that clinical trials evaluating universal vaccines and monoclonal antibody therapeutics should monitor for antibody-associated enhanced disease.

8.3. Does antibody-dependent enhancement of disease occur in influenza infections?

Man-Wah Tan, Genentech, San Francisco, USA.

MHAA4549A is a human monoclonal IgG1 antibody that binds to a highly conserved region of the HA stalk of all influenza A viruses. In a trial with an A/Wisconsin/67/2005 (H3N2) challenge, 3 intravenous doses were tested, 400, 1200 and 3600 mg (NCT01980966). The primary end point was a decrease in virus load, with the secondary endpoint a decrease in clinical symptoms. The 3600 mg dose was the only one to decrease virus load. There was no evidence of ADE, with no increase in disease severity with the lower doses.

8.4. VIS410, a broadly neutralizing antibody to influenza A: characterisation and potential for ADE

Jose Trevejo, Visterra Inc, Cambridge, USA.

To investigate concerns about ADE with suboptimal antibody concentrations, infected cells were treated with low, medium and high concentrations of the mAb VIS410. No enhancement of infection was seen at any concentration. VIS410 was further evaluated in a murine macrophage cell line. Treatment with NA removes the sialic acid receptors, resulting in decreased infection by influenza. Addition of VIS410 increased infection through Fc–FcR binding; there was no enhancement of infectivity without NA treatment. To date resistance has been seen only in group 2 influenza viruses, with all mutations abrogating VIS410 binding, but resulting in decreased virus fitness.

9. Host cell targets: factors involved in virus replication or mediating the inflammatory response

9.1. Using genetic approaches to discover host–virus interactions

Abe Brass, Ragan Institute of Massachusetts General Hospital, USA.

To elucidate the role of host proteins in rhinovirus replication, three sets of commercial RNAi pools were used with the readouts based on the numbers of cells infected. The redundancy of the sets helps address false positives and negatives. Those with more than 50% inhibition were further screened for gene expression based on microarray. The host cell transmembrane protein RNASEK was identified as necessary for replication of multiple HRV serotypes, and of influenza and dengue viruses (Perreira et al., 2015). RNASEK, in association with V-ATPase, is required for endosomal acidification and endocytosis.

9.2. Sphingosine-1-phosphate receptor modulation of cytokine amplification

Sean Studer, The Scripps Research Institute, La Jolla, USA.

Sphingosine 1-phosphate (S1P) is a pleiotropic lysophospholipid mediator present in plasma and is released in large amounts from activated platelets. There are 5 types of S1P receptor, S1PR1-5. S1PR1-3 are found on various types of cells and tissues, while S1PR 4 is found mostly in cells of the immune system, and S1PR5 mostly in the nervous system. The sphingosine analogue, AAL-R, targets types 1, 3, 4, and 5 S1PRs, blunting the Type 1 IFN response and cytokine induction upon influenza infection of mice, without affecting the antibody response (Marsolais et al., 2009; Walsh et al., 2011). In the presence of AAL-R, purified dendritic cells (DC) from spleen and lung produced lower levels of IFN-α after virus infection or stimulation with the IFN inducer CpG. There was also a marked reduction in infiltration of PML and macrophages into the lung and resultant pulmonary tissue injury. DC maturation was suppressed, limiting proliferation of specific antiviral T cells in the lung and draining lymph nodes. The S1P1-specific receptor agonist, CYM5442, caused similar effects to the broadly-active AAL-R (Walsh et al., 2011); however, S1P1 knockout is lethal in mice.

9.3. Host cell factors in influenza A virus uncoating

Ari Helenius, ETH Zurich, Switzerland.

After endocytosis, uncoating of the influenza A virus core occurs following a two-step priming process, involving the influx of protons and K^+ ions through the M2 ion channel protein, which induces irreversible conformational changes in the M1 protein, weakening M1-vRNP association (Stauffer et al., 2014). After HA-mediated membrane fusion the cores detach from the endosome surface and M1 and the vRNPs dissociate from each other in the cytosol, allowing the vRNPs to be transported through nuclear pores into the nucleus. Treatment of cells with Importazole, an importin inhibitor, or mutation of a consensus sequence in M1 through which transportin binds, inhibits post-fusion transport of the vRNPs. Using siRNA silencing of cellular targets 177 hits were obtained, with 29 affecting uncoating, and 24 nuclear import. Knockdown of histone deacetylase 6 (HDAC6), an essential component in ubiquitin-dependent aggresome formation and assembly, reduced uncoating (Banerjee et al., 2014). HDAC6 binds to free ubiquitin in the cytosol, via its C-terminal zinc-finger ubiquitin-binding domain (UBD), and is recruited to the late endosome surface after infection. The capsids mimic misfolded protein aggregates binding the unanchored ubiquitin. Free ubiquitin is also found in virus particles. HDAC6 associates with actin and myosin 10 on microtubules, leading to physical disruption of the endosome/core structure. Cleavage of the UBD by Caspase 3, prevents virus uncoating. Thus these cellular proteins offer potential targets for antiviral strategies.

9.4. Update on DAS181

Ronald Moss, Ansun Biopharma, USA.

DAS181 is a recombinant sialidase in which a catalytic domain, from *Actinomyces viscosus*, is linked to an amphiregulin glycosaminoglycan respiratory epithelium–anchoring domain, which improves cell targeting and increases its half-life. Administered via inhalation or a nebuliser, the sialidase cleaves terminal sialic acid residues from glycans on the surface of respiratory cells, thus reducing the receptors available for binding of viruses, such as influenza and parainfluenza. In H5N1-infected mice DAS181 administered at 1 mg/kg/day from 24 h pre-to 72 h post-infection reduced lethality (Belser et al., 2007). For mice infected with either oseltamivir-sensitive or -resistant H7N9 viruses, 0.3, 0.6 and 1 mg/kg all protected against death (Marjuki et al., 2014). In a phase IIb trial, patients with influenza received 10 mg daily for 3 days (multidose), or 10 mg as a single dose, or placebo (Moss et al., 2012). While both groups treated with DAS181 showed a significant decrease in virus load at 24 h, only the multidose group showed a significant decrease at 3 and 5 days. There was, however, no difference in the time to alleviation of clinical symptoms. More than 80 patients, primarily immunocompromised transplant patients, have been treated with DAS181 on a compassionate use basis. There was a decrease in virus load and less need for oxygenation in four PIV-infected children (Waghmare et al., 2015). A cystic fibrosis patient infected with an oseltamivir-resistant influenza virus showed improvement (Silveira et al., 2015), and hematopoietic cell transplant (HTC) recipients infected with PIV have also appeared to benefit (Chalkias et al., 2014). Unpublished results have shown that up to 14 of 16 patients had decreased virus load and increased survival. A phase II trial for PIV pneumonia in immunocompromised patients is currently underway. While DAS181 generally appears safe, some patients have shown elevated alkaline phosphatase levels, with transient increases in liver enzymes (Moss

et al., 2012). It has systemic immunogenicity, with anti-DAS181 antibodies induced in healthy patients (Zenilman et al., 2015). Hence, it is unlikely to be useful systemically, and it is also undergoing particle size testing to minimise systemic absorption.

9.5. A novel class of host-directed antivirals with broad spectrum activity against respiratory viruses

Kristen Bedard, Kineta Inc, Seattle, USA.

A novel class of small molecule isoflavones has been identified, that trigger a natural immune response by targeting the innate immune transcription factor, IRF-3, a critical first responder essential for suppressing virus replication and clearing infection. Compounds were selected that cause phosphorylation of IRF-3 and induce its nuclear translocation (Bedard et al., 2012). KIN269 enhanced interferon-stimulated gene 54 induction. SAR led to the production of more than 250 analogues, with improvement in EC_{50}s from 8500 to 25 nM. Lead compounds for influenza A and B caused more than a 3 $\log_{10}$ reduction in virus titers *in vitro*. Similarly the lead compound against RSV A2 reduced virus titers by more than 3 $\log_{10}$. In time of addition studies *in vitro*, treatment up to 24 h post-infection reduced virus yields. These inhibitors also act synergistically with protease inhibitors against HCV. In influenza PR8–infected mice, treatment from 24 pre-to 24 h post-infection with 10 mg/kg decreased lung virus titers by 2–3 $\log_{10}$ to 1 $\log_{10}$, respectively. Pretreatment at 24 h resulted in 50% survival at 7 days, compared to 100% lethality in the controls. While these compounds are orally available, intranasal delivery is superior. The minimum toxic dose is greater than 400 mg/kg, with no pathology or off-target receptor activation, and they have no genotoxic or cardiotoxic potential.

9.6. Potent anti-influenza and anti-inflammatory activity of Verdinexor, a selective inhibitor of nuclear export (SINE) across a broad panel of influenza strains, including avian influenza A H7N9

Margaret Lee, Karyopharm Therapeutics Inc, Newton, USA.

Verdinexor (KPT335) targets the nuclear export of influenza vRNP in infected cells mediated by exportin 1 (XPO1), which interacts with viral nuclear export protein bound to vRNP. Silencing of XPO1 results in reduced influenza virus replication (Perwitasari et al., 2014). Verdinexor binds reversibly to cysteine 528 in the XPO1 binding pocket, inhibiting vRNP export and the replication *in vitro* of various influenza A subtypes, including A(H1N1)pdm09 (IC_{50} 200 nM), A(H3N2) (IC_{50} 20 nM), avian A(H7N9) (IC_{50} 420 nM) and highly pathogenic avian A(H5N1) (IC_{50} 270 nM), and influenza B (IC_{50} 10–90 nM). Treatment from 24 h pre-to 24 h post-infection resulted in 1–2 $\log_{10}$ reductions in virus titer. In mice infected with A(H1N1)pdm09, prophylaxis with 10–20 mg Verdinexor, 1–3 days prior to infection, reduced lung virus titers. Delayed dosing at 24 and/or 72 h post-infection reduced both lung virus titers and lung pathology. Verdinexor treatment also lowered the expression of proinflammatory cytokines associated with influenza virus infection (γ IFN, TNFα, IL-1β and IL-6). In ferrets, treatment from 2 h post-infection for 3 days resulted in approximately a 3 $\log_{10}$ reduction in virus load, with minimal toxicity. Phase 1 trials of Verdinexor are planned.

9.7. Targeting sirtuins, novel viral restriction factors, to limit acquired resistance

Lillian Chiang, FORGE Life Science, Doylestown, USA.

Sirtuins are a family of seven NAD^+- dependent deacylases known to regulate numerous cellular functions, including metabolism, the cell cycle, and longevity. They act as virus restriction factors, since siRNA knock-down of each sirtuin increased virus growth (Koyuncu et al., 2014). Small molecule activators of sirtuins block *in vitro* replication of viruses, including influenza, HCMV, adenovirus, and polyomavirus. Antivirals are being developed based on Sirt1 and 2 dual inhibition, and Sirt 6 activation. The most potent inhibitor has an IC_{50} of 0.4 nM. The simultaneous inhibition of Sirt1 and 2 unexpectedly resulted in a 5-fold decrease in influenza virus titer. It appears that the initial NS1 down regulation of p53 (Terrier et al., 2013) is restored with Sirt1/2 inhibition. This results in reactivation of p53, thus inducing apoptosis, specifically in virus-infected cells. Compared to oseltamivir, novel Sirt1/2 inhibitors lead to enhanced virus clearance. Sirt/1/2 inhibitors are already in clinical trials for Huntington's Disease, and they could act synergistically with antivirals like oseltamivir.

9.8. Repurposing of signal transduction inhibitors to fight the flu – an update

Stephan Ludwig, University of Muenster, Germany.

Penetration of cells by influenza viruses is controlled by various cell signalling pathways. These include the receptor tyrosine kinases, such as PI3K involved in entry/endocytosis, IKK/NFκB and Raf/MEK/ERK involved in RNP export. Several anti-MEK inhibitors, now in clinical trials for cancer therapy, have been investigated for potential antiviral action against influenza. U0126 blocks nuclear export, leading to accumulation of RNPs in the nucleus of influenza-infected cells, and has been shown to cause an 80% decrease in lung virus titers in influenza-infected mice, and 70% survival against lethal infection (Droebner et al., 2011). Other MEK inhibitors have been shown to act synergistically with oseltamivir at suboptimal doses (Haasbach et al., 2013). The Raf inhibitor, Zelboraf (vemurafenib, PLX-4032), has shown greater efficacy than oseltamivir against A(H1N1)pdm09 infection. Currently, LASAG (lysine acetyl salicylate glycine) a derivative of aspirin, is the first molecule targeting a host intracellular protein (NF-κB) to undergo a phase II clinical trial for the treatment of severe influenza virus infection (Ludwig et al., 2014).

9.9. Eritoran (E5564), a TLR4 antagonist effective against influenza induced disease

Jorge Blanco, Sigmovir Biosystems, Rockville, USA.

Toll-like receptor 4 (TLR4) signalling can lead to lung inflammation in influenza-infected mice and TLR4–/– mice are highly refractory to influenza induced lethality. Administration of a TLR4-specific IgG also protected wild-type mice infected with a lethal dose of influenza PR8. Intravenous administration of 200 μg/day of Eritoran (E5564), a synthetic TLR4 antagonist, at 2, 4 or 6 days post-infection, increased survival of PR8-infected mice (Shirey et al., 2013). The mice showed reduced lung pathology, clinical symptoms and cytokine gene expression. Treatment with tenfold less Eritoran decreased survival from 90% to 40% and Eritoran efficacy decreased with increasing virus challenge, from 90% survival with 7500 $TCID_{50}$ to 60% and 25% survival with 10,000 and 20,000 $TCID_{50}$, respectively. Mice subsequently challenged with PR8 all survived, indicating an immune response had developed despite the Eritoran treatment. Cotton rats infected with non-adapted H3N2 (A/Wuhan/359/95) and A(H1N1)pdm09 (A/California/07/1009) influenza viruses and treated with Eritoran showed reduced lung pathology and lower levels of IL-6 and IL-10 (Shirey et al., 2013). In both the mice (lung homogenates) and the cotton rats (blood), Eritoran treatment of influenza infection showed strong reduction in concentrations of high mobility group protein 1 (HMGB1), a TLR4 agonist known to be involved in systemic inflammation.

10. Diagnostics and resistance

10.1. Detection of drug resistance in influenza: current status and future directions

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Detection of drug resistance requires knowledge of the molecular mechanisms underlying the resistance phenotype, the markers of resistance, and criteria to correlate laboratory results with clinically relevant resistance. For the M2 inhibitors, a limited number of mutations are known to correlate with resistance in cell culture and *in vivo*. However for the NAIs, cell culture sensitivity does not always correlate with reduced inhibition of the NA enzyme. Hence enzyme-based assays have been used as a surrogate assay for monitoring influenza virus susceptibility to NAIs. The WHO Expert Working Group for Surveillance on Influenza Antiviral Susceptibility has used the fold increase in IC_{50} compared with the reference IC_{50} value as an indicator of possible resistance. 'Reduced inhibition' defines influenza A viruses with a 10- to 100-fold increase in IC_{50}, or influenza B viruses with a 5- to 50-fold increase in IC_{50}, and 'highly reduced inhibition' defines influenza A viruses with >100-fold increase in IC_{50} or influenza B viruses with >50-fold increase in IC_{50} (WHO, 2012). While commercial kits do not include reference viruses, panels of resistant viruses are available from both the CDC and isirv AVG. A major limitation of the enzyme-based assay is that viruses must be amplified in culture for testing, which may result in selection for or against resistant viruses, as well as de novo mutations. A new bioluminescent assay may, however, allow direct assessment of NA enzyme inhibition in clinical samples. Various sequencing methods have been used to identify NA substitutions, including high throughput 96 well pyrosequencing or next generation sequencing. However, while sensitive, most of these methods only allow screening for known resistance mutations. Novel mutations require the use of phenotypic assays. Correlating the presence of low levels of resistant virus to wild type virus in a clinical sample with lack of clinical effectiveness is also challenging.

10.2. Characterisation of a large cluster of influenza A(H1N1) pdm09 virus cross-resistant to oseltamivir and peramivir during the 2013–2014 influenza season

Emi Takashita, National Institute of Infectious Diseases, Tokyo, Japan.

During the 2013–2014 influenza season, 2531 influenza A(H1N1)pdm09 viruses from Japan were screened for the H275Y substitution in their NA protein, which confers cross-resistance to oseltamivir and peramivir. Prior to the main influenza season, 28% of isolates in Sapporo/Hokkaido had the H275Y mutation (Takashita et al., 2015). Phylogenetic analysis suggested the clonal expansion of a single mutant virus. The virus replicated as well as the wild type virus in cell culture and in ferrets, and was transmitted as efficiently by droplet infection. H275Y-containing viruses had two additional substitutions in NA, V241I and N369K, known to increase replication and transmission fitness. However, modelling suggested a third substitution (N386K) in the NA destabilised the mutant NA structure, thus allowing the fitter wild type to compete as the dominant strain.

10.3. Six years of monitoring emergent oseltamivir resistance in patients with influenza A virus infections in the Influenza Resistance Information Study (IRIS)

Bruno Lina, University Claude Bernard, Lyon, France.

Following naturally-occurring oseltamivir resistance in 2008, a global observational study, the Influenza Resistance Information Study (IRIS; NCT00884117) was initiated to study the emergence of NAI resistance and the clinical course of influenza in non-immunocompromised treated and untreated patients (Whitley et al., 2013). Years 1–5 of the study included non-immunocompromised patients >1 year old; year 6 included only children aged <12 years. Of 3385 RT-PCR-positive patients infected with a single influenza strain in years 1–6, 2468 had influenza A of whom 1358 received oseltamivir monotherapy within 48 h of symptom onset (9 seasonal H1N1; 734H3N2; 615 A(H1N1)pdm09). After 2008 no baseline resistance was detected. Emergence of resistance during or after treatment was detected in 50 oseltamivir-treated influenza A patients (3.7% of total), mostly with a mixed genotype (44–86% resistant). Most of these isolates were from children 1–5 years old (22/157H1N1 and 10/174H3N2). Resistance did not increase over the study period, within the range 3–5%. All resistant viruses detected had either the H275Y substitution for N1 viruses (36), or the R292K substitution for N2 viruses (16). At day 10, 15/50 patients (30%) were still RT-PCR positive, although their symptoms resolved by day 6.

10.4. The changing landscape of influenza diagnostics and the effect on clinical management

Alicia Fry, Centers for Disease Control and Prevention, Atlanta, USA.

Several US guidance documents recommend influenza testing, during the influenza season, if treatment will be changed by the test result, including outpatients at high risk for influenza complications and hospitalised patients with acute respiratory illness. Similarly, antiviral treatment is recommended for hospitalised patients and outpatients at high risk for influenza complications, with suspected or confirmed influenza; providers are urged not to wait for laboratory confirmation and not to rely on results of insensitive assays before beginning treatment. However, several studies suggest that insensitive rapid influenza diagnostic assays (RIDT) are commonly used in outpatient and inpatient settings and that providers wait for the RIDT result to begin treatment. Many new sensitive molecular diagnostic assays are now commercially available. However, while very sensitive, these assays are expensive and several studies suggest empiric treatment during periods of high influenza prevalence may be more cost-effective. Additional work is needed to optimize the use of antiviral treatment for vulnerable populations, and the role of new diagnostic assays has yet to be determined.

11. Regulatory issues and clinical trial endpoints

11.1. Regulatory perspectives on antiviral drug development for influenza and endpoint considerations

Peter Miele, FDA, Silver Spring, USA.

FDA guidance for the development of drugs for the treatment and/or prophylaxis of influenza reflects experience with previous influenza drug development and participation with working groups (FDA, 2011). Well controlled phase 3 efficacy trials are necessary to support FDA approval of influenza antivirals. Other study types, e.g. human challenge studies or trials in special populations, can contribute to the development of the clinical trial but not substitute for it. Efficacy trials for serious influenza in hospitalised patients face several challenges, including a heterogeneous population on other medications, a current standard of care that varies by local practices, no current product with proven efficacy that would allow an inferiority trial, and no validated clinical

endpoint or accepted biomarker. Efficacy endpoints have not been standardised for all types of influenza trials; most trials warrant examination of multiple secondary endpoints. Virologic endpoints, e.g. virus shedding, are important secondary endpoints but not primary endpoints. FDA encourages the development of novel efficacy endpoints for trials of the treatment of serious influenza and conducting pilot studies of them before entering into phase 3 trials. Such endpoints should demonstrate improvement in how the patient feels, functions or survives. Early discussions with FDA can facilitate new drug development.

11.2. Human respiratory virus challenge models: a worthwhile challenge

Matthew Memoli, NIAID, Bethesda, USA.

Human challenge models are tools that can help to describe the natural history and pathogenesis of influenza viruses, including virus replication, shedding, and immune responses. In addition, human challenge models can inform the potential benefits of new diagnostics, vaccines and therapeutics. A new model, H1N1pdmMIST, was recently developed by genetically modifying the A(H1N1)pdm09 virus (Memoli et al., 2015). Challenge studies with H1N1pdmMIST, enrolling participants with A(H1N1)pdm09 HAI titers $\leq$1:40, demonstrated that clinical symptoms of infection and antibody responses correlated with infective dose, and that the number and severity of symptoms correlated with viral shedding. Baseline HA and NA antibody titers were inversely correlated with disease severity. This model has potential to facilitate future studies of influenza pathogenesis, animal model validation, and evaluation of novel vaccines and therapeutics. Future models will include genetically modified A(H3N2) and B viruses.

11.3. The human virus challenge model-accelerating drug and vaccine development

Anthony Gilbert, Retroscreen (hVivo), London, UK.

Retroscreen have carried out more than 40 trials in the last 15years, with 20–140 patients per trial, and more than 1970 volunteers. Most trials are randomised, double blind placebo controlled, with participants aged 18–45. Viruses tested include RSV, HRV and influenza. Primary endpoints tend to be a decrease in virus load, by RT-PCR or cell culture.

11.4. Design and conduct of a drug development program for severe/complicated influenza: lessons from the IV zanamivir experience

Amanda Peppercorn, GlaxoSmithKline, North Carolina, USA.

Challenges with conducting a phase III study to evaluate the efficacy of intravenous (IV) zanamivir in hospitalised patients include lack of a validated clinical endpoint, inability to have a placebo arm (oseltamivir had to be the comparator), and inability to use a non-inferiority design since the randomised trials for oseltamivir licensure were performed in outpatients. The phase III multisite randomised double-blind study compared two doses of IV zanamivir (300 mg and 600 mg daily) to oral oseltamivir (75 mg twice daily) in hospitalised patients aged >16 years with confirmed influenza and symptom onset within 5 days. Primary endpoints included hospital discharge or signs of clinical response (afebrile and >95% O_2 saturation plus two of the following: return to baseline oxygen needs or respiratory rate <25/min, pulse <101/min and systolic blood pressure >91 mm Hg). Secondary endpoints included mortality, time to pre-morbid functional status, length of intensive care unit and hospital stay, and several measures of virologic improvement. Enrolment began in January 2011 and was completed in March 2015 and analysis is ongoing; target enrolment was 600. Interim analysis of 445 participants demonstrated that 75% of participants had underlying medical conditions and had had symptoms for 4 days or less at enrolment. Among 348 influenza-positive participants, the median days to clinical response was 4.71 (range 0–35) days and time to improvement in respiratory status was 3.1 (range 0–32) days. In addition to the clinical trial, GlaxoSmithKline has made IV zanamivir available for compassionate use. Worldwide, approximately 2300 treatment courses have been given for compassionate use, which in some countries has affected enrolment into the clinical trial.

11.5. Challenges in designing informative clinical trials in patients hospitalized with influenza: the peramivir experience

Sylvia Dobo, BioCryst, Durham, USA.

In a study of IV peramivir (600 mg daily) compared to placebo, the primary endpoint was time to clinical resolution (normalization of temperature, pulse, respiratory rate, systolic blood pressure and oxygen saturation). Of 405 subjects enrolled, 338 had laboratory-confirmed influenza infection; however, 217 subjects received oseltamivir as part of standard care and were excluded from analysis. Thus, only 121 influenza infected participants qualified for primary efficacy analysis (43 placebo, 78 peramivir); only 28% had positive virus titers at baseline and 45% had had symptoms for >48 h. Also, inclusion of oseltamivir in standard care was often used for patients who were clinically more seriously ill. Thus, the patients included in the final analysis were less ill, and may not be representative of severe influenza, and were admitted after the optimal time for a NAI to be effective. The study was terminated early as it was deemed unlikely to enrol adequate numbers. The experience with this trial highlighted several roadblocks to conducting clinical trials for new antiviral drugs in hospitalised patients. Widespread availability and use of oseltamivir obfuscates the ability to randomly allocate to a placebo group and reasons for hospitalization vary, so that hospitalization per se may not be an appropriate case definition for severe disease; hospitalised patients represent a continuum of disease. There is a lack of validated endpoints and many variables could contribute to the resolution of the endpoints used in the peramivir study. Conducting clinical trials in hospitalised patients with influenza thus presents unique research difficulties. Future studies might be improved if enrolment were limited to patients with documented severe illness, such as those with lower respiratory tract illness.

11.6. Antibody-based therapy for influenza B

Man-Wah Tan, Genentech, San Francisco, USA.

NAI enzyme assays consistently demonstrate higher IC_{50}s for current NAIs against influenza B viruses (Burnham et al., 2013). While the clinical implication of this is unclear, a few observational studies suggest slower resolution of fever for oseltamivir-treated patients with influenza B infection compared to patients with influenza A infection. A human monoclonal antibody against influenza B HA was demonstrated to block membrane fusion, but not virus attachment, and to protect mice from lethal influenza B infection.

11.7. Supporting advanced development of novel influenza antiviral therapeutics

Michael Wathen, BARDA, Washington, USA.

BARDA's mission is to develop and provide countermeasures against CBRN threats, pandemic influenza and emerging infectious diseases by product development, stockpile acquisition/building,

manufacturing infrastructure building, and product innovation. Drug development is expensive, lengthy, and risky. Few products make it to the stage of human studies and fewer to phase III trials. The BARDA antiviral program has included the development of two NAIs (IV peramivir and inhaled long acting laninamivir), an inhaled recombinant sialidase (Fludase, DAS181), and a broad spectrum, host-targeted small molecule (nitazoxanide). Future funding will shift focus to unmet medical needs, including the emergence of antiviral resistance to current drugs, treatment of severely ill patients, drugs with a wider treatment window, and discovery and development of broadly-neutralizing antibodies. Several funding announcements focussing on these unmet needs have been or will be published. In addition, BARDA has convened an interagency working group to develop better clinical endpoints for severe influenza and improve study design of clinical trials.

11.8. Multi-center evaluation of outpatient endpoints for RSV and other respiratory virus antivirals

John DeVincenzo, University of Tennessee, Memphis, USA.

RSV infections are common and result in an estimated 2.8–4.3 million hospital admissions globally among children <5 years of age (Nair et al., 2010). No effective therapeutics for RSV are currently licensed (Simoes et al., 2015). Novel therapeutics are being developed, and challenges related to the evaluation of new agents against severe RSV disease were discussed. RSV-infected children who are hospitalised are admitted later in their illness (mean 4 days) and thus might be outside the optimal therapeutic window for most drugs. A study that included telephone surveys of parents suggested that parents recognised difficulty in breathing, elevated respiratory rate and wheezing, signs of lower respiratory illness, early in RSV infection. Approximately 56% of children were taken for medical care within 1 day of illness onset and 100% within 3 days of illness onset. Thus, studies to evaluate new therapeutics that targeted ambulatory patients may be feasible and prevention of hospitalization could be a clinical outcome.

12. Conclusions

New anti-influenza therapeutic agents could improve morbidity associated with influenza, especially those that target treatment of severe influenza and with a wider therapeutic window than 48 h. Several novel agents with the potential to add to the current armamentarium against influenza were described. Additional studies on other respiratory viruses were presented, including those targeting the proteins involved in replication, receptor binding and fusion, and host cell targets. While each is in a different phase of development, few have made it to human studies. Future challenges include identifying the best methods to evaluate efficacy against severe influenza and other respiratory diseases and to monitor for adverse effects related to antibodies.

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