



HHS Public Access

Author manuscript

Antiviral Res. Author manuscript; available in PMC 2022 April 15.

Published in final edited form as:

Antiviral Res. 2021 October ; 194: 105158. doi:10.1016/j.antiviral.2021.105158.

Influenza polymerase inhibitor resistance: Assessment of the current state of the art - A report of the isirv Antiviral group

Michael G. Ison^a, Frederick G. Hayden^b, Alan J. Hay^c, Larisa V. Gubareva^d, Elena A. Govorkova^e, Emi Takashita^f, Jennifer L. McKimm-Breschkin^{g,*}

^aDivisions of Infectious Diseases and Organ Transplantation, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA

^bDivision of Infectious Diseases and International Health, University of Virginia School of Medicine, Charlottesville, VA, USA

^cThe Francis Crick Institute, London, UK

^dInfluenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

^eDepartment of Infectious Diseases, St Jude Children's Research Hospital, Memphis, TN, USA

^fNational Institute of Infectious Diseases, Tokyo, Japan

^gDepartment of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Victoria, Australia

Abstract

It is more than 20 years since the neuraminidase inhibitors, oseltamivir and zanamivir were approved for the treatment and prevention of influenza. Guidelines for global surveillance and methods for evaluating resistance were established initially by the Neuraminidase Inhibitor Susceptibility Network (NISN), which merged 10 years ago with the International Society for influenza and other Respiratory Virus Diseases (isirv) to become the isirv-Antiviral Group (isirv-AVG). With the ongoing development of new influenza polymerase inhibitors and recent approval of baloxavir marboxil, the isirv-AVG held a closed meeting in August 2019 to discuss the impact

*Corresponding author. Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, 792 Elizabeth Street, Parkville, Victoria, 3000, Australia. jmbvirology@gmail.com.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: **MI** received research support, paid to Northwestern University, from AiCuris, GlaxoSmithKline, Janssen Inc. and Shire; he is a paid consultant for Adagio, AlloVir, Celltrion, Cidara, Genentech Inc., F. Hoffmann-La Roche Ltd, Janssen Inc., Shionogi Inc., Viracor Eurofins; he is also a paid member of DSMBs from CSL Behring, Janssen Inc., Merck, SAB Biotherapeutics, Sequiris and Takeda. **LG** No declarations. **FGH**: Personal honoraria from World Health Organization and University of Alabama Antiviral Drug Discovery and Development Consortium; charitable donations by Cidara, resTORbio, Shionogi for consulting; meeting travel costs provided by Shionogi and Roche; non-compensated consulting for Arcturus, FujiFilm/Toyama, Gilead, GSK, Janssen/JNJ, MedImmune, Merck, Regeneron, resTORbio, Ridgeback, Genentech, SAB Biotherapeutics, Vir, Visterra; Versatope, DSMB chair/member for studies by Celltrion, GSK, and Vaccitech with fees paid to the University of Virginia. **AH** No declarations. **EG** is supported with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contracts HHSN272201400006C and 75N93021C00016. **ET** No declarations. **JMB** No declarations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.antiviral.2021.105158>.

of resistance to these inhibitors. Following this meeting and review of the current literature, this article is intended to summarize current knowledge regarding the clinical impact of resistance to polymerase inhibitors and approaches for surveillance and methods for laboratory evaluation of resistance, both *in vitro* and in animal models. We highlight limitations and gaps in current knowledge and suggest some strategies for addressing these gaps, including the need for additional clinical studies of influenza antiviral drug combinations. Lessons learned from influenza resistance monitoring may also be helpful for establishing future drug susceptibility surveillance and testing for SARS-CoV-2.

Keywords

Influenza virus; Antiviral; Polymerase inhibitor; Baloxavir; Drug resistance; Fitness

1. Introduction

Antiviral treatment and prophylaxis are important interventions to minimize the morbidity and mortality of seasonal, zoonotic, and pandemic influenza virus infections. As discussed below, considerable data exist regarding resistance to the two widely available classes of influenza antivirals, the M2 inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (NAIs). The previous Neuraminidase Inhibitor Susceptibility Network (NISN) played a key role in establishing surveillance and testing strategies for resistance to the NAIs (McKimm-Breschkin et al., 2003; Monto et al., 2006; Tashiro et al., 2009; Wetherall et al., 2003), which were subsequently incorporated in the WHO Global Influenza Surveillance and Response System (GISRS) (Hay and McCauley, 2018). NISN merged with the International Society for Influenza and other Respiratory Virus Diseases (isirv) ten years ago to form the Antiviral Group (isirv-AVG). Building on its extensive experience with studying resistance to the NAIs, the isirv-AVG held a closed 2-day meeting in August 2019 to bring together academic and public health investigators and representatives from the relevant pharmaceutical companies (Fujifilm, Janssen, Roche and Shionogi) to discuss clinical, epidemiological and laboratory issues related to resistance to novel antivirals targeting the influenza virus polymerase complex. As a follow up to the presentations and discussions at the meeting, and to mark the 10th anniversary of the isirv-AVG this review aims to summarize current knowledge of the laboratory detection and clinical impact of resistance to polymerase inhibitors and address strategies for overcoming gaps in this knowledge. With the development of therapeutics for SARS CoV-2 many of the approaches used for influenza drug susceptibility surveillance and testing may provide a valuable template for monitoring resistance to novel therapeutics for SARS-CoV-2 and other future pandemic respiratory viruses.

2. Background: resistance to M2 and neuraminidase inhibitors

2.1. M2 inhibitors

Until recently, the M2 inhibitors and NAIs were the only options for the treatment of influenza in most countries. The M2 inhibitors (amantadine and rimantadine) inhibit the M2 ion channel activity of influenza A viruses and, for many years, were effective in

prophylaxis and early treatment of uncomplicated influenza. However, resistance emerged frequently (27%–50% in ambulatory children and 39%–50% in ambulatory adults) in patients treated with amantadine or rimantadine (Hall et al., 1987; Hayden et al., 1989). Resistance to amantadine and rimantadine is mediated by a single amino acid substitution at residues 26, 27, 30, 31 or 34 of the M2 protein (Deyde et al., 2007). These resistant variants showed no reduction in replicative fitness or transmissibility (Hayden et al., 1991; Sweet et al., 1991). Widespread resistance was first observed in seasonal influenza A(H3N2) viruses in China around 2000 (Bright et al., 2005), and then by 2004–2005 it was also observed in seasonal A(H1N1) viruses (Deyde et al., 2007). The 2009 pandemic A(H1N1) virus subsequently displaced the seasonal A(H1N1) virus, but it was also resistant. Hence, both subtypes of seasonal influenza A viruses currently circulating globally are resistant to amantadine and rimantadine due to the presence of the M2-S31N amino acid substitution. As a result, these antivirals are no longer recommended for prevention or treatment of influenza. It is unclear to what extent use of amantadine or related compounds in humans or birds (Parry, 2005) may have contributed to emergence of resistance or which other mutations may have contributed to the stability and transmissibility of the resistant viruses. Such data might inform the risk of resistance emergence to new antivirals, including the polymerase inhibitors.

2.2. Neuraminidase inhibitors

NAIs were rationally designed to inhibit influenza A and B neuraminidases (NAs) to prevent the release of virus from the host cell and spread within the respiratory tract (Kim et al., 1997; von Itzstein et al., 1993). Oral oseltamivir is the most widely available NAI, whereas the availability of inhaled and intravenous zanamivir, intravenous peramivir and inhaled laninamivir varies by country. Because of their safety and clinical effectiveness, the NAIs, particularly oral oseltamivir, have become the standard of care for the treatment of influenza in most clinical settings. NAI treatment is associated with approximately a 24 h reduction in time to alleviation of symptoms compared to placebo when started within 36 h of symptom onset in uncomplicated influenza, and with reductions in complications and hospitalizations (Dobson et al., 2015; Fry et al., 2014; Hayden et al., 1997; Higashiguchi et al., 2018; Kaiser et al., 2000; Watanabe et al., 2010; Whitley et al., 2015). Observational studies have also demonstrated reductions in morbidity and mortality in patients hospitalized with severe influenza, with the greatest benefit among those who were treated with oseltamivir within the first 48 h of symptom onset (Chan et al., 2012; Muthuri et al., 2014; Venkatesan et al., 2017).

Emergence of NAI resistance among influenza viruses can result from point mutations in the NA gene, the hemagglutinin (HA) gene, or both (McKimm-Breschkin, 2013). Although resistance conferred by HA mutations has been demonstrated *in vitro*, their role in clinical resistance is not established. Changes in NA protein sequence (amino acid substitutions and less often deletions) which reduce NAI susceptibility *in vitro* are available at the WHO website (WHO, 2021a).

The nature of the NA amino acid substitutions and the NA type/subtype determine the degree of inhibition of enzyme activity by the individual NAIs. Several NA substitutions

conferring reduced susceptibility to oseltamivir, for example H275Y in N1-, or E119V in N2-containing viruses, do not affect inhibition by zanamivir or laninamivir (McKimm-Breschkin, 2013). Emergence of resistance during oseltamivir therapy has generally been uncommon, but depends on the infecting virus, patient age, immune status, and illness severity. Rates are higher among outpatient children less than 5 years of age (0–16.1%) than in older children and adults (1.2–1.7%) (Lina et al., 2018; Whitley et al., 2013). Treatment-emergent oseltamivir resistance is more frequent in influenza A(H1N1) than A(H3N2) viruses, in immunocompromised hosts, and in critically ill patients, in whom detection of H275Y-containing A(H1N1) variants is associated with increased mortality (Behillil et al., 2020). Currently, the frequency of NAI resistance in community isolates (untreated persons) is exceedingly low (<1%) (Lackenby et al., 2018; Takashita et al., 2020b), although a higher frequency of resistant variant detection has been described in some seasons and clusters of apparent transmission of oseltamivir-resistant variants have emerged (Hurt et al., 2012; Takashita et al., 2015).

However, widespread oseltamivir resistance occurred in 2008–2009 when seasonal A(H1N1) viruses with the NA-H275Y amino acid substitution circulated globally (Hurt et al., 2009). Based on observational studies from Japan (Kawai et al., 2009; Saito et al., 2010), patients infected with these viruses did not respond to oseltamivir treatment, i.e., were clinically resistant. In neuraminidase inhibition (NI) assays, these viruses displayed >1000-fold decreased inhibition by oseltamivir. They spread quickly and replaced the oseltamivir-susceptible circulating A (H1N1) viruses because of a competitive advantage (associated with R222Q and V234M substitutions in the NA protein). The emergence of the transmissible drug-resistant variants occurred in a genetic lineage of A(H1N1) viruses with intrinsically higher NA activity, which offset the loss of fitness caused by the drug resistance mutation (Bloom et al., 2010). In addition, antigenic drift in the oseltamivir-resistant seasonal A (H1N1) virus is also thought to have contributed to its rapid spread and replacement of the previously susceptible virus, by so-called hitch-hiking (Gubareva and Fry, 2020). Notably, gene reassortment between co-circulating oseltamivir-resistant and amantadine-resistant lineages of A(H1N1) viruses led to the emergence of viruses carrying both H275Y in NA and S31N in M2 (Sheu et al., 2011). However, by 2010 the oseltamivir-resistant seasonal A(H1N1) viruses were replaced by the oseltamivir-susceptible A(H1N1)pdm09 virus. Modeling based on the emergence of the oseltamivir-resistant variants has provided new insight into predictors of transmission and potential approaches to limit future emergence (Chao et al., 2012). The above observations emphasize the dynamic relationship between the emergence of drug resistance and genetic variation in influenza, which may lead to the appearance and disappearance of new variants.

3. Efficacy of and resistance to polymerase inhibitors approved or in advanced clinical development

The influenza virus polymerase complex is a heterotrimer composed of three protein subunits: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA). Since the influenza polymerase complex is highly conserved and critical for virus replication, it has long been a focus of antiviral development. Recent

understanding of the structures of the components and their interactions has facilitated the development of selective inhibitors (Dias et al., 2009; Pflug et al., 2014; Reich et al., 2014). Transcription and replication of vRNA occur in the nucleus, where synthesis of viral mRNA is initiated by a “cap-snatching” process. The PB2 subunit binds to the cap structure of host nascent mRNA and the endonuclease domain of the PA subunit cleaves the mRNA 10–14 bases downstream from the 5′ cap (Fodor and Te Velthuis, 2020; Krug et al., 1979; Plotch et al., 1979). This then acts as a primer for RNA elongation by the PB1 RNA-dependent RNA polymerase (Stevaert and Naesens, 2016). Transcription ceases when the polymerase complex reaches an oligo-U tract upstream of the 5′ terminus of the vRNA resulting in addition of a polyadenylation signal to the viral mRNAs (Poon et al., 1999).

Currently, four polymerase inhibitors have undergone clinical testing: AL-794, baloxavir marboxil, favipiravir, and pimodivir. Two of these, the PB1 inhibitor favipiravir and PA inhibitor baloxavir marboxil are approved in some countries (Hayden and Shindo, 2019; Mifsud et al., 2019). The following sections briefly outline the clinical findings from studies to date.

3.1. AL-794

AL-794 (also known as ALS-033794/JNJ-64155806) is an orally active, isobutyrate prodrug of ALS-033719, which selectively binds to the endonuclease domain of the influenza virus PA protein and potently inhibits the endonuclease activity. In the human challenge model using intranasal A/Perth/16/2009 (H3N2) inoculation, the higher dose (150 mg) of AL-794 reduced the viral AUC, 87.5 vs. 142 log₁₀ TCID₅₀/mL*h for placebo, and was associated with a 32.7 h reduction in time to no detection of virus compared to placebo (Yogarathnam et al., 2019). AL-794-treated subjects also had a shorter time to symptoms resolution (median, 26.4 vs. 49.1 h) compared to placebo-treated. No variants with PA substitutions associated with loss of susceptibility were identified in paired pre- and post-treatment samples. Although AL-794 was shown to have antiviral efficacy in experimentally influenza virus-infected humans (Yogarathnam et al., 2019), its development has been abandoned because of its narrow therapeutic index, related to central nervous system adverse events.

3.2. Baloxavir

Baloxavir marboxil is an oral prodrug that is converted to the active compound baloxavir acid (both are referred to as baloxavir in this article), an inhibitor of the PA cap-dependent endonuclease of influenza A-D viruses (Mishin et al., 2019). Because of its prolonged plasma elimination half-life, the prodrug has been tested using a single-dose administration for treatment of adult and pediatric outpatients and post-exposure prophylaxis in household contacts (Baker et al., 2020; Hayden et al., 2018; Hirotsu et al., 2020; Ison et al., 2020). Initially approved in Japan and the USA in 2018, baloxavir has been approved for treatment in over two dozen countries to date. Randomized controlled trials (RCTs) demonstrated that baloxavir treatment resulted in more rapid improvement in time to symptom alleviation compared to placebo, but similar to a standard 5-day course of oseltamivir in adults and children over 1 year of age with uncomplicated influenza A infections (Baker et al., 2020; Hayden et al., 2018; Hirotsu et al., 2020; Ison et al., 2020). In high-risk outpatients, baloxavir reduced the risk of complications, and in those with influenza B infections

more rapid symptoms resolution than those given oseltamivir (Ison et al., 2020). Infectious virus titers declined much more quickly with baloxavir than with oseltamivir or placebo treatment (Ison et al., 2020). Single-dose baloxavir prophylaxis also reduced the risk of clinical influenza in household contacts of infected index patients (Ikematsu et al., 2020). In adults hospitalized with influenza, a placebo-controlled RCT showed that a combination of multiple-dose baloxavir added to standard-of-care NAI treatment was associated with greater antiviral effect than NAI treatment alone, although the combination did not significantly accelerate overall illness recovery (Kumar et al., 2020). Baloxavir has been used with apparent success in treating a highly immunocompromised patient with protracted influenza A virus illness associated with two treatment-emergent NA substitutions conferring NAI resistance (Harada et al., 2020).

Viruses with PA substitutions conferring reduced susceptibility to baloxavir, particularly substitutions in PA-I38, have been selected after passaging *in vitro* in the presence of drug and detected in baloxavir-treated patients. A summary of PA substitutions conferring greater than 3-fold reductions in baloxavir susceptibility (for at least one subtype or type) of viruses obtained from either laboratory or clinical settings is shown in Table 1. Fold changes in EC₅₀ values vary depending on the assay used and on influenza virus type and subtype. Substitutions of the highly conserved I38, in the active site of the PA and positioned to interact with both the RNA substrate and baloxavir (Omoto et al., 2018), are most commonly selected, with I38T causing the largest fold change in sensitivity of influenza A and B viruses in most phenotypic assays (Abed et al., 2020; Checkmahomed et al., 2020; Chesnokov et al., 2020; Imai et al., 2020; Ince et al., 2020; Jones et al., 2020; Koszalka et al., 2019; Noshi et al., 2018; Omoto et al., 2018; Takashita et al., 2018, 2019a, 2019b, 2019c, 2020a; Uehara et al., 2020; Yano et al., 2020). Other substitutions at residue I38 have also been detected after baloxavir treatment (Hashimoto et al., 2021; Hayden et al., 2018; Hirotsu et al., 2020; Ikematsu et al., 2020; Ince et al., 2020; Sato et al., 2021; Uehara et al., 2020) (Table 1) and may be rarely found among circulating viruses (Gubareva et al., 2019).

The effects of various PA-I38 amino acid substitutions, F, L, M, N, S, T and V on the degree of reduction in baloxavir susceptibility have been assessed in cell culture-based assays (Table 1). PA-I38V has minimal effect on baloxavir susceptibility of seasonal and animal influenza viruses (Gubareva et al., 2019; Noshi et al., 2018). Overall, substitutions by polar amino acids (N, S, T) confer greater reductions in baloxavir susceptibility than by nonpolar amino acids (F, M, L, V).

Mini replicon assays using recombinant PA (N1 subtype) proteins showed that I38 K/R substitutions, that introduce a strong positive charge, reduce polymerase activity to 44–66% of I38-WT (Jones et al., 2021). When compared with the prototypical baloxavir resistance marker I38T in the presence of 50 nM baloxavir, (normalized to 100% reduction in inhibitory activity) five substitutions (M, L, F, Y, C) at residues 38 conferred 10%–35% reductions in inhibitory activity and 11 substitutions (R, K, S, N, G, W, A, Q, E, D, H) conferred >50% reductions, while the V substituted PA remained unchanged (Jones et al., 2021).

The frequency and clinical consequences of treatment-related emergence of PA/I38X variants depend on the infecting virus, age and immune status of the patient, and possibly severity of illness. The frequency has been higher in type A viruses, especially of the A(H3N2) subtype, and relatively uncommon in type B viruses collected from baloxavir-treated individuals (Hayden et al., 2018; Ison et al., 2020). The highest frequency is seen in young children, particularly those with lower neutralizing antibody titers against the infecting virus (Hirotsu et al., 2020; Sato et al., 2021; Yokoyama et al., 2020). Some children in whom PA-I38 variants emerged have experienced a rebound in virus titers and shed infectious virus for a longer time, as well as exhibiting delays in alleviation of clinical symptoms (Hirotsu et al., 2020; Sato et al., 2020, 2021; Yokoyama et al., 2020). In baloxavir-treated adults, emergence of PA-I38X variants has been associated with transient rises in infectious virus titers, initial delays in symptom alleviation and, uncommonly, with rebound of symptoms (Ince et al., 2020; Uehara et al., 2020). In a hospital-based RCT, a combination of baloxavir and NAI tended to decrease emergence of NA variants resistant to oseltamivir, although in two immunocompromised patients, with A(H1N1)pdm09 virus infection, receiving the drug combination, dual resistant variants (with substitutions in NA and PA) were detected (Mira et al., 2020).

Of concern, transmission of PA variants to untreated patients has been documented or inferred in several instances. Among 377 influenza A-positive clinical samples collected in Japan in 2018/2019 before antiviral treatment, no influenza A(H1N1)pdm09 viruses with the PA-I38T substitution (0/129, 0.0%) and four A(H3N2) viruses with the PA-I38T substitution (4/229, 1.7%) were detected (Osada et al., 2021). Two cases were in siblings, with identical HA sequences indicating a common source of infection, and all four cases were in patients less than 10 years old. In another Japanese study in 2018/2019, PA sequence analysis of 25 A(H3N2) viruses identified a PA-I38T substitution in virus from an untreated pediatric patient (Yano et al., 2020) and in national surveillance 3 of 9 A(H1N1)pdm09 viruses and 5 of 34 A(H3N2) viruses with PA-I38T were from untreated patients (NIID, 2019) (Table 3). Viruses with PA-I38T (Takashita et al., 2019c) and PA-E23K (Takashita et al., 2020a) have also been isolated from untreated pediatric patients during outbreaks in a preschool or a primary school, indicating limited transmission of baloxavir-resistant viruses in the community. There are two reports in which an untreated child has been infected with a PA-I38T variant where a sibling has been previously treated with baloxavir (Imai et al., 2020; Takashita et al., 2019a). In one report whole genome sequences of viruses from the treated and untreated siblings were identical, confirming human-human transmission (Takashita et al., 2019a).

Because of the limited data and high frequency of mutants with reduced susceptibility to baloxavir, the Japanese Association for Infectious Diseases recommends careful consideration of the use of baloxavir for children <12 years of age. The Japan Pediatric Society does not actively recommended the use of baloxavir for the treatment of influenza in children <12 years of age, and has recommended that baloxavir should not be used for monotherapy of influenza in the severely immunosuppressed (Shionogi, 2019; Takashita et al., 2020a).

Substitutions at other PA residues – E23G, E23K, A37T and E199G – have also been detected in influenza A viruses collected post-treatment (Ince et al., 2020; Omoto et al., 2018), in contacts receiving post-exposure prophylaxis (Ikematsu et al., 2020), and in viruses with no link to baloxavir treatment (Gubareva et al., 2019; Takashita et al., 2020a) (CDC unpublished data) (Table 1), but have less effect on baloxavir susceptibility than the PA-I38 T/M/F substitutions (Omoto et al., 2018). PA-K34R, identified during influenza virological surveillance, conferred 3–4-fold reduced susceptibility to baloxavir (CDC, unpublished data).

3.3. Favipiravir

Favipiravir was approved in Japan in 2014 for treatment of novel or reemerging influenza virus infections, unresponsive or insufficiently responsive to approved agents, but is investigational elsewhere. In placebo-controlled RCTs, oral favipiravir has shown antiviral effects and clinical benefit in uncomplicated influenza (Hayden and Shindo, 2019; McKimm-Breschkin et al., 2018). An observational study in severely ill hospitalized patients in China found that a combination of favipiravir and oseltamivir provided greater antiviral effects and somewhat more rapid clinical recovery compared to oseltamivir alone (Wang et al., 2020a).

Several *in vitro* studies have not selected resistance to favipiravir (Baranovich et al., 2013; Takashita et al., 2016). However, in one study, serial passage of an A(H1N1)pdm09 virus in cell culture in the presence of favipiravir led to the emergence of amino acid substitution K229R in motif F of the PB1 subunit. This substitution conferred reduced susceptibility to favipiravir in a mini replicon assay (Goldhill et al., 2018), and caused a 30-fold reduction in susceptibility of recombinant viruses in a yield reduction assay.

In a clinical study in Japan, no resistant virus was isolated from 57 patients treated with favipiravir; however, 4 specimens collected post-treatment had viruses with amino acid substitutions in PB1, PB2 and/or PA subunits, in 2/20 A(H1N1)pdm09, 1/17 A(H3N2) and 1/20 B viruses (Takashita et al., 2016). The significance of these substitutions is presently unknown, although they might reflect substitutions due to the proposed effects of favipiravir in increasing random mutation frequency and ultimately lethality in progeny virions (Baranovich et al., 2013).

3.4. Pimodivir

Pimodivir is a PB2 inhibitor that is active only against influenza A viruses. It has been evaluated in two phase 2 studies alone or in combination with oseltamivir. In studies of acute uncomplicated influenza, pimodivir treatment was associated with significant reductions in virus load at day 8 (-3.6 and -4.5 day \cdot log₁₀ copies/mL for 300 mg and 600 mg doses respectively), with greater reductions in patients who received both pimodivir and oseltamivir (-8.6 day \cdot log₁₀ copies/mL) (Finberg et al., 2019). There was a trend towards a shorter time to symptom resolution in the combination arm. Combination therapy was also associated with a reduction in the incidence of variants with reduced susceptibility compared to monotherapy with pimodivir (1.8% vs 6.9% vs 10.5% for combination vs 300 mg vs 600 mg, respectively). However, a recent placebo-controlled RCT in hospitalized patients did not

show additional benefit of combining pimodivir with the standard of care NAI treatment in most patients, compared to the standard of care alone, and development of pimodivir has been discontinued (Janssen, 2020). However, information on resistance to pimodivir could be useful for the development of other PB2 inhibitors.

Data from an *in vitro* study (Byrn et al., 2015) and clinical trials (Finberg et al., 2019; Trevejo et al., 2018) have identified nine residues in the PB2 subunit, in mid, cap-binding, or RNA binding/linker regions, where substitutions lead to reduced pimodivir susceptibility. Passage of A/Puerto Rico/8/34 (H1N1) virus (A/PR/8/34) in cell culture in the presence of pimodivir resulted in six different amino acid substitutions in PB2 reducing sensitivity to the drug (Table 2). Five conferred >100-fold reduction in sensitivity, with the F404Y conferring the highest resistance, a 257-fold lower sensitivity to pimodivir (Byrn et al., 2015). In patients treated with pimodivir the most common substitution in PB2 has been M431I, although M431 L/R/V have also been detected (Finberg et al., 2019; Trevejo et al., 2018). M431I confers a 57-fold decrease in sensitivity to pimodivir, but also results in a 12.5-fold reduction in replication fitness compared to wild-type (Trevejo et al., 2018). Serine in position 324 also appears to be a target for resistance. PB2 variants with substitutions S324I/N/R were generated *in vitro* (Table 2) (Byrn et al., 2015), and S324 C/K/N/R have been detected in viruses from patients treated with pimodivir (Finberg et al., 2019; Trevejo et al., 2018). Naturally occurring S324C and S324R substitutions, with 20–27-fold and 317–688-fold reductions in sensitivity, respectively, were detected in seasonal influenza A viruses, in the CDC surveillance program (Patel et al., 2021). Substitutions F325L, S337P, K376 N/R, T378S and N510K in PB2 have also been associated with reduced pimodivir sensitivity, although no individual EC₅₀ values were published, only a range of fold reduction in sensitivity (9.4 to >372.0-fold decrease) (Finberg et al., 2019). CDC surveillance found a seasonal A (H1N1)pdm09 virus naturally containing PB2–N510K that displays a 273-fold reduction in pimodivir sensitivity (Patel et al., 2021). Presently, information on fitness of most of these variants is lacking. Using deep mutational analysis Soh et al. (2021) have recently revealed a third set of mutations in the PB2 N-terminal domain, clustering on the surface of the protein (E188, E192, D195, C196) the influence of which cannot be explained by the existing models of pimodivir action as they do not interact directly with pimodivir.

4. Surveillance strategies for polymerase inhibitor resistance

4.1. General points

Several key principles have been recognized throughout the use of the M2 inhibitors and NAI classes of drugs, which are likely to inform the design of surveillance strategies for polymerase inhibitor resistance:

1. Amino acid substitutions conferring resistance will primarily occur in regions impacting the site of drug binding in the target virus proteins.
2. Mutations may be influenza virus type/subtype-specific.
3. Drugs of different chemical structure which act against the same functional target may generate different escape mutations.

4. Amino acid substitutions occurring outside the drug target region may affect the competitive fitness of viruses with drug resistance mutations in the target region.
5. The emergence of viruses with drug resistance mutations is more likely to occur in patient populations with naïve or compromised immunity.
6. Drug resistance may emerge during natural virus evolution, unrelated to drug use.
7. The relationship between genotypic changes and phenotypic properties of virus isolates *in vitro* and changes in clinical effectiveness of antivirals cannot be predicted.
8. Resistance detected in pre-clinical and clinical development programs is a reasonable, but incomplete, predictor of what may occur with widespread use.

Taken together, these characteristics suggest that future laboratory surveillance strategies for polymerase inhibitors should be designed using a tiered approach. An unbiased, broadly-based sequence screen of a geographically representative pool of circulating human viruses of all types/subtypes for known/suspected molecular markers of resistance will provide a suitable baseline, season by season. A representative portion of these viruses should be tested using phenotypic methods, suited to individual antiviral targets, to confirm known drug resistance signature changes, and identify altered susceptibility, which may arise *de novo* through novel mutations. Arrangements for pooling data and regular systematic review are essential to enable understanding of patterns of resistance in diverse settings and ensure early warning of transmissible resistance emergence. In addition, regular enhanced sampling of susceptible drug-treated populations, such as children and immunocompromised patients, in areas of high drug use will provide sensitive early warning of the association of drug resistance with particular virus types/subtypes or genetic variants.

4.2. Current surveillance of antiviral resistance of influenza viruses

The WHO GISRS, composed currently of 147 national influenza centers (NICs) in 123 UN member states, 7 WHO Collaborating Centers for Influenza (CCs), 4 essential regulatory laboratories and 13 H5 reference laboratories, is responsible for global influenza surveillance. The NICs undertake virological surveillance of influenza activity and share information on the epidemiology of the circulating influenza types, subtypes and lineages with the WHO. They also share representative and unusual virus samples with the WHO CCs for more detailed antigenic and genetic characterization. Some NICs and the CCs also assess antiviral sensitivity of selected viruses. This collaborative work led to the recognition of the global emergence of amantadine resistance in 2002 and oseltamivir resistance of seasonal influenza A(H1N1) viruses in 2008 (Bright et al., 2005; Lackenby et al., 2008).

While worldwide screening of NAI susceptibility has been conducted for almost 20 years, there are still challenges in interpreting data obtained using current laboratory methods, e.g., a lack of cell culture-based assays, which can adequately predict NAI-susceptibility of virus in a human host. Instead, surveillance laboratories routinely use a surrogate phenotypic assay, which is based on assessing NA enzyme activity in the presence of NAIs. Notably, the buffer, the time and temperature of preincubation with the virus and the time and

temperature of the incubation with substrate, as well as the choice of substrate all impact the IC₅₀ measurement of the NAI (Barrett et al., 2011; Hodges et al., 2019; McKimm-Breschkin et al., 2003; Wetherall et al., 2003). To have some way of comparing results across laboratories, the WHO GISRS Working Group on Surveillance of Influenza Antiviral Susceptibility suggested that fold differences compared to the type/subtype-specific median IC₅₀ values be used when reporting testing outcomes, rather than actual IC₅₀ values (WHO, 2012). For influenza A viruses <10-fold inhibition is considered as 'normal' inhibition (NI), 10–100-fold is considered as 'reduced' inhibition (RI) and >100-fold is considered as 'highly reduced' inhibition (HRI). Because of a lower potency of NAIs towards influenza B NAs, <5-fold reduction in inhibition is classified as NI, 5–50-fold as RI and >50-fold as HRI. A panel of reference wild-type and viruses with a range of reduced inhibition is available from the US CDC (International Reagent Resource: <https://www.internationalreagentresource.org/Catalog.aspx?qCDC%20neuraminidase%20Susceptibility%20Reference%20Virus%20Panel%20FR-1755>). Defining the magnitude of reduced inhibition relevant to clinical outcomes is an ongoing challenge. Nevertheless, it is common to interpret reduced inhibition, and especially highly reduced inhibition, as an indicator of decreased susceptibility to a particular NAI.

Two countries, USA and Japan, with longstanding influenza surveillance programs also have the most intensive antiviral susceptibility testing programs for NAIs and baloxavir. A variety of methodologies are used in an algorithmic manner, including whole genome (WGS), or partial genome, sequencing using next generation sequencing (NGS) platforms combined with phenotypic and enzymatic analyses of cultured virus isolates (Patel et al., 2020). In the USA, the priority has been focused on geographically representative sampling, primarily to track virus evolution using a screening approach based on WGS as a laboratory first line investigation. This approach is based on a large number of virus samples that are collected routinely and tested by the state public health laboratories. Among about 90,000 clinical samples tested, approximately 6000 are fully sequenced. For each class of drug (M2 inhibitors, NAIs or polymerase inhibitors), different gene segments are analyzed for mutations (Zhou et al., 2009, 2014). Screening for resistance to M2 inhibitors is solely based on M2 sequence analysis to identify amino acid substitutions at residues L26, V27, A30, S31 and G34. Screening NA sequences of clinical specimens and virus isolates identifies molecular markers previously associated with NAI resistance/decreased susceptibility.

Baloxavir susceptibility monitoring is focused on detection of amino acid substitutions in the PA protein. To assist in sequence-based surveillance, a summary of the PA amino acid substitutions analyzed for their effects on baloxavir susceptibility has been posted at the WHO GISRS website (WHO, 2021b). To confirm the sequence-predicted drug phenotype, viruses are then tested using phenotypic assays such as the high-content imaging neutralization test (HINT) or focus reduction assay (FRA) (Gubareva et al., 2019; Takashita et al., 2018). In addition, subsets of virus isolates, lacking any suspected markers of resistance and representing all circulating subtypes and lineages, are tested to monitor the baseline antiviral susceptibility using phenotypic assays. Using this approach, NGS analysis of 6981 PA genes and phenotypic testing by HINT of 116 viruses collected during 2016/2017 and 2017/2018 seasons revealed very low detection of viruses with decreased baloxavir susceptibility [0.032% for A(H3N2), 0.3% for A(H1N1)pdm09, and 0% for B

viruses] prior to approval of the drug in 2018 (Gubareva et al., 2019). After drug approval, NGS analysis of 4828 PA sequences collected between October 1, 2018 to August 5, 2019 found four virus isolates [0.113% for A(H3N2) and 0.096% for A(H1N1)pdm09] with PA markers previously associated with reduced susceptibility – two A (H3N2) viruses with I38L or I38 M/I and two A(H1N1)pdm09 viruses with E199G or A36 V/A. Phenotypic testing of the virus isolates containing mixtures of variants (A36 V/A or I38 M/I) showed <3-fold change in IC₅₀ by HINT (CDC unpublished data).

The surveillance performed in Japan is crucial because of the large per capita use of influenza antivirals prescribed by clinicians in the country. During the 2018/2019 season, 5.3, 4.6, 2.9, 0.6 and 0.3 million doses of baloxavir, oseltamivir, laninamivir, zanamivir and peramivir, respectively, were supplied to medical institutions in Japan. The NIID utilizes 3000 pediatric and 2000 internal medicine clinical sites to collect samples (without clinical data); of these 500 sentinel sites perform laboratory-based antiviral susceptibility. Clinical samples are sent to 73 public health institutions to perform virus isolation, allele-specific RT-PCR for NA-H275Y and/or NA and PA sequencing. Ten to fifteen percent of these virus isolates are randomly selected and sent to the NIID for NAI susceptibility, baloxavir susceptibility (FRA) and NGS analysis. Testing of 2802 isolates for NAI and 863 isolates for baloxavir susceptibility from the 2018/19 season (Table 3) found low rates of NAI resistance (0.9% for A(H1N1)pdm09 and 0.5% for influenza B). Viruses from four patients had NAI resistance substitutions without prior NAI treatment. Slightly higher rates for PA-I38X substitutions conferring reduced susceptibility to baloxavir were detected [(2.3% for A(H1N1) pdm09, 8.0% for A(H3N2) and 0% for B viruses (NIID, 2019; Takashita, 2020)].

5. Evaluation of the susceptibility to polymerase inhibitors *in vitro*

Unlike the NAIs, the polymerase inhibitors have different targets and mechanisms of action, which may necessitate using different laboratory-based methods for susceptibility screening. As more countries approve polymerase inhibitors for clinical use, collecting and comparing data generated by various laboratories will be needed. It is therefore essential to find affordable, high throughput, reproducible, relatively low technology methods. Because the primary enzymatic function of the polymerase requires the intact complex and an RNA template, *in vitro* enzyme assays are not feasible for surveillance, as they require recombinant reagents (Noshi et al., 2018; Omoto et al., 2018; Takashita et al., 2016). Hence, in contrast to the NAIs, drug susceptibility will need to be primarily evaluated in cell culture-based assays. Due to the different mechanisms of action and pharmacokinetics, the fold reduction in susceptibility *in vitro* may not necessarily have the same relevance clinically for the different polymerase inhibitors. Furthermore, since influenza B viruses also appear to be less susceptible to baloxavir than influenza A viruses (Gubareva et al., 2019; Koszalka et al., 2019; Mishin et al., 2019; Noshi et al., 2018; Omoto et al., 2018), there may need to be different thresholds of fold-changes for classification of type A and B virus resistance to the polymerase inhibitors.

5.1. Phenotypic evaluation of resistance

5.1.1. Reagents and cell culture—The first parameter to be established is which cell line to use for susceptibility testing, since certain contemporary viruses may grow poorly using conventional cell lines, such as MDCK cells. Virus recovery from clinical samples tends to be better in the modified MDCK-SIAT1 cells due to the higher level of human-like α 2,6-sialic acid receptors on the cell surface (Matrosovich et al., 2003). More recently, a humanized MDCK cell line (hCK) has been described, which also expresses high levels of α 2,6-sialoglycans, but in contrast to MDCK-SIAT1 cells, expresses low levels of α 2,3-sialoglycans (Takada et al., 2019). Influenza A(H3N2) viruses are claimed to be more stable on passage in hCK cells. All viruses require initial titration to determine the appropriate inoculum for cell culture antiviral testing, to achieve cytopathic effect (CPE) or an appropriate number of plaques or clusters of infected cells (foci).

To assist with implementing drug susceptibility testing, the US CDC provides a baloxavir reference panel available via IRR (FR-1678 - CDC Baloxavir Susceptibility Reference Virus Panel version 1.1). Some laboratories have made reverse genetics viruses with substitutions known, from *in vitro* or clinical studies, to reduce susceptibility to baloxavir (Koszalka et al., 2019; Noshi et al., 2018; Takashita et al., 2018). However, because the assays may use different cell lines and the genetic backgrounds of viruses may differ the effects of substitutions on susceptibility to baloxavir may vary.

The appropriate form of drug needs to be used. Baloxavir marboxil is a prodrug, so baloxavir acid (dissolved in DMSO) is needed for *in vitro* assays. The PB1 inhibitor favipiravir requires activation in cells to form the active triphosphate, which may take several hours. Hence the time of some assays may be too short for it to have a noticeable antiviral effect (Mishin et al., 2019). The PB2 inhibitor pimodivir is the active compound, but it needs to be dissolved in DMSO and diluted into cell culture medium prior to use (Byrn et al., 2015).

5.1.2. Plaque reduction assay (PRA)—The PRA has been used to evaluate sensitivity of influenza viruses to baloxavir (Abed et al., 2020; Jones et al., 2020; Noshi et al., 2018; Omoto et al., 2018; Takashita et al., 2018; Uehara et al., 2020) and favipiravir (Furuta et al., 2002; Omoto et al., 2018; Sleeman et al., 2010; Takahashi et al., 2003; Takashita et al., 2016). It requires no expensive equipment but usually takes 2–3 days and relies on the viruses being cytopathic so that plaques can be counted by eye or microscopically. However, large numbers of 6 or 12-well plates are required; and although simple, it is not high throughput.

5.1.3. CPE inhibition assay—This has been a common antiviral assay for many virus/drug combinations as it only requires a single cell culture step for the drug sensitivity assay and can be carried out in a 96- or 384-well plate format, allowing multiple viruses to be tested simultaneously. It has been used to determine sensitivity to baloxavir (Noshi et al., 2018) and favipiravir by measuring cell viability after 3–6 days by commercial assays, quantified using an ELISA plate reader (Takashita et al., 2016). Similarly, sensitivity to

pimodivir has been measured after 3 days, using a commercial cell viability assay (Byrn et al., 2015; Clark et al., 2014).

5.1.4. Yield reduction assay (YRA)—Several laboratories have used the YRA to evaluate sensitivity of influenza viruses to baloxavir (Koszalka et al., 2019; Mishin et al., 2019; Noshi et al., 2018; Taniguchi et al., 2019) or favipiravir (Mishin et al., 2019; Sleeman et al., 2010; Takahashi et al., 2003; Taniguchi et al., 2019). However, a further round of cell culture is needed to determine the virus yield. As a variation of the YRA, the sensitivity to pimodivir has been measured by evaluating virus replication using the levels of HA vRNA, expressed in the treated cells at 20–22 h post-inoculation (hpi), by branched DNA hybridization (Byrn et al., 2015; Clark et al., 2014). bDNA EC₅₀ values were marginally higher than in the CPE assay and it takes a further 24 h processing, including lysis, hybridization, amplification, and addition of luminescent substrate.

5.1.5. Focus reduction assay (FRA)—The FRA has been used for evaluation of sensitivity of influenza viruses to baloxavir (Gubareva et al., 2019; Koszalka et al., 2019; Mishin et al., 2019; Takashita et al., 2018), favipiravir (Sleeman et al., 2010) and pimodivir (Beigel et al., 2019; Patel et al., 2021). It uses a viscous overlay to limit virus spread to adjacent cells, such that clusters of infected cells (foci) can be visualized and counted. Incubation is generally for 24 hpi, after which cells are fixed and immunostained with an anti-nucleoprotein (NP) antibody, followed by either a horseradish peroxidase (HRPO) conjugated secondary antibody with a tetramethyl benzidine chromogenic (TMB) substrate or a fluorescent conjugate. While it is a rapid assay, expensive imaging hardware and appropriate software are required for analysis.

The Virospot assay is similar (Omoto et al., 2018), except incubation is for 24–48 hpi, followed by fixation and anti-NP staining. Despite their similarity, mean EC₅₀ values in the Virospot assay for baloxavir were up to 10-fold higher for A(H1N1)pdm09 viruses, 5-fold higher for A(H3N2) viruses, and 2-fold higher for influenza B viruses, than in the PRA (Omoto et al., 2018).

5.1.6. High-content imaging neutralization test (HINT)—In the HINT (Jorquera et al., 2019) cells are infected for 16–24 h in the absence of trypsin, limiting replication to a single cycle. Detection is by immunostaining with an anti-NP antibody, followed by a fluorescent conjugate, and analysis by a plate imaging system that counts dually stained (anti-NP antibody and DNA dye) virus-infected cells. While sensitivity to baloxavir (Gubareva et al., 2019; Mishin et al., 2019) and pimodivir (Patel et al., 2021) was able to be evaluated, favipiravir did not produce a measurable antiviral effect by HINT because it requires several hours for intracellular activation. For baloxavir the EC₅₀ values by HINT were within two-fold of FRA and PRA values, but for pimodivir, the HINT EC₅₀ values were 8-fold higher than those determined by FRA, although the fold changes conferred by two PB2 substitutions remained consistent between the two assays (Patel et al., 2021).

5.2. Sequence-based detection of resistance

Once *in vitro* phenotypic assays have been used to establish baseline susceptibility of wild-type clinical isolates, they can be used in surveillance, and to evaluate reduced susceptibility of viruses obtained from patients treated with antivirals. However, until more cases of reduced effectiveness of antiviral therapy are seen clinically, it will be difficult to establish a benchmark for *in vitro* assays relevant to clinical resistance.

Genotypic assays are typically used for detecting known resistance-conferring markers in viral genomes. Over the years, surveillance laboratories have used an array of genotypic methods (Sanger and NGS sequencing, pyrosequencing, allelic discrimination by real-time RT-PCR, and others) to monitor resistance to M2 inhibitors and NAIs (Deyde et al., 2010; Monto et al., 2006; Nakauchi et al., 2011).

One of the advantages of genotypic testing is the ability to utilize common laboratory techniques and equipment to detect molecular markers of resistance for various antivirals. Genotypic assays also offer higher sensitivity compared to phenotypic assays in detecting minor subpopulations of resistant viruses and allow for testing to be done on original clinical specimens. However, genotypic assays may be less informative than phenotypic assays when information on molecular markers of resistance is sparse. Such situations typically occur when a new antiviral drug enters the market, or a novel influenza virus emerges. The detection of a well-established marker is not necessarily proof of antiviral resistance as other changes in the virus genome may influence drug susceptibility. For these reasons, interpretation of genotypic results require caution.

Sanger sequencing and NGS are used for comprehensive analysis of an individual viral gene or entire genome; and can aid in the discovery of novel markers conferring drug resistance. Additionally, NGS offers deep and ultra-deep sequencing options, allowing for the detection of minor subpopulations of drug-resistant viruses in mixtures (Ghedini et al., 2012). However, deep sequencing is not yet widely used by public health laboratories for antiviral resistance surveillance and diagnostic applications. In addition, NGS commonly requires sophisticated sample preparation and data analysis, which can take 2–3 days before results are acquired. Nevertheless, development of third-generation, sequence-based diagnostic technologies is underway and may reduce some of the time-consuming processes of NGS (Van Poelvoorde et al., 2020).

The assay chosen for resistance detection depends on available resources and objectives of the laboratory (e.g., surveillance, outbreak or clinical management, etc.). Using more than one assay may improve interpretation of results and enhance knowledge of drug resistance markers. For example, single nucleotide polymorphism (SNP) analysis by real-time RT-PCR can be performed for rapid testing, followed by comprehensive analysis by NGS and/or phenotypic testing.

Besides gene sequencing, two genotypic assays have been developed to detect baloxavir resistant viruses: RNase H2-based mutation dependent differential PCR amplification (rhPCR) (Nakauchi et al., 2020) and pyrosequencing (Gubareva et al., 2019; Koszalka et al., 2020; Patel et al., 2020). These assays were designed and validated for detecting amino

acid substitutions at PA-I38 in influenza A viruses (Gubareva et al., 2019; Patel et al., 2020) or both type A and B viruses (Koszalka et al., 2020; Nakauchi et al., 2020).

The rhPCR method is based on the utilization of blocked primers containing a single ribonucleotide residue that provides the cleavage site for the RNase H2 enzyme. Blocked primers prevent extension by DNA polymerase until the blocked portion is cleaved. Cleavage efficiency of RNase H2 is reduced in the presence of mismatches near the RNA residue in the DNA:RNA heteroduplex of template and primer, which is used to detect an SNP. Three independent tests were developed for A(H1N1) pdm09, A(H3N2) and type B viruses due to PA sequence differences (Nakauchi et al., 2020). Each rhPCR assay consisted of one PA gene-specific primer and two allele-specific primers. One allele-specific primer was designed to recognize I38 and was detected with a Yakima Yellow-labeled universal probe, while another allele-specific primer was designed to recognize T38 with an FAM-labeled universal probe.

Overall, rhPCR assays have been shown to accurately discriminate between viruses with I38 or T38, even in clinical samples containing mixtures (Nakauchi et al., 2020). A 5% cut-off for each rhPCR assay was determined to detect I38 or T38 in a double mixture using positive RNA controls. However, the possibility of erroneous results remains for clinical samples containing less than 10% variant in the mixture, which may be due to differences in RNA purity between positive RNA control and clinical specimens (Nakauchi et al., 2020). Additionally, Osada and colleagues have recently reported the development of a cycling probe based real-time PCR methodology using fluorescent-labeled chimeric RNA-DNA probes to detect the PA-I38T substitution in influenza A viruses (Osada et al., 2021). These PCR based assays were designed to identify only PA-I38 or -T38, and this limitation needs to be considered when interpreting results, as it may underestimate the presence of drug resistant viruses (Nakauchi et al., 2020; Osada et al., 2021).

To this end, pyrosequencing (Qiagen platform) offers more options for detecting amino acid substitutions at PA-I38 by generating short, targeted sequence readouts and pyrograms (Gubareva et al., 2019; Koszalka et al., 2020; Patel et al., 2020). Pyrosequencing can be carried out in a short turn-around time, but it is more cumbersome than rhPCR. Published pyrosequencing assays differ in their design (e.g., size of amplicon to be sequenced, forward or reverse direction of sequencing, order of nucleotide dispensation). These assays were shown to readily detect amino acid PA-I38 substitutions F, M, and T in both type A and B viruses (Koszalka et al., 2020) or F, L, M, S, T and V in type A viruses (Gubareva et al., 2019; Patel et al., 2020). However, identification of a PA-I38X variant can be challenging when it is present in a mixture with wild-type, which is common in post-treatment specimens (Takashita et al., 2019a, 2019c; Uehara et al., 2020). The detection of PA variants in double virus mixtures can be improved by customizing the order of nucleotide dispensation (Patel et al., 2020). However, identification of PA-I38X variants in triple virus mixtures remains challenging using pyrosequencing (Patel et al., 2020). Apart from detecting SNPs, pyrosequencing can also determine the relative proportions of PA variant and wild-type viruses in a mixed population. Of note, the PyroMark ID software does not support SNP analysis of triple mixtures due to consecutive changes at more than two nucleotides of the same codon. In addition, PyroMark ID software often encounters

problems when quantifying the pyrogram peak heights of homopolymers (e.g., F→TTT; AAA in reverse complement), which may lead to diminished accuracy in determining the proportions for certain mixtures (Koszalka et al., 2020). These limitations of pyrosequencing are less likely to affect testing for surveillance purposes as specimens are typically collected before antiviral treatment and unlikely to contain mixtures of PA variants.

6. Evaluation of efficacy of polymerase inhibitors in animal models

The mouse and ferret models are the most common used for evaluation of the efficacy of antiviral drugs against influenza virus infections with both wild type and resistant variants, as well as for *in vivo* generation of resistant variants, assessment of fitness, virulence and transmissibility of resistant variants. There are many variables which need to be standardized for wild type viruses, prior to investigating resistant variants, including dose of challenge virus, time of inoculation relative to drug exposure, and the dosing regimen of the inhibitor. Further, while mouse- or ferret-adapted laboratory viruses with substitutions of interest may be generated using reverse genetics, clinical isolates may have different infectivity/lethality and drug susceptibility in animal models. The challenge is to try to standardize treatment and infection protocols and to find *in vivo* correlates of resistance that are relevant to failure of antiviral therapy in patients.

6.1. Baloxavir

For baloxavir it is difficult to set a dose regimen in mice that is clinically equivalent to that in humans owing to the crucial difference in half-life of baloxavir marboxil in plasma between humans (85.9 h at 40 mg) and mice (2.26 h at 15 mg/kg). Oral administration of baloxavir marboxil in mice at 50 mg/kg twice daily for one day or 15 mg/kg twice daily for 5 days achieves plasma concentrations of baloxavir acid comparable to a single 40 mg dose in humans (Taniguchi et al., 2019). The baloxavir acid plasma concentration following an 8 mg/kg subcutaneous administration (back of neck) of baloxavir acid was also similar to that following 50 mg/kg of oral baloxavir marboxil (Ando et al., 2021).

Several groups have investigated the efficacy of treatment with baloxavir in mice challenged with various viruses (Fukao et al., 2019a, 2019b; Kiso et al., 2019, 2020; Taniguchi et al., 2019). However, as each group used a different inoculum of virus, different dosing ranges and different timing of drug administration, there is not yet a standard protocol for evaluating susceptibility of potentially resistant variants in mice (Supplementary Table A).

As ferrets have been widely used to assess the drug susceptibility of wild type influenza viruses and NAI resistant variants they are also a good model for studying susceptibility to polymerase inhibitors. In addition, transmission studies can also be used to evaluate the efficacy of antiviral treatment and fitness of resistant variants. Transmission can be by direct contact among co-housed ferrets; or by aerosol (respiratory droplet) between animals in cages separated by several cm, with a barrier preventing direct contact (Hurt et al., 2010; Kwon et al., 2018).

Due to the short half-life of baloxavir marboxil given orally to ferrets, different administration routes have been evaluated. After oral administration, via intragastric tube,

of 10 or 30 mg/kg of baloxavir marboxil maximum plasma concentrations of baloxavir acid were attained at 1.5 and 2 h respectively, with elimination half-lives of 6.91 (3.79) and 4.44 (0.67) h respectively. Ferrets given two doses on day 1 or day 2 pi had an initial decrease in nasal wash virus titers, which rebounded 24 h later, indicating insufficient levels of baloxavir in plasma (Kitano et al., 2020). Other studies used baloxavir delivered subcutaneously at four locations on the dorsal region of the ferret (4 mg/kg per animal) and showed comparable PK concentrations to those of humans receiving the standard baloxavir dose. Using this dosing regimen, baloxavir-treated donor ferrets, infected with an A(H1N1)pdm09 virus, shed less virus, based on infectious virus titers in nasal washes, compared to oseltamivir-treated or untreated ferrets. Virus was only transmitted to 1/4 respiratory droplet sentinels compared to 3/4 and 4/4 animals in the oseltamivir and untreated groups, respectively. Direct contact transmission still occurred among baloxavir-treated animals, but virus was cleared significantly earlier than in untreated donor animals (Frise et al., 2019). Using the same dosing protocol, another study found that even when baloxavir treatment was delayed by 24 or 48 hpi, it significantly reduced the duration of virus shedding in A(H1N1)pdm09 virus-infected animals. Transmission to co-housed ferrets was also reduced by 75% and 50%, respectively, compared to oseltamivir and untreated controls (Lee et al., 2020).

6.2. Favipiravir

Favipiravir when administered orally at 200–400 mg/kg four times a day at 6 h intervals for 5 days, beginning at 1 hpi completely protected A/PR/8/34 virus-infected mice from death. When delayed up to 25 hpi, treatment with 200 mg/kg still protected 71% of mice from a >1000 MLD₅₀ challenge with A/PR/8/34 (Takahashi et al., 2003). A combination of oseltamivir (20 mg/kg/day) and favipiravir (50 mg/kg/day), twice daily for 5 days by oral gavage, when delayed until 72 or 96 hpi, protected 100% of mice from a lethal infection with a highly pathogenic A/turkey/15/2006 (H5N1) virus, whereas favipiravir treatment alone protected 90% and 40% of the mice, respectively (Marathe et al., 2016).

6.3. Pimodivir

Pimodivir has favorable pharmacokinetic properties in a mouse model following a single oral administration of 10 mg/kg, with a half-life of 6.7 h. Twice daily dosing of 1, 3 or 10 mg/kg from 2 h prior to infection for 10 days protected mice from lethal infection with A/PR/8/34 (H1N1), A/California/04/2009 (H1N1)pdm09 and A/Vietnam/1203/2004 (H5N1) viruses (Byrn et al., 2015; Clark et al., 2014). Treatment could be delayed for up to 96 hpi and still provide 100% protection from death and a dose-dependent reduction in body weight. Even at 120 hpi some protection was seen, whereas oseltamivir had little effect on mortality when started at 24 hpi (Byrn et al., 2015). However, pimodivir was not as effective against A/Victoria/3/75 (H3N2) as against an A(H1N1)pdm09 virus (Smee et al., 2016). Treatment for less than 10 days was not tested.

6.4. Immunocompromised animal models

Nude mice have been used as an immunocompromised model for selection of resistant viruses with favipiravir and oseltamivir (Kiso et al., 2018). Combination therapy of favipiravir with oseltamivir or laninamivir increased survival times of mice. No favipiravir-

resistant variants were isolated, but combination therapy did not suppress the emergence of NAI-resistant variants.

In nude mice treated with baloxavir for 28 days, 5 of 6 mice survived but virus was not eliminated, as once treatment ceased the remaining mice lost body weight and died 39–72 days pi (Kiso et al., 2020). No resistant viruses were detected in 10 lung samples, but one of 45 picked plaques had a PA-I38M change, while two plaque-purified viruses possessed the PA-E199G substitution.

Other baloxavir studies used an immunocompromised model where mice were treated subcutaneously with cyclophosphamide once daily at 24 h pre-virus inoculation and for up to 9 days pi. When treatment with baloxavir was initiated at 120 hpi, virus titers were reduced within 24 h after initial treatment, and body weight loss was inhibited in the virus-infected immunocompromised mice. No mutant virus with amino acid substitutions in PA was detected in immunocompromised mice during the treatment with baloxavir for 5 days. It was noted, however, that immunosuppression may be modest in cyclophosphamide-treated mice; thus, the risk of emergence of variant viruses might be relatively low compared to the nude and SCID mouse models (Fukao et al., 2019a).

Immunocompromised ferrets (treated with a cocktail of immune-suppressive drugs mycophenolate mofetil, tacrolimus and prednisolone) infected with either wild-type, or oseltamivir-resistant A(H1N1)pdm09 (NA-H275Y) showed prolonged virus shedding, as seen with immunocompromised patients. Immunocompromised ferrets have also been useful for selecting NAI-resistant variants, e.g., in wild-type virus-infected animals treated with oseltamivir, NA-H275Y or NA-R292K variants emerged (Roosenhoff et al., 2018; van der Vries et al., 2013). Thus, this ferret model may be suitable for evaluating the propensity for resistance to emerge after treatment with polymerase inhibitors in an immunocompromised population.

6.5. Evaluation of fitness of resistant variants

Similar approaches used for evaluating replication and transmission fitness of NAI resistant mutants *in vitro* and *in vivo* are being employed for polymerase inhibitor resistant mutants. However, results can vary depending on whether *in vitro* replication kinetics are based on single or multicycle growth, the cell lines used and whether it is a laboratory strain or a patient-derived isolate. Competitive fitness experiments can also be carried out *in vitro* and *in vivo* with mutant and wild-type viruses at different ratios and different doses of viruses (Govorkova et al., 2010; Hurt et al., 2010). *In vivo* the relative ratios of wild-type and resistant variants can be determined either after replication in infected animals or after transmission to contact animals.

6.5.1. Baloxavir—Omoto et al. (2018) found that reverse genetics (rg) rg-A/WSN/33 (H1N1) and rg-A/Victoria/3/75 (H3N2) viruses with I38 T/F/M substitutions in the PA had lower replication fitness compared to the wild-type in MDCK and human RPMI2650 cells. In contrast, only I38F reduced fitness of the rg-B/Maryland/1/59 virus. However, these are old laboratory strains. Contemporary influenza rg-A/Quebec/144147/2009 (H1N1)pdm09 and rg-A/Switzerland/9715293/2013 (H3N2) influenza viruses and their rg-I38T PA mutants

were compared in single and competitive infection experiments in ST6GalI-MDCK cells and in C57/BL6 mice. The I38T substitution did not alter the *in vitro* replication kinetics of the A(H1N1)pdm09 and A(H3N2) viruses. In competition experiments in ST6GalI cells, a 50%:50% (WT: mutant) mixture evolved to 70%:30% for the A(H1N1)pdm09 and 88%:12% for the A(H3N2) viruses after a single passage, but the I38T substitution remained stable after 4 passages (Checkmahomed et al., 2020). In mice, the WT and I38T mutant induced similar weight loss with comparable lung titers for both subtypes and in those infected with a 50:50 mixture of WT: mutant, the mutant viruses tended to predominate (Checkmahomed et al., 2020).

Jones et al. (2020) generated rg-baloxavir-resistant viruses with PA-I38 T/F/M substitutions in A/California/04/2009 (H1N1)pdm09, A/Texas/71/2017 (H3N2) and B/Brisbane/60/2008 virus backgrounds and examined transmission among naïve ferrets through direct contact and airborne routes. Viruses with the I38 T/M substitutions had minimal or no reduction in contact or airborne transmission, while the I38F substitution attenuated airborne transmission of the A(H3N2) and B viruses but allowed transmission of the A(H1N1)pdm09 virus by both routes. Another study (Imai et al., 2020) reported that influenza A (H1N1)pdm09 and A(H3N2) viruses carrying a PA-I38T substitution showed replicative fitness and pathogenicity similar to those of baloxavir-susceptible viruses in hamsters and that they transmitted efficiently between ferrets by respiratory droplets.

Comparisons of replication *in vitro* in MDCK and MDCK-SIAT1 cells of recent clinical isolates showed that A/Illinois/08/2018 (H1N1) pdm09 with I38 T/S substitutions replicated more slowly than the baloxavir-susceptible virus at 24 hpi in both cell lines, but by 48 hpi titers were similar (Chesnokov et al., 2020). Replication of the PA-I38L variant was similar at all times in both cell lines compared to the baloxavir-susceptible virus. Influenza A/Bangladesh/3007/2017 (H3N2) with PA-I38T and A/Louisiana/49/2017 (H3N2) with PA-I38M also exhibited slower growth than the baloxavir-susceptible virus at 24 hpi in MDCK-SIAT1 cells, but the difference was minimal in MDCK cells (Chesnokov et al., 2020). These viruses, with the PA-I38T or PA-I38M substitutions, replicated to equivalent titers in ferrets and no reversion to I38 was observed, indicating that the PA variants are genetically stable *in vivo* (Chesnokov et al., 2020). In competitive growth experiments, ferrets were intranasally inoculated with mixtures of the two A (H3N2) viruses, baloxavir-susceptible virus and either I38T- or I38M-substituted counterpart, at ratios of 10:90, 30:70, or 70:30. The proportion of baloxavir-susceptible virus in nasal washes increased incrementally over time in both I38: I38M and I38: I38T virus pairs. Although apparently reduced in fitness, the I38-substituted subpopulations remained detectable as late as 7 dpi (Chesnokov et al., 2020). Fitness of recombinant and patient-derived A(H3N2) and A (H1N1)pdm09 variant viruses containing PA-I38T compared to wild-type viruses was recently evaluated using a competitive mixture ferret model (Lee et al., 2021). The PA-I38T variants had lower fitness and the relative fitness cost was greater in A(H1N1)pdm09 than A (H3N2) viruses.

6.5.2. Favipiravir—Favipiravir-resistant virus with a PB1–K229R substitution had reduced virus replicative fitness *in vitro*, which was restored by a compensatory substitution P653L in the PA subunit (Goldhill et al., 2018). Virus with both substitutions infected ferrets and transmitted by direct contact to 4/4 and by respiratory droplet to 3/4 animals.

Resistance was maintained, although the PB1–K229R substitution decreased in frequency in some ferrets (Goldhill et al., 2021).

7. Research gaps and priorities

7.1. General points

Multiple knowledge gaps related to polymerase inhibitor resistance need to be addressed (Table 4). In addition to issues related to laboratory testing methods, pre-clinical models, surveillance and clinical management, there are several other overarching concerns. Communication and sharing of information between pharma experts, academia, public health laboratorians and regulators remains an important limitation. Proprietary and commercial confidentiality issues specific to individual companies often preclude open communication between companies and may impact information sharing with government regulators and public health agencies. While formal channels currently exist for communication between pharma and regulators, communication between academia and both pharma and regulators have often been more ad hoc and limited in nature. This may impact ability to achieve optimal design of clinical and pre-clinical studies relevant to efficacy and resistance to influenza antivirals.

While it is recognized that there are competing priorities in sharing data with the scientific community and often delays in publication, sharing of relevant data about drugs in late-stage development is essential to ensure that clinicians are informed about proper use of new agents and investigators are able to perform studies addressing gaps in knowledge. For several polymerase inhibitors, key pre-clinical and clinical studies have been completed, in some cases for several years, and yet only limited data are available in published literature. Transparency of information about clinical trials during drug development studies is an important principle. Posting of results through online servers would help earlier dissemination of information. With commitment from regulatory agencies, medical journals and pharma, significant changes have taken place in recent years towards much more rapid publication of clinical trial data. Lack of publication of patient level data in the early stages of NAI development programs led to controversies, which have overshadowed the profile of these drugs and undermined confidence in their use (Doshi et al., 2013; Jefferson et al., 2011, 2014).

Addressing two other gaps would clearly improve understanding of the epidemiology, risk factors, consequences, and management of influenza antiviral resistance. First, alignment of regulatory requirements for pre-clinical and clinical studies would ensure more consistent approaches to collecting critical data on antiviral resistance. Second, the availability of reliable resistance testing platforms that are readily accessible to clinicians. At present, there are few laboratories that can quickly screen influenza viruses for antiviral resistance, and most are focused on identifying specific substitutions (e.g. NA-H275Y in A(H1N1)pdm09). There are currently no commercially available test systems to perform phenotypic susceptibility testing to polymerase inhibitors or to easily screen for potential substitutions that reduce susceptibility.

Predictors of the emergence of polymerase inhibitor resistant variants and their likely transmission are needed. With the increasing diversity of licensed antivirals, suitable generalizable models, which allow linkage between *in vitro* detection of resistance, competitive fitness and transmissibility of variants would provide useful tools. From a global perspective, there are clear needs for data to inform the utility of novel agents for inclusion in country-held and WHO global antiviral stockpiles. This should include modeling data on the impact of antivirals in controlling local epidemics and global pandemics.

7.2. Clinical issues related to polymerase inhibitor resistance

Clinically, there are several key issues that need to be addressed to mitigate the emergence and transmission of resistant variants. Central to this are studies to better define the pharmacokinetic-pharmacodynamic (PK-PD) relationships of baloxavir and other polymerase inhibitors in pre-clinical models and in both outpatient and hospitalized patient cohorts. Currently, there have been limited clinical PK data collected with a limited range of doses of baloxavir and favipiravir. For example, the maximum dose of baloxavir studied to date is 80 mg as a single dose, and no data have been published on its oral bioavailability and PK in seriously ill patients or on respiratory tract cell concentrations of baloxavir over time. Studies of higher doses and variable frequencies of dosing are needed to understand its optimal dosing in seriously ill patients (e.g., impact of larger loading doses and multiple-dose regimens). Similarly, the favipiravir PK changes considerably in seriously ill patients, and the optimal dose regimen is uncertain in hospitalized patients (Wang et al., 2020b). This is particularly important in countries where obesity is a major issue, as higher doses may be required for patients with increased weight. Studies of the PK of polymerase inhibitors in patients for which there is a paucity of data, including those with severe renal and hepatic dysfunction, patients on ECMO, and pregnant women are also needed to expand the use of these drugs to more seriously ill patients.

While there are studies ongoing to understand the utility of polymerase inhibitors in hospitalized patients, clinical studies are needed in other groups at high risk for influenza complications, including pregnant and immediately post-partum women, infants including premature ones and immunocompromised persons. Immunosuppressed patients, for example, may provide insight into the risk factors associated with development and the kinetics of emergence of resistant variants. Similarly, the high frequency of baloxavir resistance emergence in young children strongly argues for studies of multiple-dose regimens and combination antiviral studies. These critical studies could help define if particular antiviral combinations reliably reduce the risk of emergence of variants with reduced susceptibility and preserve the potent antiviral effectiveness of an agent like baloxavir. Such findings would support the more widespread use of antiviral combinations even if the added clinical benefits were modest.

In addition, there is need to study the new polymerase inhibitors in two unique settings: prophylaxis and treatment in low- and middle-income countries. Prophylaxis is a potential benefit of influenza antivirals and can provide immediate protection against illness. Polymerase inhibitors provide an alternative should resistance emerge with oseltamivir or other NAIs. An initial placebo-controlled study of single-dose baloxavir found that it was

highly effective for post-exposure prophylaxis in household contacts, although PA variants with reduced susceptibility were detected in a minority of recipients (Ikematsu et al., 2020). While the long plasma elimination half-life of baloxavir makes it especially attractive in high-risk settings, studies are needed to assess the frequency of dosing to provide optimal protection in those requiring longer periods of protection. First responders in a pandemic or high pathogenicity virus setting or in influenza outbreaks among nursing home residents are two examples of such high-risk populations. Active controlled studies, as done for NAIs in high-risk nursing home outbreaks (Gravenstein et al., 2005), would determine efficacy, frequency of breakthrough infection and resistant variant emergence. Likewise, studies in low- and middle-income countries are needed to inform the optimal use of these agents. Although costs may be higher than generic oseltamivir therapy, the ease of delivery, particularly for single dose baloxavir treatment of outpatients, may facilitate access and compliance. Modeling studies (Du et al., 2020) could help assess the impact of antiviral therapy in patients in resource-limited settings.

In addition to susceptibility testing of circulating strains, there is an important need for real-world longitudinal studies of the emergence of resistant variants and associated effectiveness of baloxavir in treated outpatients, similar to the methods employed by the Influenza Resistance Information Study (IRIS) (Lina et al., 2018; Whitley et al., 2013). Such studies can examine the risk factors and responses to therapy in treated patients with and without resistant variant emergence, as well as the potential for transmission to close household contacts. Further, enrichment for groups at high risk for resistance emergence, including young children and the immunocompromised, would increase the efficiency of such efforts. Optimally, such studies should be representative of countries where there is antiviral use and should have a mechanism for providing interim data in near real time to key stakeholders.

7.3. Potential impact of the response to the COVID-19 pandemic

We are currently in the midst of a global pandemic caused by SARS-CoV-2, another respiratory virus. While lessons learned from influenza resistance monitoring may be helpful for establishing future drug susceptibility surveillance and testing for this new virus, there will undoubtedly be beneficial outcomes from the urgent response to the pandemic in the development of therapeutics and vaccines, effective collaboration, more open communication and sharing of data, and accelerated publication of important developments, which may mitigate some of the deficiencies mentioned above. Changes in laboratory capabilities brought about by the SARS-CoV-2 pandemic are likely to have a lasting effect on influenza surveillance. One landmark improvement has been the explosion in the use of NGS technologies by laboratories worldwide, including low-middle income countries. Not only has the generation of WGS for SARS-CoV-2 viruses been conducted at an astonishing speed and at high throughput, but these data have also been promptly shared with the global community. Notably, the development and implementation of third-generation sequencing-based diagnostic technologies such as MinION from Oxford Nanopore are underway and may further expand the number of laboratories able to rapidly generate sequence data (Van Poelvoorde et al., 2020). MinION and similar cost-effective platforms may facilitate sequencing by GISRS laboratories in low-middle income countries and resource-poor regional or local hospitals (Rambo-Martin et al., 2020). Moreover, sequence analysis can

be expedited by establishing collaborations with academic institutions. The wide availability and use of sequencing technologies by many laboratories worldwide will undoubtedly improve the preparedness and response to the emergence of new pathogens and will have significant impact on influenza antiviral surveillance.

8. Conclusions

Understanding the epidemiology and clinical implications of the emergence of treatment-related polymerase inhibitor resistance requires systematic surveillance of circulating influenza viruses over multiple seasons and in diverse locations. In addition to pre-clinical studies, trials of the clinical consequences and transmissibility of treatment-emergent variants can be accomplished with dedicated studies focusing on higher risk populations, such as young children, immunocompromised individuals, and critically ill patients. These are also populations in whom further studies of antiviral combinations are needed. Regular meetings and effective communication between the four key entities, pharma, public health, academia and regulators, are essential to ensure thoughtful study designs, as well as real-time sharing of the data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge helpful discussions with the following: Wendy S. Barclay, Alicia M. Fry, Aeron C. Hurt, John W. McCauley, Nahako Shindo, Norio Sugaya, Reiko Saito, Maria Zambon, Mira C. Patel. **EG** is supported with Federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contracts HHSN272201400006C and 75N93021C00016.

References

- Abed Y, Fage C, Checkmahomed L, Venable MC, Boivin G, 2020. Characterization of contemporary influenza B recombinant viruses harboring mutations of reduced susceptibility to baloxavir marboxil, in vitro and in mice. *Antivir. Res.* 179, 104807. 10.1016/j.antiviral.2020.104807. [PubMed: 32343991]
- Ando Y, Noshi T, Sato K, Ishibashi T, Yoshida Y, Hasegawa T, Onishi M, Kitano M, Oka R, Kawai M, Yoshida R, Sato A, Shishido T, Naito A, 2021. Pharmacokinetic and pharmacodynamic analysis of baloxavir marboxil, a novel cap-dependent endonuclease inhibitor, in a murine model of influenza virus infection. *J. Antimicrob. Chemother.* 76, 189–198. 10.1093/jac/dkaa393. [PubMed: 33035324]
- Baker J, Block SL, Matharu B, Burleigh Macutkiewicz L, Wildum S, Dimonaco S, Collinson N, Clinch B, Piedra PA, 2020. Baloxavir marboxil single-dose treatment in influenza-infected children: a randomized, double-blind, active controlled phase 3 safety and efficacy trial (miniSTONE-2). *Pediatr. Infect. Dis. J.* 39, 700–705. 10.1097/inf.0000000000002747. [PubMed: 32516282]
- Baranovich T, Wong SS, Armstrong J, Marjuki H, Webby RJ, Webster RG, Govorkova EA, 2013. T-705 (favipiravir) induces lethal mutagenesis in influenza A H1N1 viruses in vitro. *J. Virol.* 87, 3741–3751. 10.1128/JVI.02346-12. [PubMed: 23325689]
- Barrett S, Mohr PG, Schmidt PM, McKimm-Breschkin JL, 2011. Real time enzyme inhibition assays provide insights into differences in binding of neuraminidase inhibitors to wild type and mutant influenza viruses. *PLoS One* 6, e23627. 10.1371/journal.pone.0023627. [PubMed: 21858186]
- Behillil S, May F, Fourati S, Luyt CE, Chicheportiche T, Sonnevile R, Tandjaoui-Lambiotte Y, Roux D, Guérin L, Mayaux J, Maury E, Ferré A, Georger JF, Voiriot G, Enouf V, van der Werf S, Dessap AM, de Prost N, 2020. Oseltamivir resistance in severe influenza A(H1N1)pdm09 pneumonia and

- acute respiratory distress syndrome: a French multicenter observational cohort study. *Clin. Infect. Dis.* 71, 1089–1091. 10.1093/cid/ciz904. [PubMed: 31538643]
- Beigel JH, Nam HH, Adams PL, Krafft A, Ince WL, El-Kamary SS, Sims AC, 2019. In: *Advances in Respiratory Virus Therapeutics - A Meeting Report from the 6th Isirv Antiviral Group Conference Antiviral Res.*, vol. 167, pp. 45–67. 10.1016/j.antiviral.2019.04.006.
- Bloom JD, Gong LI, Baltimore D, 2010. Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science* 328, 1272–1275. 10.1126/science.1187816. [PubMed: 20522774]
- Bright RA, Medina MJ, Xu X, Perez-Oroz G, Wallis TR, Davis XM, Povinelli L, Cox NJ, Klimov AI, 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. *Lancet* 366, 1175–1181. 10.1016/S0140-6736(05)67338-2. [PubMed: 16198766]
- Byrn RA, Jones SM, Bennett HB, Bral C, Clark MP, Jacobs MD, Kwong AD, Ledebauer MW, Leeman JR, McNeil CF, Murcko MA, Nezami A, Perola E, Rijnbrand R, Saxena K, Tsai AW, Zhou Y, Charifson PS, 2015. Preclinical activity of VX-787, a first-in-class, orally bioavailable inhibitor of the influenza virus polymerase PB2 subunit. *Antimicrob. Agents Chemother.* 59, 1569–1582. 10.1128/AAC.04623-14. [PubMed: 25547360]
- Chan PK, Lee N, Zaman M, Adisasmito W, Coker R, Hanshaoworakul W, Gasimov V, Oner AF, Dogan N, Tsang O, Phommasack B, Touch S, Bamgboye E, Swenson A, Toovey S, Dreyer NA, 2012. Determinants of antiviral effectiveness in influenza virus A subtype H5N1. *J. Infect. Dis.* 206, 1359–1366. 10.1093/infdis/jis509. [PubMed: 22927451]
- Chao DL, Bloom JD, Kochin BF, Antia R, Longini IM Jr., 2012. The global spread of drug-resistant influenza. *J. R. Soc. Interface* 9, 648–656. 10.1098/rsif.2011.0427. [PubMed: 21865253]
- Checkmahomed L, M'Hamdi Z, Carbonneau J, Venable MC, Baz M, Abed Y, Boivin G, 2020. Impact of the baloxavir-resistant polymerase acid I38T substitution on the fitness of contemporary influenza A(H1N1)pdm09 and A(H3N2) strains. *J. Infect. Dis.* 221, 63–70. 10.1093/infdis/jiz418. [PubMed: 31419295]
- Chesnokov A, Patel MC, Mishin VP, De La Cruz JA, Lollis L, Nguyen HT, Dugan V, Wentworth DE, Gubareva LV, 2020. Replicative fitness of seasonal influenza A viruses with decreased susceptibility to baloxavir. *J. Infect. Dis.* 221, 367–371. 10.1093/infdis/jiz472. [PubMed: 31541547]
- Clark MP, Ledebauer MW, Davies I, Byrn RA, Jones SM, Perola E, Tsai A, Jacobs M, Nti-Addae K, Bandarage UK, Boyd MJ, Bethiel RS, Court JJ, Deng H, Duffy JP, Dorsch WA, Farmer LJ, Gao H, Gu W, Jackson K, Jacobs DH, Kennedy JM, Ledford B, Liang J, Maltais F, Murcko M, Wang T, Wannamaker MW, Bennett HB, Leeman JR, McNeil C, Taylor WP, Memmott C, Jiang M, Rijnbrand R, Bral C, Germann U, Nezami A, Zhang Y, Salituro FG, Bennani YL, Charifson PS, 2014. Discovery of a novel, first-in-class, orally bioavailable azaindole inhibitor (VX-787) of influenza PB2. *J. Med. Chem.* 57, 6668–6678. 10.1021/jm5007275. [PubMed: 25019388]
- Deyde VM, Sheu TG, Trujillo AA, Okomo-Adhiambo M, Garten R, Klimov AI, Gubareva LV, 2010. Detection of molecular markers of drug resistance in 2009 pandemic influenza A (H1N1) viruses by pyrosequencing. *Antimicrob. Agents Chemother.* 54, 1102–1110. 10.1128/AAC.01417-09. [PubMed: 20028826]
- Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, Shu Y, Gubareva LV, Cox NJ, Klimov AI, 2007. In: *Surveillance of Resistance to Adamantanes Among Influenza A(H3N2) and A(H1N1) Viruses Isolated Worldwide*, vol. 196, pp. 249–257.
- Dias A, Bouvier D, Crepin T, McCarthy AA, Hart DJ, Baudin F, Cusack S, Ruigrok RW, 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature* 458, 914–918. 10.1038/nature07745. [PubMed: 19194459]
- Dobson J, Whitley RJ, Pocock S, Monto AS, 2015. Oseltamivir treatment for influenza in adults: a meta-analysis of randomised controlled trials. *Lancet* 385, 1729–1737. 10.1016/S0140-6736(14)62449-1. [PubMed: 25640810]
- Doshi P, Dickersin K, Healy D, Vedula SS, Jefferson T, 2013. Restoring invisible and abandoned trials: a call for people to publish the findings. *Bmj* 346, f2865. 10.1136/bmj.f2865. [PubMed: 23766480]

- Du Z, Nugent C, Galvani AP, Krug RM, Meyers LA, 2020. Modeling mitigation of influenza epidemics by baloxavir. *Nat. Commun.* 11, 2750. 10.1038/s41467-020-16585-y. [PubMed: 32487990]
- Finberg RW, Lanno R, Anderson D, Fleischhackl R, van Duijnhoven W, Kauffman RS, Kosoglou T, Vingerhoets J, Leopold L, 2019. Phase 2b study of pimodivir (JNJ-63623872) as monotherapy or in combination with oseltamivir for treatment of acute uncomplicated seasonal influenza A: TOPAZ trial. *J. Infect. Dis.* 219, 1026–1034. 10.1093/infdis/jiy547. [PubMed: 30428049]
- Fodor E, Te Velthuis AJW, 2020. Structure and function of the influenza virus transcription and replication machinery. *Cold Spring Harbor perspectives in medicine* 10. 10.1101/cshperspect.a038398.
- Frise R, Zhou J, Zwanziger E, Kuhlbusch K, Clinch B, Wildum S, Barclay W, 2019. Baloxavir Reduces Respiratory Droplet Transmission of Influenza A Virus in Ferrets, Options X for the Control of Influenza, p. 10857. Singapore.
- Fry AM, Goswami D, Nahar K, Sharmin AT, Rahman M, Gubareva L, Azim T, Bresee J, Luby SP, Brooks WA, 2014. Efficacy of oseltamivir treatment started within 5 days of symptom onset to reduce influenza illness duration and virus shedding in an urban setting in Bangladesh: a randomised placebo-controlled trial. *Lancet Infect. Dis.* 14, 109–118. 10.1016/s1473-3099(13)70267-6. [PubMed: 24268590]
- Fukao K, Ando Y, Noshi T, Kitano M, Noda T, Kawai M, Yoshida R, Sato A, Shishido T, Naito A, 2019a. Baloxavir marboxil, a novel cap-dependent endonuclease inhibitor potently suppresses influenza virus replication and represents therapeutic effects in both immunocompetent and immunocompromised mouse models. *PloS One* 14, e0217307. 10.1371/journal.pone.0217307. [PubMed: 31107922]
- Fukao K, Noshi T, Yamamoto A, Kitano M, Ando Y, Noda T, Baba K, Matsumoto K, Higuchi N, Ikeda M, Shishido T, Naito A, 2019b. Combination treatment with the cap-dependent endonuclease inhibitor baloxavir marboxil and a neuraminidase inhibitor in a mouse model of influenza A virus infection. *J. Antimicrob. Chemother.* 74, 654–662. 10.1093/jac/dky462. [PubMed: 30476172]
- Furuta Y, Takahashi K, Fukuda Y, Kuno M, Kamiyama T, Kozaki K, Nomura N, Egawa H, Minami S, Watanabe Y, Narita H, Shiraki K, 2002. In vitro and in vivo activities of anti-influenza virus compound T-705. *Antimicrob. Agents Chemother.* 46, 977–981. 10.1128/aac.46.4.977-981.2002. [PubMed: 11897578]
- Ghedini E, Holmes EC, DePasse JV, Pinilla LT, Fitch A, Hamelin ME, Papenburg J, Boivin G, 2012. Presence of oseltamivir-resistant pandemic A/H1N1 minor variants before drug therapy with subsequent selection and transmission. *J. Infect. Dis.* 206, 1504–1511. 10.1093/infdis/jis571. [PubMed: 22966122]
- Goldhill DH, Te Velthuis AJW, Fletcher RA, Langat P, Zambon M, Lackenby A, Barclay WS, 2018. The mechanism of resistance to favipiravir in influenza. *Proc. Natl. Acad. Sci. U. S. A.* 115, 11613–11618. 10.1073/pnas.1811345115. [PubMed: 30352857]
- Goldhill DH, Yan A, Frise R, Zhou J, Shelley J, Gallego Cortés A, Miah S, Akinbami O, Galiano M, Zambon M, Lackenby A, Barclay WS, 2021. Favipiravir-resistant influenza A virus shows potential for transmission. *PLoS Pathog.* 17, e1008937 10.1371/journal.ppat.1008937. [PubMed: 34061908]
- Govorkova EA, Ilyushina NA, Marathe BM, McClaren JL, Webster RG, 2010. Competitive fitness of oseltamivir-sensitive and -resistant highly pathogenic H5N1 influenza viruses in a ferret model. *J. Virol.* 84, 8042–8050. 10.1128/jvi.00689-10. [PubMed: 20519385]
- Gravenstein S, Drinka P, Osterweil D, Schilling M, Krause P, Elliott M, Shult P, Ambrozaitis A, Kandel R, Binder E, Hammond J, McElhaney J, Flack N, Daly J, Keene O, 2005. Inhaled zanamivir versus rimantadine for the control of influenza in a highly vaccinated long-term care population. *J. Am. Med. Dir. Assoc.* 6, 359–366. 10.1016/j.jamda.2005.08.006. [PubMed: 16286056]
- Gubareva LV, Fry AM, 2020. Baloxavir and treatment-emergent resistance: public health insights and next steps. *J. Infect. Dis.* 221, 337–339. 10.1093/infdis/jiz245. [PubMed: 31309982]
- Gubareva LV, Mishin VP, Patel MC, Chesnokov A, Nguyen HT, De La Cruz J, Spencer S, Campbell AP, Sinner M, Reid H, Garten R, Katz JM, Fry AM, Barnes J, Wentworth DE, 2019. Assessing

- baloxavir susceptibility of influenza viruses circulating in the United States during the 2016/17 and 2017/18 seasons. *Euro Surveill.* 24 10.2807/1560-7917.ES.2019.24.3.1800666.
- Hall CB, Dolin R, Gala CL, Markovitz DM, Zhang YQ, Madore PH, Disney FA, Talpey WB, Green JL, Francis AB, et al. , 1987. Children with influenza A infection: treatment with rimantadine. *Pediatrics* 80, 275–282. [PubMed: 3302925]
- Harada N, Shibata W, Koh H, Takashita E, Fujisaki S, Okamura H, Nanno S, Yamada K, Nakamae H, Hino M, Kakeya H, 2020. Successful treatment with baloxavir marboxil of a patient with peramivir-resistant influenza A/H3N2 with a dual E119D/R292K substitution after allogeneic hematopoietic cell transplantation: a case report. *BMC Infect. Dis.* 20, 478. 10.1186/s12879-020-05205-1. [PubMed: 32631240]
- Hashimoto T, Baba K, Inoue K, Okane M, Hata S, Shishido T, Naito A, Wildum S, Omoto S, 2021. Comprehensive assessment of amino acid substitutions in the trimeric RNA polymerase complex of influenza A virus detected in clinical trials of baloxavir marboxil. *Influenza Other Respir Viruses* 15, 389–395. 10.1111/irv.12821. [PubMed: 33099886]
- Hay AJ, McCauley JW, 2018. The WHO global influenza surveillance and response system (GISRS)- A future perspective. *Influenza Other Respir Viruses.* 10.1111/irv.12565.
- Hayden FG, Belshe RB, Clover RD, Hay AJ, Oakes MG, Soo W, 1989. Emergence and apparent transmission of rimantadine-resistant influenza. *A virus in families* 321, 1696–1702.
- Hayden FG, Osterhaus AD, Treanor JJ, Fleming DM, Aoki FY, Nicholson KG, Bohnen AM, Hirst HM, Keene O, Wightman K, 1997. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenzavirus infections. *GG167 Influenza Study Group. N. Engl. J. Med.* 337, 874–880. 10.1056/NEJM199709253371302. [PubMed: 9302301]
- Hayden FG, Shindo N, 2019. Influenza virus polymerase inhibitors in clinical development. *Curr. Opin. Infect. Dis.* 32, 176–186. 10.1097/QCO.0000000000000532. [PubMed: 30724789]
- Hayden FG, Sperber SJ, Belshe RB, Clover RD, Hay AJ, Pyke S, 1991. In: *Recovery of Drug-Resistant Influenza A Virus during Therapeutic Use of Rimantadine*, vol. 35, pp. 1741–1747.
- Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, Ishida T, Sekino H, Yamada K, Portsmouth S, Kawaguchi K, Shishido T, Arai M, Tsuchiya K, Uehara T, Watanabe A, Baloxavir Marboxil Investigators G, 2018. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. *N. Engl. J. Med.* 379, 913–923. 10.1056/NEJMoa1716197. [PubMed: 30184455]
- Higashiguchi M, Matsumoto T, Fujii T, 2018. A meta-analysis of laninamivir octanoate for treatment and prophylaxis of influenza. *Antivir. Ther.* 23, 157–165. 10.3851/IMP3189. [PubMed: 28869418]
- Hirotsu N, Sakaguchi H, Sato C, Ishibashi T, Baba K, Omoto S, Shishido T, Tsuchiya K, Hayden FG, Uehara T, Watanabe A, 2020. Baloxavir marboxil in Japanese pediatric patients with influenza: safety and clinical and virologic outcomes. *Clin. Infect. Dis.* 71, 971–981. 10.1093/cid/ciz908. [PubMed: 31538644]
- Hodges EN, Mishin VP, De la Cruz J, Guo Z, Nguyen HT, Fallows E, Stevens J, Wentworth DE, Davis CT, Gubareva LV, 2019. Detection of oseltamivir-resistant zoonotic and animal influenza A viruses using the rapid influenza antiviral resistance test. *Influenza Other Respir Viruses* 13, 522–527. 10.1111/irv.12661.
- Hurt AC, Ernest J, Deng YM, Iannello P, Besselaar TG, Birch C, Buchy P, Chittaganpitch M, Chiu SC, Dwyer D, Guigon A, Harrower B, Kei IP, Kok T, Lin C, McPhie K, Mohd A, Olveda R, Panayotou T, Rawlinson W, Scott L, Smith D, D'Souza H, Komadina N, Shaw R, Kelso A, Barr IG, 2009. Emergence and spread of oseltamivir-resistant A(H1N1) influenza viruses in Oceania, south East Asia and South Africa. *Antivir. Res.* 83, 90–93. 10.1016/j.antiviral.2009.03.003. [PubMed: 19501261]
- Hurt AC, Hardie K, Wilson NJ, Deng YM, Osbourn M, Leang SK, Lee RT, Iannello P, Gehrig N, Shaw R, Wark P, Caldwell N, Givney RC, Xue L, Maurer-Stroh S, Dwyer DE, Wang B, Smith DW, Levy A, Booy R, Dixit R, Merritt T, Kelso A, Dalton C, Durrheim D, Barr IG, 2012. Characteristics of a widespread community cluster of H275Y oseltamivir-resistant A(H1N1)pdm09 influenza in Australia. *J. Infect. Dis.* 206, 148–157. 10.1093/infdis/jis337. [PubMed: 22561367]
- Hurt AC, Nor'e SS, McCaw JM, Fryer HR, Mosse J, McLean AR, Barr IG, 2010. Assessing the viral fitness of oseltamivir-resistant influenza viruses in ferrets, using a competitive-mixtures model. *J. Virol.* 84, 9427–9438. 10.1128/jvi.00373-10. [PubMed: 20631138]

- Ikematsu H, Hayden FG, Kawaguchi K, Kinoshita M, de Jong MD, Lee N, Takashima S, Noshi T, Tsuchiya K, Uehara T, 2020. Baloxavir marboxil for prophylaxis against influenza in household contacts. *N. Engl. J. Med.* 383, 309–320. 10.1056/NEJMoa1915341. [PubMed: 32640124]
- Imai M, Yamashita M, Sakai-Tagawa Y, Iwatsuki-Horimoto K, Kiso M, Murakami J, Yasuhara A, Takada K, Ito M, Nakajima N, Takahashi K, Lopes TJS, Dutta J, Khan Z, Kriti D, van Bakel H, Tokita A, Hagiwara H, Izumida N, Kuroki H, Nishino T, Wada N, Koga M, Adachi E, Jubishi D, Hasegawa H, Kawaoka Y, 2020. Influenza A variants with reduced susceptibility to baloxavir isolated from Japanese patients are fit and transmit through respiratory droplets. *Nature microbiology* 5, 27–33. 10.1038/s41564-019-0609-0.
- Ince WL, Smith FB, O'Rear JJ, Thomson M, 2020. Treatment-emergent influenza virus polymerase acidic substitutions independent of those at I38 associated with reduced baloxavir susceptibility and virus rebound in trials of baloxavir marboxil. *J. Infect. Dis.* 222, 957–961. 10.1093/infdis/jiaa164. [PubMed: 32253432]
- Ison MG, Portsmouth S, Yoshida Y, Shishido T, Mitchener M, Tsuchiya K, Uehara T, Hayden FG, 2020. Early treatment with baloxavir marboxil in high-risk adolescent and adult outpatients with uncomplicated influenza (CAPSTONE-2): a randomised, placebo-controlled, phase 3 trial. *Lancet Infect. Dis.* 20, 1204–1214. 10.1016/s1473-3099(20)30004-9. [PubMed: 32526195]
- Janssen, 2020. Janssen to Discontinue Pimodivir Influenza Development Program. <https://www.janssen.com/janssen-discontinue-pimodivir-influenza-development-program>. (Accessed 10 September 2020). Accessed.
- Jefferson T, Doshi P, Thompson M, Heneghan C, 2011. Ensuring safe and effective drugs: who can do what it takes? *Bmj* 342, c7258. 10.1136/bmj.c7258. [PubMed: 21224325]
- Jefferson T, Jones MA, Doshi P, Del Mar CB, Hama R, Thompson MJ, Onakpoya I, Heneghan CJ, 2014. Risk of bias in industry-funded oseltamivir trials: comparison of core reports versus full clinical study reports. *BMJ open* 4, e005253. 10.1136/bmjopen-2014-005253.
- Jones JC, Pascua PNQ, Fabrizio TP, Marathe BM, Seiler P, Barman S, Webby RJ, Webster RG, Govorkova EA, 2020. Influenza A and B viruses with reduced baloxavir susceptibility display attenuated in vitro fitness but retain ferret transmissibility. *Proc. Natl. Acad. Sci. U. S. A.* 117, 8593–8601. 10.1073/pnas.1916825117. [PubMed: 32217734]
- Jones JC, Pascua PNQ, Harrington WN, Webby RJ, Govorkova EA, 2021. Multiple polymerase acidic (PA) I38X substitutions in influenza A(H1N1)pdm09 virus permit polymerase activity and cause reduced baloxavir inhibition. *J. Antimicrob. Chemother.* 76, 957–960. 10.1093/jac/dkaa527. [PubMed: 33351916]
- Jorquera PA, Mishin VP, Chesnokov A, Nguyen HT, Mann B, Garten R, Barnes J, Hodges E, De La Cruz J, Xu X, Katz J, Wentworth DE, Gubareva LV, 2019. Insights into the antigenic advancement of influenza A(H3N2) viruses, 2011–2018. *Sci. Rep.* 9, 2676. 10.1038/s41598-019-39276-1. [PubMed: 30804469]
- Kaiser L, Keene ON, Hammond JM, Elliott M, Hayden FG, 2000. Impact of zanamivir on antibiotic use for respiratory events following acute influenza in adolescents and adults. *Arch. Intern. Med.* 160, 3234–3240. 10.1001/archinte.160.21.3234. [PubMed: 11088083]
- Kawai N, Ikematsu H, Hirotsu N, Maeda T, Kawashima T, Tanaka O, Yamauchi S, Kawamura K, Matsuura S, Nishimura M, Iwaki N, Kashiwagi S, 2009. Clinical effectiveness of oseltamivir and zanamivir for treatment of influenza A virus subtype H1N1 with the H274Y mutation: a Japanese, multicenter study of the 2007–2008 and 2008–2009 influenza seasons. *Clin. Infect. Dis.* 49, 1828–1835. 10.1086/648424. [PubMed: 19911968]
- Kim CU, Lew W, Williams MA, Liu H, Zhang L, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver WG, Stevens RC, 1997. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. *J. Am. Chem. Soc.* 119, 681–690. 10.1021/ja963036t. [PubMed: 16526129]
- Kiso M, Lopes TJS, Yamayoshi S, Ito M, Yamashita M, Nakajima N, Hasegawa H, Neumann G, Kawaoka Y, 2018. Combination therapy with neuraminidase and polymerase inhibitors in nude mice infected with influenza virus. *J. Infect. Dis.* 217, 887–896. 10.1093/infdis/jix606. [PubMed: 29186472]

- Kiso M, Yamayoshi S, Furusawa Y, Imai M, Kawaoka Y, 2019. Treatment of highly pathogenic H7N9 virus-infected mice with baloxavir marboxil. *Viruses* 11. 10.3390/v11111066.
- Kiso M, Yamayoshi S, Murakami J, Kawaoka Y, 2020. Baloxavir marboxil treatment of nude mice infected with influenza A virus. *J. Infect. Dis.* 221, 1699–1702. 10.1093/infdis/jiz665. [PubMed: 31837268]
- Kitano M, Matsuzaki T, Oka R, Baba K, Noda T, Yoshida Y, Sato K, Kiyota K, Mizutare T, Yoshida R, Sato A, Kamimori H, Shishido T, Naito A, 2020. The antiviral effects of baloxavir marboxil against influenza A virus infection in ferrets. *Influenza Other Respir Viruses*. 10.1111/irv.12760.
- Koszalka P, Farrukee R, Mifsud E, Vijaykrishna D, Hurt AC, 2020. A rapid pyrosequencing assay for the molecular detection of influenza viruses with reduced baloxavir susceptibility due to PA/I38X amino acid substitutions. *Influenza Other Respir Viruses* 14, 460–464. 10.1111/irv.12725. [PubMed: 32045100]
- Koszalka P, Tilmanis D, Roe M, Vijaykrishna D, Hurt AC, 2019. Baloxavir marboxil susceptibility of influenza viruses from the Asia-Pacific, 2012–2018. *Antivir. Res.* 164, 91–96. 10.1016/j.antiviral.2019.02.007. [PubMed: 30771405]
- Krug RM, Broni BA, Bouloy M, 1979. Are the 5' ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs? *Cell* 18, 329–334. 10.1016/0092-8674(79)90052-7. [PubMed: 498272]
- Kumar D, Ison MG, Mira J-P, Welte T, Ha JH, Hui DS, Zhong N, Saito T, Katugampola L, Collinson N, Williams S, Wildum S, Ackrill A, Clinch B, Lee N, 2020. Combining Baloxavir with Standard-Of-Care (SoC) Neuraminidase Inhibitor (NAI) in Patients Hospitalised with Severe Influenza: Results from the Global Randomised Phase 3 FLAGSTONE Study ESWI.
- Kwon JJ, Choi WS, Jeong JH, Kim EH, Lee OJ, Yoon SW, Hwang J, Webby RJ, Govorkova EA, Choi YK, Baek YH, Song MS, 2018. An I436N substitution confers resistance of influenza A(H1N1)pdm09 viruses to multiple neuraminidase inhibitors without affecting viral fitness. *J. Gen. Virol.* 99, 292–302. 10.1099/jgv.0.001029. [PubMed: 29493493]
- Lackenby A, Besselaar TG, Daniels RS, Fry A, Gregory V, Gubareva LV, Huang W, Hurt AC, Leang SK, Lee RTC, Lo J, Lollis L, Maurer-Stroh S, Odagiri T, Pereyaslov D, Takashita E, Wang D, Zhang W, Meijer A, 2018. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors and status of novel antivirals, 2016–2017. *Antivir. Res.* 157, 38–46. 10.1016/j.antiviral.2018.07.001. [PubMed: 29981793]
- Lackenby A, Hungnes O, Dudman SG, Meijer A, Paget WJ, Hay AJ, Zambon MC, 2008. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. *Euro Surveill.* 13 10.2807/ese.13.05.08026-en.
- Lee LY, Zhou J, Koszalka P, Frise R, Farrukee R, Baba K, Miah S, Shishido T, Galiano M, Hashimoto T, Omoto S, Uehara T, Mifsud EJ, Collinson N, Kuhlbusch K, Clinch B, Wildum S, Barclay WS, Hurt AC, 2021. Evaluating the fitness of PA/I38T-substituted influenza A viruses with reduced baloxavir susceptibility in a competitive mixtures ferret model. *PLoS Pathog.* 17, e1009527 10.1371/journal.ppat.1009527. [PubMed: 33956888]
- Lee LYY, Zhou J, Frise R, Goldhill DH, Koszalka P, Mifsud EJ, Baba K, Noda T, Ando Y, Sato K, Yuki AI, Shishido T, Uehara T, Wildum S, Zwanziger E, Collinson N, Kuhlbusch K, Clinch B, Hurt AC, Barclay WS, 2020. Baloxavir treatment of ferrets infected with influenza A(H1N1)pdm09 virus reduces onward transmission. *PLoS Pathog.* 16, e1008395 10.1371/journal.ppat.1008395. [PubMed: 32294137]
- Lina B, Boucher C, Osterhaus A, Monto AS, Schutten M, Whitley RJ, Nguyen-Van-Tam JS, 2018. Five years of monitoring for the emergence of oseltamivir resistance in patients with influenza A infections in the Influenza Resistance Information Study. *Influenza Other Respir Viruses* 12, 267–278. 10.1111/irv.12534. [PubMed: 29265727]
- Marathe BM, Wong SS, Vogel P, Garcia-Alcalde F, Webster RG, Webby RJ, Najera I, Govorkova EA, 2016. Combinations of oseltamivir and T-705 Extend the treatment window for highly pathogenic influenza A(H5N1) virus infection in mice. *Sci. Rep.* 6, 26742. 10.1038/srep26742. [PubMed: 27221530]
- Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD, 2003. Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J. Virol.* 77, 8418–8425. 10.1128/jvi.77.15.8418-8425.2003. [PubMed: 12857911]

- McKimm-Breschkin J, Trivedi T, Hampson A, Hay A, Klimov A, Tashiro M, Hayden F, Zambon M, 2003. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. *Antimicrob. Agents Chemother.* 47, 2264–2272. 10.1128/aac.47.7.2264-2272.2003. [PubMed: 12821478]
- McKimm-Breschkin JL, 2013. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza Other Respir Viruses* 7 (Suppl. 1), 25–36. 10.1111/irv.12047. [PubMed: 23279894]
- McKimm-Breschkin JL, Jiang S, Hui DS, Beigel JH, Govorkova EA, Lee N, 2018. Prevention and treatment of respiratory viral infections: presentations on antivirals, traditional therapies and host-directed interventions at the 5th ISIRV Antiviral Group conference. *Antivir. Res.* 149, 118–142. 10.1016/j.antiviral.2017.11.013. [PubMed: 29162476]
- Mifsud EJ, Hayden FG, Hurt AC, 2019. Antivirals targeting the polymerase complex of influenza viruses. *Antivir. Res.* 169, 104545. 10.1016/j.antiviral.2019.104545. [PubMed: 31247246]
- Mira J-P, Kumar D, Ison MG, Katugampola L, Collinson N, Williams S, Wildum S, Ackrill A, Clinch B, Lee N, 2020. Low Rates of Antiviral Resistance in Hospitalised Patients Treated with Baloxavir Plus Standard-Of-Care Neuraminidase Inhibitors (NAIs) or Placebo Plus NAIs: Genotypic Analysis of the Phase 3 FLAGSTONE Study. *ESWI*.
- Mishin VP, Patel MC, Chesnokov A, De La Cruz J, Nguyen HT, Lollis L, Hodges E, Jang Y, Barnes J, Uyeki T, Davis CT, Wentworth DE, Gubareva LV, 2019. Susceptibility of influenza A, B, C, and D viruses to baloxavir(1). *Emerg. Infect. Dis.* 25, 1969–1972. 10.3201/eid2510.190607. [PubMed: 31287050]
- Monto AS, McKimm-Breschkin JL, Macken C, Hampson AW, Hay A, Klimov A, Tashiro M, Webster RG, Aymard M, Hayden FG, Zambon M, 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. *Antimicrob. Agents Chemother.* 50, 2395–2402. 10.1128/AAC.01339-05. [PubMed: 16801417]
- Muthuri SG, Venkatesan S, Myles PR, Leonardi-Bee J, Al Khuwaitir TS, Al Mamun A, Anovadiya AP, Azziz-Baumgartner E, Baez C, Bassetti M, Beovic B, Bertisch B, Bonmarin I, Booy R, Borja-Aburto VH, Burgmann H, Cao B, Carratala J, Denholm JT, Dominguez SR, Duarte PA, Dubnov-Raz G, Echavarría M, Fanella S, Gao Z, Gerardin P, Giannella M, Gubbels S, Herberg J, Iglesias AL, Hoger PH, Hu X, Islam QT, Jimenez MF, Kandeel A, Keijzers G, Khalili H, Knight M, Kudo K, Kusznierz G, Kuzman I, Kwan AM, Amine IL, Langenegger E, Lankarani KB, Leo YS, Linko R, Liu P, Madanat F, Mayo-Montero E, McGeer A, Memish Z, Metan G, Mickiene A, Mikic D, Mohn KG, Moradi A, Nymadawa P, Oliva ME, Ozkan M, Parekh D, Paul M, Polack FP, Rath BA, Rodriguez AH, Sarrouf EB, Seale AC, Sertogullarindan B, Siqueira MM, Skret-Magierlo J, Stephan F, Talarek E, Tang JW, To KK, Torres A, Torun SH, Tran D, Uyeki TM, Van Zwol A, Vaudry W, Vidmar T, Yokota RT, Zarogoulidis P, Investigators PC, Nguyen-Van-Tam JS, 2014. Effectiveness of neuraminidase inhibitors in reducing mortality in patients admitted to hospital with influenza A H1N1pdm09 virus infection: a meta-analysis of individual participant data. *Lancet Respir Med* 2, 395–404. 10.1016/S2213-2600(14)70041-4. [PubMed: 24815805]
- Nakauchi M, Takashita E, Fujisaki S, Shirakura M, Ogawa R, Morita H, Miura H, Saito S, Watanabe S, Odagiri T, Kageyama T, 2020. Rapid detection of an I38T amino acid substitution in influenza polymerase acidic subunit associated with reduced susceptibility to baloxavir marboxil. *Influenza Other Respir Viruses* 14, 436–443. 10.1111/irv.12728. [PubMed: 32064779]
- Nakauchi M, Ujike M, Obuchi M, Takashita E, Takayama I, Ejima M, Oba K, Konomi N, Odagiri T, Tashiro M, Kageyama T, 2011. Rapid discrimination of oseltamivir-resistant 275Y and -susceptible 275H substitutions in the neuraminidase gene of pandemic influenza A/H1N1 2009 virus by duplex one-step RT-PCR assay. *J. Med. Virol.* 83, 1121–1127. 10.1002/jmv.22101. [PubMed: 21567417]
- NIID, 2019. Antiviral Resistance Surveillance in Japan 2018/2019 Influenza Season. Sep. 2018-Aug. 2019). <https://www.niid.go.jp/niid/en/influ-resist-e/10258-flu-r-e20210325.html>. (Accessed 6 April 2021). Accessed.
- Noshi T, Kitano M, Taniguchi K, Yamamoto A, Omoto S, Baba K, Hashimoto T, Ishida K, Kushima Y, Hattori K, Kawai M, Yoshida R, Kobayashi M, Yoshinaga T, Sato A, Okamatsu M, Sakoda Y, Kida H, Shishido T, Naito A, 2018. In vitro characterization of baloxavir acid, a first-in-class

- cap-dependent endonuclease inhibitor of the influenza virus polymerase PA subunit. *Antivir. Res.* 160, 109–117. 10.1016/j.antiviral.2018.10.008. [PubMed: 30316915]
- Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, Kawai M, Kawaguchi K, Uehara T, Shishido T, Naito A, Cusack S, 2018. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. *Sci. Rep.* 8, 9633. 10.1038/s41598-018-27890-4. [PubMed: 29941893]
- Osada H, Chon I, Phyu WW, Wagatsuma K, Nagata N, Kawashima T, Sato I, Saito T, Kodo N, Masaki H, Asoh N, Tsuchihashi Y, Shirahige Y, Ono Y, Shimada Y, Hamabata H, Saito K, Saito R, 2021. Development of cycling probe based real-time PCR methodology for influenza A viruses possessing the PA/I38T amino acid substitution associated with reduced baloxavir susceptibility. *Antivir. Res.* 188, 105036. 10.1016/j.antiviral.2021.105036. [PubMed: 33577807]
- Parry J, 2005. Use of antiviral drug in poultry is blamed for drug resistant strains of avian flu. *Bmj* 331, 10. 10.1136/bmj.331.7507.10.
- Patel MC, Chesnokov A, Jones J, Mishin VP, De La Cruz JA, Nguyen HT, Zanders N, Wentworth DE, Davis TC, Gubareva LV, 2021. Susceptibility of widely diverse influenza A viruses to PB2 polymerase inhibitor pimodivir. *Antivir. Res.* 188, 105035. 10.1016/j.antiviral.2021.105035. [PubMed: 33581212]
- Patel MC, Mishin VP, De La Cruz JA, Chesnokov A, Nguyen HT, Wilson MM, Barnes J, Kondor RJG, Wentworth DE, Gubareva LV, 2020. Detection of baloxavir resistant influenza A viruses using next generation sequencing and pyrosequencing methods. *Antivir. Res.* 182, 104906. 10.1016/j.antiviral.2020.104906. [PubMed: 32798601]
- Pflug A, Guilligay D, Reich S, Cusack S, 2014. Structure of influenza A polymerase bound to the viral RNA promoter. *Nature* 516, 355–360. 10.1038/nature14008. [PubMed: 25409142]
- Plotch SJ, Bouloy M, Krug RM, 1979. Transfer of 5'-terminal cap of globin mRNA to influenza viral complementary RNA during transcription in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 76, 1618–1622. 10.1073/pnas.76.4.1618. [PubMed: 287003]
- Poon LL, Pritlove DC, Fodor E, Brownlee GG, 1999. Direct evidence that the poly (A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. *J. Virol.* 73, 3473–3476. [PubMed: 10074205]
- Rambo-Martin BL, Keller MW, Wilson MM, Nolting JM, Anderson TK, Vincent AL, Bagal UR, Jang Y, Neuhaus EB, Davis CT, Bowman AS, Wentworth DE, Barnes JR, 2020. Influenza A virus field surveillance at a swine-human interface. *mSphere* 5. 10.1128/mSphere.00822-19.
- Reich S, Guilligay D, Pflug A, Malet H, Berger I, Crepin T, Hart D, Lunardi T, Nanao M, Ruigrok RW, Cusack S, 2014. Structural insight into cap-snatching and RNA synthesis by influenza polymerase. *Nature* 516, 361–366. 10.1038/nature14009. [PubMed: 25409151]
- Roosenhoff R, van der Vries E, van der Linden A, van Amerongen G, Stittelaar KJ, Smits SL, Schutten M, Fouchier RAM, 2018. Influenza A/H3N2 virus infection in immunocompromised ferrets and emergence of antiviral resistance. *PloS One* 13, e0200849. 10.1371/journal.pone.0200849. [PubMed: 30024940]
- Saito R, Sato I, Suzuki Y, Baranovich T, Matsuda R, Ishitani N, Dapat C, Dapat IC, Zaraket H, Oguma T, Suzuki H, 2010. Reduced effectiveness of oseltamivir in children infected with oseltamivir-resistant influenza A (H1N1) viruses with His275Tyr mutation. *Pediatr. Infect. Dis. J.* 29, 898–904. 10.1097/INF.0b013e3181de9d24. [PubMed: 20442686]
- Sato M, Takashita E, Katayose M, Nemoto K, Sakai N, Fujisaki S, Hashimoto K, Hosoya M, 2021. Detection of variants with reduced baloxavir marboxil and oseltamivir susceptibility in children with influenza A during the 2019–2020 influenza season. *J. Infect. Dis.* 10.1093/infdis/jiab196.
- Sato M, Takashita E, Katayose M, Nemoto K, Sakai N, Hashimoto K, Hosoya M, 2020. Detection of variants with reduced baloxavir marboxil susceptibility after treatment of children with influenza A during the 2018–2019 influenza season. *J. Infect. Dis.* 222, 121–125. 10.1093/infdis/jiaa061. [PubMed: 32034420]
- Sheu TG, Fry AM, Garten RJ, Deyde VM, Shwe T, Bullion L, Peebles PJ, Li Y, Klimov AI, Gubareva LV, 2011. Dual resistance to adamantanes and oseltamivir among seasonal influenza A(H1N1) viruses: 2008–2010. *J. Infect. Dis.* 203, 13–17. 10.1093/infdis/jiq005. [PubMed: 21148491]

- Shionogi, 2019. Shionogi Provides Update on Japanese Association for Infectious Diseases Statement and the Revised Guidelines of the Japanese Pediatric Society for the Treatment of Influenza. <https://m.marketscreener.com/quote/stock/SHIONOGI-CO-LTD-6493659/news/Shionogi-nbsp-Provides-Update-on-the-Japanese-Association-for-Infectious-Diseases-Statement-and-th-29465708/>. (Accessed 18 June 2021). Accessed.
- Sleeman K, Mishin VP, Deyde VM, Furuta Y, Klimov AI, Gubareva LV, 2010. In vitro antiviral activity of favipiravir (T-705) against drug-resistant influenza and 2009 A(H1N1) viruses. *Antimicrob. Agents Chemother.* 54, 2517–2524. 10.1128/aac.01739-09. [PubMed: 20350949]
- Smee DF, Barnard DL, Jones SM, 2016. Activities of JNJ63623872 and oseltamivir against influenza A H1N1pdm and H3N2 virus infections in mice. *Antivir. Res.* 136, 45–50. 10.1016/j.antiviral.2016.10.009. [PubMed: 27771390]
- Soh YQS, Malone KD, Eguia RT, Bloom JD, 2021. Comprehensive profiling of mutations to influenza virus PB2 that confer resistance to the cap-binding inhibitor pimodivir. *Viruses* 13. 10.3390/v13071196.
- Stevaert A, Naesens L, 2016. The influenza virus polymerase complex: an update on its structure, functions, and significance for antiviral drug design. *Med. Res. Rev* 36, 1127–1173. 10.1002/med.21401. [PubMed: 27569399]
- Sweet C, Hayden FG, Jakeman KJ, Grambas S, Hay AJ, 1991. Virulence of rimantadine-resistant human influenza A (H3N2) viruses in ferrets. *J. Infect. Dis.* 164, 969–972. 10.1093/infdis/164.5.969. [PubMed: 1940477]
- Takada K, Kawakami C, Fan S, Chiba S, Zhong G, Gu C, Shimizu K, Takasaki S, Sakai-Tagawa Y, Lopes TJS, Dutta J, Khan Z, Kriti D, van Bakel H, Yamada S, Watanabe T, Imai M, Kawaoka Y, 2019. A humanized MDCK cell line for the efficient isolation and propagation of human influenza viruses. *Nature microbiology* 4, 1268–1273. 10.1038/s41564-019-0433-6.
- Takahashi K, Furuta Y, Fukuda Y, Kuno M, Kamiyama T, Kozaki K, Nomura N, Egawa H, Minami S, Shiraki K, 2003. In vitro and in vivo activities of T-705 and oseltamivir against influenza virus. *Antivir. Chem. Chemother* 14, 235–241. 10.1177/095632020301400502. [PubMed: 14694986]
- Takashita E, 2020. Influenza polymerase inhibitors: mechanisms of action and resistance. *Cold Spring Harbor perspectives in medicine.* 10.1101/cshperspect.a038687.
- Takashita E, Abe T, Morita H, Nagata S, Fujisaki S, Miura H, Shirakura M, Kishida N, Nakamura K, Kuwahara T, Mitamura K, Ichikawa M, Yamazaki M, Watanabe S, Hasegawa H, 2020a. Influenza A(H1N1)pdm09 virus exhibiting reduced susceptibility to baloxavir due to a PA E23K substitution detected from a child without baloxavir treatment. *Antivir. Res.* 180, 104828. 10.1016/j.antiviral.2020.104828. [PubMed: 32574689]
- Takashita E, Daniels RS, Fujisaki S, Gregory V, Gubareva LV, Huang W, Hurt AC, Lackenby A, Nguyen HT, Pereyaslov D, Roe M, Samaan M, Subbarao K, Tse H, Wang D, Yen HL, Zhang W, Meijer A, 2020b. Global update on the susceptibilities of human influenza viruses to neuraminidase inhibitors and the cap-dependent endonuclease inhibitor baloxavir, 2017–2018. *Antivir. Res.* 175, 104718. 10.1016/j.antiviral.2020.104718. [PubMed: 32004620]
- Takashita E, Ejima M, Ogawa R, Fujisaki S, Neumann G, Furuta Y, Kawaoka Y, Tashiro M, Odagiri T, 2016. Antiviral susceptibility of influenza viruses isolated from patients preand post-administration of favipiravir. *Antivir. Res.* 132, 170–177. 10.1016/j.antiviral.2016.06.007. [PubMed: 27321665]
- Takashita E, Ichikawa M, Morita H, Ogawa R, Fujisaki S, Shirakura M, Miura H, Nakamura K, Kishida N, Kuwahara T, Sugawara H, Sato A, Akimoto M, Mitamura K, Abe T, Yamazaki M, Watanabe S, Hasegawa H, Odagiri T, 2019a. Human-to-Human transmission of influenza A(H3N2) virus with reduced susceptibility to baloxavir, Japan, february 2019. *Emerg. Infect. Dis.* 25, 2108–2111. 10.3201/eid2511.190757. [PubMed: 31436527]
- Takashita E, Kawakami C, Morita H, Ogawa R, Fujisaki S, Shirakura M, Miura H, Nakamura K, Kishida N, Kuwahara T, Mitamura K, Abe T, Ichikawa M, Yamazaki M, Watanabe S, Odagiri T, On Behalf Of The Influenza Virus Surveillance Group Of, J., 2019b. Detection of influenza A(H3N2) viruses exhibiting reduced susceptibility to the novel cap-dependent endonuclease inhibitor baloxavir in Japan, December 2018. *Euro Surveill.* 24 10.2807/1560-7917.ES.2019.24.3.1800698.

- Takashita E, Kawakami C, Ogawa R, Morita H, Fujisaki S, Shirakura M, Miura H, Nakamura K, Kishida N, Kuwahara T, Ota A, Togashi H, Saito A, Mitamura K, Abe T, Ichikawa M, Yamazaki M, Watanabe S, Odagiri T, 2019c. Influenza A(H3N2) virus exhibiting reduced susceptibility to baloxavir due to a polymerase acidic subunit I38T substitution detected from a hospitalised child without prior baloxavir treatment, Japan, January 2019. *Euro Surveill.* 24 10.2807/1560-7917.ES.2019.24.12.1900170.
- Takashita E, Kiso M, Fujisaki S, Yokoyama M, Nakamura K, Shirakura M, Sato H, Odagiri T, Kawaoka Y, Tashiro M, 2015. Characterization of a large cluster of influenza A(H1N1)pdm09 viruses cross-resistant to oseltamivir and peramivir during the 2013–2014 influenza season in Japan. *Antimicrob. Agents Chemother.* 59, 2607–2617. 10.1128/AAC.04836-14. [PubMed: 25691635]
- Takashita E, Morita H, Ogawa R, Nakamura K, Fujisaki S, Shirakura M, Kuwahara T, Kishida N, Watanabe S, Odagiri T, 2018. Susceptibility of influenza viruses to the novel cap-dependent endonuclease inhibitor baloxavir marboxil. *Front. Microbiol.* 9, 3026. 10.3389/fmicb.2018.03026. [PubMed: 30574137]
- Taniguchi K, Ando Y, Nobori H, Toba S, Noshi T, Kobayashi M, Kawai M, Yoshida R, Sato A, Shishido T, Naito A, Matsuno K, Okamatsu M, Sakoda Y, Kida H, 2019. Inhibition of avian-origin influenza A(H7N9) virus by the novel cap-dependent endonuclease inhibitor baloxavir marboxil. *Sci. Rep.* 9, 3466. 10.1038/s41598-019-39683-4. [PubMed: 30837531]
- Tashiro M, McKimm-Breschkin JL, Saito T, Klimov A, Macken C, Zambon M, Hayden FG, Neuraminidase Inhibitor Susceptibility N, 2009. Surveillance for neuraminidase-inhibitor-resistant influenza viruses in Japan, 1996–2007. *Antivir. Ther.* 14, 751–761. 10.3851/IMP1194. [PubMed: 19812437]
- Trejejo JM, Asmal M, Vingerhoets J, Polo R, Robertson S, Jiang Y, Kieffer TL, Leopold L, 2018. Pimodivir treatment in adult volunteers experimentally inoculated with live influenza virus: a Phase IIa, randomized, double-blind, placebo-controlled study. *Antivir. Ther.* 23, 335–344. 10.3851/IMP3212. [PubMed: 29244026]
- Uehara T, Hayden FG, Kawaguchi K, Omoto S, Hurt AC, De Jong MD, Hirotsu N, Sugaya N, Lee N, Baba K, Shishido T, Tsuchiya K, Portsmouth S, Kida H, 2020. Treatment-emergent influenza variant viruses with reduced baloxavir susceptibility: impact on clinical and virologic outcomes in uncomplicated influenza. *J. Infect. Dis.* 221, 346–355. 10.1093/infdis/jiz244. [PubMed: 31309975]
- van der Vries E, Stittelaar KJ, van Amerongen G, Veldhuis Kroeze EJ, de Waal L, Fraaij PL, Meesters RJ, Luider TM, van der Nagel B, Koch B, Vulto AG, Schutten M, Osterhaus AD, 2013. Prolonged influenza virus shedding and emergence of antiviral resistance in immunocompromised patients and ferrets. *PLoS Pathog.* 9, e1003343 10.1371/journal.ppat.1003343. [PubMed: 23717200]
- Van Poelvoorde LAE, Saelens X, Thomas I, Roosens NH, 2020. Next-generation sequencing: an eye-Opener for the surveillance of antiviral resistance in influenza. *Trends Biotechnol.* 38, 360–367. 10.1016/j.tibtech.2019.09.009. [PubMed: 31810633]
- Venkatesan S, Myles PR, Leonardi-Bee J, Muthuri SG, Al Masri M, Andrews N, Bantar C, Dubnov-Raz G, Gerardin P, Koay ESC, Loh TP, Memish Z, Miller E, Oliva ME, Rath BA, Schweiger B, Tang JW, Tran D, Vidmar T, Waight PA, Nguyen-Van-Tam JS, 2017. Impact of outpatient neuraminidase inhibitor treatment in patients infected with influenza A(H1N1)pdm09 at high risk of hospitalization: an individual participant data metaanalysis. *Clin. Infect. Dis.* 64, 1328–1334. 10.1093/cid/cix127. [PubMed: 28199524]
- von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Van Phan T, Smythe ML, White HF, Oliver SW, et al. , 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 363, 418–423. 10.1038/363418a0. [PubMed: 8502295]
- Wang Y, Fan G, Salam A, Horby P, Hayden FG, Chen C, Pan J, Zheng J, Lu B, Guo L, Wang C, Cao B, 2020a. Comparative effectiveness of combined favipiravir and oseltamivir therapy versus oseltamivir monotherapy in critically ill patients with influenza virus infection. *J. Infect. Dis.* 221, 1688–1698. 10.1093/infdis/jiz656. [PubMed: 31822885]
- Wang Y, Zhong W, Salam A, Tarning J, Zhan Q, Huang JA, Weng H, Bai C, Ren Y, Yamada K, Wang D, Guo Q, Fang Q, Tsutomu S, Zou X, Li H, Gillesen A, Castle L, Chen C, Li H, Zhen J, Lu

- B, Duan J, Guo L, Jiang J, Cao R, Fan G, Li J, Hayden FG, Wang C, Horby P, Cao B, 2020b. Phase 2a, open-label, dose-escalating, multi-center pharmacokinetic study of favipiravir (T-705) in combination with oseltamivir in patients with severe influenza. *EBioMedicine* 62, 103125. 10.1016/j.ebiom.2020.103125. [PubMed: 3232871]
- Watanabe A, Chang SC, Kim MJ, Chu DW, Ohashi Y, Group MS, 2010. Long-acting neuraminidase inhibitor laninamivir octanoate versus oseltamivir for treatment of influenza: a double-blind, randomized, noninferiority clinical trial. *Clin. Infect. Dis.* 51, 1167–1175. 10.1086/656802. [PubMed: 20936975]
- Wetherall NT, Trivedi T, Zeller J, Hodges-Savola C, McKimm-Breschkin JL, Zambon M, Hayden FG, 2003. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. *J. Clin. Microbiol.* 41, 742–750. 10.1128/jcm.41.2.742-750.2003. [PubMed: 12574276]
- Whitley R, Laughlin A, Carson S, Mitha E, Tellier G, Stich M, Elder J, Alexander WJ, Dobo S, Collis P, Sheridan WP, 2015. Single dose peramivir for the treatment of acute seasonal influenza: integrated analysis of efficacy and safety from two placebo-controlled trials. *Antivir. Ther.* 20, 709–719. 10.3851/IMP2874. [PubMed: 25318121]
- Whitley RJ, Boucher CA, Lina B, Nguyen-Van-Tam JS, Osterhaus A, Schutten M, Monto AS, 2013. Global assessment of resistance to neuraminidase inhibitors, 2008–2011: the influenza resistance information study (IRIS). *Clin. Infect. Dis.* 56, 1197–1205. 10.1093/cid/cis1220. [PubMed: 23307766]
- WHO, 2012. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility - Geneva, November 2011 and June 2012. *Wkly. Epidemiol. Rec.* 87, 369–374.
- WHO, 2021a. Laboratory Methodologies for Testing the Antiviral Susceptibility of Influenza Viruses: Neuraminidase Inhibitor (NAI). https://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/nai_overview/en/. (Accessed 26 April 2021). Accessed.
- WHO, 2021b. Laboratory Methodologies for Testing the Antiviral Susceptibility of Influenza Viruses: Polymerase Acidic (PA) Inhibitor. Baloxavir. https://www.who.int/influenza/gisrs_laboratory/antiviral_susceptibility/baloxavir_overview/en/. (Accessed 26 April 2021). Accessed.
- Yano T, Ochiai H, Akachi S, Matsumura Y, 2020. Polymerase acidic subunit I38T mutant influenza A(H3N2) virus isolated from a pediatric patient without prior baloxavir marboxil treatment in mie prefecture (November 2018). *Jpn. J. Infect. Dis.* 73, 383–385. 10.7883/yoken.JJID.2019.210. [PubMed: 32475868]
- Yogarathnam J, Rito J, Kakuda TN, Fennema H, Gupta K, Jekle CA, Mitchell T, Boyce M, Sahgal O, Balarathnam G, Chanda S, Van Remoortere P, Symons JA, Fry J, 2019. Antiviral activity, safety, and pharmacokinetics of AL-794, a novel oral influenza endonuclease inhibitor: results of an influenza human challenge study. *J. Infect. Dis.* 219, 177–185. 10.1093/infdis/jiy410. [PubMed: 30053042]
- Yokoyama T, Sakaguchi H, Ishibashi T, Shishido T, Piedra PA, Sato C, Tsuchiya K, Uehara T, 2020. Baloxavir marboxil 2% Granules in Japanese children with influenza: an open-label phase 3 study. *Pediatr. Infect. Dis. J.* 39, 706–712. 10.1097/inf.0000000000002748. [PubMed: 32433222]
- Zhou B, Donnelly ME, Scholes DT, St George K, Hatta M, Kawaoka Y, Wentworth DE, 2009. Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza A viruses. *J. Virol.* 83, 10309–10313. 10.1128/JVI.01109-09. [PubMed: 19605485]
- Zhou B, Lin X, Wang W, Halpin RA, Bera J, Stockwell TB, Barr IG, Wentworth DE, 2014. Universal influenza B virus genomic amplification facilitates sequencing, diagnostics, and reverse genetics. *J. Clin. Microbiol.* 52, 1330–1337. 10.1128/JCM.03265-13. [PubMed: 24501036]

Table 1PA substitutions conferring >3-fold reduced susceptibility to baloxavir compared to wild-type viruses.^a

PA amino acid substitution	Fold change in susceptibility of influenza virus				Reference
	A (H1N1)	A (H1N1) pdm09	A (H3N2)	B	
E23G	NI	4–7	NI	NI	(Gubareva et al., 2019; Takashita et al., 2020b)
E23K	4.7	7–9	5.5	1–3	(Abed et al., 2020; Omoto et al., 2018; Takashita et al., 2020a)
A36V	3.6	NI	6	<3 ^b	Omoto et al. (2018)
A37T	NI	>5.2 ^c	8	NI	(Omoto et al., 2018; Sato et al., 2021)
I38F	8–11	7–17	16–20	2.4–8	(Jones et al., 2020; Koszalka et al., 2019; Omoto et al., 2018)
I38L	6.3	7–9	2–4.1	NI	(Chesnokov et al., 2020; Gubareva et al., 2019; Hashimoto et al., 2021; Takashita et al., 2020b) (CDC unpublished data)
I38M	13	7–29	4–21	2–8	(Chesnokov et al., 2020; Gubareva et al., 2019; Ince et al., 2020; Jones et al., 2020; Kiso et al., 2020; Koszalka et al., 2019; Omoto et al., 2018; Takashita et al., 2020b)
I38N	23.7	NI	10.3	NI	(Hashimoto et al., 2021; Ince et al., 2020)
I38S	12	31–112	5.6	NI	(Chesnokov et al., 2020; Hashimoto et al., 2021; Sato et al., 2021; Takashita et al., 2020a)
I38T	22–54	44–124	20–391	5–15	(Abed et al., 2020; Checkmahomed et al., 2020; Chesnokov et al., 2020; Imai et al., 2020; Ince et al., 2020; Jones et al., 2020; Koszalka et al., 2019; Noshi et al., 2018; Omoto et al., 2018; Takashita et al., 2018, 2019a, 2019b, 2019c, 2020a; Uehara et al., 2020; Yano et al., 2020)
E119D E199G	6.5	NI 1	4.5 4.5	2 ^d	Omoto et al. (2018) (Kiso et al., 2020; Omoto et al., 2018)

NI No information.

^aor to median (Takashita et al., 2019c).^bCorresponds to F36V in influenza B PA.^c96%: 4% variant: WT.^dCorresponds to E120D in influenza B PA.

Table 2

Influenza A/PR/8/34 (H1N1) PB2 variants with reduced susceptibility to pimodivir, selected by passage *in vitro* (Byrn et al., 2015).

PB2 amino acid substitution	Pimodivir susceptibility (mean EC ₅₀ ± SD, μM) ^a	Fold change
WT ^b	0.003 ± 0.002	1
Q306H	0.56 ± 0.71	186
S324I	0.47 ± 0.071	157
S324N	0.38 ± 0.68	127
S324R	0.19 ± 0.23	63
F404Y	0.77 ± 0.50	257
N510T	0.40 ± 0.05	133

^aEC₅₀ values were determined in a cell viability assay in MDCK cells.

^bWT, wild-type, pimodivir-susceptible.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 3

Frequency of reduced susceptibility to antivirals of selected clinical isolates from Japan (2018)/2019 season.^a

Type/subtype	Drug	Total frequency	Age group (years)					Unknown
			0–11	12–19	20–64	>65		
A(H1N1)pdm09	NAI	0.9% ^b (21/2252) ^c	1.1% (14/1329) ^d	0% (0/271)	0.6% (3/527)	3.7% (4/109)	0% (0/16)	
	Baloxavir	2.3% (9/395) ^e	2.7% (7/264)	2.3% (1/44)	1.6% (1/63)	0% (0/21)	0% (0/3)	
A(H3N2)	NAI	0% (0/357)	0% (0/231)	0% (0/48)	0% (0/54)	0% (0/24)	0% (0/0)	
	Baloxavir	8.0% (34/424) ^f	10.8% (26/240)	9.6% (5/52)	1.1% (1/90)	5.6% (2/36)	0% (0/6)	
B	NAI	0.5% (1/193)	0% (0/104)	0% (0/48)	2.9% (1/35)	0% (0/4)	0% (0/2)	
	Baloxavir	0% (0/44)	0% (0/25)	0% (0/8)	0% (0/7)	0% (0/3)	0% (0/1)	

^a (NIID, 2019; Takashita, 2020; Takashita et al., 2020a).

^b All have an NA-H275Y substitution (N1 numbering).

^c Number of resistant viruses per total viruses tested.

^d 4 patients without prior treatment with NAIs.

^e 3 patients shed A(H1N1)pdm09 PA-I38X variants without prior baloxavir treatment.

^f 5 patients shed A(H3N2) PA-I38X variants without prior baloxavir treatment.

Table 4

Knowledge gaps related to polymerase inhibitors resistance.

General Issues

- Communication gaps between pharma, academia, public health laboratories and regulators.
- Optimizing the timing of communication (i.e. with influenza IRIS-like study, when will data be made available).
- Alignment of FDA, EMEA and other regulatory authority surveillance requirements.
- Need to publish pre-clinical and clinical data on all agents, especially favipiravir.
- More studies needed on the likelihood of resistance development and spread within circulating viruses.
- How to improve access to drug and reduce delay in starting therapy.
- Need for clinically available resistance testing.
- Address regulatory hurdles associated with the clinical development of combination therapy for influenza.
 - Basic PK data on various approaches (higher initial dose, change in dosing regimen).
 - Animal studies to inform, which approach is associated with the greatest reduction in emergence of resistance.

Methodology

- Need easy phenotypic testing methods to address resistance profile of influenza viruses.
- Limited capacity for viral culture.
- Standardization of techniques.
- Reference viruses with confirmed susceptibility and resistance.
- Testing against influenza C and D viruses.
- Testing against a broad array of zoonotic and variant viruses.
- Studies on development of resistance in avian viruses [A(H5Nx), A(H7N9)].
- Studies on resistance emergence in influenza B viruses.

Pre-Clinical Models

- Need structural models to understand why baloxavir resistance is more common for A(H3N2) than for A(H1N1)pdm09 and B viruses.
- Experiments to understand fitness of resistant variants, models of fitness evaluation that can be standardized.
- Experiments to understand stability and competitive fitness of resistant variants.
- Ferret experiments with influenza A and B wild-type and resistant viruses.
- Models to inform optimal approach to prevent resistance emergence (i.e. variation of dose and dosing regimen).
- Animal and *in vitro* studies with highly pathogenic influenza viruses [A(H5Nx), A (H7N9)].
- Screen for and understand the role of compensatory substitutions in the setting of therapy.
- Studies of mutations in all 3 polymerase subunit genes and their clinical significance.
- Animal studies with immunocompromised animals to enhance understanding of resistance emergence.

Surveillance Strategies

- Many NICs are not doing phenotypic susceptibility testing, which may result in missing localized clusters of resistance.
- Clinical data not available to NICs.
- Treatment data are not collected as part of strategies to understand clinical effectiveness of therapy.
- Variability of screening methods used at NICs, genotypic vs phenotypic.
- How to scale up antiviral surveillance with expanded use of the anti-influenza drugs?
 - Who to share results with?
 - Complexities of the Nagoya Protocol.
 - Information sharing and global databases/Informatics approaches to compensatory mutation identification.

Clinical Data

- Obtain PK data to understand optimal dosing regimens for baloxavir and favipiravir in key target populations, particularly hospitalized and critically ill influenza patients.
 - Obtain PK data for baloxavir in influenza-infected pregnant women.
 - Undertake controlled studies to assess strategies to reduce baloxavir resistance emergence in young children (e.g., repeated dosing, combination therapy with NAI).
 - Assess a wider range of individual baloxavir doses, especially in higher BMI patients.
 - Studies to inform the use of newer agents to treat influenza, particularly severe disease, in low- and middle-income countries.
 - Controlled clinical study to inform how to prevent and manage polymerase inhibitor resistance emergence in immunocompromised hosts (i.e., combination antiviral).
 - Studies of baloxavir for prophylaxis in high-risk settings where longer course of prophylaxis may be needed.
 - More studies of combination therapy of antivirals with different mechanism of action.
 - Prospective IRIS-like studies of baloxavir in outpatients, including young children, to determine the frequencies and clinical impact of resistant variant emergence.
-

NIC: National Influenza Center.