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Detection and treatment strategies for influenza viruses with reduced susceptibility to baloxavir marboxil

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Abstract

Influenza viruses circulate globally and lead to seasonal epidemics and occasionally pandemics. In humans the virus causes a respiratory illness, a cough often with fever are the main symptoms and illness is usually self-limited. However, in those that are at high-risk of complications such as the very young, elderly or the immunocompromised, infection may lead to severe illness or death. Vaccination for influenza is a major strategy to prevent severe illness and antiviral drugs are also available for prophylaxis and treatment. In 2018, the antiviral drug baloxavir marboxil was first licensed which expanded options for influenza antiviral treatment. Baloxavir inhibits the polymerase acidic (PA) protein subunit of the influenza polymerase complex, which blocks the endonuclease function of this protein. The neuraminidase inhibitors (NAIs) have been licensed since the early 2000's and include oseltamivir which is the most commonly administered influenza antiviral and the standard of care for treatment of severe influenza illness in many countries.

The selection of viruses with reduced susceptibility to antiviral drugs is a major concern as these viruses can potentially limit therapeutic options available if they are sufficiently fit to spread from person to person. As a result of clinical and preclinical studies it was known viruses isolated from some patients in clinical trials had reduced susceptibility to baloxavir, most frequently due to substitutions at position 38 of the PA gene. The over-arching aim of this thesis was to characterise and investigate viruses with reduced susceptibility to baloxavir.

First, we sought to develop phenotypic and genotypic methods to characterise viruses with reduced baloxavir susceptibility and establish a baseline baloxavir half maximal effective concentration (EC_{50}) for currently circulating seasonal influenza viruses. Reverse genetics viruses with PA/I38T, I38M and I38F engineered substitutions were generated to characterise the impact of the various substitutions on the EC_{50} relative to wild-type (drug-sensitive) viruses. In this chapter we showed that the PA/I38T virus in the A(H3N2) subtype led to the greatest reduction in baloxavir susceptibility.

Second, we aimed to study the *in vivo* effectiveness of baloxavir treatment in ferrets infected with viruses that had reduced baloxavir susceptibility due to PA/I38T and PA/E23K amino acid substitutions, this was compared to combination therapy with baloxavir and oseltamivir. These experiments showed that while PA/I38T and PA/E23K substitutions lead to a high and low reduction in baloxavir EC_{50} *in vitro*, the effectiveness of baloxavir was reduced to both viruses *in vivo* and that the combination of baloxavir and oseltamivir was additive in antiviral effectiveness. Combination treatment led to a lower selection of viruses with reduced susceptibility compared to baloxavir monotherapy.

Finally, we utilised information from our *in vivo* study and applied this to generate a mathematical modelling framework to study the spread of pandemic influenza virus in the population. Spread of influenza in the population was decreased similarly by baloxavir and baloxavir + oseltamivir in combination, even when viruses with reduced susceptibility could emerge following treatment.

These studies aid our understanding of viruses with reduced susceptibility to baloxavir and show that the spread of viruses with PA/I38T and other substitutions that cause reduced drug susceptibility are of significant public health concern. Viruses with reduced susceptibility to baloxavir should continue to be monitored and further investigation into combination therapies for influenza are warranted.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications during enrolment

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer reviewed journals and 2 submitted publications. The core theme of the thesis is influenza antiviral drugs. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Department of Microbiology and Immunology under the supervision of Professor Kanta Subbarao, Professor Steven Turner, Dr Aeron Hurt and Dr Vijaykrishna Dhanasekaran.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of chapters *1, 2, 3, 4, 5,* my contribution to the work involved the following:

Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
1	Preclinical and clinical developments for combination treatment of influenza	*Submitted*	*80%. Review literature, manuscript writing and editing*	*1) Dr. Kanta Subbarao 10%, Manuscript review and editing* *2) Dr. Mariana Baz 10% Manuscript review and editing*	*N*
2	Baloxavir marboxil susceptibility of influenza viruses from the Asia-Pacific, 2012–2018	*Published*	*70% Study design, performed experiments, data analysis and preparation of manuscript*	*1) Dr. Danielle Tilmanis 10%, Study design and experimental advice* *2) Ms. Merryn Roe 5%, Experimental support* *3) Dr. Dhanasekaran Vijaykrishna 5% Manuscript review and editing* *4) Dr. Aeron Hurt 10%, Study design, Manuscript review and editing*	*N*
3	A rapid pyrosequencing assay for the molecular detection of influenza viruses with reduced baloxavir susceptibility due to PA/I38X amino acid substitutions	*Published*	*70% Study design, performed experiments, data analysis and preparation of manuscript*	*1) Dr. Rubaiyea Farrukee 10% Experimental support* *2) Dr. Edin Mifsud 5% Experimental support* *3) Dr. Dhanasekaran Vijaykrishna 5% Manuscript review and editing* *4) Dr. Aeron C. Hurt 10%, Study design, Manuscript review and editing*	*N*

4	Effect of baloxavir and oseltamivir in combination on infection with influenza viruses with PA/I38T or PA/E23K substitutions in the ferret model	*Submitted*	*70% Study design, performed experiments, data analysis and preparation of manuscript*	*1) Ms. Ankita George 5% Experimental Support 2)Dr. Dhanasekaran Vijaykrishna 5% Manuscript review and editing 3) Dr. Aeron C. Hurt 10%, Study design, Manuscript review and editing 4) Dr. Kanta Subbarao 10%, Study design, Manuscript review and editing*	*N*
5	Adapted SEIR model with baloxavir and oseltamivir combination treatment to determine the effectiveness of antiviral treatment on the spread of influenza in the population	*Unpublished*	*75%, Study design, data collection, data analysis and preparation of chapter*	*1) Dr. James McCaw 10%, Study design, Manuscript review and editing 2) Dr. Pengxing Cao 10%, Study design, Manuscript review and editing 3) Dr. Kanta Subbarao 5% Manuscript review and editing*	*N*

I have not renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

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Abbreviations

AHMPPI	Australian health management plan for pandemic influenza
AUC	Area under the curve
BID	bis in die
DMSO	Dimethyl sulfoxide
EC_{50}	50% effective concentration
FRA	Focus reduction assay
HA	Hemagglutinin
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
IC_{50}	50% inhibitory concentration
LLOD	Lower limit of detection
M1	Matrix protein 1
M2	Matrix protein 2
MDCK-SIAT	Madin-Darby Canine Kidney-human 2,6-sialtransferase
MEM	Minimum essential medium
mRNA	Messenger RNA
NA	Neuraminidase
NAI	Neuraminidase inhibitor
NP	Nucleoprotein
NS1	Non-structural protein 1
OwH	Other-wise healthy
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffered saline
R_0	Basic reproduction number
RDRP	RNA-dependant-RNA-polymerase
RG	Reverse genetics
SFCA	Surfactant-free Cellulose Acetate
SD	Standard deviation
SEIR	Susceptible-exposed-infected-recovered
SOC	Standard of care
TCID50	50% tissue culture infectious dose
vRNP	Viral ribonucleoprotein
WGS	Whole genome sequencing
WHO	World health organisation
WHO-AVWG	WHO-Antiviral working group
WHO-GISRS	WHO-Global influenza surveillance and response system
WT	Wild type

Amino acid codes

Amino acid	Three letter code	One letter code
alanine	ala	A
arginine	arg	R
asparagine	asn	N
aspartic acid	asp	D
asparagine or aspartic acid	asx	B
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glutamine or glutamic acid	glx	Z
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S
threonine	thr	T
tryptophan	trp	W
tyrosine	tyr	Y
valine	val	V

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Chapter 1: Introduction

1.1 Influenza viruses

The influenza virus contains a negative sense, segmented RNA genome and belongs to the *Orthomyxoviridae* family[1]. In humans the virus causes a respiratory infection and symptoms include a cough, fever, sore throat, runny nose and myalgia. There are four types of influenza viruses, termed A, B, C and D. Influenza A has a natural reservoir in wild aquatic birds where infection occurs via the gastrointestinal route; both influenza A and B viruses are responsible for seasonal epidemics in humans and influenza A viruses cause periodic pandemics[2]. Influenza C viruses cause mild disease in humans and influenza D cause disease in cattle[3, 4]. Influenza A viruses are divided further into subtypes based on the hemagglutinin (HA) and neuraminidase (NA) glycoproteins present on the surface of the virus. There are eighteen and eleven known HA and NA subtypes, that are termed H1-H18 and N1-N11, respectively [5, 6]. The influenza A subtypes that are currently circulating in humans are A(H3N2) and A(H1N1pdm09); and can even further be classified by clade and sub-clade. Influenza B viruses are categorised by lineages, the currently circulating influenza B viruses are B/Yamagata and B/Victoria.

In addition to seasonal epidemics, there have been four influenza pandemics in the last century; the 1918 pandemic was caused by a H1N1 virus and resulted in an estimated 50 million deaths, at the time this was approximately 3% of the global population[7]. In 1957, a H2N2 virus emerged and replaced H1N1. This pandemic went on to infect 25% of the world's population and caused approximately 2 million deaths[8]. The H2N2 virus was later replaced in 1968 by H3N2 viruses that continue to circulate in humans[9]. The 1968 pandemic resulted in approximately one million deaths, making it relatively less severe than the two preceding pandemics[10]. The most recent influenza pandemic occurred in 2009, caused by an H1N1 virus (genetically distinct to the 1918 H1N1) that led to up to half a million deaths[11]. Further influenza pandemics in the future are unpredictable but

inevitable and may increase in frequency as the human population grows and further urbanisation leads to greater incursion at the human-animal interface.

Severe disease can result from human infections with avian influenza viruses, such as H5N1 and H7N9 subtypes [12]. These events are of concern as a pandemic threat and infections tend to lead to high mortality from the aggressive symptoms that ensue from infection. An H5N1 outbreak was first reported in Hong Kong in 1997 and resulted in 18 human infections and six deaths[13]. H5N1 viruses continue to circulate in bird populations and infections have been identified in humans, particularly in people who had close contact with avian populations such as poultry workers. Since 2013 H5N1 viruses have become endemic in poultry in some countries[14]. Notably, since October 2021 there have been an unprecedented number of H5N1 viruses identified in North American and Europe (1050 outbreaks to date), largely in poultry and wild populations. These viruses are genetically different to previous isolates from 1997 and 2003-04. Human H7N9 virus infections were first identified in China in 2013 and the fatality rate of the initial outbreak was 36% [15]. H7N9 infections have caused five waves of infection since 2013 but vaccination of poultry contained infections in poultry and humans[16].

1.2 Public health burden of seasonal influenza in Australia

Due to public health measures and strict border closures used to control the current SARS-CoV-2 pandemic, laboratory confirmed influenza in Australia is at a historical low with 550 notifications recorded in 2021 as of the 10th of October. In comparison there were 21,266 laboratory confirmed influenza infections in 2020 (impacted by the pandemic), 313,033 in 2019 and the 5-year average prior was 2014-2018 was 113,861. Importantly, in years with a high burden of influenza, such as 2019, the number of deaths reported in patients with laboratory confirmed influenza was 953 and there were 3,913 admissions with confirmed influenza to sentinel hospitals in 2019 (1st April to 6th October). The global

burden of influenza is around 3-5 million cases and 250,000 to 650,000 deaths each year[17].

1.3 Influenza virus structure, replication and genetics

The influenza virus contains eight viral RNA segments that encode for the polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix 1 and 2 proteins (M1 and M2) and non-structural proteins 1 and 2 (NS1 and NS2) and several accessory proteins[18].

The virion is comprised of an outer lipid layer that is derived from host cells with viral HA and NA glycoproteins that are essential for virus attachment and exit, presented on the surface of the virus particle[19, 20]. The trimeric HA protein mediates attachment by binding to sialic acid receptors on the host cell surface[21]. Human influenza viruses preferentially bind to sialic acids with terminal α-(2, 6) linkages whereas avian influenza viruses have a preference to α-(2, 3) linkages[21]. Following attachment, the virus enters the host cell by receptor-mediated endocytosis. The M2 ion channel facilitates the acidification of the endosome. The low pH then induces a conformational change in the HA protein. Viral and host cell endosome membranes then fuse and this results in the subsequent release of viral ribonucleoproteins (vRNPs) into the cytoplasm[19].

The vRNPs comprised of NP, PA, PB1,PB2 proteins, associated with viral RNA gene segments are transported to the nucleus via nuclear localisation signals where the viral RNA dependent RNA polymerase (RDRP) initiates viral genome replication[22]. The structure and function of the polymerase complex will be discussed further in section 1.3.1. Newly formed vRNPs are exported from the nucleus and transported through the cytoplasm to the host cell surface along with membrane proteins HA, NA and M2 that have been post-translationally modified in the endoplasmic reticulum and Golgi apparatus[23]. There is some debate on whether the mechanism of influenza vRNP packaging occurs

through a random or specific model[24]. Following packaging, the virus buds from the host cell plasma membrane; the tetrameric NA protein cleaves sialic acids from the host membrane to allow the final release of virions from the host cell[25].

1.3.1 The influenza polymerase complex

The influenza virus genome is segmented and virions contain eight distinct, single stranded, negative sense vRNPs. Viral mRNA are transcribed into viral proteins and full length complementary RNA (cRNA) are produced for new vRNPs to be packaged in the virus progeny. Each vRNP contains a polymerase heterotrimer; the genomic ends of the vRNP form a double-stranded structure that is coated with NP and is associated with the viral polymerase complex[26]. Therefore, due to complementarity of the 5’ and 3’ termini, the final molecule consists of a polymerase complex that is bound to vRNA in a double-helical loop twisted into a hairpin structure [22].

The influenza virus polymerase complex is comprised of three interacting subunits, PB2, PB1 and PA that associate via extensive protein interactions. The overall structure of the complex is likened to a “right hand” with distinctive finger, palm and thumb structures; several viruses have this characteristic RDRP structure[27]. The synthesis of viral RNA occurs in two stages, beginning with the primary transcription of vRNA to mRNA, this process occurs in the nucleus and is facilitated by a cap-snatching mechanism where mRNA caps are stolen from the host cell mRNA[28]. The cap-binding domain of PB2 specifically binds the 5′, 7-methylguanosine cap of the host nuclear pre-mRNA. The PA endonuclease domain then cleaves 9–15 nucleotides, which are utilised as a primer for transcription[28]. Following this process, the PB1 subunit is responsible for chain elongation[29]. The resultant mRNA is transported to the cytoplasm for translation[29]. In the replication step, new viral RNA copies are synthesised by the RNA polymerase via complementary RNA intermediates, independently of primers, and these are organised

into new vRNPs by packaging signals that are present at the 5’ and 3’ ends of vRNA segments[30, 31].

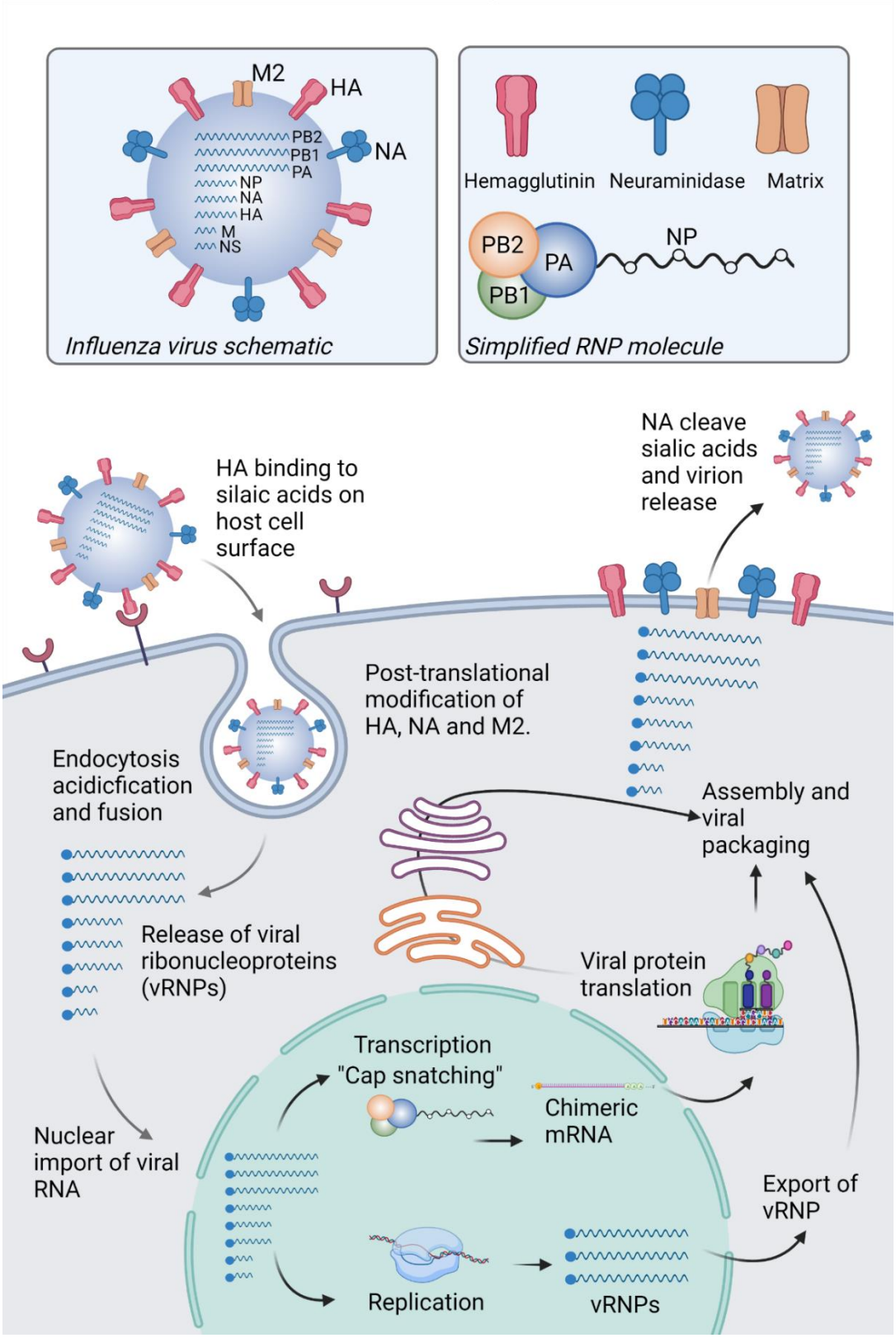

Figure 1. Influenza A virus replication cycle and simplified representation of virion and polymerase complex. Figure generated with BioRender.

1.4 Antigenic shift and drift

Antigenic shift and drift are important mechanisms of genetic change and evolution for influenza. During viral replication point mutations accumulate because the influenza RDRP lacks a proofreading function[32]. Although some of these point mutations will be synonymous or may even be deleterious, the gradual accumulation of amino acid changes in the HA and NA proteins can alter important antigenic sites and therefore result in immune escape, even if a person has had a prior infection or has been vaccinated against previously circulating influenza strains[33]. Antigenic drift occurs at a higher frequency in influenza A viruses than influenza B viruses[34]. Due to antigenic drift certain amino acid substitutions may be present and selection pressures like antiviral treatment may provide an evolutionary advantage and increase the proportion of variant over time[35].

The segmented genome structure of influenza means that gene segments can be exchanged between viruses that can result in antigenic shift[36]. If two influenza virions co-infect a cell, a reassortment event can occur as the host cell will contain a mixture of genes from either infecting influenza virus[37]. If a human influenza virus acquires a novel HA and NA combination, humans will be immunologically naïve and a pandemic virus can emerge (the 1957 H2N2 and 1968 H3N2 pandemic influenza A viruses emerged by reassortment events)[38]. Swine have been described as “mixing vessels” for avian and human influenza viruses as both viruses can infect pigs because both α-(2,3) and α-(2,6) sialic acid receptors are present[21]. An example of this occurred in 2009, a H1N2 and H1N1 reassortment event lead the extinction of seasonal A(H1N1) and the replacement with A(H1N1pdm09)[8].

1.5 Strategies for the control of influenza: Vaccines

Vaccination is an important public health measure to reduce the number of influenza infections in the population. There are three currently licensed vaccine types that primarily target the HA protein; these are inactivated, live attenuated or recombinant. The WHO

annually updates the recommendations for influenza strains to be included in the vaccine to reflect changes in circulating influenza viruses due to antigenic drift; the current vaccine formulation includes a representative A(H1N1pdm09), A(H3N2) and either one or both influenza B virus lineages (B/Victoria, B/Yamagata). The WHO recommendations for the northern hemisphere are announced in February and in September for the southern hemisphere. As the virus strains are selected several months prior to the annual influenza season, ongoing circulation of influenza viruses can result in mismatch(es) between circulating strains and those included in the vaccine formulation. Vaccine effectiveness can also vary year to year and in different populations. The effectiveness against influenza tends to be around 50-60%, but can be as low as 33% in some years especially for the A(H3N2) subtype [39]. In the event that a pandemic influenza strain emerges, the production of a vaccine would require several months.

1.6 Strategies for the control of influenza: Antiviral drugs

Antiviral drugs can supplement vaccination for control of influenza in the population. They are important for the treatment of those that are severely ill or hospitalised due to influenza. The risk of complications due to influenza illness may be greater in unvaccinated people and vaccine effectiveness tends to be lower in elderly or immunosuppressed people than in healthy adults. In this situation, antiviral drug treatment can be used to reduce the risk of complications due to illness. Immunocompromised individuals may shed virus for extended periods and therefore can benefit from antiviral treatment to clear the virus[40, 41]. Prophylaxis either pre-exposure or post-exposure is a further strategy that can limit the transmission of the virus especially in a household settings[42]. Countries may also stockpile antiviral drugs as part of pandemic preparedness plans, to be used in the period before a vaccine is available[42]. Several antiviral drug classes are licensed for the treatment of influenza including the adamantanes, neuraminidase and endonuclease inhibitors.

1.6.1 Drugs in pre-clinical and clinical development for influenza

The process to develop and license a new antiviral drug is long and expensive and only around 12% of drug candidates (all drugs, not just for influenza) that enter Phase I clinical trials reach licensure. For a compound of interest it is estimated that it takes 15 years and 2 billion USD from the initial identification to licensure[43]. The complexity of licensing new drugs is due to many factors including the high cost of human clinical trials to demonstrate safety, tolerability and efficacy. In some cases, drugs can be repurposed and the time and cost of development can be reduced compared to a novel compound. Nonetheless, in addition to the currently licensed antiviral drugs, there are a large number of antiviral drug candidates for influenza in various stages of development from *in vitro* investigations to Phase III clinical trials. Drugs with novel mechanisms of action are needed to broaden therapeutic options and ensure that treatment is available for viruses resistant to other drug classes. Antiviral drugs may target the virus or the host; a drug targeting the virus is likely to exert a greater pressure to select for resistant viruses. There are no currently licensed host-targeted drugs for the treatment of influenza, however, the combination of host and virus-targeted drugs is a potential strategy to circumvent the development of drug resistance during antiviral treatment. A greater arsenal of antiviral drugs will expand the scope of antiviral therapies for influenza and novel antiviral drugs for influenza continue to be assessed in clinical trials.

1.6.2 M2 ion channel blockers

The M2 ion channel is critical for the acidification of the influenza virion, facilitating the entry of protons to create a low pH environment that triggers a conformational change in the HA protein that causes virus and endosomal membrane to fuse. Once this fusion occurs, vRNA can be released into the cytoplasm. The activity of the ion channel can be blocked by the adamantane drugs, amantadine and rimantadine. Due to structural differences in the M2 protein, the adamantanes are active against influenza A but not

influenza B viruses[44]. Other non-adamantane inhibitors have also been shown to block M2 ion channel activity, but are not licensed for use in humans[45]. Amantadine was first licensed in 1966 for prophylaxis for H2N2 infection. However, there was limited use of the drug at the time due to central nervous system side effects including headaches, confusion and hallucinations. Rimantadine, an analogue of amantadine, was licensed in 1994 but also resulted in neurological side effects[46]. Clinical trials showed that prophylaxis with 200 mg per day for six weeks of either amantadine or rimantadine led to an equal reduction in influenza infection however fewer adverse effects were reported with rimantadine[47]. A Cochrane review on the effectiveness of these compounds summarised the results of clinical trials from 1996 to 2003 and identified that use of amantadine prevented 61% of influenza A cases and that duration of fever was reduced by about one day compared to no treatment[48]. The effectiveness for rimantadine was similar, but with fewer adverse events.

Adamantane drugs only inhibit influenza A viruses and have side effects. However, the greatest barrier to their use has been the widespread emergence of resistant influenza viruses. There are several amino acid substitutions in the M2 protein that are known to cause resistance. These include S31N, L26F, V27A, A30T and G34E. Of these, S31N, L26F, V27A have been identified in circulating influenza viruses but S31N is predominant and occurs in >95% of resistant viruses[49]. The S31N substitution first emerged in A(H3N2) viruses in 2003-2004 in China and continued to spread globally until most circulating A(H3N2) viruses contained the S31N mutation. When the novel A(H1N1pdm09) virus emerged, they already contained the S31N substitution in the M2 protein[50]. Therefore, all currently circulating influenza A viruses are resistant to the adamantanes and this class of drugs is not currently recommended for clinical use.

1.6.3 Neuraminidase inhibitors

In 1982, the crystal structure of sialic acid complexed with the NA protein was solved and this advance led to the design of a sialic acid analogue that inhibits NA enzymatic function[51, 52]. Rational drug design was used to develop a class of antiviral drugs termed neuraminidase inhibitors (NAIs) that include: zanamivir, oseltamivir, peramivir and laninamivir. The NAIs are active against both influenza A and B viruses. Zanamivir was the first of this class of drugs to be licensed for clinical use in 1999 and is administered as 10 mg inhaled twice daily for five days[53]. Zanamivir is administered by inhalation because of limited bioavailability on oral administration. This prompted the development of oseltamivir in 2000 an orally available drug that is administered in doses of 75 mg twice a day for five days[54]. Zanamivir at a 600 mg intravenous infusion has been licensed for compassionate use in some countries. Peramivir and laninamivir are both administered as a single dose; peramivir is administered intravenously (600 mg) and laninamivir via inhalation (40 mg)[55, 56]. Zanamivir and oseltamivir are approved globally, whereas peramivir and laninamivir are only licensed in some countries. Meta-analysis of oseltamivir has identified conflicting results that have led to debates on the effectiveness of treatment, particularly on the reduction of hospitalisation and secondary complications[57-60]. Nonetheless, due to the ease of administration, oseltamivir is the current standard of care (SOC) for severe influenza illness. Amino acid substitutions that can cause reduced susceptibility to the NAIs tend to occur in the NA where the drug binds and amino acid substitutions can either cause a catalytic or framework change in the NA [61, 62]. Amino acid changes in catalytic residues of the NA protein are more likely to result in a fitness cost to the virus due to a change in enzymatic function[63]. The NA/H275Y substitution is the most commonly reported substitution in N1 viruses and NA/E119V in the N2 NA[64].

1.6.4 Polymerase inhibitors

The heterotrimeric polymerase complex is highly conserved in influenza viruses and is essential for virus replication, making this an attractive target for drugs. Favipiravir, pimodivir and baloxavir are key drugs that have been developed for the inhibition of the influenza polymerase complex and all three have been assessed in late-phase clinical trials.

Favipiravir (formerly T-705) is a purine nucleoside analogue that induces lethal mutagenesis during viral replication and is a competitive inhibitor of PB1 RDRP mechanism [65]. Due to teratogenicity concerns in animals, favipiravir is conditionally licensed in Japan and remains an investigational drug elsewhere. Favipiravir is a broad spectrum antiviral that has also been investigated for a range of RNA viruses including filoviruses, arenaviruses, coronaviruses and bunyaviruses[66-68]. A PB1/K229R amino acid substitution has been identified as a key amino acid substitution that leads to reduced susceptibility to favipiravir[69].

Pimodivir (also known as JNJ-63623872; formerly VX-787) is a PB2 subunit inhibitor that was under investigation following oral and intravenous delivery methods; however the development program has recently been discontinued by Janssen[70]. Pimodivir blocks PB2 from interaction with 7-methyl-GTP caps of host mRNA and prevents their utilisation as primers for viral transcription [71]. Due to structural differences of the PB2 protein, pimodivir is active against influenza A but not influenza B viruses. Amino acid substitutions in the PB2 protein that confer reduced susceptibility to pimodivir have been identified in a phase IIa trial, including S324C, K376R, M431L/R/V, and M431I [72]. In a phase IIb trial, viruses with PB2 substitutions at S324K/N/R, F325L, S337P, K376N/R, T378S, and N510K were identified[73].

1.6.5 Baloxavir marboxil

In 2018, baloxavir marboxil (also known as S-033188; trade name Xofluza), a polymerase inhibitor drug with a novel mechanism of action was licensed in Japan and the US for the

treatment of uncomplicated influenza (patients > 12 years)[74]. Since 2018, baloxavir has been licensed in a further 60 countries for the treatment of patients at high risk of complications. Baloxavir is administered orally and the dose administered depends on the patient's body weight. Single doses of 40 mg and 80 mg are administered to patients that weigh 40 to 79 kg and more than 80 kg, respectively[75]. During the 2018-2019 influenza season in Japan, baloxavir was administered to 5.3 million people[76]. Baloxavir is effective against both influenza A and B viruses and has also been shown to be effective *in vivo* against avian influenza viruses such as H5N1 viruses[77]. Phase III clinical trials showed that administration of a single dose of baloxavir resulted in a rapid reduction in viral load. The median time to cessation of viral shedding was 24 hours, compared to 72 and 96 hours in oseltamivir and placebo treated patients, respectively[74]. The time to reduction of symptoms was similar for baloxavir and oseltamivir. In a study where baloxavir treatment was given to an index patient, transmission in the household was half as likely to occur as when treatment with oseltamivir occurred[78]. Baloxavir continues to be investigated in clinical trials for different patient cohorts and in household transmission studies (Table 1).

Baloxavir marboxil is a prodrug that is hydrolysed by arylacetamide deacetylase in the blood, small intestine and liver to the active form, baloxavir acid (Figure 1). Baloxavir acid is then metabolised by UGT1A3 and has a half-life in the plasma of 80-100 hours after a single oral dose[79]. Baloxavir binds to five key residues in the PA endonuclease. For influenza A viruses these residues are: A20, Y24, K34, A37 and I38 and in influenza B viruses: A20, F24, M34, N37 and I38[80]. Baloxavir inhibits the endonuclease function of the PA protein that therefore blocks the cap-snatching mechanism that is essential for transcription of viral genes.

1.7 Viruses with reduced susceptibility to baloxavir

Clinical studies with baloxavir have shown that amino acid substitutions in the PA protein can lead to reduced susceptibility, particularly at sites where the drug binds. Position 38 of the PA protein is the most common residue for amino acid substitutions that lead to reduced susceptibility. In post-treatment samples obtained from clinical trials amino acids, T, F, M, N, S, R, V, L were identified in place of isoleucine at amino acid position 38 of the PA protein; demonstrating that variants at this residue are a major pathway to reduced drug susceptibility[80]. Other amino acid substitutions that also led to reduced drug susceptibility include: PA/A20S, PA/E23K, PA/L28P, PA/A36V, PA/E119D/G. Of these amino acid changes, PA/I38F/M/T lead to the greatest change in drug susceptibility (Table 2)[80]. PA/I38T is the amino acid change of greatest concern, leads to the greatest fold-reduction in drug susceptibility and it is the most common change present in post-treatment clinical isolates. In clinical trials in children and adolescents, the frequency of PA/I38X variants was greater than in adults at 25% (Table 2). The crystal structure of the PA protein of influenza A and B viruses with the PA/I38T substitution bound to baloxavir acid shows that compared to wild-type, Van der Waals interactions are altered and conformational changes are required for drug binding, resulting in reduced drug susceptibility[80].

In patients in whom PA/I38X substitutions emerged following treatment, analysis of viral shedding showed that there was also a transient increase in viral titre on days three to six post-treatment compared to patients in whom these substitutions were not detected. It was also shown that the time to alleviation of symptoms was 12 hours longer in patients in whom PA/I38X viruses were selected, but this was still 17.2 hours shorter than in placebo-treated patients[81]. Viruses with reduced susceptibility to baloxavir that have equal or greater fitness to a wild-type strain could result in global circulation of a drug-resistant virus. *In vivo* studies have shown that PA/I38F substitutions lead to attenuated fitness.

However, PA/I38T and PA/I38M are approximately equal to wild-type in fitness. For this reason, circulating influenza viruses are monitored for reduced susceptibility to antiviral drugs. Since the licensure of baloxavir in 2018, viruses with reduced susceptibility to baloxavir have only been identified infrequently (0-1%) among circulating viruses. There are some case studies of human-to-human transmission of viruses with reduced baloxavir susceptibility in the absence of antiviral treatment, suggesting that these viruses may be sufficiently fit to transmit between humans.

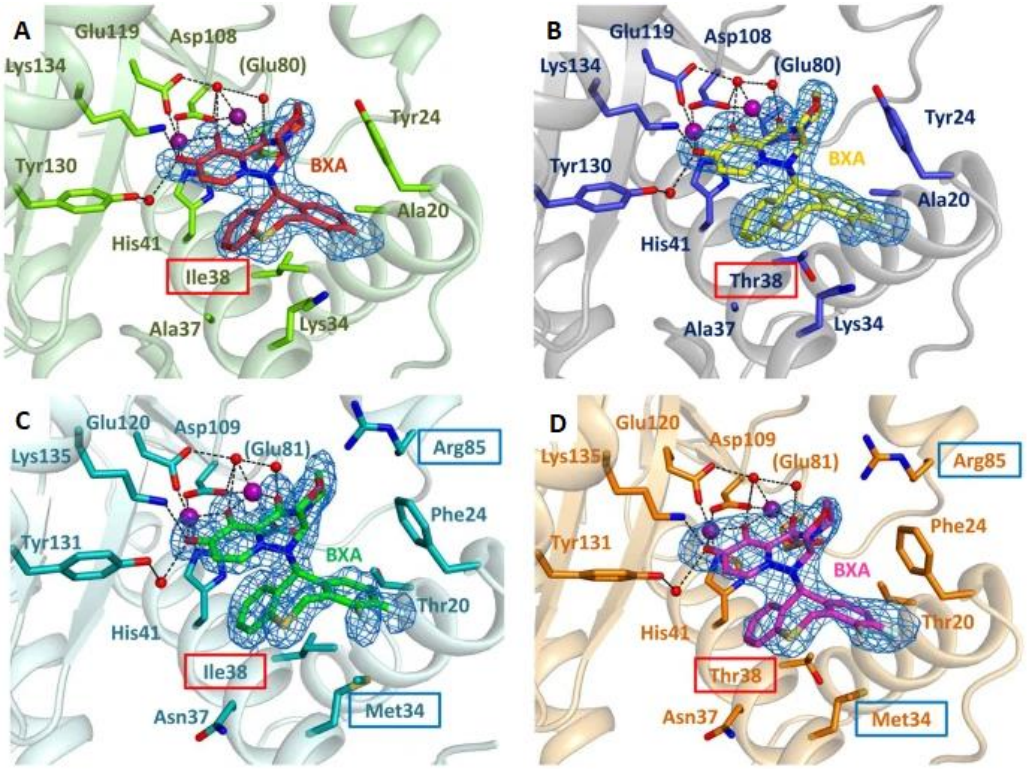

Figure 2. 3D structure of baloxavir binding in influenza A and B endonuclease, a) influenza a wild-type (I38), b) influenza A I38T, c) influenza B wild-type (I38), d) influenza B I38T. Adapted from Omoto et al, 2018[80].

Table 1. Summary of clinical trials that have been completed or are currently recruiting for the study of baloxavir marboxil treatment

Patient cohort (participants enrolled)	Clinical trial phase	Objective	Primary outcome	Year	Study identifier
Completed					
Healthy participants aged 20-59 (n=32)	I	Pharmacokinetics, safety and tolerability of a single oral dose of baloxavir marboxil	Maximum plasma concentration	2019-2020	NCT03959332
Healthy paediatric participants with influenza-like symptoms (n=173)	III	Pharmacokinetics, safety, and efficacy of baloxavir compared to oseltamivir in otherwise healthy paediatric participants (<12 years) with influenza-like symptoms	Percentage of participants with adverse events	2018-2020	MiniSTONE-2 NCT03629184
Hospitalised participants with severe influenza (n=363)	III	Efficacy, safety and pharmacokinetics of baloxavir in combination with standard of care neuraminidase inhibitor drugs in patients hospitalised with influenza	Time to clinical improvement	2019-2020	NCT03684044
Healthy patients with influenza (n=1463)	III	Evaluate efficacy of a single, oral dose of baloxavir for time to alleviation of symptoms in patients with uncomplicated influenza infection	Time to alleviation of symptoms	2016-2017	CAPSTONE 1 NCT02954354
Households, aged 3 months or older (n=481)	IIII	Evaluate a home-based approach for influenza self-test kits, telemedicine and rapid baloxavir delivery	To initiate antiviral therapy within 48 hours of symptom onset	2019-2021	pCHIMES NCT04141930
Patients with influenza at high risk of complications (n=2184)	III	Evaluate a single dose of baloxavir in patients for the improvement of influenza symptoms	Time to improvement of influenza symptoms	2017-2018	CAPSTONE 2 NCT02949011
Recruiting					
Nursing home residents, aged 18 to 120 years, estimated n=1000	IIII	To compare oseltamivir with baloxavir for the treatment of an index case of influenza in nursing homes	Total number of influenza-like-illness identified following index case notification in nursing homes	2021-2023 (estimated)	NCT05012189
Aged birth to <1 year, estimated n=30	III	Efficacy, safety and pharmacokinetics in healthy paediatric participants	Percentage of participants with adverse events	2019-2022 (estimated)	NCT03653364
Households, aged 5-64, estimated n=3160	III	Households are assigned oseltamivir or baloxavir if an index patient becomes unwell with influenza	Virological transmission by day 5	2019-2023 (estimated)	NCT03969212
Hospitalised patients with influenza, estimated n=60	II, III	Oseltamivir and baloxavir combination therapy for patients hospitalised with influenza	Time to clearance of viral shedding	2020-2022 (estimated)	COMBO 1 NCT04327791
Severe influenza infection in hospitalised patients, estimated n=60	II	Patients with Hematopoietic and Lymphoid Cell Neoplasm and influenza, combination treatment with baloxavir and oseltamivir	Main clinical outcome and length of hospital stay	2021-2023 (estimated)	NCT04712539

Table 2. Influenza viruses with PA/I38 substitutions identified in clinical trials

	Age group	Total	A(H1N1pdm09)	A(H3N2)	B
Phase 3: otherwise healthy patients (Japan)	<6 years	(13/41)	20% (1/5)	52.2% (12/23)	0% (0/13)
	6-12 years		0% (0/2)	18.9% (10/53)	0% (0/3)
Phase 3: Capstone 1 otherwise healthy patients	20-64 years	9.7% (36/370)	0% (0/4)	10.9% (36/330)	2.7% (1/37)
Phase 3: Capstone 2 patients a high risk of complications	>12 years	5.2% (15/290)	5.6% (1/18)	9.2% (13/141)	0.8% (1/131)
Phase 3: Paediatric study (Japic CTI-163417)	6 months-11 years	23.4% (18/77)	0% (0/2)	25.7% (18/70)	0% (0/6)

1.8 Combination therapy for the treatment of viral infections

Combination antiviral therapies for other viral infections such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV) have been shown to decrease the emergence of resistant viruses and reduce viral loads in patients to elimination or below the limit of detection, depending on the infection [82, 83]. In comparison, the study of antiviral combinations for influenza has been relatively limited because only a small number of licensed drugs were available and there was little variety in the modes of action.

The licensure of baloxavir adds an additional treatment option for influenza, including as a combination treatment. As oseltamivir is the SOC for severe influenza infection, it is reasonable that baloxavir and oseltamivir should be tested in combination. Different population groups should be included in clinical studies, particularly those that are hospitalised or at high risk of complications. High-risk groups include the elderly, pregnant women, patients on immunosuppressive treatments, individuals with chronic conditions such as obesity, asthma and diabetes. Prolonged viral replication and severe disease may increase the risk of complications and death in these populations. Diverse co-morbidities pose a challenge for the design of randomised controlled trials in hospitalised patients and it may be considered unethical to withhold the SOC from a severely ill patients but investigational drugs may be added to SOC and compared to SOC alone[84].

1.8.1 Reduced selection of antiviral resistance mutations

The selection of resistant viruses following antiviral treatment can lead to poor clinical outcomes [81, 85], viral spread in geographic clusters [86, 87], or if the viruses are sufficiently fit, they can circulate globally and limit the usefulness of antiviral treatment [88]. Resistant viruses have been detected for all licensed influenza antivirals. In theory, if two or more antiviral drugs with different mechanisms of action are used in combination, a virus will require two independent selections of amino acid substitutions that cause reduced susceptibility; a virus that is resistant to one antiviral drug but sensitive to the paired antiviral drug(s) will still face a barrier to the selection of resistance. There is clinical evidence that treatment with an antiviral drug with an alternative mechanism of action should remain effective in patients infected with a drug resistant virus [89, 90]. In immunocompromised or severely ill patients the duration of viral shedding is prolonged compared to an otherwise healthy (OwH) individual [91-93] and continued antiviral therapy over extended periods (sometimes several weeks or months) can increase the likelihood that resistant viruses are selected [94-98]. Combination treatment regimens may reduce the selection of resistant viruses in such patients. It has also been shown that in pregnant, obese and asthmatic mice, the rate of viral point mutations is increased compared to mice that are not obese, pregnant or asthmatic. This highlights that such individuals may be more likely to select for antiviral resistance upon treatment; however this needs to be studied further experimentally [99-101].

1.8.2 Improved clinical outcomes

The effect of drug interactions can be defined by the following terms– antagonistic, additive or synergistic. Antagonistic or synergistic effects are lower or greater, respectively, than the sum of the drug effects alone – which would be additive [102]. Combinations of antiviral drugs may show synergy on the reduction of viral replication in cell-based or preclinical studies; however, in clinical trials the primary endpoint of antiviral drug

treatment is often a clinical parameter such as the time to improvement of symptoms while reduction in viral titre is a secondary endpoint. Virological endpoints are unlikely to be considered sufficient to approval a combination therapy, therefore clinical endpoints are critical. Studies have shown a positive correlation between viral loads and severity of influenza symptoms [103-106]. Limited evidence is available on whether combination antiviral treatment results in a greater reduction of symptoms and viral load compared to monotherapy. Importantly, combination therapy may show superior efficacy in reduction in viral titre but not for clinical improvement compared to the SOC, thus may result in the abandonment of a combination treatment under investigation.

1.8.3 Reduction in severe outcomes or complications of infection

Antiviral treatment may have limited effectiveness in immunocompetent individuals if not administered within 48 hours of symptom onset, because the incubation period of influenza is short and the duration of symptoms and course of illness can be relatively short and mild. On the other hand, antiviral treatment may reduce complications that result in hospitalisation or death. For example, a study in pregnant women with severe disease showed that the length of hospital stay could be greatly reduced with timely antiviral treatment[107]. Retrospective studies have shown that antiviral treatment of influenza in children can decrease progression to severe disease [108]. Historically, the evaluation of antiviral treatment for severe influenza was limited. However, recently more studies have been published that compared combinations of antiviral drugs with SOC antivirals in severe influenza [109-111]. Clinical trials in these patient cohorts aid in our understanding on potential benefits of combination therapy compared to monotherapy.

1.8.4 Reduced drug dose that results in decreased drug toxicity or adverse effects

Drug interactions occur through complex pharmacodynamics and pharmacokinetics and can occur at several stages including drug absorption, elimination or metabolism. Adverse interactions between drugs can lead to undesirable effects and therefore drug-drug

interactions must be carefully assessed. Combining antiviral drugs may mean that a lower dose of either drug can be administered, leading to decreased dose-related drug effects.

1.9 Antiviral combinations: Favipiravir

Synergistic interactions between favipiravir and the NAIs have been shown *in vitro*[112]. Smee and colleagues showed *in vivo* synergy in mice infected with A(H1N1) and A(H3N2) viruses that were treated 24 hours post-infection with favipiravir + oseltamivir but there was only a small improvement in survival with A(H5N1) challenge[113]. Delayed (96 hours post-challenge) treatment with favipiravir and peramivir twice daily for five days in mice infected with A/California/4/2009 (A(H1N1pdm09)), showed synergistic interactions with favipiravir at 20 mg/kg combined with 0.025, 0.05 or 0.1 mg/kg/day of peramivir [114]. The combination of peramivir + favipiravir was beneficial at sub-optimal doses and led to greater improvement in survival and body weight, but not in lung viral titres relative to monotherapy using higher doses[114]. Favipiravir or peramivir treatment in mice infected with an oseltamivir-resistant A(H1N1pdm09) virus showed that monotherapy with either drug resulted in severe weight loss in all mice [115], but the combination was synergistic at higher doses of favipiravir (20 and 40 mg/kg) in reducing mortality. Favipiravir monotherapy at 40 mg/kg was as effective as favipiravir (20 mg/kg) + peramivir (50 or 100 mg/kg) in combination [115]. Delayed treatment of A(H5N1) infection, showed that favipiravir (50 mg/kg/day) + oseltamivir (20 mg/kg/day) protected 100% of mice from mortality and reduced weight loss more effectively than monotherapy [116]. Several amino acid substitutions in the PB1 gene emerged following treatment, but these mutations did not change the *in vitro* drug susceptibility[116].

Treatment was commenced 48 hours post-infection with oseltamivir (20 mg/kg) and a low or high dose of favipiravir (20 or 40 mg/kg/day) twice daily for five or ten days in immunosuppressed mice infected with an A(H1N1pdm09) virus [117]. A low dose of favipiravir + oseltamivir did not reduce mortality compared to monotherapy, however lung

viral titres were lower on days eight and 12 post-infection. A high dose of favipiravir (40 mg/kg/day) resulted in equivalent survival rates in all treatment groups (monotherapy and combination therapy)[117]. Oseltamivir + favipiravir reduced lung viral titres to a similar magnitude as favipiravir monotherapy and both were more effective than oseltamivir alone[117].

A phase IIa, open-label trial, tested pharmacokinetics of favipiravir and oseltamivir in combination in patients with severe influenza [118, 119]. The dosing regimens were well-tolerated, but higher doses may be required to achieve the desired favipiravir concentration in plasma[118]. A separate retrospective analysis from this study compared oseltamivir + favipiravir treatment (NCT03394209) to oseltamivir alone in 40 and 128 patients, respectively (Table 2)[119]. Both treatment groups had the same median time to clinical improvement; but combination treatment reduced the number of severe outcomes compared to monotherapy[119]. Ten days post-treatment, the proportion of patients with no detectable RNA was 67.5% and 21.7% for combination and oseltamivir monotherapy, respectively[119].

In a case study of a severely immunocompromised child with influenza B virus infection, a combination of oseltamivir, zanamivir and nitazoxanide failed to clear infection[120]. With an alternative treatment combination of favipiravir, oseltamivir and zanamivir, the patient tested negative for influenza for two months, after which influenza B was detected again[120]. The infection cleared after further treatment with favipiravir + zanamivir for two weeks[120]. This combination failed to prevent the emergence of zanamivir resistance [120].

1.10 Antiviral combinations: Pimodivir

A phase Ia safety and pharmacokinetic study with pimodivir and oseltamivir showed no clinically relevant drug-drug interactions or safety concerns (Table 2)[121]. A phase II double blind trial in patients with uncomplicated influenza compared three treatment

groups; pimodivir monotherapy at either 300 or 600 mg and the combination of pimodivir (600 mg) + oseltamivir (75 mg) (NCT02342249) (Table 2)[122]. The time to viral clearance relative to placebo based on qPCR was reduced by 31%, 13%, 18% for oseltamivir + pimodivir, 300 mg pimodivir and 600 mg pimodivir, respectively[122]. The primary endpoint of reduction of viral load was met and the study was terminated early [122]. Viruses with PB2 substitutions or reduced susceptibility to pimodivir were detected in 6.9%, 10.5% and 1.8% of 300 mg pimodivir, 600 mg pimodivir and pimodivir +oseltamivir patients, respectively[73].

A phase II clinical trial compared combination treatment with pimodivir + oseltamivir versus oseltamivir alone in hospitalised patients with influenza (NCT02532283) (Table 2)[109]. In elderly and non-elderly patients the time to patient-reported symptom resolution for pimodivir + oseltamivir was 72.45 hours versus 94.15 hours for the oseltamivir group[109]. Viral clearance was faster in the pimodivir + oseltamivir group than the oseltamivir monotherapy group (72 and 96 hours, respectively)[109].

Two phase III placebo-controlled trials were initiated for pimodivir, one was in hospitalised adolescents and adults (NCT03376321) and the second was in high risk outpatients (NCT03381196) (Table 2). The study in hospitalised patients was terminated early based on interim analysis but the results have not been published yet. Summary results for both trials are available on the *clincialtrials.gov* website. The primary outcome was clinical status based on a hospital recovery scale; there was no difference between pimodivir and SOC treatment with either antivirals or supportive care based on the local practice; for avoiding hospitalisation (48.03% and 47.59%, respectively) or other measures on the recovery scale. The time to hospital discharge in the pimodivir group was 113 hours and 108 hours for SOC. The viral load over time, measured by qPCR, showed no significant differences between the treatment groups. The viruses from study participants were also analysed for “mutations of interest” in the NA and PB2 genes that are known to confer

drug resistance. Participants treated with pimodivir + SOC had no “mutations of interest” in the NA gene and 1.3% (2/159) participants had mutations of interest in the PB2 gene. 1.9% (3/159) patients treated with SOC, had mutations of interest in the NA gene and none in the PB2 gene.

The second phase III study was conducted in adolescent, adult and elderly patients at high risk of developing complications (NCT03381196) where the primary endpoint was the resolution of influenza-related symptoms. The median time was 92 and 105 hours in the pimodivir and SOC treatment groups, respectively. Mutations of interest in the pimodivir + SOC treatment group occurred in 5.6% (4/71) of viruses isolated from participants while none occurred (0/108) in the group treated with SOC.

1.11 Antiviral combinations: Baloxavir *in vitro* and pre-clinical studies

In vitro and *in vivo* synergy of baloxavir and the NAIs has been shown for A(H1N1pdm09), A(H3N2) and influenza B viruses[123, 124]. For influenza A viruses, baloxavir also shows *in vitro* synergy with favipiravir, whereas the combination of baloxavir with ribavirin shows variable results [123]. Fukao and colleagues studied delayed treatment (96 hours post-infection) with baloxavir, oseltamivir or the combination of both drugs in a mouse model infected with a lethal dose of influenza A/PuertoRico/8/34[124]. Baloxavir monotherapy was administered in a dose escalating manner (0.5, 15 and 50 mg/kg) BID (twice a day) , oseltamivir at 10 mg/kg BID, or a combination of 0.5 mg/kg baloxavir + 10 mg/kg or 50 mg/kg of oseltamivir BID [124]. Combination therapy was just as effective as monotherapy with 15 or 50 mg/kg of baloxavir in reduction of viral lung titres and mouse lung pathology [124]. However, for reduction in mortality baloxavir (0.5 mg/kg) + oseltamivir (either 10 or 50 mg/kg) was more effective than monotherapy.

In an immunodeficient mouse model, antiviral treatment with favipiravir, baloxavir or oseltamivir was commenced 48 hours post-infection with a mouse-adapted

A/Switzerland/9715293/2013 (H3N2) [125]. All drug treatments were administered for ten days: oseltamivir (20 mg/kg BID), baloxavir (40 mg/kg once a day) or favipiravir (100 mg/kg BID) monotherapy or combination therapy with equivalent doses with oseltamivir + favipiravir, oseltamivir + baloxavir or a triple combination of oseltamivir + favipiravir + baloxavir[125]. Significant weight loss was observed with oseltamivir and favipiravir monotherapy, while the combination treatment prevented weight loss to levels similar to baloxavir monotherapy. Oseltamivir and favipiravir monotherapy offered no protection against mortality. While, baloxavir monotherapy, oseltamivir + baloxavir and oseltamivir + favipiravir + baloxavir reduced mortality equally [125]. The greatest reduction in viral lung titres were observed with any treatment that contained baloxavir (including monotherapy); there was no added benefit of favipiravir or oseltamivir in combination with baloxavir [125]. Interestingly, there was no increase in antiviral resistance in mice treated with the antiviral combinations. While viruses recovered from 50% of the mice (two of four) that received oseltamivir monotherapy had an NA/E119V substitution[125].

A serial passage experiment in mice compared the effect of treatment with baloxavir monotherapy, oseltamivir monotherapy or baloxavir + oseltamivir in combination on selection of resistant viruses [126]. Bronchoalveolar lavage fluid harvested from mice five days post-infection with A/Korea/CNH1/2016 (A(H1N1pdm09)) was serially passaged in mice ten times[126].At each passage, mice were given a sub-optimal single dose of baloxavir (1 or 5 mg/kg), oseltamivir (25 mg/kg for 5 days) or a combination of both drugs (1 mg/kg or 5 mg/kg baloxavir + 25 mg/kg oseltamivir) [126]. With oseltamivir monotherapy, no amino acid substitutions were identified in viruses from passages four, seven or ten[126]. In the low (1 mg/kg) and high dose (5 mg/kg) baloxavir groups, two of three mice had viruses with PA/I38X substitutions. Following treatment with baloxavir + oseltamivir (1mg/kg; 25 mg/kg or 5 mg/kg; 25 mg/kg) an NA/N274Y substitution was identified, but no amino acid substitutions were noted in the PA protein[126].

1.12 Antiviral combinations: Baloxavir clinical studies

The potential for drug interaction between baloxavir and oseltamivir was studied in 18 healthy patients; baloxavir or oseltamivir monotherapy were compared to baloxavir + oseltamivir combination therapy. No significant adverse effects or meaningful drug-drug interactions were observed[75]. A retrospective study in Japan studied the benefit of baloxavir and peramivir in combination on reduction of mortality in patients hospitalised with influenza, compared to peramivir treatment alone[127]. Ten patients were treated with a combination of peramivir + baloxavir and there were no deaths, however sample size was small. The mortality rate was 4.5% in the peramivir only treatment group (132 patients). Albeit limited, this study suggests that a combination of the two drugs could reduce mortality from severe influenza and a larger multi-centre study would be required to confirm this.

A phase III clinical trial (Flagstone; NCT03684044) in hospitalised patients with severe influenza compared the combination of SOC NAIs with baloxavir or placebo for time to clinical improvement (Table 2)[128]. The combination of baloxavir with an NAI did not add clinical benefit compared to NAI alone; the median time to clinical improvement was 97.5 hours for baloxavir + NAI and 100.2 hours for SOC. A secondary endpoint of the study was time to cessation of viral shedding; the median time was 23.9 hours for baloxavir + NAI and 63.7 hours for SOC, respectively[128]. Dual drug resistance emerged in viruses isolated from two immunocompromised patients enrolled in the trial, in one patient NA/H275Y and PA/I38T were identified following peramivir treatment from day one to five, with a dose of baloxavir administered on days one and four. Peramivir treatment was stopped after day five and oseltamivir treatment was given on days 10 to 19. In the second patient, a dose of baloxavir was administered on days one, four and seven in combination with oseltamivir from days one to 11. This patient also developed antiviral resistance due to two amino acid substitutions NA/H275Y and PA/I38T[128].

1.13 Scope of thesis

The licensure of an influenza antiviral drug with a novel mechanism of action, such as baloxavir, is important as it expands treatment options and provides a new choice for combination treatment. The importance of having antiviral drugs available for the treatment of viral infections has become evident during the SARS-CoV-2 pandemic; where there is a currently a clear unmet need for effective therapeutic options. The usefulness of an antiviral drug can be limited by the emergence and spread of antiviral resistant viruses and therefore investigation of such viruses is warranted. This thesis had three aims regarding viruses with reduced susceptibility to baloxavir:

- To develop phenotypic and genotypic methods to identify and characterise viruses with reduced susceptibility to baloxavir and to test these methods with viruses that contain PA/I38X substitutions generated by reverse genetics.
- Second, to determine the effectiveness *in vivo* of antiviral treatment following infection with viruses with different amino acid substitutions associated with reduced susceptibility to baloxavir (PA/I38T and PA/E23K). In addition, we aimed to determine the effectiveness of combination treatment with baloxavir and oseltamivir for the treatment of infection with wild-type and viruses with reduced baloxavir susceptibility.
- Third to utilise our *in vivo* data to establish a mathematical model to determine the impact of a virus with reduced drug susceptibility spreading in the community and the potential benefit of utilising antiviral treatment, such as oseltamivir or baloxavir, on limiting the spread of virus in the population.

Chapter 2: Baloxavir marboxil susceptibility of influenza viruses from the Asia-Pacific, 2012–2018

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Baloxavir marboxil susceptibility of influenza viruses from the Asia-Pacific, 2012–2018

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Antiviral activity

ABSTRACT

Baloxavir Marboxil (BXM) is an influenza polymerase inhibitor antiviral that binds to the endonuclease region in the PA subunit of influenza A and B viruses. To establish the baseline susceptibility of viruses circulating prior to licensure of BXM and to monitor for susceptibility post-BXM use, a cell culture-based focus reduction assay was developed to determine the susceptibility of 286 circulating seasonal influenza viruses, A(H1N1)pdm09, A(H3N2), B (Yamagata/Victoria) lineage viruses, including neuraminidase inhibitor (NAI) resistant viruses, to Baloxavir Acid (BXA), the active metabolic form of BXM. BXA was effective against all influenza subtypes tested with mean EC_{50} values (minimum-maximum) of 0.7 ± 0.5 nM (0.1–2.1 nM), 1.2 ± 0.6 nM (0.1–2.4), 7.2 ± 3.5 nM (0.7–14.8), and 5.8 ± 4.5 nM (1.8–15.5) obtained for A(H1N1)pdm09, A(H3N2), B(Victoria lineage), and B(Yamagata lineage) influenza viruses, respectively. Using reverse genetics, amino acid substitutions known to alter BXA susceptibility were introduced into the PA protein resulting in EC_{50} fold change increases that ranged from 2 to 65. Our study demonstrates that currently circulating viruses are susceptible to BXA and that the newly developed focus reduction assay is well suited to susceptibility monitoring in reference laboratories.

1. Introduction

Despite increased vaccination rates in many parts of the world, influenza continues to cause high levels of morbidity and mortality in high-risk groups (Thompson et al., 2009), particularly when influenza seasons are dominated by A(H3N2) viruses (Sullivan et al., 2017). Influenza antivirals are available for the short-term prophylaxis of individuals to prevent influenza infection, but their primary use has been to treat severely ill patients, many of whom are hospitalised. Two classes of influenza antivirals have been licensed for many years, the M2 ion channel inhibitors and neuraminidase inhibitors (NAIs). Clinical use of the M2 ion channel inhibitors, amantadine and rimantadine, is limited as close to 100% of circulating influenza A viruses contain an amino acid (AA) substitution at residue 31 of the M2 protein (S31N) that confers resistance to these compounds (Dong et al., 2015). Four NAIs are licensed in different parts of the world, of which oseltamivir is the most widely available and commonly used. Oseltamivir resistance has become widespread amongst certain groups of viruses in different periods of time (e.g. seasonal H1N1 between 2007 and 2009 (Hauge et al., 2009; Matsuzaki et al., 2010)), but for the last seven years the frequency of viruses that circulate with reduced NAI susceptibility has remained at less than 5% (Lackenby et al., 2018). The licensure of alternative antivirals, especially those with different modes of action to NAIs, is likely to be of benefit if oseltamivir resistant viruses emerge. In addition, combination therapy may be a strategy to improve clinical effectiveness compared to NAI monotherapy (de Mello et al., 2018).

Baloxavir marboxil (S-033188, BXM) is an influenza polymerase inhibitor that was licensed for the treatment of uncomplicated influenza in Japan and the US in 2018 (Noshi et al., 2016). BXM is a prodrug that is hydrolysed by the enzyme arylacetamide deacetylase to the active form baloxavir acid (S-033447, BXA) (Kawaguchi et al., 2018). BXA is a small molecule inhibitor of the highly conserved cap dependant endonuclease (PA_N) in the PA protein of influenza A and B viruses (Noshi et al., 2018). Inhibition of the PA_N disrupts endonuclease function and

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as a consequence the cap-snatching mechanism of the influenza polymerase (Noshi et al., 2018). Treatment of uncomplicated influenza with BXM as a single oral dose was shown in a Phase III clinical trial to reduce influenza symptom duration by 26.5 h compared to placebo, a similar reduction time achieved with oseltamivir (Hayden et al., 2018). However, 24 h post-drug administration the reduction in viral load was twice as large in BXM treated patients compared to oseltamivir treated patients (Hayden et al., 2018).

In post-treatment samples obtained from phase II and III BXM clinical trials, A(H3N2) and A(H1N1)pdm09 viruses with reduced BXA susceptibility were detected and shown to carry AA substitutions at position 38 of the PA_N including I38T, I38M and I38F (Omoto et al., 2018). In the phase II clinical trial (which involved predominantly A(H1N1)pdm09 viruses) an I38 variant emerged in 4 of 182 patients (2.2%), while the phase III study (which involved predominantly A(H3N2) viruses) showed a frequency of an I38 variant in 36 of 370 (9.7%) of BXM recipients (Hayden et al., 2018). The highest frequency of viruses with reduced BXM susceptibility has been reported from a paediatric study, where 18 of 77 (23.4%) of patients had treatment-emerged PA_N/I38 variants (Omoto et al., 2018). The transmissibility of the PA_N/I38 variants between patients in the absence of drug treatment is currently unknown, but *in vitro* studies suggest that these viruses have reduced replication compared with equivalent wildtype strains (Noshi et al., 2018).

Given the frequency of viruses detected post BXM treatment with reduced BXA susceptibility, and potentially the large use of the drug in clinical practice in coming years, it is important to conduct surveillance of circulating strains for BXM susceptibility. This study aimed to develop a high-throughput and reproducible phenotypic assay for surveillance purposes to determine the BXA susceptibility of recently circulating influenza viruses in the Asia-Pacific, thereby proving baseline susceptibility data for which prospective samples can be compared to in the future.

2. Materials and methods

2.1. Antiviral compounds, cells and viruses

20 mM stocks of Baloxavir acid (S-033447; BXA) (kindly provided by Shionogi & Co., Ltd.) were prepared in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA), filtered with a 0.2 µm surfactant-free cellulose acetate (SFCA) filter (ThermoFisher, USA) and stored in aliquots at −20 °C.

COS-7 African green monkey kidney cells (ATCC, CRL-1651), HEK-293T (ATCC, CRL11268), Madin-Darby canine kidney (MDCK)-TMPRSS2 cells (kindly provided by Dr. Jesse Bloom (Böttcher et al., 2009)) and MDCK-SIAT cells (MDCK cells that overexpress α2,6-linked sialic acids, kindly provided by Dr M. Matrosovich (Matrosovich et al., 2003)), were cultured at 37 °C in a 5% CO_2 gassed incubator in Dulbecco's Modified Eagle Medium (DMEM) (SAFC Biosciences, US). DMEM growth media (DMEM GM) used for the culture of COS-7, HEK-293T and MDCK-TMPRSS2 cell lines was supplemented with: 10% foetal bovine serum (Bovogen Biologicals, Australia), 1x GlutaMAX (Gibco, USA), 1x MEM non-essential amino acid solution (Gibco, USA), 0.06% sodium bicarbonate (Gibco, USA), 20 µM HEPES (Gibco, USA) and 100 U/mL penicillin-streptomycin solution (Gibco, USA). Growth media for MDCK-SIAT cells (DMEM GM SIAT) was supplemented as described for DMEM GM with the addition of 1 mg/mL Geneticin (Gibco, USA).

The influenza viruses used in this study were submitted through the WHO Global Influenza Surveillance and Response System (GISRS) to the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia. Viruses tested were collected during 2013–2018 from Australia (n = 158), Singapore (n = 58), Malaysia (n = 34), Cambodia (n = 19), Thailand (n = 7), Sri Lanka (n = 3), New Zealand (n = 3), New Caledonia (n = 2) and Fiji (n = 2). All viruses grown for assay purposes were propagated in MDCK-SIAT cells, using a DMEM maintenance media (DMEM MM) supplemented as DMEM GM, with the addition of 4 µg/mL TPCK-treated trypsin (Worthington, USA) but without foetal bovine serum.

2.2. Site directed mutagenesis and the generation of recombinant viruses

The reverse genetics plasmid pHW2000 (kindly provided by Richard Webby) containing each of the eight gene segments of A/Perth/261/2009 (A(H1N1)pdm09), A/Perth/16/2009 (A(H3N2)) or B/Yamanashi/166/98 virus (B/Yamagata lineage) were utilised for reverse genetics. The Gene Art® Site-Directed Mutagenesis Kit (Life Technologies, USA) and relevant primer pairs were used for site-directed mutagenesis to introduce AA substitutions within the PA gene of each virus. Sanger sequencing was used to confirm AA changes in each plasmid. To generate recombinant viruses an eight-plasmid reverse genetics method adapted from Hoffman et al. was used (Matrosovich et al., 2003). Alterations to the methods include, HEK-293T and MDCK-TMPRSS2 were seeded at an 8:1 ratio with a total of 5×10^5 cells and COS-7 and MDCK-TMPRSS2 at a 3:1 ratio with a total of 3×10^5 cells, for influenza A and influenza B virus experiments, respectively. GeneJuice® Transfection reagent (Merck Millipore, USA) was used for transfection. MDCK-TMPRSS2 were infected 72 h post-transfection using 1 mL of supernatant from the co-culture. Virus growth was determined using a haemagglutination assay in 1% (v/v) turkey red blood cells and the PA protein sequence was confirmed using Sanger sequencing.

2.3. BXA cytotoxicity assay

The cytotoxicity of BXA was measured to identify non-toxic drug concentrations suitable for use *in vitro*. The inner wells of 96 well plates (Corning, USA) were seeded with MDCK-SIAT cells at a concentration of 2.5×10^5 cells/mL (100 µl/well) and incubated overnight at 37 °C in a 5% CO_2 gassed incubator. The BXA concentration range of 50 µM–0.4 µM was obtained from a two-fold serial dilution of BXA in DMSO (Sigma Aldrich, US) and further diluted in the final MM supplemented with 2 µg/mL TPCK-trypsin overlay. One well was left free of BXA as a negative control. Treated cells were incubated at 35 °C in a 5% CO_2 gassed incubator for 24, 48 and 72 h. Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay as per manufacturer's instructions (Promega, USA) and luminescence was measured using a FLUOstar Optima luminometer (BMG Labtech, Germany). The BXA concentration that reduces cell viability by 50% compared to the cell only control (CC_{50}) was calculated using non-linear regression analysis (GraphPad Prism, USA).

2.4. Virus titration

Virus titration is required to select a suitable virus dilution for the focus reduction assay (FRA). MDCK-SIAT cells were seeded in the inside wells of 96 well plates (Corning, USA) at a concentration of 2.5×10^5 cells/mL (100 µl/well) and incubated overnight at 37 °C in a 5% CO_2 gassed incubator. The experiment was only continued if the cell monolayer was 100% confluent the following day. MDCK-SIAT cells were infected and immunostained with previously described methods (Tilmanis et al., 2017). Briefly, nine half-log dilutions of viruses to be tested in the FRA were prepared in MM. MDCK-SIAT cell monolayers were removed of DMEM GM SIAT and washed once with PBS. 50 µl of each virus dilution was added to the appropriate wells on each plate with the tenth well mock-infected with MM to serve as a cell-only control. The plates were incubated at 35 °C in a 5% CO_2 gassed incubator for 90 min. The virus inoculum was then removed, cells washed once with PBS and overlayed with 100 µl of infection media (IM). IM contained equal parts 3.2% carboxymethyl cellulose (CMC) (1.6% final) (Sigma Aldrich, US) and 2x MEM (1x final) (Sigma Aldrich, USA) and

was supplemented with 2 µg/mL trypsin. The 2x MEM was supplemented with 20 µM HEPES (Gibco, USA), 100 U/mL Penicillin-Streptomycin (Gibco, USA), 0.06% Sodium Bicarbonate (Gibco, USA). Plates were incubated at 35 °C in a 5% CO_2 gassed incubator for 24 h. Following the incubation period, the cells were fixed with 10% formalin (Sigma Aldrich, US) and permeabilised with 0.5% Triton X-100 (Sigma Aldrich, US). Plates were washed three times in wash buffer (0.05% Tween20 (Sigma-Aldrich, US) in PBS) and incubated for one hour with mouse anti-influenza monoclonal antibody against influenza A virus nucleoprotein (Millipore, USA, Cat#MAB8251) or influenza B virus nucleoprotein (Millipore, USA, Cat#MAB8661), diluted 1: 10,000 in 2% skim milk. Plates were then washed and incubated for one hour with goat anti-mouse IgG-horse radish peroxidase (Biorad, US) secondary antibody, diluted 1:1000 in 2% skim milk. Plates were again washed and then incubated for ten minutes in the dark with TrueBlue™ Peroxidase Substrate (KPL, US) and plates were then washed three times with distilled water, the water was then removed and plates allowed to dry. Focus forming units (FFU) were quantified using the Immunospot BioSpot 5.1.36 (CenturyLink Inc, US).

2.5. BXA focus reduction assay

The concentration of BXA required for a 50% reduction in FFU (EC_{50}) was used to determine susceptibility of influenza viruses to BXA. MDCK-SIATs were seeded and infected as described in section 2.4, however, virus was diluted such that there was 1000–2000 FFU/well, as previously determined by virus titration. Cell monolayers were washed with PBS and eight wells were overlaid with 4-fold serial dilutions of BXA (200–0.01 nM) in 100 µl IM. IM only was added to virus and cell control wells. Plates were incubated and immunostained as described in Section 2.4. Each virus was tested in duplicate wells, the foci were determined as an average of duplicate wells as described above. The EC_{50} was only calculated if the FFU count was between 500 and 2500 FFU in the virus control well. Using the mean FFU, the percentage inhibition of FFU was calculated with use of the following formula:

$$Percent\ inhibition = \left(100 - \frac{X - CC}{VC - CC}\right) \times 100$$

where,

CC = FFU in cell control wells (no virus, no drug)
VC = FFU in virus control wells (virus, no drug).
X = Mean FFU

Using the percent inhibition, the EC_{50} for BXA of each virus was determined using non-linear regression analysis (GraphPad Prism, USA).

2.6. Yield reduction assay

The yield reduction assay is an alternative method to determine influenza virus BXA susceptibility and was utilised to confirm the data from the FRA. MDCK-SIAT cells were seeded in 96 well plates as described in section 2.4. Test viruses were inoculated quadruplicate in 96 well plates with a MOI of 0.01 $TCID_{50}$/per well and the viruses were then adsorbed for 1 h at 35 °C. The virus inoculum was then removed, the wells were washed once with PBS and eight wells were overlaid with 4-fold serial dilutions of BXA (200–0.01 nM) in 100 µL MM, with a ninth well as a virus only control and tenth well as a cell only control. Following 24 h the quadruplicate virus samples were pooled and a $TCID_{50}$ was carried out as previously described (Hurt et al., 2010). The EC_{50} was derived as described in section 2.5, with FFU substituted with a $TCID_{50}$ titre.

2.7. Statistical analysis

The Linear regression analysis and unpaired student's t-tests were performed using GraphPad Prism (USA) where p-values < 0.05 were considered statistically significant. To evaluate assay reproducibility, FRA was performed with replicate (n = 48) wells of positive and negative controls (± virus) on a 96-well plate and Z factors were calculated using the equation outlined in (Zhang et al., 1999).

3. Results

3.1. Cytotoxicity of BXA

To determine the maximum working drug concentration for use in the *in vitro* assays, a CellTiterGlo assay was used to measure cell viability in the presence of increasing concentrations of BXA in MDCK-SIAT cells at 24, 48 and 72 h. The MDCK-SIAT cell cytotoxicity (CC_{50}, the 50% reduction of cell cytotoxicity compared to a cell only control) of BXA was 34.1 ± 1.9 µM, 10.1 ± 2.1 µM and 7.8 ± 0.9 µM at 24, 48 and 72 h, respectively.

3.2. Reproducibility of BXA focus reduction assay

Several reproducibility factors were tested to ensure the FRA method was suitable for use as a robust, high throughput screening assay. Z scores were determined at 18, 24 and 30-h post-infection times for three viruses, A/Perth/169/2017 [A(H1N1)pdm09], A/Victoria/189/2017 [A(H3N2)] and B/Sydney/42/2016 [B/Victoria lineage]. The closer a Z score is to the value 1, the more reproducible an assay. For all influenza viruses the exclusion of the outside wells increased the Z-score by 10–30% and therefore was used for subsequent assays. For a 24-h infection time, the Z score was 0.62, 0.73 and 0.69 for A(H1N1) pdm09, A(H3N2) and influenza B test viruses, respectively. For influenza B, the Z score was similar for all infection times (0.58–0.69). In addition to these assays, two control viruses, A/Perth/16/2009 and A/Perth/16/2009-PA/I38M (reduced BXA susceptibility), were tested in n = 12 assays in biological replicates in distinct assays. The minimum and maximum EC_{50} values and the coefficients of variation were 0.13–1.19 nM (80%) and 6.06–12.49 nM (45%), respectively. The EC_{50} values obtained in each experimental repeat are shown in Fig. 1. The EC_{50} values obtained from the yield reduction and FRA were found to be highly comparable for the viruses tested (Table 1). Compared with the respective wildtype virus, the PA_N/I38T variant had a 62-fold increase in EC_{50} in the FRA and a 73-fold increase in the yield reduction assay (Table 1).

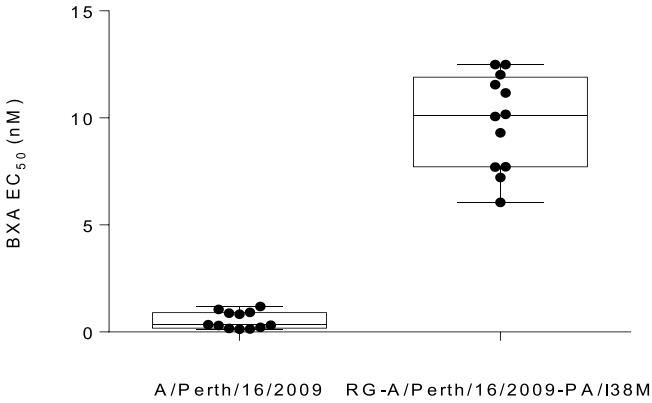

Fig. 1. Control influenza virus EC_{50} values for 24-h FRA used to measure BXA susceptibility. Data was derived from 12 independent experiments, A/Perth/16/2009 had a mean ± standard deviation EC_{50} value of 0.5 ± 0.4 nM and the RG-A/Perth/16/2009-PA/I38M virus had an EC_{50} of 8.5 ± 3.8 nM.

Table 1
Comparison of EC_{50} values obtained from the yield reduction assay and focus reduction assay.

Virus Designation	Subtype/Lineage	BXA EC_{50} (nM)	
		Yield Reduction Assay	Focus Reduction Assay
RG-A/Perth/261/2009	A(H1N1)pdm09	0.3 ± 0.1	0.6 ± 0.5
RG-A/Perth/261/2009-PA_N/I38T	A(H1N1)pdm09	22.1 ± 9.3	37 ± 18.2
RG-A/Perth/261/2009-PA_N/I38M	A(H1N1)pdm09	2.1 ± 1.7	13.3 ± 7.3
A/Victoria/189/2017	A(H3N2)	0.6 ± 0.14	0.91 ± 0.32
B/Christchurch/558/2015	B/Victoria	1.7 ± 0.8	1.2 ± 0.2
B/Sydney/46/2017	B/Yamagata	4.1 ± 3.1	1.3 ± 0.8
B/South Australia/2/2015	B/Yamagata	2 ± 0.8	1.9 ± 0.8

Mean EC_{50} values and standard deviations are based on a minimum of three independent experiments.

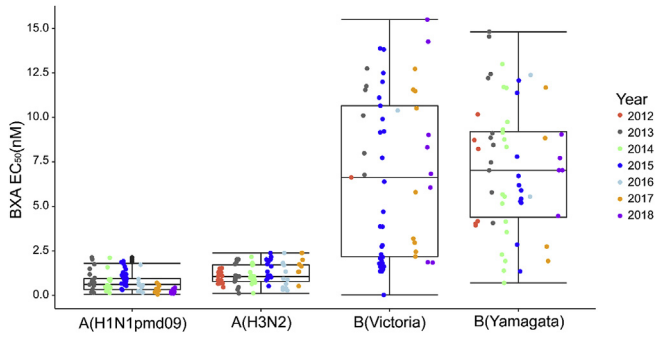

Fig. 2. BXA Susceptibility of seasonal influenza viruses circulating in the Asia Pacific region between 2012 and 2018. Influenza A(H1N1pdm09) (n = 89), A(H3N2) (n = 88) and B(Victoria Lineage) (n = 53) and B(Yamagata Lineage) (n = 56) were tested in a 24 h FRA in MDCK-SIAT cells. The EC_{50} was determined using the percentage inhibition of FFU compared to a no-drug but infected virus control well. The mean ± SD EC_{50} values for A(H1N1pdm09), A(H3N2), B/Victoria lineage and B/Yamagata lineage viruses are 0.7 ± 0.5 nM, 1.2 ± 0.6 nM, 7.2 ± 3.5 nM and 5.8 ± 4.5 nM, respectively, and are grouped based on virus subtype/lineage and the year of circulation. Based on a student's T test the mean EC_{50} of all influenza B viruses was significantly higher than that of influenza A viruses ($P < 0.0001$).

3.3. Susceptibility of circulating influenza viruses to BXA

BXA EC_{50} values were obtained for influenza viruses circulating in the Asia-Pacific region between the years 2012 and 2018 (Fig. 2). The mean BXA EC_{50} of A(H1N1)pdm09 viruses (n = 89) was 0.7 ± 0.5 nM, with maximum and minimum values of 0.1 and 2.1 nM, while n = 88 A(H3N2) viruses had a mean EC_{50} of 1.2 ± 0.6 nM with minimum and maximum values of 0.1 and 2.4 nM. The mean EC_{50} value of A(H3N2) viruses were significantly greater than that of A(H1N1)pdm09 viruses ($P < 0.0001$) based on an unpaired student's T test. B/Victoria lineage viruses (n = 53) had a mean EC_{50} of 7.2 ± 3.5 nM, with minimum and maximum values of 0.7 and 14.8 nM, while B/Yamagata lineage viruses (N = 56) had a mean value of 5.8 ± 4.5 nM and the minimum and maximum values of 1.8 nM and 15.5 nM, respectively. Based on a student's T test there was no significant difference between the mean of influenza B viruses from the two lineages. Taken together the mean EC_{50} of all influenza A viruses (1.0 ± 0.6 nM) was approximately 6-fold lower than that of influenza B viruses (6.6 ± 4.1 nM). This difference was statistically significant ($P < 0.0001$) based on an unpaired student's T test.

3.4. Susceptibility of NAI resistant viruses to BXA

Eleven viruses with neuraminidase AA substitutions that confer reduced susceptibility to oseltamivir, zanamivir, peramivir or laninamivir were screened for susceptibility to BXA using the FRA (Table 2). All viruses tested had EC_{50} values within the expected range for influenza A (0.1–2.4 nM) and influenza B (0.7–15.5 nM). This data demonstrates that BXA is active against influenza strains which have reduced susceptibility to NAIs.

3.5. Susceptibility and phenotypic screening of PA_N I38 amino acid substitution to BXA

Substitutions PA_N/I38T/I38M/I38F have been identified in some influenza viruses in BXM clinical trials from patients post-treatment (Omoto et al., 2018), and therefore it is useful to evaluate the BXA susceptibility of viruses with these substitutions in the FRA. The substitutions PA_N/I38T, PA_N/I38M and PA_N/I38F in an A(H1N1)pdm09 viruses had a 65, 23 and 17 fold increases in EC_{50} compared to respective wild type viruses, respectively, while PA_N/I38M and PA_N/I38F in an A(H3N2) virus both conferred a 16 fold increase in EC_{50} compared to respective wildtype viruses (Table 3). The PA_N/I38 AA substitutions conferred smaller increases in EC_{50} in an influenza B virus than they did in the influenza A strains, 5-fold for PA_N/I38T and 2 fold for PA_N/I38M (Table 3). Of the three AA substitutions, PA_N/I38T resulted in the greatest BXA EC_{50}. RG-A/Perth/16/2009-PA_N/I38T and RG-B/Yamanashi/166/98-PA_N/I38F were not able to be rescued by reverse genetics.

4. Discussion

This study aimed to establish a robust and reproducible assay to determine the susceptibility of influenza viruses circulating in the past seven years to the PA_N inhibitor drug BXA. In addition, we investigated the impact of substitutions at position I38 of the PA_N in contemporary A(H1N1)pdm09, A(H3N2) and influenza B viruses on BXA susceptibility to confirm the ability of the assay to detect such variants. Using the FRA method, BXA was shown to be active against all 286 influenza viruses tested that had circulated in the Asia-Pacific from 2012 to 2018. The mean EC_{50} values obtained in this study for each type/subtype of influenza virus were similar to those described in other studies (Noshi et al., 2016; Takashita et al., 2018; Gubareva et al., 2019).

When comparing the BXA EC_{50} values of influenza A viruses with influenza B viruses, two noteworthy observations can be made. Firstly, mean BXA EC_{50} values are approximately 6-fold higher for influenza B viruses than for influenza A viruses. BXA forms a “wing” shaped structure that binds to five key AAs (20, 24, 34, 37 and 38) in a V-shaped conformation within the PA_N active site. However, aside from residue I38, which is conserved across both influenza A and B viruses, the other four positions have different residues in influenza A or B viruses (influenza A viruses: A20, Y24, K34 and A37; influenza B viruses: T20, F24, M34, N37 and I38) (Omoto et al., 2018), which is likely to be the reason for the difference in binding and EC_{50} values. Lower susceptibility in influenza B viruses compared to influenza A viruses is also observed for oseltamivir, where IC_{50} values for influenza B viruses are 15–20 fold higher than that of influenza A viruses (Farrukee et al., 2015; Escuret et al., 2008). This difference in *in vitro* oseltamivir susceptibility translates into an *in vivo* effect, where

Table 2
Susceptibility of NAI resistant viruses to BXA.

Virus Designation	Type/subtype/lineage	NA Mutation	NAI IC_{50} (nM) [Fold Change to Wild Type]				BXA EC_{50} (nM)
			Oseltamivir	Zanamivir	Peramivir	Laninamivir	
A/Victoria/2500/2016	A(H1N1)pdm09	H275Y	256.8 [642]	0.59 [1]	17.71 [112]	1.6 [3]	1.4 ± 0.1
A/Malaysia/2/2014	A(H1N1)pdm09	I223R	2.86 [19]	19 [1.4]	7 [0.4]	7.6 [0.4]	0.2 ± 0.1
A/Victoria/1031/2010	A(H3N2)	E119V	24 [120]	0.63 [1.6]	0.18 [0.9]	0.78 [1.3]	1.2 ± 0.1
A/Sydney/236/2014	A(H3N2)	Q136K	0.21 [0.57]	9.8 [11]	0.97 [2.9]	2.8 [2.7]	0.1 ± 0.01
B/Sydney/25/2017	B/Vic	I221T	252.3 [10]	16.8 [7]	15.6 [18]	15.3 [6]	1.1 ± 0.6
B/Malaysia/0471/2016	B/Vic	G104E	1539 [87]	3330.4 [120]	16838 [17724]	2536 [701]	1.3 ± 0.2
B/Christchurch/558/2015	B/Vic	H134Y	63.5 [4]	2.3 [1]	72 [76]	1.7 [2]	1.2±0.2
B/Sydney/726/2017	B/Yam	M464T	25.6 [1]	2.2 [1]	8.6 [10]	1.5 [0.5]	1.9±0.9
B/Brisbane/21/2017	B/Yam	I115T	47 [2]	2.7 [1]	7.8 [9]	3.8 [1.5]	4.9 ± 1.1
B/Sydney/46/2017	B/Yam	V131A	21.3 [1]	32.3 [14]	1.1 [1]	3.1 [1]	1.3 ± 0.8
B/South Australia/2/2015	B/Yam	D197N	89.9[7]	6.3 [3]	19.4 [28.8]	7.4 [3]	1.9 ± 0.8

Values shaded in light grey indicate "reduced NAI inhibition" (Influenza A viruses 10-100 fold and influenza B viruses 5-50 fold greater than the median), and dark grey indicate "highly reduced NAI inhibition" (Influenza A viruses >100 fold and influenza B viruses >50 fold above the median). NAI IC_{50} values based on single experiments and mean BXA EC_{50} values and standard deviations are based on three independent experiments.

Table 3
BXA susceptibility of reverse genetics derived influenza viruses with I38 PA_N amino acid substitutions.

Virus Designation (Subtype/Lineage)	PA_N Protein AA Substitution	BXA EC_{50} (nM) [Fold Change from wild type]
A/Perth/261/2009 (A(H1N1pdm09))	Wild Type	0.6 ± 0.5
	I38T	37 ± 18.2 [65] **
	I38M	13.3 ± 7.3 [23] **
	I38F	9.6 ± 5.3 [17] **
A/Perth/16/2009 (A(H3N2))	Wild Type	0.5 ± 0.4
	I38M	8.5 ± 3.8 [16] ****
	I38F	8.5 ± 3.7 [16] ****
B/Yamanashi/166/98 (B/Yamagata Lineage)	Wild Type	5.3 ± 4.2
	I38T	26.3 ± 18.1 [5] ns
	I38M	9.1 ± 4.1 [2] ns

Mean EC_{50} values and standard deviations are based on a minimum of six independent experiments. Fold change in EC_{50} is compared to wild type virus for each corresponding subtype.
Unpaired students T Test was used to compare mean EC_{50} values of variants to corresponding wild type virus, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns P > 0.05.

numerous studies have reported reduced clinical effect of oseltamivir against influenza B infections compared with influenza A infections (Sugaya et al., 2007; Kawai et al., 2006; Heinonen et al., 2010; Singh et al., 2003). However based on clinical trial data in patients at high-risk of severe influenza, BXM seems to have a comparable clinical effect against both influenza A and B virus infections (Ison et al., 2018). The second observation of note from this study was that the range of BXA EC_{50} values for influenza B viruses was considerably larger than it was for influenza A viruses, which may be due to greater variation amongst framework residues in the influenza B PA_N than for influenza A viruses, resulting in subtle impacts on drug binding.

The interaction of PA_N/I38 with BXA is present in both influenza A and B viruses and based on BXM clinical trials, this residue appears prone to selection pressure in both virus types. The I38T AA substitution, which confers the largest change in EC_{50}, results in the loss of a methyl group present in wild type viruses. The absence of a methyl group reduces van der Waals interactions between BXM and influenza PA_N. For drug binding, the presence of T38 also requires a rotational change in the PA_N that is not necessary in wild type viruses (Omoto et al., 2018). Although the substitutions I38M and I38F in A(H1N1) pdm09 and A(H3N2) viruses conferred 16–25 fold increase in EC_{50}, it is important to note that the EC_{50} values for these viruses range from 8.5 to 13.3 nM, which is not substantially higher than the mean EC_{50} of wildtype influenza B viruses (6.6 ± 4.1 nM). Therefore understanding how these *in vitro* findings impact clinical effectiveness and which of the I38 variants are likely to result in reduced clinical effectiveness will be important. The median C_{24} of BXA in clinical trial patients was 59.7 ng/mL (189 nM) (Koshimichi et al., 2018) and therefore at this level it is likely that the drug will still inhibit I38X variants described in this study and those reported elsewhere (Omoto et al., 2018). However, BXA concentrations at 72 h post-treatment decline to a level that is similar to the EC_{50} levels of I38T variants, suggesting that these variant viruses may not be readily inhibited by the drug at this time point (Koshimichi et al., 2018).

One disadvantage of the FRA is that it may not be suitable for front-line diagnostic laboratories, where molecular-based genotypic assays are more commonly used due to time, equipment and labour constraints. To date, data indicates that PA_N/I38 AA substitutions are expected to be the most common AA substitutions that confer reduced BXA susceptibility. While the I38 residue appears to be a 'hot-spot' for AA substitutions under BXA pressure, there are a small number of other substitutions in the PA_N that have also been reported to reduce susceptibility *in vitro* (such as E199G) (Omoto et al., 2018), and it is likely that additional sites will be detected as clinical use of the drug increases.

This study provides information on the baseline susceptibility of a large number of recently circulating influenza viruses across all relevant subtypes and lineages in the Asia-Pacific. It will be important to continue to test circulating viruses for BXM susceptibility as the antiviral continues to be licensed and used more widely to better understand the molecular determinants of BXA susceptibility, the frequency that such viruses occur, and whether they have the capacity to transmit amongst the community in the absence of drug selective pressure.

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Chapter 3: A rapid pyrosequencing assay for the molecular detection of influenza viruses with reduced baloxavir susceptibility due to PA/I38X substitutions

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SHORT ARTICLE

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A rapid pyrosequencing assay for the molecular detection of influenza viruses with reduced baloxavir susceptibility due to PA/I38X amino acid substitutions

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Abstract

Baloxavir marboxil is a novel endonuclease inhibitor licensed for treatment of otherwise healthy or high-risk individuals infected with influenza. Viruses with reduced baloxavir susceptibility due to amino acid substitutions at residue 38 of the PA have been detected in some individuals following treatment. Here, we describe a genotypic pyrosequencing method that can be used to rapidly screen circulating influenza A and B viruses for substitutions in the PA/I38 codon and to quantify mixed viral populations. This method is suitable for surveillance of baloxavir susceptibility and to analyse samples from hospitalised patients undergoing baloxavir treatment to aid in clinical decision making.

KEYWORDS
antiviral resistance, baloxavir marboxil, I38T, influenza viruses, pyrosequencing

1 | INTRODUCTION

Baloxavir marboxil is a small molecule inhibitor of the endonuclease region in the polymerase acidic (PA) protein of influenza viruses and was recently licensed for treatment of influenza in otherwise healthy and high-risk individuals in Japan and the United States.[1] Analysis of viruses obtained from patients following treatment revealed that amino acid substitutions at residue 38 of the PA protein (I38T, I38M or I38F, referred to as PA/I38X) confer 10-fold to 68-fold reductions in baloxavir susceptibility in vitro.[2,3] These substitutions are detected at variable frequencies in baloxavir-treated patients, with the highest rates in adolescents infected with A(H3N2) viruses, where PA/I38X substitutions were identified in 23.4% of patients.[2] To date, PA/I38T is the most commonly detected substitution and is associated with the largest reduction in baloxavir susceptibility (50-fold to 68-fold compared with wild-type virus).[2,3]

In the 2018/19 influenza season, over six million people were treated with baloxavir in Japan and PA/I38X substitutions were reported in 6/335 (1.5%) of A(H1N1pdm09) viruses, 34/356 (9.6%) of A(H3N2) and 0/42 of influenza B viruses by The National Institute of Infectious Diseases (NIID, Japan). Viruses that contain PA/I38T substitutions were also detected in four patients who had not been treated with baloxavir, suggesting that variant viruses had transmitted between people.[4] Given the current rates of PA/I38X variants

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obtained from baloxavir-treated patients and the potential transmissibility of these viruses, surveillance is important to monitor for the emergence of PA/I38X variants in the community. Importantly, rapid detection of viruses with reduced antiviral susceptibility in hospitalised patients can aid clinicians in selecting appropriate antiviral drugs and improve patient management.

Point-of-care tests are available for the rapid detection of influenza infection, however, these tests do not have the capacity to provide information on the presence of specific amino acid substitutions. Therefore, laboratory assays are utilised to determine antiviral susceptibility. Phenotypic assays that directly measure baloxavir susceptibility have been developed [5-7]; however these assays typically require cultured isolates, are slow (3-5 days) and relatively low throughput. Consequently, rapid genotypic assays which can be performed directly on clinical specimens are required. Pyrosequencing has been previously utilised to detect amino acid substitutions that are known to confer reduced susceptibility to M2 ion channel inhibitors and neuraminidase inhibitors.[8] Here, we outline a pyrosequencing method for the detection of PA/I38X variants in A(H3N2), A(H1N1pdm09) and influenza B viruses and report on the accuracy of sequence analysis and estimated mixture proportions.

Full-length PA nucleotide sequences for all circulating influenza subtypes/types submitted to the Global Initiative on Sharing All Influenza Data (GISAID) database from 2009 to 2018 were downloaded. For each virus type/subtype, nucleotide sequences were aligned using MAFFT and primer sets were designed such that they bound to regions of high similarity (>90% conservation of sequences)[9] (Table 1). RNA was extracted using the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's protocol, and RT-PCR was conducted using the MyTaq One-Step RT-PCR kit (Bioline) and standard thermocycling conditions.[10] The PyroMark vacuum prep workstation, PyroMark ID Q96 and PyroMark gold reagents (Qiagen) were used as previously described.[11]

The workflow for identifying PA/I38X variants is depicted in Figure 1, where the sequence of the PA/I38 codon is determined using the "sequence analysis" (SQA) mode of the PyroMarkID Q96. A biotinylated PCR product will yield a pyrogram and a nucleotide sequence for a short region (approximately 15-30 base pairs) that encompasses the single nucleotide polymorphism (SNP) of interest. As a result, the presence of an amino acid substitution can be identified. As biotin is tagged on the forward primer of the RT-PCR reaction, the codons depicted in Figure 1 are in the reverse complement. It is also important to note that the codon sequence for the A(H1N1pdm09) wild-type PA/I38 was TAT (ATA, forward direction) prior to 2015 but has since changed to AAT (ATT, forward direction). Once the nucleotide sequence for a virus is obtained and an amino acid substitution is detected, the relative proportion of the wild type and variant mixture proportion can be assessed using the "Allele Quantitation (AQ)" mode. The AQ mode will estimate the proportion of the two nucleotides of interest based on the relative height of the pyrogram peak. This additional step is only necessary if detailed mixture analysis is required (Figure 1).

The specificity of the pyrosequencing primers was tested with 60 representative cultured virus isolates (including A(H1N1)pdm09, A(H3N2), B/Victoria, B/Yamagata) collected as part of the WHO Global Influenza Surveillance and Response System from various geographic locations between 2011 and 2018. All viruses were successfully amplified by the primers, and SQA analysis indicated that all viruses contained the PA/I38 wild-type codon. As part of routine surveillance, 71 influenza virus isolates circulating in 2019 were tested, and all were successfully amplified and shown to contain a wild-type codon at PA/I38.

To evaluate the sensitivity and limit of detection of the pyrosequencing assay, three virus isolates A/Tasmania/501/2018 (H1N1pdm09), A/Sydney/21/2019 (H3N2) and B/Perth/14/2018 (B/Yamagata lineage) were titrated in 10-fold dilutions from 10^5 to 10^1 RNA copy number/mL. PyroMarkID SQA software will provide a "pass," "check" or "fail" quality score for each sequence. SQA analysis was successfully performed with a "pass" quality check on all virus types/subtypes until 10^3 RNA copies/mL. A "check" quality score was obtained at 10^2 RNA copies/mL for all virus types; however, a nucleotide sequence was still generated by the PyroMark ID software. For sequences generated with the "check" quality score, pyrograms need to be visually checked for markers of poor quality sequence such as wide peaks, spurious peaks, initial baseline drift or overall low signal. At 10^1 RNA copies/mL, the pyrosequencing method we have described cannot obtain a sequence for all influenza virus types and a "fail" quality score was obtained.

Mixed viral populations of wild type (WT) and variant viruses can occur in patients undergoing antiviral treatment.[12] The accuracy of the pyrosequencing assay to detect PA/I38X:WT mixtures was tested using DNA plasmids containing the PA segment from A/Perth/261/2009 (H1N1pdm09), A/Perth/16/2009 (H3N2) and

TABLE 1 RT-PCR and pyrosequencing primer sequences

Influenza type/subtype	RT-PCR forward	RT-PCR reverse	Sequencing
A(H1N1)pdm09	Biotin-CAATCCAATGATCGTCGAGC	GGTGCTTCAATAGTGCATTTGG	CAAACTTCCAAATGTGTGCA
A(H3N2)	Biotin-TTGTCGAACTTGCAGAAAAGGC	GCCATTGTTCTGTCTCTCCCCT	CATACCTCCAAGTGAGTGCA
Influenza B	Biotin-ATACAAAAGGCCAAAAACACAATG	GTTCTTTCCCTTGTCCTTCTAATGC	GCAAACCTCTAGATGGACRCA

Note: All primers in 5′-3′ orientation. Sequencing primers are in the reverse complement.

Pyrosequencing assays require a standard RT-PCR reaction in conjunction with specific primers designed for amplification of the PA segment that encodes codon 38, specifically, nucleotide 38-260 (A(H3N2)), 27-223 (A(H1N1pdm09)) or 40-211 (Influenza B). The forward primer is biotinylated to enable binding to streptavidin beads later in the assay.

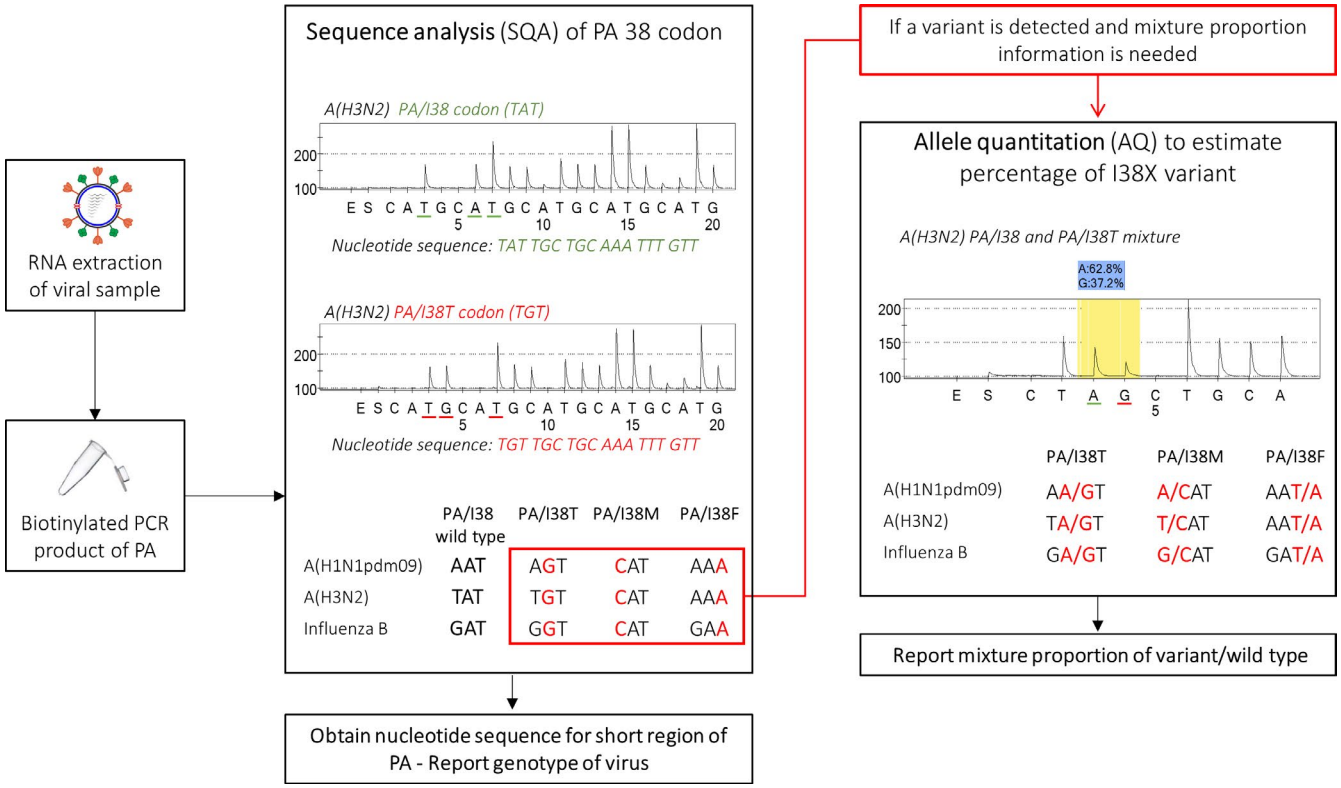

FIGURE 1 Workflow for the identification of PA/I38X variants using pyrosequencing. The PyromarkID system and Pyromark Gold reagents in conjunction with the primers in Table 1 are used for the identification of PA/I38X variants in influenza samples. RNA is extracted from influenza virus samples and RT-PCR with biotin-tagged primers is used to amplify an approximately 100 base pair segment that encompasses the region of interest in the PA protein. Using the sequence analysis mode of the Pyromark ID, a nucleotide sequence surrounding codon 38 is obtained. The sequence analysis panel of the diagram depicts PA/I38 and PA/I38T pyrogram, with the y-axis as a luminescence measure and the x-axis showing the addition of enzyme (E), substrate (S) and nucleotides A, T, G, C. The peak height is representative of nucleotide addition. If a variant is identified, the sample may be further characterised for mixture proportion using the allele quantitation mode of the Pyromark ID. Using half volumes of the initial biotinylated PCR product allows the same PCR product to be used for both sequence analysis and allele quantitation. Codons are depicted as the reverse complement as the biotin tag for the pyrosequencing primers will sequence in the reverse direction

B/Yamanashi/166/1998 (B Yamagata lineage) with either a WT or variant codon that was generated through site-directed mutagenesis. Plasmids were quantified using the Quant-iT™ PicoGreen™ assay and tested as unmixed pure stocks or mixtures of WT/variant codon plasmids. Samples were quantified using the AQ mode to determine the percentage of variant (Table 2). For all viruses, the assay was accurate in estimating PA/I38T:WT and PA/I38M:WT mixtures, for example the 50% A/Perth/261/2009 PA/i38T mixture was detected as 48.8%. Poor accuracy was detected for PA/I38F:WT mixtures, where the B/Yamanashi/166/1998 PA/I38F 50% mixture was detected as 35.8%. The PyroMark ID software does not always have the ability to accurately quantify the chromatogram peak height of homopolymers, this may cause the poor accuracy shown for the influenza A PA/I38F codon for which the nucelotide sequence is AAA (reverse complement).[13] Mixture proportions were generated for the influenza B PA/I38F:WT but the estimate was 10%-15% lower than expected. The error associated with estimating pure populations of PA/I38 or PA/I38X is such that percentages that are <5% or >95% cannot be accurately quantified (Table 2).

To evaluate the accuracy of the mixture proportion estimates at low RNA levels, a PA/I38T:WT virus mixture (reverse genetics derived A/Perth/261/2009) was prepared and titrated 10-fold from 10^7 to 10^1 RNA copy number/mL. AQ analysis of these samples showed that mixture proportions remained consistent until 10^3 copy number/mL (Table 3).

The main analysis to determine the accuracy of the assay was conducted with varying mixtures of plasmids rather than viruses to improve the accuracy of mixture preparation. However, when equivalent mixtures of live viruses have been prepared, the assay has performed similarly (data not shown). Laboratories that are establishing this assay may wish to validate the primers and assay on clinical material from patients.

Pyrosequencing has several benefits for the detection of SNPs to infer antiviral susceptibility. It is rapid, high-throughput, easy to analyse and can provide the quantification of wild type and variant mixtures. The relatively high sensitivity of our assay also allows for detection of PA/I38X variants and mixture estimates in clinical specimens with low RNA copy numbers and does not require a cultured isolate. This allows for a turn-around time of

TABLE 2 Accuracy of mixture estimates by the AQ pyrosequencing assay across different influenza virus types/subtype and PA/I38 substitutions

	Expected variant (%)	Percentage of variant detected by pyrosequencing from a mixed population of WT and indicated PA/I38X variant		
		PA/I38T	PA/I38M	PA/I38F
A(H1N1pdm09)	0	2.4 ± 0.3	0 ± 0	ND
A/Perth/261/2009	25	23.8 ± 0.4	23.3 ± 0.3	ND
	50	48.8 ± 0.5	47.8 ± 0.4	ND
	75	72.7 ± 0.6	72.1 ± 0.3	ND
	100	98.3 ± 0.2	100 ± 0	ND
A(H3N2)	0	0.5 ± 0.7	1.3 ± 0.1	ND
A/Perth/16/2009	25	27.3 ± 0.5	27.2 ± 0.5	ND
	50	52.6 ± 0.3	51.9 ± 0.6	ND
	75	73.9 ± 0.4	74.3 ± 0.1	ND
	100	98.3 ± 0.4	99.3 ± 0.5	ND
B/Yamagata lineage	0	1.2 ± 1.7	0.4 ± 0.6	0 ± 0
B/Yamanashi/166/1998	25	31.7 ± 0.4	25.8 ± 0.5	9.5 ± 1.3
	50	49.7 ± 0.4	47.5 ± 0.5	35.8 ± 5.5
	75	71.6 ± 0.2	72.1 ± 0.5	63.7 ± 3.2
	100	98.4 ± 1.7	100 ± 0	100 ± 0

Note: Not determined (ND) - AQ analysis could not be performed by PyroMarkID software. Variant percentages are the average ± standard deviation of three experiments.

TABLE 3 Limit of accuracy for AQ analysis

RNA copy number/mL	Proportion of PA/I38T (%)	PyroMark ID quality score
10^7	36.6 ± 0.8	Pass
10^6	36.6 ± 0.9	Pass
10^5	38.0 ± 2.5	Pass
10^4	38.6 ± 0.4	Pass
10^3	39.3 ± 0.2	Pass
10^2	59.2 ± 2.0	Check
10^1	27.2 ± 25.7	Fail

Note: PA/I38T proportions are the average ± standard deviation of three experiments.

<4 hours, meaning antiviral treatment of hospitalised patients with influenza can be altered in a clinically relevant window if a variant is detected. This is important for patients who are treated with antivirals for prolonged periods (such as immunocompromised patients) as continual viral shedding under drug selection pressure has been shown to select variants with reduced drug susceptibility.[12]

Amino acid substitutions known to confer reduced susceptibility to antiviral compounds can also be determined with other genotypic methods such as qPCR, Sanger sequencing and next-generation sequencing (NGS). Although qPCR is a high-throughput method that is widely established in laboratories and is relatively inexpensive, the variety of PA/I38X substitutions already observed in clinical specimen means that separate assays would be required for each potential substitution and the sequence of the PCR products is not usually obtained so novel changes at PA/I38X would not be identified.[14] Sanger sequencing is of moderate cost and is accessible but requires several days to generate sequences and is relatively inaccurate in assessing viral mixtures.[14] NGS has higher accuracy for viral mixture quantification but requires extensive sample preparation and data analysis.

Pyrosequencing is frequently performed for identifying reduced susceptibility to the neuraminidase inhibitors (NA/H275Y) and the M2 ion channel inhibitors (M2/S31N), although the equipment is not as commonly available in laboratories as real-time PCR thermocyclers.[15] The pyrosequencing method described in this study can be used for high-throughput screening of circulating influenza viruses for PA/I38X amino acid substitutions, and further analysis for mixtures of PA/I38X and wild-type sequences provides information on the emergence of variant viruses in patients receiving treatment.

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Chapter 4: Effect of baloxavir and oseltamivir in combination on infection with influenza viruses with PA/I38T or PA/E23K substitutions in the ferret model

4.1 Introduction

Antiviral drugs are important for the control of influenza, particularly for treatment of patients who are hospitalised or outpatients who are at a high-risk of complications due to infection. Four neuraminidase inhibitors (NAIs) were licensed in the early 2000s; oseltamivir is the most commonly prescribed of these drugs [129]. Baloxavir marboxil (herein baloxavir) was licensed for the treatment of uncomplicated influenza in Japan and the US in 2018. To date, baloxavir has been licenced in over 27 countries for the treatment of uncomplicated influenza and in some countries for those at high-risk of complications or for post-exposure prophylaxis. Baloxavir inhibits the polymerase acidic protein (PA) endonuclease function of the heterotrimeric influenza polymerase complex[130, 131]. The utility of an antiviral drug is lost or reduced if a virus acquires amino acid substitutions that decrease drug binding; these have been identified for both the NAIs and baloxavir. For oseltamivir such substitutions are typically identified in 0.4 to 4% of post-treatment isolates from adults and 3 to 37% from children[132]; the NA/H275Y substitution is most common in A(H1N1pdm09) viruses and NA/E119V in A(H3N2) viruses[132]. Amino acid changes at PA/I38 are associated with reduced susceptibility to baloxavir and have been identified following treatment in 2.3 to 9.7% of adults and 23% of children in separate Phase III clinical trials[133, 134]. PA/I38T is the most common substitution associated with reduced baloxavir susceptibility and is found at the highest frequency post-treatment in adolescents infected with A(H3N2) viruses. Other amino acid substitutions including PA/E23K, PA/A37T and PA/E119D have been identified at lower frequencies post-treatment (less than 1% of patients) and are also associated with reduced susceptibility to baloxavir *in vitro,* but to a lower extent than PA/I38T[134-136]. Due to viral fitness or drug selection pressure, the proportion of a variant in the total viral population can increase or decrease in frequency over the course of infection. Clinically, the emergence of viruses with reduced baloxavir susceptibility can also lead to a transient increase in viral titre and an increased duration of viral shedding[137]. In two case studies, viruses with either PA/I38T and

PA/E23K substitutions were detected in children who had not been treated with baloxavir but were household contacts of baloxavir-treated children, this suggests the potential for these viruses to transmit from person-to-person[138-140].

Combinations of antiviral drugs with different mechanisms of action have been used to reduce the emergence of drug-resistant viruses in patients with HIV or Hepatitis C virus infection[141, 142]. The availability of anti-influenza drugs with different mechanisms of action (such as oseltamivir and baloxavir) permits a consideration of combination therapy. The drugs are synergistic *in vitro* and the combination of oseltamivir and baloxavir was more effective than oseltamivir monotherapy in reducing viral lung titres in a mouse model, even when treatment was delayed to 96 hours post-infection[143, 144].

The effectiveness of baloxavir or oseltamivir monotherapy or a combination of both drugs, against influenza viruses with amino acid substitutions associated with reduced susceptibility to baloxavir *in vitro* (e.g. PA/I38T and PA/E23K) is currently unknown. The primary aim of this study was to compare the effectiveness of oseltamivir and baloxavir combination therapy with either drug alone for the treatment of ferrets infected with wild-type (drug-sensitive) influenza viruses or paired post-treatment isolates that contained either a PA/I38T or PA/E23K substitution that is known to reduce baloxavir susceptibility *in vitro*. We hypothesised that combination antiviral therapy would offer additional virological benefit over monotherapy for infection with influenza viruses with reduced susceptibility to baloxavir.

The secondary aim of the study was to use a mixed infection with baloxavir-sensitive and reduced susceptibility variants, to model a scenario that could occur in patients, where a virus with reduced drug susceptibility may emerge and increase in relative proportion under drug treatment pressure. Ferrets were co-infected with the paired clinical isolates to determine the change in proportion of variant and wild-type viruses over the duration of viral shedding under selection pressure with drug monotherapy and combination therapy.

We hypothesised that combination therapy would exert a lower selective pressure than baloxavir monotherapy on the relative proportion of viruses with reduced baloxavir susceptibility over time, as the viruses should remain sensitive to oseltamivir because it has a different mechanism of action.

To achieve these aims, antiviral treatment with baloxavir and oseltamivir was studied in ferrets infected with influenza viruses obtained from patients before and after treatment with baloxavir; one was an A(H3N2) PA/I38T variant and the second was an A(H1N1pdm09) virus that contained a PA/E23K amino acid substitution.

4.2 Materials and methods

4.2.1 Cells

Madin-Darby Canine Kidney (MDCK)-SIAT1 cells were cultured at 37 °C and 5% $C0_2$ in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA). The DMEM was supplemented with: 10% foetal bovine serum (Bovogen Biologicals, Australia), 1x GlutaMAX (Gibco, USA), 1x MEM non-essential amino acid solution (Gibco, USA), 0.06% sodium bicarbonate (Gibco, USA), 20 µM HEPES (Gibco) and 100 U/mL penicillin-streptomycin solution (Gibco, USA) and 1 mg/mL Geneticin (Gibco, USA).

4.2.2 Antiviral compounds

Shionogi & Co. Ltd. synthesised and kindly provided baloxavir acid, the active form of baloxavir marboxil, for these studies. For *in vitro* experiments baloxavir acid was prepared in dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA), filtered with a 0.2 µm surfactant-free cellulose acetate (SFCA) filter (ThermoFisher, USA) and stored in aliquots at −80 °C at a concentration of 20 mM. For *in vivo* studies, a 1 mg/mL suspension of baloxavir acid was prepared using an agate mortar and pestle in 0.5 w/v % methyl cellulose (Sigma-Aldrich, Australia). This suspension was prepared immediately prior to administration in ferrets[145]. For *in vivo* studies, oseltamivir phosphate (Thermofisher, USA), was prepared at 10 mg/mL by dilution in sterile 0.5% (v/v) sugar/phosphate-buffered solution (PBS) solution.

4.2.3 Viruses

The clinical isolate pairs utilised in this study were kindly provided by Shionogi & Co. Ltd and were obtained from nasopharyngeal swabs in patients enrolled in the Phase III clinical trial (CAPSTONE-1: NCT02954354) and [BLOCKSTONE: JapicCTI-184180]. The first clinical isolate pair is termed "344103" and was previously described in [146] and [137]. The pre-treatment isolate is "wild-type" (WT; i.e lacking a PA/I38T substitution) and post-treatment isolate contains a PA/I38T substitution. The second clinical isolate pair, termed

PNA508 and PNA012, were a pre- and post-treatment isolate that contain a PA/E23K substitution[147]. PA, NA and HA genes in the clinical isolate pairs were Sanger sequenced and contained no other amino acid substitutions. The viruses were propagated from nasopharyngeal swabs in MDCK-SIAT1 cells.

4.2.4 In vitro characterisation of viruses

The sensitivity of pre- and post-treatment isolates to baloxavir was determined by a focus reduction assay, as has been previously described [148]. The susceptibility of viruses was determined by measuring the concentration of baloxavir acid required to reduce viral focus forming units by 50% (EC_{50}). To achieve this, the percentage of inhibition at each concentration of baloxavir acid was determined and analysed by non-linear regression analysis (GraphPad Prism, USA). The sensitivity of the isolates to the neuraminidase inhibitor drugs was determined by a fluorometric NA inhibition assay with the substrate 2'-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA; Sigma-Aldrich, Castle Hill, NSW, Australia). The enzymatic activity was measured and the relative inhibition of enzyme activity by oseltamivir carboxylate using methods described previously[149]. The NAI concentration that inhibited 50% of NA activity (IC_{50}) was determined using JASPR (version 1.2, CDC, USA) software program.

4.2.5 *In vitro* synergy experiments

A yield reduction assay was utilised for synergy experiments, as the effect of oseltamivir is not reliably measured by focus reduction assay. To perform the yield reduction assay, MDCK-SIAT cells were seeded into a 96-well plate and cultured overnight as described above. Cell monolayers were washed with PBS and infected with 100 virus particles (calculated based on virus titre determined by median tissue culture infectious dose ($TCID_{50}$)) of A(H3N2) or A(H1N1pdm09) wild type or matched baloxavir resistant virus for 90 minutes. Following this incubation time, the viral inoculum was removed and the cells were overlaid with baloxavir (0 to 25 nM, four-fold serial dilution) or oseltamivir (0 to 10,000

nM, ten-fold serial dilution) alone or in full factorial combination. The plates were incubated for 24 hours at 35 °C and the supernatant was collected and quantified for infectious viral titre by $TCID_{50}$ assay.

Synergy was determined using Combenefit software, the viral titre was expressed as percentage inhibition compared to a no drug control (0 nM baloxavir, 0 nM oseltamivir). The software scores the percent inhibition difference between the single drug titre and inhibition by the combined drug. If the reduction in titre is greater than either drug alone, the combination is synergistic.

4.2.6 Ethics statement

Ferret experiments were conducted under the guidelines of the University of Melbourne Biochemistry & Molecular Biology, Dental Science, Medicine, Microbiology & Immunology, and Surgery Animal Ethics Committee, in accordance with the NHMRC Australian code of practice for the care and use of animals for scientific purposes (8th edition). These experiments were listed under AEC#20033.

4.2.7 Ferrets

Outbred male and female ferrets (*Mustela putorius furo*), greater than 6 months old were obtained from independent vendors for the purpose of this study. Ferrets weighed 600 grams at minimum and sera from all ferrets were tested by hemagglutination inhibition assay prior to study commencement for sero-negativity to recently circulating influenza viruses. Ferrets were provided with *ad libitum* food and water for the duration of the experimental period.

4.2.8 Infection of ferrets

In this study, the experiments were organised into two series. In the first series we utilised the A(H3N2) clinical isolate pair; in the second series we utilised the A(H1N1pdm09) clinical isolate pair. Virus stocks for ferret inoculation were diluted to 10^4 and 10^5 $TCID_{50}$/500 µL in PBS for the A(H1N1pdm09) and A(H3N2) clinical isolate pairs,

respectively. For the 100% wild-type pre-treatment isolate and 100% post-treatment isolate pure populations of virus were used for infection. In co-infected ferrets (20% variant: 80% wild-type) the mixtures were prepared based on $TCID_{50}$ titre. A total of 36 ferrets were used in each experiment (72 ferrets for the complete study) with 12 ferrets in each infection group: 100% wild-type, 100% variant or 20% variant: 80% wild-type. An 20:80 mixture was utilised to match a previous study with the A(H3N2) clinical isolate pair and the feasibility of detecting variant proportions by pyrosequencing For viral inoculation, the ferrets were given a reversible anaesthesia via an intramuscular injection using a mixture of ketamine (10 mg/kg, Troy Laboratories), midazolam (0.5 mg/kg, Troy Laboratories) and medetomidine (0.02 mg/kg, Troy Laboratories), that was antagonised following the procedure by atipamezole (0.01 mg/kg, Troy Laboratories). During anaesthesia, the ferrets were inoculated via the intranasal route with 250 µL of virus suspension in each nostril.

4.2.9 Antiviral treatment of ferrets

Ferrets in each infection group of twelve were randomly allocated to an antiviral treatment. Three ferrets of the twelve were each assigned to placebo, baloxavir monotherapy, oseltamivir monotherapy or baloxavir and oseltamivir combination therapy. All antiviral treatment was commenced at 24 hours post-infection. Baloxavir treatment was administered as previously described in [145]. Briefly, treatment was delivered under reversible anaesthesia, as a single dose. The baloxavir acid suspension, described above, was administered by four subcutaneous injections on the dorsal region (1 mg/kg per site: 4 mg/kg baloxavir acid per ferret). Placebo treated ferrets received a single dose of methyl-cellulose as a subcutaneous injection on the dorsal region (1 mL/kg per site: 4 mL/kg methyl cellulose per ferret). Oseltamivir treatment was administered orally for 5 days at 5 mg/kg twice daily (bis in die (BID)) in non-sedated ferrets (10 mg/kg/day). Combination treated ferrets were administered both a single dose of baloxavir acid and oseltamivir

phosphate for 5 days BID, as described. Baloxavir and oseltamivir treatment doses are based on prior pharmacokinetic analyses that achieved similar plasma concentration-time curves that occur in humans[145, 150].

4.2.10 Ferret monitoring and sample collection

The body temperature and weight of ferrets was measured daily. Nasal washes were collected daily for ten consecutive days from sedated ferrets (intramuscular injection of xylazine at 5 mg/kg) by instilling 1 mL of sterile PBS through the nostril. Ferrets were sacrificed at 14 days post infection, anaesthesia was first administered by intramuscular injection (Ketamine (≥25 mg/kg) and Xylazine (≥5 mg/kg)) followed by an overdose of pentobarbitone sodium (Lethabarb; 0.5 mL/kg). Blood was collected by cardiac puncture 14 days post infection and the antibody response was measured by hemagglutination inhibition assay to a recently circulating virus that matched the viral subtype. Two ferrets were sacrificed on days 7 and 8 post infection under guidance of the animal ethics committee (Ferret 2, baloxavir treated, A(H3N2)-PA/I38T and Ferret 3, placebo treated, A(H1N1pdm09)-WT).

4.2.11 Virological analysis

Infectious viral titre was determined by titration in MDCK cells (LLOD at $2\log_{10}$ $TCID_{50}$/mL) as previously described [151]. Viral RNA was extracted from nasal wash samples with the NucleoMag VET isolation kit (Macherey Nagel) on the KingFisher Flex (ThermoFisher Scientific) platform according to manufacturer's instructions. For pyrosequencing analysis, primers and reaction conditions described in [152] were utilised for the PA/I38T substitution. For the PA/E23K substitution, we designed a new set of pyrosequencing primers. PCR amplification was performed with specific primers (Forward: GCTTCAATCCAATGATCGTC, Reverse: 5'Biotin-CATGAAACAAACTTCCAAATGTG). The pyrosequencing reaction was performed with a TGCGGAAAAGGCAATGAA primer. For the NA/H275Y substitution in influenza A(H1N1pdm09) virus, primers and reaction

conditions as previously described[153].The lower and upper limit of detection for either pyrosequencing analysis is 5% and 95% of variant.

4.2.12 Whole genome sequencing

The original virus stocks for ferret inoculation and nasal wash samples from day 5 post-infection (or the final day of viral shedding) in co-infected ferrets (20% variant: 80% wild-type) were selected for further analysis by whole genome sequencing. The samples were passaged once in cell culture prior to preparation for sequencing to ensure the viral load was sufficient for WGS. Viral RNA was extracted with QIAamp viral RNA mini kit (QIAGEN) and one-step RT-PCR was performed with universal influenza A primers using the qSCRIPT XLT one-step kit (Quanta). The polymerase genes, PB2, PB1 and PA, were amplified in separate reactions to ensure gene coverage. Sequencing libraries were generated using the Nextra library preparation kit and sequencing was performed on the illumina iSeq.

4.2.13 Statistical Analysis

Data analysis was performed using GraphPad Prism (GraphPad Software, v6). The AUC for $TCID_{50}$, was compared by unpaired student's t-test with Welch's correction. Samples below the LLOD were assigned zero values for graphing and statistical analyses. A value of $p < 0.05$ was considered statistically significant.

4.3 Results

4.3.1 *In vitro* drug susceptibility and drug interactions

The two pairs of clinical isolates were tested for *in vitro* susceptibility to baloxavir and oseltamivir by determining the 50% effective concentration (EC_{50}) and 50% inhibitory concentration (IC_{50}), respectively. The A(H3N2)-PA/I38T and A(H1N1pdm09)-PA/E23K viruses had a 77-fold and 17-fold higher EC_{50} for baloxavir compared to the corresponding pre-treatment virus, respectively, confirming that the PA/E23K substitution results in a lower fold-change reduction in baloxavir susceptibility than PA/I38T (Table 1). All four viruses were susceptible to the NAI drugs: oseltamivir, zanamivir, peramivir and laninamivir based on a neuraminidase enzyme inhibition assay (Table 1, Table S2).

The viruses were tested *in vitro* against combinations of baloxavir and oseltamivir in a yield reduction assay to determine whether the drugs would be synergistic in their antiviral activity. Increasing concentrations of baloxavir and oseltamivir were analysed alone and in combination; the synergistic potential was determined by the Bliss independence model. All four viruses were more effectively inhibited by a combination of baloxavir and oseltamivir, compared to either drug alone (Figure 1). Therefore, the drugs were synergistic even against viruses with reduced susceptibility to baloxavir. The greatest region of drug synergy occurred at low concentrations of either drug (0.006 to 1.56 nM baloxavir).

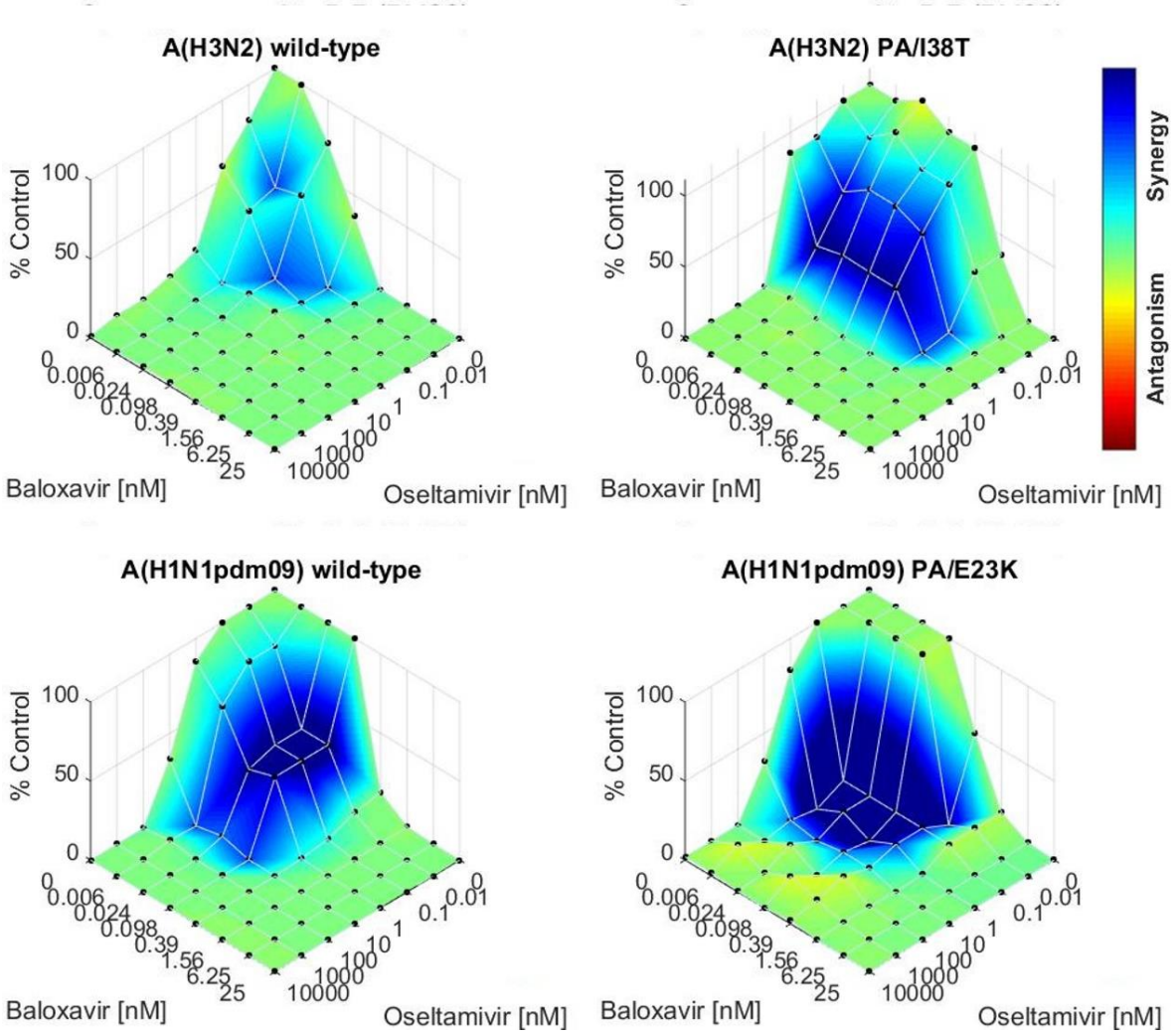

Figure 1. Surface plot to show interactions between baloxavir and oseltamivir against the growth of A(H3N2)-WT or PA/I38T and A(H1N1pdm09)-WT or PA/E23K viruses using the Bliss independence synergy model. The z-axis depicts the percent inhibition of viral growth relative to no drug (0 nM baloxavir + 0 nM oseltamivir). The baloxavir and oseltamivir concentrations were tested in full factorial combination and the coloured shading on the plot depicts additive (green), synergistic (blue) and antagonistic (red) effects.

4.3.2 Effectiveness of baloxavir and oseltamivir combination therapy in ferrets infected with wild-type or viruses with reduced baloxavir susceptibility

Ferrets infected with the pairs of clinical isolates were treated with placebo, baloxavir or oseltamivir alone or in combination and nasal washes were collected for ten days to determine virus titres in the upper respiratory tract (Figure 2). An area under the curve (AUC) analysis was performed to incorporate the viral titre and duration of viral shedding for ferrets in each antiviral treatment group.

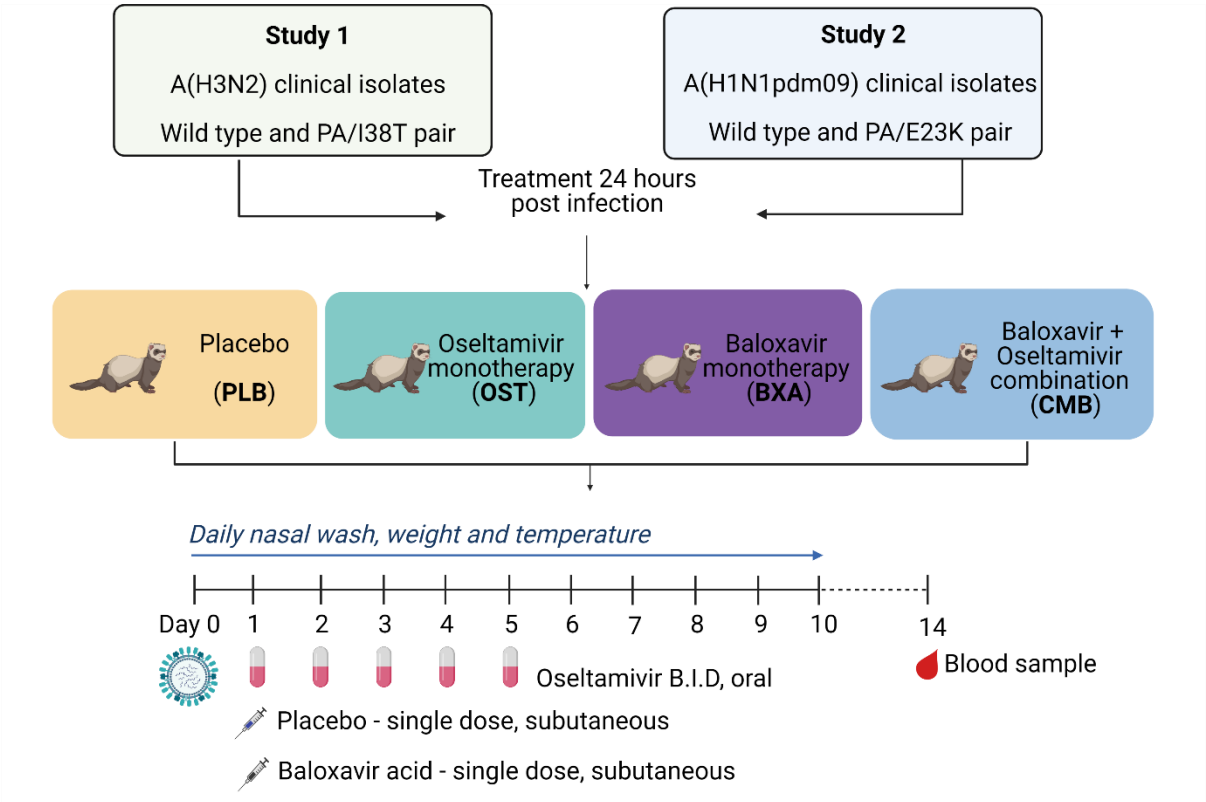

Figure 2. Schematic of the experimental model used to assess baloxavir and oseltamivir combination therapy against influenza viruses with reduced antiviral susceptibility in ferrets.

Ferrets were infected with 10^5 $TCID_{50}$/500 µL (A(H3N2) virus) or 10^4 $TCID_{50}$/500 µL (A(H1N1pdm09) virus) via the intranasal route and antiviral treatment was commenced 24 hours later. Antiviral treatment included 4 mL/kg placebo (subcutaneous single dose, methylcellulose vehicle), 10 mg/kg/day oseltamivir monotherapy (oral, twice a day BID), 4 mg/kg baloxavir monotherapy (subcutaneous, single dose) or a combination of baloxavir and oseltamivir (doses as described for each monotherapy). Nasal washes were collected daily for 10 days and animal's weight and temperature were monitored for 14 days. On day 14 ferrets were sacrificed and a blood sample was obtained.

4.3.2.1 A(H3N2)-wild-type and A(H3N2)-PA/I38T clinical isolate pair

In ferrets infected with the A(H3N2)-WT virus, oseltamivir monotherapy (mean ± standard deviation AUC: 19.7 ± 1.6, $p = 0.046$) and combination treatment (AUC: 20.4 ± 1.2, $p < 0.01$) both reduced the AUC by 24% and 22% compared to placebo (AUC: 25.9 ± 1.3), whilst baloxavir monotherapy (AUC: 18.7 ± 1.1, $p < 0.01$) led to the greatest reduction in AUC relative to placebo (28%) (Figure 3a). Combination treatment was not more effective at reducing viral shedding than either monotherapy. The duration of viral shedding was 1.7 days shorter in ferrets treated with baloxavir monotherapy (4.3 days) than in ferrets that received either oseltamivir monotherapy or combination treatment (5.3 and 5 days, respectively), or those that received placebo (6 days) (Table 1).

In ferrets infected with the A(H3N2)-PA/I38T virus, oseltamivir (AUC: 16.5 ± 1.1, $p = 0.03$) and combination treatment (AUC: 16.7 ± 1.8, $p = 0.03$) performed similarly and reduced the AUC by 37% and viral shedding by 2.7 days relative to placebo (AUC: 26 ± 1) (Figure 3b, Table 1). Whereas, baloxavir monotherapy (AUC: 23.4 ± 1.9, $p = 0.087$) resulted in only a 10% reduction in AUC and 0.6 day reduction in viral shedding, indicating that although some effect of baloxavir was retained, it was significantly reduced compared to that achieved against the WT virus (Figure 3b).

4.3.2.2 A(H1N1pdm09)-wild-type and A(H1N1pdm09)-PA/E23K clinical isolate pair

We sought to explore the *in vivo* effectiveness of baloxavir in ferrets infected with a clinical A(H1N1pdm09) isolate containing the PA/E23K substitution obtained after treatment, paired with a pre-treatment isolate. In ferrets infected with the A(H1N1pdm09)-WT virus, baloxavir (AUC: 21.8 ± 1.7, $p = 0.01$) and combination therapy (AUC: 21.7 ± 3, $p < 0.01$) reduced the average AUC by 32%, compared to placebo (AUC: 29.3 ± 3.2) (Figure 4a), and reduced the duration of viral shedding by an average of 2.3 days than placebo-treated animals (Table 1). Oseltamivir (AUC: 30 ± 1.4, $p = 0.21$) treatment was less effective than baloxavir monotherapy or combination therapy in reducing viral shedding, with an AUC only 6% lower than in placebo-treated animals (Figure 4a). This contrasts with the

A(H3N2) virus, where oseltamivir monotherapy reduced the average AUC by 24% compared to the corresponding placebo group (Figure 3a). A 1000-fold reduction in mean viral titre was observed in baloxavir monotherapy treated ferrets one day following antiviral treatment (Figure 4a), compared to a 100-fold reduction in ferrets receiving placebo or combination treatment and no reduction in oseltamivir treated ferrets. In addition, the NA/H275Y substitution was identified by pyrosequencing in all ferrets treated with oseltamivir monotherapy (3/3) but was not present in any other antiviral treatment group. In ferrets infected with the A(H1N1pdm09)-E23K virus, a rapid reduction in viral load was not observed in the first 24 hours following baloxavir treatment. In addition, the effectiveness of baloxavir monotherapy (AUC: 28.6 ± 1.1, $p = 0.59$) was greatly reduced, with no significant difference in AUC compared to the placebo-treated group (AUC: 27.9 ± 1.9 (Figure 4b), confirming that the PA/E23K amino acid substitution confers reduced susceptibility to baloxavir *in vivo*. Oseltamivir monotherapy (AUC: 26.2 ± 1.7, $p = 0.49$) and combination therapy (AUC: 25.7 ± 1.6, $p = 0.39$) resulted in an average AUC reduction of only 6% and 8%, respectively compared to placebo (Figure 4b). In these ferrets, the NA/H275Y amino acid substitution was present in all ferrets treated with oseltamivir monotherapy (3/3) three to five days after antiviral treatment was commenced and in one ferret treated with oseltamivir + baloxavir combination (1/3), on the final day of infectious virus shedding.

Table 1. Influenza viruses utilised in study with in vitro susceptibility (EC_{50}) and upper respiratory tract viral shedding characteristics in ferrets

	A(H3N2)			A(H1N1pdm09)		
	100% Wild-Type	*80% Wild-Type: 20% PA/I38T*	*100% PA/I38T*	*100% Wild-Type*	*80% Wild-Type:20% PA/E23K*	*100% PA/E23K*
Baloxavir EC_{50} (nM) [#]						
	0.6 ± 0.2	N/A	46 ± 6.9	1.2 ± 0.2	N/A	20.8 ± 10.2
Oseltamivir IC_{50} (nM) [#]						
	0.22 ± 0.2	N/A	0.15 ± 0.11	0.3 ± 0.03	N/A	0.33 ± 0.04
Duration of Viral Shedding (Days) [#]						
Placebo	6 ± 0	6.3 ± 0.5	6.3 ± 0.5	6.3 ± 0.5	6 ± 0	6 ± 0
Oseltamivir	5.3 ± 0.5	5.3 ± 0.5	3.7 ± 1.2	6 ± 0	6.7 ± 0.5	6.3 ± 0.5
Baloxavir	4.3 ± 0.9	4.7 ± 0.5	5.7 ± 0.5	4 ± 0.8	5.3 ± 0.5	6.3 ± 0.5
Combination	5 ± 0	3.7 ± 1.7	3.7 ± 0.9	4 ± 0.8	5.7 ± 1.2	5.7 ± 0.5
Area Under the Curve [#]						
Placebo	25.9 ± 1.3	25.1 ± 2.9	26 ± 1	32 ± 1.6	26.3 ± 1.2	27.9 ± 1.9
Oseltamivir	19.7 ± 1.6*	19.3 ± 1.8	16.5 ± 1.1*	30 ± 1.4	26.9 ± 1.7	26.2 ± 1.7
Baloxavir	18.7 ± 1.1**	18.6 ± 1.4	23.4 ± 1.9	21.8 ± 1.7*	24.7 ± 2.4	28.6 ± 1.1
Combination	20.4 ± 1.2**	16.8 ± 3.4	16.7 ± 1.8*	21.7 ± 3**	23.9 ± 3.3	25.7 ± 1.6

[#] *Data is represented as Mean ± Standard Deviation*

** P < 0.05, ** P < 0.01, *** P < 0.001, for the treatment group compared to placebo in area under the curve analysis (Unpaired t test with Welch's correction)*

N/A not applicable

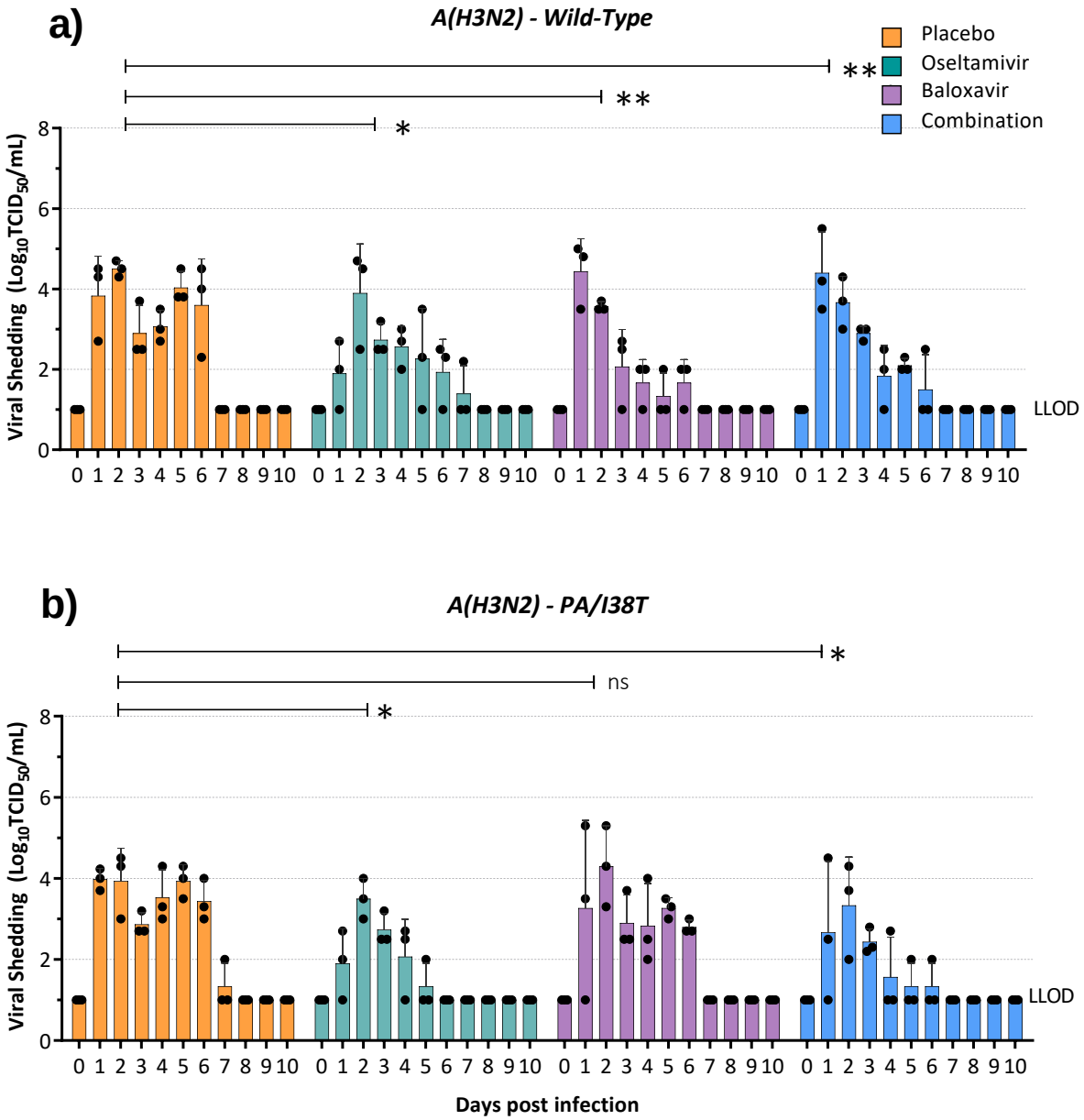

Figure 3. Effect of antiviral treatment on viral shedding in ferrets infected with A(H3N2)-WT or A(H3N2)-PA/I38T. Ferrets were inoculated intranasally with 10^5 $TCID_{50}$/500 µL with a) a pure population of A(H3N2)-WT or b) a pure population of A(H3N2)-PA/I38T. Antiviral treatment was commenced 24 hours post infection with 1 mL/kg placebo (subcutaneous single dose, methylcellulose vehicle), 10 mg/kg/day oseltamivir monotherapy (oral, twice a day BID), 1 mg/kg baloxavir monotherapy (subcutaneous, single dose) or a combination of baloxavir and oseltamivir (doses as described for each monotherapy). Nasal washes were collected daily for ten days and infectious virus titre was determined in MDCK-SIAT cells. The viral titre in the nasal washes of ferrets in each antiviral treatment group is represented by the mean ± standard deviation and the area under the curve for each group is shown above the bar graph. Statistical analysis compares the area under the curve for each antiviral treatment to the corresponding placebo (unpaired t test with Welch's correction) where: ns = non-significant, * $P < 0.05$, ** $P < 0.01$. The lower limit of detection (LLOD) for the assay is 10^1 $TCID_{50}$/mL.

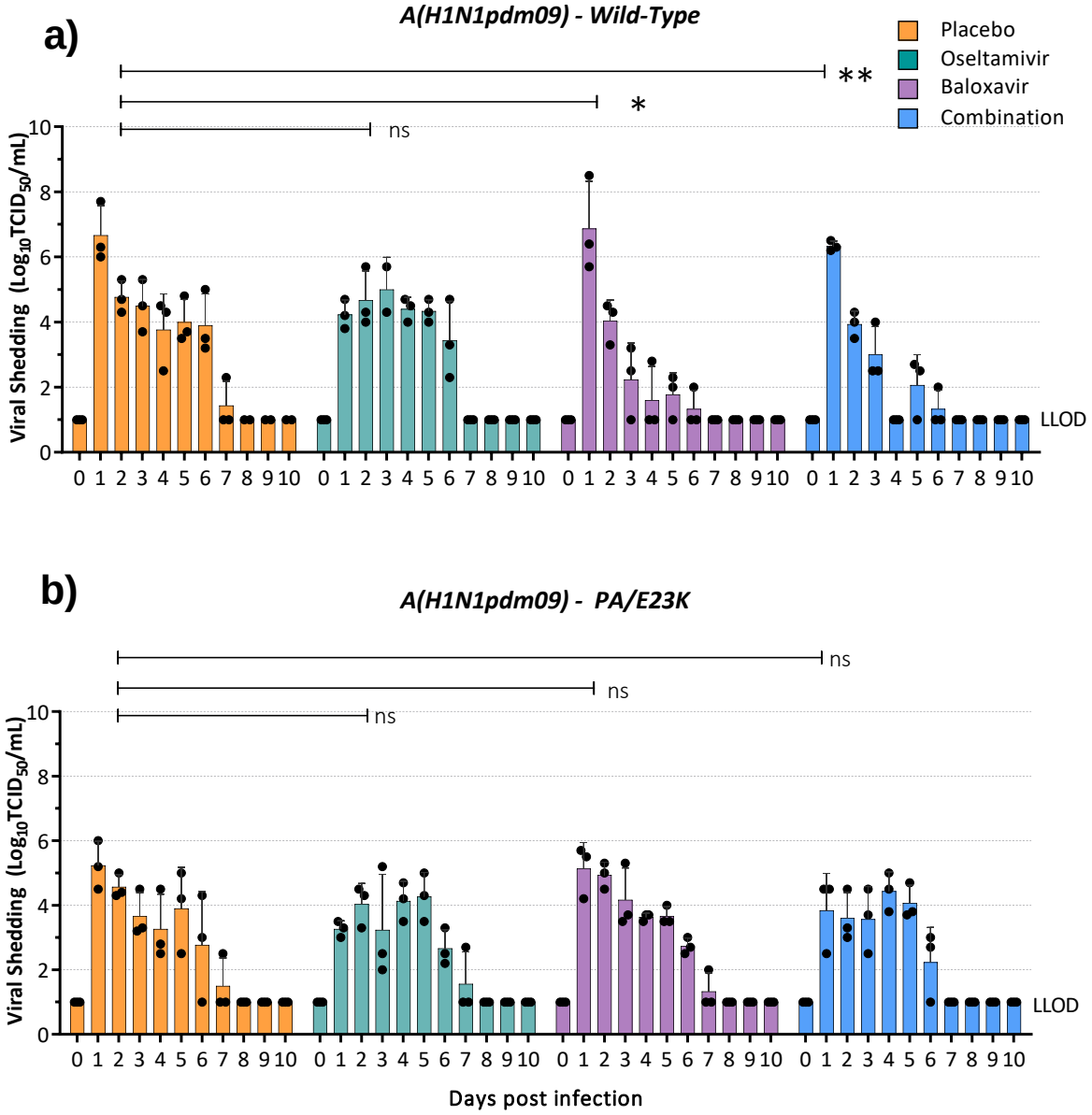

Figure 4. Effect of antiviral treatment on viral shedding in ferrets infected with A(H1N1pdm09)-WT or A(H1N1pdm09)-PA/E23K. Ferrets were inoculated intranasally with 10^5 $TCID_{50}$/500 µL with a) a pure population of A(H1N1pdm09)-WT or b) a pure population of A(H1N1pdm09)-WT. Antiviral treatment was commenced 24 hours post infection with 1 mL/kg placebo (subcutaneous single dose, methylcellulose vehicle), 10 mg/kg/day oseltamivir monotherapy (oral, twice a day BID), 1 mg/kg baloxavir monotherapy (subcutaneous, single dose) or a combination of baloxavir and oseltamivir (doses as described for each monotherapy). Nasal washes were collected daily for ten days and infectious virus titre was determined in MDCK-SIAT cells. The viral titre in the nasal washes of ferrets in each antiviral treatment group is represented by the mean ± standard deviation and the area under the curve for each group is shown above the bar graph. Statistical analysis compares the area under the curve for each antiviral treatment to the corresponding placebo (unpaired t test with Welch's correction) where: ns = non-significant, * $P < 0.05$, ** $P < 0.01$. The lower limit of detection (LLOD) for the assay is 10^1 $TCID_{50}$/mL.

4.3.3 Effectiveness of baloxavir and oseltamivir combination therapy in ferrets infected with mixed populations of clinical isolates

In patients, viruses with reduced susceptibility to baloxavir tend to emerge from a minor population that is selected for and increases in proportion over time during treatment. We modelled this situation by co-infecting ferrets with an 20:80 mixture of pre- and post-treatment isolates and studied the effectiveness of antiviral treatment on the competitive viral mixture estimated by pyrosequencing.

4.3.3.1 A(H3N2)-Wild-type and A(H3N2)-PA/I38T clinical isolate pair

In ferrets infected with a mixture of 20% A(H3N2)-PA/I38T:80% A(H3N2)-WT, treatment with placebo or oseltamivir did not select for a significant increase (or decrease) in viruses that contained PA/I38T over the duration of viral shedding. However, the proportion of PA/I38T increased rapidly following baloxavir monotherapy to 72-83% on the final day of viral shedding (Figure 5c). Even though baloxavir monotherapy increased the propensity of PA/I38T, both oseltamivir and baloxavir monotherapy reduced viral shedding to similar levels with a 23% (AUC: 19.3 ± 1.8, p = 0.065) and 26% (AUC: 18.6 ± 1.4, p = 0.072) reduction in AUC compared to placebo treated ferrets, respectively (Figure 5b, 5c)

In ferrets receiving combination treatment, two had a final proportion of PA/I38T of 34-46%, while the third ferret had a higher final proportion of 95%, which was comparable to the PA/I38T proportion in ferrets treated with baloxavir monotherapy (Figure 5d). In these ferrets receiving combination treatment, the average duration of viral shedding was 2.7 days shorter than in placebo-treated ferrets (Table 1). The two ferrets with a low proportion of PA/I38T ceased shedding infectious virus on days 3 and 4 post-infection (days 2 and 3 post-baloxavir treatment) (Figure 5d), while the third ferret with a high proportion of PA/I38T had an extended duration of viral shedding that lasted 6 days post-infection (Figure 5d). Viral shedding in ferrets treated with the combination of oseltamivir and baloxavir gave an average reductions of 34% (AUC: 16.8 ± 3.4, p = 0.064) relative to

placebo (AUC: 25.1 ± 2.9) but the difference did not reach statistical significance (Figure 5a).

Whole genome sequence (WGS) analysis was conducted on all ferrets to determine if any additional amino acid substitutions were selected under antiviral pressure and to investigate if the ferret receiving combination treatment with extended viral shedding had acquired any amino acid substitutions associated with reduced oseltamivir susceptibility. Several non-synonymous amino acid changes were identified, of note PA/E677K, NA/V240I and NA/D251H were identified under combination treatment pressure (Table 2). However, these substitutions did not lead to a phenotypic change in drug susceptibility (data not shown). We did not identify any amino acid changes to explain why the single ferret treated with the antiviral drug combination had extended viral shedding.

4.3.3.2 A(H1N1pdm09)-Wild-type and A(H1N1pdm09)-PA/E23K clinical isolate pair

Treatment with placebo or oseltamivir did not select for a significant increase (or decrease) in viruses that contained PA/E23K over the duration of viral shedding. For ferrets treated with baloxavir monotherapy, the proportion of PA/E23K ranged from 8-40% on the final day of virus shedding (Figure 6c). In ferrets treated with the combination, the proportion of PA/E23K ranged from 15-40%, values that were similar to that seen for baloxavir monotherapy (Figure 6d). Even though the overall duration of virus shedding for the PA/E23K mixture was longer than for the PA/I38T mixture, thereby allowing a longer duration for selection of viruses with reduced antiviral susceptibility, the proportion of the PA/E23K virus was not as strongly selected as PA/I38T was in ferrets in the competitive mixture experiments. Baloxavir monotherapy and combination therapy resulted in similarly minor reductions in AUC of 6% (AUC: 24.7 ± 2.4, p = 0.31) and 9% (AUC: 23.9 ± 3.3, p = 0.43), respectively compared to placebo, even in the presence of PA/E23K (AUC: 26.3 ± 1.2) (Figure 6a, 6c, 6d).

WGS was performed on samples obtained from ferrets with a mixed infection on day five for genetic analysis. Three PA substitutions, S272R, P325S, R496Q, were identified in the

combination treated ferrets. Strikingly all three ferrets treated with oseltamivir monotherapy had viruses with an NA/H275Y substitution which is known to reduce susceptibility to oseltamivir (Table 2). This was associated with prolonged viral shedding in ferrets in the oseltamivir treatment group, with an AUC that was 2% greater than placebo (AUC: 26.9 ± 1.7, p = 0.27) (Figure 6b).In the combination treated ferrets, the NA/H275Y substitution was only identified in one ferret on the final day of infectious viral shedding (1/3). There were no NA/H275Y substitutions identified in the placebo or baloxavir only treated ferrets.

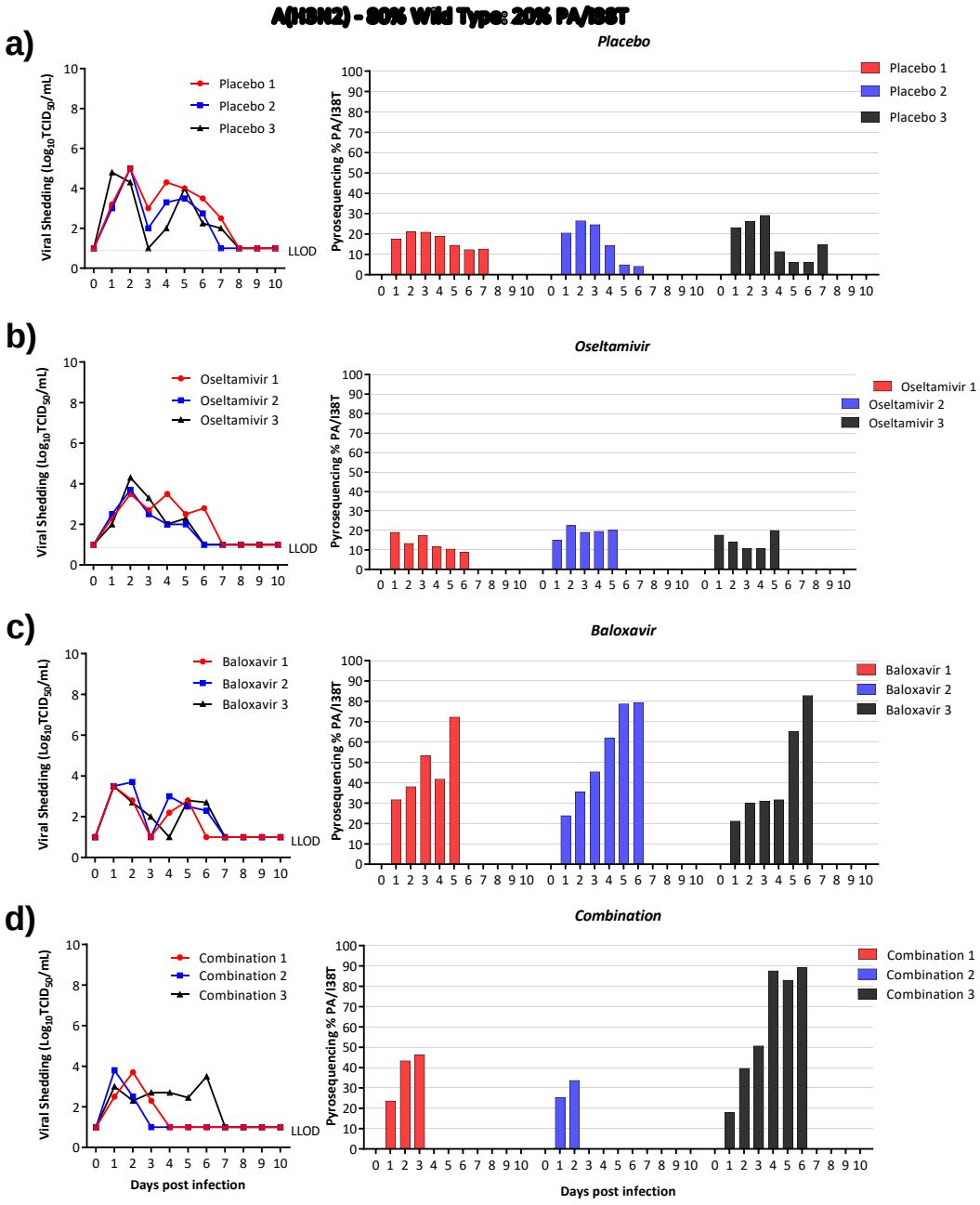

Figure 5. Effect of antiviral treatment on the relative proportion of A(H3N2)-PA/I38T in ferrets infected with a competitive mixture of WT:PA/I38T. Ferrets were intranasally inoculated with 10^5 $TCID_{50}$/500 µL of 20% A(H3N2)-PA/I38T: 80% A(H3N2)-WT. Antiviral treatment was commenced 24 hours post-infection with a) placebo (subcutaneous single dose, methylcellulose vehicle), b) oseltamivir monotherapy (oral, twice a day BID), c) baloxavir monotherapy (subcutaneous, single dose) or d) combination therapy with oseltamivir and baloxavir (doses as described for each monotherapy). Nasal washes were collected daily for ten days and the infectious virus titre was determined by titration in MDCK cells (left panel) and the percentage of PA/I38T in the nasal wash was determined by pyrosequencing for the duration of viral shedding (right panel). The average area under the curve is represented above the viral shedding for each treatment group. The pyrosequencing plots are shown for each individual ferret and the colours correspond to the virus titre in $TCID_{50}$. Whole genome sequencing was performed on samples obtained from day five or the final day of viral shedding. The lower limit of detection (LLOD) for the $TCID_{50}$ assay is 10^1 $TCID_{50}$/mL.

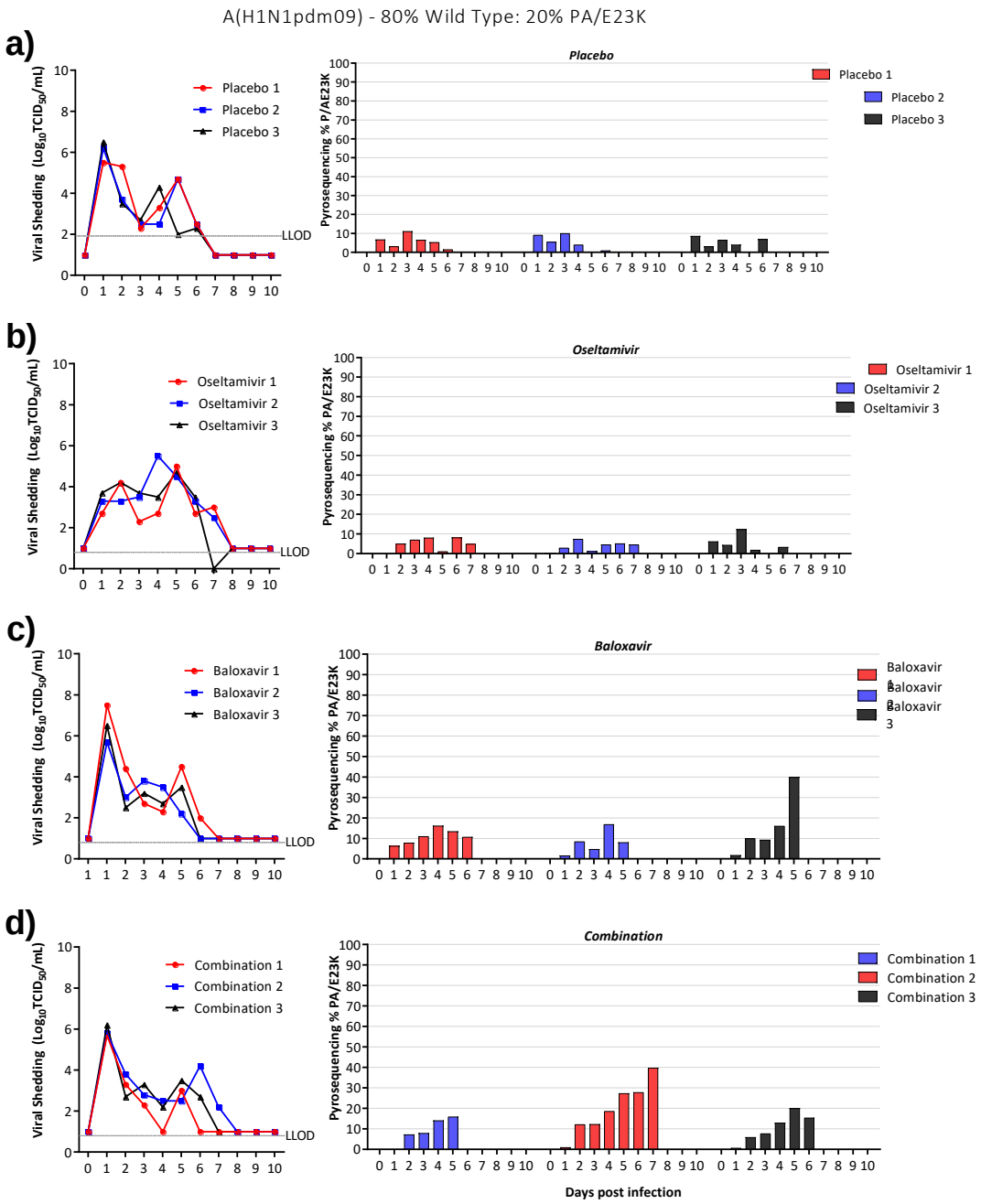

Figure 6. Effect of antiviral treatment on the relative proportion of A(H1N1pdm09)-PA/E23K in ferrets infected with a competitive mixture of WT:PA/E23K. Ferrets were intranasally inoculated with 10^5 $TCID_{50}$/500 µL of 20% A(H1N1pdm09)-PA/E23K: 80% A(H1N1pdm09)-WT. Antiviral treatment was commenced 24 hours post-infection with a) placebo (subcutaneous single dose, methylcellulose vehicle), b) oseltamivir monotherapy (oral, twice a day BID), c) baloxavir monotherapy (subcutaneous, single dose) or d) combination therapy with oseltamivir and baloxavir (doses as described for each monotherapy). Nasal washes were collected daily for ten days and the infectious virus titre was determined by titration in MDCK cells (left panel) and the percentage of PA/I38T in the nasal wash was determined by pyrosequencing for the duration of viral shedding (right panel). The average area under the curve is represented above the viral shedding for each treatment group. The pyrosequencing plots are shown for each individual ferret and the colours correspond to the $TCID_{50}$ shedding. Whole genome sequencing was performed on samples obtained from day five. The lower limit of detection (LLOD) for the $TCID_{50}$ assay is 10^1 $TCID_{50}$/mL.

Table 2. Summary of amino acid substitutions identified from whole genome sequencing in mixed infection ferrets treated with placebo, oseltamivir, baloxavir or a combination of oseltamivir and baloxavir

	Amino acid substitutions in A(H3N2) infected ferrets and number of ferrets in which the substitution was detected out of the total number in each antiviral treatment group			
Gene segment	**Placebo**	**Oseltamivir**	**Baloxavir**	**Combination**
PB2			S107G (1/3)	
PA	I38T (3/3)	I38T (3/3)	I38T (3/3)	I38T (3/3) E677K (1/3)
NP	G384R (1/3)	S450N (1/3)		
HA	N181K (1/3) T176K (1/3)	T327I (1/3)	N181K (1/3)	
NA			E259K (1/3) L163Q (1/3) E41G (1/3)	V240I(1/3) D251H (1/3)
NS			N176I (1/3)	
	Amino acid substitutions in A(H1N1pdm09) infected ferrets and number of ferrets in which the substitution was detected out of the total number in each antiviral treatment group			
Gene segment	**Placebo**	**Oseltamivir**	**Baloxavir**	**Combination**
PA	E23K (3/3)	E23K (3/3)	E23K (3/3)	E23K (3/3) S272R(1/3) P325S (1/3) R496Q (1/3)
NA		H275Y (3/3)		
NS				A257T (1/3)

4.4 Discussion

In this study we evaluated the effectiveness of baloxavir and oseltamivir combination therapy on pairs of influenza clinical isolates that have reduced *in vitro* susceptibility to baloxavir due to two different amino acid substitutions, PA/I38T and PA/E23K, and asked whether treatment with a drug combination could reduce the selection of these viruses. To our knowledge, this study is the first to assess the PA/E23K substitution *in vivo* and the first to study the effectiveness of baloxavir against viruses with either substitution (PA/I38T and PA/E23K) in the ferret model.

Our results demonstrated *in vitro* drug synergy for the combination of oseltamivir and baloxavir, even when tested against viruses with reduced baloxavir susceptibility. *In vivo* studies showed that for the treatment of baloxavir-sensitive viruses, the combination of oseltamivir and baloxavir offered no major virological benefit over either monotherapy. We confirmed that baloxavir monotherapy provides only a minor benefit in the reduction of viral shedding for ferrets infected with PA/I38T *in vivo* and that baloxavir + oseltamivir provided no added benefit over monotherapy. Baloxavir was also not effective in reducing viral shedding for the PA/E23K virus relative to placebo treatment.

A previous study in mice showed that baloxavir treatment (10 mg/kg and 30 mg/kg) 24 hours after infection with a PA/I38T virus led to three-fold (0.47 $\log_{10}$ $TCID_{50}$/mL, rgA/WSN/33) and five-fold (0.78 $\log_{10}$ $TCID_{50}$/mL, rgA/WSN/33) reduction in viral lung titres, compared to the control[154]. This demonstrates that the PA/I38T substitution leads to a significant reduction of baloxavir effectiveness *in vivo*, as we observed in our study. Furthermore, baloxavir monotherapy provided only a small reduction in AUC for the A(H3N2)-PA/I38T virus and no effect on the A(H1N1pdm09)-PA/E23K virus, although the *in vitro* baloxavir EC_{50} is 4.5-fold lower for PA/E23K than PA/I38T. A potential explanation is that the substitutions were present in different influenza A subtypes (H1N1pdm09 versus H3N2) and it is known that A(H1N1pdm09) viruses tend to replicate more efficiently than

A(H3N2) viruses in ferrets [155]. This may have resulted in a greater ‘barrier’ for antiviral activity to the A(H1N1pdm09) virus.

For oseltamivir monotherapy, previous studies have not shown an antiviral effect in a treatment model (i.e. when treatment is commenced after viral infection); alternative study designs such as prophylactic oseltamivir treatment, or donor and recipient viral transmission models have been used[156-159]. In a clinical setting, antiviral drug treatment for influenza is recommended within 48 hours of symptom onset for optimal effectiveness. Our study design of antiviral treatment commencing one day post infection recapitulates a more realistic clinical scenario than providing drug at the time of (or prior to) infection, but resulted in low oseltamivir effectiveness against A(H1N1pdm09) viruses.

In vitro studies, including ours, have shown that the combination of baloxavir and oseltamivir is synergistic[160]. However, a synergistic effect on the reduction of viral titre *in vivo*, in mice or ferrets, has not been demonstrated. In mice infected with influenza A/PR/8/34. The reduction in viral lung titre following combination treatment with baloxavir (0.5 mg/kg, B.I.D) and oseltamivir (10 mg/kg, B.I.D) was approximately equal to the reduction from baloxavir monotherapy alone[144]. In an A(H3N2) immunodeficient mouse model, the reduction in lung viral titre was the same in mice treated with baloxavir monotherapy (40 mg/kg, single dose) was similar to that in mice treated with a combination of baloxavir and oseltamivir (40 mg/kg single dose and 20 mg/kg. B.I.D, respectively)[161]. Treatment with baloxavir has been demonstrated to result in a rapid reduction of virial load in preclinical and clinical studies; based on our current knowledge the addition of oseltamivir does not provide added benefit[74, 162].

From a clinical perspective, baloxavir monotherapy for a virus that harbours a PA/I38T or PA/E23K substitution is unlikely to provide a significant virological benefit to patients. Oseltamivir monotherapy or oseltamivir + baloxavir combination therapy may be useful, but only if oseltamivir is effective

In the mixed viral infections, antiviral treatment pressure on the proportion of variant virus over time was compared to the virological outcome. In the PA/I38T mixture experiments, combination treatment reduced the rapid selection of PA/I38T and was virologically more effective than baloxavir monotherapy in two out of the three ferrets. Notably, PA/I38T increased to a high proportion following baloxavir monotherapy but the reduction in viral shedding was similar to oseltamivir monotherapy and was reduced relative to placebo. There was a lower propensity to select for PA/E23K compared to PA/I38T under baloxavir selection pressure (either monotherapy or combination therapy) and interestingly, the *de novo* selection of NA/H275Y was greatly reduced with combination therapy compared to oseltamivir monotherapy. A small increase in the proportion of PA/E23K still led to reduced viral shedding with baloxavir monotherapy and combination therapy relative to placebo.

If a virus with reduced susceptibility to baloxavir emerges in a patient, treatment decisions may depend on whether the patient is hospitalised or not. As baloxavir is administered as a single dose, further adjustments to the antiviral treatment course may not be feasible. Our study in ferrets suggests that although the proportion of virus with reduced drug susceptibility may increase in proportion over time in an otherwise-healthy, host it may not lead to a significant change in the duration and amplitude of viral shedding. In this case, a change in drugs or commencement of combination therapy may have negligible benefit, particularly if the duration of viral shedding is short. Given that our study has shown reduced drug effectiveness with a pure population of PA/I38T or PA/E23K the risk of transmitting a drug-resistant variant should also be considered.

On the other hand, prolonged viral shedding in immunocompromised individuals increases the risk of selecting resistant viruses during antiviral treatment; several case reports have described this phenomenon for the NAIs[163-165]. Investigators may alter antiviral drug treatment regimens if variants emerge in patients[166, 167]. Combination therapy may be useful from the outset of treatment as our results suggest that baloxavir + oseltamivir may

be effective in decreasing the selection of viruses with reduced susceptibility. A serial passaging study in mice has also shown that baloxavir + oseltamivir can reduce the *de novo* emergence of variants with reduced susceptibility to either drug[168].

A Phase III clinical trial in hospitalised patients with influenza (Flagstone; NCT03684044) showed the addition of baloxavir to 'standard of care' oseltamivir compared to placebo did not affect the time to clinical improvement: 97.5 and 100.2 hours, respectively. Baloxavir and oseltamivir in combination reduced viral shedding more effectively in patients infected with a drug-sensitive virus as the time to cessation of viral shedding was 39.8 hours shorter in the NAI + baloxavir (23.9 hours) compared to the NAI + placebo treated group (63.7 hours)[169]. In patients infected with an A(H3N2) virus, no PA/I38X or NA/H275Y substitutions were identified following NAI + baloxavir treatment. For A(H1N1pdm09), a PA/I38X variant was selected in 2% of patients (3/134) treated with NAI + baloxavir and NA/H275Y occurred in 2.5% of patients (5/199) treated with an NAI (combined with either placebo or baloxavir). Notably, combination treatment led to a dual amino acid change (NA/H275Y + PA/I38T) in two immunocompromised patients. In previous trials, monotherapy with baloxavir or oseltamivir for A(H1N1pdm09) viruses, resulted in 9.7% and 5% of post-treatment isolates with PA/I38X or NA/H275Y, respectively. Therefore, in the Flagstone trial, the combination treatment in a hospitalised treatment group had an overall lower selection of viruses with reduced susceptibility and a more rapid reduction of viral shedding, but the emergence of variants with reduced susceptibility to both drugs in two patients is of concern[169].

Viral fitness is a consideration for the emergence of viruses with reduced susceptibility as it influences the likelihood that a virus will transmit efficiently from person to person. The conclusions for the fitness of influenza viruses with PA/I38X substitutions in animal models vary, but tend to have similar fitness to matched baloxavir-sensitive viruses[170-173]. Here we continued the analysis of a previously studied clinical isolate pair where it was

determined that the amino acid substitution A(H3N2)-PA/I38T results in some within-host attenuation of viral fitness in ferrets[146]. If only placebo treated ferrets are considered, the ferrets infected with a A(H1N1pdm09)-PA/E23K virus have a small reduction in AUC relative to wild-type, suggesting this substitution may reduce viral fitness. Additional *in vivo* studies are needed to determine the fitness of viruses with substitutions in the PA gene other than at the PA/I38X to understand this further.

In conclusion we have shown that amino acid substitutions PA/I38T and PA/E23K lead to reduction of baloxavir effectiveness *in vivo*, and that baloxavir and oseltamivir used in combination did not result in a synergistic benefit. We did, however, show that combination treatment reduced the selection of PA/I38T viruses, relative to baloxavir monotherapy. Given that such variants are of significant concern, further investigation of combination treatment with baloxavir and oseltamivir as a method to reduce the selection of viruses are warranted.

Chapter 5: Adapted SEIR model with baloxavir and oseltamivir combination treatment to determine the effectiveness of antiviral treatment on the spread of influenza in the population

5.1 Introduction

Influenza viruses circulate globally and cause respiratory illness in humans; in the absence of stay-at-home measures and border restrictions due to the on-going SARS-CoV-2 pandemic, infection can lead to 250,000 to 650,000 lives lost globally per year[17]. There are four influenza types, classed A, B, C and D, of which types A and B cause seasonal epidemics[174]. Two subtypes of influenza A, termed A(H3N2) and A(H1N1pdm09) and two lineages of influenza B, B/Yamagata and B/Victoria cause seasonal influenza epidemics[2].

Vaccination and antiviral treatment are public health measures that can be used to control the burden of infectious disease in the population and annual vaccination for influenza is encouraged in many countries. In the event that a novel strain of influenza emerges and causes a pandemic, a vaccine will require approximately six months to be manufactured and during this time antiviral drugs are likely to play an important role for the treatment of influenza. The neuraminidase inhibitor (NAI) antiviral drugs have been licensed since the early 2000's, this drug class inhibits the neuraminidase protein on the surface of the virus that is involved in viral release from the host cell. Of the NAIs, oseltamivir (Tamiflu®) is the most frequently used and the standard of care for treatment of severe influenza illness[175]. When administered within 48 hours of symptom onset, oseltamivir can reduce the duration of symptoms by 1.5 days and the likelihood of severe outcomes in patients[57]. In 2018, an antiviral drug with a novel mechanism of action, baloxavir marboxil (Xofluza®), was licensed for clinical use[176]. This drug inhibits the polymerase acidic (PA) protein involved in viral replication[176]. In a clinical trial that compared treatment with baloxavir to oseltamivir and placebo in otherwise healthy individuals, the median time to cessation of viral shedding was 24, 72 and 96 hours, for baloxavir, oseltamivir and placebo treatment respectively[74]. The time to cessation of symptom resolution was similar between baloxavir and oseltamivir treatment[74].

Although baloxavir is effective in reducing viral shedding in patients, drug resistant viruses are frequently selected during treatment. In clinical trials, amino acid changes that lead to baloxavir resistance occurred in 2.2% of adult patients infected with A(H1N1pdm09) and 9.8% of patients infected with A(H3N2)[74]. The rate of resistance to oseltamivir is lower, amino acid substitutions that cause resistance are present in approximately 1-2% of circulating influenza viruses[177]. Resistance to influenza antiviral drugs usually occurs due to a single amino acid substitution in viral proteins. For example, the most common amino acid substitution to cause resistance to oseltamivir is a histidine at position 275 of the NA protein changing to a tyrosine (i.e. NA/H275Y)[177]. The most common substitution for baloxavir is PA/I38T, though other substitutions such as PA/E23K have been identified[80]. Such amino acid changes can cause drug treatment to be partially or completely ineffective against the virus and if they are adequately fit, drug resistant viruses can circulate globally and render a drug class obsolete as happened with the adamantanes[178].

Antiviral drugs are used in combination for the treatment of other RNA viruses such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) to reduce the selection of resistance and improve the effectiveness of antiviral treatment[179]. Viruses resistant to one class of antiviral drugs, are generally sensitive to other classes of drugs with a different mechanism of action. Options for antiviral combinations for influenza as a limited number of drugs were licensed for treatment. The combination of oseltamivir and baloxavir is an attractive treatment option; the different mechanisms of action and the antiviral potency of baloxavir mean that when used together there may be greater antiviral effectiveness and lower selection of resistance.

It is currently unknown how routine and widespread antiviral treatment with baloxavir, and the subsequent increased selection pressure for antiviral resistance, may affect the transmission of drug sensitive and resistant influenza A viruses in the human population.

The aim of this study is to utilise data from ferret studies and human clinical trials to compare the difference in transmission of two different influenza A subtypes (H3N2 and H1N1pdm09) when baloxavir monotherapy, oseltamivir monotherapy, or combination therapy with both drugs are used. The ferret model is the gold standard animal model to study influenza infection, as they have a similar sialic acid distribution to humans, exhibit clinical signs such as sneezing and can be directly infected with human influenza strains[180].

To approach this aim we developed a deterministic mathematical model that incorporated antiviral effectiveness and the selection of resistant viruses following antiviral treatment. This model was used to simulate low, moderate and high virus transmission of drug-sensitive or drug-resistant influenza viruses by increasing the basic reproduction number (R_0). For each simulation, the relative effect on the time to the epidemic peak, duration and cumulative number of infections was determined. In addition, the proportion of infections due to a resistant virus at the end of the epidemic following monotherapy or combination therapy was compared, when resistance to either antiviral drug could emerge following treatment.

Based on the virological differences of A(H3N2) and A(H1N1pdm09) influenza subtypes, the proportion of resistant viruses selected following treatment and the effectiveness of oseltamivir and baloxavir may vary. Therefore we hypothesised that the viral subtype may be an important factor in the spread of resistant virus in the population under antiviral pressure and that antiviral treatment with a combination of baloxavir and oseltamivir from the outset could be a potential strategy to reduce the selection of resistant virus.

5.2. Methods

5.2.1 SEIR model for influenza with antiviral treatment

To achieve these aims we adapted a deterministic susceptible (S), exposed (E), infectious (I), recovered (R) model in a fixed population (no births or deaths) for an influenza pandemic scenario.

5.2.2 Model 1 Equations

For the first model (Model 1) symptomatic infections with a pure population of baloxavir-sensitive or resistant virus can be treated with an antiviral drug to allow for direct comparison of treatment with baloxavir or oseltamivir or a combination of both (Figure 1).

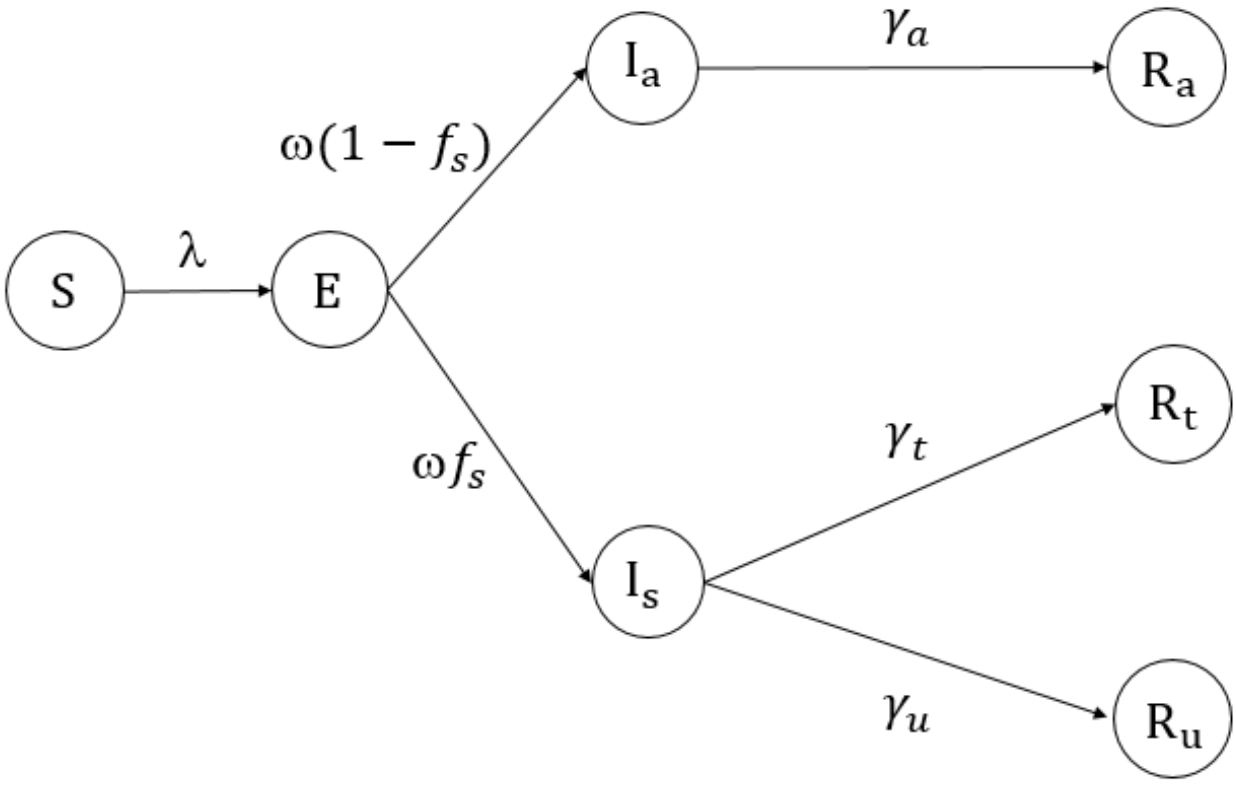

Figure 1. SEIR model for influenza where antiviral treatment can be administered to the symptomatic class. The force of infection (λ) determines the transition rate from susceptible (S) to exposed (E). Following the latent period (ω; mean duration given by 1/ω), a fraction will develop symptoms (f_s). The infectious classes can then be split into asymptomatic (I_a) and symptomatic (I_s) infections. Asymptomatic patients recover at a rate γ_a while symptomatic patients recover at rate γ_t if treated or γ_u if untreated.

The model equations are:

$$\lambda = \beta(I_a + I_s) \quad (1)$$

Where $\beta = \Phi\psi(1-\alpha)$ (2)

$$\frac{dS}{dt} = -\lambda S \quad (3)$$

$$\frac{dE}{dt} = \lambda S - (1-f_s)\omega E - f_s\omega E \quad (4)$$

$$\frac{dIa}{dt} = (1-f_s)\omega E - \gamma_a I_a \quad (5)$$

$$\frac{dI_s}{dt} = f_s\omega E - \gamma_t I_s - \gamma_u I_s \quad (6)$$

$$\frac{dRa}{dt} = \gamma_a I_a \quad (7)$$

$$\frac{dRt}{dt} = \gamma_t I_s \quad (8)$$

$$\frac{dRu}{dt} = \gamma_u I_s \quad (9)$$

5.2.3 Model 2 equations

The second model (Model 2) extends Model 1 by including the processes of the emergence of antiviral resistance and re-infection of the population by the emerging resistant strain (Figure 2; an illustrative diagram is shown in Figure 3). In both models all classes recover and are assumed to have sufficient immunity to prevent re-infection.

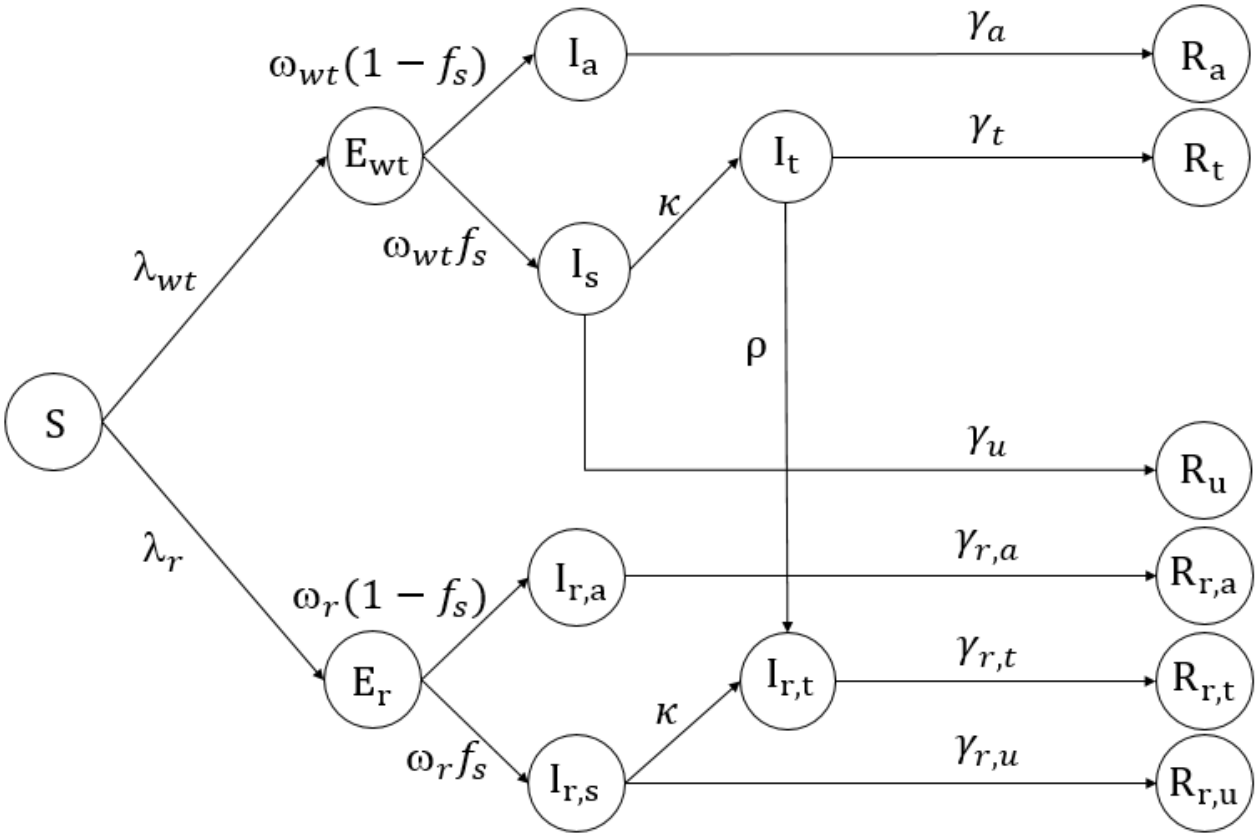

Figure 2. SEIR model for influenza where antiviral treatment can be administered to the symptomatic class and resistant viruses can then be selected in patients treated with an antiviral drug. The susceptible class (S) can be infected with either a drug-sensitive (wild-type; wt) or resistant virus (r), each of these will have a force of infection (λ_{wt} or λ_r) that will determine the rate between S and exposed (E). Following the latent period (E_{wt} or E_r) a fraction (f_s) will have a symptomatic infection and asymptomatic infection (1-f_s), regardless if the infection is drug-sensitive or resistant. If the infection is symptomatic (I_s), there can be antiviral treatment at rate, κ, however mutations that result in antiviral resistance can be selected at the rate ρ.

The model equations are:

$$\lambda_{wt} = \beta_{wt}(I_a + I_s + I_t) \quad (10)$$

Where $\beta_{wt} = \Phi_{wt}(1-\alpha_t^{wt})$ (11)

$$\lambda_r = \beta_r\psi\left(I_{r,a} + I_{r,s} + I_{r,t}\right) \quad (12)$$

Where $\beta_r = \Phi_r(1-\alpha_t^r)$ (13)

$$\frac{dS}{dt} = -(\lambda_{wt} + \lambda_r)S \quad (14)$$

$$\frac{dE_{wt}}{dt} = \lambda_{wt}S - (1-f_s)\omega_{wt}E_{wt} - f_s\omega_{wt}E_{wt} \quad (15)$$

$$\frac{dE_r}{dt} = \lambda_r S - (1-f_s)\omega_r E_r - f_s\omega_r E_r \quad (16)$$

$$\frac{dIa}{dt} = (1-f_s)\omega_{wt}E_{wt} - \gamma_a^{wt}I_a \quad (17)$$

$$\frac{dI_s}{dt} = f_s\omega_{wt}E_{wt} - \kappa I_s - \gamma_u^{wt}I_s \quad (18)$$

$$\frac{dI_t}{dt} = \kappa I_s - \gamma_t^{wt} I_t - \rho I_t \quad (19)$$

$$\frac{dI_r}{dt} = \kappa I_{r,s} + \rho I_t - \gamma_t^{r} I_{r,t} \quad (20)$$

$$\frac{dIr,a}{dt} = (1 - f_s)\omega_r E_r - \gamma_a^{r} I_{r,a} \quad (21)$$

$$\frac{dI_{r,s}}{dt} = f_s \omega_r E_r - \kappa I_{r,s} - \gamma_u^{r} I_{r,s} \quad (22)$$

$$\frac{dRa}{dt} = \gamma_a^{wt} I_a \quad (23)$$

$$\frac{dRt}{dt} = \gamma_t^{r} I_t \quad (24)$$

$$\frac{dRu}{dt} = \gamma_u^{wt} I_s \quad (25)$$

$$\frac{dRr,a}{dt} = \gamma_a^{r} I_{r,a} \quad (26)$$

$$\frac{dRr,t}{dt} = \gamma_t^{r} I_{r,t} \quad (27)$$

$$\frac{dRr,u}{dt} = \gamma_u^{r} I_{r,s} \quad (28)$$

5.2.4 Latent period and fraction of infections that are symptomatic

Following exposure to influenza virus, there is a latent period prior to the onset of infectiousness. The median duration of the latent period is from 1 to 2 days, with a range from 1 to 4 days[181-183], however there are studies that suggest a longer duration between 2 and 4 days[184, 185]. In our model we assumed a duration of 2 days for the latent period and assumed that the duration for A(H1N1pdm09) and A(H3N2) drug-sensitive and matched resistant viruses in our simulation are the same (Table 2). Seasonal and pandemic influenza studies demonstrate that a significant proportion of individuals experience an asymptomatic infection[14]. Asymptomatic infections are difficult to identify unless there is large scale testing in the population; therefore, seroconversion rates can aid in providing estimates. However such studies show a high degree of variability in estimates on asymptomatic infection, in part due to differences in study design and influenza strains. A recent systematic review summarised point estimates of asymptomatic infection derived from influenza outbreaks, to range from 4-28% (16% was the pooled mean) and for multiple epidemics the range was 65-85%[186]. Previous modelling studies have estimated that 30-50% of infections are asymptomatic[187-189]. In our model, we set the number of asymptomatic infections at 60% but also made several

additional assumptions: (i) that the asymptomatic and symptomatic classes are equally infectious, (ii) that asymptomatic individuals will not be treated with an antiviral drug due to the lack of symptoms (i.e. no post or pre-exposure prophylaxis), (iii) that asymptomatic infections are just as likely to occur with a drug-sensitive or resistant virus and (iv) will have the same rate of recovery as a symptomatic infection (Table 2).

5.2.5 Basic reproduction number

The basic reproduction number (R_0) is defined to be the number of secondary cases from each infectious case if all contacts are fully susceptible to infection. This is an important parameter characterising the ability of a virus to spread in a fully susceptible population. The basic reproduction number for seasonal influenza has been estimated from past influenza seasons (2016 to 2019) to range from 1.1 to 1.5 [190-192]. However, these estimates are derived from seasonal epidemics and therefore are in the context of a partially immune population. In a pandemic, prior immunity may provide less (or no) protection from a novel influenza strain and it is plausible that novel strains may have a greater R_0 value than a seasonal influenza virus. In our study, we use the estimates of R_0 that are derived from Du et al[193], where a transmission scaling factor is derived from a within-host model of surveillance data from the past influenza season and stochastic simulations were repeated for different R_0 values to estimate values for this parameter. It should be noted that the degree of prior immunity to a novel pandemic strain and the attack rate are difficult to predict so three scenarios are modelled with increasing R_0 values (and therefore the transmission scaling factor) from 1.2, 1.4 and 1.6 to represent a low, moderate or high transmissibility of the virus (Table 2).

5.2.6 Effectiveness of antiviral treatment for baloxavir-sensitive and resistant strains

The infectious and symptomatic class in our model can be treated with an influenza antiviral drug one day after the onset of symptoms. The antiviral treatments available are baloxavir monotherapy, oseltamivir monotherapy or a combination of both drugs. For Model 1, only one influenza virus circulates, either an A(H3N2) or A(H1N1pdm09) subtype,

that is sensitive or resistant to baloxavir (Figure 1). In Model 2, antiviral treatment can select for resistant viruses (Figure 2), therefore treatment with baloxavir or oseltamivir selects resistance at a specified rate (see section 2.7).

For our parameter selection we utilised two sources of information for the effectiveness of antiviral treatment; published clinical data from phase II and III clinical trials[74] and data from our ferret antiviral treatment model (discussed in chapter 4 of this thesis). From phase II and III clinical trials, data are available on the reduction of viral shedding with baloxavir monotherapy and oseltamivir monotherapy for drug-sensitive (i.e., wild-type) infections. Two key parameters are not available from this trial; the first is the effectiveness of baloxavir + oseltamivir in combination for the treatment of drug-sensitive influenza infection, the second is the effectiveness of monotherapy and combination therapy for the treatment of a drug-resistant infection. Therefore, we will utilise information from *in vivo* experiments to estimate parameters of antiviral effectiveness for these two factors. The study outline is summarised in Figure 3.

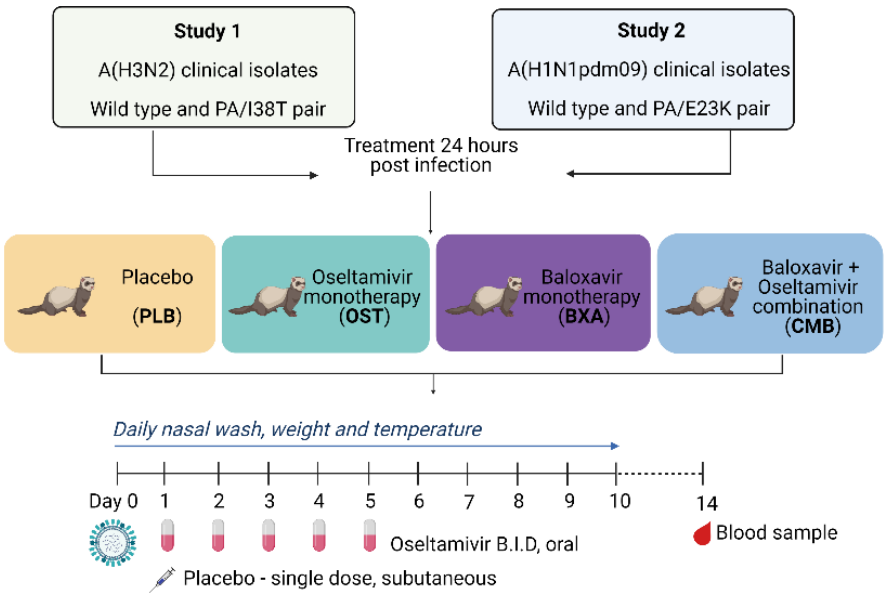

Figure 3. Study outline for source of antiviral effectiveness data. Ferrets were inoculated via the intranasal route with one of four influenza viruses: an A(H3N2) baloxavir-sensitive or resistant virus (due to a PA/I38T) or a A(H1N1pdm09) baloxavir-sensitive or resistant virus (due to PA/E23K). Antiviral treatment was commenced 24 hours post infection with a placebo, baloxavir, oseltamivir or a combination of baloxavir + oseltamivir. Daily nasal washes were collected for ten days from animals and infectious virus titre was determined by titration in MDCK cells and expressed in 50% tissue culture infectious dose ($TCID_{50}$) units.

Due to virological differences, the effectiveness of antiviral treatment will be considered separately for A(H3N2) and A(H1N1pdm09) (Tables 1,2). From the infectious virus titre data obtained from ferrets, the antiviral effectiveness of drug treatment relative to placebo was estimated as summarised in Table 1. To incorporate *in vivo* data into our population model, we introduce the following terms:

$$\beta = \Phi\psi(1-\alpha) \quad (29)$$

that are dependent on Φ, the baseline transmission rate in the absence of antiviral treatment and α, which is the antiviral effectiveness relative to placebo (i.e., no treatment). The effectiveness of antiviral treatment is simplified here to be defined by the difference in the mean area under the curve (AUC) of viral load between placebo and antiviral treated ferrets. A summary of α values and the derivation is shown in Table 1. When α is 0, this represents placebo (i.e. no treatment) and β is then equal to the baseline transmission rate (Φ). The transmission rate can be further tuned by ψ that represents the relative fitness of a virus strain. When ψ is 1, there is no fitness cost on the virus. However, we note that antiviral effectiveness is likely to be linked further to several factors including, but not limited to, the duration and peak viral titre of viral shedding.

Table 1. Effectiveness of antiviral treatment administered 24 hours post infection in the ferret model

	A(H3N2) Baloxavir-sensitive		**A(H3N2) PA/I38T Baloxavir-resistant**	
	Area Under the Curve (AUC) mean ± standard deviation	Antiviral effectiveness (α_t^{wt}relative to placebo	Area Under the Curve (AUC) mean ± standard deviation	Antiviral effectiveness (α_t^{r}relative to placebo
Placebo	22 ± 1.2	0	22.3 ± 1.3	0
Oseltamivir	15 ± 3.1	0.32	10.2 ± 4.3	0.55
Baloxavir	13.1 ± 2.3	0.4	19 ± 2	0.15
Combination	15.4 ± 1.8	0.3	10.3 ± 4.5	0.54
	A(H1N1pdm09) Baloxavir-sensitive		**A(H1N1pdm09) PA/E23K Baloxavir-resistant**	
	Area Under the Curve	Antiviral effectiveness (α_t^{wt}relative to placebo	Area Under the Curve	Antiviral effectiveness (α_t^{r}relative to placebo
Placebo	29.25 ± 3.2	0	23.9 ± 1.3	0
Oseltamivir	25.8 ± 2.2	0.12	22.5 ± 1.5	0.07
Baloxavir	15.8 ± 3.8	0.46	24.9 ± 1.2	0
Combination	15.7 ± 3	0.46	21.4 ± 3.5	0.12

5.2.7 Selection of antiviral resistance following drug treatment

Viruses resistant to baloxavir have been identified in clinical trials and the most common amino acid substitutions occur at position 38 of the PA gene, however other substitutions can also emerge, including PA/E23K, PA/A37T and PA/E119G[80].

The rate at which amino acid substitutions are selected for in patients following antiviral treatment varies depending on viral subtype and age. Clinical trial data show that viruses isolated from children infected with the A(H3N2) subtype have the highest rates of antiviral resistance. Although baloxavir is not currently licensed outside of Japan for patients under the age of 12, we have included some analysis in children where the rate of resistance is higher than in adults. In our model, resistance is only considered for treatment of otherwise-healthy populations infected with influenza. The rate of resistance is related to the probability that a fraction of participants in a clinical trial will develop resistance by,

$$P(t) = 1 - e^{-\rho t}, \tag{30}$$

where t is time and ρ is the rate at which participants develop resistance due to antiviral treatment. Different treatments may lead to different probabilities of resistance (ρ), as shown

in Table 2. The values used are derived from a phase II clinical trials in which a majority of A(H1N1pdm09) infections (2.2% of adult patients; 14.3% of children) and a phase III trial that had mostly A(H3N2) (9.7% of adult patients; 25.7% of children)[74]. For oseltamivir, which has been used clinically for a longer duration, we have derived the rate of resistance based on the most common substitution for each subtype, NA/H275Y in A(H1N1pdm09) and NA/E119V in A(H3N2), and estimated that these occur in 1% of viruses for each (Table 2)[177].

There are insufficient data from clinical trials to estimate the selection of resistance following combination treatment in otherwise healthy patients. Therefore, we utilised our ferret experiment, where ferrets were infected with a mixed infection with 20% resistant + 80% sensitive virus, either A(H3N2)-PA/I38T or A(H1N1pdm09)-PA/E23K. To derive the relative rate of resistance, the difference in the final proportion of resistant virus in the nasal wash on the final day of viral shedding following baloxavir treatment is compared to baloxavir + oseltamivir (Table 2).

5.2.8 Fitness of resistant strains

The fitness of a virus relative to the wild-type strain is dependent on between-host transmission efficiency of a virus and the within-host replication efficacy. The fitness of viruses that harbour PA/I38T substitutions has been studied by several groups[194]. In general, there is mild or no fitness cost for between or within host transmission or replication for viruses with a PA/I38T amino acid substitution *in vivo*. To our knowledge, there are no published studies on the fitness impact of PA/E23K substitutions. For this reason and for continuity with our estimates of antiviral effectiveness, within-host fitness of resistant strains is considered relative to wild-type (drug-sensitive) virus. We derived our fitness impacts for PA/I38T and PA/E23K from the relative AUC of drug-sensitive and matched resistant viruses in ferrets; which provides an estimate of the within-host fitness for this virus (Table 2). For oseltamivir resistant viruses, we assume that there is negligible

fitness cost associated with the NA/H275Y and NA/E119V substitutions, consistent with previous studies (Table 2)[195].

5.2.9 Recovery rate

Based on epidemiological studies we estimate that with no intervention the average duration of infectiousness for influenza infection is 4 days[185]. Given information from the baloxavir clinical trial from which we have derived other parameters, baloxavir treatment reduced the mean time for infectious virus shedding by 75% compared to placebo and by 25% compared to oseltamivir treatment[6]. Therefore, for baloxavir monotherapy we assume a duration of infectiousness of 1 day and 3 days for oseltamivir monotherapy. For baloxavir + oseltamivir combination treatment, we assume that the duration of infectiousness equal to baloxavir monotherapy (Table 2). Additional assumptions we made are that asymptomatic infections and infections with a drug-resistant strain have an equal duration of infectiousness as symptomatic infection.

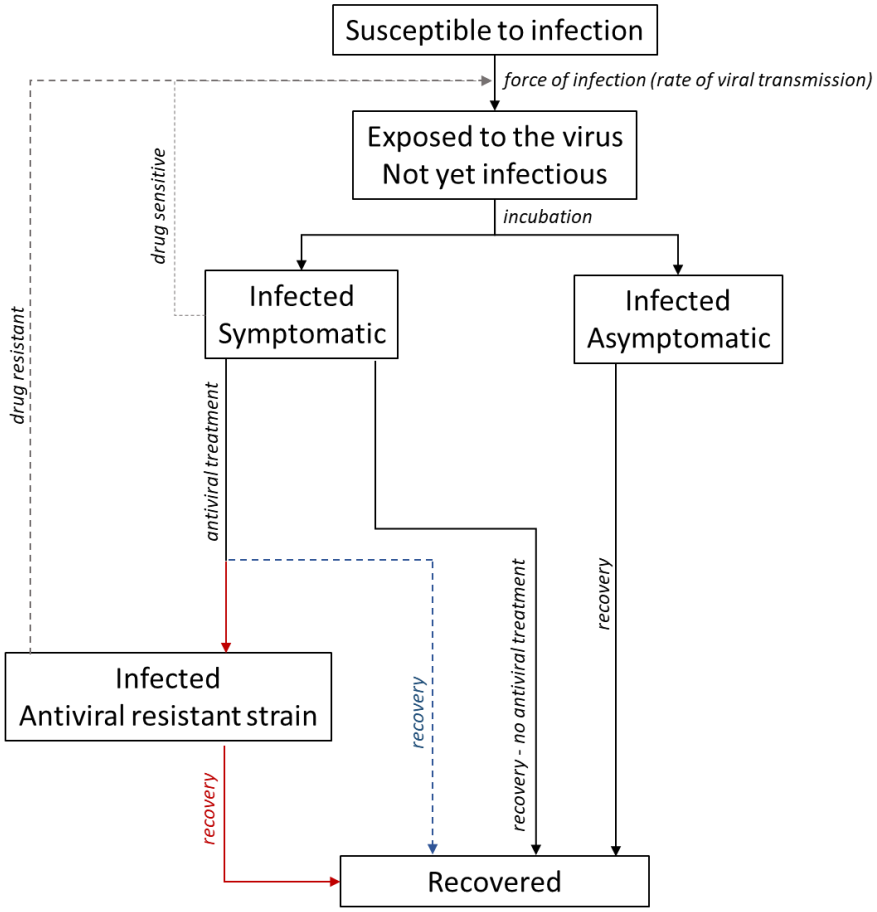

Figure 4. Illustration of the models with antiviral treatment and emergence of resistance. Red and blue lines represent antiviral-resistant and sensitive infections, respectively.

Table 2. Summary of model parameter values

Parameter & Unit	Description	Value		Reference and comments
		A(H3N2)	**A(H1N1pdm09)**	
Φ_{wt} (unit less)	Baseline transmission rate for R_0 1.2, 1.4 and 1.6 (infection with a wild-type strain)	3.03, 3.56, 4.08	3.9, 4.58, 5.25	The estimates for this parameter are derived from Du et. al[196].
Φ_{r} (unit less)	Baseline transmission rate for R_0 1.2, 1.4 and 1.6 (infection with a drug resistant strain)	3.03, 3.56, 4.08	3.9, 4.58, 5.25	These parameters are tentatively assumed to be the same as for wild-type, but are adjusted in the model based on viral fitness (Ψ).
κ (days)	Rate of antiviral treatment for the symptomatic class (days)	1	1	We assume a duration of one day from developing a symptomatic infection to antiviral treatment. This timeframe for treatment also matches our ferret model (see reduction in infectiousness from antiviral treatment section).
α_t^{wt} (unit less)	Reduction of infectiousness of a drug sensitive infection due to antiviral treatment	0.4 (Baloxavir) 0.32 (Oseltamivir) 0.3 (Oseltamivir + Baloxavir)	0.46 (Baloxavir) 0.12 (Oseltamivir) 0.46 (Oseltamivir + Baloxavir)	Estimated from data generated *in vivo* with a ferret model. Antiviral treatment (baloxavir, oseltamivir or baloxavir + oseltamivir) was commenced in ferrets one day following infection with antiviral sensitive (wild-type) viruses obtained from human clinical isolates. Values are estimated from viral shedding measured in ferrets from samples collected from the upper respiratory tract. See text section 2.4.
α_t^{r} (unit less)	Reduction of infectiousness of baloxavir resistant infection due to antiviral treatment	0.15 (Baloxavir) 0.55 (Oseltamivir) 0.54 (Oseltamivir + Baloxavir)	0 (Baloxavir) 0.07 (Oseltamivir) 0.12 (Oseltamivir + Baloxavir)	Estimated from data generated *in vivo* in a ferret model. Antiviral treatment (baloxavir, oseltamivir or baloxavir + oseltamivir) was commenced in ferrets one day following infection with baloxavir resistant viruses matched to the wild-type human clinical isolates. Values are estimated from viral shedding measured in ferrets from samples

				collected from the upper respiratory tract. See text section 2.4.
p (days^{-1})	Rate of resistance emerging following antiviral treatment (Greater than 12 years old)	0.02 (Baloxavir) 0.002 (Oseltamivir) 0.018 (Oseltamivir + Baloxavir)	0.0044 (Baloxavir) 0.002 (Oseltamivir) 0.004 (Oseltamivir + Baloxavir)	The rate of resistance following antiviral treatment in adults treated with baloxavir, oseltamivir or a combination of both. The values for baloxavir are estimated from phase II and III clinical trials[74] and for oseltamivir on circulating seasonal influenza viruses[64]. The rate of resistance emerging from combination antiviral treatment has not been published in clinical trials and is therefore estimated from *in vivo* data obtained from the ferret model. The clinical trials and *in vivo* data provided information on the frequency of resistant viruses being selected following antiviral treatment for specified duration. See section 2.5.
	Rate of resistance emerging following antiviral treatment (In patients older than 12 years of age)	0.059 (Baloxavir) 0.002 (Oseltamivir) 0.053 (Oseltamivir + Baloxavir)	0.031 (Baloxavir) 0.002 (Oseltamivir) 0.028 (Oseltamivir + Baloxavir)	The rate of resistance following antiviral treatment in adolescents treated with baloxavir, oseltamivir or a combination of both. Selection of these values is as outlined for adults.
ω_{wt} (days^{-1})	Rate of progression from the exposed to infectious class for a wild-type virus	0.5	0.5	Estimates from clinical data indicate a range for the exposure period $(1/\omega_{\text{wt}})$ of one to four days[197, 198] We assumed a period of 2 days.
ω_{r} (days^{-1})	Rate of progression from the exposed to infectious class for an antiviral resistant virus	0.5	0.5	There is insufficient evidence to show that the incubation period differs for infection with a resistant virus; given similar fitness and viral shedding kinetics for drug sensitive and resistant viruses we assume that the rate of this parameter is equivalent to the wild-type virus.
f_s (unit less)	Fraction of infections that are symptomatic	0.6	0.6	The fraction of people with influenza infections who develop symptoms varies widely between previous modelling studies (30-50%[188, 189, 199]), retrospective epidemiological studies

				(65-85%[184, 198]) and outbreak studies (4-28%[186, 200]). Taking this into account, we estimate that 60% of infections develop symptoms.
Ψ (unit less)	Relative fitness of a resistant strain compared to the equivalent wild-type	1	0.75	Previous studies of the fitness of baloxavir-resistant viruses tend to show that viruses with a PA/I38T substitution have similar replicative fitness *in vivo* as matched wild-type viruses[194]. Our laboratory has shown that the A(H3N2) wild-type and matched A(H3N2)-PA/I38T virus has limited attenuation of within-host viral fitness. There is no *in vivo* data, aside from our ferret study, available on the fitness impact of the PA/E23K mutation. Therefore our within-host (or replicative) fitness estimate for viruses that harbour these mutations are based on the results of placebo (i.e. untreated) ferrets in our ferret study, see text section 2.6.
γ_u^{wt} $(days^{-1})$	Rate of recovery of a symptomatic, wild-type infection that is not treated with an antiviral drug	0.25	0.25	In the absence of interventions we estimate the duration of the symptomatic period for influenza infection is four days (D_u^{wt} = 4) ($\gamma_u^{wt} = \frac{1}{D_u^{wt}}$), therefore the corresponding rate of recovery is $\gamma_u^{wt} = \frac{1}{D_u^{wt}}$= 0.25
γ_a^{wt} $(days^{-1})$	Rate of recovery for a wild-type and asymptomatic infection	0.25	0.25	For a wild-type virus, we assume the infectious duration for asymptomatic infection to be four days (assumed to be the same as symptomatic infection).
γ_t^{wt} $(days^{-1})$	Rate of recovery for a symptomatic, wild-type infection that is treated with antiviral drug	1 (Baloxavir) 0.33 (Oseltamivir) 1 (Oseltamivir + Baloxavir)	1 (Baloxavir) 0.33 (Oseltamivir) 1 (Oseltamivir + Baloxavir)	Median duration of infectious virus shedding in clinical trials show that the patients treated with baloxavir or oseltamivir was reduced by 75% and 25% compared to placebo-treated patients respectively; for combination treatment there is no data available from otherwise healthy adults therefore we assume an equal rate of recovery as baloxavir treatment alone[74].

γ_a^r (days^{-1})	Rate of recovery of an asymptomatic, baloxavir resistant infection	0.25	0.25	Due to similar fitness of wild-type and resistant viruses, we assume that the duration of the symptomatic period is the same for an asymptomatic infection with a baloxavir-resistant virus as it is for a wild-type virus.
γ_t^r (days^{-1})	Rate of recovery for a baloxavir resistant infection that is treated with an antiviral drug	0.25 (Baloxavir) 0.25(Oseltamivir) 0.33 (Oseltamivir + Baloxavir)	0.25 (Baloxavir) 0.25 (Oseltamivir) 0.33 (Oseltamivir + Baloxavir)	Following baloxavir treatment, a baloxavir-resistant virus is assumed to be infectious period for the same period as an untreated infection. Oseltamivir treatment retains its reduction on the rate of recovery and combination treatment is assumed to be equally effective as oseltamivir monotherapy.
γ_u^r (days^{-1})	Rate of recovery for a baloxavir resistant infection that is untreated	0.25	0.25	Assumed to equal the rate of recovery for an untreated, wild type infection.

5.3. Results

5.3.1. Modelling the treatment of baloxavir-sensitive and baloxavir-resistant viruses with baloxavir monotherapy, oseltamivir monotherapy or combination therapy

To demonstrate the relative effectiveness of antiviral treatments for drug-sensitive A(H3N2) or A(H1N1pdm09) viruses or matched baloxavir-resistant viruses (PA/I38T or PA/E23K), we developed a compartmental model where a single virus strain circulates in the population. Antiviral treatment can occur after the infected class develop symptoms (i.e., Model 1 in Figure 1). Two key variables included in this model, were antiviral effectiveness (α) and viral fitness (ψ). Cumulative infections expressed as a proportion of the population and the relative impact of increasing these parameters where all other parameters are held equal, is shown in Figure 5. To achieve the same change in the final cumulative number of infections for a virus with greater relative fitness, requires increased antiviral effectiveness (Figure 5).

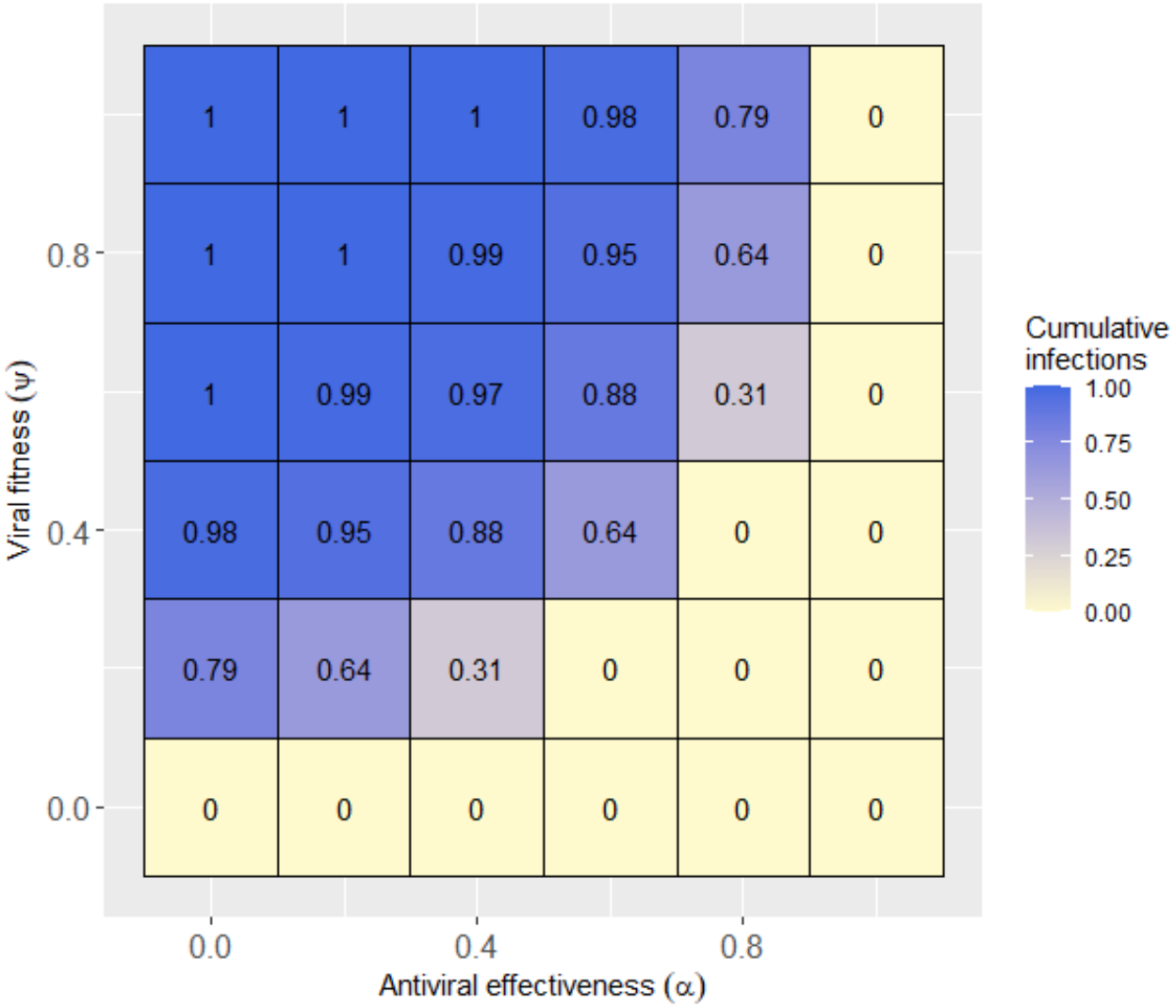

Figure 5. Cumulative infections for different values of the antiviral effectiveness (α) and viral fitness (ψ). Results for cumulative infections represented are relative to a virus with no reduction in fitness treated with an antiviral drug that has no effectiveness (the top left-hand corner of the grid).

Table 3. Characteristics of the epidemic curve with a single virus model following antiviral treatment, relative to no treatment*

	Baloxavir			Oseltamivir			Combination		
	Percentage change in cumulative infections								
R_0	**1.2**	**1.4**	**1.6**	**1.2**	**1.4**	**1.6**	**1.2**	**1.4**	**1.6**
A(H3N2) baloxavir-sensitive									
Cumulative infections	-27%	-27%	-26%	-6%	-6%	-6%	-26%	-26%	-26%
Peak infections	-53%	-53%	-49%	-22%	-21%	-17%	-47%	-44%	-43%
Time to peak of epidemic curve	+69%	+58%	+55%	+31%	+25%	+27%	+46%	+42%	+36%
A(H3N2) baloxavir-resistant									
Cumulative infections	0%	0%	0%	-9%	-7%	-7%	-8%	-7%	-7%
Peak infections	-6%	-6%	-6%	-44%	-38%	-34%	-41%	-35%	-31%
Time to peak of epidemic curve	+8%	+8%	+9%	+77%	+67%	+64%	+77%	+67%	+64%
A(H1N1pdm09) baloxavir-sensitive									
Cumulative infections	-27%	-26%	-26%	-6%	-6%	-6%	-27%	-26%	-26%
Peak infections	-50%	-46%	-46%	-9%	-9%	-11%	-50%	-46%	-46%
Time to peak infections	+73%	+55%	+60%	+9%	+0%	+10%	+73%	+55%	+60%
A(H1N1pdm09) baloxavir-resistant									
Cumulative infections	0%	0%	0%	-6%	-6%	-6%	-6%	-6%	-6%
Peak infections	0%	0%	0%	-9%	-9%	-6%	-12%	-9%	-9%
Time to peak of epidemic curve	+0%	+0%	+0%	+8%	+8%	+9%	+15%	+8%	+9%

*Cumulative proportion of infections (i.e. the AUC of the time series of infected population) where a positive or negative percentage corresponds to an increase or decrease in the quantity of interest compared to no treatment.

5.3.1.1. Baloxavir-sensitive virus

Baloxavir or oseltamivir monotherapy and baloxavir + oseltamivir combination therapy, were then assessed with each virus when the R_0 increased from 1.2-1.6. The epidemic curves for baloxavir-sensitive and resistant A(H1N1pdm09) and A(H3N2) viruses with antiviral monotherapy and combination therapy when the R_0 is 1.4 are summarised in Figure 6a and 6c. Relative to no antiviral treatment, baloxavir monotherapy reduced the peak infections (range 46 to 53%) more effectively than oseltamivir monotherapy (range 9 to 22%) for drug-sensitive A(H3N2) and A(H1N1pdm09) viruses (Table 3). The reduction in the cumulative number of infections for baloxavir-sensitive viruses was similar for all R_0 values with baloxavir monotherapy (26% to 27%) and was 6% for oseltamivir monotherapy (Table 3). Compared to monotherapy, the combination of both drugs had a similar reduction in the peak of infections (43 to 50%) as baloxavir monotherapy and the reduction in cumulative infections (26 to 27%) was the same as for baloxavir treatment alone (Table 3).

5.3.1.2. Baloxavir-resistant virus

For baloxavir-resistant viruses, baloxavir treatment caused no change (or a small change) in the characteristics of the epidemic curve, showing that the effectiveness of this antiviral treatment is indeed lost when a baloxavir-resistant virus circulates alone (Figure 6b and d). Oseltamivir was effective against the baloxavir-resistant A(H3N2) virus (Figure 6b) and treatment reduced the relative peak number of infections (34-44%) and increased the time to the peak number of infections by 64-77%. However, oseltamivir was not effective for the baloxavir-resistant A(H1N1pdm09) virus (Figure 6d), resulting in less than 10% change relative to no treatment for all parameters. When the R_0 is increased from 1.2 to 1.6 the subsequent characteristics of the epidemic curves are summarised in Table 3. Increasing the R_0 value had a minimal effect on cumulative infections for all viruses and antiviral

treatments tested, although the time to the peak number of infections decreased by between 10-15% as the R_0 increased. The R_0 value 1.4 will be used for subsequent analyses.

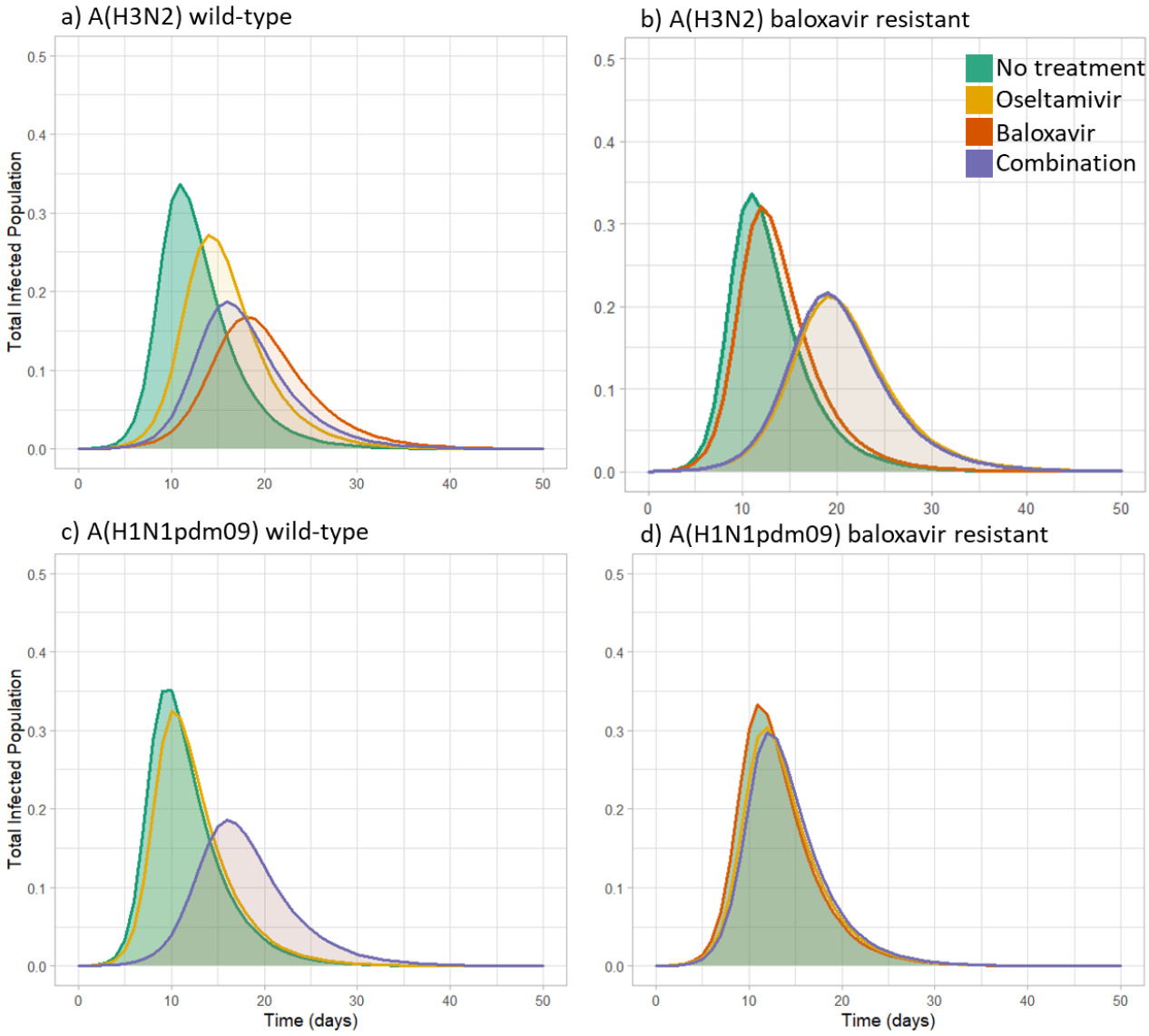

Figure 6. Epidemic curves for A(H1N1pdm09) or A(H3N2) sensitive or resistant viruses when the R_0 is set at 1.4, treated with baloxavir or oseltamivir monotherapy or a combination of both drugs.

5.3.2 Modelling the emergence of antiviral resistance following drug treatment

Antiviral resistant viruses are typically selected as a result of antiviral treatment in patients and thus we extend Model 1 to include the emergence of antiviral resistance

after treatment (Model 2 in Figure 2). The rate that resistant viruses are selected is greater for baloxavir treatment than oseltamivir and we assume that combination treatment has a lower selection of resistance compared to baloxavir monotherapy (as the virus remains sensitive to the second antiviral used in combination). The antiviral effectiveness estimates are the same as those utilised for Model 1 and the A(H1N1pdm09)-PA/E23K virus has a fitness cost whereas the A(H3N2)-PA/I38T does not. Figure 7 depicts the epidemic curves using this model for two viruses, A(H3N2) and A(H1N1pdm09), where different antiviral treatments are administered in adults (7a and 7b) or children (7c and 7d).

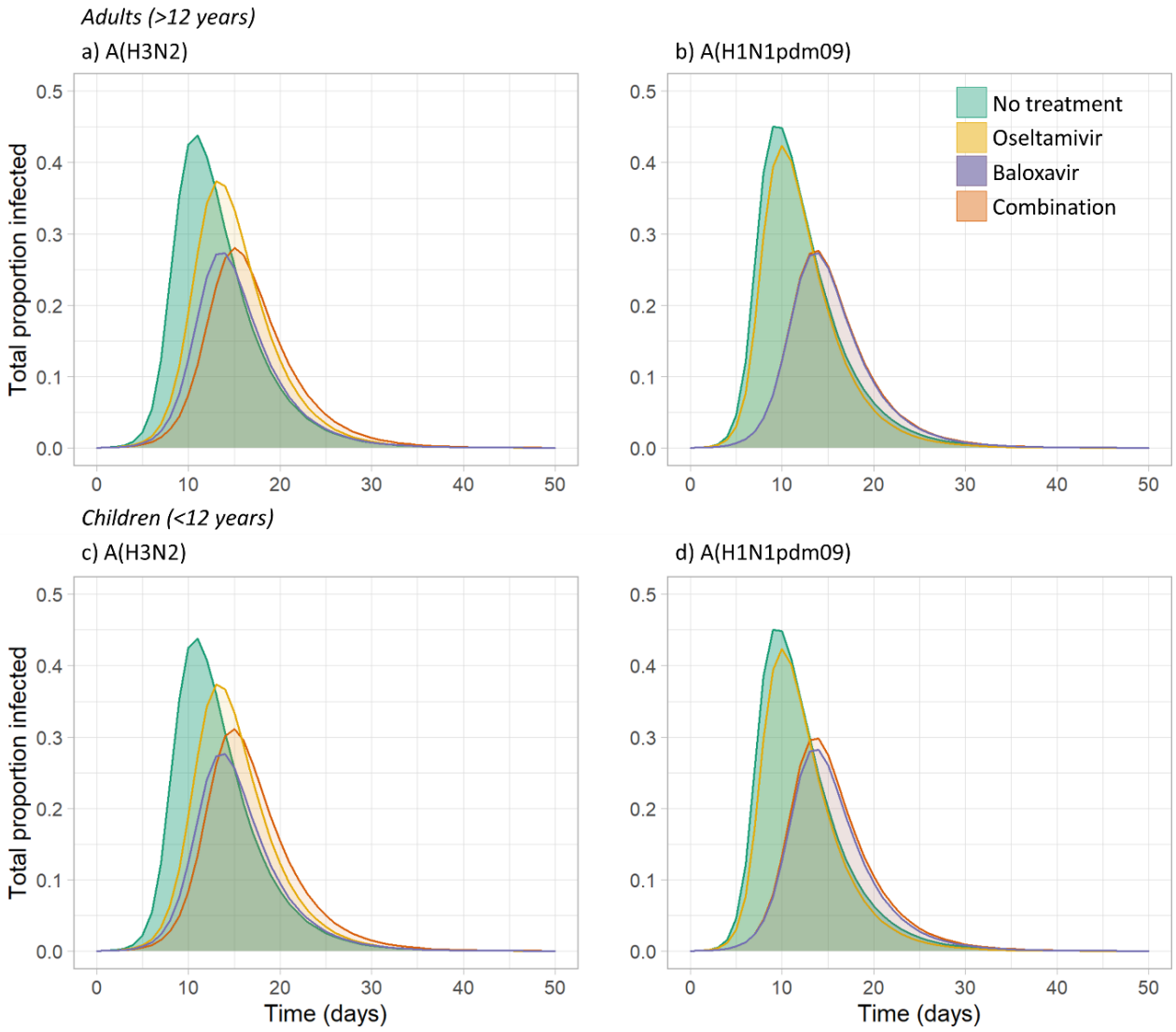

Figure 7. Epidemic curves where antiviral resistance is selected following antiviral treatment in A(H3N2) and A(H1Npdm09) viruses for adults and children.

5.3.2.1 A(H3N2) virus

For the A(H3N2) virus simulation in adults there is a similar reduction in total cumulative infections for baloxavir monotherapy and combination therapy relative to no treatment, 29% and 35% respectively (Table 4). However, the final proportion of resistant virus is 19.5% higher in the monotherapy treatment than the combination treatment (Table 4). Oseltamivir monotherapy exerts a low selection of resistance, but the reduction in total cumulative infections is modest (11%) compared to either baloxavir monotherapy or combination therapy, despite the high selection of resistance with baloxavir (Table 4). For children, there is an 18% increase in the number of resistant infections compared to adults when only baloxavir monotherapy is considered (Table 4). There is also a major reduction in the total number of resistant infections with combination treatment compared to baloxavir monotherapy alone, in both adults and children (Figure 8).

5.3.2.2 A(H1N1pdm09) virus

For the A(H1N1pdm09) virus, both baloxavir monotherapy and combination therapy reduced the cumulative total infections by 35%; there was a 9% increase in resistant infections with baloxavir monotherapy (Table 4). The cumulative total infections were reduced by 11% compared to no treatment for oseltamivir, the same reduction as for the A(H3N2) infection. The greatest selection of resistant virus occurred with baloxavir monotherapy, however treatment in combination with oseltamivir more than halves the final number of resistant viruses selected following antiviral treatment (Figure 9). In children, the use of baloxavir and combination treatment led to a similar reduction in the peak of the epidemic curve relative to no treatment (Table 4; Figure 7) but combination treatment reduced the cumulative number of resistant infections by half (Figure 9).

Table 4. Characteristics of the epidemic curve where resistance can emerge following antiviral treatment, relative to no treatment*

	Change relative to no treatment				
	Cumulative sensitive	**Cumulative resistant**	**Cumulative total infections**	**Peak infections**	**Time to peak infection**
Adult > 12 years					
A(H3N2)					
Baloxavir	-48%	+19%	-29%	-35%	+25%
Oseltamivir	-12%	+0.4%	-11.5%	-14%	+10%
Combination	-37%	+1.8%	-35%	-37%	+25%
A(H1N1pdm09)					
Baloxavir	-38%	+3.4%	-35%	-38%	+50%
Oseltamivir	-12%	+0.5%	-11.5%	-4.4%	+10%
Combination	-37%	+1.4%	-36%	-40%	+50%
Children <12 years					
A(H3N2)					
Baloxavir	-60%	+37%	-22%	-28%	+25%
Oseltamivir	-12%	+0.4%	-11%	-13%	+12.5%
Combination	-38%	+4%	-34%	-37%	+20%
A(H1N1pdm09)					
Baloxavir	-48%	+19.5%	-29%	-33%	+50%
Oseltamivir	-12%	+0.5%	-11.5%	-6.6%	+10%
Combination	-42%	+8.7%	-33%	-38%	+50%

***** For example, +25% for cumulative resistant infections means a 25% increase in the proportion of resistant infections (i.e. the AUC of the time series of infected and resistant population) due to antiviral treatment

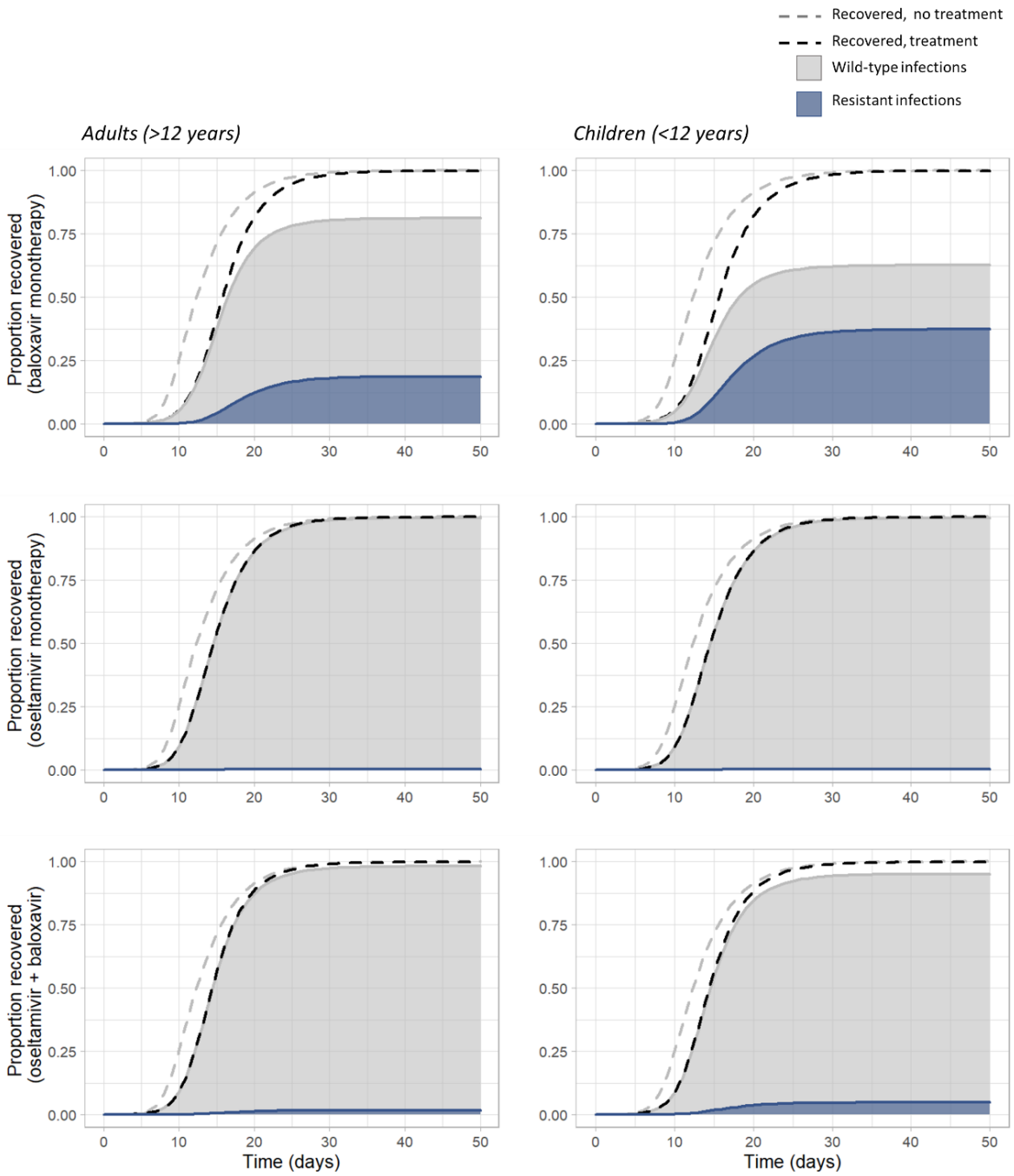

Figure 8. Cumulative infections for A(H3N2) viruses when antiviral resistance is selected following antiviral treatment. The grey line is the cumulative infections where no antiviral treatment is administered, the black line shows total cumulative infections for the treatment group. The grey shading represents the proportion of baloxavir-sensitive infections, and the coloured shading shows the proportion of resistant infections.

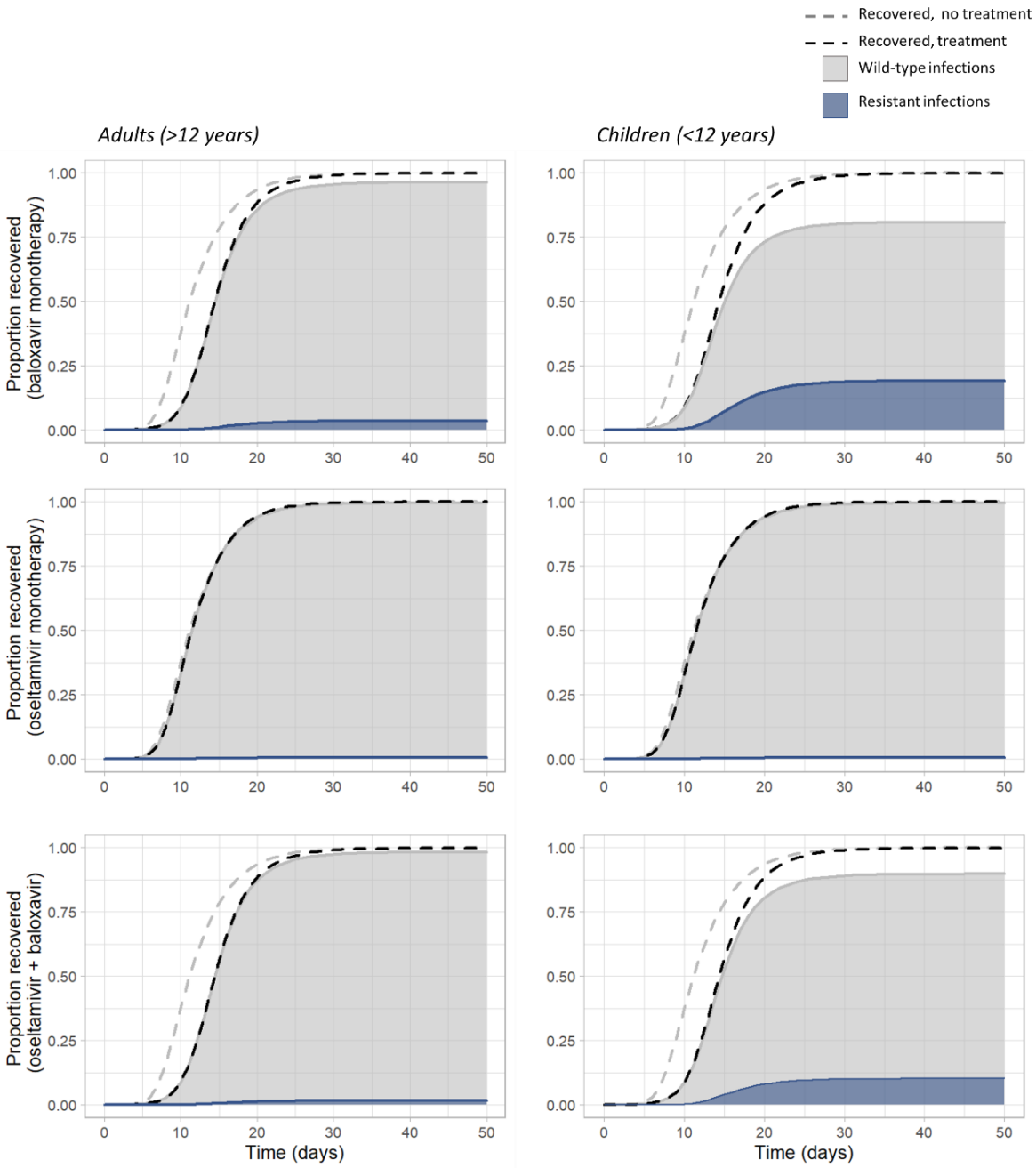

Figure 9. Cumulative infections for A(H1N1pdm09) when antiviral resistance can be selected following antiviral treatment. The grey line is the cumulative infections where no antiviral treatment is administered, the black line shows total cumulative infections for the treatment group. The grey shading represents the proportion of baloxavir-sensitive infections, and the coloured shading shows the proportion of resistant infections.

5.4 Discussion

In an influenza pandemic, antiviral treatment is likely to play a key role in the public health response, particularly for the period when vaccines are not yet available. A major limitation of antiviral drugs is the potential circulation of antiviral-resistant strains. Here we have constructed a mathematical model that utilises information from *in vivo* studies to incorporate into a between-host population model. Our study includes antiviral effectiveness data on baloxavir and oseltamivir, both alone and in combination, derived from experiments in the ferret model and allows for co-circulation of antiviral sensitive influenza A strains (either H3N2 or H1N1pdm09) with antiviral-resistant viruses. An earlier modelling study has shown that the use of baloxavir in the population can significantly reduce morbidity and mortality due to influenza infection compared to oseltamivir[196]. We extend this study by incorporation of combination treatment and the additional consideration of resistant viruses.

We show that if a baloxavir resistant virus alone circulates (Figure 6); oseltamivir is beneficial for the A(H3N2) subtype but not for A(H1N1pdm09). Baloxavir is not effective for either influenza A subtype when the virus harbours an amino acid substitution that leads to reduced susceptibility, which is similar to the current scenario with the adamantanes because all currently circulating influenza A viruses are resistant to this drug class[88]. Baloxavir is highly effective in reducing the peak of infections and the cumulative number of infections when only a baloxavir-sensitive virus circulates. The combination of oseltamivir and baloxavir was beneficial for all scenarios except the circulation of baloxavir resistant A(H1N1pdm09). In our second model, antiviral resistance could be selected following treatment; baloxavir and combination therapy had similar effectiveness in reducing the cumulative number of infections in the population. This is an interesting result, because both treatment options had similar effects relative to no treatment even though the total proportion of the population infected with a resistant virus was greater in the baloxavir monotherapy model compared to combination treatment. We believe this

warrants further investigation in future studies. Furthermore, combination treatment in children is highly effective in reducing the proportion of the population that is infected with a resistant virus and may be a useful treatment strategy to reduce the selection of resistant viruses in this age cohort. It has been shown in a previous modelling study that combination treatment is in important treatment strategy for reducing the spread of influenza resistant viruses in the population[201].

The selection of viruses with reduced susceptibility or resistance is of major concern and is monitored by regular surveillance of circulating influenza viruses[64]. The spread of resistant viruses in the population is likely to be influenced by two factors; the viral fitness and the relative effectiveness of antiviral drugs. These factors may be linked to the subtype of influenza virus in which the resistant mutation occurs. Furthermore, it is clear from clinical trial data that the selection rate of antiviral resistant viruses is different for H3N2 and H1N1pdm09 influenza A subtypes[76]. It should be noted that the fitness of a virus is linked to replicative and transmission fitness. Differences in the selection of parameters for A(H3N2) and A(H1N1pdm09) were therefore included in our model and the transmission term is increased or decreased based on these parameters. For example, in our simulation the relative fitness of the A(H1N1pdm09) strain is estimated to be lower than the A(H3N2) strain. Cumulative infections were similar for both subtypes when no antiviral treatment was administered, but, if the replicative fitness of the resistant virus is higher than our chosen estimate, the reduction in the epidemic curve relative to no treatment will not be as great. Previously identified influenza viruses that have gained an amino acid change that confer antiviral resistance show there is often a reduction in replicative or transmission fitness associated with the gained mutation[194]. If a virus were to have equivalent or greater fitness to the drug-sensitive virus this is likely to result in greater transmission.

The effectiveness of an antiviral treatment may be different for seasonal influenza viruses compared to a potential pandemic scenario. For novel influenza strains, assumptions must be made such as fitness, transmission potential, antiviral effectiveness, severity of the illness and the degree of prior immunity in the population. These parameters will influence the size and duration of the epidemic curve, but will also have different effects on mortality which was not included in this model. The number of hospitalisations and deaths associated with the illness would be an important extension of this model as these factors have important public health implications, particularly how many can be avoided by widespread antiviral treatment and whether there is a difference given treatment with oseltamivir or baloxavir (or a combination)[202, 203]. In addition to antiviral treatment, other social interventions or vaccination are important public health measures to consider[204].

A limitation of this work is that the antiviral effectiveness data was sourced from our ferret model rather than clinical data. The ferret model is the gold standard animal model for influenza but will not exactly recapitulate human influenza infection or the pharmacokinetics of antiviral drugs in humans and has the additional limitation of small samples sizes. For this reason, the effectiveness of the antiviral treatments explored here may be over or underestimated. Clinical trials of combinations of oseltamivir and baloxavir have only recently been completed in hospitalised patients, but not in otherwise healthy outpatients. In the absence of clinical trial data on the effectiveness of treatment for baloxavir-resistant viruses, we have to rely on the data collected from the ferret model to make model-based predictions, providing the first insight into the effect of baloxavir on viral transmission at the population-level. Our model and results can be modified when clinical trial data become available.

We have also only taken into account treatment following a symptomatic infection at an average of 48 hours after symptoms emerge; however, antiviral drugs can also be used

prophylactically. To introduce a new compartment into this model and include prophylactic antiviral use would be an interesting extension of this work. Post-exposure or household prophylaxis could be a good strategy to not only reduce the onward transmission of virus, as we have demonstrated in the ferret model, but will also reduce the selection of resistant virus as there is no selection pressure for such viruses in an uninfected contact[199, 205]. We have also assumed an average treatment time of 48 hours, but it has been previously been shown that the effectiveness of antiviral treatment beyond this window may be limited. Therefore timely administration of antiviral drugs is required[206].

In conclusion, we have established a framework based on within-host estimates that can be modified to assess the effects of antiviral treatment, from, on transmission of influenza in the population.

Based on the results and parameters in this model, we believe that combination treatment could be explored as a strategy for antiviral treatment of influenza and that with use of mathematical modelling that the most efficient distribution of antiviral drugs can be estimated. Extensions of this work include testing which treatment strategy provide the greatest public health benefit including targeted antiviral distribution to certain groups of the population and estimating changes in mortality or disability-adjusted life years (DALYs). There is likely to be a significant public health benefit of antiviral drugs if a new influenza strain was to emerge, particularly in the period before vaccines are available. Our models have the flexibility to integrate new features from novel data including *in vivo* and population data. This will accelerate the translation from novel laboratory/clinical findings to an impact on population transmission.

Chapter 6: General discussion and concluding remarks

The emergence of SARS-CoV-2 has given rise to a monumental era of history and highlighted and reaffirmed the health and economic impacts of a pandemic. The Australian Health Management Plan for Pandemic Influenza (AHMPPI) outlines that vaccines and antiviral drugs are key pharmaceutical measures to prevent and treat influenza infections in a pandemic scenario. While it is highly likely that a pandemic due to an influenza virus will occur in the future; the circulation of seasonal influenza viruses in the meantime is an on-going public health concern and leads to significant health and economic burden. Antiviral drugs have been shown to reduce consequences of infection and can be administered to outpatients or those that are hospitalised[207, 208]. Antiviral drugs for the treatment of influenza may be required for individuals at a high-risk of complications and have an increased risk of hospitalisation and death due to influenza infection including those that have co-morbidities, are at extremes of age or are immunocompromised[209]. Antiviral drugs may also be utilised as part of pre- or post-exposure prophylaxis, as is outlined in the AHMPPI for the treatment of healthcare workers and close contacts[210]. Some countries, including Australia, stockpile antiviral drugs (typically oseltamivir) as part of pandemic preparedness plans[210, 211].

There are three classes of licensed antiviral drugs for influenza, that include the adamantanes, NAIs and a PA inhibitor, but other drugs with a range of mechanisms of action are in various stages of clinical development. Due to widespread resistance, the adamantanes are of limited clinical use. The NAIs are commonly utilised for the treatment of influenza and are the SOC for severe influenza illness due to the ease of administration and low cost, though there has been some debate on the effectiveness of oseltamivir treatment in different clinical settings[212]. Since the early 2000’s the NAI’s have been the main drug type utilised to treat influenza but reliance on one drug class poses risks for widespread transmission of viruses with reduced susceptibility (more simply, drug resistance) which could spread and limit treatment options. Additional therapeutic options,

such as baloxavir marboxil, are a welcome addition to the influenza antiviral drug arsenal. As with other influenza antiviral drugs, the selection of viruses that are resistant to baloxavir are of concern and have been identified in 2.2-9.7% of adults treated with baloxavir and up to 25% of treated adolescents[76]. These are high frequencies of resistance and therefore in this thesis, we sought to study viruses resistant to baloxavir further. We developed virus characterisation methods, evaluated effectiveness of treatment of infection with resistant viruses and explored whether selection of these viruses could be reduced with combination treatment with baloxavir and oseltamivir.

6.1 Phenotypic and genotypic characterisation of viruses with reduced susceptibility to baloxavir due to PA/I38X substitutions

When baloxavir was licensed (2018) a proportion of post-treatment isolates from clinical trials were identified to have gained amino acid changes associated with baloxavir resistance[80]. The most common amino acid substitutions occurred at position 38 of the PA gene, PA/I38T was the most frequent substitution but PA/I38F and PA/I38M were also identified[80]. Viruses with other amino acid changes in the PA gene, such as PA/A20S, PA/E23K and PA/E199G/D, also occurred but at a lower frequency than PA/I38X[80]. In 2018, there was little published information on the *in vitro* characterisation of viruses with baloxavir treatment acquired amino acid changes, our laboratory did not have an established method to characterise such viruses.

The *in vitro* investigations outlined in chapters two and three of this thesis include protocols for phenotypic and genotypic assays that are capable of determining reduced susceptibility to baloxavir. Therefore there were three keys aims, to establish baseline EC_{50} values for circulating viruses to baloxavir that could be used as a benchmark in the surveillance of circulating viruses with reduced susceptibility. The second was to measure the relative reduction in baloxavir susceptibility due to PA/I38X viruses. Lastly, we

developed a high-throughput method for identifying PA/I38X amino substitutions by pyrosequencing.

By FRA, seasonal influenza A viruses had a baloxavir EC_{50} in the range of 1.2 to 2.4 nM and influenza B viruses had a range of 0.7 to 15.5 nM. Phenotypic analysis of viruses with PA/I38X, showed that the PA/I38T substitution is a major path for reduced susceptibility to baloxavir and resulted in a 65-fold change in EC_{50} in A(H1N1pdm09) viruses compared to wild-type viruses. PA/I38M and PA/I38F had a 16-23 fold change in EC_{50}, which was lower than for PA/I38T. These *in vitro* data showed that not only is PA/I38T identified at the highest frequency following treatment in patients, it also causes the greatest change in baloxavir susceptibility *in vitro*. The pyrosequencing assay was validated to be effective in determining sequence mixtures of PA/I38T and PA/I38M for influenza A and B viruses, and at PA/I38F for influenza B viruses only. The limit of detection for the pyrosequencing method to determine a viral mixture at PA/I38T was measured to be 10^3 RNA copy number/mL.

Antiviral resistance in circulating influenza strains is monitored in laboratories of the WHO Global Influenza Surveillance and Response System (WHO GISRS). Such surveillance is important because if a drug-resistant virus is sufficiently fit, person-to-person transmission may occur. Such transmission may lead to small outbreaks in geographical clusters or potentially may lead to widespread resistance in circulating viruses. Both scenarios will limit therapeutic options. A key example of this is the adamantane class of drugs, as all circulating influenza viruses are resistant to the drug[213]. This has also occurred with oseltamivir. A somewhat serendipitous outcome of the 2009 H1N1 pandemic was that the virus is oseltamivir-sensitive and it replaced the previously circuiting A(H1N1) viruses that were resistant to oseltamivir[214]. Since 2009, localised clusters of oseltamivir resistant viruses have been identified, but these viruses have not spread globally[86, 215]. For the neuraminidase inhibitors (NAIs), standardised WHO guidelines utilise an enzymatic assay

and stipulate a five-fold increase in IC_{50} as reduced inhibition and 50-fold for highly reduced inhibition[64].

The purpose of establishing a baseline EC_{50} for baloxavir is to allow for viruses with reduced susceptibility to baloxavir to be identified in phenotypic screening of circulating influenza viruses. The WHO GISRS Expert Working Group for Surveillance of Antiviral Susceptibility (WHO-AVWG) have reported results of NAI susceptibility in circulating viruses from each participating laboratory since 2012–13 and have begun to report susceptibility of viruses to baloxavir since the 2017-2018 influenza season[64]. Unlike for the NAIs, there is currently no standardised protocol or minimum fold change outlined by the WHO for the definition of reduced susceptibility to baloxavir. It has been tentatively suggested that a three-fold change in EC_{50} should be the threshold for defining baloxavir-resistance, however this may change in the future when more data are collected[216]. Other groups have also developed phenotypic assays to determine baloxavir susceptibility and the results show similar baseline EC_{50} values for influenza A and B viruses and comparable fold-changes in EC_{50} for PA/I38X substitutions compared to matched wild-type viruses[64, 216]. Surveillance for baloxavir resistant viruses, especially PA/I38X substitutions, using phenotypic and genotypic methods is ongoing. However, the reduced circulation of influenza viruses during the SARS-CoV-2 pandemic has limited the number of available viruses.

An interesting result from this study was that the baseline EC_{50} for baloxavir was higher for influenza B than influenza A viruses. We hypothesise that this is because baloxavir binds to different sites in the PA endonuclease of influenza A and B viruses, leading to a lower affinity in drug binding to influenza B viruses *in vitro*. For oseltamivir, the clinical effectiveness is lower in influenza B than influenza A infections; the duration of fever and viral shedding was significantly higher in patients infected with influenza B viruses and treated with oseltamivir in clinical trials[217, 218]. In clinical trials with baloxavir, there was

no difference in the time to resolution of symptoms or viral shedding between influenza A and B viruses, even though we observed higher EC_{50} values *in vitro*[74].

The results from the phenotypic baloxavir assay have also shown that the median EC_{50} of PA/I38M and PA/I38F (8.5 to 13.3 nM) falls in the same range as wild-type (baloxavir sensitive) influenza B viruses (0.7 to 15.5 nM), this may mean that baloxavir remains effective in patients at these “low” EC_{50} values. Additionally, given that the drug is administered in a single dose, waning baloxavir acid concentrations in patient plasma in low nM may reduce the selection barrier for resistant viruses to emerge[79]. However, care should be taken when directly comparing *in vitro* assays to *in vivo* or clinical settings, as the EC_{50} values may not necessarily correlate with clinical effectiveness. Interestingly, the recently published clinical trial assessing oseltamivir and baloxavir in combination (FLAGSTONE) was the first study to test multiple doses of baloxavir (on days 1, 4 and 7 if symptoms were not resolved)[128]. This clinical trial was performed in a hospitalised cohort, but we look forward to further information in other patient cohorts.

The benefits of phenotypic assays for drug susceptibility is that they can be used to identify novel amino acid changes that cause resistance and to derive numerical values on fold-change in susceptibility. Genetic sequence analysis can also be used to monitor for resistance, but only for amino acid changes that are already known. For example, as the majority of amino acid changes that cause resistance occur at PA/I38X, a high-throughput screening with a method such as pyrosequencing, can be used. Pyrosequencing is already utilised for the identification of NA/H275Y that causes resistance to oseltamivir. While only one locus is tested using this method, it is significantly faster and cheaper than whole genome sequencing. It will be interesting to see how whole genome sequencing will be utilised in the future for antiviral surveillance as it becomes less expensive and more ubiquitous.

Another benefit of pyrosequencing is that this method is able to discern viral mixtures in a sample. When viruses with reduced susceptibility emerge under drug selection pressure in a patient or animal, this often occurs as a minor proportion that increases over the duration of viral shedding, especially if a patient is unable to clear infection due to an immunocompromised state. In viral fitness experiments we have conducted in the ferret model, we used “competitive mixtures” where a ferret is infected with a mixture of a wild-type and variant virus of interest. Pyrosequencing was used determine whether the variant virus increased or decreased in proportion over the duration of viral shedding to infer viral fitness[219]. We also use this method in chapter four of this thesis.

6.2 *In vivo* effectiveness of baloxavir and oseltamivir, alone and in combination, for the treatment of ferrets infected with viruses that contain PA/I38T and PA/E23K substitutions

In chapters two and three, we generated fundamental tools for *in vitro* surveillance and characterisation of viruses resistant to baloxavir. In chapter four we sought to study viruses resistant to baloxavir further by utilising the ferret model to test whether combination antiviral treatment was an effective method to reduce the selection of resistant viruses. The aim of this study was to assess whether baloxavir treatment retained any antiviral effect against a virus that displayed reduced susceptibility/resistance *in vitro* and whether the relative EC_{50} of these resistant viruses led to different clinical effectiveness of baloxavir. i.e., whether increasing EC_{50} proportionally decreases the *in vivo* effectiveness of a drug. A similar study has shown that for oseltamivir resistant viruses, greater IC_{50} values correlated with lower effectiveness in reducing viral shedding in ferrets [220]. Secondly, we were interested in assessing the effectiveness of combination treatment with oseltamivir and baloxavir for the treatment of baloxavir sensitive and resistant viruses. The relative selection pressure on baloxavir resistant viruses was assessed using a competitive mixture of either PA/I38T or PA/E23K and corresponding baloxavir sensitive virus in the presence of baloxavir monotherapy and combination therapy.

Combination therapy with baloxavir and oseltamivir provided no additional virological benefit over monotherapy and the effectiveness of antiviral treatment with baloxavir monotherapy was substantially reduced for viruses that have PA/I38T or PA/E23K amino acid substitutions. Infection with competitive mixtures of baloxavir sensitive and resistant viruses, showed that the rapid selection of PA/I38T with baloxavir monotherapy, could be reduced with combination therapy and the proportion of PA/E23K remained low over the duration of viral shedding.

This study showed that while phenotypic baloxavir susceptibility was greater for PA/I38T than PA/E23K *in vitro*, baloxavir was not more effective for the treatment of PA/E23K, suggesting that *in vitro* EC_{50} values may not necessarily correlate with *in vivo* effectiveness. As discussed in chapter 4, there may also be differences in antiviral effectiveness due to the replication kinetics of different viral subtypes in ferrets. Due to the high frequency of PA/I38X viruses in clinical trials, the majority of the available literature is on baloxavir resistant viruses with these amino acid substitutions. However, due to the major reduction in baloxavir effectiveness we have shown in ferrets infected with the PA/E23K virus, amino acid changes at other positions of the PA gene are also of concern even if they occur at lower frequencies. Indeed, a case study on a child infected with a virus containing PA/E23K has been reported[221]. Further studies of the fitness and treatment effectiveness for viruses that contain substitutions like PA/A20S and PA/E199D/G should be undertaken.

There have been a number of studies by different groups to understand the fitness of viruses that bear PA/I38X substitutions in the absence of antiviral treatment [80, 221-227]. The fitness of a virus can be defined by two factors, the within-host replication fitness and between-host transmission fitness[228]. Within-host fitness refers to the replicative ability of a virus to establish an infection and replicate efficiently in a host. This will impact the likelihood that a virus will transmit between hosts. The effectiveness of antiviral treatment

on PA/I38T and PA/E23K is inherently interrelated with the fitness of these viruses; if a virus with these substitutions were to be severely compromised in replication fitness, the concern for transmission between humans would be lower. This has previously been demonstrated with the NA/R292K amino acid substitution that causes resistance to oseltamivir and was identified in an A(H3N2) virus but the mutation also severely compromised viral fitness[229, 230].

In our study, viruses bearing either PA/I38T or PA/E23K were able to replicate efficiently in ferrets in the presence and absence of antiviral treatment. It has been previously determined that a PA/I38T substitution has similar or reduced transmission of virus between hosts compared to matched viruses[219, 223, 226, 231]. While a PA/E23K virus was isolated from a patient, there is limited information on the fitness of PA/E23K viruses. In chapter four of this thesis we have reported a novel finding that the PA/E23K virus was able to replicate efficiently in the ferret model. Transmission of the PA/E23K virus by direct contact or aerosols PA/E23K virus in the ferret model would be an interesting extension of this study.

A limitation of our study is that we have used an “artificial” infection model, where ferrets are directly infected with a high volume and concentration of virus delivered via the intranasal route. Further investigations could use an infection route that recapitulates a more natural infection, such as co-housing naïve ferrets with an infected donor ferret. Either the host or recipient ferret can be treated with an antiviral drug, depending on the research question being addressed. The effectiveness of antiviral treatment may be more pronounced with this experimental approach and could also include prophylaxis.

In a previous study, we have shown in ferrets that baloxavir treatment is effective in reducing the onward transmission of A(H1N1pdm09) viruses from a donor to an untreated recipient ferret, but oseltamivir treatment was not[232]. A clinical trial in household settings has shown that treatment with baloxavir of an “index” case was twice as effective in

preventing infections in a household compared to oseltamivir[233]. Experiments in the future could test whether a virus with either PA/I38T or PA/E23K transmits effectively between ferrets, when either the donor or recipient is treated with baloxavir or oseltamivir.

A final point to address on fitness of drug resistant viruses is the acquisition of compensatory mutations. In our study, we studied a subset of viruses for additional acid substitutions gained in the polymerase genes (PB2, PB1 and PA) with baloxavir treatment selection pressure. We did not identify any notable amino acid changes in these genes. The polymerase complex is heterotrimeric and the function relies on all three components being functional. Although the PA active site is the target of baloxavir, amino acid substitutions that cause resistance could occur in genes other than the PA, especially if they are permissive or compensatory mutations that can restore or enhance the fitness cost of a mutation. For example the PA/P653L substitution was shown to restore the fitness cost of the PB1/K229R substitution that causes favipiravir resistance[234]. There have not yet been any reports of compensatory mutations in addition to the already known amino acid substitutions that cause resistance to baloxavir. However, there is a rich literature on compensatory substitutions for NAI resistant viruses; a notable example are compensatory mutations that restored the fitness cost of viruses that bear a NA/H274Y substitution[195, 235].

If permissive or compensatory amino acid substitutions improve the fitness of baloxavir resistant viruses to be greater than wild-type/baloxavir sensitive viruses, there is a greater risk of these viruses spreading and widely circulating. This highlights the importance of on-going surveillance of circulating influenza viruses for antiviral drug resistance as it is likely any compensatory or permissive substitutions would only be identified after they have already emerged in patients or spread in the community. Methods to predict permissive mutations before they emerge include a mutational library approach, as has been described by Bloom and colleagues in previous studies[235, 236].

The combination of oseltamivir and baloxavir did not provide additional virological benefit in our study over monotherapy but did reduce the selection of resistant viruses. Other combinations with baloxavir could be explored further, such as with other polymerase inhibitors pimodivir or favipiravir which have only to our knowledge been studied in combination with oseltamivir to date. Drug combinations with different mechanisms could also be tested, such as host-targeted or anti-inflammatory drugs. Clinically, antiviral drug combinations are more likely to be used or studied in hospitalised patients compared to otherwise-healthy outpatients.

6.3 Mathematical modelling framework to study the spread of influenza in the population with antiviral treatment with baloxavir alone or in combination with oseltamivir

Epidemics can be represented with compartmental models that divide a population into disease states which in the simplest form are susceptible (S), infected (I) and recovered (R), with an additional compartment, exposed (E), usually added for influenza studies. The compartments included in a model can be based on any variable with an epidemiological relevance, such as being susceptible to or infected with a pathogen. The dynamics of movement from one compartment to the next will be linked to the parameters defined for the model, which in turn will govern the size and duration of an epidemic. SIR/SEIR models are very useful for the study of infectious diseases and a range of variables can be accommodated into the model depending on the application. This may include vaccination or antiviral treatment of a population. SIR models can be modified to fit the purpose or aims of a study, in chapter five of this thesis we have derived a mathematical model to explore the application of antiviral treatment with baloxavir or oseltamivir alone or in combination.

As baloxavir is orally administered as a single dose, it is an attractive option to stockpile for use in the case of a pandemic, compared to an antiviral drug that requires a five day course, such as oseltamivir, may have additional challenges of drug

adherence/compliance. Baloxavir has also been shown in clinical trials to result in a rapid reduction of viral load in patients; the median time to sustained cessation of viral shedding was 24 hours post-treatment in the baloxavir group, compared to 72 and 96 hours in the oseltamivir and placebo groups, respectively[74]. This was a major motivation for our mathematical modelling study, as it was hypothesised that the rapid reduction in viral shedding following antiviral treatment may directly correlate with reduced transmission of virus in the population. Finally, in our ferret study and study of baloxavir treatment with an index case infected with influenza has been shown to reduce the transmission of virus [232, 233, 237]. These three factors make baloxavir of interest to use in the event of a pandemic.

The approach used to construct the model to simulate the spread of influenza in a closed population was a deterministic SEIR compartmental model that was used. Using the SEIR model, we showed that baloxavir monotherapy and baloxavir and oseltamivir combination therapy are effective strategies to reduce the cumulative number of infections in the population and are more effective than oseltamivir. We also showed that baloxavir monotherapy and combination therapy were equally effective in reducing cumulative infections in the population, even when antiviral resistant viruses could emerge and spread following antiviral treatment.

There are several inherent assumptions in a deterministic model, the population in question is assumed to be large and homogeneously mixed, it is assumed that outbreaks occur from a single initial case, that there is no prior immunity to the pathogen in the population and that individuals recover at a constant rate. For seasonal influenza, the assumption that the population is immunologically naïve to the virus is flawed, as most people are either vaccinated or have been exposed to the virus. However, in a pandemic, this assumption is reasonable because the population is likely to have little or no immunity to the novel virus. Even with these underlying assumptions, a well-constructed

mathematical model will be as simple as possible and will be transparent and adaptable with accurate parametrisation from available data.

Mathematical models can be used to allocate limited resources or to target control measures more efficiently. In such models, transmission of a pathogen between infectious and susceptible individuals will be altered by the control measure that is implemented. For example, with antiviral drugs, models can be designed to assess which population may be offered the greatest benefits from antiviral treatment, such as healthcare workers, close contacts, household contacts or vulnerable members of the population[42, 238]. The trade-off between antiviral treatment after infection or prophylaxis can also be compared[205, 239].

A previous study has also shown the benefit of administering baloxavir in an influenza epidemic using population modelling based on previous influenza seasons, but this study did not incorporate the selection of resistance following treatment[196]. We believe that the exclusion of this parameter was a limitation of the study, due to the high likelihood of emergence of resistant viruses in patients following treatment. Not accounting for resistance may over-estimate the benefit of an antiviral drug. Further studies on the emergence and spread of antiviral resistant viruses could add a stochastic (rather than deterministic) component for resistant viruses[201, 240]. We have also included the analysis of baloxavir and oseltamivir in our study, as an extension on previous work. A limitation of our study is that our estimates for antiviral effectiveness are based on experiments in a small number of ferrets. Therefore we are likely to have included biases that result from small experimental numbers or differences in viral kinetics or pharmacokinetics between ferrets and humans.

While we have sourced other information from epidemiological studies or large scale clinical trials, there was not sufficient information on the effectiveness of antiviral treatment of baloxavir resistant strains and the relative effectiveness of combination therapy with

oseltamivir and baloxavir compared to monotherapy for sensitive and resistant strains (as outlined in chapter four of this thesis). While a combination treatment study has now been completed in hospitalised patients, this is not applicable to a population-scale study where the majority of individuals would be otherwise healthy. We look forward to further data on combination treatment in otherwise healthy patient cohorts if studied in these populations, however it is more likely that these trials will be conducted in those that are hospitalised. Future work could explore the administration of baloxavir as pre- or post-exposure prophylaxis. This may be a more effective strategy to reduce the number of infections in the population than antiviral treatment of already infected individuals, however targeted prophylaxis may be required to preserve pandemic stocks. The model we have built could also be used to study the potential reduction in mortality and hospitalisations at a population level when an antiviral treatment is applied. The allocation of antiviral drugs is an interesting (and important) logistical problem when applied to a large population and when limited supplies of drug need to be used effectively. Examples of groups in the population that may be targeted include healthcare workers, high-risk contacts, otherwise healthy outpatients and outpatients at risk of complications. Our study builds a mathematical framework to explore some of these research questions further.

6.4 Concluding remarks

In this thesis, we aimed to explore baloxavir resistance at the *in vitro*, *in vivo* and population level. Baloxavir resistance is a significant concern, particularly when clinical trials show that viruses with resistance are selected in one in four children treated with baloxavir. Overall, our results suggest that the effectiveness of baloxavir monotherapy in the ferret model is severely compromised for viruses that are resistant to baloxavir due to substitutions at PA/I38T or PA/E23K. Circulating influenza viruses must be monitored in the future for resistance to baloxavir and further work on the combination of baloxavir with other drugs may reveal the best strategy to reduce the emergence of resistance. While

there is a significant body of knowledge on the fitness of PA/I38T viruses, further work is needed to elucidate the fitness potential of other amino acid changes that cause resistance to baloxavir and to study permissive substitutions that may increase the fitness of resistant viruses. This is especially important in patient cohorts that have a higher propensity to select for resistant influenza viruses, such as those that are immunocompromised. Baloxavir should be considered as an option for antiviral pandemic stockpiles, as it is administered with a single dose and has shown to have a potent antiviral effect compared to the neuraminidase inhibitors. The decision to administer baloxavir in combination with another antiviral drug such as oseltamivir is likely to reduce the selection of resistance however needs to be carefully considered with a cost-benefit analysis and stockpile depletion. While this thesis did not focus on influenza B viruses, there seems to be a lower propensity to select for resistance in influenza B viruses following baloxavir treatment than influenza A. Public health measures that have been implemented for the control of SARS-CoV-2, such as social-distancing, quarantine and travel restrictions, have led to a drastic reduction of seasonal influenza around the world over the last two years. As global travel resumes, it is uncertain how much influenza will circulate in the coming years, however with increased influenza activity there will also be increased antiviral drug use – we need to continue to understand the selection of antiviral drug resistance to protect our antiviral treatment options.

Supplementary figures – Chapter 4

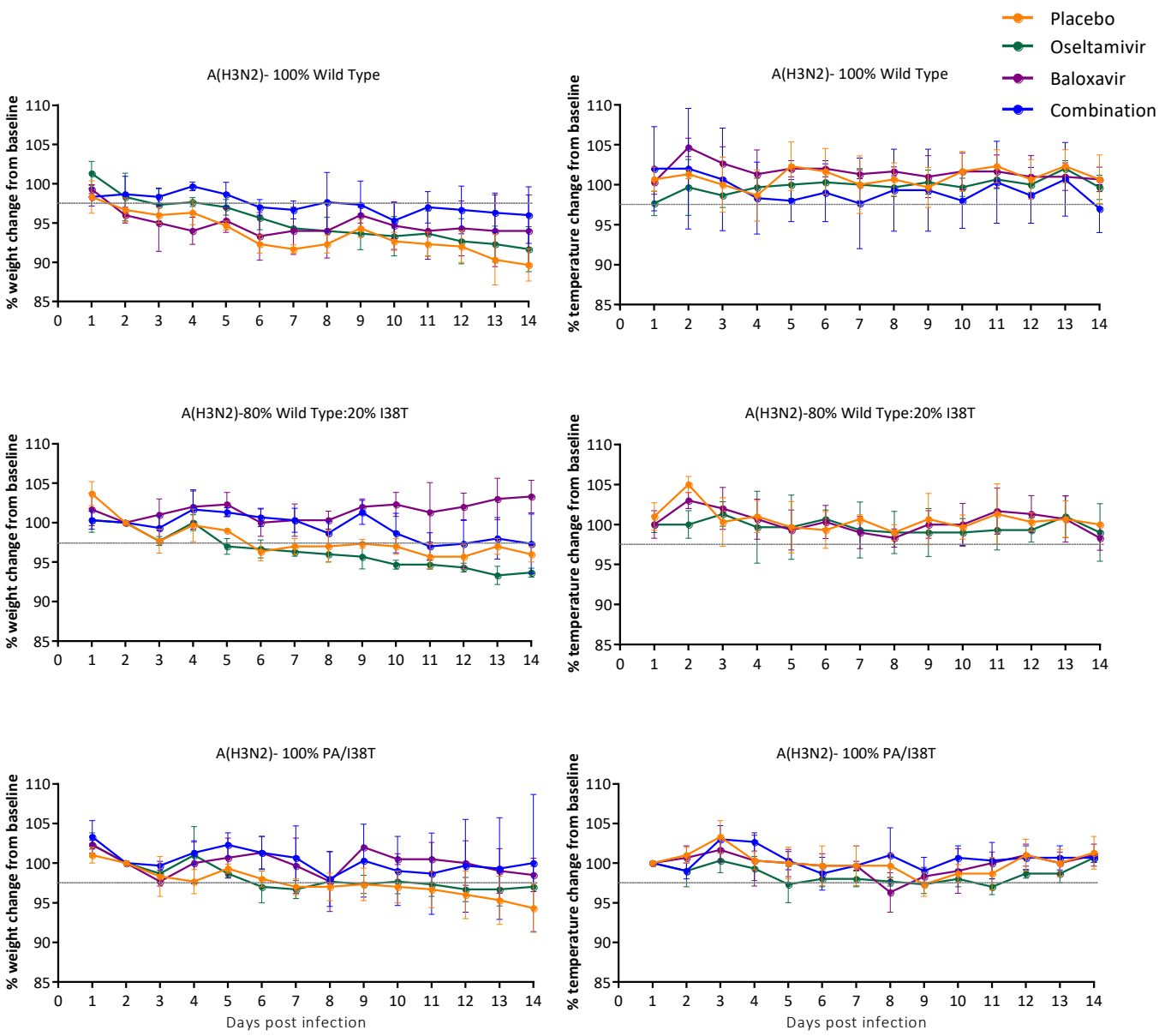

Figure S1: Summary of change in % weight and % temperature of ferrets infected with the A(H3N2) clinical isolate pair compared to the starting baseline. Weight and temperature of ferrets were measured daily and the line plot depicts the mean and standard deviation in each ferret treatment group.

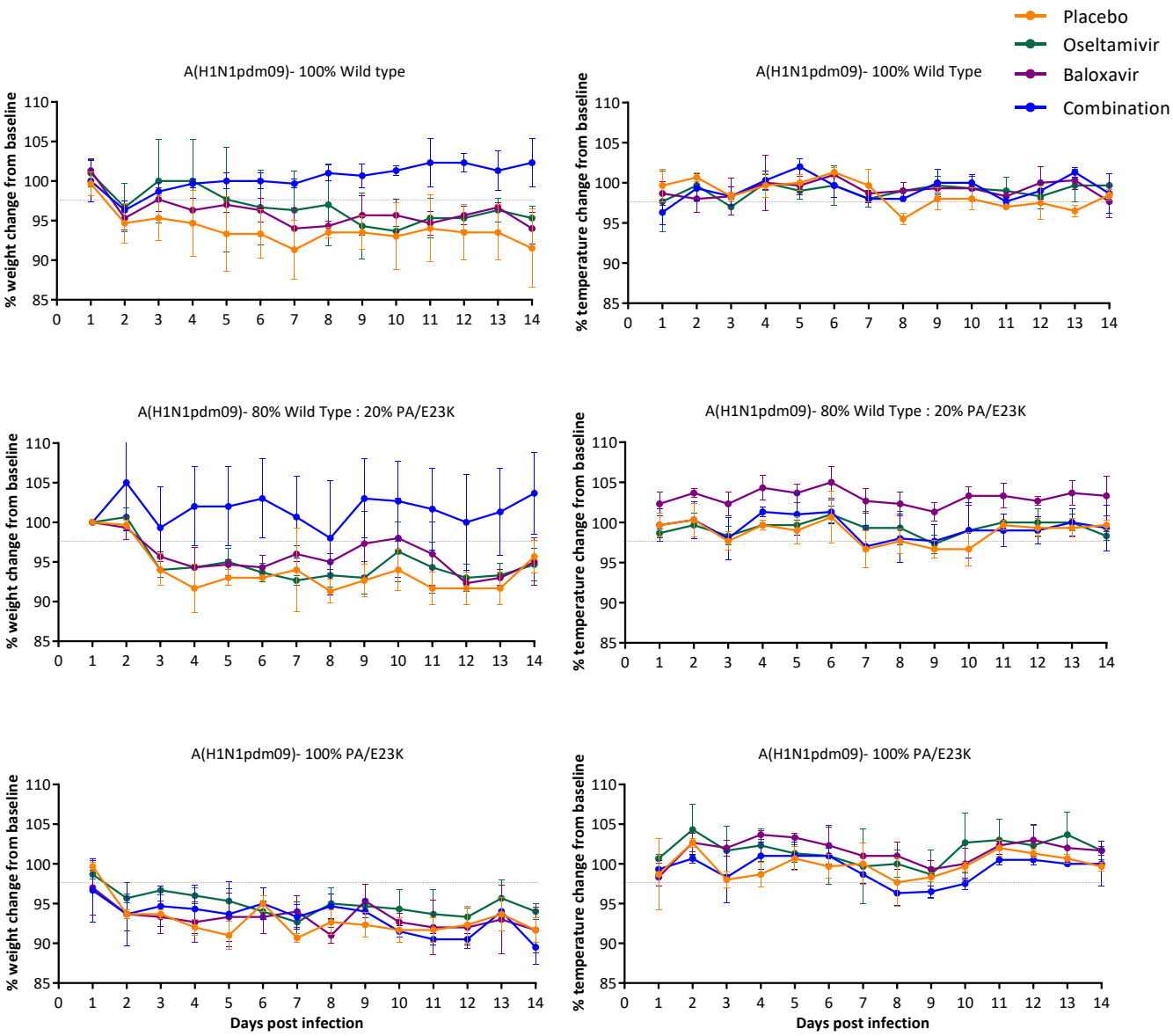

Figure S2: Summary of change in % weight and % temperature of ferrets infected with the A(H1N1pdm09) clinical isolate pair compared to the starting baseline. Weight and temperature of ferrets were measured daily and the line plot depicts the mean and standard deviation in each ferret treatment group.

Supplementary Table 1. Serum antibody hemagglutinin inhibition (HAI) responses 14 days post-infection

	Treatment group			
	Placebo	**Oseltamivir**	**Baloxavir**	**Combination**
Virus/Ferret number				
A(H3N2)-WT				
Ferret 1	160	320	160	160
Ferret 2	160	160	80	80
Ferret 3	80	320	160	160
80% A(H3N2)-WT : 20% A(H3N2)-PA/I38T				
Ferret 1	160	80	640	80
Ferret 2	160	80	160	160
Ferret 3	160	320	160	160
A(H3N2)-PA/I38T				
Ferret 1	320	160	320	320
Ferret 2	160	320	80*	320
Ferret 3	160	160	160	320
A(H1N1pdm09)-WT				
Ferret 1	1280	2560	1280	1280
Ferret 2	2560	2560	1280	2560
Ferret 3	320**	2560	2560	2560
80% A(H1N1pdm09)-WT : 20% A(H1N1pdm09)-PA/E23K				
Ferret 1	2560	2560	2560	640
Ferret 2	1280	2560	2560	2560
Ferret 3	1280	2560	1280	1280
A(H1N1pdm09)-PA/E23K				
Ferret 1	2560	1280	2560	2560
Ferret 2	1280	640	1280	2560
Ferret 3	1280	1280	1280	2560

*Ferret culled and cardiac bleed obtained on day 8 post-infection

** Ferret culled and cardiac bleed obtained on day 7 post-infection

Supplementary Table 2. *In vitro* susceptibility of viruses to neuraminidase inhibitors

	Neuraminidase Inhibitor*			
	Zanamivir IC_{50} (nM)	**Oseltamivir IC_{50} (nM)**	**Peramivir IC_{50} (nM)**	**Laninamivir IC_{50} (nM)**
Virus				
A(H3N2)-WT	0.57 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.96 ± 0.02
A(H3N2)-PA/I38T	0.36 ± 0.3	0.15 ± 0.11	0.12 ± 0.1	0.62 ± 0.5
A(H1N1pdm09)-WT	0.33 ± 0.04	0.3 ± 0.03	0.15 ± 0.03	0.41 ± 0.015
A(H1N1pdm09)-PA/E23K	0.39 ± 0.03	0.33 ± 0.04	0.17 ± 0.01	0.46 ± 0.02

***** IC_{50} values represented as Mean ± Standard Deviation, results from three independent experiments

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