

Division of Investigative Sciences

Faculty of Medicine

**Inhibiting influenza virus replication using novel
and established antiviral compounds.**

Submitted for the degree of

Doctor of Philosophy

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Declaration

I confirm that all work presented here is my own and that the use of all material from other sources has been properly and fully acknowledged.

Daniel Brookes

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Abstract

Circulating influenza viruses have the potential to cause pandemics, as seen in 2009 with the emergence of a novel H1N1 virus that rapidly disseminated worldwide. In such an event, huge reliance is placed upon antiviral drugs for the prophylaxis and treatment of influenza infections. To date, only two different classes of antiviral drug are licensed for the treatment of influenza and only one of these, oseltamivir, has been stockpiled by major world governments in preparation for an influenza pandemic. However, widespread resistance to this drug has been documented in seasonal circulating strains, which emerged in the absence of intense drug use and predominated over drug-sensitive phenotypes. Furthermore, examples of oseltamivir resistant viruses have been found sporadically in pandemic A/H1N1 2009 influenza strains. The need for novel antivirals is therefore essential for effective infection control of influenza viruses in future years. A potential source of novel therapies are licensed compound libraries. We begin with the screening of two compound libraries, resulting in the identification of several compounds which inhibited influenza virus replication *in vitro*. After completing a traditional library screen, a novel approach to high-throughput compound screening using reporter plasmids expressed in a stable cell line was attempted. Although these cell-lines did not prove maintainable in the long-term, this work resulted in the generation of a reporter plasmid that is directly initiated by influenza infection *in vitro* and is thus a useful tool for assaying polymerase fitness of different influenza strains. The investigation then focused on one of the newly discovered hits and attempted to identify the spectrum of activity of this compound, a glycosylation inhibiting molecule, which was shown to be efficacious against influenza A strains only. The drug was shown to have low toxicity and proved active against the recently emerged pandemic influenza virus. The thesis then documents the effects of a common oseltamivir-resistant mutation, H275Y, on the neuraminidase protein of a representative 2009 pandemic influenza virus. This mutation was well tolerated *in vitro* by the virus and did not handicap replication in a human airway epithelial model. However, subtle impediments to the neuraminidase activity of the mutant were observed biochemically which may suggest why this mutation has not emerged more readily in the field.

List of acronyms and abbreviations used in this project

ATP	Adenosine triphosphate
β -gal	β -galactosidase
BAL	Bronchial Alveolar Lavage
BSA	Bovine serum albumin
BVDV	Bovine Diarrhoea Virus
CCLR2	CC Chemokine Ligand 2
CDC	Centres for Disease Control and Prevention
cDNA	Complementary DNA
cRNA	Complementary RNA
CMV	Cytomegalovirus
CN	Cyanide
COX-2	Cyclooxygenase-2
CPE	Cytopathic effect
CRD	Carbohydrate Recognition Domain
CVN	Cyanovirin-N
DANA	2-deoxy-2,3-didehydro-N-acetylneuraminic acid
DI	Defective Interfering

DMEM	Dulbecco's modified eagle medium
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNJ	Deoxynojirimycin
dNTPs	Deoxynucleotide triphosphates
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EMEM	Eagle's minimal essential medium
ER	Endoplasmic Reticulum
EUA	Emergency Use Authorisation
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GTP	Guanosine triphosphate
HA	Haemagglutinin
HAART	Highly active antiretroviral therapy

HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus - 1
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPA	Health Protection Agency
HPAIV	Highly pathogenic avian influenza virus
HSV	Herpes simplex virus
ID ₅₀	Infectious Dose of 50% of cells
Ig	Immunoglobulin
IKK	IκB kinase
IL-6	Interleukin-6
I-κB	Inhibitor of NF-κB
IMPDH	Inosine monophosphate
Kg	Kilogram
LB	Lysogeny Broth
LDL	Low-Density Lipoprotein
M1	Matrix protein 1
M2	Matrix protein 2
MDBK	Madin-Darby Bovine Kidney

MDCK	Madin-Derby Canine Kidney
MES	2-(N-morpholino)ethanesulfonic acid
Mg	Milligram
ml	Millilitre
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MLV	Murine Leukaemia Virus
MuNANA	2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid
NA	Neuraminidase
NAI	Neuraminidase Inhibitor
NDV	Newcastle disease virus
NEP	Nuclear export protein
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NI	Neuraminidase Inhibiting
NIAID	National Institute of Allergy and Infectious Disease
NK	Natural killer
NLS	Nuclear localisation signal
NP	Nucleoprotein

NPFS	National Pandemic Flu Service
NV	New variant
NS1	Non-structural protein 1
ONPG	<i>ortho</i> -Nitrophenyl- β -galactoside
ORFs	Open reading frames
ORV	Oseltamivir-resistant virus
PA	Polymerase acidic protein
PAMP	Pathogen-associated molecular patterns
PB1	Polymerase basic protein 1
PB1-F2	Polymerase basic protein 1-Frame 2
PB2	Polymerase basic protein 2
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PRR	Pattern recognition receptor
PPAR	Peroxisome proliferator-activated receptor
PTX3	Pentraxin 3
RFU	Relative Fluorescence Units
RNA	Ribonucleic acid
RNP	Ribonucleoprotein

RMP	Ribavirin monophosphate
RTP	Ribavirin triphosphate
RT-PCR	Reverse-transcriptase Polymerase Chain Reaction
RSV	Respiratory Syncytial Virus
SP-D	Surfactant Protein-D
SDS	Sodium dodecyl sulphate
SF	Serum-free
TCID ₅₀	Tissue Culture Infectious Dose of 50% of cells
TD ₅₀	Toxic Dose of 50% of cells
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2
TRP	Tamiflu Reserves Programme
UK	United Kingdom
US	United States of America
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
VLP	Virus Like Particle
VSV	Vesicular Stomatitis Virus

WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
XMP	Xanthosine monophosphate

Amino acid code

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

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1. Introduction

The influenza virus is an important pathogen of humans and animals across the globe. As the etiological agent of influenza, these viruses are associated with significant morbidity amongst the general population and mortality amid risk groups, such as the elderly, immunocompromised and the very young. Influenza is a highly contagious, acutely febrile respiratory ailment which is often diagnosed on the following symptomatology: sudden onset fever, cough, headaches, debility, muscle pain and anorexia (Eccles, 2005). Influenza viruses are enveloped RNA viruses belonging to the Orthomyxoviridae family, more specifically, the Influenzavirus A, B and C genera (Palase & Shaw, 2007). Types A and B present a major clinical burden worldwide in the form of seasonal outbreaks, resulting in 3-5 million cases and up to 500,000 deaths during any given influenza season (WHO, 2003). Influenza A viruses are comprised of a single-stranded RNA genome fragmented into eight segments that encode eleven or twelve separate proteins (Lamb & Krug, 2001; Wise *et al.*, 2009). The Influenza A viruses are further subtyped based on the haemagglutinin (HA) and neuraminidase (NA) glycoproteins present on the outer membrane of the virus particle, of which there are currently 16 recognised HA subtypes (1-16) and 9 different NA variations (1-9) (Alexander, 2007; Fouchier *et al.*, 2005). Each virus particle adopts one HA and one NA antigen in potentially any combination. It is by virtue of this segmented genome that influenza viruses pose a constant pandemic threat, as reassortant viruses can emerge which introduce novel surface antigens into a naive population. If these viruses are able to transmit readily between humans, then a pandemic is likely to occur. The pandemics of 1918, 1957, 1968 and most recently 2009, were all associated with significant illness, due in part to the absence of pre-existing immunity against the novel influenza strain, and in the case of the 20th century pandemics, this contributed to vastly increased mortality (Table 1.1).

Year	Subtype	Estimated deaths	Case fatality rate
1918-1919	H1N1	40-100 million	>2.5%
1956-1958	H2N2	2 million	<0.1%
1968-1969	H3N2	1 million	<0.1%
1997-Present	H5N1	300	>59%
2009-Present	H1N1	~20,000 confirmed	lab <0.03%
Seasonal Influenza	H1N1, H3N2 & B	~500,000	<0.1%

Table 1. 1 Significant influenza epidemics and pandemics of the last one hundred years.

Extrapolated from (Johnson & Mueller, 2002; Taubenberger & Morens, 2006; WHO, 2005; 2010a; b)

Given the toll influenza viruses continue to exact on the human population, control of both seasonal and pandemic infections is a challenge of significant clinical importance. There are currently two established infection control methods employed to reduce and prevent the spread of the influenza viruses: seasonal influenza vaccines and antiviral drugs.

1.1. Inhibiting influenza virus spread: Vaccination

The principle prophylactic method is immunisation using an annually updated, trivalent influenza vaccine. This vaccine contains antigens from two different type A influenza strains (H3 and H1) and one type B strain, which are chosen according to circulating viruses detected by the World Health Organisation's global influenza surveillance network. Although the influenza vaccine is well tolerated in the general population, sole reliance on a vaccine to control the spread of the virus is unfeasible given the antigenic drifting and shifting potential of the influenza genome. Due to the high mutation rate of the virus, the annual vaccine remains effective for approximately one year, providing a constant need for

vaccine reformulation and readministration to at-risk groups. This provides a unique challenge to public health officials as enthusiasm for the immunisation programme and public attitudes towards vaccination change over time. The influenza vaccine is unlikely to ever eradicate influenza due to the broad host-range utilised by Influenza A viruses – able to infect humans, pigs, sheep, felids, equines and birds (Amonsin *et al.*, 2006; Keawcharoen *et al.*, 2004). – and the fact that the complete virus reservoir exists entirely in free-flying avian populations, ensuring the risk of zoonotic infection is ever present (Capua & Alexander, 2002). Furthermore, there is a risk that the World Health Organisation's recommended vaccine strains result in a poor match to the drift variants which eventually circulate during the ensuing influenza season. Such a mismatch occurred in 1997 when the H3N2 variant A/Sydney05/97 emerged and caused severe disease outbreaks in Europe and the US. Of the collected H3N2 samples analysed by the WHO during this season, only 23% of the isolates matched the selected H3N2 vaccine strains of A/Nanchang/933/95 or A/Wuhan/359/95, with the remaining 77% matching the Sydney strain. This vaccine failure is thought to have resulted in a drop in vaccine effectiveness from 65% to 31% at preventing all-cause mortality in the elderly that season (Nordin *et al.*, 2001). Finally, the current vaccine manufacturing technique utilises growth of the candidate viruses in chicken eggs, thus precluding those with egg-related allergies from receiving the vaccine. Seasonal influenza vaccines are also unlikely to protect against novel, pandemic influenza isolates, as witnessed with the pandemic A/H1N1 2009 outbreak to which the seasonal vaccine was not seen to offer cross-protection (Iuliano *et al.*, 2009; Kelly & Grant, 2009). This pandemic outbreak required a new vaccine to be generated, tested and marketed *de novo* after the pandemic was declared.

1.2. Inhibiting influenza virus spread: Antiviral Drugs

Antiviral drugs constitute the second branch of influenza infection control. Antiviral drugs, which target ongoing viral replication, are employed for both treatment purposes and as

emergency prophylactics, and their efficacy is not determined by the antigenic constitution of circulating viruses. Antiviral drugs were used extensively following the outbreak of pandemic A/H1N1 2009 and represented the only clinical option in the interim time period between the initial declaration of a global pandemic, and the marketing of an effective vaccine against this virus five months later. Before the outbreak of pandemic H1N1 influenza in 2009, the scientific community had anticipated the emergence of a new influenza pandemic, with the highly pathogenic avian influenza H5N1 virus being considered the most likely outbreak candidate for over a decade. Since the emergence of H5N1 in 1997, research has intensified in the field of influenza antivirals given their essential role in the management of pandemic and seasonal influenza outbreaks. However, to date only a limited number of authorised anti-influenza compounds exist.

Presently, there are two classes of antiviral drug licensed for the treatment of influenza; the M2 inhibitors - amantadine and rimantadine and the neuraminidase inhibitors - zanamivir and oseltamivir. The M2 inhibitors ('adamantanes') represent the oldest class of influenza antiviral, with amantadine being discovered during the early 1960s. The drug was first officially approved for use against all influenza A infections in 1976, whilst its congener, rimantadine, was licensed in 1993 in the United States (Hayden & Aoki, 2005). M2 inhibitors are effective against influenza A viruses only, due to the absence of the M2 protein from the influenza B genome. By blocking the M2 protein, adamantane drugs prevent proton flux across the ion channel and inhibit viral uncoating in the endosome (reviewed below). The clinical effectiveness of M2 inhibitors has substantially diminished in recent years due to the rapid emergence of resistant influenza isolates that remain genetically stable and fully transmissible in reservoir populations, including man. Seasonal influenza viruses now boast widespread resistance to this drug class and the 2009 A/H1N1 pandemic virus is also resistant. In the early years, the adamantane drugs were widely used due to their low cost and long-term stability, with early studies suggesting that only 0.8% of

isolates emerged with drug resistance between 1992-1995 (Ziegler *et al.*, 1999). However, ongoing surveillance reported that by 2004, 12.3% of worldwide H3N2 seasonal isolates had acquired drug resistance, with individual countries such as China and South Korea reporting that 96% and 72% of all H3N2 isolates were adamantane resistant respectively (Bright *et al.*, 2005). Although resistance appeared to proliferate primarily and most rapidly in South East Asia, the United States also saw the emergence of a predominant drug resistant H3N2 phenotype, as surveillance reported <2% of viruses bearing drug resistance in 2004, 14.5% in 2004-2005 and 92.3% in 2005-2006 (Bright *et al.*, 2006). Between 2001 and 2007, H1N1 isolates represented the minority of circulating strains worldwide; however, by 2006 15.5% of all isolates were resistant to the adamantane drugs (Deyde *et al.*, 2007). Meanwhile, individual regions such as Japan reported >62% of H1N1 isolates as conferring resistance during the 2006-2007 season (Saito *et al.*, 2008). Resistance is almost ubiquitously conferred by a mutation in the M2 ion channel protein, S31N, although five mutations in total are known to each confer resistance to the adamantanes; at positions 26, 27, 30, 31 and 34. By the 2006-2007 season, the unprecedented emergence of drug resistance across both H1 and H3 seasonal subtypes effectively ruled out the entire drug class as a therapeutic option for influenza. Unsurprisingly, since 2006 it has been recommended that adamantane drugs no longer be considered as a treatment for influenza A infections in the United States (CDC, 2006).

As well as the emergence of viral resistance towards this drug class, use of the adamantanes has also been linked to a number of adverse side-effects. The most commonly reported of these include gastrointestinal complaints such as nausea, vomiting, and dyspepsia, and central nervous system disturbances, including anxiety, insomnia, depression and confusion (Dolin *et al.*, 1982). Long term use of amantadine therapy has also been linked to vision loss, hypotension, urinary retention, peripheral oedema and

congestive heart failure, which could greatly undermine the effectiveness of using the drug as a long term prophylactic measure against susceptible influenza strains (Prober, 2002).

The neuraminidase inhibitors (NAI) are the only drug class licensed for use against both type A and type B influenza viruses and represent the most reliable anti-influenza drug available for treatment today. NAIs inhibit the neuraminidase enzyme and thus prevent the final step of the virus replication cycle by prohibiting viral release from an infected cell after budding. This enzyme is responsible for cleaving sialic acid residues which otherwise remain omnipresent on both the cell surface and on the surface of adjacent budding virions. Deprived of neuraminidase activity, nascent virions reattach to these sialic acid residues via the more abundant surface glycoprotein, haemagglutinin, causing viral aggregation and preventing progeny virus dissemination.

Both licensed NAI drugs are structurally similar (Figure 1.1), though zanamivir (trade name Relenza) is administered as an inhalant, whilst oseltamivir (Tamiflu) is administered as an oral dose. Relenza was marketed as the first major breakthrough in influenza treatment in over thirty years. The U.S Food and Drugs Administration (FDA) approved Relenza for use in 1999, and in doing so, the first anti-influenza drug was licensed since rimantadine in 1993 (Miller, 1999). Administration via an inhaler is necessary due to the poor absorbance of the drug from the gastrointestinal tract. Due to a negatively charged group on the zanamivir molecule, only 1-5% of an oral dose is efficiently absorbed. This necessitates topical delivery as a dry powder via an inhaler, which directs the drug directly to the oropharynx, the primary site of influenza infection. Although this advantageously directs the antiviral rapidly to the site of infection, it may result in bronchospasm, making treatment of asthmatics a risk and of those with pulmonary destructive diseases altogether unfeasible.

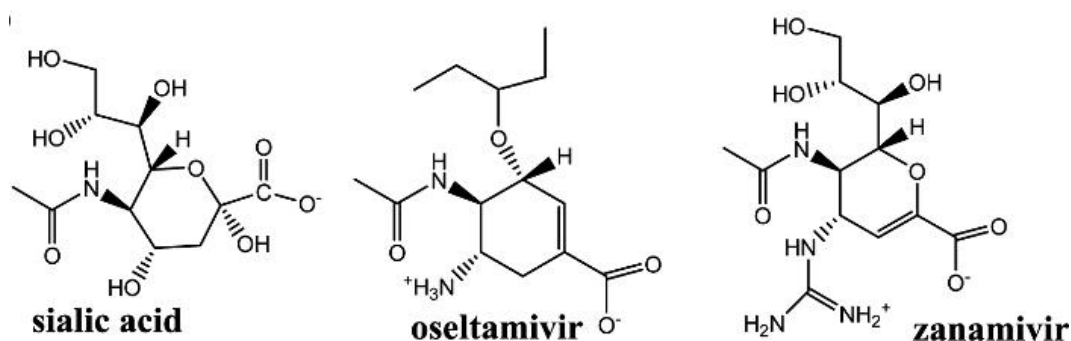


Figure 1.1. The molecular structure of sialic acid and the neuraminidase inhibitors, oseltamivir and zanamivir.

Oseltamivir is administered as an inactive prodrug, oseltamivir phosphate, which is metabolised into its active form, oseltamivir carboxylate in the liver. Although both drugs are administered in different ways, their clinical effectiveness is equally comparable and both treatments alleviate influenza symptoms by approximately 0.5-1 day (Burch *et al.*, 2009). The development of the first orally administered neuraminidase inhibitor allowed for the treatment of younger children, and provided a more convenient drug delivery method for those with respiratory complications. In anticipation of an influenza pandemic, oseltamivir has become the focus of stockpiling strategies due to its low toxicity and convenient oral formulation (Meijer *et al.*, 2007). Between 2004 and the outbreak of pandemic H1N1 influenza in 2009 it has been suggested that 350 million treatment regimens were stockpiled by governments worldwide, which equates to approximately enough doses to treat 5% of the global population (Reddy, 2010). Compared to the adamantane drugs, NAIs are less inclined to generate resistant viruses and until 2007, only 0.2% of all human epidemic influenza viruses monitored possessed natural resistance to oseltamivir (Sheu *et al.*, 2008). However, the effectiveness of oseltamivir as a therapeutic drug was significantly reduced during the 2007/2008 influenza season, which saw the unexpected emergence of H1N1 viruses containing the mutation H275Y (N1 numbering) in the N1 neuraminidase protein. The H275Y mutation is well characterised in H1N1 viruses

and confers resistance to both oseltamivir, and the experimental neuraminidase inhibitor peramivir, but not to zanamivir (Abed *et al.*, 2006). The detection of NAI-resistant viruses by surveillance networks occurred simultaneously across several countries and did not correlate with drug use (Eshaghi *et al.*, 2009; Hurt *et al.*, 2009; Meijer *et al.*, 2009; Njouom *et al.*, 2010). The following 2008/2009 season saw this trend continue as drug resistant viruses predominated over susceptible seasonal H1N1 strains, with some countries reporting up to 100% of isolates possessing resistance to oseltamivir via H275Y (Baranovich *et al.*, 2010; Correia *et al.*, 2010; Kawai *et al.*, 2009a; Kawai *et al.*, 2009b). Pandemic nvH1N1 viruses remain largely susceptible to the NAI class of drug, even with oseltamivir being prescribed on a wide scale as a pandemic control measure, though resistant isolates containing H275Y have been detected (Chen *et al.*, 2009; Harvala *et al.*, 2010; Memoli *et al.*, 2010; Valinotto *et al.*, 2010). Had pandemic nvH1N1 emerged with the same invulnerability to oseltamivir as seasonal H1N1 viruses, then stockpiles of this drug would have been rendered redundant, leaving only limited supplies of zanamivir available for infection control measures. Reliance on a single drug, against which substantial resistance presides in circulating viral strains, is dangerous and could prove costly in terms of human health and finance. Thus, the need for novel inhibitors of influenza viral replication is an urgent priority in preparation for future epidemics and pandemics.

Subtype	Oseltamivir	Zanamivir	Adamantanes
Seasonal H1N1	Resistant	Susceptible	Mostly Susceptible
Seasonal H3N2	Susceptible	Susceptible	Resistant
Influenza B	Susceptible	Susceptible	Resistant
HP H5N1	Mostly Susceptible	Susceptible	Mostly Resistant
Pandemic H1N1	Susceptible	Susceptible	Resistant

Table 1. 2. Susceptibility of currently circulating influenza isolates to antiviral drugs.

(Correct as of June 2010. Adapted from (WHO, 2010c))

Thankfully, this observation has not gone unnoticed and research into antiviral compounds against influenza has generated many previously undescribed molecules with novel mechanisms of action. This review seeks to give a broad overview of the numerous compounds presented in the literature and draw attention to the potential for each of these drugs to complement our current clinical armamentarium.

1.3. Drugs which target the influenza virion

Both of the currently licensed classes of antiviral drug target components of the influenza virion in order to inhibit replication of the virus. By specifically targeting the influenza virion, antiviral drugs should ideally succeed in having low toxicities in humans and avoid causing adverse side effects. Novel drugs which interact with and inhibit the proper function of influenza proteins have thus remained popular in the area of antiviral research. In order to understand where and how potential new drugs can interfere with the virus replication cycle, a knowledge of virion structure and the mechanism by which it reproduces itself is required. The following section seeks to introduce the influenza virion and then elaborate on the well-characterised methods by which the influenza virus infects host cells, replicates its genome, and then passes progeny virus on to other cells. Potential targets for therapeutic intervention by novel compounds will be reviewed at each step of the replication cycle.

1.3.1. The influenza virion

The influenza viral envelope consists of a lipid bilayer contributed by the host cell, containing three transmembrane proteins on the outside and matrix protein (M1) on the inside (Figure 1.2.). The three transmembrane proteins: haemagglutinin (HA), neuraminidase (NA) and M2 (ion channel) are anchored in the lipid bilayer, with haemagglutinin the dominant presence covering approximately 80% of the virion surface.

Underlying the lipid bilayer is the M1 protein, a major structural component of the virion. The M1 shell surrounds the viral core, consisting of helical ribonucleocapsids (RNP) which contain the negative stranded RNA genome of the virus, coiled to nucleoprotein (NP) and three polymerase proteins (3P proteins: PB1, PB2 and PA) (Portela & Digard, 2002). Minor quantities of NEP (Nuclear Export Protein, formerly known as NS2) are also present in the virion complex. Other proteins encoded by the influenza genome include NS1, which is expressed intracellularly and absent from the final virion and in some strains, PB1-F2, which is also excluded from the virion and is not essential for virus replication (Chen *et al.*, 2001).

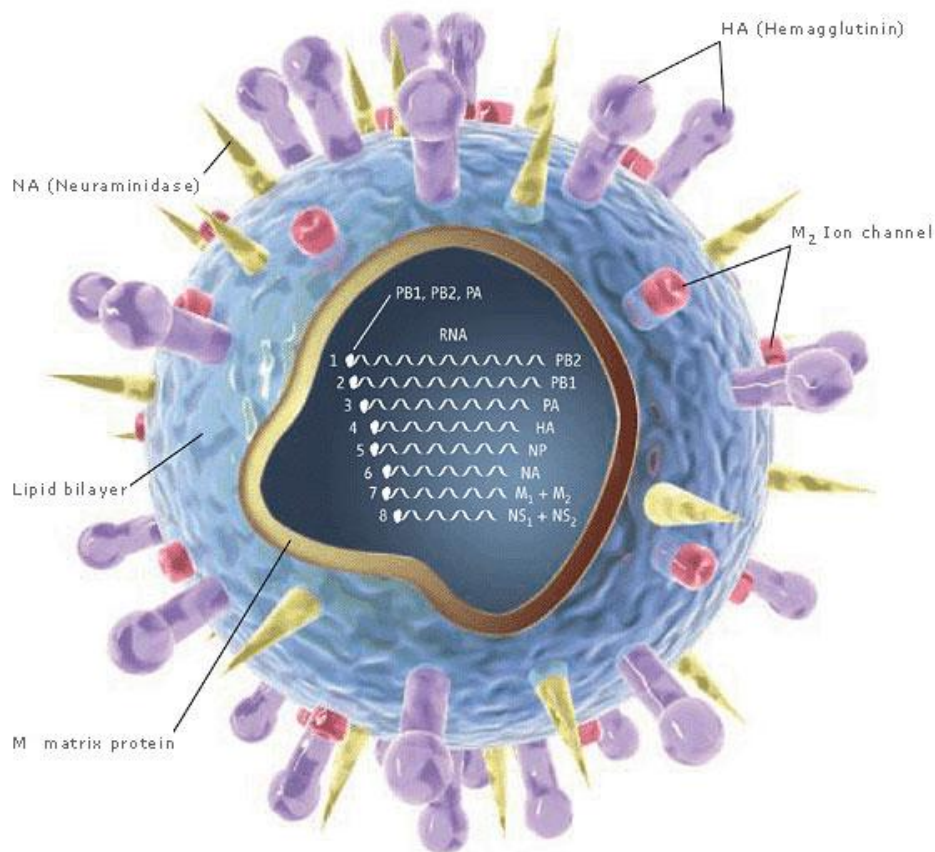


Figure 1.2. The influenza A virion.
(Image credit: (Kaiser, 2006))

1.3.2. Attachment

The first step of the replication cycle is attachment of the virus to the host cell, a process mediated by the haemagglutinin glycoprotein. Haemagglutinin is a homotrimer which binds to sialic acid residues, which are found ubiquitously on the surface glycoproteins of cells, via a shallow receptor binding site at the distal end of the protein. The conformation of sialic acid present on the cell surface (α 2,3-linked or α 2,6-linked) is an important determinant of species-specific restriction of certain influenza viruses, with the viral haemagglutinin of human influenza viruses traditionally preferring the α 2,6-linkage of sialic acid, compared to the α 2,3-linkage preferred by avian strains (Gambaryan *et al.*, 2005; Rogers & Paulson, 1983). This observation likely explains the tendency of human influenza viruses to infect tracheal epithelial cells abundant in α 2,6-linked sialic acid and cause a localised respiratory infection, compared to avian influenza viruses which replicate in the α 2,3-linked rich area of the avian gut and remain enteric (Ito & Kawaoka, 2000). It should be noted that this observed viral specificity is not absolute, however, and that certain cells in the human lower airway have been shown to present α 2,3-linked sialic acid moieties (Shinya *et al.*, 2006). Moreover, ciliated cells in human airway cultures also display α 2,3-linked sialic acid and are vulnerable to infection with avian influenza viruses (Matrosovich *et al.*, 2004; Thompson *et al.*, 2006). The interaction between the distal tip of a haemagglutinin spike and the sialic acid receptor is one of low-affinity, and the high avidity of the influenza virion for cell surfaces is achieved through multiple low-affinity interactions. Influenza attachment, and in particular the haemagglutinin protein, represents an attractive target for drug design due to its highly conserved active site and its tendency to be maintained even through antigenic drift events. Furthermore, neutralising antibodies generated by the host immune system are effective by targeting the haemagglutinin protein, and the ability to inhibit viral replication and provide therapeutic intervention before pathogen proliferation is an obvious incentive.

1.3.2.1. Inhibiting viral attachment

The innate immune system of humans pre-dates clinical science in attempting to inhibit viral replication through the prevention of initial attachment. Components of the host sera and mucosal secretions are capable of binding the surface glycoproteins of influenza viruses. Such components include collagenous lectins (collectins) which bind glycosylated regions of influenza glycoproteins and can inhibit haemagglutination, as well as virus replication *in vitro* and *in vivo*. Collectins are inhibitory via both steric hindrance of the HA protein and by enhancing immune cell opsonisation (Benne *et al.*, 1995; Hartshorn *et al.*, 1994; LeVine *et al.*, 2001). Furthermore host proteins present in the saliva, such as mucins, are highly sialated and restrict the spread of influenza viruses by providing false receptors to which viral haemagglutinin will bind instead of susceptible cells (White *et al.*, 2009). Our increasing understanding of the pulmonary secretory system has led some to speculate that the individual peptides utilised by our innate immune response, refined and selected over many evolutionary generations, may represent therapeutic potential (Doss *et al.*, 2009). If such a drug was developed that utilised non-specific host proteins as its active element, then it is likely that the therapy would have a very broad-spectrum of activity across multiple microorganisms and theoretically, few toxicity issues. An early attempt to create a compound that utilises collectins resulted in the generation of a trimeric molecule that contains the truncated neck, and carbohydrate recognition domain (CRD) of surfactant protein-D (SP-D); an innate defence collectin found in the BAL of humans. This compound was initially shown to be less effective at neutralising haemagglutination and *in vitro* infectivity of MDCK cells by influenza A viruses when compared with the natural multimeric form of SP-D (Hartshorn *et al.*, 2007). However, the neutralisation ability of this molecule could be restored by replacing the neck and carbohydrate recognition domain with that of bovine SP-D rather than the human form (Hartshorn *et al.*, 2010). These compounds were shown to be antiviral via several mechanisms including inhibition of the viral neuraminidase

protein, by enhancing uptake by neutrophils and causing enhanced virus aggregation. These early studies highlight the potential of using trimeric collectins as a therapy, however the advantageous effects have only been measured *in vitro* to date and it remains to be seen whether they can be recapitulated in a relevant animal model.

A similar high molecular weight protein, pentraxin 3 (PTX3), has also been shown to exhibit antiviral activity and plays a role in recognising and binding a range of pathogens. PTX3 is an innate, long-chain protein that is rapidly upregulated in response to primary inflammatory signals by several cell types, including endothelial and dendritic cells, as well as mononuclear phagocytes (Bottazzi *et al.*, 2006). Human PTX3 was shown to inhibit haemagglutination by an H3N2 virus *in vitro* and also improved the survival of mice from 40 to 100% when administered daily as a treatment. It was also shown that infection of mice lacking the PTX3 gene generated higher viral titres in the BAL than wild type mice that retained PTX3 function (Reading *et al.*, 2008). Interestingly, PTX3 only inhibited older subtypes of virus, with H3N2 viruses isolated after 1975 being resistant to the antiviral effects of the protein. This observation reflects the change in sialic acid binding preferences of H3N2 viruses which over time have evolved to bind preferentially to α 2,6-linked sialic acid, whereas PTX3 is antiviral due to its abundance of α 2,3-linked glycoconjugates, which operate as false receptors for the virus. Indeed, most recent evidence suggests that both the pentraxin and collectin families are ineffective against the A/H1N1 pandemic influenza virus (Job *et al.*, 2010). These observations probably precludes PTX3 as an effective antiviral, though it again highlights the potential of innate molecules as therapeutic interventions.

Another mechanism for preventing viral attachment is via steric inhibition. Cyanovirin-N (CVN) is an example of a drug which inhibits in this manner, and was initially discovered as an anti-HIV drug, before showing activity against several other enveloped viruses including

Ebola and influenza (Barrientos *et al.*, 2003; Boyd *et al.*, 1997; O'Keefe *et al.*, 2003). CVN is a mannose-binding protein and binds to glycosylation sites on the haemagglutinin. Treatment with CVN resulted in a 1000-fold reduction in viral titre in the mouse model when treatment began 4 hours pre-infection. This was superior to ribavirin, which only reduced viral titres 30-fold. In ferrets, the drug also succeeded in reducing nasal viral titres and in doing so showed greater efficacy than oseltamivir (Smee *et al.*, 2008). Although the drug showed efficacy in the mouse and ferret models, the compound suffers from a similar disadvantage to PTX3 in that only certain viral strains are susceptible. Mutants that abrogate glycosylation sites in the haemagglutinin protein can restrict drug binding and allow the virus to withstand the inhibitory effects of CVN. In general, mouse-adapted influenza viruses are resistant to CVN due to the occlusion of glycosylation sites in the haemagglutinin protein as part of the adaptation process. Resistance to the drug has also been demonstrated in cell culture, suggesting that the selective pressure of the drug could select for isolates that are naturally resistant to CVN (Smee *et al.*, 2007).

Since the structure of viral haemagglutinin in complex with sialic acid receptors was resolved in the late 1980s (Weis *et al.*, 1988), there have been numerous attempts to generate synthetic inhibitors which target the active site of this protein. Initial research attempted to generate low-molecular weight, monovalent, sialic-acid-containing receptor mimics that it was hoped would have sufficient affinity to the receptor binding site to competitively inhibit the haemagglutinin protein. Such molecules were found to inhibit the ability of an H3 haemagglutinin to adsorb human erythrocytes in a dose-dependent fashion. However, large concentrations of compound in the millimolar range (15mM) were required to guarantee complete inhibition (Pritchett *et al.*, 1987). Conversely, utilisation of the high molecular weight 'glycoprotein 2' from bovine erythrocytes, artificially modified to present approximately 200 sialic acid residues on its surface was shown to inhibit haemagglutination by influenza viruses at micromolar concentrations (1-4µM) (Suzuki *et*

al., 1987). These experiments reveal that influenza haemagglutinins bind preferentially to multivalent substrates and suggest that any attempt to inhibit binding should be made with larger compounds which present multiple sialic acid residues. Multivalent substrates are much more representative of the receptors that viruses have evolved to bind during the course of an infection, and given the low affinity individual haemagglutinin proteins display for single receptors, it is unlikely that monovalent molecules will compete effectively with the multivalent interactions which occur between the virus and cell surface. Polyvalent inhibitory molecules have undergone continued development since the late 1980s and as a result, multiple structural variations of sialic-acid-containing polymers have been reported. To date, polymeric inhibitors have appeared as sialic-acid containing dendritic polymers (Reuter *et al.*, 1999), sialyllactose covered polystyrene molecules (Tsuchida *et al.*, 1998) and glutamic-acid-bound sialyloligosaccharides (Totani *et al.*, 2003), each with the capability to bind potently to influenza surface glycoproteins and prevent haemagglutination *in vitro*. However to date the effect of these compounds on the influenza replication cycle, and indeed use of these compounds *in vivo*, has not been reported and thus this therapy remains at an early stage of development. It should also be mentioned that this class of drug has typically exhibited subtype specific inhibition depending on which sialic acid residues (α 2,6- or α 2,3-linked) were presented by the inhibitory polymer. Furthermore it is prudent to assume that these compounds may face the problem of irreversible destruction by the influenza neuraminidase enzyme and thus their success may depend on the neuraminidase activity of the target virus.

Alternative mechanisms for inhibiting influenza virus entry have been proposed which do not involve inhibiting the haemagglutinin protein at all. Instead of mimicking the sialic acid receptor, the novel antiviral drug Fludase (also DAS181) purports to prevent attachment by completely removing sialic acid receptors from the cell surface. Of all the attachment inhibiting compounds mentioned so far, Fludase is the closest to clinical use. Billed as a

'broad spectrum therapeutic agent for all annual and pandemic variations of influenza', Fludase emerged as a result of Biodefense funding from the National Institute of Allergy and Infectious Diseases (NIAID). The drug is further unique compared to the aforementioned compounds in that it targets the host cell, rather than viral components in order to prevent influenza infection. More specifically, Fludase is a sialidase fusion protein which removes the sialic acid receptors used by influenza to bind to the host cell and thus prevents attachment and endocytosis of the virion into the host cell. The sialidase component of Fludase is acquired from *Actinomyces viscosus*, a commensal microorganism of humans, linked to an epithelial anchoring domain to ensure drug concentrations remain localised to the trachea. The compound is mass produced in *Escherichia coli*, leaving the potential for easy up-scaling should increased demand arise (Malakhov *et al.*, 2006). The bacterial sialidase functions analogously to influenza neuraminidase and actively cleaves both α 2,3-sialic acid-linked receptors, traditionally utilised by avian influenza viruses and α 2,6-sialic acid-linked receptors, preferred by human influenza viruses (Teufel *et al.*, 1989). The mechanism of action of Fludase has led to concerns against its potential use in the clinic (Zhang, 2008); namely that the removal of sialic acid receptors has been shown to increase susceptibility to secondary infection by pneumonia-causing bacteria in a number of different animal models (Braun *et al.*, 2007; McCullers & Bartmess, 2003; van der Sluijs *et al.*, 2004). Bacterial pneumonia has long been associated with influenza virus infection and it is believed that secondary bacterial pneumonia was responsible for more deaths during the influenza pandemics of the 20th century than acute influenza infection itself. However, a recent study has moved to alleviate this concern by suggesting that Fludase treatment did not exacerbate secondary bacterial infection in mice by *S. Pneumoniae* and in fact those mice receiving the drug were protected from other opportunistic bacterial pathogens compared to those receiving vehicle formulations (Hedlund *et al.*, 2010). It has been suggested that removing sialic acid receptors is not enough to completely block

influenza virus infection and that while sialic-acid enhances the efficiency of viral entry, it was ultimately 'dispensable' as sialidase treatment did not abolish replication of influenza *in vitro* (Stray *et al.*, 2000). However, a recent study has highlighted the importance of sialic acid on the cell surface in the role of successful viral replication. It was revealed that by suppressing sialic acid production in cells using siRNA against key sialyltransferases involved in the biosynthesis of α 2,3-linked sugar moieties, subsequent attempts to infect these cells with an H5N1 virus resulted in attenuation of virus replication by approximately 2 logs (Monteerarat *et al.*, 2010). Furthermore, Fludase has been shown effective at reducing viral titres of a number of recent seasonal oseltamivir-sensitive and -resistant H1N1 viruses *in vitro* and was efficacious as a prophylactic and therapeutic measure in mice challenged with highly pathogenic avian influenza A/Vietnam/1203/2004 (H5N1) (Belser *et al.*, 2007; Triana-Baltzer *et al.*, 2009). An isomeric precursor molecule of Fludase (DAS178) was well tolerated in ferrets when administered twice daily for seven days over the course of the experiment, and improved the outcome of an H1N1 challenge infection, protecting 25% of the animals from any detectable influenza infection at all (Malakhov *et al.*, 2006). Having a therapeutic drug that targets the host cell should ensure less frequent emergence of viral resistance and thus gives Fludase a potential benefit over other novel anti-virals. The results of an ongoing phase one clinical trial are now highly anticipated.

Finally, a broad-spectrum antiviral compound was recently described with potency against several enveloped viruses, including Ebola, HIV, West Nile, Yellow Fever, HCV and influenza (Wolf *et al.*, 2010). It is known that, unlike the host cell, viruses lack the ability to repair or recycle plasma membranes and are entirely reliant on the lipids accumulated from the host during the budding process. This therefore leaves the viral membrane susceptible to specific disruption by antiviral drugs. The compound described, LJ001, exploits the virion's inability to restore lipid membrane function and irreversibly binds to the virion envelope. It is hypothesised that after intercalating into the lipid bi-layer, the polar pharmacophore

group of the compound remains free to disrupt lipid membranes and subsequently disturb fusion of the viral and cell membranes during viral entry. Although this drug is capable of chelating with host-cell membranes, it remains largely nontoxic to the cell, though the presence of inhibitors of fatty-acid biosynthesis markedly enhance the toxicity of the drug, suggesting that the host cell's membrane reparation ability is only sufficiently able to cope with the potentially toxic mechanism of the drug under normal conditions. Although this compound has exhibited a broad-spectrum activity against several enveloped viruses in vitro, it has thus far only exhibited prophylactic activity and must interact with the virus before it contacts the cell to be effective.

1.3.3. Entry and fusion

Influenza virions bound to cell surface sialic receptors are then internalized via receptor-mediated endocytosis (Matlin *et al.*, 1981). Following endocytosis, the virion must escape from the endosome before it can deposit its genetic material in the nucleus. Once inside an endosome, the virus is exposed to a low pH environment thus facilitating the next step of the replication cycle; fusion. Fusion of the virion with the endosome is initiated by an irreversible conformational change in haemagglutinin glycoproteins on the surface of the internalised virion, provided that the HA0 precursor molecule has been cleaved into two subunits, HA1 and HA2 (Lazarowitz & Choppin, 1975). This fusion event results in the exposure of the hydrophobic HA2 N-terminal fusion peptide core of the haemagglutinin protein, while the HA1 receptor binding domain remains structurally intact (reviewed in (Stegmann, 2000)). The exposed domain can now interact with the endosomal membrane, resulting in the HA2 component having contact with both the endosomal membrane via its N-terminus and the virus membrane via its C-terminus. Once several HA molecules have undergone this structural change, the opposing membranes are essentially pulled together and then fused with each other, creating a fusion pore (Figure 1.3.). Cleavage of HA is thus

an absolute requirement for infectivity. Without proper HA cleavage, virions may bind to and enter the cell, but will remain in the endosome and are essentially non-infectious.

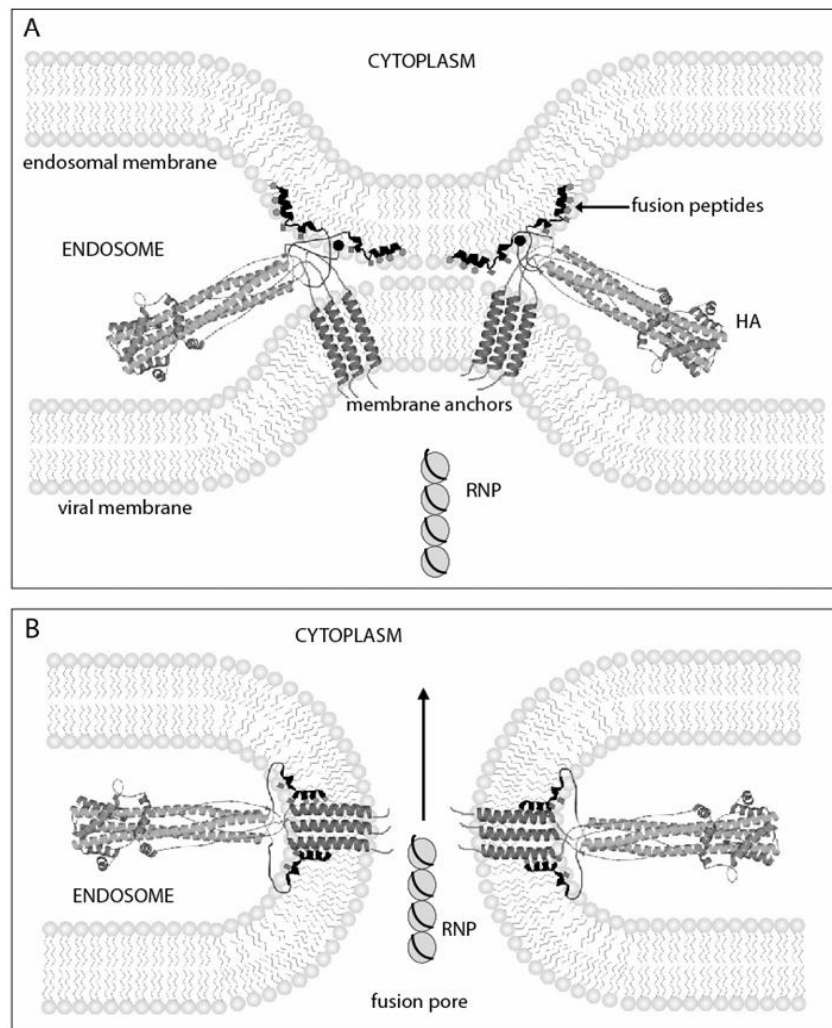


Figure 1.3. Fusion of the viral and endosomal membranes.

(A) HA spike undergoes a conformational change due to the low pH in the endosome, resulting in exposure of the hydrophobic core which then interacts with the membrane of the endosome. (B) The force of several HA spikes undergoing the same changes pulls the membranes apart, resulting in a fusion pore, via which the viral RNP can exit the endosome.

The acidic conditions of the endosome also facilitate conformational changes in the M2 ion channel protein, which opens and allows the influx of H⁺ ions that result in the acidification of the virion core. This shift in pH disrupts protein-protein interactions and results in the release of vRNPs from the M1 structural protein, enabling them to exit through the fusion pore (Bui *et al.*, 1996). The dissociation of genome from particle is essential for the successful replication of influenza A viruses and has unsurprisingly been targeted by antiviral drugs in the past.

1.3.3.1 Inhibiting viral entry and fusion

The ion channel blockers amantadine and rimantadine specifically target the M2 protein of influenza A viruses and inhibit the acidification of the virion core (Hay *et al.*, 1979; Skehel *et al.*, 1978). Interestingly, the M2 protein also plays a role in the latter part of the virus lifecycle, particularly of viruses with readily cleaved HA0, such as H5, H7 and HPAIV. Here M2 regulates the pH in the Golgi apparatus during protein translation, preventing premature cleavage of the newly folded haemagglutinin protein. This theoretically presents two phases of the virus lifecycle that could be targeted by inhibitors of the ion channel protein. Despite many years as first-choice antiviral drugs, the mechanisms by which amantadine and rimantadine block the ion channel have only recently been elucidated. Structural data published in 2008 suggested amantadine bound inside the ion channel pore and prevented the flow of ions via simple physical obstruction (Figure 1.4. Right) (Stouffer *et al.*, 2008).

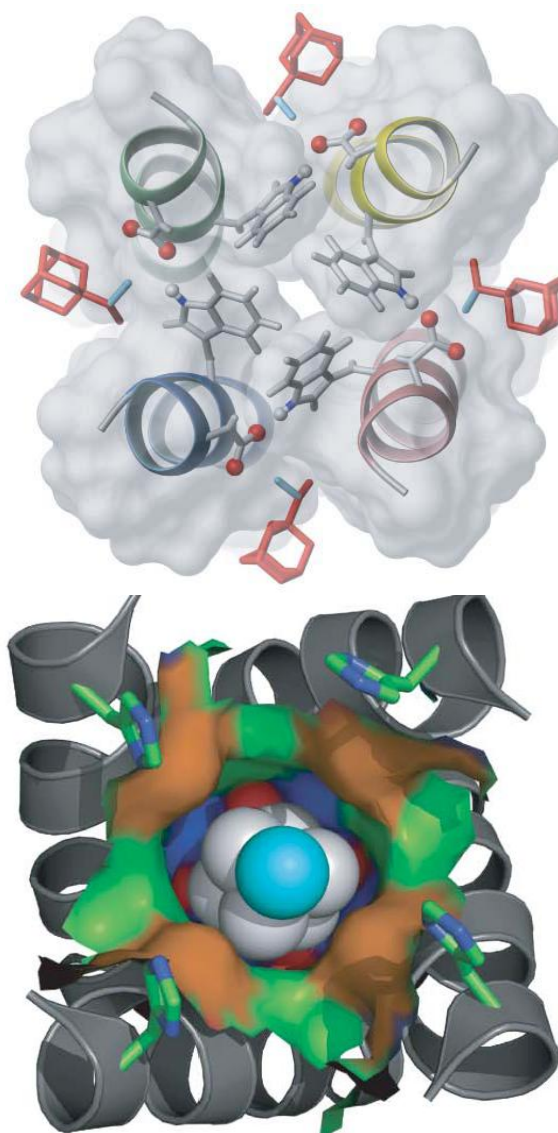


Figure 1.4. Proposed mechanisms of ion channel inhibition.

Left: A transverse view of the C-terminus of the M2 ion channel is shown with Rimantadine molecules binding on the outward facing exterior of the protein (shown in red), modelled by Schnell *et al.* **Right:** Amantadine (shown in sky blue and white) modelled to the interior of the ion channel by Stouffer *et al*, 2008.

This proposed mechanism is supported by the fact that amantadine was shown to attach in close proximity to residues 27, 30, 31 and 34 in the ion channel, which have all been shown confer resistance to this class of drug when mutated in clinical influenza isolates. A

simultaneous publication proposed an alternative inhibitory mechanism for the adamantane drugs. The surprising structural findings suggested that multiple rimantadine molecules bound to the ion channel at the outward facing intermembrane portion of the ion channel. It was proposed that rimantadine could 'block' the ion channel allosterically by keeping the homotetramer locked in its closed conformation (Figure 1. 4., Left (Schnell & Chou, 2008)). Supporting evidence for this binding mechanism comes from the fact that amantadine binds much more easily to the ion channel in its closed conformation than its open conformation, which does not make intuitive sense for a channel blocking molecule (Wang *et al.*, 1993). Furthermore, several of the drug resistance mutations that line the ion channel pore have actually been shown to retain drug binding, which would not correlate with a loss of susceptibility to the drug if binding was all that is required for drug affectivity (Astrahan *et al.*, 2004). Further studies on the M2 protein revealed that amantadine does indeed bind to both the lumen of the ion channel, and on the outward facing surface of the protein, though is more likely to bind in the former confirmation due to an increased affinity of the drug for this region of the protein. Indeed, drug binding to the outside of the protein was seen to be an interaction of low-affinity and was only observed when high doses of amantadine were recorded inside the lipid bilayer (Cady *et al.*, 2010). This last study also observed that binding of the drug to the outer face of M2 did not affect binding of other amantadine molecules to the lumen.

Due to the pervading problem of drug resistance, the association of these drugs with adverse side effects, and the subtype specific restriction of adamantane use, the ion channel inhibitors might not seem like an obvious candidate for further development. However, these structural studies have inspired the development of novel and next generation M2 protein inhibitors that may be of some therapeutic use. The search for effective ion channel inhibitors has since branched into two arms; (i) the refinement of amantadine-based molecules and (ii) the development of unrelated inhibitory compounds.

It is known that amantadine binding to the lumen of the ion channel is sub-optimal and thus there is room for the development of rationally-derived amantadine analogues which may bind more efficiently to the protein. Modification of both amantadine and rimantadine molecules has resulted in a multitude of drugs which potently inhibit influenza viruses at micromolar concentrations *in vitro*, with most of these compounds displaying favourable cytotoxicity and more potent IC50 values than their parent drugs (Kolocouris *et al.*, 1994; Kolocouris *et al.*, 1996; Stamatiou *et al.*, 2003; Zoidis *et al.*, 2006; Zoidis *et al.*, 2003). The subtle changes introduced to the precursor adamantanes are represented in Figure 1.5.

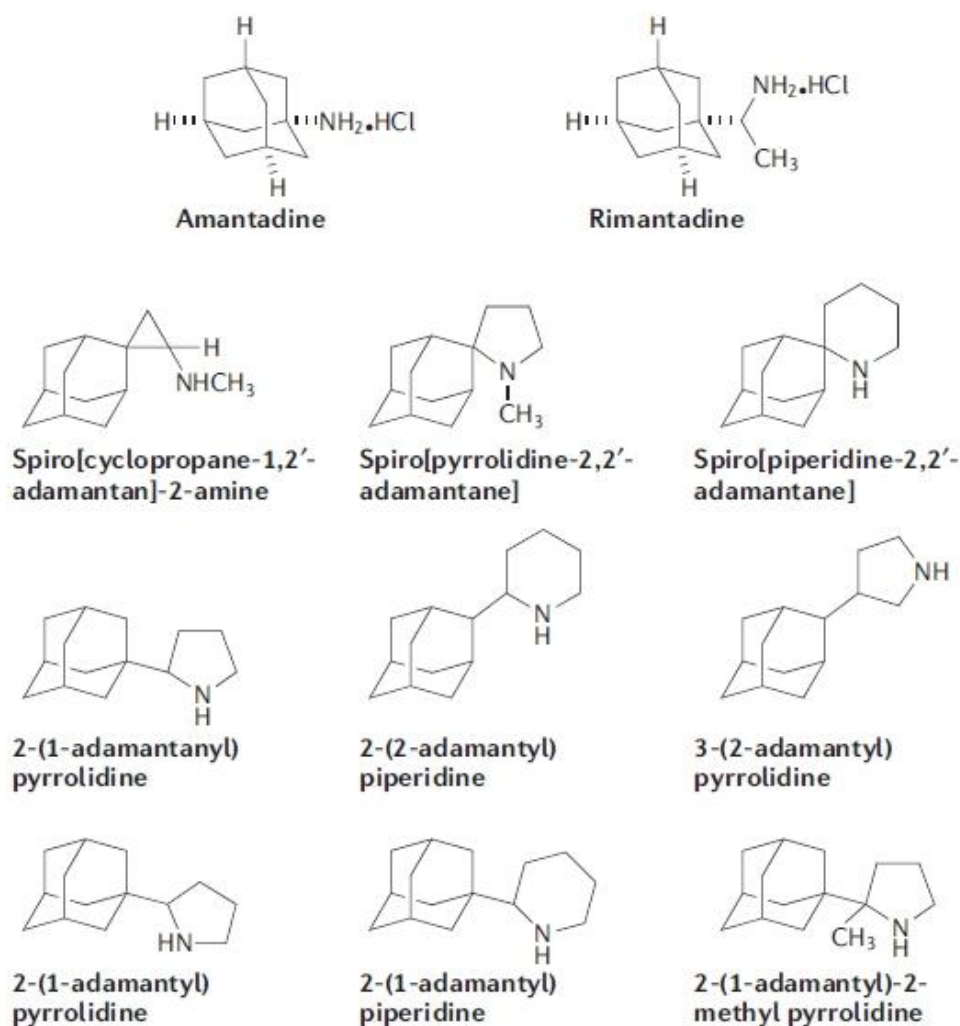


Figure 1.5. Chemical structure of Amantadine and Rimantadine, and derivative compounds which inhibit influenza replication *in vitro*.

Amantadine and rimantadine share structural similarities which enable both to exert an antiviral effect against the viral M2 ion channel protein. By maintaining these common structural features, the adamantane backbone can be modified to incorporate novel side-groups which alter the targeting and binding properties of the drug. With this approach it is hoped that novel antiviral drugs will be identified which supersede the established adamantane drugs in terms of clinical efficiency and resistance profiles (Reviewed in (De Clercq, 2006)).

Although it seems that these drugs are abundant and diverse, to date they have only been screened against H3N2 and H2N2 isolates *in vitro* and have not yet been assayed in animal models, nor against recent clinically relevant strains of influenza. It thus remains to be seen whether these drugs could evolve into effective anti-influenza therapies. Another group has proposed that modification of the amantadine molecule to contain more than one active pharmacophore group could result in more efficient inhibition of the ion channel, when taking into account the proposed exterior facing binding site on the M2 protein (Du *et al.*, 2010). As M2 is a homotetramer, four amantadine molecules are required to bind each constituent helix of the protein; however, a multivalent amantadine could be engineered to bind multiple neighbouring helices at once, thus requiring fewer drug molecules to inhibit the protein and increasing avidity. At present this potential drug type remains a hypothetical concept and no experiments using multi-valent adamantanes have yet been described.

This drug class continues to evolve via the discovery of novel inhibitors of the ion channel which are not based on the adamantane scaffold. In 1995, a novel inhibitor of M2 function was identified using a screen in *Saccharomyces cerevisiae* cells (Kurtz *et al.*, 1995). Briefly, it was observed that transient expression of the M2 ion channel in these cells impaired growth, but that this impairment could be alleviated with amantadine. This led to the development of a high-throughput screen which assayed for compounds which also restored growth to *S. cerevisiae* cultures and presumably negated the toxic effects of the ion channel in a similar manner to amantadine. This screen identified BL-1743, a spirene-containing lipophilic molecule which was then shown to inhibit growth of a H3N2 influenza virus in a plaque reduction assay, albeit at higher concentrations than amantadine. Regrettably, BL-1743 did not inhibit a virus containing the S31N amantadine resistance mutation now prevalent in many circulating influenza strains. Further characterisation of this compound revealed that whereas amantadine binding results in irreversible inhibition,

the effects of BL-1743 could be reversed; and that other amantadine resistance mutations at positions 27, 30 and 34 in the M2 protein also conferred resistance to BL-1743 (Tu *et al.*, 1996). Since its identification, BL-1743 has undergone derivative changes at the hands of structural chemists, much like the adamantane drugs mentioned previously, in the hope of finding superior inhibitory compounds. A more recent study identified a derivative of BL-1743 from a family of newly synthesised analogues, which exhibited the promising ability to potently inhibit H3N2 viruses containing the known L26F and V27A amantadine resistance mutations in plaque reduction assays. However, once again known resistance mutations built into the M2 protein at positions 31, 30 and 34 diminished the ability of these drugs to inhibit the ion channel (Balannik *et al.*, 2009). This study moves to both highlight the potential of developing analogous molecules that improve upon their parent drugs, and also the difficulties faced in inhibiting a protein that can seemingly mutate multiple residues to escape drug inhibition without incurring an overwhelming fitness costs. The ease at which influenza viruses can acquire and maintain resistance to ion channel inhibiting drugs is clearly a cause for valid concern, and coupled with the fact that drugs of this class are typically specific to treating only influenza A subtypes, may make future development of these drugs less appealing. It is worth remembering, however, that the M2 inhibitors enjoyed over thirty years of active service as an only option influenza therapy during a period of time that saw only sporadic emergence of resistant variants, and that the influenza A subtypes are arguably the most important influenza viruses and indeed the strains with pandemic potential. One may even argue that a novel M2 inhibitor, that lacked the ability to inhibit influenza B subtypes and therefore precluded itself as a reliable seasonal influenza therapy, might actually represent an ideal stockpiling candidate in preparation for future pandemic emergencies.

Finally, an anti-influenza therapy currently licensed in Russia is known to inhibit fusion of the viral membrane with the host cell early on during infection. Arbidol has been shown to

inhibit the replication of multiple influenza A and influenza B subtypes *in vitro* as well as reducing the duration of illness in infected patients by 1.7-2.6 days in clinical studies (Boriskin *et al.*, 2008). The drug is reported to be well tolerated and exhibit no severe side effects in the general population, however, details regarding the size and experimental design of these clinical trials are scant and thus all published results must be treated with a degree of caution. Although Arbidol was first approved for the treatment and prophylaxis of seasonal influenza viruses in 1990, use of the drug outside of Russia and China is rare and it is likely that the non-rigorous nature of published clinical data has restricted the acceptance of this drug in other parts of the world. Indeed, the WHO concluded that there was insufficient data on either the efficacy or safety of arbidol and thus the drug could not be adopted as a recommended treatment for the A/H1N1 pandemic influenza virus (WHO, 2010c). Recent studies have suggested that arbidol is active against the HA2 portion of the viral haemagglutinin and by binding to this region, stabilises the HA in a manner that reduces its ability to undergo the necessary conformational change usually induced by low pH in the endosome (Leneva *et al.*, 2009). By generating arbidol-resistant viruses, the researchers observed that mutations in the haemagglutinin that conferred resistance were successful at de-stabilising the HA protein in a manner that made it re-susceptible to conformational change at a low pH. Furthermore, arbidol has been accredited with immunomodulatory activity, in particular the ability to stimulate interferon and activate phagocytes, as well as possessing antioxidant activity (Silin *et al.*, 2009). These findings have led to the assumption that arbidol could function as a broad spectrum antiviral and indeed evidence has shown that the drug inhibits acute and chronic HCV infection *in vitro* (Boriskin *et al.*, 2006; Pecheur *et al.*, 2007). Other human respiratory viruses such as RSV, rhinovirus 14 and parainfluenza virus type 3 have also been shown to be inhibited by arbidol *in vitro*, suggesting the therapeutic potential of arbidol is at least sufficient enough to warrant a stringent clinical trial in the near future (Brooks *et al.*, 2004).

1.3.4. Nuclear import and RNA synthesis

All viral RNA synthesis of the influenza segments occurs in the cell nucleus and no nascent viral RNA is ever exposed in the cytoplasm due to the complex it forms with the 3P polymerase proteins and NP. The vRNP complexes, once released from the virion, are too large to rely on passive diffusion in order to reach the nucleus and instead contain nuclear localisation signals which facilitate their interaction with nuclear import machinery (Jones *et al.*, 1986; Mukaigawa & Nayak, 1991; Nath & Nayak, 1990; Nieto *et al.*, 1994). Once inside the nucleus, transcription of the negative sense viral RNA (vRNA) into messenger RNA (mRNA) occurs. A 5' methylated mRNA cap is cleaved off of host-cell pre-mRNAs and then used to 'prime' transcription of the vRNA into mRNA (Plotch *et al.*, 1981). It is the PB2 component of the vRNP which binds the 5' cap of host mRNAs, and the PA component which provides endonuclease activity to cleave caps of 10-13 nucleotides (Dias *et al.*, 2009). Each viral segment contains a string of 5-7 uracil residues located approximately 17 nucleotides from the 5' end, and the PB1 protein responsible for chain elongation (Braam *et al.*, 1983) will continue synthesizing complementary mRNA in the direction of the terminal 5' end until it reaches this tract. The RdRp then generates a poly-A tail for the mRNA transcript thanks to the process of 'reiterative slipping' in which the polymerase will stutter on this poly-U sequence and generate a string of A residues in the mRNA strand (Poon *et al.*, 1999). This results in mRNA transcripts which are not full length when compared to their vRNA template. The newly transcribed mRNA thus comprises a proper methylated 5' cap and a poly-adenylated tail and is treated as a regular host-cell transcript.

This primary round of transcription is followed by production of full length copies of the original vRNA as cRNA. cRNAs differ from mRNAs in that they are full length transcripts that do not have poly-A tails nor methylated 5' caps structures. The cRNAs are then used as templates to produce more vRNA transcripts which are subsequently used as the genomic

constituents of new virions, or used as templates for more mRNA (reviewed in (Portela & Digard, 2002)).

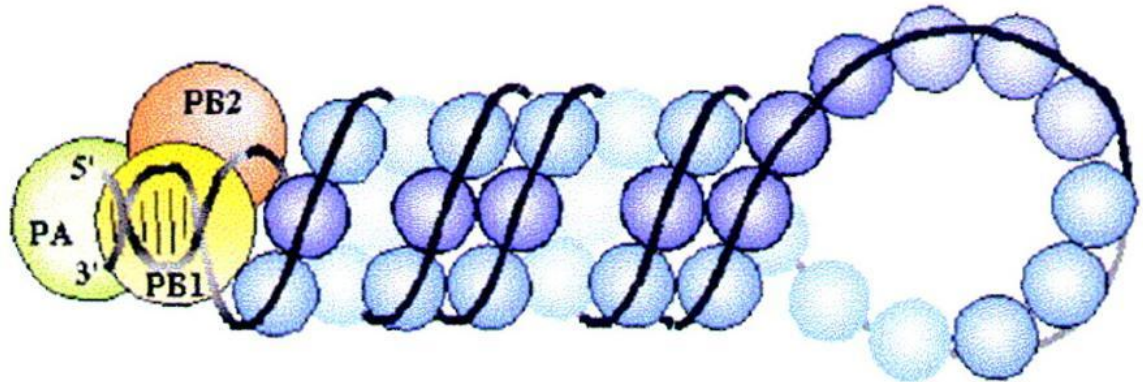


Figure 1.6. Viral RNP complex

Each segment of the influenza genome forms its own RNP. Here, the 3P complex can be seen, comprised of PB1, PB2 and PA. The bold black line indicates vRNA coiled around a series of polymeric NP proteins (blue spheres) (Adapted from Portela & Digard, 2002).

1.3.4.1. Inhibiting replication of the viral genome

The vRNP complex is a highly specialised polymerase system and its function is essential for virus propagation. It therefore represents an attractive target for inhibition by antiviral drugs. Several compounds which target the replicative machinery of the virus are known and these will be described here beginning with the most recently discovered nucleoside analogues. Favipiravir (also T-705), is currently under development by Toyama Chemicals and has exhibited inhibitory effects against a wide array of influenza viruses both *in vitro* and *in vivo*. Favipiravir has potent inhibitory activity against all three types of Influenza, A, B and C (Furuta *et al.*, 2002) and has shown efficacy against seasonal oseltamivir-resistant

and -sensitive H1N1 isolates, pandemic H1N1/09 isolates and highly pathogenic H5N1 viruses *in vitro*, as well as mouse-adapted H5N1 isolates in the murine model (Kiso *et al.*, 2009; Sleeman *et al.*, 2010). The mechanism of action for this drug is reasonably well understood; in the host cell, Favipiravir undergoes ribosylation and consequent phosphorylation and subsequently functions as a nucleoside analogue. The favipiravir anti-metabolite then specifically inhibits the formation of viral RNA and not cellular DNA or RNA. This specificity accounts for a much lower toxicity than another known nucleoside analogue, ribavirin (reviewed later), a drug which is also less effective at inhibiting RNA synthesis than favipiravir (Furuta *et al.*, 2005). By virtue of its mechanism of action, favipiravir has also shown efficacy against other RNA viruses, such as bunyavirus, arenavirus and west Nile virus, making it an attractive target for further development given its obvious potential as a broad-spectrum, non-toxic drug (Furuta *et al.*, 2009).

Ribavirin functions as a nucleoside analogue, much like T-705, and can incorporate into a replicating viral genome causing lethal mutations in any virus which utilises RNA-dependant replication machinery. Ribavirin has been licensed for the treatment of severe RSV infection in children (Rodriguez, 1999), and since 1998 has been used in combination with interferon- α as a therapy for HCV infection (Lerebours *et al.*, 2002). Although the anti-influenza effect of ribavirin has been known since the early 1970's (Sidwell *et al.*, 1972) it has never been widely marketed as an influenza treatment and the exact mechanism of action remains controversial. The drug is thought to inhibit influenza viral replication in several ways; firstly in the host cell, ribavirin is metabolised into ribavirin triphosphate (RTP), a nucleotide-resembling molecule capable of selectively inhibiting influenza polymerase (Eriksson *et al.*, 1977). Secondly, in its monophosphate form (RMP), ribavirin inhibits the intracellular enzyme Inosine Monophosphate Dehydrogenase (IMPDH), preventing the conversion of inosine monophosphate (IMP) into xanthosine monophosphate (XMP) which under normal conditions is then metabolised into guanine

metabolites such as GTP – an essential precursor of RNA synthesis (Streeter *et al.*, 1973). Thirdly, during polio infection, RTP has been shown to incorporate into viral genomes during replication causing ambiguous base-pairing (Crotty *et al.*, 2000). These multiple mechanisms of action have resulted in very few examples of viral resistance across the panel of RNA viruses susceptible to this drug; to date, ribavirin-resistant isolates of HCV and poliovirus have been detected with resistance being conferred by mutations in the RdRp proteins of each virus (Pfeiffer & Kirkegaard, 2003; Young *et al.*, 2003). To date, no ribavirin-resistant influenza isolates have been reported in the literature. However, given the indiscriminate activity of the drug, use of ribavirin has been associated with significant toxicities and teratogenicity, and common side effects of prolonged treatment include haemolytic anaemia (Kowdley, 2005). An obvious fact that has so far precluded ribavirin from receiving widespread authorisation for the treatment of influenza viruses is that clinical trial data regarding its use has been inconsistent and insignificant. An early trial in 1988 noted no significant benefit between ribavirin treated or placebo treated patients suffering from influenza B infections (Bernstein *et al.*, 1988), however this was preceded by a trial in which inhaled ribavirin was associated with improved outcome (reduction in duration of fever, more rapid clearance of influenza virus from respiratory secretions) in students suffering from H1N1 and H3N2 seasonal isolates in 1981 (Knight *et al.*, 1981). In one of the largest clinical trials of ribavirin to date (n=67), the aerosolised form of the drug was shown to reduce fever duration in children by approximately 14 hours, though no other improvements were observed between the drug- and placebo- treated groups (Rodriguez *et al.*, 1994). Clinical trials for ribavirin repeatedly feature unsubstantial sample sizes (rarely more than 50 patients), which make statistical conclusions about the efficacy of the drug difficult to obtain. It is also often administered as a last-line treatment option for the chronically ill and thus it may not be surprising that ribavirin is unable to improve outcomes in those suffering from particularly severe infections.

In an attempt to circumvent the known toxicity issues associated with ribavirin use, analogous compounds have been devised which are significantly less toxic than the parent drug. Taribavirin (viramidine) is a prodrug of ribavirin and is currently under development as a ribavirin-alternative for the treatment of HCV. Viramidine resembles adenosine, and is rapidly converted into ribavirin by the hepatic enzyme adenosine deaminase. Compared to ribavirin, viramidine remains concentrated to the liver after adsorption, which although desirable for HCV treatment, is unlikely to prove beneficial in the treatment of influenza. However, phase II clinical trials have identified a definite advantage of viramidine treatment over ribavirin from the fact that viramidine is less likely to cause haemolytic anaemia, due to a lower efficiency of the compound to uptake into red blood cells (Gish *et al.*, 2004; Lin *et al.*, 2003). This observation may allow for increased dosages of viramidine to be administered in the clinic, which may translate to a more potent antiviral effect. Viramidine has been shown *in vitro* to inhibit replication of influenza viruses of H1N1, H3N2, H5N1 and influenza B subtypes, as well as protecting mice from a H1N1 challenge *in vivo*, with viramidine exhibiting three-fold less toxicity than ribavirin (LD₅₀ of 610mg/kg/day and 220mg/kg/day respectively)(Sidwell *et al.*, 2005). Viramidine was shown to be effective even when administered 48 hours post-infection, as well as when administered at high-doses in drinking water. Although no human clinical trials have been undertaken to date to assess the efficacy of viramidine against influenza, the revelation from other clinical trials that the drug is well tolerated in patients trials is reassuring.

Recently, a novel molecule with inhibitory mechanisms not related to nucleoside mimicry was published by Wunderlich and colleagues (Wunderlich *et al.*, 2009). Building on the knowledge that PB1 and PA interaction is necessary for functional polymerase activity (Fodor *et al.*, 2002; Perales & Ortin, 1997), they attempted to prevent it by synthesizing a short peptide based on the PA-binding domain of the PB1 protein. By synthesising a short 25 amino acid peptide synonymous with the highly conserved PA binding domain of the

PB1 protein of influenza A viruses, and introducing a single influenza B specific amino acid change, they identified a molecule which could recognise and bind to the PA protein of both types. In binding the PA protein and blocking the interface between PA and PB1, the drug was shown to have dose-dependent antiviral activity against influenza A and B viruses *in vitro*, demonstrating that inhibiting protein-protein interactions necessary for viral replication has potential as an antiviral mechanism.

Finally, the nucleoprotein of influenza A has recently been described as a target for novel inhibitory compounds. The nucleoprotein represents the most abundant protein transcribed by influenza viruses during infection and serves to stabilise the RNA genome as the major protein component of vRNPs, and potentially plays an important role in export of the polymerases complexes from the nucleus of infected cells (reviewed by (Portela & Digard, 2002)). Kao and colleagues observed that the treatment with the commercially sourced compound nucleozin dose-dependently inhibited the infection of MDCK cells by H3N2 and H5N1 test viruses, and was also effective when administered 6 hours post-infection (Kao *et al.*, 2010). The antiviral mechanism of this compound was shown to be via potent inhibition of NP accumulation in the nucleus. During the course of investigation, a resistant viral phenotype emerged carrying an amino acid change in the NP protein (Y289H). This virus was still replication competent, though replication activity of the viral genome was approximately 20% less in the mutant than in the wild type virus. Nucleozin did not disrupt the binding of RNA to NP, nor disrupt or block the nuclear import signal. Instead it is thought that nucleozin treatment results in the formation of large NP aggregate complexes that are functionally useless and too large to enter the nucleus. The compound also demonstrated efficacy *in vivo* by protecting 50% of mice infected with a highly virulent avian influenza strain and lead to a ten-fold reduction in lung titres when compared to the infected but untreated group. However, although the Y289H mutation was found to be rare in circulating human isolates prior to 2009, the pandemic A/H1N1

virus was found to naturally contain this mutation and demonstrated a natural resistance to the drug.

1.3.5. Virus assembly

Influenza viruses are known to assemble and bud from the apical membrane of polarized cells and consequently, no complete influenza virus particles are found inside an infected cell. The M1 protein, as well as binding to the nuclear RNP complex, can also associate with lipid membranes, suggesting that M1 plays a key role in assembly by recruiting newly synthesised genomes to the assembly site at the plasma membrane (Schmitt & Lamb, 2005). Following synthesis on membrane-bound ribosomes, the integral membrane proteins of influenza enter the ER. Here, NA and HA are glycosylated, HA is trimerised and NA and M2 form tetramers. The proteins are then transferred to the Golgi apparatus and eventually directed to the virus assembly site via their apical membrane sorting signals (Barman *et al.*, 2001). These same sorting signals also promote the association of HA and NA with lipid rafts. Lipid microdomains are located in the plasma membrane and are rich in sphingolipids and cholesterol (reviewed by (Ono & Freed, 2005)). Completed influenza virions selectively bud from these lipid raft domains – which is evident from the lipid content of purified virus particles (Scheiffele *et al.*, 1999).

The HA molecule is particularly vulnerable to changes in pH at this point in the replication cycle. Once synthesised, the HA protein is transported to the *trans*-Golgi network and accompanied by the M2 protein. The role of M2 is to prevent acidification of the Golgi lumen and transportation vesicles, thus preventing premature conformational changes in the HA protein. A low pH will cause a conformational change in the HA molecule, similar to that which occurs during endosome fusion with the virion membrane during viral entry. Because of the involvement of M2 as an ion channel in this part of the replication cycle, this step is susceptible to inhibition via adamantane drugs (Hay, 1992).

1.3.6. Viral budding and release

Haemagglutinin and neuraminidase have been recently suggested as critical proteins in the budding process (Chen *et al.*, 2007) as opposed to the M1 protein, which was previously thought to be the driving force behind bud formation (Gomez-Puertas *et al.*, 2000). The importance of M2 in bud morphogenesis has also been alluded to, suggesting this process likely requires multiple interactions between several structural proteins (Iwatsuki-Horimoto *et al.*, 2006). Influenza viruses face the problem of grouping all eight vRNP segments together in the correct location before successful budding can take place and because the structural proteins alone can initiate virus-like particle (VLP) budding, it is not uncommon to have incomplete or non-infectious particles budding out from the infected cell. This can lead to the generation of 'defective interfering (DI)' viruses, which are generally defined as particles that exist without critical genomic material necessary for functionality. Such viruses are generally non-functional without co-infection with other viruses to provide the missing genomic material (Dimmock, 1985).

Fusion of the host cell membrane at the base of the bud completes the budding process, though the new virion cannot escape the cell immediately because of the interaction of its own HA proteins with sialic acid receptors still abundant on the infected cell surface. Neuraminidase, as a receptor destroying enzyme, is required for the release of the virion from the infected cell and without the hydrolysing activity of NA, influenza viruses 'clump' together on the cell surface, unable to depart the cell, resulting in a loss of infectivity (Colman, 1994).

1.3.6.1 Inhibiting the release of progeny viruses

The neuraminidase glycoprotein is the target for oseltamivir and zanamivir. Neuraminidase consists of four identical monomers anchored into the viral membrane and each monomer possesses a deep cleft which houses a binding site for the sialic acid substrate (Figure 1.7).

This cleft is highly conserved throughout various strains of influenza which makes it an obvious choice for drug targeting (Colman, 1994).

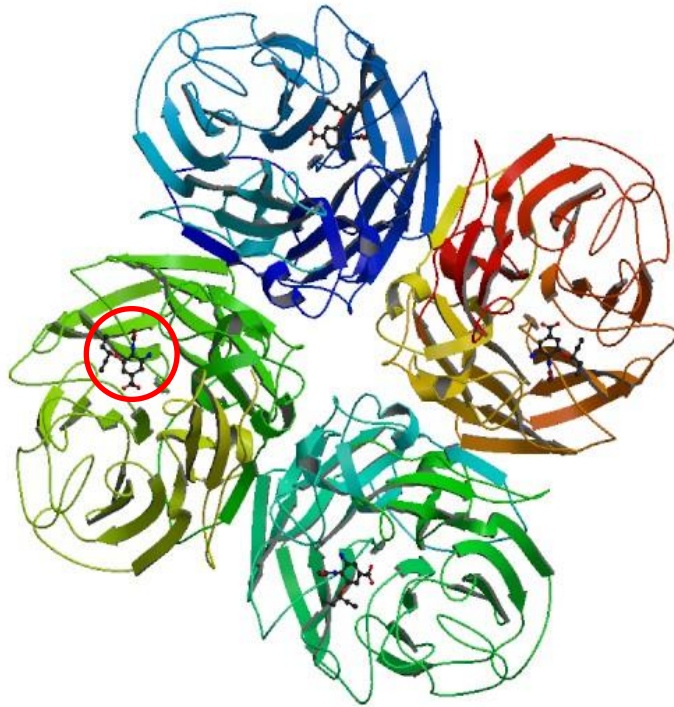


Figure 1.7. Structure of N1 Neuraminidase

A top-down schematic of the viral neuraminidase tetramer. Each of the monomers is shown complexed with a zanamivir molecule (highlighted on the left-most monomer with a red ring). Image adapted from (Russell *et al.*, 2006).

It was long-since known that synthetic analogues of sialic acid such as 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA) could inhibit neuraminidases of a wide range of species (Meindl & Tuppy, 1969). However, the non-specific inhibition of human, bacterial and viral neuraminidases exhibited by DANA and early derivatives of this analogue was

regarded as undesirable for a therapeutic against influenza (Meindl *et al.*, 1974). Furthermore, animal studies involving the most potent neuraminidase inhibiting DANA derivative (2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid) found no beneficial effect of the drug against influenza infection (Palese & Schulman, 1977). Utilising the crystal structure of N2 group influenza neuraminidases and the computer-aided design software, 'GRID', researchers then synthesised novel compounds which were predicted to inhibit viral neuraminidase with a higher affinity and specificity than DANA (von Itzstein *et al.*, 1993). It was observed that when docked in the neuraminidase binding site, the hydroxyl group at position 4 of the C-ring of DANA directly aligned with a negatively charged Glutamic Acid at position 119, thus it was suggested that replacing the hydroxyl group with a positively charged amino group would lead to an increased affinity of the inhibitor for the active site. Indeed, this substitution led to the formation of a stable salt bridge between Glu119 and the 4-amino substituted group of DANA, resulting in an inhibitory effect approximately 100-fold greater than the parent analogue. GRID then suggested that position 4 could be further altered to contain a guanidinyll group instead of a hydroxyl or amino group to further enhance the affinity between drug and active site. In this conformation, the drug bound even more tightly due to interactions of the guanidinyll group with both positions Glu119 and Glu227 of the active site (von Itzstein *et al.*, 1993). 4-guanidino-Neu5Ac2en showed significant inhibition of viral neuraminidase activity and plaque formation, as well as dose-dependent inhibition of influenza virus replication in mice (Woods *et al.*, 1993). This compound came to be called zanamivir, and represented a successful example of rational, computer-assisted drug development. Zanamivir's positively-charged guanidinyll group ensures a high-affinity binding directly into the neuraminidase cleft (Figure 1.7.) and results in binding without any induced conformational change in the enzyme. The alterations to the initial DANA template resulted in a molecule which binds with specificity to influenza neuraminidases and although analogous sialidases

are utilised by other species, zanamivir has been shown to exhibit a one million times greater affinity to influenza NA than other types of non-viral neuraminidase (Gubareva *et al.*, 2000).

The discovery of zanamivir was followed rapidly by the marketing of a second neuraminidase inhibitor, oseltamivir. Following the example of structure-led drug design, researchers set out to generate another neuraminidase inhibiting compound. A neuraminidase inhibitor which could be taken orally was still commercially desirable and thus the ultimate aim was to create a more stable compound which could be administered as a pill. As seen in figure 1.1., both sialic acid and zanamivir contain a dihydropyran ring as their core structure. Oseltamivir differs from these compounds by containing a carbocyclic template at its core, deliberately chosen for its enhanced stability and structural malleability. Using X-ray crystallographic analysis it was observed that a hydrophobic pocket inside the neuraminidase binding site could potentially accommodate lipophilic groups, absent from both zanamivir and sialic acid, but which would be desirable for enhancing the bioavailability of a novel drug and allow greater absorption through the intestinal tract (Kim *et al.*, 1997). Although this novel compound was found to be more lipophilic than zanamivir, it exhibited poor oral bioavailability in the animal model. In order to remediate the problem of low bioavailability, further modifications to the lead compound were made in order to deliver it as an esterised prodrug. This modification led to higher and more sustained concentrations of the drug in multiple animal models following oral administration (Li *et al.*, 1998). Today, oseltamivir is administered orally as oseltamivir phosphate and rapidly metabolised to the active form oseltamivir carboxylate by esterases in the liver, with >75% of the dose entering systemic circulation (Gubareva *et al.*, 2000).

The discovery of neuraminidase inhibitors which so closely mimicked the natural substrate of influenza sialidase led to the hope that significant resistance mutations would not readily arise in circulating isolates. However, as mentioned previously, resistance to the neuraminidase inhibiting class of antiviral has been reported.

One of the principle mechanisms of drug resistance is a mutation in the neuraminidase protein of N1 subtypes at position 275, where histidine is replaced by tyrosine. This mutation confers resistance against oseltamivir and peramivir *in vivo* (Weinstock *et al.*, 2003), however, other mutations at position 119 and 292 are also known confer resistance to neuraminidase inhibitors in N2 and N9 neuraminidase subtypes (Gubareva, 2004). This observation suggests that there are numerous mechanisms of resistance to NI drugs that are not yet well characterised.

Although nine different neuraminidase proteins are utilised by influenza viruses, and amino acid sequences vary substantially between these different subtypes, the active site of the neuraminidase protein is highly conserved and oseltamivir has been shown to inhibit all nine known neuraminidases (Thomas *et al.*, 1994). The fact that neuraminidase inhibition can successfully lead to broad cross-protection against potential pandemic subtypes has ensured that the active site of neuraminidase has therefore remained an attractive drug target, and has led to the continued development of neuraminidase inhibiting compounds such as the experimental drugs peramivir and CS-8958.

Peramivir, like oseltamivir and zanamivir, is a potent inhibitor of the neuraminidase enzyme at nanomolar concentrations *in vitro* (Gubareva *et al.*, 2001). Furthermore, peramivir has demonstrated inhibitory efficacy in the animal model, contributing to improved outcomes in both mice (44% increase in survival rates) and ferrets (40-63% increase in survival rates) when challenged with a robust H5N1 infection (Yun *et al.*, 2008). Importantly, peramivir is administered either intramuscularly or intravenously, and thus represents a novel

mechanism of drug delivery for seriously ill patients who are unable to commit to oral or inhaled dosing regimens. Following the outbreak of nvH1N1 pandemic influenza in 2009, peramivir was granted an Emergency Use Authorisation (EUA) by the FDA for 'last-line' treatment of pandemic influenza, making it the first unapproved drug to be recommended for use in patients in this fashion (Birnkrant & Cox, 2009). Unfortunately, despite still being an unlicensed drug, resistance to peramivir already exists in the community with the common H275Y mutation in H1N1 viruses conferring resistance to this inhibitor in the same manner as oseltamivir (Abed *et al.*, 2006). Furthermore, both R292K, R152K and E119V/D/G/A have been shown to grant resistance to Influenza B viruses against peramivir *in vitro*, suggesting that even once licensed, the drug may already have diminished efficacy (Jackson *et al.*, 2005).

A second novel neuraminidase inhibiting drug, laninamivir (also known as CS-8958 & R-118958), is also under development, which like zanamivir is an inhaled NAI, but is long-acting and remains in the host for many hours after administration. Laninamivir has shown efficacy against neuraminidase subtypes 1-9 *in vitro* and in a mouse model has prolonged the survival of animals challenged with lethal doses of A/Puerto Rico/8/34 (H1N1). When laninamivir and zanamivir were administered singly, seven days pre-infection, 100% of mice were dead in the zanamivir group by day 8, compared to a 90% survival rate seen in the laninamivir group on this day, confirming the long-acting properties of the drug (Yamashita *et al.*, 2009). This study also highlighted the efficacy of laninamivir against oseltamivir-resistant seasonal influenza viruses, including H1N1 isolates carrying H275Y mutation, suggesting that the widespread prevalence of this mutation will not impact on the drug's effectiveness in the clinic. This finding has been reinforced *in vivo* in a study which accredits laninamivir with reducing the duration of shedding of oseltamivir-resistant influenza viruses by 30 hours compared to oseltamivir in humans (Sugaya & Ohashi, 2010). Laninamivir has also demonstrated efficacy against the pandemic H1N1 virus, and H5N1 isolates in the

mouse model (Itoh *et al.*, 2009; Kiso *et al.*, 2010a). Pharmacokinetic analysis of laninamivir suggests that the drug is eliminated slowly from the body, still being present in the urine of patients after 144 hours, and that even with repeat and high concentration dosing regimens, no adverse effects are recorded in healthy human males (Ishizuka *et al.*, 2010). The benefits of a long-acting drug are obvious. Presently, both oseltamivir and zanamivir are administered twice daily for five days as a treatment, or once daily for ten+ days as a prophylaxis, for influenza infection in uncomplicated patients. If such laborious treatment regimens can be replaced by a single-dose therapy, then far fewer stockpiled doses would be needed for pandemic protection, and there will be a reduced risk of patient non-compliance with long-term prophylaxis.

1.4. Inhibiting influenza spread by targeting the innate immune response

The innate immune system of the host is capable of mounting a powerful and complex antiviral response to a myriad of viral infections. Influenza viruses have had to evolve mechanisms to circumvent the full complement of secreted chemokines and cytokines utilised by the host to limit viral replication and spread. The NS1 protein of influenza in particular is crucial in constraining the interferon response of the host in an attempt to maintain optimal replication conditions in the cell (reviewed in (Hale *et al.*, 2008)) and thus represents a potential target for antiviral intervention. The NS1 protein is anti-inflammatory via several mechanisms, including via the sequestering of viral double-stranded RNA (a pathogen associated molecular pattern (PAMP)), thus preventing intracellular detection and degradation of viral genomic material by the host cell and by blocking the nuclear export of cellular proteins involved in the interferon response. Basu and colleagues recently described several novel antimicrobials that dose-dependently reduced viral titres of lab-adapted isolates in MDCK cells (Basu *et al.*, 2009). Treatment with

one compound in particular resulted in a reduction in viral protein synthesis, as observed by the absence of viral NP in infected cells 24 hour post-infection, and the restoration of the interferon- β signal in virally infected cells, which was otherwise inhibited by the presence of untreated virus.

1.4.1 Modulating the host immune response as an influenza therapy.

With the host cell capable of mounting such an effective antiviral response to influenza infection, it may seem intuitive to suggest that both artificial stimulation and/or upregulation of the system may prove beneficial in treating viral infection. Indeed, a recent study by Dudek and colleagues reported that *in vitro* treatment of cells with the known proteasome inhibitor PS-341 (also known as Bortezomib and Velcade) resulted in a reduction of viral titres (Dudek *et al.*, 2010). Ironically, the researchers were attempting to inhibit the pro-inflammatory effects of the well characterised transcription factor, nuclear factor κ B (NF- κ B), due to their previous observation that activation of the NF- κ B pathway was essential for efficient influenza replication (Wurzer *et al.*, 2004). However, rather than resulting in the inhibition of NF- κ B signalling, the drug instead was shown to activate this transcription factor and lead to low-level induction of the interferon response early in the course of infection. It is likely that the drug gave cells a head start on interferon induction which caused the reduction of progeny viral titres. This drug also benefits from current clinical use as an anti-cancer therapy, so may be somewhat tolerated in humans, and has displayed no cytotoxic effect in human cells *in vitro*.

Early work intending to explain the high pathogenicity of human H5N1 infections observed that infection with this virus resulted in rapid accumulation of proinflammatory cytokines and chemokines and that this hyperactivity of the immune system actually correlated with higher viral loads in pharyngeal secretions (Cheung *et al.*, 2002; de Jong *et al.*, 2006). This

observation was also true of another highly pathogenic virus, the 1918 pandemic strain, and is likely a major determinate of viral pathogenesis (Kash *et al.*, 2006). The discovery that certain viruses could not only induce high levels of cytokines, but also replicate extensively in their presence (Seo *et al.*, 2002) led to the suggestion that clinical management of such patients should focus on managing the intense 'cytokine storm' induced by viral replication. The host proinflammatory response can potentially be mediated by immunomodulatory compounds which are both relatively well characterised and tested in humans and also hugely abundant across the globe. Many immunomodulators exist in generic forms and are especially cheap to acquire with long shelf-lives, making them an appealing alternative to specific and heavily patented antiviral drugs which are currently stockpiled in insufficient quantities to benefit the majority of the global population (Fedson, 2008).

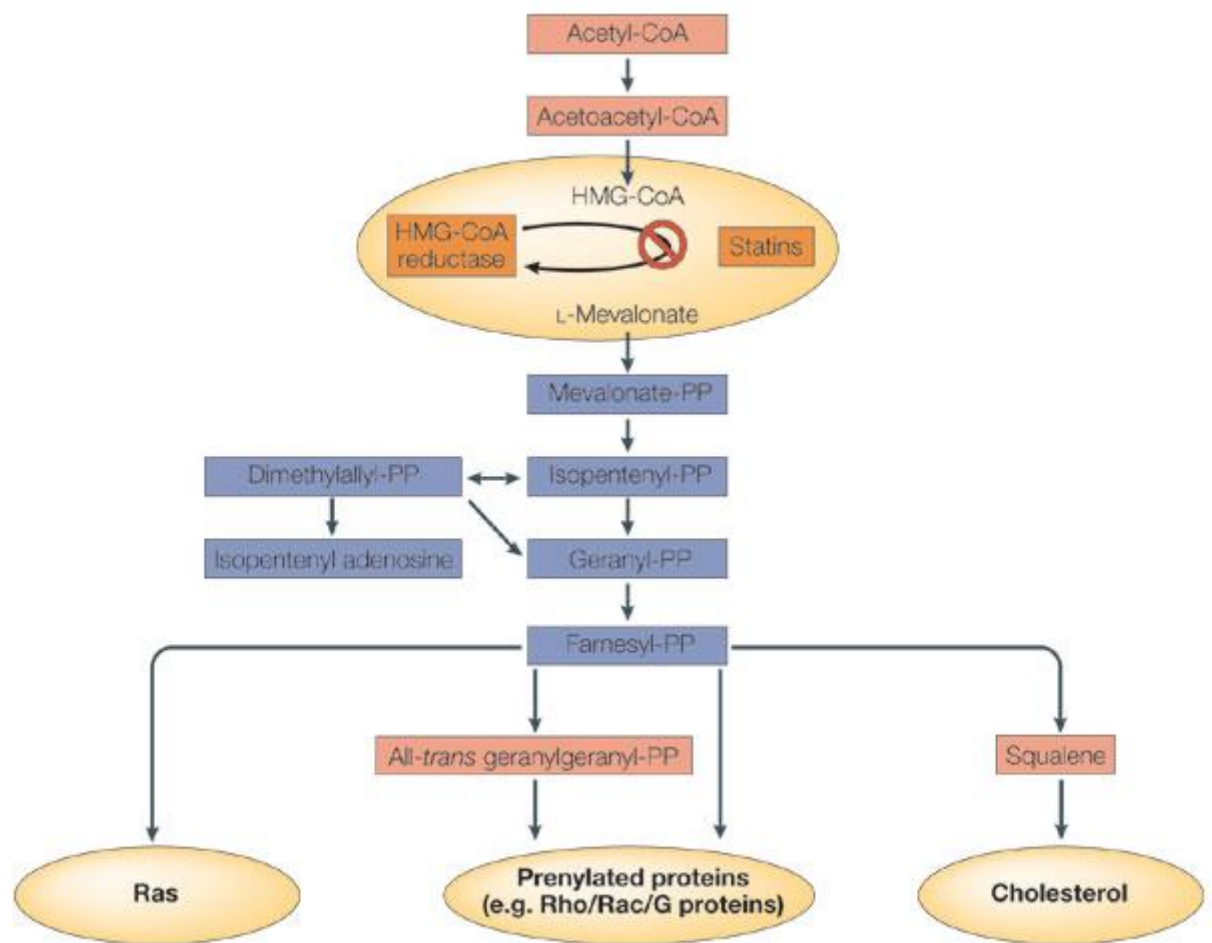
1.4.2. The case against using immunomodulatory compounds

Early mouse studies suggested that hypercytokinemia alone was not sufficient to explain the high pathogenicity and fatal outcome of H5N1 infection. TNF- α , IL-6 and CC Chemokine ligand 2 (CCLR2) are three proinflammatory cytokines known to be elevated in humans and mice during H5N1 infection. Salomon and colleagues observed that knockout mice genetically deficient in either TNF- α (or its receptors TNFR1 or TNFR2), IL-6 or CCLR2, all succumbed to H5N1 infection in a similar manner to wild-type mice with functional cytokine responses (Salomon *et al.*, 2007). Furthermore, treatment of wild-type mice with anti-inflammatory glucocorticoids did not reduce lethality of H5N1 challenge, suggesting that suppression of inflammatory cytokines does not result in improved outcomes in mice. A similar study by Szretter and colleagues also found that mice lacking individual cytokine responses were just as likely to succumb to highly pathogenic H5N1 infection as wild-type

mice. This study also highlighted that mice lacking Interleukin 1 receptor (IL-1R) exhibited heightened mortality and morbidity to a low pathogenic H5N1 challenge (Szretter *et al.*, 2007). Another obvious criticism of relying solely on immunomodulatory molecules to control influenza infection is that not every influenza isolate induces cytokine storms, indeed the A/H1N1 2009 pandemic isolate was not associated with significant cytokine dysregulation in the majority of cases and thus reliance on immunomodulatory treatments that do not impact on viral replication might be unhelpful.

1.4.3. The case for using immunomodulatory compounds

The principal focus on anti-inflammatory therapies that might be beneficial in controlling pandemic infection has been the statins. These agents are currently widely used as cholesterol-lowering drugs, though laboratory studies have identified their pleiotropic and anti-inflammatory effects (Terblanche *et al.*, 2007). Statins may hypothetically exhibit direct antiviral effects through their cholesterol depletion properties. Influenza viruses are known to require cholesterol for efficient viral replication, as they bud from cholesterol-rich lipid microdomains at the apical plasma membrane of infected cells (Nayak & Barman, 2002). Indeed, approximately 44% of the lipid content of influenza virions is comprised of cholesterol (Lenard & Compans, 1974). Statins generally inhibit the enzyme HMG-CoA reductase, which is the first dedicated enzyme of the cholesterol biosynthesis pathway, resulting in a reduction of cellular cholesterol concentrations and all upstream intermediates of this pathway (Figure 1.8.). Many of these upstream intermediates are involved in protein prenylation and serve as post-translational modification molecules for various membrane-bound proteins involved in inflammatory signalling. By slowing the rate of prenylation, statins interfere with the proper intracellular trafficking of signalling proteins involved in inflammation and thus exhibit an immunomodulatory effect.



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Figure 1.8. Intermediates of the cholesterol biosynthesis pathway

Image credit: (Menge *et al.*, 2005)

Statins are effective as cholesterol lowering drugs because they force the host to upregulate low density lipoprotein (LDL) receptors and subsequently attract cholesterol back into the cell from circulation (Ma *et al.*, 1986). It is unclear whether this would result in any hindrance to viral replication as the cell attempts to source more cholesterol or whether it is sufficient to inhibit virus propagation. Laboratory studies documenting the direct effect of statins on influenza replication are rare, however one such study observed the effect of a combination of caffeine and statin against influenza challenges of H1N1, H3N2 and H5N1 subtypes in mice. The combination of 200µg caffeine and 50µg of statin

inhibited viral replication and ameliorated lung damage by all isolates with a similar potency to oseltamivir (Liu *et al.*, 2009). However, several retrospective cohort studies have been performed to assess the effect of statin prescription with morbidity: Over ten influenza seasons in Canada, statin use was associated with small protective effects against pneumonia hospitalisation and mortality, as well as all-cause mortality. The researchers concluded that although the minimal protective effect against influenza morbidity was statistically significant, 'clinicians should focus on other measures to reduce morbidity and mortality from the next influenza pandemic' (Kwong *et al.*, 2009). Two further studies also noted that statin use did not correlate with positive respiratory infection outcomes and suggested that the beneficial effects of statin use are limited to their established area of action; vascular disease (Fleming *et al.*, 2010; Smeeth *et al.*, 2009).

Other classes of immunomodulators include PPAR agonists. An animal study on mice identified a significant mortality reduction from H2N2 challenge virus when animals received gemfibrozil (a fibrate that acts as a PPAR- α agonist) as late as four days post-infection (Budd *et al.*, 2007). Gemfibrozil is also a long-established lipid-lowering compound with noted pleiotropic effects, including the reduction of TNF- α and Interferon- γ in serum (Madej *et al.*, 1998). Although no direct antiviral effect of this drug is known, the observation that mice survival was enhanced by an anti-inflammatory compound may provide a proof of principle for future research into immunomodulators as a complementary arm of influenza treatment.

Research has also shown that a combination of a neuraminidase inhibitor (zanamivir) with two anti-inflammatory drugs, celecoxib (an inhibitor of cyclooxygenase-2 (COX-2)) and mesalazine, increased survival rates of mice infected with extremely high doses (1000 LD₅₀) of H5N1 virus (Zheng *et al.*, 2008). Following triple combination therapy administered 48 hours post-infection, mice survival was increased from 0 to 53% after 20 days. The triple

combination was more effective than zanamivir with a single anti-inflammatory, though the effects of either celecoxib or mesalazine alone after 48 hours were not examined. When treatment was administered 4 hours post-infection, only zanamivir alone was sufficient to prevent mouse mortality, with neither celecoxib, mesalazine or a combination of the two improving the outcome in mice, suggesting the timing of treatment with such regimes may be critical.

1.5. Utilising combination therapies to treat influenza

As previously described, drug resistant strains of influenza exist against both of the licensed classes of antiviral therapy. The ability of influenza viruses to acquire and propagate drug-resistance mechanisms can often preclude the use of entire classes of drug against particular subtypes of virus, particularly when no fitness cost is associated with the resistance mutation (Table 1.2.). The standard course of treatment for uncomplicated influenza infections has always centred on the prescription of a single drug. With the adamantanes now largely ineffective, and oseltamivir resistance emerging amongst seasonal H1N1s, it is obvious that the monotherapy strategy will continue to breed resistant variants and force clinicians to move onto alternative drugs, which are in very short supply. It is likely that the future of influenza treatment strategies lies in combination therapies. Although there is an obvious financial burden associated with prescribing and stockpiling multiple drugs, the probability of higher treatment success rates and a much slower accumulation of resistant variants should ensure a long-term cost-efficiency of this strategy. As antiviral research into novel compounds continues, we will invariably discover compounds which target alternative areas of the viral replication cycle, or indeed the host cell, which may then have the potential to act synergistically if prescribed together. It then stands to reason that the virus will be unable to generate resistance if several of its

proteins are blockaded at the same time. Combination therapy has proven effective in the treatment of retroviral infections, with highly active antiretroviral therapy (HAART) being particularly effective against HIV infection (Palella *et al.*, 1998). However, the complexity of such treatment regimens has also highlighted the problems of serious side-effects borne from taking multiple drugs, the potential that two drugs will have negative synergies, and the possibility that patient non-adherence to the regimen may lead to the generation of resistant viruses. It may be likely that combination therapy against influenza virus infection would not be so complex given the self-limiting nature of the infection, with one assuming that patients would be medicated over the course of several days rather than years.

Currently, the most obvious choice for influenza combination therapy would be to administer an M2 inhibitor simultaneously with a neuraminidase inhibitor. The combination of these two drugs has been shown to synergistically and additively inhibit the replication of several influenza A isolates in MDCK cells (Govorkova *et al.*, 2004). The presence of a neuraminidase inhibitor was also shown to delay the emergence of amantadine-resistant influenza variants when viruses were grown in the presence of both drugs, whereas viruses in the presence of amantadine alone rapidly generated resistance after five passages (Ilyushina *et al.*, 2006). This result was recapitulated *in vivo* using a mouse model, where it was shown that whereas oseltamivir or amantadine alone would provide 50-60% survival against H3N2 and H1N1 challenge infections, a combination of both drugs would confer 100% survival (Masihi *et al.*, 2007). This study also noticed that 15-fold less oseltamivir was required to confer complete protection. Given that drug resistance emerges rapidly to M2 inhibitors and that most circulating and recently emerged viruses are already resistant to the adamantanes, as well as the fact that influenza B viruses are immune to the effects of ion channel blockers, it is unlikely that this combination would be clinically useful. This has led researchers to consider other combinations of drugs, such as a neuraminidase inhibitor with ribavirin. In one study using a recent mouse-adapted H1N1

(A/New Caledonia/20/99) isolate, oseltamivir failed to protect mice unless administered 4 hours before infection. Intriguingly, when administered 1-4 days post infection, oseltamivir granted no protection to mice, whereas ribavirin treatment prevented mortality by 50-80%. However, when an influenza B challenge was used, both ribavirin and oseltamivir afforded 50-80% and 30-40% survival respectively when administered 2-4 days post infection, and in combination survival rates were superior to either compound alone (Smee *et al.*, 2006). Similar results were obtained when oseltamivir was issued in combination with the experimental polymerase inhibitor favipiravir. When administered alone, oseltamivir and favipiravir protected 41% and 8% of mice from H1N1 challenge respectively. When these two combinations were administered in combination however, survival was increased to 100%. When tasked with protecting against an H5N1 challenge, oseltamivir and favipiravir protected 0% and 40% respectively, but when combined protected 90% of mice (Smee *et al.*, 2010). Further studies have highlighted the success of using a triple combination therapy of amantadine, ribavirin and oseltamivir to successfully treat a panel of influenza A viruses *in vitro*. The triple combination of all three drugs were significantly more potent at inhibiting viral replication than either combination used singularly or in a double combination (Nguyen *et al.*, 2009). This triple combination regimen was then shown to inhibit drug-resistant isolates of influenza, including A/H1N1 09 pandemic virus, with amantadine contributing to the overall synergy of the triple therapy even if the virus isolate was resistant to drug (Nguyen *et al.*, 2010). These experiments highlight the potential for combination therapy to inhibit influenza replication with greater potency, and diminish the effect and emergence rate of drug-resistance mutations. Interestingly, this study also utilised combinations of zanamivir/oseltamivir and zanamivir/peramivir to treat A/H1N1 09 pandemic influenza isolates, but found no synergism as a result. This is likely because all three drugs target the identical substrate binding pocket of neuraminidase and essentially compete with one another. However, the question of whether a combination of two

neuraminidase inhibitors would actively prevent the emergence of drug resistant mutations remains unanswered.

1.6. Other compounds that exhibit anti-influenza activity.

Finally, as interest in the field of influenza antivirals continues to grow, it becomes increasingly common to read of established drugs and familiar molecules having newly discovered activity against the virus. The macrolide antibiotic clarithromycin, used traditionally to treat bacterial infections of the respiratory tract, was found to inhibit influenza A virus replication *in vitro* (Miyamoto *et al.*, 2008). The antibiotic did not inhibit any stage of viral attachment or entry but was shown to prevent virus-induced apoptosis of infected cells, suggesting that the viral replication cycle is directly inhibited by the drug. Clarithromycin is an effective antimicrobial via its ability to inhibit bacterial protein synthesis, and with the observation that the drug is effective between the 4th-7th hours of influenza infection, it is suggested that the mechanism of action against influenza is also through the prevention of protein synthesis.

A separate study found that polyphenolic catechin compounds, abundantly found in green tea, were able to inhibit influenza virus replication in MDCKs of H1N1, H3N2 and Influenza B viruses (Song *et al.*, 2005). The compounds inhibited haemagglutination and viral RNA synthesis in cell culture. Catechin compounds were also shown to inhibit viral neuraminidase activity at high concentrations, further enhancing their potency against the virus.

Another polyphenolic compound, Resveratrol, which is synthesised by many plant species including grapes, was found to strongly inhibit influenza replication in MDCK cells (Palamara *et al.*, 2005). The mechanism by which Resveratrol inhibits replication is via the prevention of ribonucleoprotein translocation out of the nucleus into the cytoplasm, and

the reduction of late protein expression by the virus. The compound is nontoxic and found in clinically relevant concentrations in red wine. This drug also improved the survival of mice infected with H1N1 PR8 challenge.

Of these compounds, resveratrol is the only one to be assayed *in vivo* and thus it remains to be seen whether they represent genuine potential as future antiviral compounds or not. Whether *in vivo* experiments with the other compounds have been performed with ineffectual results, or whether any further work has simply not been undertaken after the initial publication is not clear.

Finally, it has also been suggested that defective-interfering influenza particles could play a role in the treatment of influenza viruses. DI particles exist as partially-intact virions which can competently enter cells, but whose genomes contain internal deletions of sequences required for the transcription of necessary viral proteins. This usually results in the presence of genetic material that contains the usual terminal promoter regions, meaning they are recognised by viral polymerases from another replicating virion in the same cell. The suggestion that DIs may have a therapeutic benefit stems from the observation that they act as competitive inhibitors of 'genuine' virus replication, as their usually miniature genomes can be replicated rapidly and preferentially by influenza polymerases at the expense of longer, whole influenza segments. Early studies showed that mice could be protected from a lethal WSN challenge if they were treated with DI particles of WSN (Morgan *et al.*, 1993). But since then, literature on the subject has been sparse, which is surprising given the advances in reverse genetics technology which would allow for a much more convenient and regulated method for generating DI particles.

1.7. Lessons learned from the 2009 pandemic outbreak

The 2009 A/H1N1 pandemic outbreak offered insights into how we prepared and then tackled the outbreak, and with retrospect, suggests how we can better prepare ourselves

for future pandemics. We witnessed the alarming rate at which the pandemic spread from its source in Mexico to the rest of the world. After emerging in March 2009, the virus has spread to North America and Europe by April and by June had been declared a global pandemic. This is despite widespread attempts by the Mexican government to close down public and private facilities across the country. Thus, it is likely that should a new pandemic virus emerge, it would be extremely difficult to contain and quarantine the pathogen before it rapidly spread worldwide, a problem exacerbated by the popularity of air travel across the globe. This knowledge, coupled with the fact that influenza vaccines can take months to assemble and license, suggests that antiviral drugs will certainly be required to treat a future pandemic virus and that stockpiles should be maintained.

We know that the attack rate of the pandemic virus was higher in children than in adults, and that the virus was predominantly transmitted by children (Ghani *et al.*, 2009). It would therefore be beneficial to target children with a drug that is effective and convenient for younger people to take, particularly given the unpopularity of closing schools and the fact that school closure does not prevent children socially interacting with one another. Of the list of drugs reviewed in this introduction, perhaps the most convenient drug on the list is the long-acting neuraminidase inhibitor, laninamivir, which if licensed would only require a single dose administered at the beginning of each week to guarantee long-term prophylaxis, which is an obvious advantage when treating any patients, particularly young children.

We were also fortuitous in that our sole stockpiled drug was mostly effective against the pandemic strain. Stockpiles of anti-influenza drugs should be augmented to contain multiple drugs, preferably with different mechanisms of action in the event of virus acquiring resistance to one particular compound. In an ideal world, multiple therapies would be stockpiled and administered in combination to patients in order to enhance the

potency of our treatment regimes. Such combination therapies should also be restricted to those who are more severely at risk from influenza infection, such as the immunocompromised, pregnant and at risk age groups. More stringent control of the drug stockpile should clearly be implemented and administration strategy should be based on the severity of the pandemic isolate. Thankfully, the 2009 pandemic virus resulted in mostly uncomplicated and largely asymptomatic infections, which throws into doubt why so much oseltamivir was administered throughout the outbreak.

Another key feature for any stockpiled drug would be its shelf-life. According to Roche, the shelf-life of oseltamivir is five years, which following the outbreak of the pandemic was extended to seven years (Reddy, 2010). Thus, if oseltamivir was available following the last influenza pandemic in 1969 and we maintained a stockpile ever since, we would have had to have completely replaced the entire stockpile five times before the 2009 outbreak. This obviously represents a huge financial burden for any government, and one wonders whether the stockpile would have been maintained as a precautionary measure, or whether continued investment in the drug would be regarded as unfeasible. A drug set that was therefore stable, cheap and conveniently administered would therefore be the ideal therapeutic answer to pandemic influenza outbreaks.

1.8 Aims of thesis

This work will focus on the discovery and characterisation of novel antiviral compounds against influenza isolates, and assess the suitability of relying on oseltamivir as the sole stockpiled drug for pandemic influenza emergencies.

- A novel compound library was screened for antiviral activity against influenza viruses.
- A selection of hits from this screen were then further categorised for their ability to inhibit a range of influenza viruses.
- Compound library hits from a previous library screen were also probed as to discover their efficacy as potential future therapies.
- A novel cell screening tool was generated with the potential to improve on the current high throughput screening techniques used to identify the aforementioned compounds.
- A pandemic representative virus was generated using reverse genetics and a mutation conferring drug resistance to NAIs was artificially mutated into this virus.
- The effect of harbouring this mutation on viral fitness was then investigated in these pandemic isolate viruses.
- A Human Airway Epithelial cell line was assessed in its ability to provide a more human-like and challenging growth strata on which to assay for differences in viral growth kinetics.

2. Materials and Methods

2.1. Materials

2.1.1. Cell lines and primary cultures

Cell Line	Comments	Source
A549	Human lung epithelial cell line derived from carcinoma.	ATCC
HAE	Fully differentiated human airway epithelial model comprised of primary cells cultured from the bronchus, trachea and nasal cavity of humans.	Epithelix-Sàrl
MDCK	Madin Darby Canine Kidney cells	ATCC
MDCK-SIAT1	MDCK cells which express enhanced levels of α -2,6-linked sialic acid (Matrosovich <i>et al.</i> , 2003).	Mikhail Matrosovich, Phillips University, Marburg, Germany
293T	Human kidney cells expressing large T-antigen of SV40	ATCC

Table 2. 1 Cell lines used in this study.

2.1.2. Virus strains

Virus	Type	Comments	Source
A/Victoria/3/75	H3N2	Reverse genetics derived.	This project
A/Puerto Rico/8/34	H1N1	Reverse genetics derived.	This project
A/England/195/2009	H1N1	Reverse genetics derived.	This project
A/England/195/2009^{H275Y}	H1N1	Reverse genetics derived, containing H275Y mutation in the NA gene.	This project
A/England/195/2009	H1N1	Pandemic human isolate.	HPA Colindale
B/Beijing/1/87	B	Wild type virus.	Professor Wendy Barclay, Imperial College London
A/Duck/England/1/62	H4N6	Avian wild type virus	Dr. Anna Hayman, Reading University
PR8 (Victoria NA)	H1N2	Recombinant virus generated using reverse genetics. Seven segments of PR8 were rescued with segment 6 of Victoria.	This project
PR8 (Victoria HA)	H3N1	Recombinant virus. Seven segments from PR8 with segment 4 of Victoria.	This project
PR8 (Victoria HA & NA)	H3N2	Recombinant virus. Six segments from PR8 and segments 4 and 6 of Victoria.	This project
RG14	H5N1	Recombinant virus. Six segments of PR8 and segments 4 and 6 of A/Vietnam/1194/04 (minus multi-basic site in segment 4).	NIBSC
RD3	H7N1	Recombinant virus. Six segments of PR8 and segments 4 and 6 of A/Chicken/Italy/4789/1999	Professor Wendy Barclay, Imperial College London

Table 2. 2 Viruses used in this study.

2.1.3. Plasmid vectors

All plasmids used in this study contain genetic antibiotic resistance to Ampicillin unless otherwise stated.

Plasmid	Comments	Source
pCMV-VictoriaPB2	Helper plasmid for rescuing reverse genetics viruses, containing segment 1 of A/Victoria/3/75	Dr. T. Zurcher, GlaxoSmithKline
pCMV-VictoriaPB1	Helper plasmid for rescuing reverse genetics viruses, containing segment 2 of A/Victoria/3/75	Dr. T. Zurcher, GlaxoSmithKline
pCMV-VictoriaPA	Helper plasmid for rescuing reverse genetics viruses, containing segment 3 of A/Victoria/3/75	Dr. T. Zurcher, GlaxoSmithKline
pCMV-VictoriaNP	Helper plasmid for rescuing reverse genetics viruses, containing segment 5 of A/Victoria/3/75	Dr. T. Zurcher, GlaxoSmithKline
pPol.I-VictoriaPB2	pPol.I vector containing segment 1 of A/Victoria/3/75	Dr. T. Zurcher, GlaxoSmithKline
pPol.I-VictoriaPB1	pPol.I vector containing segment 2 of A/Victoria/3/75.	Dr. T. Zurcher, GlaxoSmithKline.
pPol.I-VictoriaPA	pPol.I vector containing segment 3 of A/Victoria/3/75..	Dr. T. Zurcher, GlaxoSmithKline.
pPol.I-VictoriaHA	pPol.I vector containing segment 4 of A/Victoria/3/75.	Dr. T. Zurcher, GlaxoSmithKline.
pPol.I-VictoriaNP	pPol.I vector containing segment 5 of A/Victoria/3/75.	Dr. T. Zurcher, GlaxoSmithKline.
pPol.I-VictoriaNA	pPol.I vector containing segment 6 of A/Victoria/3/75.	Dr. T. Zurcher, GlaxoSmithKline.
pPol.I-VictoriaM	pPol.I vector containing segment 7 of A/Victoria/3/75.	Dr. T. Zurcher, GlaxoSmithKline.
pPol.I-VictoriaNS	pPol.I vector containing segment 8 of A/Victoria/3/75..	Dr. T. Zurcher, GlaxoSmithKline.

pPol.I-PR8PB2	pPol.I vector containing segment 1 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8PB1	pPol.I vector containing segment 2 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8PA	pPol.I vector containing segment 3 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8HA	pPol.I vector containing segment 4 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8NP	pPol.I vector containing segment 5 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8NA	pPol.I vector containing segment 6 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8M	pPol.I vector containing segment 7 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-PR8NS	pPol.I vector containing segment 8 of PR8.	Dr. Alison Whiteley, Reading University.
pPol.I-England195PB2	pPol.I vector containing segment 1 of A/England/195/09.	GeneArt AG.
pPol.I-England195PB1	pPol.I vector containing segment 2 of A/England/195/09.	GeneArt AG.
pPol.I-England195PA	pPol.I vector containing segment 3 of A/England/195/09.	GeneArt AG.
pPol.I-England195HA	pPol.I vector containing segment 4 of A/England/195/09.	GeneArt AG.
pPol.I-England195NP	pPol.I vector containing segment 5 of A/England/195/09.	GeneArt AG.
pPol.I-England195NA	pPol.I vector containing segment 6 of A/England/195/09 (Kanamycin resistance).	GeneArt AG.
pPol.I-England195NA^{H275Y}	pPol.I vector containing segment 6 of A/England/195/09 (Kanamycin resistance).	This project.
pPol.I-England195M	pPol.I vector containing segment 7 of A/England/195/09.	GeneArt AG.

pPol.I-England195NS	pPol.I vector containing segment 8 of A/England/195/09.	GeneArt AG.
p-β-galactosidase	β-galactosidase reporter plasmid.	Steve Goodbourn, St. George's, London.
pPol.I-Luc	Luciferase reporter plasmid flanked by non-coding regions of influenza A segment 8.	Steve Goodbourn, St. George's, London.
pPol.I-358Luc	Luciferase reporter plasmid flanked by non-coding regions of influenza A segment 8, containing triple up-promoter mutations in the 3'end.	This project
pPol.I-mGFP	GFP reporter plasmid flanked by non-coding regions of influenza A segment 7.	Dr. Manuela Mura, Imperial College London.
pPol.I-358mGFP	GFP reporter plasmid flanked by non-coding regions of influenza A segment 7, containing triple up-promoter mutations in the 3'end.	This project
PCDNA3-358mGFP	GFP reporter plasmid flanked by non-coding regions of influenza A segment 7, containing triple up-promoter mutations in the 3'end. Provides eukaryotic antibiotic resistance to zeocin.	Dr. Manuela Mura, Imperial College London.
pCMV-mCherryRed	Expression plasmid encoding Cherry Red signal under a CMV promoter. Provides eukaryotic antibiotic resistance to geneticin.	Dr. Manuela Mura, Imperial College London.
pCAG-E195NA	Expression plasmid encoding FLAG-tagged neuraminidase protein from A/England/195/09.	Deena Blumenkrantz, Imperial College London.

Table 2. 3. List of plasmid vectors used in this study.

2.1.4. Antibodies

Antibody	Comments	Source
Mouse α-NP	Primary monoclonal antibody raised against an H7N1 avian isolate and reactive with influenza A nucleoprotein. (Diluted 1:300 for blue cell assay)	HPA, Colindale.
Goat α-mouse IgG- β-galactosidase	Secondary antibody conjugated to β -galactosidase for use in blue cell assay. (Diluted 1:400 for blue cell assay)	Immunologicals Direct, Oxford, UK
Mouse αFLAG	Primary M2 monoclonal antibody used to probe for FLAG tag. (Diluted 1:300 for FACS analysis)	Sigma (F3165)
Goat αMouse IgG-FITC	Secondary antibody FITC tagged. (Diluted 1:1000 for FACS analysis)	Oxford Biotechnology

Table 2. 4. List of antibodies used in this study.

2.1.5. Oligonucleotides

Name of Primer	Sequence	Use
NA Fwd 1	CCC CGG GCG CTC CGT GTG TGG CTG CGA T	Sequencing primer of A/England/195/2009 NA
NA Rev 1	GGT GTT TGG ATA GGG AGA ACT AAA AGC	Sequencing primer of A/England/195/2009 NA
NA Fwd 2	CTC CCT ATC CAA ACA CCA TTG CCG	Sequencing primer of A/England/195/2009 NA
NA Rev 2	GGA ATT TCT GGC CCA GAC AAT GGG GCA GTG G	Sequencing primer of A/England/195/2009 NA
NA Fwd 3	CCA CTG CCC CAT TGT CTG GGC CAG AAA TTC C	Sequencing primer of A/England/195/2009 NA
NA Rev 3	GGA TTA GCC ACT CAA TTC AAC TTG GG	Sequencing primer of A/England/195/2009 NA
NA Fwd 4	CCC AAG TTG AAT TGA GTG GCT AAT CC	Sequencing primer of A/England/195/2009 NA

NA Rev 4	CTC GAT CCG GAT ATA GTT CCT CCT TTC	Sequencing primer of A/England/195/2009 NA
H275Y MexNA Fwd	GAA TGC CCC TAA TTA TTA CTA TGA GGA ATG CTC C	Mutagenic Primer to introduce H275Y mutation into A/England/195/2009
H275Y MexNA Rev	GGA GCA TTC CTC ATA GTA ATA ATT AGG GGC ATT C	Mutagenic Primer to introduce H275Y mutation into A/England/195/2009
NSLuc 35 F	GAC CAT GCC GGC CAG TAG AAG CAG GGT GAC AAA G	Mutagenic Primer to introduce up-promoter mutations at positions 3 and 5 in the 3' end of pPol.I-Luc vector.
NSLuc 35 R	CTT TGT CAC CCT GCT TCT ACT GGC CGG CAT GGT C	Mutagenic Primer to introduce up-promoter mutations at positions 3 and 5 in the 3' end of pPol.I-Luc vector.
NSLuc 8 F	CCG GCC AGT AGA AAC AGG GT	Mutagenic Primer to introduce up-promoter mutation at position 8 in the 3' end of pPol.I-Luc vector.
NSLuc 8 R	ACC CTG TTT CTA CTG GCC GG	Mutagenic Primer to introduce up-promoter mutation at position 8 in the 3' end of pPol.I-Luc vector.

Table 2. 5. List of oligonucleotides used in this report.

2.1.6. Buffers, diluents and culture media.

Solution	Recipe	Uses
TAE Buffer	40mM Tris-acetate pH 8 1mM EDTA	DNA gel electrophoresis
6x DNA loading buffer	0.25% Bromophenol blue 40% (w/v) sucrose. Used at 1x.	Loading of DNA samples
Phosphate Buffered Saline (PBS)	8% Sodium chloride 0.2% Potassium chloride 1.15% Na ₂ HPO ₄ 0.2% Na ₂ HPO ₄ pH 7.4	Used for washes, dilutions and short-term storage of cellular monolayers at 4°C
LacZ Buffer	60mM Na ₂ HPO ₄ ·7H ₂ O 40mM NaH ₂ PO ₄ ·H ₂ O 10mM KCl 1mM MgSO ₄	Developing β-galactosidase assays
Methylene Blue	2% Formaldehyde (36.5%) 10% Methanol 0.175% Methylene Blue (1.4%) PBS to 100ml	Methylene Blue cytotoxicity and cell viability assay
Virus Diluent	0.35% BSA 1% Penicillin-Streptomycin PBS	Collecting virus for timepoint sampling
Antibody Diluent	0.35% BSA 0.2% Sodium Azide PBS	Antibody dilutions (Blue cell assay)
2x MuNANA Assay Buffer	13g 2-(N-morpholino)ethanesulfonic acid (MES) 8mL 1M Ca Cl ₂ Distilled Water (Filter sterilise)	MuNANA assays
10x CN Buffer	30mM Ferricyanide 30mM Ferrocyanide 10mM MgCl ₂	Blue cell assays
5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal) Substrate	Prepared to 2% in Dimethylformamide (DMF).	Blue cell assays

Lysogeny Broth (LB)	1% Oxoid tryptone 0.5% Oxoid yeast extract 0.5% NaCl 0.1% glucose	Culturing transformed bacterial cells
L Agar (LA)	LB + 1.5% (w/v) Difco Agar Supplemented with 1% Ampicillin or 2% Kanamycin	Culturing transformed bacterial cells
SOC Medium	2% Oxoid tryptone 0.5% Oxoid yeast extract 10mM NaCl 2.5mM KCl 10mM MgCl ₂ 10mM MgSO ₄ 20mM glucose	Recovery medium used during transformation of <i>Escherichia coli</i> TOP10 cells.
NZY+ Broth	1% NZ Amine 0.5% Yeast extract 0.5% NaCl 15mM MgCl ₂ 15mM MgSO ₄ 10mM glucose	Recovery medium used during transformation of <i>Escherichia coli</i> XL-10 Gold.
Serum Free DMEM	Dulbecco's modified Eagles medium (DMEM) (Invitrogen) 1% glutamine (200mM) 2% non-essential amino acids 1% penicillin-streptomycin (5000IU/ml) (no pen-strep in media used for cells undergoing transfection)	Mammalian cell culture and virus propagation
10% DMEM	Serum Free DMEM 10% Fetal calf serum (FCS)	Mammalian cell culture
Plaque assay overlay	100ml 10 x Earle's minimal essential medium (EMEM) 28ml 7.5% BSA 1 % glutamine (200mM) 20ml 7.5% NaHCO ₃ 10ml 1M HEPES 5ml 1% DEAE Dextran (Sigma) 1% penicillin-streptomycin (5000IU/ml) 0.6% Agarose (Oxoid)	Plaque assays

Avicel overlay	(Used in place of 0.6% agarose above) 2.6% avicel in 186ml distilled H ₂ O, sterilised by autoclave.	Plaque assays
Epithelix Culture Medium	Ready-to-use MucilAir Culture Medium (EP04MM)	Culturing Human Airway Epithelial cells.
Trypsinising solution	8ml 10x 2.5% Trypsin 2mM EDTA PBS	Detaching cells from plasticware
Saponin permeabilisation buffer	0.05% Saponin 1% BSA 0.2% Na Azide PBS	Permeablising cells, antibody dilutions.

Table 2. 6. List of reagents used in this study.

2.2. Methods

2.2.1. Cell and virus culturing techniques

2.2.1.1. Cell culture

Madin – Darby canine kidney cells (MDCKs), 293T and A549 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 1% non-essential amino acids plus 1% penicillin and streptomycin and supplemented with 10% fetal calf serum (FCS) (v/v) (Gibco BRL). All cells were grown at 37°C with 5% atmospheric CO₂.

HAE cells (Epithelix-Sàrl MucilAir™) were maintained at an air-liquid interface with Epithelix culture medium being replenished underneath the wells every 4-5 days. At the same time a single wash of the apical surface was performed with Phosphate Buffered Saline (PBS) to remove mucus secretions.

To create frozen stocks of immortalised cell lines, all cells from a T75 flask were detached from plasticware using Trypsin and centrifuged at 80xg for 5 minutes. The cells were then resuspended in 5ml PBS and centrifuged again at the same speed. The cells were then slowly resuspended in 1ml of medium containing 10% DMSO to 90% FCS and 0.5ml aliquots were prepared into cryovials. These cryovials were kept in a container carrying room-temperature isopropanol and placed at -80°C overnight, before being transferred to liquid nitrogen for long-term storage.

To thaw immortalised cells from liquid nitrogen storage, frozen cryovials were warmed in a 37°C water bath until the ice pellet melted, and then all 0.5ml of cells were transferred into a T75 tissue culture flask. Slowly, 10ml of DMEM supplemented with 10% FCS was added to the cells, and the flask was cultured overnight at 37°C/5% CO₂.

2.2.1.2. Virus propagation

Wildtype viruses were propagated in MDCK cells at 37°C/5% CO₂. Cells were infected at an MOI of 0.01 in serum free DMEM (SF DMEM) containing 1µg/ml of Trypsin (Worthington). To harvest viruses, supernatant fluid was collected 3 days post infection. Collected supernatant was clarified by centrifugation at 1000xg for 10 minutes, distributed into aliquots, and stored at -80°C.

2.2.1.3. Virus infections

MDCK cells were infected with influenza virus at the relevant multiplicity of infection (MOI) in SF DMEM. After 1 hour incubation at 37°C, medium was removed and cells washed twice with PBS. Where appropriate, cells were acid-washed with PBS pH3.3 for 1 minute followed by a further wash with PBS to inactivate extracellular virus. Serum free DMEM containing 1µg/ml Trypsin was added, and cells incubated at 37°C. In experiments involving drug treatment, the overlaying SF DMEM was supplemented with drug at the appropriate concentration.

For HAE cells, the apical surface was washed once with PBS directly prior to infection. Virus was then added to the apical cell surface of the well in volumes of 50µl (viral titre permitting) for 1 hour. Following infection, the virus inoculum was removed and the cells washed 3 times with PBS, leaving an air-liquid interface with no fluid at the apical surface. To collect time points from these wells, 200µl virus diluent (PBS containing 0.35% BSA and 1% Penicillin-Streptomycin) was added to the well for 2 minutes, before being recovered and stored for future use in viral titre assays. These cells were then incubated at 37°C/5% CO₂.

2.2.1.4. Determining virus yields

Viral yields were determined by haemagglutination and plaque assays. Haemagglutination assays were performed by mixing serial two-fold dilutions of virus-containing supernatant with equal volumes of 1% chicken or turkey red blood cell suspensions, in PBS in 96 well V-bottom plates (Greiner bio-one). These were incubated for 1 hour on ice. The HA titre of the virus was determined as the well prior to the first concentration displaying a red blood cell pellet.

Plaque assays were performed by incubating 200µl of 10-fold serial dilutions of samples onto MDCK monolayers for 1 hour at 37°C/5% CO₂. Medium was then removed and 2.6% avicel overlay medium containing 1µg/ml Trypsin was added to the wells. For plaque reduction assays, this overlay medium was supplemented with antiviral drugs at appropriate concentrations. These were incubated at 37°C/5% CO₂ for 3 to 4 days and then visualised by either crystal violet staining or in the case of A/England/195/2009 virus, immunostaining ('blue cell assays'). The number of plaques were then counted and used to determine virus titres.

Blue cell assays were performed by removing the overlaying supernatant from plaque assays and fixing the monolayer with 1:1 methanol:acetone for 5 minutes. The methanol:acetone was then aspirated from the cells and the cells washed three times with PBS. A primary mouse α-NP antibody was diluted 1:300 in antibody diluent (PBS containing 0.35% BSA and 0.2% sodium azide)

and 200µl added to individual wells of a 12-well plate. The plates were incubated at room temperature with shaking for 1 hour. Primary antibody was then removed from the plates and the plates washed twice with PBS. A secondary Goat-αMouse antibody, conjugated to β-galactosidase, was then used to probe the cells at a 1:400 dilution for 1 hour, with

shaking, at room temperature. The secondary antibody was then removed from the cells by flick of the wrist, and the plates washed twice with PBS. A developing solution was then prepared containing 0.25ml 2% 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal) in Dimethylformamide (DMF), 1ml 10x cyanide buffer (30mM Ferricyanide, 30mM Ferrocyanide and 10mM $MgCl_2$ in water) and 9.75ml PBS. This developing solution was added to cells in 400 μ l volumes overnight at 37°C. Plaques were then counted to calculate viral titre per millilitre.

2.2.1.5. Assaying viral neuraminidase activity

Viral desialylation ability was detected by establishing a haemagglutination assay, but following 1 hour on ice, the plate was transferred to 37°C for 1-2 hours until haemagglutination reversal could be observed.

The neuraminidase activity of viral isolates was assessed biochemically using the 2-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MuNANA) substrate (Potier *et al.*, 1979). Black 96-well plates (Greiner bio-one) were used for the assay. Influenza virus NA activity was initially assessed by incubating viruses 2-fold serially diluted in 1x MuNANA assay buffer (MES buffer containing 8mM $CaCl_2$, mixed 1:1 with distilled water), in the presence of 0.15mM working stock of MuNANA substrate at 37°C for 1 hour. The plate was then read for fluorescence using the UV optic of the BMG Fluoscan plate reader (Excitation 360, Emission 450). A set dilution of virus was then chosen for K_m and drug IC_{50} assays. To assess K_m values, a single concentration of virus was added to the plate in the presence of increasing 2-fold concentrations of MuNANA substrate (highest concentration 0.15mM). The plate was then transported to the Fluoscan plate reader and measurements of fluorescence were taken every five minutes for sixty minutes. K_m and V_{max} calculations were then calculated using Michaelis-Menten enzyme kinetic calculations. To assess NAI

IC₅₀ values, a single virus dilution was incubated in the presence of increasing concentrations of either oseltamivir or zanamivir, serially diluted in 1x Mutafluor assay buffer, for 1 hour at 37°C with shaking. 0.15mM Mutafluor substrate was then added to each well and the plate re-incubated at 37°C for 1 hour. The plate was then read as described above.

2.2.1.6. Cytotoxicity assays

Cytotoxicity of Phytopure compounds was measured on MDCK cells using the ToxiLight® Non-Destructive Cytotoxicity BioAssay Kit (Lonza) according to manufacturer's protocol. Briefly, cells were seeded onto 96-well plates (Greiner bio-one) and incubated in the presence of Phytopure compound for 3 days. At 24 hour time points, AK detection reagent was added to cells, incubated for 5 minutes and then 20µl of supernatant was measured for luminescence. Drugs were assayed in triplicate with each time point being conducted on individual plates. This kit measured the release of the enzyme adenylate kinase from dead cells. This enzyme actively phosphorylates ADP into ATP, which was then measured using a bioluminescent firefly luciferase reaction. Luminescence readings were compared to those obtained from a control group that underwent full lysis, and expressed as a percentage of this value.

Cytotoxicity of Summit Plc. compounds was determined using a Methylene Blue assay. Confluent MDCK cells were grown on 96 well plates in the presence of serially diluted compound and at 24 hour time points, supernatant was removed and replaced with 40µl working stock methylene blue solution. Following a 2 hour room temperature incubation, methylene blue was removed by a flick of the wrist and cells were washed 3 times in PBS before being incubated for a further 30 minutes in PBS at room temperature. Then, 100µl

of 1% SDS was added to cells and plates were incubated with shaking for 1 hour at room temperature before being read for absorbance at 660nm using a spectrophotometer.

2.2.2. Molecular biology

2.2.2.1. vRNA extraction

Total vRNA was extracted from virus-containing supernatants using the QIAamp viral RNA mini kit (QIAGEN) following manufacturer's instructions. Briefly, 280µl of virus stock were lysed using the AVL buffer containing RNAsin. This was bound to silica membrane, washed and eluted in 30µl of AVE elution buffer. The vRNA was stored at -20°C.

2.2.2.2. RT-PCR and first-chain amplification

First-strand synthesis using reverse transcription PCR (RT-PCR) of vRNA was performed using the following reaction procedure. A total of 2µg of isolated RNA was added to the forward segment-specific primer (3.2pmol) in a volume of 11µl, and heated to 70°C for 5 minutes. The solution was then added to ice for 5 minutes to cool, before being added to a reaction mixture (AMV Reverse Transcriptase kit, Promega) containing the following: 5µl AMV Reverse Transcriptase 5x Reaction buffer, 2.5µl 10mM dNTP mix, 1µl (30 units) of AMV Reverse transcriptase, and nuclease free water to a final volume of 25µl.

The mix was incubated at 37°C for 1 hour. The RT PCR products were amplified as follows: 5µl 10x Platinum *Pfx* DNA polymerase reaction buffer (Invitrogen), 1.5µl 10mM dNTPs (ABI), 1 µl 50mM MgSO₄ (Invitrogen), 5µl cDNA from RT PCR, 50pmoles of segment specific forward and reverse primers, made up to 50µl with sterile water (Sigma), were amplified using an initial denaturation step of 95°C for 2 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 1 minute and elongation for

1:45 minutes. An elongation time of 10 minutes was used on the final cycle. The PCR products were purified using a QIAquick gel extraction kit (QIAGEN), following manufacturers' instructions. Products were eluted in 30µl sterile water and stored at -20°C.

2.2.2.3. Site directed mutagenesis

Site-directed mutagenesis was performed using the Quikchange XL site directed mutagenesis kit (Stratagene), following the manufacturers protocol. The mutagenic PCR reaction mixtures comprised of 10ng of DNA template, 125ng of each of forward and reverse primers, 1µl 10µM dNTPs, 1x reaction buffer, 3µL Quikchange solution, 2.5 units of Pfu turbo DNA polymerase and made up to 50µl with sterile water. The PCR was carried out using an initial denaturation stage of 2 minutes at 94°C, followed by 18 cycles of denaturation at 94°C for 50 seconds, annealing at 78°C for 50 seconds and elongation at 68°C for 5 minutes. The final elongation step occurred at 68°C for 7 minutes. The PCR products were digested with 10 U (1µl) of DpnI restriction enzyme to remove the methylated parental (non-mutated) DNA prior to transformation. Plasmids were transformed into XL10-Gold Ultracompetent cells (Stratagene) following manufacturers' instructions, and plated out onto L Agar + 100µg/mL ampicillin or 200µg/ml kanamycin plates.

2.2.2.4. Small scale plasmid purification

A single bacterial colony was picked from agar plates containing a selection antibiotic (ampicillin or kanamycin) and grown overnight in 10ml Lysogeny Broth (LB) containing the same antibiotic at 37°C, with shaking. Bacteria were pelleted from 1.5 ml of the bacterial suspension in a 1.5ml Eppendorf using a microcentrifuge at 14000xg for 3 minutes. The supernatant was discarded, 1.5mls fresh bacterial suspension added and the process repeated. Plasmid DNA was purified using the QIAprep spin miniprep kit (QIAGEN)

following the manufacturers protocol. This involves alkaline lysis of bacterial cells followed by neutralisation and binding of plasmid DNA to a silica membrane. The membrane was washed and plasmids eluted in 30µL of sterile water. Plasmids were stored at -20°C to prevent DNA degradation. In some cases, 300µl of the original suspension was mixed with 300µl of sterile glycerol and stored at -80°C as a glycerol stock.

2.2.2.5. Large scale plasmid purification

A 100µl sample from either an overnight culture or a glycerol stock was inoculated into 100ml of LB containing selection antibiotic at the appropriate concentration, and grown overnight at 37°C with shaking. Plasmids were recovered using the high-speed plasmid purification maxi kit (QIAGEN) according to the manufacturer's instructions. Briefly, bacterial cells weight-adjusted to match using LB broth and then pelleted by centrifugation at 2300xg for 15 minutes at 4°C. The supernatant was discarded and cells resuspended in 10mls of resuspension buffer containing RNaseA. Alkaline lysis buffer containing SDS was added to lyse the cells and denature the chromosomal DNA. The suspension was neutralised, precipitating contaminants, and passed through a resin containing column that binds plasmid DNA. The columns were washed and plasmid DNA eluted. Isopropanol was added to desalt and precipitate plasmid DNA, which was then ethanol washed and eluted in 500µl of TE (elution) buffer (QIAGEN). Plasmid preparations were stored at -20°C.

2.2.2.6. Agarose gel electrophoresis and product purification

DNA fragments were separated on 2% agarose gels diluted with 1x TAE buffer (0.04M Tris-acetate buffer + 0.001M EDTA) and supplemented with 1µg/ml Gel Red (Sigma). Gels were submerged in TAE buffer. Samples were run simultaneously with a commercially available DNA size marker at 80-100V until the bands had separated. DNA was visualised using a UV

transilluminator. Slices containing the required DNA fragments were excised, and DNA extracted using QIAquick gel extraction kit (QIAGEN) following manufacturer's instructions.

2.2.2.7. DNA Sequencing

Constructs were sequenced using the 'in-house' sequencing service by Ivan Andrew (MRC Clinical Sciences Centre, Hammersmith Campus, Du Cane Road, London, W12 0NN). Samples are subjected to automated fluorescent DNA sequencing using the ABI3730xl instrument. The reaction mixture was dispatched as a 10µl solution containing 3.2pmol primer, 200ng DNA and nucleotide-free water.

2.2.3. Transfection of immortalised cell lines.

2.2.3.1. A549 cell transfections

The A549 cell-line was seeded to achieve ~80% confluence the next day. Cells were transfected using Polyfect as described in the product manual. Briefly, 1.75µg of total plasmid DNA was mixed with 100µl serum-free DMEM, 14µl Polyfect and 0.6ml 10% FCS DMEM and added drop-wise to monolayer containing fresh 10% FCS medium. For stable cell line experiments, an initial killing curve was performed to determine antibiotic selection sensitivity. These A549 cells were plated into 6-well plates and allowed to reach 50% confluency overnight. The supernatant was then removed and replaced with fresh DMEM containing zeocin or geneticin at varying concentrations ranging from 0 to 1000µg/ml. The selection medium was replaced every 3-4 days with fresh DMEM containing the desired concentration of antibiotic. The concentration of antibiotic which killed approximately 90% of cells within 14 days was chosen as the concentration to use in stable cell line generation experiments. Fresh A549 cells were then plated onto 6 well plates and allowed to reach a confluency of ~80% before being co-transfected with mCherry Red and m358GFP plasmids. Twenty four hours later, the medium was removed

and the cells detached from the plate using Trypsinising solution. Cells were seeded onto fresh 100mm dishes at varying densities and grown in 10% DMEM containing both selection antibiotics. Monolayers were analysed daily for surviving foci over the course of several weeks.

2.2.3.2. Transfection of 293T cells.

All transfections involving 293T cells were performed on Poly-L-Lysine treated plasticware. Poly-L-Lysine was diluted to 0.0001% in PBS and added to tissue culture plates for five minutes before being removed and the plates washed once with PBS. 293T cells were then seeded to achieve 95% confluency the next day. For 24-well plate transfections, the medium was changed to 0.25ml/well 2% DMEM (without antibiotics) prior to transfection. The ratio of Lipofectamine:DNA used throughout this study was 3 μ l:1 μ g and a master mix containing lipofectamine in 125 μ l OptiMem per well was incubated for 5 minutes at room temperature. Simultaneously, 0.125 μ g plasmid DNA was diluted into 125 μ l OptiMEM per well and incubated in the same manner, before both solutions were mixed and incubated for a further 20 minutes at room temperature. The Lipofectamine:DNA mix was then added dropwise to 293T cells. The quantity of DNA used to transfect per well was scaled up or down depending on the size of the plate. For experiments involving fluorescent signal read-outs, cells were seeded onto black 96-well plates with translucent bottoms (Costar).

2.2.3.3. Rescuing recombinant influenza virus using a 12-plasmid transfection.

Sub-confluent 293T cells (70-80% confluency) were transfected in 12-well plates using Eugene6 (Roche Biochemicals, cat. No. 1815091). Two hours prior to transfection, medium was replaced to 0.5ml DMEM with 2% FCS. The 12 reverse genetics plasmids were aliquotted into Eppendorf tubes, each at 0.5 μ g apart from pCMV-NP, which was added at 1 μ g. Then, 130 μ l of serum-free DMEM was added to 20 μ l Eugene6 and incubated for 5

minutes. DNA was added to the medium/Fugene, mixed, incubated for 15 minutes and added dropwise to the 293T cells. Transfected 293T cells were incubated overnight at 37°C. The following day, confluent MDCK cells were treated with Trypsin and resuspended in 20ml DMEM with 10% FCS. Four drops of 1mg/ml Trypsin solution was added to each well of 293T cells, together with 1 ml of MDCK cell suspension. This was transferred to a 25cm² flask together with a further 4ml of MDCK suspension. This co-culture was incubated for 3-4 hours at 37°C until the cells attached, then washed in 5ml of SF DMEM and incubated with 5ml of fresh serum-free DMEM containing 2.5µg/ml of porcine Trypsin. After 3-4 days, successful rescues resulted in cytopathic destruction of the monolayer and this was used as an indicator to harvest the virus and seed a new flask of MDCKs to propagate this virus stock. In all rescue procedures, a PR8 virus was rescued alongside as a control measure to determine whether any rescue failures were caused by a faulty procedure, or rather incompatible virus gene segments.

2.2.4. Analysis of protein expression

2.2.4.1. Luciferase and β -galactosidase

The supernatants of transfected or infected wells were removed by aspiration and the monolayers lysed with 1x Cell Culture Lysis Reagent (CCLR, Perkin Elmer). For 24-well plates, 150µl/lysis reagent per well was used. Cell lysates were left on the plate and frozen at -80°C to aid in the lysis process, before being thawed and spun at 14000xg in a centrifuge. Whilst avoiding the cell pellet, 20µl of supernatant fluid was removed and added to a Starstedt tube which was automatically mixed with 100µl of luciferase assay reagent (LAR; Promega cat. No. E1500), and the luminescence measured with a 10-second read using a Luciferase Reader. Transfection efficiency was controlled with the co-transfection of a β -galactosidase plasmid, and the signal was measured by adding 20µl of

sample to 100µl LacZ Buffer and 20µl ONPG at 37°C until a yellow colour developed. Absorbance was then read at 420nm in a spectrophotometer.

2.2.4.2. GFP and Cherry Red

Transfections involving these proteins were carried out in black 96-well plates with translucent bottoms (Greiner bio-one). This allowed for fluorescent readouts to occur without terminating the experiment. Cells were simultaneously read through the bottom or top optic using two different filter sets. Cherry Red was assayed using the excitation: 570, emission: 610 filter set, whilst GFP was assayed using excitation: 485, emission: 520. Background readings of untransfected cells were automatically subtracted from positive signals using BMG Mars Data Analysis software.

2.2.4.3. FACS analysis

Confluent 293T cells in 12-well plates were transfected with 0.25µg of pCAG-E195NA FLAG tagged plasmid and left to transfect using lipofectamine for 3 hours. Medium was then removed and replaced with 2% DMEM containing 100µM of drug or drug vehicle and incubated overnight at 37°C/5% CO₂. The following day, the medium was removed and cells detached from the plastic ware using PBS/EDTA. Triplicate wells were then centrifuged at 2500xg for 5 minutes in Eppendorf tubes, before being resuspended in PBS, pooled and centrifuged again. Supernatant was discarded and cells were resuspended in 2% paraformaldehyde in PBS and fixed for 15 minutes at room temperature. Cells were centrifuged again and resuspended in PBS containing 0.05% Saponin, 1%BSA, 0.2% Na Azide and permeabilised for 10 minutes at room temperature. Cells were then centrifuged and resuspended in the above buffer (either with or without Saponin, depending on whether permeabilised or non-permeabilised cells were being treated) containing primary antibody. Cells were left for 1 hour at room temperature, and washed once, before the

secondary antibody was added for 45 minutes at room temperature. Cells were washed 3 times and then analysed by FACS.

2.2.5. Drug maintenance and screening assays

2.2.5.1. Compound library reconstitution

Phytopure and Summit Plc compound libraries were supplied as lyophilised powders and were reconstituted using Dimethyl sulfoxide (DMSO) to a concentration of 10mM (100% DMSO for Phytopure compounds, 70% DMSO for Summit Plc compounds). Compounds were then kept at -20°C in 20µl aliquots to prevent repeated freeze-thawing. Compounds were diluted in SF-DMEM to a concentration of 40µM for each experiment.

2.2.5.2. Compound library screen

Fully confluent MDCK cells were infected with one of three viruses: A/Victoria/3/75, B/Beijing/1/87 or RG14 for 1 hour at 37°C or 33°C at TCID₅₀ concentrations which had been shown previously to destroy the cell monolayer over three days in a TCID₅₀ assay. Virus inoculum was removed and replenished with SF DMEM containing an individual Summit compound at 40µM and 1µg/ml of Trypsin. Each of the triplicate plates contained one untreated and one uninfected column as negative controls. Plates were incubated at either 37°C or 33°C, depending on virus, for 3 days. The supernatant was then removed and the cells stained with crystal violet solution.

2.2.5.3. Drug treatment of transfected cells

293T cells were transfected with appropriate plasmids overnight and then infected with A/Victoria/3/75 for 1 hour at 37°C. Virus was then removed and medium replaced with fresh SF DMEM containing drug at the concentrations specified. For multicycle infections

on 293T cells, overlaying medium was supplemented with 0.1µg/ml of exogenous Trypsin. In all experiments, mock-treated conditions were included by incubating an equivalent concentration of diluted DMSO to control cells.

Chapter 3: Development of screening methods suitable for screening a compound library for novel inhibitors of influenza viruses.

3.1. Introduction

Novel antiviral drugs which effectively inhibit influenza viruses are a potentially lucrative investment opportunity. Influenza epidemics are a guaranteed annual global occurrence and with such a high seasonal morbidity rate, there will always be a market for therapeutic interventions. Moreover, most governments have a 'pandemic plan' that includes the stockpiling of anti-influenza drugs. Even before the recent 2009 pandemic outbreak, 350 million treatment courses of oseltamivir had been supplied as stockpiles to worldwide governments, and the drug's manufacturer, Roche, reported profits of £1.9 billion at the end of 2009 following increased demand during the 'swine flu' outbreak. Both oseltamivir and zanamivir were generated using a targeted approach which relied on the modification of existing compounds to inhibit a pre-determined viral component (von Itzstein & Thomson, 2009). An alternative method used to identify novel antiviral compounds is high throughput screening. Compound libraries of structurally related molecules may be commercially available or owned privately by industrial companies and are a potential source of new drugs. The financial incentive for a private company to screen compounds, to which it owns the patents, against influenza is obvious. High throughput screens may be an advantageous method for screening large libraries thanks to their ability to unearth inhibitory compounds with novel mechanisms of action against the virus.

There are numerous examples in the scientific literature of high throughput screens being utilised to hunt for novel molecules which inhibit the replication of influenza and/or other viruses (Hoffmann *et al.*, 2008; Patkar *et al.*, 2009; Yao *et al.*, 2010; Yu *et al.*, 2009).

Two privately owned compound libraries were provided to the laboratory by small companies who wished them screened for antiviral activity against a human influenza virus.

The first was a library of 1600 Phytopure compounds provided by MNL pharma, and the second was a library of 196 iminosugar molecules acquired from Summit plc. Both libraries were screened for ability to inhibit the replication of an H3N2 challenge virus (A/Victoria/3/75). The method used for the initial screen involved infecting MDCK cells with a concentration of virus that was guaranteed to destroy the entire monolayer over three days, and testing whether this still occurred in the presence of test compounds. Seven of the test compounds from the Phytopure library inhibited the cytopathic effect of the virus and maintained monolayer integrity. From the iminosugar library, three molecules which protected the monolayer from virus cytopathic effect were discovered. To begin with, the seven hits from the Phytopure compound library were taken forward in an attempt to discover mechanisms of action and select compounds for further development.

3.2 Results

3.2.1. High throughput screen of iminosugar library against A/Victoria/3/75 (H3N2) influenza virus.

Prior to the library screen, A/Victoria/3/75 virus was titrated in MDCK cells using a TCID₅₀ assay to determine viral titre. A challenge dose of 31.6TCID₅₀ was chosen for the screen to assure a concentration of virus that would yield a microtitre plate well devoid of cell staining through cytopathic destruction under normal viral growth conditions, and would represent a viral challenge for the drugs that was not too stringent. The Phytopure library was screened with the drugs reconstituted in 100% DMSO at 40µM. This work was performed by a previous lab member, Chris Bull. The iminosugar library obtained from Summit Plc. was reconstituted in 70% DMSO on the company's instruction and tested against Victoria virus at a final concentration of 40µM as part of this project. Virus was left to propagate for three days before the supernatant was removed and the viable cells that remained in each well stained with crystal violet solution. Each drug was assayed in triplicate and positive samples – 'hits' – were scored only if the monolayer had been protected by the cytopathic effect of the virus in all three replicates (Figure 3.1.). From a screen of 196 compounds, three hits which protected the monolayer in triplicate were identified. These hits were described by their Summit catalogue number as compounds: SMT014275, SMT014350, SMT014453. The Phytopure library had yielded 7 hits (Table 3.1.)

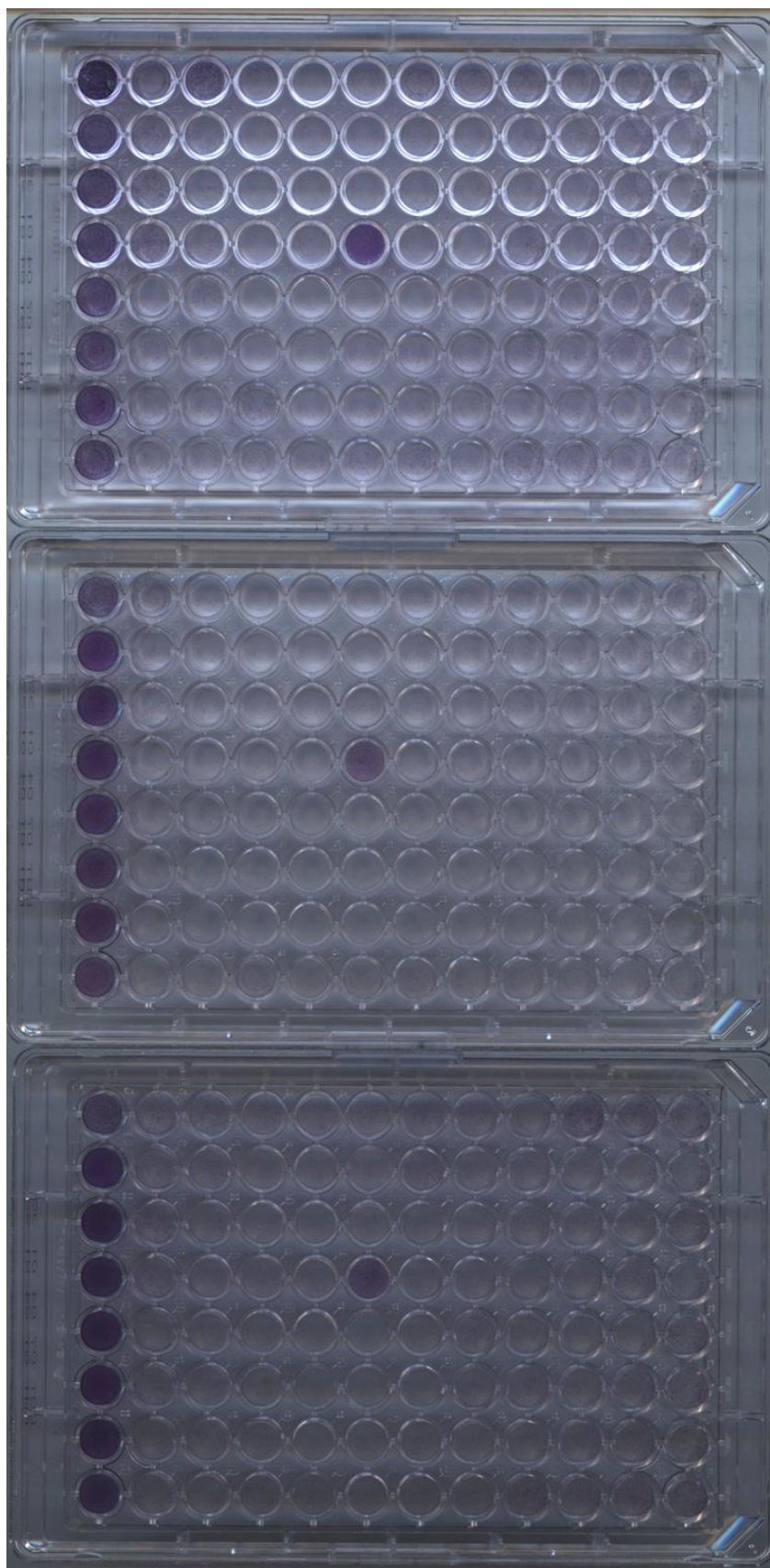


Figure 3. 1. High throughput screening hit from iminosugar library.

(Figure legend overleaf)

Figure 3.1.

MDCK cells at full confluency were infected with 31.6TCID₅₀ of challenge strain A/Victoria/3/75 for one hour, before being incubated at 37°C for three days in the presence of drug. After three days the supernatant was removed and all wells were stained with crystal violet for one hour. The left hand column corresponds to column 12 of the 96-well plate and represents MDCK cells which were not infected with virus. Column 1 of the plate (right hand column) contained untreated wells in which the challenge virus replicated in the absence of any drug. Wells without drug treatment were also supplemented with an equivalent amount of DMSO as a control measure. These controls were repeated on each plate to ensure cell and virus viability. The drug at position D7 (SMT014453) successfully protected the monolayer from viral cytopathic effect in triplicate, and was scored as a hit. This image represents eighty compounds assayed in triplicate on three separate plates.

Following the initial screen of the Phyt PURE library, MNLpharma resupplied the seven identified compounds so that further characterisation experiments could be performed. The next step was to identify the most potent compound to take forward for further drug development. The Summit Plc. compounds were not resupplied until much later and form the basis of experiments in the next chapter.

3.2.2. Cytotoxic effect of Phyt PURE compounds on MDCK cells.

Initial screens of the Phyt PURE compound library suggested that the compounds chosen for further screening were non-cytotoxic because MDCK cell monolayers were not compromised when incubated in their presence. To elucidate the cytotoxicity profile of each compound more stringently, 40µM concentrations of each drug were assayed for cytotoxicity using a Toxilight Bioassay kit (Lonza). The Toxilight bioassay quantitatively measures the release of Adenylate Kinase (indicative of membrane damage in mammalian cells) in a bioluminescent format. Control monolayers were lysed, using Lonza lysis reagent, to provide a value that represented 100% lysis at each time point. Raw data was then expressed as a percentage of the 100% lysis control to determine the cytotoxicity of Phyt PURE compounds (Figure 3.2.). After 24 hours of drug exposure, six of the seven compounds did not appear cytotoxic. However at 72 hours, only four candidates shared cytotoxicity equal to or less than untreated cells. Compounds 48, 230 and 994 were dismissed from further study due to their apparent toxicity. This same pattern of results was also observed when using 80µM of drug (data not shown).

MNLpharma		Summit Plc.	
Compound:	Molecular weight:	Compound:	Molecular weight:
48	820	SMT014275	259
80	272	SMT014350	189
230	406	SMT014453	189
397	229		
453	330		
994	316		
1243	354		

Table 3. 1. Positive hits identified from MNLpharma and Summit Plc. screens.

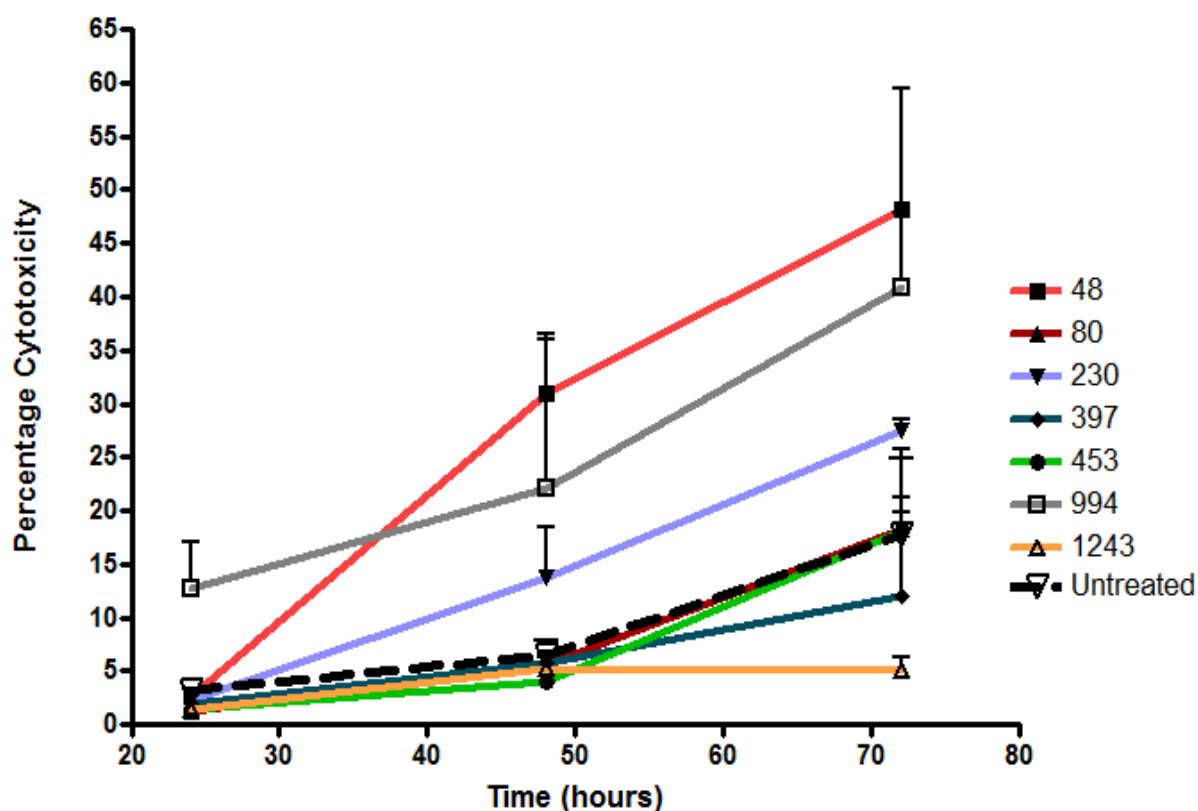


Figure 3. 2. Results of Toxilight Bioassay cytotoxicity assay.

The Toxilight Bioassay kit was used to assess cytotoxicity of Phytopure compounds. Compounds were tested at 40 μ M concentrations over a 72 hour period on MDCK cells. Samples of supernatant were collected after 24, 48 and 72 hours and bioluminescence was quantified using a luminometer. Control wells were lysed completely at each time point using a lysis reagent and then read using a luminometer. This value was taken as 100% lysis and results are expressed as a percentage cytotoxicity of this value. Error bars correspond to standard deviation between triplicate experiments.

In order to assess the commercial potential of these compounds, and attempt to narrow down the potential mechanism of action, the remaining four Phytopure compounds were screened against the H3N2 influenza A virus (A/Victoria/3/75), an Influenza B virus (B/Beijing/87) and an avian influenza virus (A/Duck/England/1/62) of H4N6 subtype, to see if they were active against different viral types or subtypes (Figure 3.3.). Cells were incubated for 16 hours in the presence of virus and individual Phytopure compounds at 40µM and then the supernatants were subjected to plaque assay on MDCKs. Compound 80 was shown to be the least potent of the four experimental drugs and only inhibited the avian influenza strain under these conditions, it was subsequently expelled from further experiments. Compounds 397, 453 and 1243 were potent across all three virus subtypes, with 1243 being the most potent molecule of the three. These results suggested that the remaining three drugs had a generic mechanism of action that worked across multiple virus types. By exhibiting activity against avian and human influenza A and B viruses, these compounds demonstrated commercially desirable broad-spectrum activity. Furthermore, it ruled out the possibility of the drugs targeting individual proteins that are influenza A or B type specific (for example, M2).

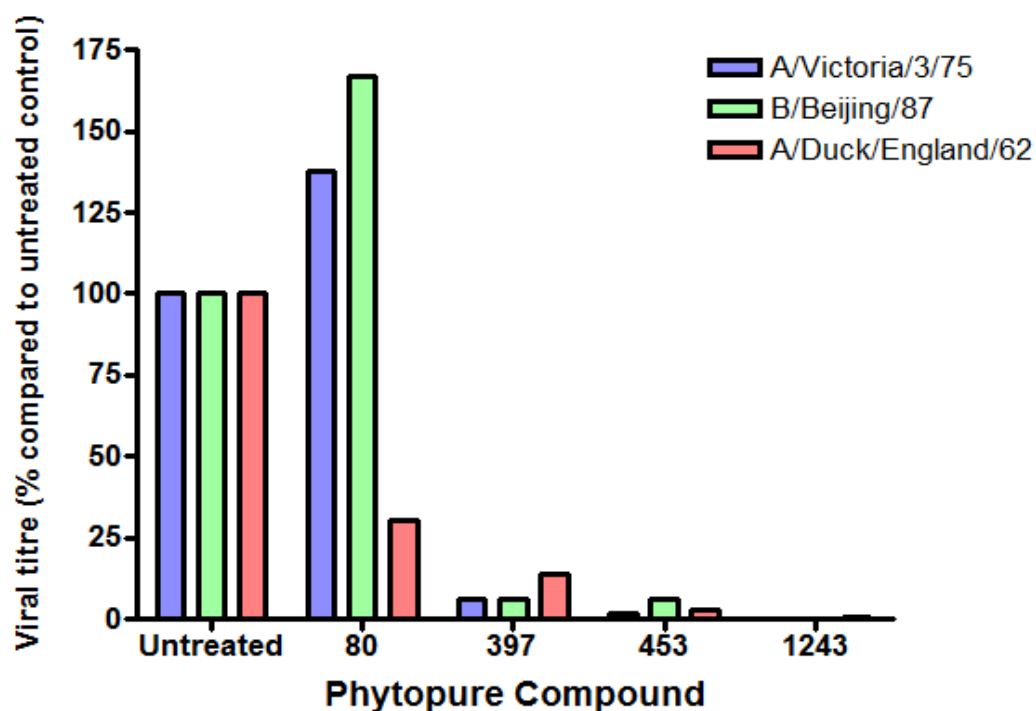


Figure 3. 3. Effect of Phytopure compounds on the replication of influenza A and B viruses.

Four Phytopure compounds were assayed for their ability to inhibit the replication of three distinct viruses: An influenza A virus (A/Victoria/3/75), an influenza B virus (B/Beijing/87) and an avian virus (A/Duck/England/62). These viruses were used to infect MDCK cells at an MOI of 1 in the presence of 40µM compound and the viral titre released at 16 hours post infection calculated by plaque assay and expressed as a percentage of the untreated control titre. Data shown represents the mean of duplicate wells.

3.2.3. Assessing the effect of Phytopure compounds on influenza and host-cell polymerase activity

The influenza polymerase proteins represent an ideal target for novel antiviral drugs given the importance of these proteins in successful viral replication. The effect of Phytopure compounds on influenza polymerase activity was then assessed using reconstituted polymerases to drive *in situ* amplification and expression of a viral-like minigenome encoding a luciferase reporter. The viral polymerase proteins PA, PB1, PB2 and NP were transfected into 293T cells in a pPol.II plasmid backbone. Inside the cell, these plasmids are transcribed by host cell machinery before assembling into the influenza polymerase complex, the minimum required proteins necessary for viral transcription and replication. An initial experiment to assess the effect of Phytopure compounds on influenza polymerase activity revealed that viral expression of a luciferase reporter gene was reduced in the presence of all three compounds (Figure 3.4). Compound 397 was the least potent drug of the three. As a transfection control, β -galactosidase was transfected into cells to report on host-cell polymerase activity and gene expression. The results indicated that the Phytopure compounds also inhibited the β -galactosidase signal and subsequently host-cell polymerase activity. This result stresses the importance of including a reporter gene that can assay host cell function as well as viral replication. The fact that these compounds inhibited host-polymerases was deemed an undesirable trait from a commercial perspective and subsequently MNLpharma withdrew interest in the drugs.

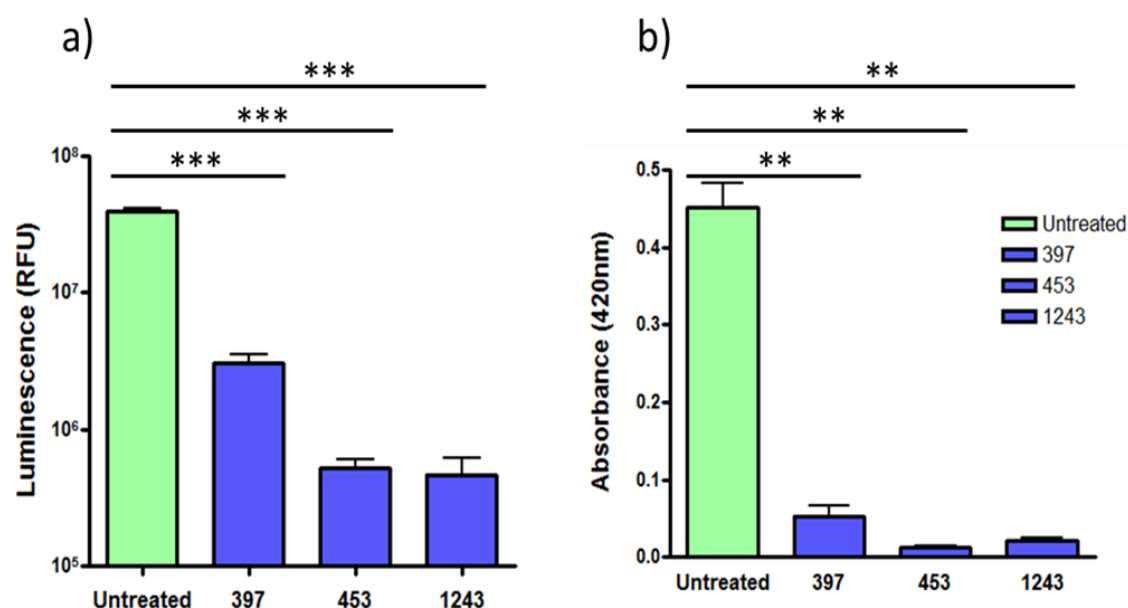


Figure 3. 4. Effect of Phytopure compounds on influenza polymerase activity and host-cell gene expression.

a) Plasmids encoding the three Influenza A virus polymerase genes and NP (with a human, Pol.II promoter) were transfected into 293T cells alongside a plasmid encoding luciferase (with a Pol.I promoter, to make viral-like RNA) and left for 30 minutes at 37°C. Phytopure compounds were then added at 40µM concentration to the monolayer and incubated for 24 hours at 37°C. Cells were lysed using CCLR buffer and luciferase activity measured with a luminometer. **b)** β-galactosidase was also co-transfected into the same cells as above under the control of a cellular promoter (Pol.II). The extracts from the above experiment were also used to assay for β-galactosidase activity using a photospectrometer. The error bars correspond to the Standard Deviation between triplicate samples. A two-tailed unpaired t-test was used to calculate P values (** = $p < 0.01$, *** = $p < 0.005$).

3.2.4.Using reporter plasmids as indicators of viral replication

In an attempt to provide a more efficient screening assay, we attempted to engineer a reporter system which could be used simultaneously to assess the cytotoxicity and influenza inhibiting potential of novel compounds using fluorescent reporter constructs at the stage of the initial screening step. It has previously been published that influenza virus superinfection can be used to transcribe intracellular reporter plasmids that are flanked by the non-coding regions of influenza segments. Work conducted by Lutz and colleagues showed that GFP and Luciferase reporter genes flanked by the non-coding regions of segment 5 (NP), could be driven by both intracellularly reconstituted vRNP complexes, and by subsequent superinfection with a range of influenza A viruses (Lutz *et al.*, 2005). Our laboratory possesses three similar reporter constructs (CAT, Luciferase and GFP flanked by NS, NS and M segment non-coding regions, respectively), but in conflict with the Lutz published results, we found that reporter gene expression from these constructs could only be driven by reconstituted viral polymerases and not by whole virus infection. This is demonstrated with the luciferase reporter construct in Figure 3.5.

In order to determine the suitability of our 'wild type' pPol.I reporter plasmids as screening tools, we assessed the ability of A/Victoria/3/75 infection to drive expression of the reporter gene in cell culture. The data in Figure 3.5. shows that the wild type reporter does not express a measurable amount of luciferase in cells infected with influenza virus as the source of viral polymerase, and the signal at 24 hours was lower than that at 9 hours. Furthermore, the signal-to-noise ratio obtained at each time point with each MOI was extremely low (Table 3.2.) and the luciferase signal did not depend on virus input.

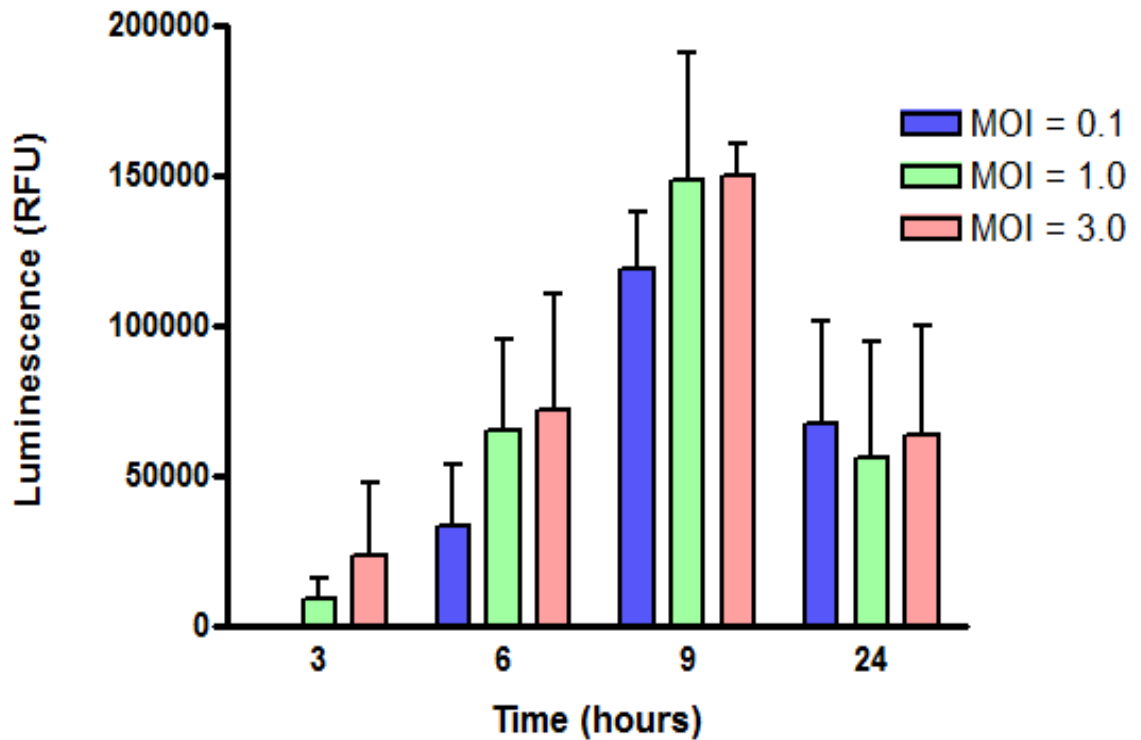


Figure 3. 5. Expression of pPol.I wild type luciferase by A/Victoria/3/75 virus infection in 293T cells.

A/Victoria/3/75 influenza virus was used to drive expression of a luciferase reporter construct at three different MOIs. Each condition was tested in triplicate and the mean of three uninfected wells containing a transfected luciferase plasmid was subtracted as the background reading from the raw data obtained. Each well was then normalised to its individual β -galactosidase value, and the mean of this normalised data is represented. The error bars represent the Standard Error of the Mean (SEM). This graph is representative of data that has been obtained on three separate occasions using luciferase as the reporter gene. The 24 hour time point has also been repeated using wild type GFP and CAT reporter genes and shows similarly low and MOI-independent signals when driven by A/Victoria/3/75 virus infection (data not shown).

3.2.5. Generating the 3-5-8 triple mutation GFP and Luciferase reporter plasmids.

The data obtained from the previous experiment suggested that our reporter plasmids were poorly and unpredictably expressed by influenza virus superinfection and would not be effective as components of a screening cell line. In an attempt to improve the signal generated during virus driven mini-genome replication, we engineered a triple mutation into the 3' non-coding region of the viral promoters that flank the reporter gene (Figure 3.6.). Work by Neumann and Hobom identified that this triple mutation resulted in increased expression when driven by a mouse-adapted influenza strain in a murine cell line (Neumann & Hobom, 1995). Using a two-step mutagenesis PCR, the triple mutation was engineered into reporter plasmids encoding both the luciferase reporter and the GFP reporter. The first mutagenesis successfully introduced the double nucleotide changes at positions 3 and 5, and this product then underwent a further mutagenic PCR to introduce the final mutation at position 8. Several attempts were made to introduce all three nucleotide changes in a single mutagenic PCR, however this proved unsuccessful. The luciferase reporter gene is flanked by the non-coding regions of influenza segment 8, whereas our GFP reporter gene is flanked by non-coding regions of segment 7. Due to the segment specific nucleotides which follow the conserved regions at the 5' and 3' ends of each influenza segment, a separate set of mutagenic primers was required for each reporter plasmid.

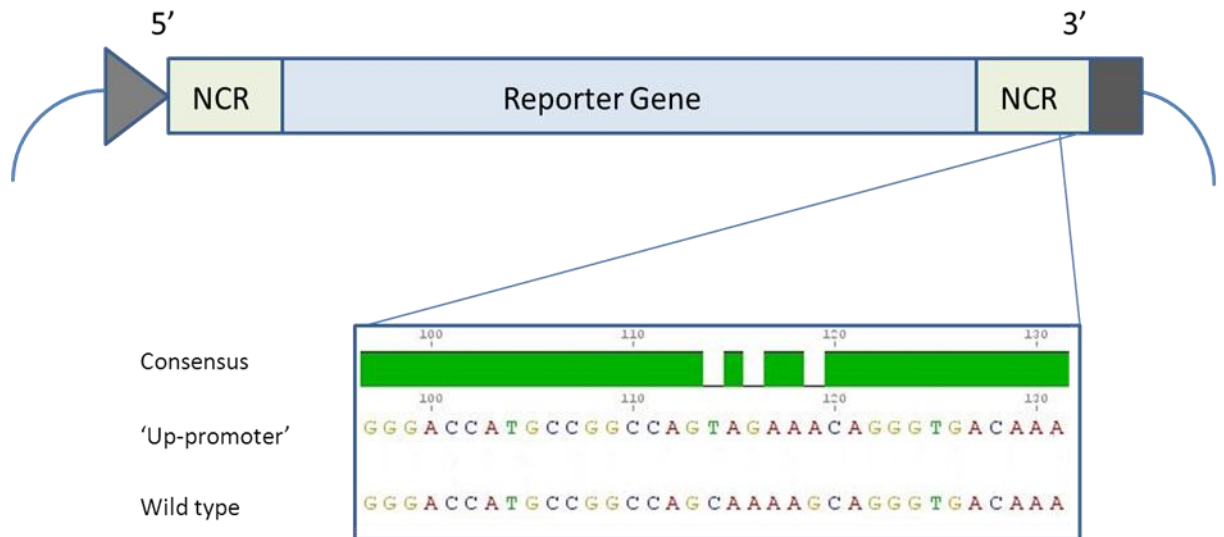


Figure 3. 6. Three point mutations introduced to 3' end of luciferase reporter mini-genome construct.

Three point mutations were introduced to the 3' end of a luciferase reporter mini-genome construct using site-directed mutagenesis. Nucleotide 112 in the sequence chromatogram corresponds to the beginning of the 3' non-coding region. Position 3 (nucleotide 114) is mutated from C → T, position 5 from A → G and position 8 from G → A. The primers used for reporter gene mutagenesis are listed in the methods section.

3.2.6. Effect of the 3-5-8 triple mutation on luciferase gene expression

The ability of either virus infection or reconstituted viral polymerase proteins to drive the expression of the reporter gene within the mini-genome flanked by wild type noncoding regions or by non-coding regions containing the 3-5-8 triple mutation was assayed. The four plasmids to reconstitute influenza polymerase components were transfected into 293T cells simultaneously with either the wild type or mutated luciferase reporter gene, along with a β -galactosidase plasmid as a transfection control. The expression of luciferase was observed at 3, 6, 9, or 24 hours post-transfection. The results shown in Figure 3.7 confirm that both viral infection and reconstituted polymerases are capable of driving 3-5-8 reporter gene synthesis. Although reconstituted influenza polymerases drove higher expression of the reporter gene flanked by the 3' triple mutant NCR, these differences were not significantly different. However, when reporter gene expression was driven by virus infection, significantly higher signals were generated from the triple mutant reporter when compared with the wild type reporter at each time point (Figure 3.7., lower panel). With the triple mutant construct, the luciferase signal was cumulative over time and had a superior signal-to-noise ratio compared to the wild type reporter plasmid (Table 3.2.). These data suggest that the 3-5-8 triple mutant construct was detected and transcribed by viral polymerases during infection and would be a suitable quantitative read-out to assess virus replication inhibition mediated by antiviral compounds.

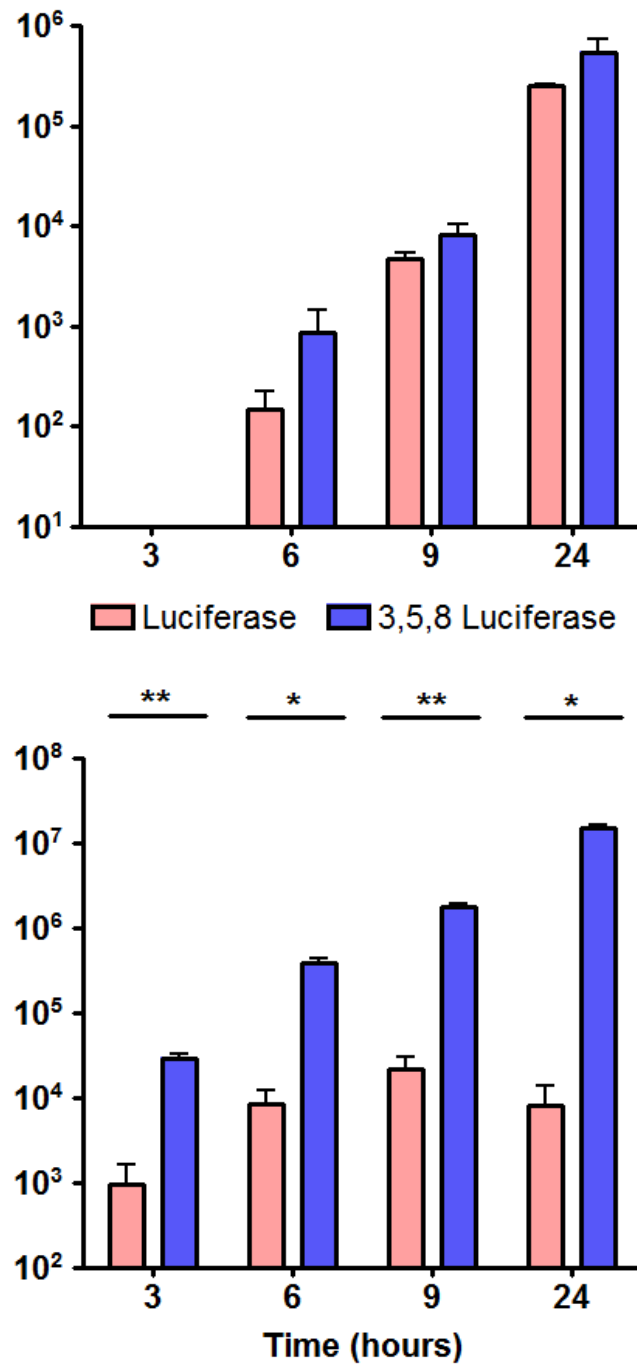


Figure 3. 7. Comparison of luciferase reporter gene expression from wild type and triple mutant 3-5-8 viral-like minigenomes by reconstituted viral polymerases or whole virus.

a) The viral polymerase complex was reconstituted *in situ* following transfection of plasmids containing the three influenza polymerase genes and NP into 293T cells. At 3, 6, 9 and 24 hours post-transfection, cells were lysed and luminescence detected using a

luminometer. A two-tailed unpaired t-test was used to assess statistical significance between the luciferase signal driven by wild-type and 3,5,8 luciferase reporters, however the difference in expression levels was not statistically significant. **b)** Viral infection was used to drive replication of the wild type or triple mutant construct, which was transfected into 293T cells 24 hours prior to infection. The error bars correspond to the Standard Error of the Mean across triplicate samples. A two-tailed unpaired t-test was used to calculate P values (* = $p < 0.05$, ** = $p < 0.01$).

MOI	Luciferase	3-5-8 Luciferase
0.1	1 : 1.7	1 : 185
1.0	1 : 2.4	1 : 236
3.0	1 : 2.4	1 : 188

Table 3. 2. Noise-to-Signal ratio of wild type and triple mutant luciferase reporter gene driven by A/Victoria/3/75 virus infection.

The ratios of signal-to-noise were calculated in triplicate at each time point by dividing the mean 'background' normalised reading of three uninfected wells, which contained a luciferase reporter, with the values generated by wells which contained the reporter and were infected. The 3-5-8 triple mutant reporter results in greater signal-to-noise readouts than the wild type reporter plasmid. The data presented in this table are the average of three calculations per MOI.

A follow up experiment was conducted to assess the effect of different virus MOIs on the expression of the 3-5-8 luciferase (3-5-8luc) reporter construct (Figure 3.8.). For its intended use, the reporter construct should be able to detect differences in the amount of replication, here driven by changing the input multiplicity of virus. 293T cells were transfected with the 3-5-8luc reporter and β -galactosidase plasmids overnight and then infected the following day with A/Victoria/3/75 at various MOIs. The luciferase signal was measured after twenty-four hours and normalised to the β -galactosidase reading. Figure 3.8. shows a viral dose-dependent luciferase signal obtained between MOIs of 0.1 down to 0.0001. To guard against the possibility that screened library compounds are cytotoxic to the host cell – an observation which may result in a reduced luciferase signal due to cytopathic destruction of viable cells that the influenza virus would otherwise infect – we require the permanent presence of a reporter of host-cell polymerase viability. If the viral reporter signal is reduced, yet the host reporter signal remains normal, it is likely that a genuine hit will have been scored in the assay. Without the host cell reporter, it is possible that a cytotoxic compound could relay a reduction in luciferase signal and resemble a false positive hit in the screen.

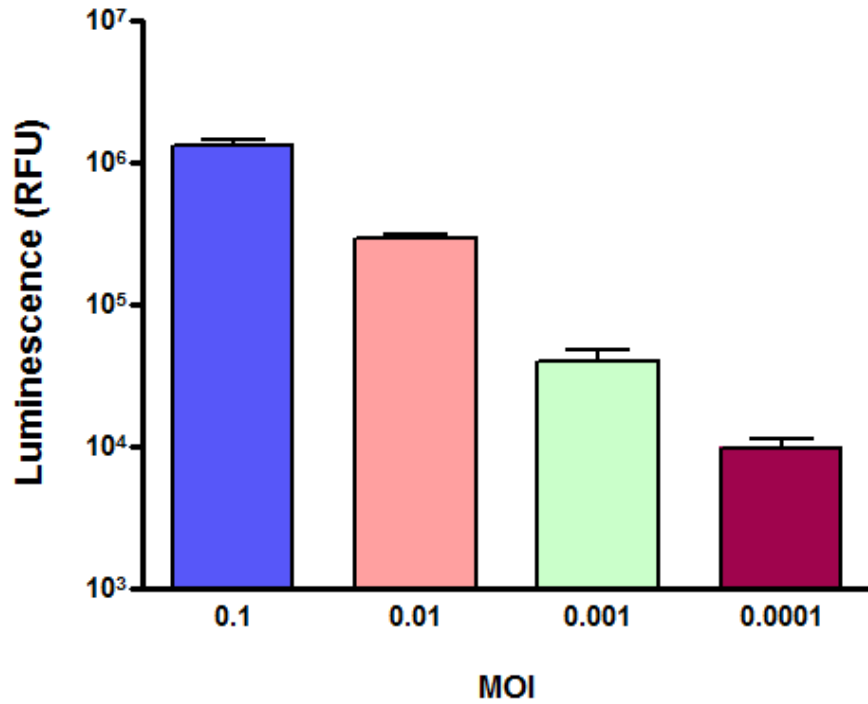


Figure 3. 8. 3-5-8luc signal generated by different multiplicities of virus.

293T cells were transfected with the 3-5-8luc and β -galactosidase plasmids overnight and subsequently infected with A/Victoria/3/75 virus for one hour at four different MOIs. Luciferase expression was measured 24-hours post-infection and normalised to β -galactosidase readings. An uninfected control containing 3-5-8luc plasmid was also assayed and subtracted from the 'background' reading from each condition. Data shown represents the mean of triplicate wells and error bars correspond to the standard deviation between samples.

3.2.7. Assessing the effect of antiviral drugs on reporter signalling.

In order to assess the suitability of the 3-5-8luc reporter plasmid as a drug screening tool, the ability of the system to detect the antiviral activity of established antiviral compounds was tested. Oseltamivir, Ribavirin and a hit identified in initial drug screen 3.2.1., SMT014350, were added to cell medium following overnight transfection of the 3-5-8luc plasmid into 293T cells and subsequent infection with A/Victoria/3/75 virus. The drug was present in the medium for 24 hours post-infection, after which time the cells were lysed and the luciferase signal read. The drug concentrations used were chosen either based on published EC₅₀ data of the compounds, or from concentrations that were known to inhibit viral replication from previous experiments. The novel compound SMT014350 was included as a way of validating the reporter plasmid as an antiviral drug identifying tool (Figure 3.9a.). The drug resulted in a small reduction of luciferase signal although this difference was not significantly different. Ribavirin, a known inhibitor of influenza polymerase, was effective at inhibiting influenza-driven expression of the luciferase signal in a dose-dependent fashion. Oseltamivir did not significantly inhibit the luciferase signal, as would be expected since the drug is effective at a later stage of the infectious cycle and does not affect polymerase activity. This experiment demonstrates that drugs which are active at later stages of the replication cycle would not be identified by this primary screen system.

In an attempt to extend the assay to find drugs that act later at stages of assembly or budding, we removed the supernatant from the first round of infection and used it to infect and drive a second round of luciferase expression (Figure 3.9b.). Now we saw a significant effect of oseltamivir, which completely ablated the luciferase signal in the second cell monolayer, as did the high doses of ribavirin. The effect of SMT014350 was also evident since the drug significantly decreased luciferase signal in the second cell monolayer ($p < 0.01$.)

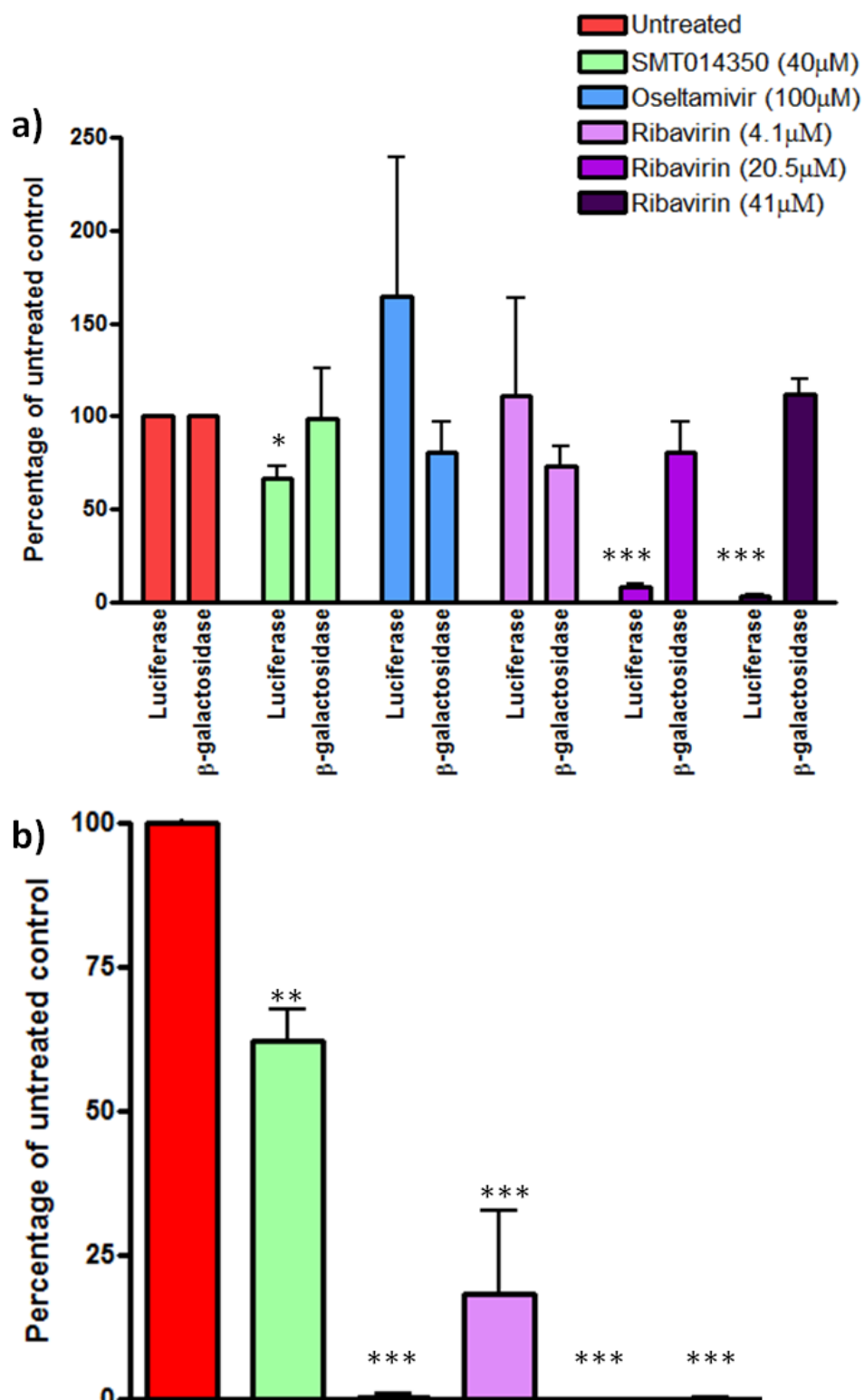


Figure 3. 9. The effect of known drugs and a previously discovered screening hit on host-cell and viral polymerase activity.

(Figure legend overleaf)

Figure 3. 10.

a) 293T cells containing the 3-5-8luc and β -galactosidase reporter plasmids were infected with A/Victoria/3/75 virus at an MOI of 1 and incubated overnight in the presence of several drugs. The luciferase signal was not significantly inhibited by either oseltamivir, or a low concentration of ribavirin (4.1 μ M). Higher concentrations of ribavirin (20.5 μ M and above) did significantly reduce the luciferase signal generated by influenza polymerases supplied from infecting virus ($p = <0.0001$). Experimental compound SMT014350 was also shown to significantly reduce the expression of the 3-5-8luc reporter signal although to a lower level of significance. None of the drugs inhibited the expression of the host-cell transcribed reporter plasmid. **b)** The supernatant from the above experiment was then treated with Trypsin for two hours at 37°C in order to activate the HA proteins on progeny virions, and then used to infect new cells containing the 3-5-8Luc plasmid overnight. The oseltamivir and ribavirin treated supernatants were all significantly inhibited in their ability to drive expression of the reporter plasmid. The effect of compound SMT014350 was also statistically significant. The data shown is the result of triplicate samples expressed as a percentage of an untreated control and is a representative data set of two individual experiments. The expression of the host-driven β -galactosidase signal was not affected by any of the drug treatment conditions, as above (data not shown). The error bars correspond to the standard deviation between triplicate samples. A two-tailed, unpaired students t-test was used to assess statistical significance between drug treated and untreated samples, with **= $p < 0.01$ and *** = $p < 0.0001$.

3.2.8 Using fluorescent reporter mini-genomes to detect efficacy of drugs against influenza and simultaneously monitor host driven gene expression.

Finally, in order to assess the potential of our constructs as high-throughput screening tools, we transfected 293T cells with plasmids containing the fluorescent variants of pPol.I (3-5-8-mGFP) and pPol.II (mCherryRed) reporter genes and challenged the cells with a low multiplicity of A/Victoria/3/75 virus (0.1) in the presence of several compounds. We then measured the fluorescence generated over time to observe the effects of these drugs on viral replication and host-cell survival. Figure 3.10 shows how each of the compounds affected the expression of virally transcribed GFP and host-driven Cherry Red. Compounds. Oseltamivir **a)**, SMT014350 **b)**, Ribavirin **c)** and Amantadine **d)** all inhibited the production of GFP when compared with untreated virus infection, whilst maintaining robust Cherry Red signal. Over time, drug treated cells generated a higher cherry-red output than the cells undergoing an untreated viral infection, possibly due to the protection from the effects of viral replication such as host cell shut off offered by these drugs. Compounds 1243 **e)** and cyclohexamide **f)** however resulted in a knockdown of both GFP and Cherry Red, suggesting they were cytotoxic to global protein expression. Cyclohexamide is a known inhibitor of eukaryotic protein-synthesis, and was included here as a control to illustrate the detection of effects on host gene expression. Experimental compound 1243 (identified in 3.2.1.) was included to confirm its activity as a drug which inhibited both viral and host-cell protein synthesis. The exact mechanism of this compound remains unknown, though its profile in this assay resembles the toxic profile of cyclohexamide which suggests it is unlikely to be pursued as a serious candidate for an anti-influenza drug. The data generated by this experiment results in a very clear visual format to illustrate compounds that are non-toxic inhibitors of influenza viral replication.

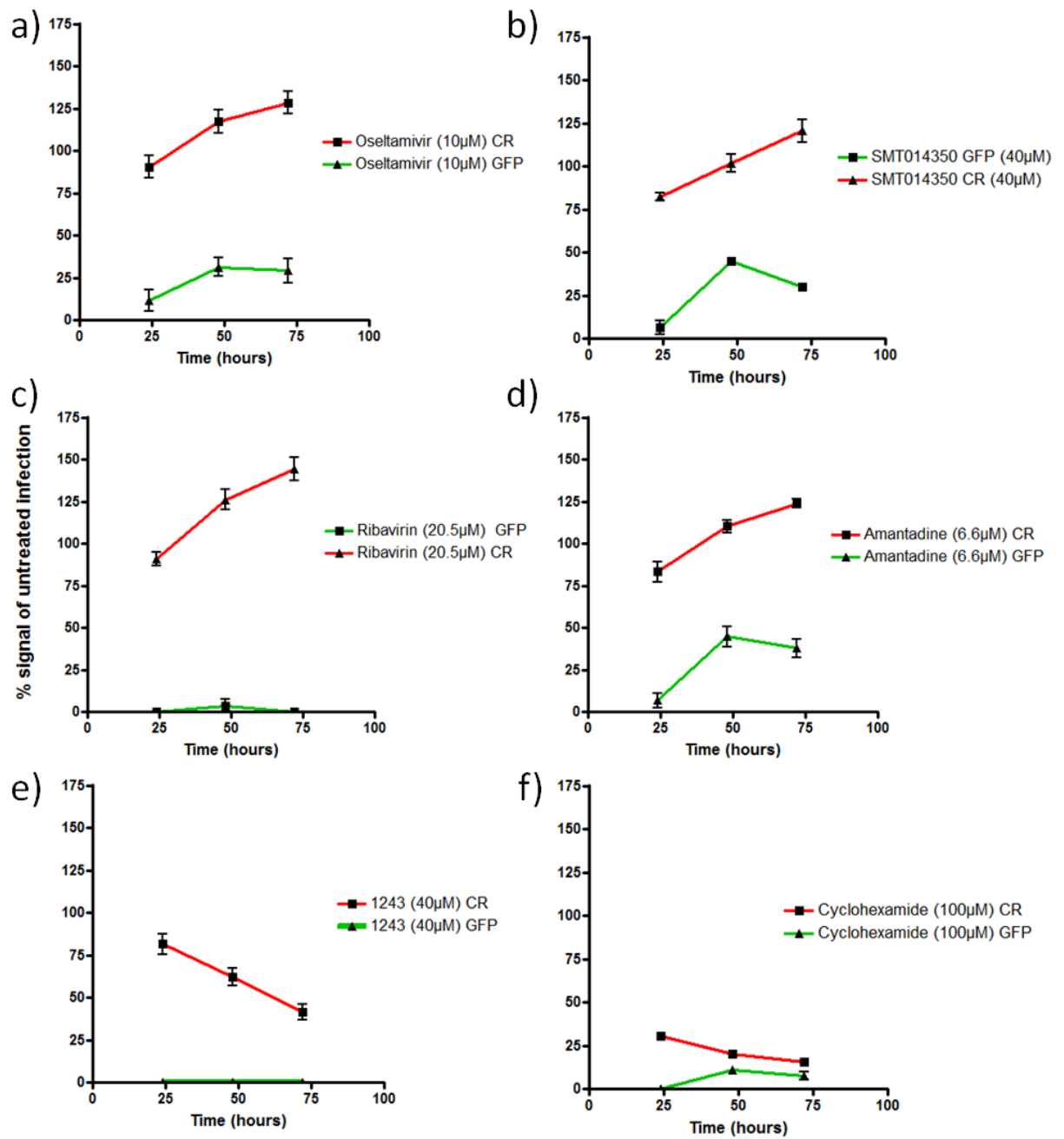


Figure 3. 11. Effect of antiviral drug treatment on the expression of host cell- and virally-driven fluorescent reporter plasmids.

(Figure legend overleaf)

Figure 3.10.

293T cells were seeded into black 96-well plates with translucent bottoms and allowed to proliferate overnight. mCherry Red and GFP-containing reporter plasmids were then co-transfected into the cells for a further 24 hours. A/Victoria/3/75 virus was then infected onto cells at an MOI of 0.1 and removed after one hour. The medium was then replaced with SFDMEM containing: **a)** oseltamivir, **b)** SMT014350, **c)** Ribavirin, **d)** Amantadine, **e)** 1243, **f)** cyclohexamide at the concentrations shown in the side bars to each figure or no drug, and supplemented with 0.1µg/ml of Trypsin. The plate was read at 24, 48 and 72 hours post-infection using a FluoStar plate reader (BMG Labtech), for GFP and Cherry Red signal. GFP and Cherry Red signals from untransfected wells were then subtracted from all other well readings as a background measurement. A transfected but uninfected well was then further subtracted from GFP signals, whereas an untransfected well measurement was used to normalise mCherry Red signal, across the rest of the plate. Data shown is expressed as a percentage of the signal generated by the untreated A/Victoria viral infection and displays the average of quadruplicate wells. The error bars correspond to the SEM between samples.

3.2.9 Generating a reporter cell line containing fluorescent reporter genes.

The previous section highlights the potential of these reporter genes to be used in a high-throughput screening assay. We next attempted to implement this technology into a cell-line that could be infected instantly when required, thus removing the necessary transfection step of the reporter plasmids each time. We also decided a change in cell-line from 293T cells to A549 cells would be desirable, as 293T cells are less adherent and less-tolerant of exogenous Trypsin which is a required addition for multicycle virus infections. A549 cells were transfected with the mCherry Red and 3-5-8mGFP plasmids and grown in the presence of two antibiotics: geneticin and zeocin, at concentrations which were individually shown to result in cell death of approximately 90% of cells over fourteen days, prior to the transfection. The mCherry Red and 3-5-8mGFP backbone vectors encode for geneticin and zeocin resistance, respectively and thus cells containing both plasmids were expected to survive the co-treatment. Despite several attempts to generate a cell-line that constitutively contained both plasmids, we could not select for a stable colony that survived serial passage following initial selection. The reasons for this failure are unclear, though selection failure seemed to map to the inclusion of the 3-5-8mGFP plasmid, as cells containing mCherry Red alone could be passaged. The entire 3-5-8GFP plasmid was sequenced and the geneticin resistance gene found to be intact.

3.3. Discussion

Using a rudimentary screening assay, several compounds were identified that prevented cytopathic destruction of an MDCK monolayer by A/Victoria/3/75 influenza virus during an *in vitro* experiment to assess the potential antiviral activity of 2 compound libraries. Three of these hits subsequently form the focus of the following chapter in which attempts are made to elucidate their mechanism of action. Seven other hits were investigated here and it was found that the most potent compounds showed adverse effects on host gene expression, that had not been identified using an initial simple screen that relied on them preserving the integrity of an MDCK cell monolayer. In light of the limitations encountered during initial screening by cytopathic effect, we envisaged a reporter cell line which could be utilised as a screening tool for high-throughput library screening assays, which would utilise fluorescent reporter genes to simultaneously quantify influenza replication and cell viability status in the presence of our compounds.

The work presented in this chapter has shown that reporter genes flanked by segment-specific non-coding regions of influenza genes are inefficiently transcribed by influenza virus during super-infection. Although these reporter plasmids are routinely used to gauge the transcriptional activity of intracellularly reconstituted influenza RNA-dependant RNA polymerases produced from transfected plasmids, they are transcribed poorly when those polymerases are delivered via whole virus infection. However, the modification of three nucleotides in the 3' end of the non-coding region resulted in a significant increase in reporter signal generated by viral superinfection. Interestingly, the reconstituted polymerase complex of A/Victoria/3/75 did not express the 3-5-8luc mini-genome more efficiently than that of the wild type reporter (Figure 3.7. a). It has been reported that a similar minigenome expressing CAT as the reporter gene was poorly transcribed by reconstituted A/Victoria/3/75 polymerases in COS-1 cells, and indeed in that study the 3-5-

8 triple mutation exhibited a 'down promoter' effect. This observation appeared to be specific for the origin of the viral polymerase as other human virus complexes tested also failed to upregulate transcription of the triple mutant, whereas avian virus polymerase complexes did enhance transcription of the 3-5-8 reporter (Crescenzo-Chaigne *et al.*, 2002). It is thus intriguing that whole A/Victoria/3/75 virus is capable of driving a significantly enhanced reporter signal from the viral-RNA like segment containing the 3' end triple-mutation. The 3' and 5' ends of vRNA segments show partial complementarity in their conserved regions, which is essential for functional promoter activity. Evidence exists which suggests that the 3' and 5' ends do not simply fold back flat onto one another, but instead a secondary 'corkscrew' motif is formed whereby a stem-loop structure is formed in both ends of the promoter (Flick & Hobom, 1999). These hairpin loop structures at the 3' and 5' ends are a requirement for efficient endonuclease activity of the influenza polymerase, which is essential for cap-snatching and subsequent transcription of the vRNA into mRNA (Leahy *et al.*, 2001a; Leahy *et al.*, 2001b). The three nucleotide changes in the 3' region of the triple-mutant reporter plasmid would hypothetically maintain a stem-loop in the corkscrew model at the 3' end as complementary base-pairing is conserved between positions 3 – 8. However, the triple mutations would further enhance the complementarity between the 3' and 5' ends of the vRNA segment were these ends to pair into a flat pan-handle configuration. This complementarity essentially results in both the 3' and 5' ends having identical promoter regions. Initiation of transcription thus could occur at the same intensity from the 3' end of a vRNA chain as it would from the 5' end of a cRNA chain (Figure 3.11.).

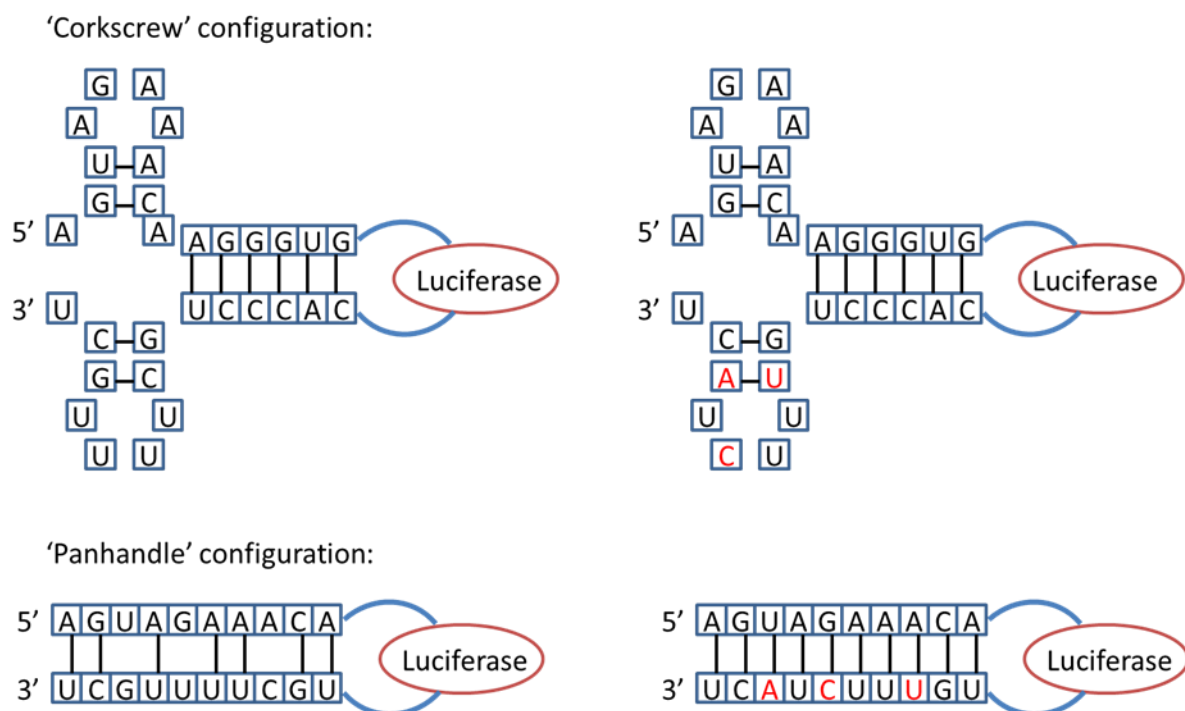


Figure 3. 12. Predicted panhandle and corkscrew promoter regions of wild type (left) and triple-mutant (right) luciferase reporter genes.

The diagram above represents the suggested 'corkscrew' or 'panhandle' structures of a negative-sense influenza NS segment, whereby the linear strand has been doubled back on itself and exhibits partial base-pairing, as suggested by Flick and colleagues (Flick *et al.*, 1996), for the corkscrew, or complete base-pairing for the panhandle.

The reason as to why reconstituted polymerase is unable, where whole virus infection is able, to drive transcription of the reporter plasmid is unknown, but one may speculate that the cellular environment during whole virus infection is markedly different than during plasmid transfection and polymerase gene expression. Whole influenza infection would likely drive expression of different host factors that may affect the function of viral polymerase (Geiss *et al.*, 2002). An alternate possibility is that the binding of polymerase to RNA is affected and that another viral factor such as NS1, not present in the plasmid transfections, modulates the polymerase promoter complex. These possibilities were not tested here.

The 'up promoter' reporter minigenome was able to detect different concentrations of influenza virus and generated lower signals when fewer virions were present to drive transcription of the reporter, providing virus MOI was ≤ 1 . This indicates that the constructs could potentially be used to detect decreases in viral titres over multi-cycle infections, should an experimental drug result in viral inhibition.

To eliminate the need for original transfections of reporter plasmids and transfer this technology to a more stable cell-type, we attempted to generate a reporter A549 cell line which would constitutively express an mCherry Red fluorescence signal to report on host-cell transcription status, as well as a 3-5-8mGFP fluorescence signal which would be activated upon successful viral replication. Both reporter plasmids were transfected into A549 cells and contained individual antibiotic resistance genes to ensure their inclusion into cells cultured in the presence of geneticin and zeocin. Despite numerous attempts to culture a cell line which included both plasmids, a sustainable cell line could not be generated. It is unlikely that the antibiotic selection is to blame for the observed cell death as the concentrations of each drug used was determined individually using concentration killing curves. We questioned whether a faulty geneticin resistance gene was responsible

for failing to protect cells under the selection of this antibiotic, but found no defect in the resistance gene after sequencing of the plasmid. A similar cell-line containing viral like mini-genome reporter RNAs under the expression of pPol.I promoters have been reported in the literature, as described in the paper by Lutz et al though no experimental data generated using the cell line is presented in the manuscript (Lutz *et al.*, 2005). A recent publication also utilised a reporter cell line harbouring a virus inducible reporter mini genome to screen an RNAi library, using the similar technique of driving a second round of reporter gene replication with the supernatant of previously treated cells as we have described in 3.2.7. (Karlsson *et al.*, 2010). It is not clear from this paper whether the cell lines constitutively expresses the viral like RNA. Furthermore, a high throughput screen using the 358 up-promoter technology was described by Hoffmann and colleagues (Hoffmann *et al.*, 2008). Once again a careful read of the methodology reveals that transfections for the reporter minigenomes were performed *de novo* for each screen suggesting difficulties in obtaining a constitutive cell line. The screening methodology of Hoffmann et al. resulted in the successful identification of several novel classes of anti-influenza compounds, though required two simultaneous screens: one to detect the effect of various compounds on virus replication using a 358 luciferase reporter plasmid, and a second to assess the cytotoxicity of the library. The screening tools presented here could reduce the workload given the ability to detect antiviral activity and cytotoxicity simultaneously.

Although we were unsuccessful in our attempt to generate a stable reporter cell line capable of real-time drug screening, the generation of a triple-mutant reporter gene has resulted in a useful experimental tool which has been used in other applications in our laboratory, such as assaying and comparing the polymerase activity of different virus strains during the recent A/H1N1 2009 outbreak. These fluorescent mini-genome reporter plasmids could also be used to transiently transfect alternate cell lines, such as A549s, should a more robust screen involving multiple manipulations of the monolayer be

required. Whilst 293T cells are particularly sensitive to the addition of exogenous Trypsin, they were shown here to tolerate a concentration that was sufficiently high to propagate A/Victoria/3/75 infection. It is likely that other, Trypsin-independent viruses, such as the lab-adapted WSN strain, could be used to drive these plasmids with some success. Furthermore, we have shown how the system can be used in two stages in a manner that may help to elucidate mechanisms of action for novel compounds during a library screen. If cells containing reporter plasmids are incubated in the absence of Trypsin, then in the primary round of infection, compounds which interfere with viral entry, uncoating or polymerase activity could be detected. Supernatants from this initial screen could then be Trypsin-treated and transferred to a second set of freshly transfected cells, where drugs which affect virus assembly and release could be detected (Figure 3.9.).

Chapter 4: Assessing the spectrum of activity of a drug screen hit.

4.1 Introduction

Work described in Chapter 4 identified three hits from the iminosugar library provided by Summit that inhibited replication of influenza A/Victoria virus. Following the relay of initial results to Summit Plc, they informed us that two of the compounds were already known in the literature. Compound SMT14350 was Castanospermine, whereas SMT014453 was Celgosivir, a 'prodrug of Castanospermine'. Due to concerns over whether these compounds would be reliably resupplied by Summit Plc, we chose to concentrate further experiments on Castanospermine, as the compound was readily available from a commercial source (Sigma). Castanospermine is a known inhibitor of glucosidase enzymes (Saul *et al.*, 1983). Glucosidase type I and II enzymes play a role in the post-translational transport of glycoproteins and are responsible for trimming N-linked oligosaccharide side chains on the surface of glycoproteins in the ER. It is known that proper trimming of these N-linked oligosaccharides is important for successful glycoprotein maturation. For example, the intracellular chaperones calnexin and calreticulin interact with incompletely folded proteins and progresses the maturation process by allowing proteins to acquire proper 3D structure. These chaperones also protect nascent proteins from degradation (Hebert *et al.*, 1996). The manner in which newly synthesised glycoproteins are recognised by such chaperones is via their oligosaccharide side chains (Hebert *et al.*, 1995), and thus by antagonising the production of this side-chain signal, these drugs may deny glycoproteins protection along the maturation pathway.

A literature search for 'Influenza [AND] Castanospermine' on PubMed returned 13 publications, ranging in publication date from 1983-2000. In 1983, Pan and colleagues reported that haemagglutinin glycoproteins of an H1N1 virus, NWS, were susceptible to

treatment of MDCK cells with Castanospermine, as witnessed by the unprocessed glucose residues abundant on the glycoprotein in the presence of drug, but also that this observation did not correspond to a decrease in viral plaque titre (Pan *et al.*, 1983). Most recently, Saito & Yamaguchi showed that the surface glycoproteins of influenza differed in their dependency on proper oligosaccharide trimming, and suggested that NA transport to the cell surface was inhibited by 50% in the presence of Castanospermine, whereas HA transport was not affected (Saito & Yamaguchi, 2000). This paper also highlighted a reduction in viral titres in the presence of Castanospermine, but it remained unclear whether the effect was viral subtype specific, a point that is particularly important to address given their choice to study such an uncommon H1N8 recombinant virus.

Research described in this chapter aimed to test the susceptibility of different influenza viruses to this drug to assess its utility as a broad spectrum anti-influenza compound.

4.2. Results

4.2.1. Assessing the cytotoxicity of Castanospermine.

The iminosugars had been screened at 40µM in the initial screening procedure and had not shown any destruction of the MDCK monolayer at this concentration. An industrially favoured cytotoxicity assay was performed with the commercially available compound Castanospermine, to determine a) that the commercially sourced compound was also non-toxic to cells and b) the highest concentration of drug that could be used for characterisation studies (Figure 4.1.). In the previous chapter, we showed that Castanospermine (SMT014350) at high concentrations did not affect host-cell polymerase activity and was thus less toxic than the hits from the Phyt PURE library (Figure 3.10). Now, we used a Methylene Blue cytotoxicity assay to determine the TD₅₀ of Castanospermine. Following the cytotoxicity assay, all further experiments were carried out using a Castanospermine concentration of either 40µM or 100µM, as indicated in individual figure legends.

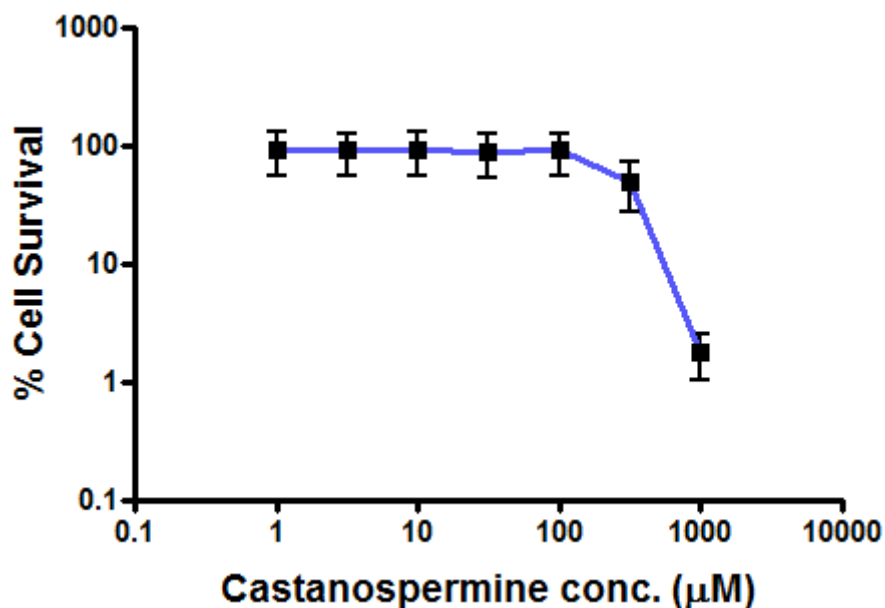


Figure 4. 1. Effect of Castanospermine concentration on MDCK cells survival

Octuplet wells on triplicate plates of MDCK cells were incubated at 37°C in the presence of serially-diluted Castanospermine for three days. At 24, 48 and 72 hours cells were removed and subjected to methylene blue treatment. Briefly, supernatant was removed and cells incubated for two hours in methylene blue solution. Cells were then washed with PBS and subjected to 1% SDS treatment for one hour before being read for absorbance using a spectrophotometer at 660nm. A control group which remained untreated throughout was also assayed at each time point and represented 100% cell viability. Drug treated wells were averaged and are plotted as a percentage of the untreated group. Error bars correspond to the standard deviation between octuplet samples.

4.2.2. Assessing the effectiveness of Summit hits against A/Victoria/3/75 and B/Beijing/1/87 in a multicycle growth curve.

The initial screen suggested that three test compounds were effective at protecting MDCK cells from A/Victoria/3/75. We next assessed the efficacy and spectrum of activity of all three iminosugars against A/Victoria/3/75 or B/Beijing/1/87 viruses by assaying for viral titre over time (Figure 4.2.). The concentration of drug used in this experiment remained at 40µM, but the challenge dose of virus was increased to an MOI of 1. The results in Figure 4.2. show that all three iminosugar compounds were inhibitory against this challenge dose of A/Victoria/3/75. However it should be noted that this effect only reached statistical significance at early time points following infection. Compound SMT014275 was the most potent compound of the three against A/Victoria virus, maintaining significant potency until 24 hours post-infection. None of the drugs significantly inhibited the replication of the influenza B virus, thus this virus was insensitive to these three drugs. At this higher challenge dose it was concluded that although these three compounds inhibited the destruction of a cell monolayer from A/Victoria virus, overall their potency was low.

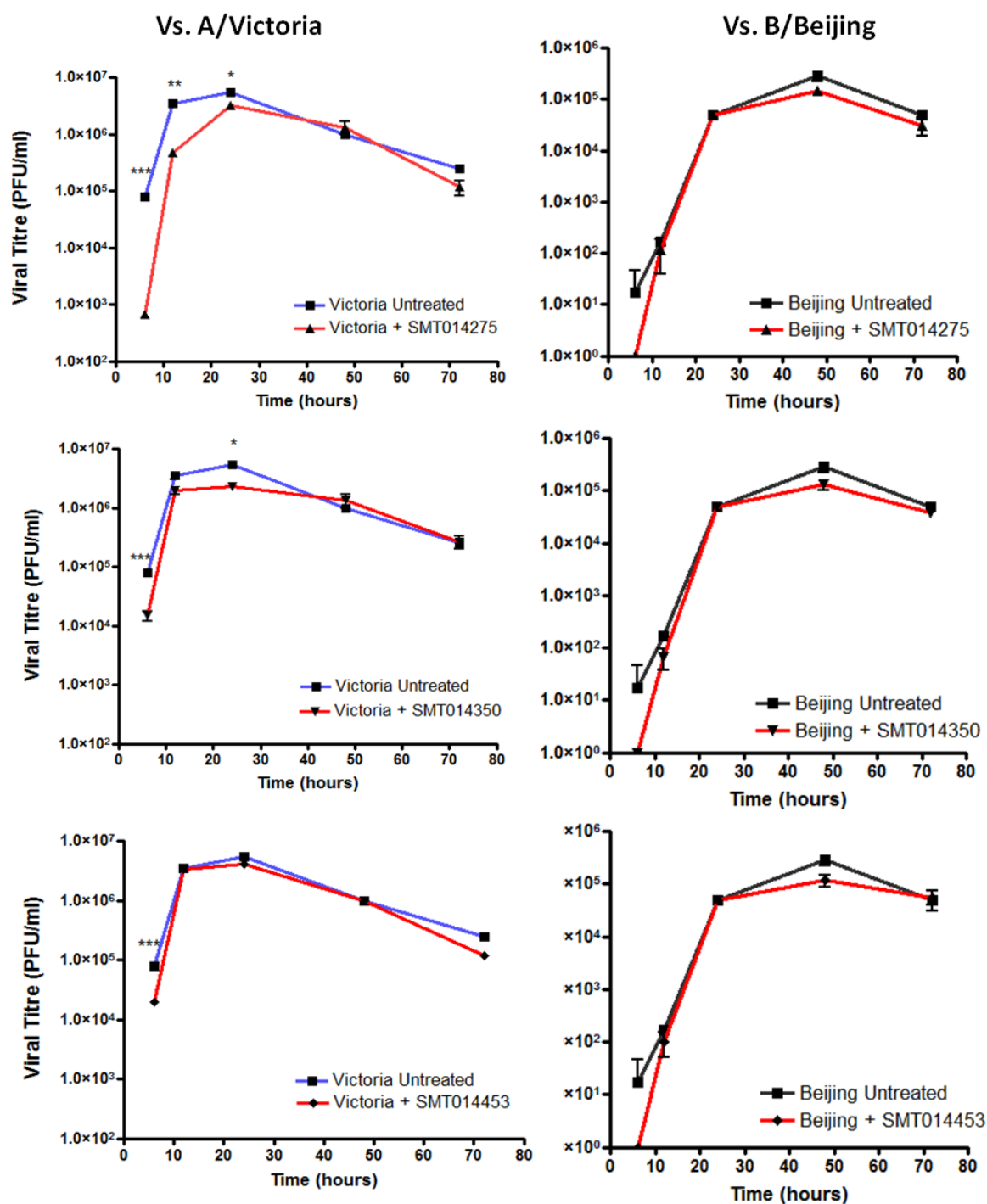


Figure 4. 2. The effect of iminosugar library compounds on the replication of A/Victoria and B/Beijing viruses.

MDCK cells were infected at an MOI of 1.0 with either A/Victoria or B/Beijing viruses and incubated for one hour at 37°C or 33°C, respectively. Virus was removed and the cells acid-washed briefly with PBS at pH 3.3, before SFDMEM containing 40µM of an individual

iminosugar compound and exogenous Trypsin was added. Samples were collected from the same well at corresponding time points, at which time the quantity of supernatant removed was replaced with an equivalent amount containing drug and Trypsin. Virus content of these samples was then enumerated by plaque assay on MDCK cells. The untreated wells were incubated with the same volume of overall DMSO as was present in the drug treated wells to control for any side effect of DMSO treatment. Data shown are the average of triplicate samples and the error bars correspond to the standard deviation between samples. A two-tailed, unpaired student's t-test was used to assess for statistical significance with * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.

4.2.3. Effect of Castanospermine on a range of influenza A viruses.

Following the observation in Figure 4.2. that Castanospermine was effective against an influenza A isolate, but ineffective against an influenza B isolate, we tested the effect of the drug against a wider spectrum of influenza A viruses, including the new pandemic H1N1 influenza strain, first of all at a lower MOI of 0.001. As a measure of drug efficacy, we assayed the supernatants of infected cells for reproductive virus with a conventional plaque assay, and also measured the neuraminidase activity of released virus with a MuNANA assay. The MuNANA assay is a sensitive measurement which assays the efficiency of influenza neuraminidase at cleaving a small sialic-acid-like substrate into a fluorescent product. We began by ensuring that virus NA enzyme activity itself was not directly inhibited by Castanospermine treatment. When each virus was incubated in the presence of Castanospermine for one hour, each virus type retained the ability to cleave the MuNANA substrate, indicating that there was no direct inhibitory effect of the drug on NA activity (Figure 4.3). This is in contrast to the observation that NA activity was substantially reduced in the presence of oseltamivir. Interestingly, the NA activity of B/Beijing was less affected by oseltamivir than the influenza A viruses.

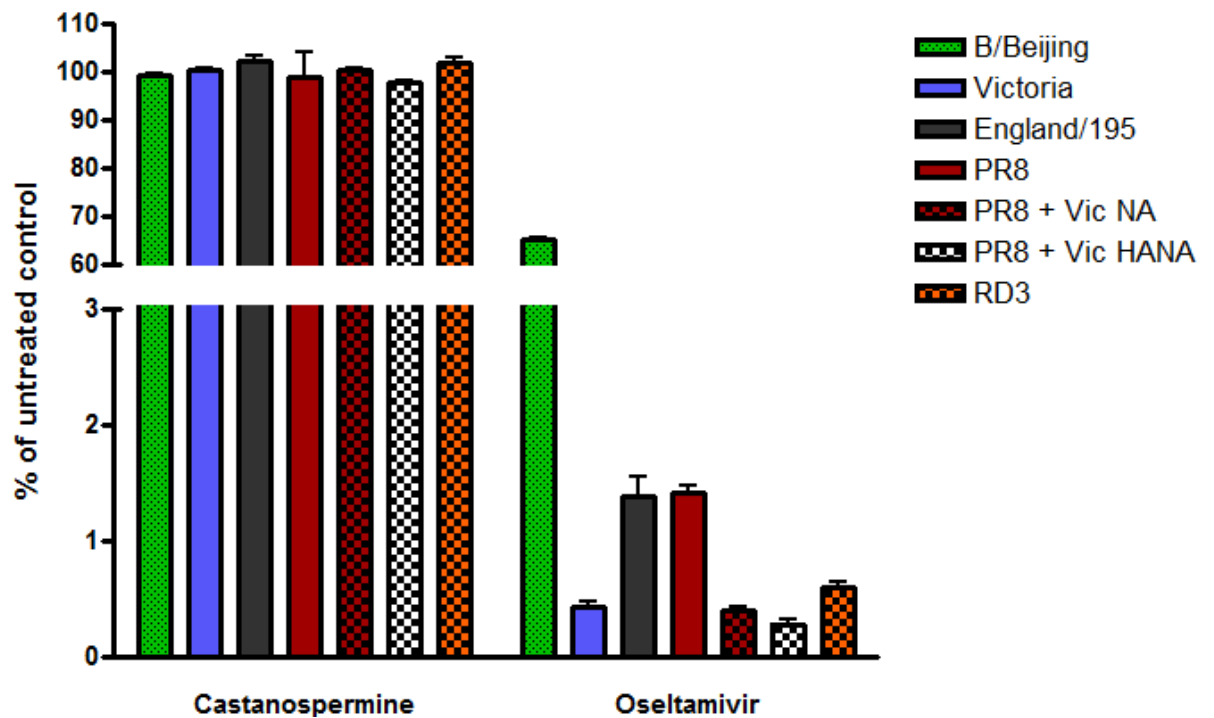


Figure 4. 3. Effect of Castanospermine and Oseltamivir on NA activity as measured by MuNANA substrate catabolisation.

Using black 96 well plates, to triplicate wells were added 50µl of virus and 50µl of Castanospermine or Oseltamivir at a concentration of 100 or 1µM respectively. The plate was incubated at 37 C for 1 hour before MuNANA substrate was added and the plate incubated at 37 C for a further hour. Fluorescence intensity was measured. Wells which possessed MuNANA substrate and buffer alone were read as background values and the average of these wells was subtracted from all other wells. The average of triplicate samples is shown here with error bars representing the standard deviation between samples.

We then inoculated MDCK cells with the same range of influenza A viruses at an MOI of 0.001. A lower MOI was chosen in order to detect differences resulting from drug treatment following the initial revelation that Castanospermine only inhibited replication early during infection (when fewer virions were present) as seen in figure 4.2. Following an overnight infection in the presence of Castanospermine, virus supernatant was removed and subjected to plaque assay (a) to determine viral titre, and MuNANA assay (b) to determine viral neuraminidase activity as a second measure of released virus. The viruses chosen for this study are shown in Table 4.1.

Virus	Comments
B/Beijing/1/87	A representative influenza B isolate.
A/Victoria/3/75	A representative influenza A H3N2 human virus with filamentous morphology (Elleman & Barclay, 2004).
A/England/195/09	A representative pandemic influenza A H1N1 isolate also shown to have filamentous morphology (Itoh <i>et al.</i> , 2009).
A/PR8	A laboratory adapted influenza A H1N1 virus with spherical morphology
A/PR8 & Victoria NA	A 7:1 recombinant virus generated by reverse genetics in this study, containing all internal segments of PR8 and the N2 NA gene of Victoria.
A/PR8 & Victoria HA+NA	A 6:2 recombinant virus generated in this study by reverse genetics containing all internal segments of PR8 and the H3 HA and N2 NA of Victoria.
RD3	A 6:2 recombinant virus described by (Whiteley <i>et al.</i> , 2007) generated by reverse genetics containing all internal segments of PR8 and the H7 (modified) HA and N1 NA of A/Chicken/Italy/4789/99

Table 4. 1 List of viruses screened against Castanospermine.

Our panel of viruses included B/Beijing and A/Victoria as resistant and sensitive viral isolates respectively. We included A/England/195/09 as the pandemic isolate strain to ask whether Castanospermine was effective against a currently circulating influenza virus. Because we suspected that surface glycoproteins would be important determinants of susceptibility, we included A/PR8 virus and a number of recombinant viruses that were rescued specifically for this project and contained the surface proteins of other isolates built upon a PR8 backbone. A/PR8 and A/Victoria viruses are associated with different budding strategies that results in different virion morphologies, with PR8 forming conventional spherical virions, whereas Victoria virus forms longer, filamentous particles which may be more or less affected by glycosylation interference. Finally, we used a reverse genetics virus containing the internal proteins of PR8 with the H7N1 surface glycoproteins of A/Chicken/Italy/4789/99 originally generated by (Whiteley *et al.*, 2007). This virus displays surface antigens of an avian isolate with pandemic potential and, if affected, could potentially validate the use of this drug as a future pandemic stockpiling solution.

The recombinant viruses PR8 + Vic NA and PR8 + Vic HA and NA were generated as part of this project by reverse genetics. Individual plasmids containing all eight segments of the desired recombinant virus were transfected into 293T cells under the expression of a polymerase I promoter. Four plasmids containing the individual polymerase genes of influenza, plus NP were concurrently transfected under the expression of a polymerase II promoter. These four plasmids are transcribed by host-cell polymerases and form a vRNP complex, the minimum proteins required for influenza virus segment replication and translation. The further eight plasmids are then transcribed by the vRNP complex and bud as whole viruses. Each of the recombinant viruses rescued successfully, though the PR8 virus containing the surface HA and NA of Victoria replicated to a 100-fold lower titre in MDCK cells, suggesting a natural attenuation in the fitness of this isolate.

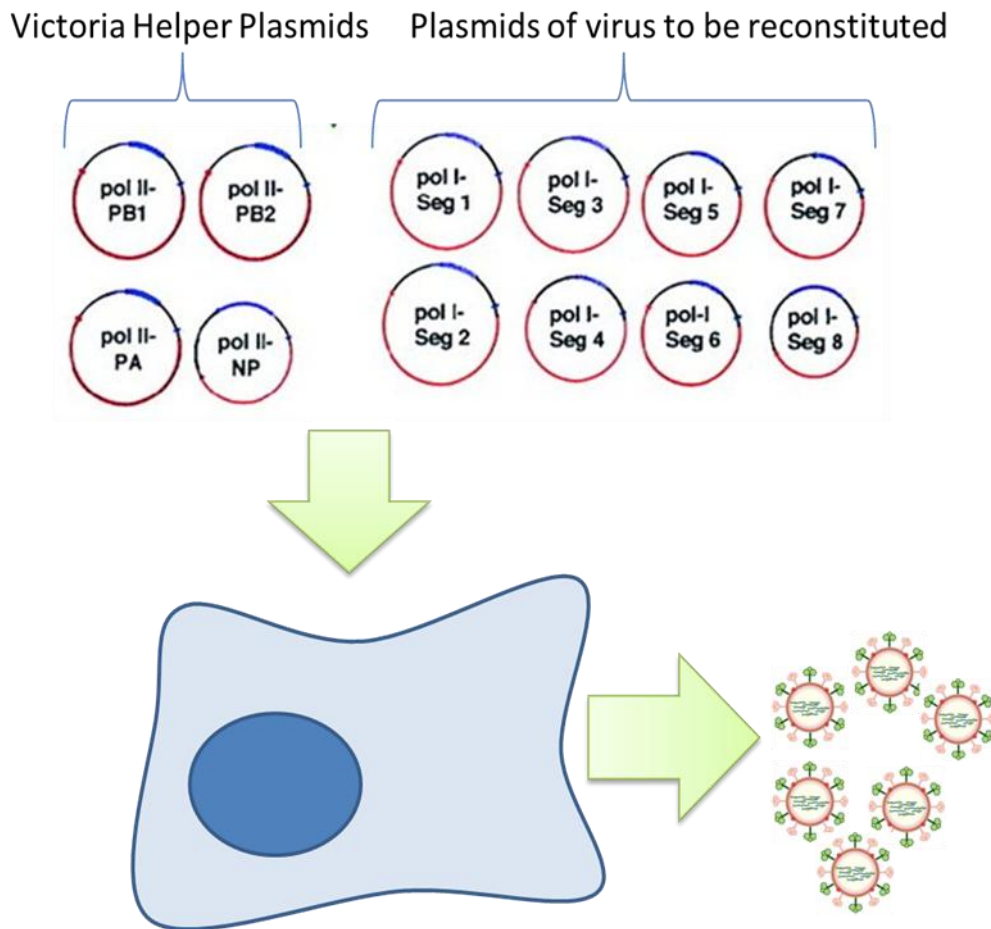


Figure 4. 4. Schematic of influenza virus rescue.

293T cells were transfected simultaneously with 12 plasmids. Four plasmids, under a host-cell polymerase recognised Pol.II promoter are transcribed by the host cell and assemble into the viral polymerase machinery (NP, PB1, PB2 and PA). The reconstituted viral polymerase then recognises the remaining 8 plasmids, under a Pol.I promoter, and transcribes the viral proteins encoded within. These proteins then assemble into new virions and are released as progeny virions. As viral release from 293T cells is inefficient, 293T cells are co-cultured with MDCK cells to boost viral titre of reconstituted viruses. MDCK cells are infected as usual by novel virus and replication occurs more efficiently in the more-permissive cell line.

We observed that all influenza A subtypes subjected to drug treatment starting with a low initial MOI of 0.001 underwent a reduction in viral titre when virus was grown in the presence of Castanospermine, whereas the influenza B isolate remained impervious to drug treatment, even at this low initial MOI. Generally, the reduction of viral titre corresponded with a reduction in fluorescence generated by the cleavage of MuNANA, though the MuNANA assay generated more statistically significant data.

A/Victoria was particularly susceptible to Castanospermine treatment, as was pandemic A/England/195/09 virus (Figure 4.5 a)) and for both of these viruses both assays reached statistical significance. However for the other 4 viruses tested the results were less dramatic and did not uniformly reach statistical significance.

For example, although the NA content of released virus after PR8 infection in presence of drug was reduced, this did not have a statistically significant effect on the viral titre. This may suggest that the PR8 virus can tolerate a certain amount of reduction in NA expression on the cell surface or on virus particles with little compromise to infectivity. Indeed, when the NA gene from the highly susceptible Victoria strain was transferred to PR8, the effect of drug on virus infectivity became significant.

However neither of the recombinant viruses in which paired HA and NA were present on the PR8 body, PR8 + Vic HA and NA nor RD3 virus, showed statistical significance in the effect of drug on either NA activity of released particles nor infectivity. It is possible that with further repeats of this assay, statistical significance of drug inhibition on all four PR8 based viruses could be established but time did not permit this.

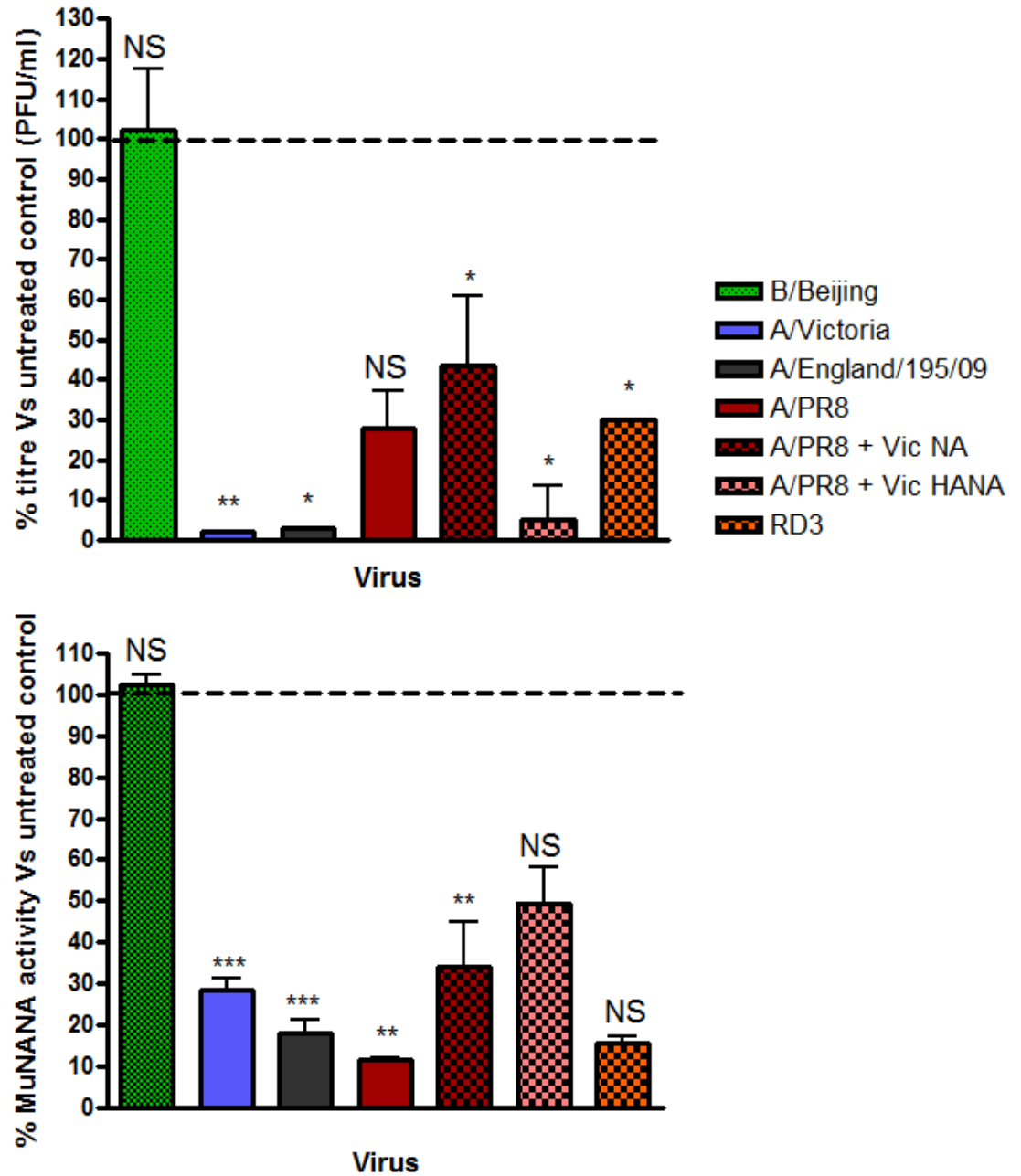


Figure 4. 5. The effect of Castanospermine on viral titre and neuraminidase activity of a panel of influenza viruses.

(Figure legend overleaf)

MDCK cells were infected at an MOI of 0.001 and allowed to replicate for 24 hours in the presence of 100 μ M Castanospermine, or drug vehicle. Following 24 hours infection, supernatants were removed and subjected to plaque assay to determine viral titre **(a)**, or MuNANA assay **(b)** to analyse for NA activity. Data shown is the average of triplicate wells and error bars correspond to the standard deviation between these samples. A two-tailed unpaired student's t-test was used to calculate statistical significance between untreated and treated control groups with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ and NS = Not significant

At higher viral challenge of MOI = 1 the Influenza B isolate remained uninhibited by Castanospermine as expected (Figure 4.6a). Furthermore, the effects of drug against influenza A isolates was no longer significantly inhibited by the action of Castanospermine at this concentration. Only A/Victoria virus suffered a reduction in viral titres. Conversely, NA activity of all isolates was significantly reduced by Castanospermine treatment (Figure 4.6b.). This suggests that although viral titres are generally unaffected, the amount of NA on the surface of those virions that are present is decreased. It is possible that this is due to improperly formed or fewer functional NA tetramers appearing on the surface of the virions which bud from the cell.

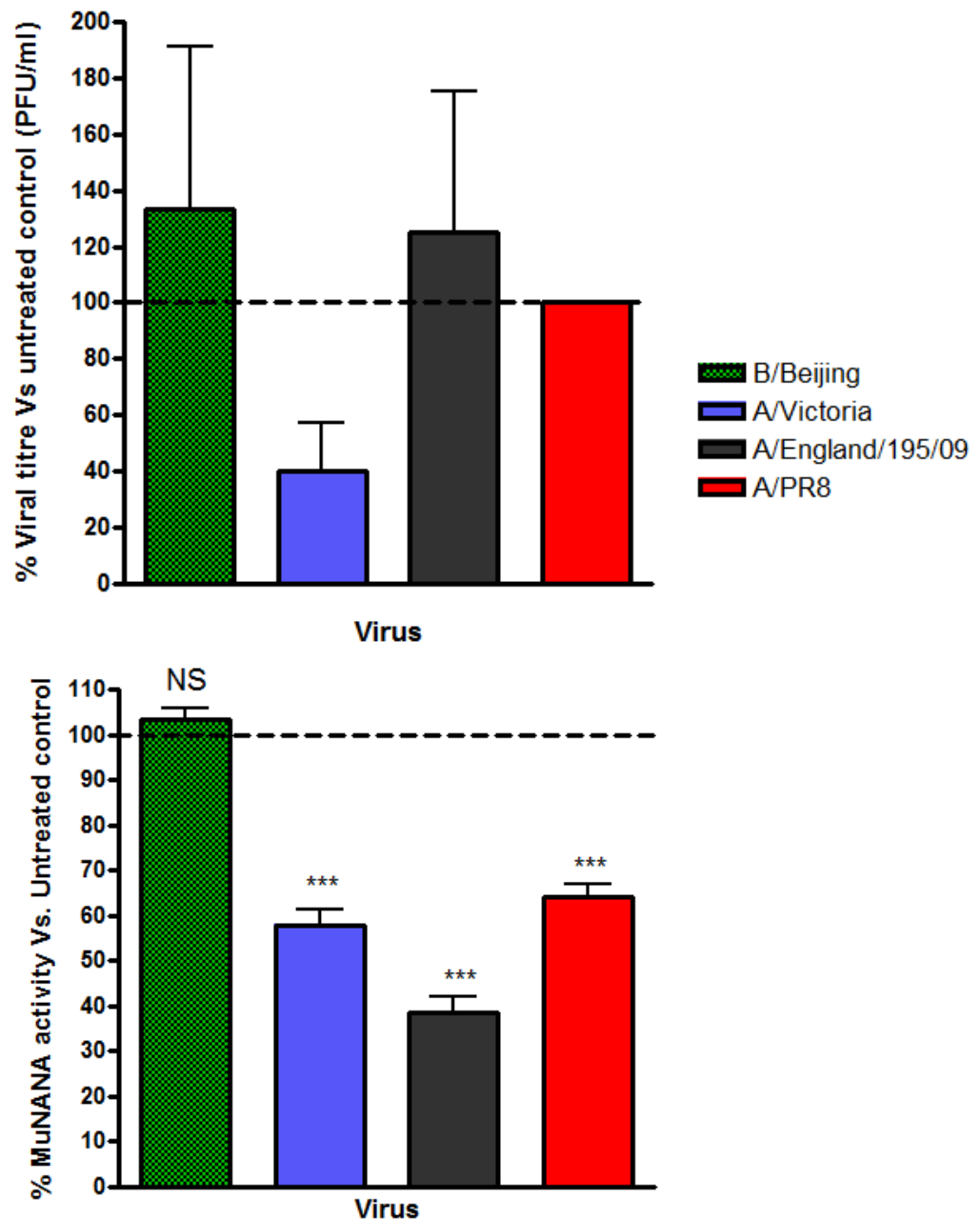


Figure 4. 6. The effect of Castanospermine on viral titre and neuraminidase activity of wild type viruses inoculated at a high MOI.

MDCK cells were infected at an MOI of 1.0 and allowed to replicate for 24 hours in the presence of 100 μ M Castanospermine, or drug vehicle. Following 24 hours infection,

supernatants were removed and subjected to plaque assay to determine viral titre **(a)**, or MuNANA assay **(b)** to analyse for NA activity as above. Data shown is the average of triplicate wells and error bars correspond to the standard deviation between these samples. A two-tailed unpaired student's t-test was used to calculate statistical significance between untreated and treated control groups with * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, NS = Not significant

4.2.4. Defining the basis of susceptibility to Castanospermine

Building on the observation that A/England/195/2009 virus was uninhibited at higher MOIs of virus, but suffered from a reduction in its ability to process the MuNANA substrate, we hypothesised that inhibition of neuraminidase transport to the surface of the cell was likely a general effect of the drug on influenza, but that some virus were tolerant of such an inhibition. To test whether Castanospermine directly affected the total number of NA molecules that reached the surface of the cell, we used a FLAG-tagged neuraminidase construct and transfected it into 293T cells. Three hours post-transfection, the medium was removed and replaced with medium containing either 100µM of drug, or the equivalent DMSO containing control. Gene expression was then allowed to proceed overnight before cells were immunostained and analysed by FACS (Table 4.2.). Our results show that the quantity of FLAG-tagged NA particles which reach the cell surface (non-permeablised wells) was not affected by the presence of Castanospermine. Furthermore, when comparing permeablised and non-permeablised cells for fluorescence, we saw no difference between drug treated and untreated cell populations, suggesting that the concentration of FLAG-containing NA proteins was not significantly altered in the presence or absence of drug either intra-cellularly or on the cell surface.

	Permeablised	Non-Permeablised
A/England/195/2009	97.9 %	98.7 %

Table 4. 2. Percentage of drug treated cells positive for FLAG-tagged NA in permeablised and non-permeablised cell populations as compared to an untreated control.

Triplicate wells of 293T cells were transfected with a plasmid containing the FLAG-tagged neuraminidase protein of A/England/195/2009 under the expression of a polymerase II promoter. Three hours post-transfection, medium was removed and replaced with drug- or vehicle-containing 2% DMEM, and cells were incubated overnight at 37°C. The following day, triplicate wells were detached from the plate, pooled and fixed using 2% paraformaldehyde. One group underwent permeabilisation with saponin, and then both groups were stained with a primary mouse α FLAG antibody, and secondary Goat α Mouse FITC antibody. Cells were then subjected to FACS analysis. Control wells containing untransfected cells, and transfected cells subjected to only secondary antibody treatment were used to subtract background readings from the final analysis.

4.2.3. Sequence analysis of influenza surface glycoproteins

With the knowledge that SMT014350 was the glycosidase inhibiting drug, Castanospermine, we then sought to identify a genetic basis for the susceptibility or resistance of influenza A or B viruses respectively to inhibition by the drug. Based on previous publications, it was assumed that the surface glycoproteins were the drug targets. Early research into Castanospermine observed effects on the HA protein of NWS virus, with drug treatment preventing haemagglutinin from associating with host-cell proteins, resulting in premature folding and oligomerisation which led to overall less efficient maturation (Hebert *et al.*, 1996). However, work by Saito & Yamaguchi suggested that the NA protein was more susceptible to Castanospermine inhibition, using a reassortant H1N8 virus (Saito & Yamaguchi, 2000). We performed a bioinformatic analysis to see whether the glycoproteins of the susceptible influenza A virus strains differed in the number of potential glycosylation sites from those of the relatively resistant influenza B virus strain used here (Table 4.3.). N-linked-glycans are attached to glycoproteins at specific amino acid residues which contain the consecutive code Asn-X-Ser (NxS) or Asn-X-Thr (NxT), with X forming any amino-acid other than proline. These glycosylation sites are known as sequons, and serve to highlight locations on the protein which can potentially undergo N-linked-glycan attachment. Generally speaking, the more sequons contained in a protein, the more highly glycosylated the protein. We noted from our genetic analysis that the Victoria HA and NA proteins contained more consensus glycosylation sites than the HA and NA of PR8 or of influenza B Beijing, both of which appeared relatively resistant to the drug.

	NxS	NxT	TOTAL
A/Victoria HA	2	7	9
A/Victoria NA	3	6	9
PR8 HA	4	3	7
PR8 NA	2	3	5
B/Beijing/1/87 HA	1	5	6
B/Beijing/1/87 NA	2	2	4
A/England/195/2009 HA	3	5	8
A/England/195/2009 NA	3	3	6
A/Chicken/Italy/13474/99 HA	0	6	6
A/Chicken/Italy/13474/99 NA	3	1	4

Table 4. 3. Number of potential glycosylation sites in the glycoproteins of our test viruses

As a final validation of the reporter cell line, developed in the previous chapter, we sought to show whether the spectrum of influenza A viruses tested above by traditional means would be inhibited by Castanospermine in our mCherry Red/358-GFP expression model of virus replication. The previous chapter characterised A/Victoria alone as the sole driver of GFP expression, but once attempts were made to use other viral isolates under the same initial MOI infection conditions, it was observed very low GFP expression across the range, except for A/Victoria virus (data not shown.) To overcome this, cells were infected with an initial MOI of 2.5 instead of 0.1 used previously and left to replicate in the presence of low concentrations of Trypsin (0.3µg/ml). Given that the viral promoter associated with these reporter plasmids is influenza A-specific, Influenza B was excluded from this assay.

When using this high MOI, it was questionable whether we would see any sufficient knockdown given the generally low potency of Castanospermine observed in figure 4.2 and also given that a drug that inhibited glycosylation of nascent surface glycoproteins should only show inhibitory activity on viral titres accumulated over multiple cycles of infection. However, as the data presented in Figure 4.6. shows, all influenza viruses, with the

exception of RD3, were inhibited in their ability to drive GFP expression. However, only for A/Victoria and A/PR8 containing Victoria HA or HA and NA, did these results reach statistical significance (Figure 4.7.). The overarching pattern of the results in this chapter suggests that A/Victoria is much more sensitive to the presence of Castanospermine than A/PR8, and indeed when a recombinant PR8 virus is supplemented with the viral surface proteins of Victoria, it becomes more susceptible to inhibition by the drug. It is possible that this is due A/Victoria surface glycoproteins being more highly glycosylated and thus more likely to be caught in an aberrant cell pathway if proper glycoprotein processing and transport is inhibited by the drug.

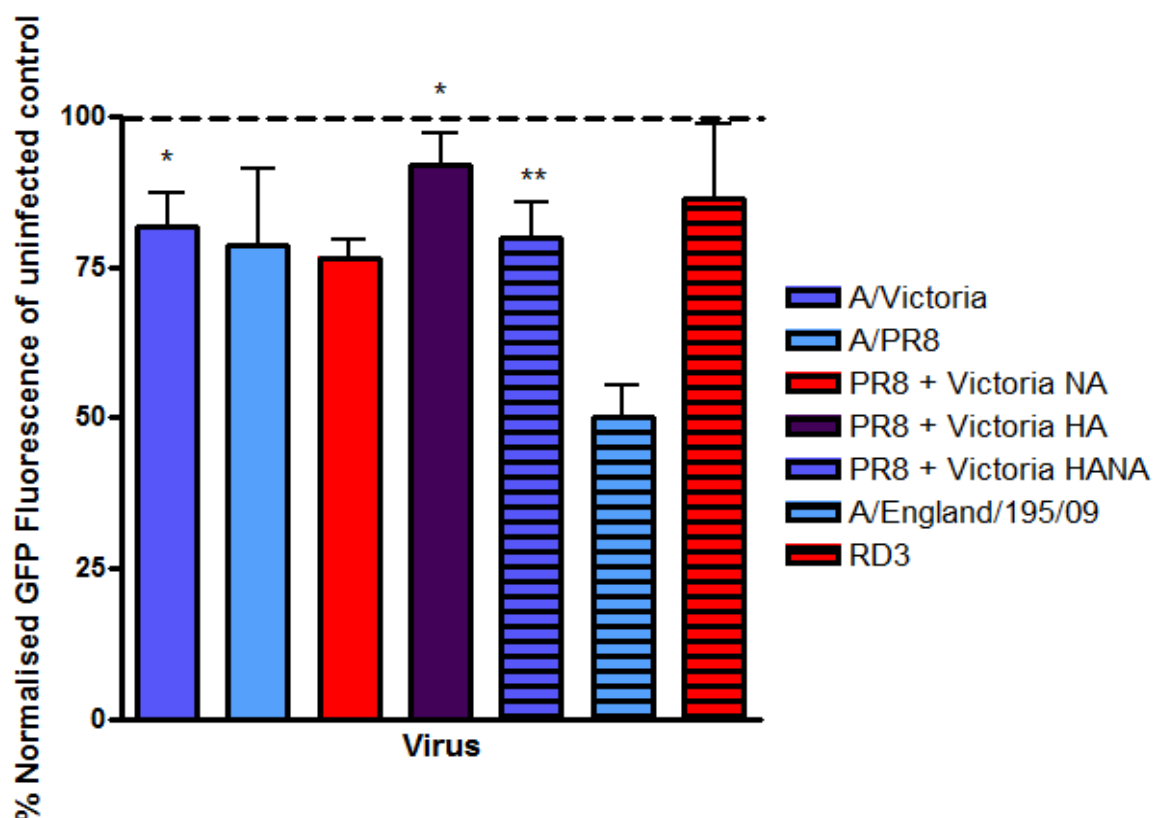


Figure 4. 7. Ability of a panel of Influenza A viruses to drive expression of GFP under control of a viral promoter.

Black 96-well plates with translucent bottoms were seeded with 293T cells, and transfected with mCherry Red and 358-GFP plasmids overnight. Medium was then removed and viruses inoculated at an MOI of 2.5 for one hour. Medium was then replaced with SF DMEM (not containing phenol red) containing either 100 μ M of drug, or the drug vehicle, and 0.3 μ g/ml Trypsin. Cells were incubated at 37°C/5% CO₂ for three days before red and green signal was read. Data presented shows the average of quadruplicate wells, where the GFP signal has been normalised to Cherry Red signal. Data is expressed as the average percentage of untreated wells. Background wells were set up and values obtained from these wells were subtracted from the green and red values. A one-tailed, unpaired student's t-test was undertaken to assess statistical significance with * = $p < 0.05$ and ** = $p < 0.01$. Where not stars are present, differences were not statistically significant.

4.3. Discussion

This chapter began with the knowledge that three compounds from the Summit iminosugar library protected MDCK cells from cytopathic destruction by A/Victoria virus. Attempts were made to elucidate whether the protective effects of these three compounds extended to an influenza B virus, though it was found that none of the drugs inhibited multicycle replication of this virus. Once these findings were relayed to Summit Plc., it was revealed that two of the drugs had known biological and antiviral activity already. Compound SMT14350 was Castanospermine, whereas SMT014453, which shared the same molecular weight, was a derivative form of Castanospermine, Celgosivir. Of these two drugs, Castanospermine could be purchased commercially and thus formed the basis of future experiments which sought to further address the affectivity of the drug against a range of influenza A viruses. In choosing Castanospermine, the most potent drug of the initial three hits, SMT014453 (Figure 4.2.) was lost from further characterisation studies. However, Summit Plc were not forthcoming with the molecular classification of this drug, nor did they make available future stocks for further academic study.

Castanospermine (Figure 4.8.) is naturally found in chestnut trees (*Castanospermum australe*) and can be easily purified and isolated in large quantities. The drug acts as an inhibitor of the α -glucosidase I enzyme in the ER and has been shown to interfere with replication of an array of viruses *in vitro*, including HIV-1 (Montefiori *et al.*, 1988) and Dengue (Courageot *et al.*, 2000), whereas Castanospermine derivatives such as Celgosivir have shown activity against the surrogate HCV model, BVDV (Whitby *et al.*, 2004). Castanospermine has also shown *in vivo* activity against HSV (Bridges *et al.*, 1995), Dengue (Whitby *et al.*, 2005) and MLV (Ruprecht *et al.*, 1989). Moreover, Castanospermine has been utilised in combination with other established therapies with some success. Furthermore, other iminosugars which inhibit α -glucosidase enzymes, such as

Deoxynojirimycin (DNJ) have shown potency against a similar wide range of viruses. When added in combination with interferon and ribavirin, DNJ showed potent activity against BVDV in MDBK cells and resulted in the permanent eradication of virus from the system, as opposed to ribavirin and interferon administered as a dual therapy (Woodhouse *et al.*, 2008). When used in combination with amantadine, DNJ also achieved a reduction in viral titres of the BVDV infection model (Steinmann *et al.*, 2007). Taken together it would appear that inhibition of the enzymes affiliated with N-linked glycosylation could form the basis of broad-spectrum antiviral therapy.

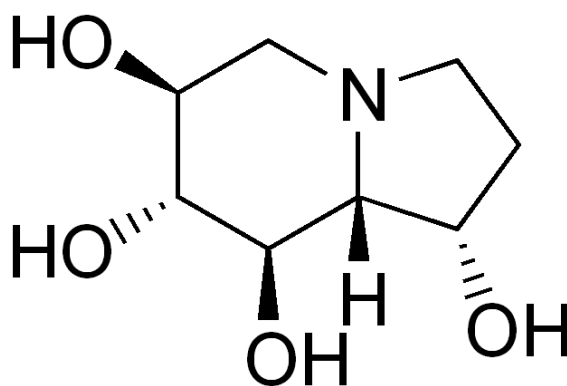


Figure 4. 8. Structure of Castanospermine (Image: creative commons)

It was observed immediately that an Influenza B isolate appeared less susceptible to the effects of Castanospermine treatment than did an Influenza A isolate. The finding that similar viruses adopt selective sensitivity to the drug has been shown by Whitby and colleagues, who credit the drug for inhibiting four different dengue virus serotypes, whereas the other flavivirus species Yellow Fever Virus and West Nile virus were partially and completely immune to the effects of the drug respectively (Whitby *et al.*, 2005). It was then observed that the more numerous glycosylation sites on the surface glycoproteins of A/Victoria virus may account for the apparent increased sensitivity of the virus to Castanospermine, and the relative paucity of sequons for B/Beijing, may explain its

resistance. However, it is not a given that all sequons are glycosylated during protein processing nor that the effect of inhibiting one sequons is equal to the effect of inhibiting another. For example, although the surface glycoproteins of RD3 virus shared equivalent numbers of potential glycosylation sites to B/Beijing, mean viral titres for this virus were reduced in cell culture. We performed the bioinformatic analysis of surface glycoproteins after we had chosen the viral strains to examine, to observe whether resistance and susceptibility mapped to the number of sequons associated with each glycoprotein. However, it remains to be seen whether drug susceptibility could actually be 'predicted' on the basis of the number of glycosylation sites contained within a protein. Future work with this experiment could incorporate viruses which contain more heavily and more sparsely glycosylated glycoproteins at the surface to see if there is a true map between sequons and susceptibility.

It has been reported in the literature that some cell lines encode for an intracellular enzyme capable of cleaving sugar-side chains that would otherwise remain uncleaved by Castanospermine inhibition. That is to say, viral glycoproteins could undergo proper glucose residue trimming thanks to the auxiliary enzyme endomannosidase, which can perform an identical job to glucosidase I, but is not inhibited by Castanospermine (Karaivanova *et al.*, 1998). Although MDCK cells are thought to express this enzyme, we saw clearly that some viral strains retained susceptibility to this drug. However, one may speculate that the folding and transport of Influenza A and B viruses occur in different manners, and indeed it may be possible that Influenza B glycoproteins spend a greater amount of time in the Golgi and thus has a greater chance to interact with this 'rescuing' endomannosidase.

The drug was only significantly effective at low doses of challenge virus, but did inhibit the replication of the pandemic A/England/195/09 isolate. Although transport of the viral NA

protein to the cell surface was not directly affected by Castanospermine treatment, the function of this protein in egressed virions was. This may suggest that some viruses are less concerned with the concentration of functional NA in their virion than others, or that the drug affects the budding or morphology of the virus particle irrespective of the actual amount of NA at the cell surface, perhaps by affecting HA transport instead. This observation could be relevant when assessing virus susceptibility to oseltamivir. The traditional NA mutation which confers resistance to oseltamivir, H275Y, is known to result in a natural attenuation of some influenza viruses, presumably due to a loss of NA functionality (Ives *et al.*, 2002). If viruses exist which can tolerate attenuations in overall NA activity that do not adversely affect overall virus titre, then one may speculate that these viruses may be primed to include mutations such as H275Y in their neuraminidase proteins without significant detriment to overall fitness.

Future work should seek to elucidate whether other influenza B isolates are also resistant to the effects of Castanospermine. Furthermore, it has not been possible to map drug sensitivity to a particular glycoprotein. It would be prudent to repeat the FACS analysis to observe the difference in HA transport to the surface under the presence of Castanospermine, and indeed to perform the analysis on cells undergoing whole virus infection as opposed to transiently transfected proteins. Suitable reagents for this were not available at the time of study. It was decided to look at viruses with different budding morphologies to assess whether the shape of the final virion would be more or less susceptible to Castanospermine treatment, however our virus panel contained different numbers of glycosylation sites between the proteins. To attempt to map drug sensitivity to morphology, we should analyse matched virus pairs that have identical numbers of sequons in their surface glycoproteins, but which still bud with distinctly different morphologies. Such viruses have been described in (Elleman & Barclay, 2004). Although overall the observed potency of Castanospermine was seen to be low in this experiment,

the observed broad-spectrum activity of the drug against a range of other viruses, and the fact that the drug targets a host-cell process suggests it would be worthy to conduct follow up experiments. The structure of Castanospermine also allows for significant chemical modification which could generate derivatives that possess greater active site selectivity, which may result in more potent antiviral activity (Aguilar-Moncayo *et al.*, 2009).

Chapter 5. Assessing the effects of the neuraminidase inhibitor resistance mutation H275Y on the neuraminidase protein of pandemic influenza H1N1 2009

5.1. Introduction

March 2009 saw the emergence of a novel strain of influenza with the ability to transmit readily between humans. This isolate was rapidly identified as an A/H1N1 virus and was comprised of a combination of gene segments previously undescribed in both humans and swine and included a neuraminidase gene derived from a Eurasian lineage swine virus that itself had crossed from birds in Europe into pigs in the late 1970s (Garten *et al.*, 2009). The novel influenza virus went on to cause the first influenza pandemic in over 40 years (Fraser *et al.*, 2009). Two therapies are currently licensed to treat influenza infections: the adamantane drugs (amantadine and rimantadine) and the neuraminidase inhibitors (oseltamivir and zanamivir). Over recent years, anticipation of an impending pandemic has prompted many countries to stockpile antiviral drugs as part of preparedness plans intended to minimise the impact of a pandemic influenza outbreak (Meijer *et al.*, 2007). The neuraminidase inhibitor drug, oseltamivir, has been the focus of stockpiling strategies due to its convenient oral formulation, superior bioavailability and suitability for use amongst paediatric and elderly populations. Pandemic influenza A/H1N1 2009 virus crossed into humans carrying a well characterised amantadine-resistance mutation, S31N, within the M2 ion channel protein, rendering the adamantane class of antiviral drug ineffective against the virus. Thankfully, initial isolates were susceptible to neuraminidase inhibitors, including the heavily stockpiled oseltamivir and consequently this drug was used extensively in the treatment and prophylaxis of pandemic influenza (Centre for Disease Control and Prevention (CDC), 2009). Oseltamivir resistance has emerged infrequently in pandemic A/H1N1 2009 influenza viruses (Baz *et al.*, 2009; Chen *et al.*, 2009). In contrast,

seasonal H1N1 influenza viruses from the 2007/2008 season onwards were predominantly resistant to oseltamivir with resistance conferred by the H275Y mutation in the viral neuraminidase (Meijer *et al.*, 2009).

Traditionally, the H275Y mutation was associated with a compromise in viral fitness amongst H1N1 isolates (Ives *et al.*, 2002). However, isolates from the 2007/2008 season with this mutation suffered no attenuation (Baz *et al.*, 2010). It is likely that certain other sequence variations, such as the D344N change found within the NA gene of oseltamivir-resistant viruses from the 2007/8 season, counteract the decrease in enzyme function that H275Y confers (Collins *et al.*, 2009; Rameix-Welti *et al.*, 2008). Residue 344 is a tyrosine in most avian influenza NA genes but mutated to aspartic acid in the N1 and N2 NAs of the 1918 and 1957 pandemic viruses during their circulation in humans. A mutation to asparagine occurred in circulating human H1N1 viruses prior to the 2007 season and led to increased NA activity. The NA enzyme of the novel swine origin 2009 H1N1 pandemic virus is derived from the avian virus-like Eurasian lineage H1N1 swine virus, and harboured asparagine at residue 344 at the time it crossed into humans, suggesting it may tolerate mutations such as H275Y that would concomitantly decrease NA activity and confer oseltamivir resistance.

Given the importance of the neuraminidase inhibiting drugs to pandemic preparation, their effectiveness is routinely monitored *in vitro* in many countries across the globe. Generally, influenza isolates are assayed for sensitivity to neuraminidase enzyme inhibition by NAIs using a 'MuNANA' assay, in which the ability of NA to cleave a monomeric, sialic-acid like substrate into fluorescent product is measured. The use of this fluorometric assay has led to the identification of several resistance mutations in the neuraminidase enzyme, as scored by an increase in the IC₅₀ of the NAI. Many of these are recorded by the Neuraminidase Inhibitor Susceptibility Network (NISN, 2010). However, this assay gives no

inclination as to whether a virus has the potential to adopt a resistance mutation, nor does it analyse the effects of a resistance mutation on viral growth kinetics. In this chapter the effect *in vitro* of the H275Y mutation in the NA of a prototypic A/H1N1 2009 isolate was assessed.

5.2. Results

5.2.1. Generating the H275Y mutation in the neuraminidase protein of a pandemic isolate

The representative pandemic isolate used in this study was A/England/195/2009, one of the first fully sequenced new variant (nv) A/H1N1 2009 influenza isolates in the UK. The eight segments of A/England/195/2009 were synthesised individually and cloned into a custom vector that flanked them with a pPol.I (virally-driven) promoter and a ribozyme, by GeneArt AG, in order to constitute a reverse genetics system for the virus. The plasmids for each segment were designed by Dr. Lorian Hartgroves (Imperial College London). The H275Y mutation required a single nucleotide change in the neuraminidase protein, which was then introduced using site-directed mutagenesis. Two viruses containing all seven 'backbone' segments of a laboratory-adapted influenza strain A/Puerto Rico/8/34 (PR8), and one of either the wild type neuraminidase gene of A/England/195/09, or the NA gene which contained H275Y were rescued for the first set of experiments described here. The decision to rescue a pair of isogenic viruses using an established PR8 body was made in order to circumvent stringent containment protocols which were in place during the initial stages of the pandemic that required wild type virus to be handled in CL3 facilities. It also gave the opportunity to study a completely uncharacterised protein in the well characterised PR8 system that would likely guarantee successful rescue and replication in cell culture. Both viruses were successfully rescued using previously described methods (Figure 4.4, (Elleman & Barclay, 2004)) and after a single passage, replicated to identical titres (Figure 5.1a.). These viruses were then assayed using a multi-cycle growth curve to

look for differences in viral growth kinetics. Both the wild type neuraminidase- and mutant neuraminidase- containing viruses displayed identical growth kinetics over 72 hours (Figure 5.1b.). From these early assays it was concluded that the presence of a H or Y at position 275 had no effect on the overall fitness of virus with pandemic origin NA gene *in vitro*.

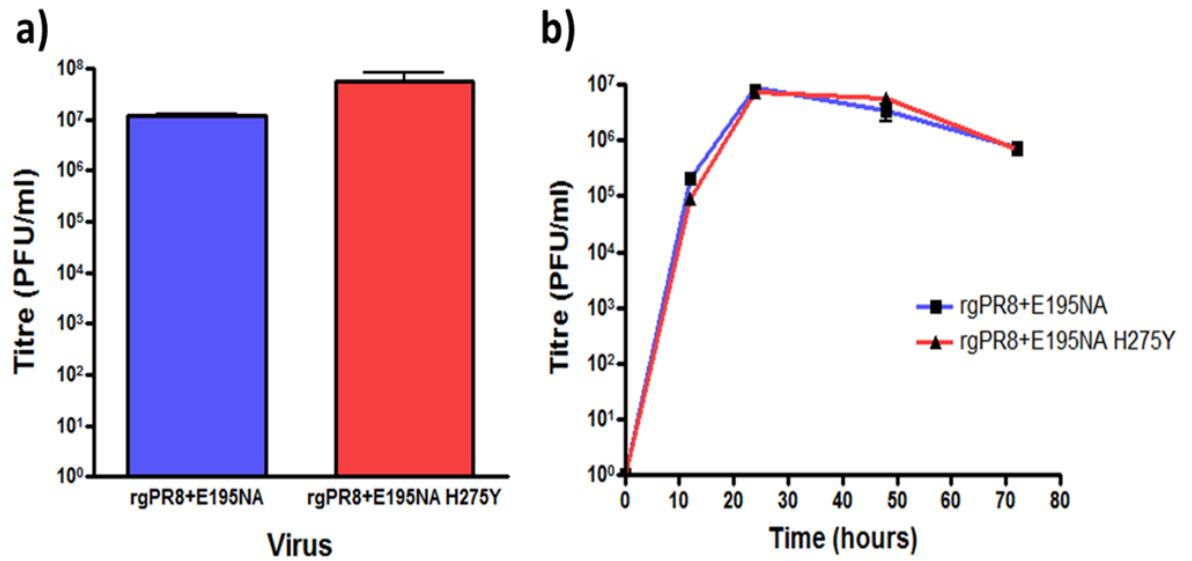


Figure 5. 1. Initial titre and growth kinetics of rescued recombinant viruses.

Two isogenic viruses, differing only at position 275 in the neuraminidase, were rescued using conventional techniques and assayed for differences in viral replication. **a)** Both viruses rescued successfully and when passaged, replicated to equivalent titres. **b)** The viruses were then assayed on MDCKs using a multi-cycle growth curve at a starting MOI of 0.1, and displayed identical growth characteristics to one another. Data shown is the mean of triplicate samples, with error bars corresponding to the standard deviation of the mean.

5.2.2. Assessing the effect of H275Y on NAI tolerance and the sialidase activity of A/England/195/09 neuraminidase.

The sialidase enzyme properties of both wild type and the mutant H275Y NA were assessed using two separate techniques. Firstly, a fluorescent substrate, 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MuNANA), was used as previously described (Potier *et al.*, 1979) to assess the ability of the neuraminidase proteins to catabolise a small monomeric substrate, and to consequently calculate the enzyme kinetics and sensitivity to NAI drugs of the two neuraminidase proteins. Then, to assess the ability of the NA enzymes to desialylate more complex, multivalent substrates, a chicken erythrocyte elution assay was established, whereby neuraminidase is required to cleave sialic acid from the abundantly sialylated cell surface of red blood cells. Using the MuNANA substrate, there was an approximate 300-fold increase in oseltamivir IC₅₀ values caused by the presence of Y275 (Table 5.1.). This mutation did not confer resistance against zanamivir. There was a 25% decrease in the binding affinity of the enzyme for its substrate resulting from the resistance mutation, as indicated by the difference in Km values. These results show that the H275Y mutation was able to confer resistance to oseltamivir in pandemic influenza isolates, and that the presence of Y275 slightly impeded the affinity of neuraminidase for its substrate. Also included in the MuNANA experiment was the human isolate of pandemic H1N1 virus, A/England/195/09, which presented comparable Km and IC₅₀ values to the rgPR8 + E195 virus. Both of these viruses contained the same NA gene which suggests that the PR8 reverse genetics backbone did not alter the neuraminidase activity in this assay. This MuNANA assay was carried out by Shah Miah at the Health Protection Agency in Colindale.

Next, equivalent titres of both viruses were titrated in a haemagglutination assay. Both viruses induced 'haemagglutination' (held erythrocytes in suspension) chicken red blood cells to a 1/128 dilution (Figure 5.2.). The plate was then incubated at 37°C, causing wells

containing higher titres of virus in which haemagglutination had previously been observed, to display ‘haemagglutination reversal’ (erythrocytes sediment out of solution). This is accounted for by the irreversible digestion of the sialic acid receptor from the erythrocyte surface by active NA enzyme. The ability of the wild type A/H1N1 2009 NA to elute virus from red blood cells was approximately 4-fold greater than that of the H275Y isogenic mutant (Table 5.1.). This suggests that neuraminidase activity in the oseltamivir resistant virus is compromised when compared to the wild type neuraminidase.

Substrate	MuNANA			Red Blood Cells	
	IC ₅₀ (nM) Oseltamivir	IC ₅₀ (nM) Zanamivir	K _m Value (μM)	Haemagglutination end point titre:	Desialylation end point titre:
Virus					
A/England/195/2009	0.4	0.1	48.35	ND	ND
rgPR8 + E195NA	1.1	0.8	49.7	1/128	1/128
rgPR8 + E195NA ^{H275Y}	294.4	0.6	64.9	1/128	1/16

Table 5. 1 The susceptibility of pandemic A/H1N1 2009 NA to antiviral drugs and activity against small and large substrates.

Reverse genetics viruses containing the NA from representative pandemic influenza H1N1 virus, A/England/195/2009, were assessed for their ability to catabolise mono- and multivalent substrates. The 2’-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MuNANA) substrate was used to determine IC₅₀ values against two neuraminidase inhibitors and assess enzyme K_m values. The red cell elution method is described below.

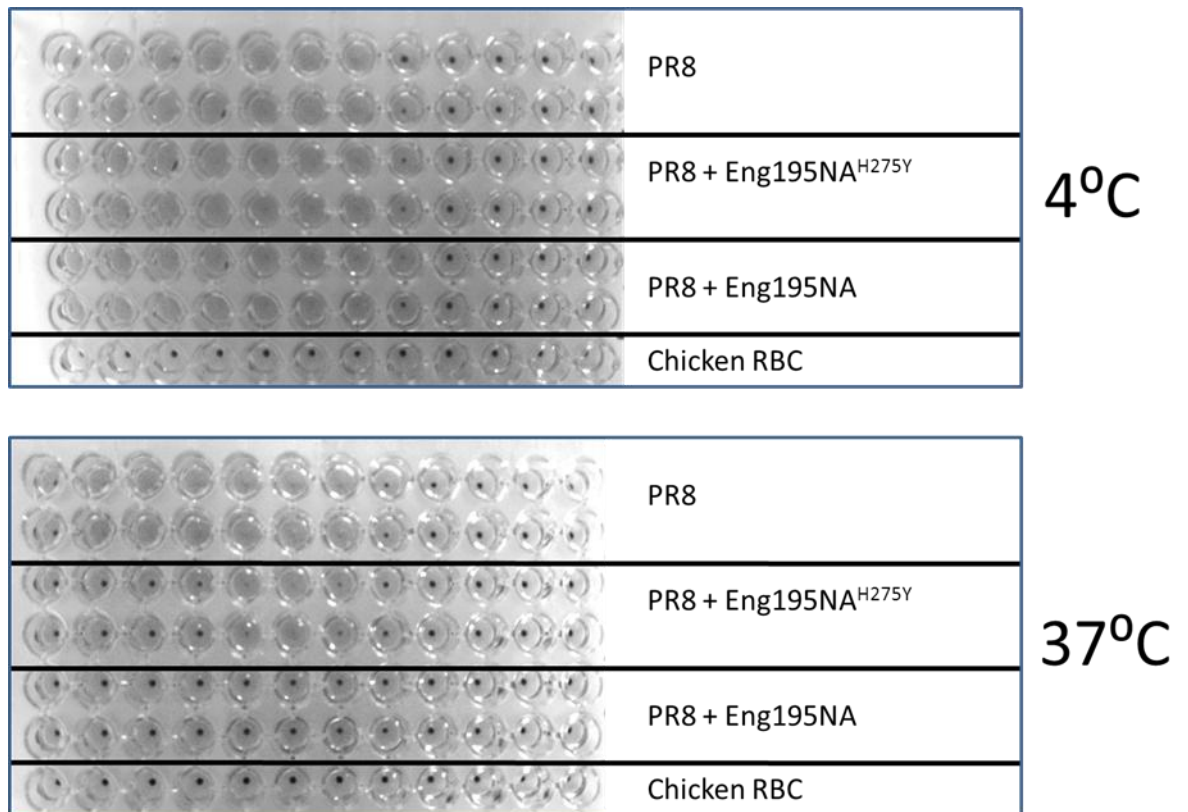


Figure 5. 2. Haemagglutination and 'reverse' haemagglutination ability of PR8-based viruses.

The haemagglutination activity of rgPR8+E195NA and rgPR8+E195NA^{H275Y} viruses were tested along with A/PR/8/34 virus as a control. All three viruses agglutinated chicken red blood cells to a titre of 1/128 units when left at 4°C for one hour (top panel). The plate was then moved to 37°C in order to increase neuraminidase activity. After one hour, agglutination had been eliminated by England 195NA as witness by the regression of agglutination from the left hand side of the plate to the right. England 195NA^{H275Y} did not regress across the plate as far as wild type A/England/195 NA.

Next, the effect of H275Y on oseltamivir tolerance in a cell infection assay, was assessed. The IC₅₀ values of NAIs calculated in the MuNANA assay do not translate to effective doses in an *in vitro* infection due to the more complex interplay between neuraminidase molecules and their substrates. The concentration of oseltamivir present in the overlay medium of plaque assays involving these two viruses was titrated to assess how much oseltamivir was required to abrogate viral infection. The results show that 100nM concentrations of oseltamivir were sufficient to eradicate viral replication of the virus containing drug-sensitive neuraminidase (Figure 5.3.). Drug-resistant variants were able to tolerate oseltamivir concentrations far beyond this, and finally succumbed to drug only when 100µM concentrations were used. The plaque morphology of each virus was also compared and even under a low dose (10nM) of oseltamivir was not noticeably different. Plaques of drug sensitive virus were totally abrogated at 100nM concentrations of oseltamivir in the overlay. The finding that pandemic neuraminidases containing H275Y can tolerate up to 1000-fold higher concentrations of drug *in vitro* is likely to have major clinical repercussions. The concentration of oseltamivir reached in circulation in patients undergoing treatment is estimated to be as low as 5µM (Fuke *et al.*, 2008), suggesting treatment of a resistant isolate with standard clinical dosing regimens will likely result in drug failure.

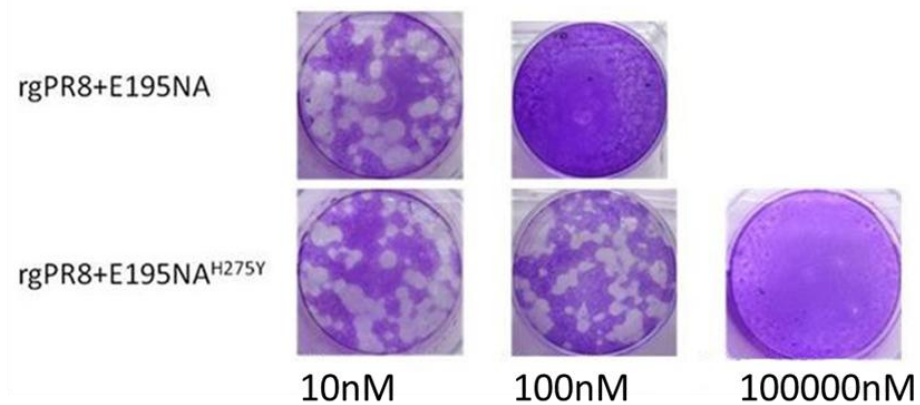
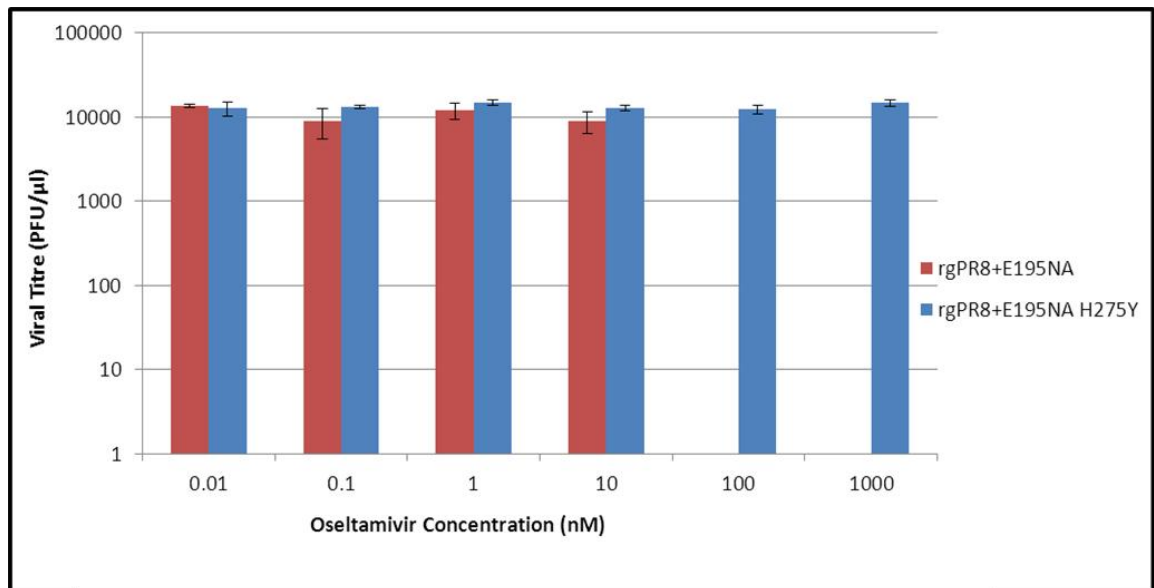


Figure 5. 3. The effect of osetamivir on plaque formation *in vitro*.

Plaque assays were performed on MDCK cells using rgPR8+E195NA and rgPR8+E195NA^{H275Y} viruses in the presence of increasing concentrations of osetamivir. MDCK cells were infected with concentrations of virus that were known to generate ~50 plaques in a 6 well plate and left for 3 days before being stained with crystal violet. Plaques were then counted and photographed. The histogram data represent the mean of three triplicate samples with the error bars corresponding to standard deviation between samples.

5.2.3. Assessing the effect of H275Y-containing neuraminidase in a recombinant pandemic strain.

Following the preliminary experiments conducted using a virus with a PR8 backbone, we moved to ascertain the effect of drug-sensitive and drug-resistant neuraminidases in the context of its other properly matched pandemic segments. When assessing the role of neuraminidase, it is important to account for the effects of the other surface glycoprotein, haemagglutinin, considering both proteins perform opposing jobs. The fact that HA is a virus receptor binder and NA is a receptor destroyer often results in evolutionary changes in these genes to accommodate reciprocal changes in the other (Kaverin *et al.*, 1998; Wagner *et al.*, 2002)). If pandemic 2009 N1 neuraminidase was weakened by the presence of H275Y, this weakness may be exacerbated when paired with a haemagglutinin protein whose activity is only usually tolerated by the whole virus due to a compensating highly active NA protein.

Using reverse genetics, two isogenic viruses were rescued comprising all eight segments of the A/England/195/09 pandemic isolate, differing only at position 275 in the NA. Both viruses successfully rescued and replicated to equivalent titres, as seen previously with our 7 + 1 recombinant viruses. The growth characteristics of these isogenic viruses in MDCK cell was not significantly different in either cell type at any time point, indicating that the small compromises in enzyme activity observed in Table 5.1. were not sufficient to result in significantly lower titres, even in the context of the entire pandemic virus genome (Figure 5.4. a)). These viruses were then assayed in their ability to infect and spread in highly differentiated Human Airway Epithelial (HAE) cells. The HAE cells represent a more difficult environment for viral replication than the laboratory standard MDCK cells due to the presence of stratified specialised cells (e.g. ciliated and non-ciliated cells) and due to the presence of mucus which is routinely secreted by goblet cells present in the 3D culture.

These cells are also maintained at an air-liquid interface, which provides further impediments to viral dissemination. Initially, cells were inoculated onto HAE cells or MDCK cells as control at low multiplicities and their growth kinetics compared side-by-side (Figure 5.3.) Although the mutant virus (dashed line) appeared to replicate less efficiently, particularly in HAE cultures, these differences were not statistically relevant.

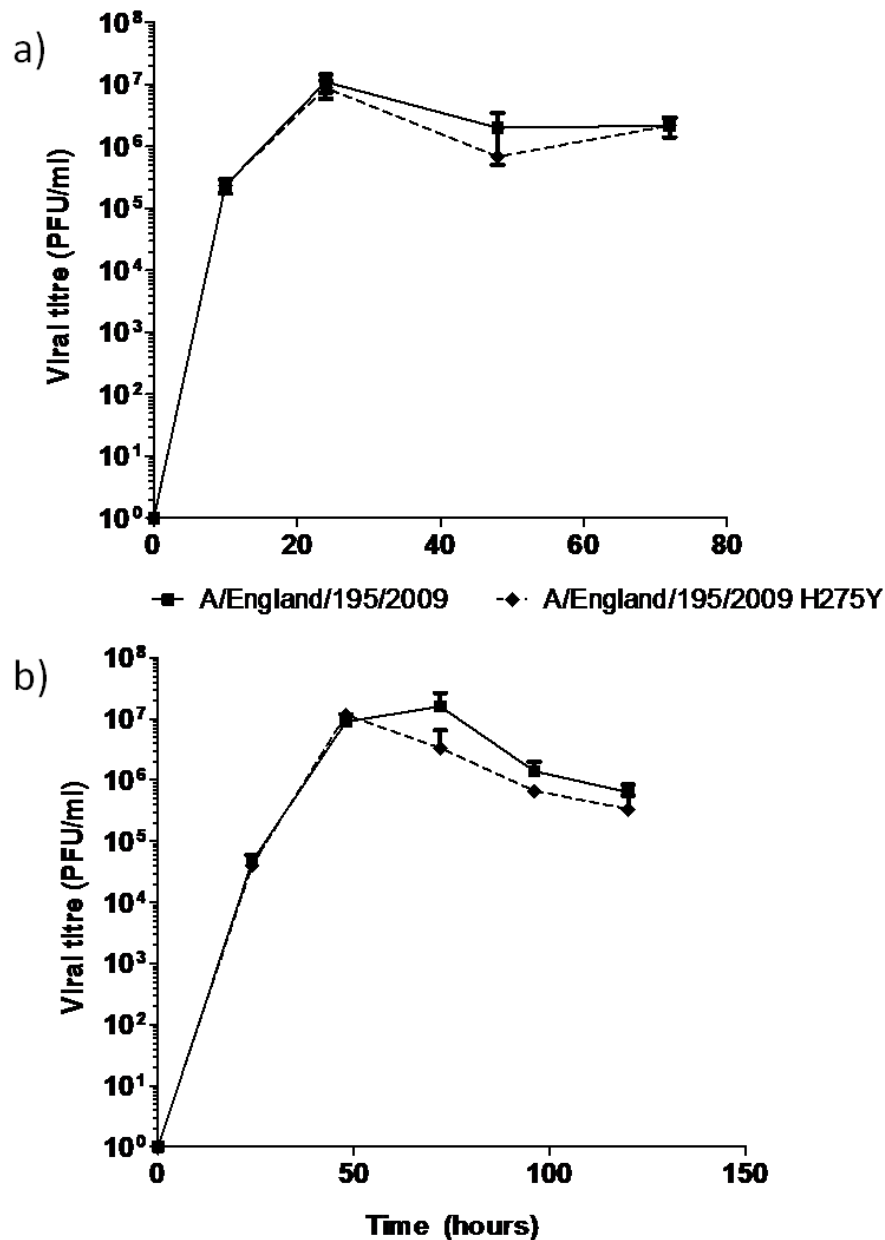


Figure 5. 4. Growth of drug-sensitive and drug-resistant pandemic influenza in MDCK and HAE cells.

Recombinant A/England/195/2009 viruses containing wild type or mutated H275Y NA segments were inoculated onto cells. **a)** Viruses were inoculated in triplicate onto 6-well plates of MDCK cells at an MOI of 0.01. Samples of supernatant were collected at various time points and then subjected to plaque assay on new MDCK cells to assess viral titre. **b)** Viruses were inoculated in triplicate onto the apical surface of HAE cells suspended on

transwell inserts on a 24-well plate for one hour at an MOI of 0.01 before being removed and washed once with PBS. Cells were left at an air/liquid interface for the remainder of the experiment and virus released from the apical surface at various time points was collected by adding 'virus diluent' (0.35% BSA, 1% Penicillin/Streptomycin in PBS) to the cells for 5 minutes. These virus harvests were then used in a plaque assay on fresh MDCK cells as above. Each of these figures is representative of data obtained in triplicate on 2 separate occasions.

5.2.4. Co-infections of drug-sensitive and drug-resistant isolates

Both viruses were then used to co-infect either MDCK or HAE cells at low MOIs and with the two viruses at defined ratios. Following a 72 hour infection, virus released was collected, the RNA extracted, and then used to produce cDNA of the neuraminidase segment which was sequenced. Analysis of the sequence chromatograms showed that in MDCK cells, there was no growth advantage for virus with either H or Y at residue 275. The output sequence exactly reflected the input ratio of the two viruses (Figure 5.5. a and b). In contrast, after 72 hours propagation in HAE cells where mucus could accumulate, the wild type oseltamivir-sensitive virus with 275H NA accumulated to a higher degree than did the drug resistant variant (Figure 5.5 c).

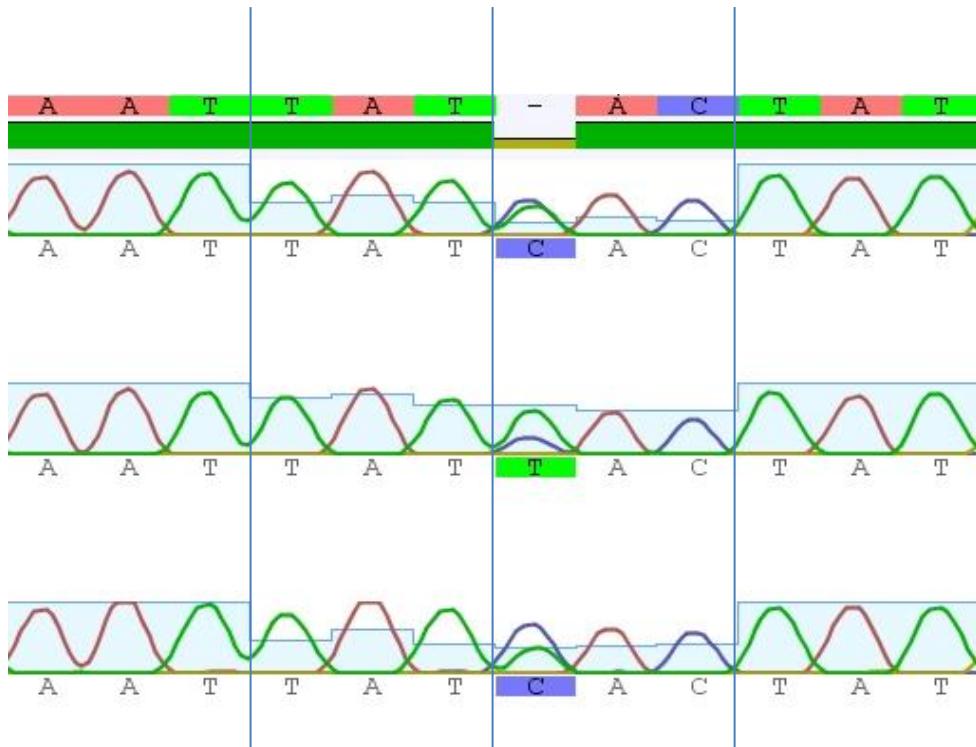


Figure 5. 5. Sequencing chromatogram of co-infection experiment

MDCK or HAE cells were infected at a total MOI of 0.01 with defined mix (50:50) or (80:20) of the isogenic A/England/195/2009 viruses with either 275H or 275Y in NA. After 72 hours virus released from the cells was collected, the RNA extracted and the NA gene amplified by RT-PCR. PCR products were then sequenced. The sequence chromatogram shows the region spanning codon 275 in which C nucleotide results in the wild type 275H amino acid and T nucleotide results in the drug resistant 275Y genotype.

- a) Virus released from MDCK cells infected with a 50:50 mix of 275H and 275Y virus.
- b) Virus released from MDCK cells infected with an 80:20 mix of 275Y and 275H virus.
- c) Virus released from HAE cells infected with a 50:50 mix of 275H and 275Y virus.

This experiment was conducted in triplicate and all three triplicates were sequenced individually. The results shown are single wells chosen arbitrarily (the middle well from each triplicate was used) from each condition and is representative of all three wells. The

input virus was also sequenced from the 50:50 and 80:20 ratios and produced identical chromatograms to rows a) and b) in this figure (data not shown).

Next, an attempt was made to quantify the observed differences in these chromatograms by using a plaque assay to determine the ratio of drug resistant and drug sensitive viruses in the supernatants of co-infected wells. The supernatants from each condition were diluted equally to a set concentration and a plaque assay was performed in the presence and absence of oseltamivir. The concentration of oseltamivir used was known to inhibit all plaque formation from wild type drug-sensitive virus. From Table 5.2. we can assume that influenza viruses with the H275Y mutation are able to form plaques in the presence of this concentration of oseltamivir, but overall they form fewer plaques when subjected to 10 μ M drug treatment – in this case, 7 in the presence of oseltamivir, 12 in the absence. Thus where we would expect to see 100% drug resistant plaques present in the drug treated wells, we only see 55% of those plaques under 10 μ M oseltamivir selection. The key comparison in this table is between the 50:50 mixtures of viruses in MDCK and HAE cells. What we can see from the results is that even though this assay has under-scored the number of drug-resistant virus plaques, there is still a substantial difference between the supernatants derived from MDCKs compared to those derived from HAE cells. The number of drug resistant virus plaques obtained from the co-infection experiment on HAE wells was ~50% less than those counted from the same co-infection performed in MDCK cells (13 plaques from the HAE Vs 27 plaques from MDCK cells). Thus, this analysis suggested an enrichment of wild type virus after replication in HAE, as implied by the sequencing chromatogram above, but it was still difficult to enumerate the amount of different NAs from the co-infections.

Virus	- Oseltamivir	+ Oseltamivir (10µM)	% Resistant plaques
A/England/195/09	22	0	0
A/England/195/09^{H275Y}	12	7	55
50:50 MDCK	43	12	27
80:20 MDCK	27	16	57
50:50 HAE	14	2	13

Table 5. 2. Number of drug-sensitive and drug-resistant plaque forming units in the supernatant of co-infected cells.

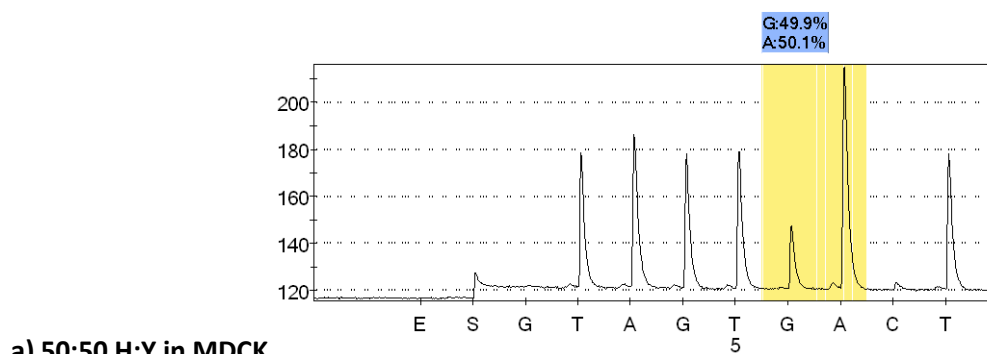
The cell supernatants from 5.2.4. were diluted 100,000-fold and a plaque assay was carried out in triplicate wells in the presence or absence of 10µM oseltamivir. Plaques were then enumerated 3 days post-infection and the average number of plaques is shown in the table.

To elucidate the total quantity of H275 or Y275 genomes present in the released virus from co-infected wells of MDCK and HAE cells more reliably, a pyrosequencing analysis of the supernatants was performed. Briefly, total RNA was extracted from the supernatants and subjected to the pyrosequencing methodology as described in (Lackenby *et al.*, 2008a). This assay directly quantifies the total number of neuraminidase strands containing either the drug-sensitive nucleotide C (shown as reverse complement – G in Figure 5.6.) or the drug-resistant nucleotide T (shown as reverse complement – A) in the NA protein. The percentage readouts of triplicate wells are given in Table 5.3. and a representative readout from this assay is shown in Figure 5.6. This data matches that obtained from the chromatograms presented in Figure 5.5 and highlights that whereas growth of drug-resistant and drug-sensitive virus in MDCK cells resulted in an even distribution of both neuraminidase genotypes, as seen by the 50.3:49.7 overall percentages of each, the replication of drug-resistant virus in the more clinically relevant HAE cell line was impaired, as shown by the 59:41% advantage conferred by H275.

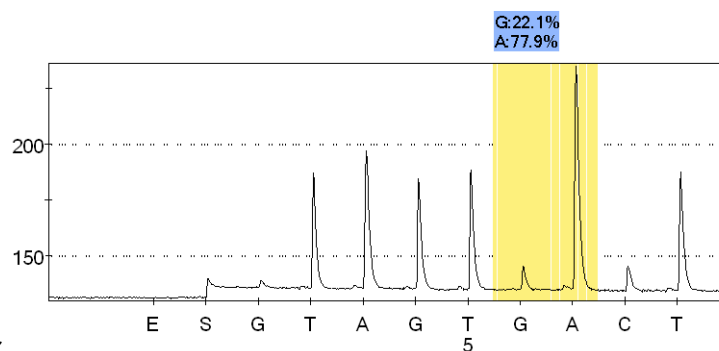
MDCK				HAE	
50:50 H:Y		80:20 Y:H		50:50 H:Y	
G	A	G	A	G	A
49.4	50.6	22.1	77.9	60.2	39.8
49.9	50.1	21	79	51.4	48.6
51.5	48.5	21.2	78.8	65.5	34.5
50.3	49.7	21.4	78.6	59.0	41.0

Table 5. 3. Pyrosequencing results of co-infected supernatants.

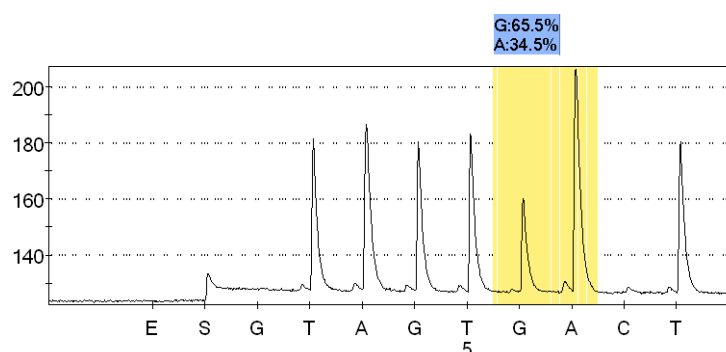
The individual results of triplicate samples are shown. The bottom row denotes the average of the triplicates.



a) 50:50 H:Y in MDCK



b) 80:20 Y:H in MDCK



c) 50:50 H:Y in HAE

Figure 5. 6. Pyrosequencing chromatogram results of co-infection supernatants

Each figure is representative of a single pyrosequencing run conducted on the co-infected supernatants of **a) 50:50 H:Y in MDCK cells**, **b) 80:20 Y:H in MDCK cells**, **c) 50:50 H:Y in HAE cells**. The highlighted yellow area shows the mixture and quantity of both drug-resistant (A) and drug-sensitive (G) nucleotide at this position. Note: The nucleotides shown here are the reverse complement of those shown in Figure 5.5. The pyrosequencing step for this assay was performed by Shah Miah at the HPA.

5.3. Discussion

The biological consequences of the H275Y mutation in the NA gene of A/H1N1 2009 influenza virus, that confers resistance to oseltamivir, are important to know because the drug is the first line treatment for patients who present with pandemic influenza infection. Drug resistance was already observed in infected individuals in the community and in the clinic during the first and second waves of the swine flu pandemic (Baz *et al.*, 2009; CDC, 2009; Chen *et al.*, 2009) although it did not spread widely. Whether oseltamivir-resistant nvH1N1 viruses might disseminate in subsequent waves through the community is key to future public health planning.

The NA gene of the new pandemic H1N1 virus was acquired from Eurasian swine influenza H1N1 virus, a lineage of virus that crossed from avian species into swine in the late 1970s, and retains some characteristics of avian virus NAs. For example, like many avian virus NA enzymes, A/H1N1 2009 NA is inherently more active than NAs of recently circulating human influenza viruses, for which elution of virus from red blood cells is rarely observed (for example, comparing PR8 wild type virus to both PR8 viruses containing pandemic NA in Figure 5.2., lower panel). In this background, the small compromise in enzyme affinity for sialic acid substrate (observed by a 1.3 fold increase in the neuraminidase K_m value) and the decrease in cell surface expression that result from the H275Y mutation (Bloom *et al.*, 2010), had no effect on virus growth in MDCK cell culture. On the other hand, *in vivo* NA must cleave complex substrates to mediate virus release from an infected airway cell and gain access through a complex layer of mucins to the new target cell. Subtle decreases in NA activity or cell surface expression may have more profound consequences in the airway than in monoculture. To probe this, we tested the NA activity of the mutated virus in two other assays that presented large sialated substrates. We detected a 4-fold compromise in the ability of the virus with H275Y mutation to mediate desialylation of chicken erythrocytes, although the biological significance of this assay is not entirely clear. In

differentiated cultures of human airway cells, the mutant virus showed a small, reproducible, but statistically insignificant decrease in infectious titre released at later time points of the multicycle infection. Importantly, in competition assays in HAE cultures, but not in MDCK monolayers, the wild type virus outcompeted growth of the drug resistant strain suggesting that in the absence of drug, the 275Y motif carries a fitness cost in the environment of the human airway.

In both recent seasonal H1N1 strains and in H5N1 highly pathogenic viruses, mutations that increase the NA activity, protein stability or cell surface transport likely compensate for the effects of the mutation at 275 that would otherwise decrease the function of the enzyme. Examples of such mutations are D344N, (Rameix-Welti *et al.*, 2008), and V235M and/or R223Q (Bloom *et al.*, 2010). Neither of the latter two mutations currently exist in the neuraminidase protein of A/H1N1 2009 isolates, but further circulation of the novel H1N1 virus in humans may select for these or other NA or HA mutations that better prime the virus to accommodate or even select for the H275Y mutation.

For contemporary A/H1N1 2009 viruses, the cost or advantage of drug resistance is so subtle that different groups have come to different conclusions about its relevance. In a hospital setting there have been reports that suggest patient-to-patient transmission of drug-resistant virus has occurred amongst immunocompromised individuals (Moore *et al.*, 2010). Hamelin and colleagues have recently shown that oseltamivir-resistant A/H1N1 2009 virus was as equally virulent as its wild-type counterpart in mice and ferrets and did transmit to co-housed animals (Hamelin *et al.*, 2010). Likewise, Seibert and colleagues showed that in two different viral genetic backgrounds the H275Y mutant was as virulent and as transmissible as the wild type virus in both ferrets and guinea pigs which are an alternative animal model for influenza transmission (Seibert *et al.*, 2010). Similarly Kiso and

co-workers found that two sets of oseltamivir resistant pH1N1 viruses were equally virulent in mice and ferrets as their wild type counterparts and transmitted equally well between ferrets even via the droplet route using a competitive assay whereby the animals were co-infected with a 50:50 ratio of the two viruses (Kiso *et al.*, 2010b). However, careful analysis of the data from Kiso shows that there was a two-day delay in transmission of the resistant virus, O180r, compared to its matched sensitive partner, suggesting some compromise that may translate into a substantial advantage to wild type virus spread in the community. In addition, Duan *et al.* found that their strain of drug resistant virus did not transmit between ferrets by the respiratory droplet route and that in co-infected animals, the wild type virus outgrew the resistant mutant and was uniquely transmitted to contact animals (Duan *et al.*, 2010). The difference in results and interpretation of data between these groups highlights that within this very active field of experimental research, environmental conditions and both host and strain specific effects may influence the outcome of experiments where fitness costs are minimal. Factors such as the distance between exposed animals, the relative humidity and temperature within the facility (Lowen & Palese, 2009) and the genetic background of the virus may all vary. Indeed table 5.3 shows the genetic differences between some of the strains used in these studies. Sequence information on other strains was not available for analysis. Although it is difficult to discern from table 5.3. a particular amino acid that may affect the results, the comparison does serve to show that there are many differences in the viral genetic background of the strains used that could affect the results. The discrepant conclusions from the plethora of recent papers on this subject also suggest that animal models, even the ferret 'golden standard', that may be optimized to detect transmission of influenza virus, may not be ideal as currently devised to discern subtle differences between strains. It may be that in future adaptations of these assays, competition assays involving mixed viruses, lower inoculum doses, larger distances between cages and testing for chains of transmission will help refine the ferret model, to

that it may recapitulate the field situation more accurately. On the other hand we have presented here a simple *in vitro* system that is at least based on human airway cells rather than animal cells, and is amenable to a higher throughput and increased number of replicated if developed in the future. Finally the data in these papers is derived from very few animals, sometimes as few as 2 per group and may not adequately reflect the possibility that in the community small fitness costs may preclude dominance of a virus variant. Anecdotal evidence from clinical or epidemiological studies in humans shows that, in most instances, contemporary drug-resistant variants of A/H1N1 2009 were replaced by drug-sensitive variants when the selective pressure of oseltamivir was removed, suggesting that wild-type isolates are fitter *in vivo* in man (Campanini *et al.*, 2010). Likewise, under competitive conditions incorporating long incubations, during which mucus accumulated in the HAE culture system, we found that the wild type virus out-competed the drug resistant virus, in the absence of drug selection pressure. Interestingly, several recent papers have also favoured the use of competitive assays to discern viral fitness advantage between two strains of influenza virus in animal models (Duan *et al.*, 2010; Hurt *et al.*, 2010). The HAE culture system offers an alternative biologically relevant model as a useful adjunct to animal studies that may adequately reflect the environment in which virus replicates in healthy humans. Information from a variety of model systems should be combined to guide the appropriate use of oseltamivir. Such knowledge clearly needs to be revised specifically for each novel influenza virus that emerges either as a seasonal strain by drift or as a pandemic viruses by antigenic shift.

HA	Position:	49	169	220
	A/Quebec	L	V	T
	A/Denmark	L	V	T
	A/Osaka	L	I	S
	A/England	I	V	S

NA	Position:	106	248
	A/Quebec	I	D
	A/Denmark	I	D
	A/Osaka	I	N
	A/England	V	N

PB2	Position:	649	677	700
	A/Quebec	V	E	E
	A/Denmark	V	E	E
	A/Osaka	I	E	K
	A/England	V	G	E

NP	Position:	100
	A/Quebec	I
	A/Denmark	I
	A/Osaka	I
	A/England	V

PA	Position:	3	364	529	626
	A/Quebec	D	N	N	G
	A/Denmark	D	S	D	R
	A/Osaka	D	S	D	R
	A/England	G	S	D	R

PB1	Position:	189	353	667	730
	A/Quebec	G	K	I	F
	A/Denmark	R	K	I	S
	A/Osaka	R	K	T	F
	A/England	R	R	I	F

NS2	Position:	63		
	A/Quebec	Normal length (121)		E
	A/Denmark	Truncated to 112		E
	A/Osaka	Normal length (121)		K
	A/England	Normal length (121)		E

NS1	Position:	93	123
	A/Quebec	M	V
	A/Denmark	I	V
	A/Osaka	M	I
	A/England	M	I

M2	Position:	Identical
	A/Quebec	
	A/Denmark	
	A/Osaka	
	A/England	

M1	Position:	33	150	227
	A/Quebec	A	N	T
	A/Denmark	A	T	A
	A/Osaka	T	T	A
	A/England	A	T	A

Table 5. 4. Sequence analysis of viruses used by recent publications to date that assess drug-resistant pandemic influenza transmission.

(Table legend overleaf)

Sequence variation between drug resistant viruses used in some of the publications documenting pandemic influenza drug resistance to date: A/Quebec/141447/2009 (Hamelin *et al.*, 2010), a resistant mutant that did transmit to co-housed animals, though droplet transmission was not tested, A/Denmark/528/2009 (Duan *et al.*, 2010), a resistant mutant which showed compromised transmission, A/Osaka/180/2009 (Kiso *et al.*, 2010b), a resistant mutant which did transmit by respiratory droplet but showed a 2 day delay in sentinels becoming virus positive and A/England/195/2009 (this study), a resistant mutant which showed a compromise in fitness in competition studied performed on human airway epithelial cells.

6. General Discussion

6.1. Overview of work presented

The work presented in this thesis has dealt largely with two aspects of antiviral chemotherapy of influenza infections. Firstly, we highlighted the limitations and difficulties associated with traditional drug screening technologies and indeed the further elucidation of antiviral compounds which present from screening hits. Secondly, we have challenged the validity of relying on oseltamivir as the sole stockpiled drug in pandemic planning agendas by asking what consequences a common drug-resistance mutation would exact on the pandemic influenza virus.

Several antiviral compounds were discovered using a rudimentary drug screening technique which assayed cell monolayer protection as a successful indication of antiviral activity. Following the discovery that seven Phytopure compounds showed activity, substantial time was invested at the beginning of the project in attempting to identify their mechanisms of action. Buoyed by the knowledge that these hits did not destroy monolayer integrity alone, and displayed negative toxicity according to a commercial cytotoxicity assay, we proceeded with antiviral studies designed to specify the antiviral mechanism of action of these compounds. Ultimately, it was observed that antiviral activity was attributable to wholesale inhibition of gene expression in the host cell, a distinctly undesirable trait for a potential therapeutic drug intended for treating the majority of influenza infections that are usually mild and self-limiting. We successfully attempted to overcome the limitations associated with our primitive screening technique by generating a novel screening tool, which has the potential of industrial scale-up for high throughput screening, and possesses the capability of identifying compounds which exhibit inhibitory

toxicity towards host-cell polymerases. Whilst in the future it would be prudent to subject novel screening hits to a battery of different cytotoxicity assays before embarking on further characterisation studies, it was heartening to discover that our cell screening technique identified immediately the cytotoxicity associated with our most potent Phytopure library hit.

The year 2009 provided a unique opportunity for influenza virologists to experience firsthand the outbreak and consequences of an influenza pandemic. As was widely expected, the principal prophylactic measure of seasonal vaccination did not protect against this new virus and whilst work commenced in generating a new specific vaccine which would protect against pandemic H1N1 influenza, the antiviral drug oseltamivir was relied upon extensively as the only widely available preventative and treatment measure, (reviewed in (Hayden, 2009)). Prior to the pandemic outbreak, H1N1 viruses from the 2007/2008 season extensively acquired resistance to oseltamivir, with 90-100% of isolates containing the molecular resistance signature mutation H275Y by the beginning of the 2008/2009 season (Correia *et al.*, 2010; Meijer *et al.*, 2009). Although the novel pandemic influenza strain was also of H1N1 phenotype, this virus did not harbour H275Y, or any other oseltamivir resistance mutation and remained fully susceptible to the drug (Centre for Disease Control and Prevention (CDC), 2009). We identified that a representative pandemic isolate could replicate with the H275Y mutation engineered into its neuraminidase protein without significant detriment to its overall viral fitness. However, we also observed minor impediments in neuraminidase activity, and in the ability of drug-resistant virus to compete with drug-sensitive virus, in a biologically relevant cell line.

It is highly likely that antiviral drugs will continue to form an important arm of our influenza defences long into the future. Although vaccine technologies continue to evolve (reviewed

in (Horimoto & Kawaoka, 2006; 2009)), humanity's biggest successes through vaccination have been against viruses which have a narrow host-range, such as Polio and Smallpox. The fact that human co-exist so closely with the natural avian reservoir for influenza, and that influenza viruses are largely asymptomatic in avian populations, means influenza viruses will be very difficult to eradicate through vaccination alone. To this end, the screening of compound libraries for novel antivirals is a worthy endeavour. In this project we developed a novel screening tool which allows for simultaneous antiviral and toxicity readouts would be beneficial to future high-throughput screens. As previously discussed, reporter cell lines have proved instrumental in identifying novel antiviral compounds from high throughput screens (3.3.). To build on this available technology, it is conceivable that the dual fluorescent read-out system engineered in this project could be invaluable for screening combinations of novel or established compounds, to observe whether a particular compound mix is synergistic, or even toxic to host cells.

Furthermore, work presented in the introduction of this project detailed potentially new chemotherapeutics against influenza, with novel mechanisms of action. However, certain candidates, such as the pentraxin and collectin-like molecules (1.3.2.1.) had not been tested in higher cell-based or animal models. A likely cause for this is that certain research groups do not have access to specialist animal housing facilities, nor the budgets or experienced personnel to cover the expense of maintaining and handling animals. Given that the licensing of new drugs requires several rounds of animal-testing before a approval for human consumption is attained, these compounds may never progress out of the *in vitro* experimental stage. To this end, use of the human airway epithelial cell line could prove useful for providing supplementary data on novel compounds, isolated from rudimentary high-throughput screens, and aid in assessing the potential of these molecules to be taken forward for *in vivo* experimentation. These cells do not require specialist facilities and are substantially cheaper than acquiring animals for experimentation. They

provide an opportunity to study the effects of lead compounds on human innate cytokine responses, as well as demonstrate the toxicity and efficacy profile of novel molecules on human tissue.

Addendum: The effect of pandemic influenza on antiviral drug use.

The revelation that pandemic H1N1 influenza was susceptible to oseltamivir was fortuitous for public health policy and meant that the stockpiled supply of drug could be used following the outbreak. It is estimated that enough drug had been stockpiled to supply 25% of the UK's population with a five day treatment course and following the outbreak of the pandemic, the National Pandemic Flu Service (NPFS) was given executive control to distribute the drug. This was the first time that prescription drugs had been handed out *en masse* without the need for patients to consult a doctor, and in the aftermath of the pandemic, questions have been raised regarding the effectiveness of the NPFS. Estimates suggest that 1.1 million doses of oseltamivir were dispensed under the scheme (NHS Direct, 2010), roughly 7% of the total stockpile, yet whether these doses actually went to sick patients, or were procured to generate a personal stockpile (as often happens when the media interest in influenza is heightened (Gasink *et al.*, 2009)), is unknown. The rationale of the NPFS was to protect NHS hospitals and GPs from an influx of non-emergency patients during the outbreak, yet the course of disease of the pandemic itself was overall very mild. The formation of a rapid drug-dispensation service ensured that drugs were readily available for patients to initiate treatment within the 48 hours of symptom onset that is recommended for successful therapy. Furthermore, the service will have technically reduced the number of virus-shedding individuals from leaving their houses, providing a possible physical barrier to pandemic spread. However, it is a scheme which was obviously designed with a far more virulent pandemic influenza virus in mind, and one might question why the service was activated, and indeed ran for as long as it did once the severity of the pandemic had become clear. The NPFS was activated on the 23rd of July, 2009, following epidemiological data that was published suggesting 100,000 new swine flu cases had been reported in the week prior resulting in 26 deaths, 17 of which occurring in patients suffering from severe underlying illness (Siva, 2009). Although the mortality rate of

the virus was thus low, public interest in the service was so intense that the website crashed within four minutes of activation and traffic was recorded at an equivalent of 9.2 million hits per hour. Britain's Chief Medical Officer, Liam Donaldson's press statement, that was (mis)interpreted as a claim that the pandemic could result in 20,000-65,000 deaths over the winter months, several days before the launch of the website, assumedly aided in generating such hysteria over the launch. With such claims it is likely that the 'worried well' as well as the genuinely ill would all attempt to sequester oseltamivir 'just in case'.

Detractors of the scheme will also claim that patients suffering from more severe afflictions, who present with flu-like symptoms, may go untreated or even be allocated oseltamivir for an unrelated disease (Newton *et al.*, 2010). Furthermore, whilst the emergence of resistance to oseltamivir in the 2007/2008 season did not correlate with heightened drug use (Lackenby *et al.*, 2008b), it is likely that the unregulated use of amantadine by poultry farmers in South-East Asia contributed to the proliferation of drug resistance against this M2 inhibitors amongst influenza viruses, which has been generally maintained in the circulating virus population since (Deyde *et al.*, 2007). One may speculate that a system designed to alleviate the burden on health-care professionals could have conversely over-burdened them should the virus have acquired resistance to our only stockpiled antiviral drug. It is arguable that the risk of generating widespread drug-resistance of a pandemic influenza strain to oseltamivir, coupled with the likely myriad of previously unnoticed side-effects which patients would be subjected to (Strong *et al.*, 2010), did not outweigh the positives of treating so many uncomplicated cases of a mild illness.

To better inform public healthcare officials as to the likelihood of a virus acquiring drug-resistance, we propose the use of human airway epithelial cells could provide a more reliable experimental tool than standard cell culturing techniques, and a cheaper and more rapidly available alternative to animal experimentation in assessing the replication kinetics and drug sensitivity of influenza viruses. We were able to observe a replication disadvantage in the resistant isolate when drug-resistant and -sensitive viruses were infected onto HAE cells and the resulting viral RNA output analysed by pyrosequencing. Furthermore, by generating a reverse genetics system for the pandemic influenza virus, we have a powerful tool which may be used to introduce any mutation into the genome of the virus and study its effects on whole virus behaviour. This could be particularly useful for ensuring that our representative virus remains representative, as the circulating pandemic will continue to adapt in humans and will require periodic updating. Table 5.4. lists numerous changes which have already emerged in circulating pandemic viruses during the course of a single year. A focused use of the reverse genetics pandemic virus system would be to introduce specific mutations, which confer known effects in other subtypes, into the pandemic virus to study their effects. Of particular interest to this study would be the introduction of mutations into the NA gene which have been suggested to result in enhanced transport of NA molecules to the cell surface *in vitro* (Bloom *et al.*, 2010). It would be interesting to observe whether such mutations enhanced the replication kinetics of our drug-resistant isolate in HAE cells. Furthermore, a mutation which has often been seen emerging in tandem with H275Y in drug resistant pandemic isolates is I223V, which may be implicated in one of the rare incidences of human-to-human transmission of oseltamivir resistant pandemic influenza (Deyde *et al.*, 2010). The effects of this mutation on viral fitness in HAE cells would be interesting to observe in future experiments. In order to validate that this HAE competition system can indeed detect virus mutants that will predominate in the community, similar assays to those described in chapter 5 could be set

up using a pair of viruses from the 2007/2008 winter season, during which the H275Y resistant mutant eventually dominated. It has already been shown that using animal experiments, the advantage conferred by this mutation within the seasonal H1N1 influenza viruses could be detected (Baz *et al.*, 2010).

Whilst the debate as to how best to use a substantial stockpile of antiviral drugs is essential, it is also a luxury that many third-world countries do not have. In 2005, Roche donated 3 million doses of oseltamivir to the WHO for use as an emergency response stockpile, and then further donated two million treatment courses for use in developing countries. The Tamiflu Reserves Programme (TRP) was also initiated by Roche as an insurance policy that the poorest countries in the world were guaranteed oseltamivir supplies at a reduced cost following the outbreak of a pandemic (Reddy, 2010). Whilst this is a promising start, the sheer quantity of drug set aside for developing countries is not enough, particularly when acknowledging that very few poor countries have routine influenza vaccinations to supplement wholesale population protection against the virus. Furthermore, should a pandemic virus emerge with oseltamivir resistance, there would be no such support for developing nations. The need for an abundant antiviral is illustrated by these points. Antiviral research often focuses on the discovery of novel, organic or synthetic compounds which can be used to inhibit influenza. However, an alternative approach may be to focus on generic agents which are already readily available throughout the world. David Fedson is a champion of such research and argues that even if all vaccine technologies were focused on generating as much novel vaccine as possible over a six month period, they would only manage to produce 1.5 billion doses. This knowledge, combined with the lack of meaningful third-world antiviral stockpiles would mean that '88% of the world's population' could be left without any therapeutic intervention against

influenza at all (Fedson, 2008; 2009a; b). This could have particularly devastating consequences if the pandemic virus was highly virulent in man. This project sought to identify novel inhibitors of influenza from scratch, but ultimately resulted in highlighting the antiviral effect of a known compound. Castanospermine is naturally found in chestnuts of *C. australe*, and can be readily extracted in large quantities, and synthetically altered into other potent antiviral forms. The observation that this compound holds broad spectrum activity against many other viruses, as well as influenza A viruses, and inhibits viral replication by targeting a host-cell mechanism, are all highly desirable traits for an antiviral drug that might be stockpiled by governments to protect communities against a range of emerging viruses. We showed that Castanospermine had a direct inhibitory effect on the viral titres of several influenza A viruses. It may be prudent to assess the activity of this drug on HAE cells as a more faithful representation of how the virus replicate in the presence of the drug *in vivo*, and perform *in vivo* studies if the *in vitro* work so justifies.

A further debate should be initiated over who genuinely needs antiviral therapy during seasonal and mild pandemic influenza virus outbreaks. There is no doubt that patients with underlying health problems or in 'at-risk' groups, such as pregnant women, the very young or the clinically obese, will be more susceptible to serious complications derived from influenza infections, and this was particularly evident during the pandemic outbreak (Jamieson *et al.*, 2009; Miller *et al.*, 2010; Nguyen-Van-Tam *et al.*, 2010; Webb *et al.*, 2009). Estimates suggest that around 70% of individuals who died from severe complications following pandemic influenza infection presented with underlying health problems (Donaldson *et al.*, 2009; Louie *et al.*, 2009), suggesting that healthy individuals generally fared better with the disease. It would therefore be prudent to ensure that in future, only the severely ill and at-risk groups receive oseltamivir treatment, rather than every patient

who presents with flu-like symptoms. This would ensure that less of the drug stockpile is committed to non-emergencies.

Interestingly, although immunosuppressed patients are the most needing of antiviral therapy, they are also the group most likely to generate resistance to drugs due to the prolonged shedding of virus in their system (WHO, 2009). The implications of this are extremely significant given the importance of effective viral control in these patients. Whilst other individual therapeutic agents such as inhaled or intravenous zanamivir could be a substituted therapy, it would make more intuitive sense to initiate a combination of treatment regimens which could inhibit viral replication on multiple fronts. Recent in vitro data has emerged which suggests a triple combination therapy containing amantadine, ribavirin and oseltamivir is synergistic in treating pandemic A/H1N1 2009 infection, and that surprisingly, the amantadine component of the regimen contributes to the overall efficacy despite the virus being resistant to this drug (Nguyen *et al.*, 2010). This result is extremely interesting and given the abundance of these drugs, and the relatively well understood toxicities of each compound, it would be prudent to quickly conduct a human clinical trial with the regimen. Other combinations of antiviral drugs have also been described in the literature (Kim *et al.*, 2010; Zheng *et al.*, 2008). Whilst there would be a significant increase in costs associated with stockpiling multiple compounds for a future influenza pandemic, perhaps only frontline hospitals would require the combination of drugs, rather than 25% of the country's population, particularly if future influenza pandemics are as mild as the 2009 outbreak. What is certain is that it is unacceptable practice in the modern era to repeatedly use a single drug class against an infectious disease until the target organism develops resistance.

Perhaps one final interesting point to consider with regard to antivirals is the idea that the WHO are about to administer a seasonal vaccine containing the 2009 A/H1N1 virus in place of previous seasonal H1N1 viruses as the representative H1N1 strain. Historically, we have seen that pandemic events replace circulating strains with the prevailing new isolate (Ferguson *et al.*, 2003), and recent surveillance would suggest that the same thing is happening this year, as old seasonal H1N1 isolates have not been identified. However, it is worth bearing in mind that the previous seasonal H1N1 viruses are all resistant to oseltamivir and are routinely handled in most influenza laboratories. One may speculate that if these viruses emerged again and no effective drug is widely available, nor the population sufficiently vaccinated, then a potentially severe influenza season could ensue.

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8. Publications

Brookes, D. W., Miah, S., Lackenby, A., Hartgroves, L. & Barclay, W. S. (2010). Pandemic H1N1 2009 influenza virus with the H275Y oseltamivir resistance neuraminidase mutation shows a small compromise in enzyme activity and viral fitness. *The Journal of antimicrobial chemotherapy* **66**, 466-470. (Brookes *et al.*, 2010)