Assessment of the potential of a narrow spectrum kinase inhibitor in the treatment of influenza infection

Division of Infectious Diseases
Faculty of Medicine

Submitted for degree of
Doctor of Philosophy

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Supervised by Professor Wendy Barclay
(Imperial College London)

September 2014
To Audrey

An inspiration.
Author’s 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.

Jonathan William Ashcroft

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Last, but certainly not least, I must thank and acknowledge the never-ending support I have received from my mum who has stoically put up with my random scientific ramblings over these last few years.

Thank you all.
Abstract

Severe outcome following influenza infection has been linked to the over induction of the host innate immune response resulting in a “cytokine storm.” In certain patient populations, a therapeutic approach to help control the innate immune response may be of benefit. However concerns have been expressed that suppression of the host immune response might also lead to an increase in virus replication and directly enhance viral induced pathology. Using cultures of primary well-differentiated human airway epithelium (HAEs), the ability of a small molecule, narrow spectrum kinase inhibitor, developed by RespiVert (RV1088) to inhibit the extent of influenza virus induced expression of an array of human cytokines including IL6, IL8, IP10, and RANTES was investigated. The virus-induced response following infection with influenza virus was found to be diminished at the mRNA and protein level in the presence of the drug. Importantly, drug treatment did not adversely increase viral replication. In contrast, treatment with a steroid did not suppress the cytokine/chemokine response and resulted in increased viral titres. RV1088 inhibited the viral induction of transcription from the interferon promoter acting at or below the level of MAVS, preventing nuclear translocation of both IRF-3 and NFkB. Used alone, RV1088 inhibited cytokine production by all currently circulating subtypes and lineages of influenza A and B virus. Used in combination with currently licenced antivirals, the virus titre released from HAE cells was suppressed even further than for either drug alone, suggesting a synergistic antiviral effect. Finally, a novel murine model of influenza infection using nebulized virus to infect the mouse airways was developed. Drug also administered through the nebulised route suppressed the interferon response in the mouse lung and did not result in increased viral lung titre or weight loss. Administered intranasally with or without Relenza to mice infected with pH1N1 2009 virus, RV1088 suppressed interferon levels in mouse lung and reduced weight loss and mortality. This, or similar molecules, may represent a new generation of compounds suitable for the treatment of respiratory virus infections.
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<td>ARDS</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Complementary RNA</td>
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<td>ISGs</td>
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<td>Mitochondrial antiviral signaling protein</td>
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<td>MDCK</td>
<td>Madin-Derby Canine Kidney</td>
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<td>Neuraminidase inhibitors</td>
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<td>Newcastle disease virus</td>
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<td>NFkB</td>
<td>Nuclear factor-kappa B</td>
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<td>PBS</td>
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<td>pfu</td>
<td>Plaque forming unit</td>
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<td>Single-stranded RNA</td>
</tr>
<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>vRNP</td>
<td>Viral ribonucleoprotein</td>
</tr>
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<td>WHO</td>
<td>World Health Organisation</td>
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Chapter 1. Introduction

1.1 The influenza virus

The Orthomyxoviruses, which include influenza A, B, and C viruses, are responsible for annual human respiratory infections. Current epidemiological data suggests that each flu season affects 10-15% of the UK population, and can be associated with 12,000 deaths (Donaldson, 2005). While a number of factors contribute to the general cost burden of the disease, the economic impact to society is primarily associated with the large number of lost work hours and overall productivity by infected individuals. In a review of published studies examining absenteeism due to influenza, Keech & Beardsworth (2008) found that the mean number of days lost per episode of illness in laboratory confirmed cases was 3-5 days. In Europe, it has been estimated that influenza accounts for around 10% of sickness absence from work, with the cost of lost productivity in France and Germany alone estimated at around €6.84bn and €10.37bn annually (OECD, 2011). In terms of a pandemic, the economic burden of disease as well as the cost with respect to “Years of Life Lost” can be significant. Vibound et al., (2010) examined the effects of the first wave of the 2009 H1N1 pandemic (May-December, 2009) and reported that in the United States alone between 334,000 and 1,973,000 years of life were lost. This high estimate results from the majority of pandemic deaths occurring in children and young adults (85% of laboratory-confirmed H1N1 deaths occurred in individuals under the age of 60 with 37 being the mean age of death). A proportion of fatal cases have been linked to an aberrant, over-induction of the hosts’ innate immune response. As such, it has been suggested that suppressing this over-induction could prove beneficial to the host in the case of such infections.
1.2 The influenza virus structure and components

The three genera (types) of influenza (A, B, and C) are classified based on the antigenic differences exhibited by two internal structural proteins, the matrix (M) and the nucleocapsid protein (NP). Influenza A infects humans, pigs, horses and birds and through antigenic shift, has the potential to cause major epidemics and even pandemics (Matrosovich et al., 2004; Nobusawa et al., 2006). As influenza types B and C primarily infect humans (with minor reports of type B infecting seals and type C in pigs), antigenic shift does not occur (Jackson et al., 2011; Osterhaus et al., 2000; Kimura et al., 1997).

The negative sense single-strand (ss)RNA genomes of influenza A and B viruses occur as eight separate segments; influenza C viruses contain seven segments of RNA, lacking a neuraminidase gene (Racaniello and Palese, 1979). Members within any of the three influenza types can readily exchange genetic material via reassortment; however, the exchange of genetic material among the three genera has not been demonstrated to date, probably due to incompatibility of the different polymerase complexes as a result of evolutionary divergence (Iwatsuki-Horimoto, et al., 2008).

The high error rate associated with RNA viral replication combined with ecological, population and immune selective pressures continuously drive the selection for evolutionary advantageous changes to the genetic make-up of the influenza virus. Ongoing antigenic changes in the haemagglutinin (HA) and the neuraminidase (NA) surface glycoprotein antigens of the influenza virus is a process referred to as antigenic drift. The RNA polymerases encoded by the influenza virus lacks proofreading capability and thus, gives rise to a high rate of mutation that, in part, is responsible for the antigenic drift associated with this group of viruses.
1.2.1 The virion

The influenza A virion is highly pleomorphic and consists of a protein capsid containing the viral ribonucleoprotein complex (RNP), which is encapsulated by a lipid membrane derived from the host cell. This membrane harbours the glycoproteins haemagglutinin (HA) and neuraminidase (NA) which protrude from the envelope surface by ~10-14nm and are responsible respectively for viral attachment and viral release (Chu, 1949). Also projecting from the membrane is the ion channel matrix protein (M2). The function of the M2 is to modulate the pH across the membrane and allowing protons to enter the core where acidification leads to the core’s disassembly so that viral replication can commence. The influenza virion has been further characterised with the aid of electron microscopy as existing primarily as a sphere with a diameter of ~100nm, although clinical isolates can often be found to be filamentous with a length of ~300nm (Calder et al., 2010; Choppin et al., 1960; Chu, 1949).

The eight RNA genetic segments contained within the core are each folded into a rod-shaped, double-helical RNP (Arranz et al., 2012; Moeller et al., 2012; Ye et al., 2012). Each RNP complex contains a viral RNA, a heterotrimeric viral polymerase (consisting of PB1, PB2 and PA) and numerous copies of the viral-encoded nucleoprotein (NP) which associated directly with the viral RNA in a stoichiometric manner (Zheng et al., 2013).

Each viral segment has non-coding regions at the 3’- and 5’-ends that are partly complementary to each other and form the viral promoter (Desselberger et al., 1980). In total, the eight gene segments encode for a minimum of 12 viral proteins, namely PB1, PB2, PA, NP, HA, NA, M1, M2, NS1, NEP/NS2 and PB1-F2 with recently reported PA-X and M42 further increasing the proposed number of virally-encoded proteins up to 14 (Wise et al., 2012; Jagger et al., 2012; Palese & Shaw, 2007).
Table 1. The viral segments and associated proteins of the three different influenza viruses A, B & C. All segments are discussed further below.

<table>
<thead>
<tr>
<th>Viral Gene Segment</th>
<th>Influenza Type A</th>
<th>Influenza Type B</th>
<th>Influenza Type C</th>
<th>Known Function</th>
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</thead>
<tbody>
<tr>
<td>One</td>
<td>PB2</td>
<td>PB2</td>
<td>PB2</td>
<td>Polymerase</td>
</tr>
<tr>
<td>Two</td>
<td>PB1 &amp; PB1-F2</td>
<td>PB1</td>
<td>PB1</td>
<td>Polymerase</td>
</tr>
<tr>
<td>Three</td>
<td>PA &amp; PA-X</td>
<td>PA</td>
<td>P3</td>
<td>Polymerase &amp; suppressor of cellular gene expression</td>
</tr>
<tr>
<td>Four</td>
<td>HA</td>
<td>HA</td>
<td>HEF</td>
<td>Attachment glycoprotein</td>
</tr>
<tr>
<td>Five</td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>Six</td>
<td>NA</td>
<td>NA &amp; NB</td>
<td>*******</td>
<td>Viral Release (NB function not known)</td>
</tr>
<tr>
<td>Seven (C=6)</td>
<td>M1, M2 &amp; M42</td>
<td>M1 &amp; BM2</td>
<td>M1 &amp; CM2</td>
<td>Matrix &amp; ion channel proteins</td>
</tr>
<tr>
<td>Eight (C=7)</td>
<td>NS1 &amp; NEP</td>
<td>NS1 &amp; NEP</td>
<td>NS1</td>
<td>Modulates immune response &amp; nuclear export</td>
</tr>
</tbody>
</table>

1.2.2 The haemagglutinin (HA) glycoprotein

First described in 1941 by George Hirst, this protein’s name was derived from the observation that it possesses the ability to agglutinate erythrocytes by binding to specific sialic acid structural motifs (Hirst, 1941). Currently, 16 different hemagglutinin subtypes (H1-H16) have been identified in type A influenza strains circulating in wild waterfowl (Galloway et al., 2013; Fouchier et al., 2005) with two novel subtypes, H17 and H18, reportedly found in bats (Tong et al., 2012). Thus far, however, only viruses with either the H1, H2 or H3 HA subtype have been sustained in humans (Galloway et al., 2013).

Structurally speaking, the HA protein forms a rod-shaped trimeric complex. The precursor protein HA0 is cleaved by a trypsin-like serine protease, found either on the plasma membrane or in the extracellular spaces of the respiratory tract, into HA1 and HA2 subunits that remain linked by disulphide bonds (Galloway et al., 2013; Kido, et al., 2012). The HA1 subunit forms a globular domain which is responsible for
binding to sialic acids of glycosylated cell surface proteins or lipids (discussed further in section 1.3.1). Following attachment, the virus is internalized via pH-dependent endocytosis (Stegmann et al., 1985). The acidification of the host cell endosomes initiates a dramatic conformational change in the HA$_1$/HA$_2$ structure which exposes a hydrophobic fusion peptide in the HA$_2$ subunit (Skehel & Wiley, 2000). The fusion peptide promotes fusion of the viral membrane with the endosomal membrane (Figure 1) permitting the release of the vRNP into the cytoplasm (Steinhauer, 1999; Taubenberger, 1998).

In addition to its role in receptor-binding and endosomal fusion, the HA protein represents a major antigen for the stimulation of an adaptive immune response (Brandenburg et al., 2013; Fouchier et al., 2005). The resulting selection pressure created by neutralizing antibodies can drive changes in the antigenic nature of the HA$_1$ domain giving rise to the generation of escape mutants (Martinez et al., 2009; Hensley et al., 2009). This gradual and step-wise accumulation of changes in the antigenic structure of HA$_1$ in viruses as they circulate in humans is referred to as antigenic drift. The consequences of this process is that it allows closely related viruses of the same subtype to re-infect individuals, necessitating the annual production of new vaccines to match the new antigenic character of circulating strains.
1.2.3. The neuraminidase (NA) glycoprotein

Encoded by the 6th RNA segment, the NA protein was named for its ability to catalyze the cleavage of glycosidic linkages between terminal sialic acids of glycoproteins and glycolipids on the surface of target host cells (Gamblin & Skehel, 2010). Currently, there are nine described NA subtypes (N1-N9) identified for type A in wildwater fowls (Gamblin & Skehel, 2010) with a novel subtype, N10, reportedly found in bats (Tong et al., 2012). The NA protein forms a homo-tetrameric complex that has a globular domain on top of a short stem region (Shtyrya et al., 2009).

The cleavage of sialic acid from the cell surface by the NA protein allows for efficient release of mature, newly-synthesized virions. This function is vital as viruses lacking a functional NA protein would be unable to spread from a host cell and would simply be bound to the same cell from which the virions were attempting to
egress (Shtyrya et al., 2009). Thus, a balance between the functionality of HA and NA is necessary for efficient influenza virus replication (Mitnaul et al., 2000).

The ability of newly-synthesized virus particles to escape from the host cell is a crucial step in viral propagation, making the NA a tempting target for therapeutic intervention (von Itzstein et al., 1993). The structure of the NA active site is highly conserved across all subtypes, (von Itzstein et al., 1993) and indeed the only class of directly acting antiviral drugs against influenza at present are neuraminidase inhibitors that bind into the enzyme active site (see section 1.8.1). Like the HA protein, the NA protein can also generate an immune response that results in the production of non-neutralizing antibodies by the adaptive arm of the host's immune response (Marcelin et al., 2011). Although less potent than HA antibodies, NA antibodies restrict viral spread to a certain degree, potentially allowing the host time to moderate the viral infection (Marcelin et al., 2011).

1.2.4 The small transmembrane proteins M2/BM2 and NB
1.2.4.1 The M2 protein

The M2 protein forms a tetrameric complex that is embedded in the lipid bilayer of the virion and functions as an ion channel; being activated at low pH (Pinto et al., 1992; Sugrue & Hay, 1991). It has been implicated in having both an early and late role in the viral life cycle (Helenius, 1992).

In the early stages of infection, the M2 protein channel allows the flow of protons from the acidified endosome into the virion resulting in a lowering of the pH within the capsid (Ivanovic et al., 2012). This weakens the binding of the M1 protein to the vRNPs; allowing their release into the cytosol after fusion of viral and endosomal membranes (Ivanovic et al., 2012; Martin & Helenius, 1991). The late-infection-stage role of the M2 is associated with regulating the pH in the trans-Golgi network (Henkel et al., 2000). By allowing the flow of protons out of the Golgi network, the M2 channel raises the intra-vesicular pH thus preventing a premature
conformational change of the HA$_1$/HA$_2$ protein which is transported to the budding site (Henkel et al., 2000). This activity of M2 is thought to be of more importance for viruses with HA0 that is cleaved intracellularly such as the highly pathogenic avian influenza viruses of H5 or H7 subtypes.

A histidine (His) at position 37 in the M2 protein, serves as a pH sensor (Wang et al., 1995) while a tryptophan (Trp) at position 41 acts as the “gate-keeper” occluding the C-terminal end of the closed channel (Tang et al., 2002). The side chains of both these residues converge to form the narrowest point in the channel, through which no molecules can pass (Shuck et al., 2000; Pinto et al., 1997).

The protonation of His37 in the low pH environment on the endosome initiates an important cascade of events that ultimately leads to the opening of the M2 channel (Wang et al., 1995). This process occurs by means of electrostatic repulsion between the positively charged imidazole rings of His37 leading to the destabilization of the helix-helix packing and resulting in widening of the channel (Wang et al., 1995). It is theorized that once the channel is open, the pH gradient drives the protons from the low pH side to the high pH side by simple diffusion (Leiding et al., 2010). The necessity for a replicating virus to possess functional M2 proteins makes the M2 yet another attractive target for antivirals (Discussed further in section 1.8.2).

Figure 2. 3d stick model of M2 influenza A protein. Viewed from (a) above and (b) sideways complexed with the M2-inhibitor, rimantadine (balls) (Figure from: Schnell et al., 2008)
1.2.4.2 The BM2 protein

The BM2 protein is encoded on the 7th segment of the type B viruses and is produced via a stop-start strategy reading the bicistronic mRNA transcript which also encodes for the M1 protein of the type B viruses (Hiebert et al., 1986; Briedis et al., 1982). The presence of BM2 in all type B strains and the highly conserved nature of BM2s amino acid sequence suggests that this protein plays a crucial role in the life cycle of the virus (Imai et al., 2004). Studies have demonstrated that BM2 is an oligomeric integral membrane protein that associates with the viral envelope via the trans-Golgi network (Paterson et al., 2003; Watanabe et al., 2003). Further studies conducted by Mould et al., (2003) have indicated that BM2 has an ion channel activity, similar to that seen with the M2 protein of type A viruses through which protons are allowed to enter the virions during the uncoating of particles in the endosomes facilitating the release of vRNPs.

1.2.4.3 The NB protein

The NB protein is encoded within the neuraminidase (NA) gene (sixth vRNA segment) of influenza B viruses (Brassard et al., 1996; Betakova et al., 1996). Influenza A viruses do not encode an NB homologue and the function of NB is currently not known.

Sequencing of the sixth vRNA segment indicated the presence of two overlapping open reading frames with the two AUG initiation codons separated by only four nucleotides (Shaw et al. 1982). Williams & Lamb, 1989 demonstrated that in influenza B infected cells, NB and NA accumulate in roughly equimolar amounts (NB:NA = 0.6:1.0). This observation suggests that the ribosome pre-initiation complex bypasses the first AUG initiation codon more often than expected and that ~60% of the ribosome pre-initiation complexes on the sixth vRNA segment bypass the first AUG codon and use the second AUG codon four nucleotides downstream of the first.
It was for many years believed that NB was the functional analogue of the influenza A virus M₂ protein. This belief was based on several factors including: a) the similarity in size (100 and 97 amino acids for NB and M₂ respectively), b) both are integral membrane protein expressed on the surface of influenza-infected cells, c) both M₂ and NB are synthesized in virus-infected cells in amounts comparable to those of other viral membrane proteins but are incorporated into the viron membrane in relatively small, yet similar, amounts and, d) both proteins possess potential ion channel activity.

However, important differences between the two proteins also exist. Firstly, NB is glycosylated whereas the M₂ is not. Secondly, NB and M₂ do not share any primary sequence homology (Zebedee et al., 1985). In addition, the characterization of the influenza B viral BM2 protein has shown it to be far closer in structure and function to the influenza A M2 protein and like the M2 protein, is vital for viral replication (Hatta et al., 2004; Jackson et al., 2004; Imai et al., 2004).

In order to determine whether the NB protein has any role in the replication of influenza B virus, NB deleted viruses were constructed via a reverse genetics system. The resultant viruses growth characteristics and other properties was then examined in cell culture and animal models. Interestingly, NB was shown not to be essential for replication of the influenza B virus in vitro but was required for efficient viral replication in animal models (Hatta & Kawaoka, 2003). These results strongly suggest that the NB protein has been highly conserved because its existence and possible function(s) are somehow advantageous to the virus even though not essential for viral replication.

1.2.5 The matrix protein (M1)

Along with the NP protein, the M₁ protein is the most abundant viral protein found in an infected cell. In the virion, the M₁ protein is associated with the lipid membrane and although the protein lacks a trans-membrane domain, it has been
suggested that this association with the lipid membrane is mediated by M1s interaction with the cytosolic domains of the surface transmembrane proteins NA, HA and M2 (Veit & Thaa, 2011). In addition to its interactions with the lipid membrane, an interaction of M1 with the NP protein of packaged RNPs has been described (Elton et al., 2001; Martin & Helenius, 1991). Specifically, in the infected cell, M1 is necessary for the export of RNPs out of the nucleus (Elton et al., 2001). This is achieved by the M1/RNP-complex binding, via M1 and the NEP/NS2 protein to Crm1 (Chase et al., 2011; Elton et al., 2001). As a cellular export receptor, Crm1 is a vital component involved in directing the export of the M1/RNP/NEP-complex into the cellular cytosol (Chase et al., 2011; Elton et al., 2001; Watanabe et al., 2001) (Figure 3).

Figure 3. Diagram of the proposed mechanism of influenza viral RNP nuclear export. The nuclear export of newly synthesized viral RNP complexes is mediated by the viral NEP. It interacts with the cellular export factor Crm1 and with the viral RNPs via the M1 protein. The interaction with the cellular export machinery facilitates the transport of the viral genome through the nuclear pore into the cytoplasm (Adapted from Paterson & Fodor, 2012).
1.2.6 The Nucleoprotein (NP)

One of the most abundant viral proteins found within an infected cell, NP is involved in a variety of viral processes including intracellular trafficking of the viral genome, vRNA replication, viral genome packaging, and virus assembly. A highly conserved protein, an alignment of the predicted amino acid sequences of the NP genes of the three influenza virus types (A, B and C) reveals significant similarity among the three proteins, with the NPs of type A and B showing the highest degree of conservation (Shu et al., 1993). Importantly, NP has demonstrated an ability to interact with a variety of factors, of both viral and cellular origins (Portela & Digard, 2002). The NP protein is a major component of the RNP complex, binding genomic vRNA segments and forming helical, rod-like RNP structures (Figure 4) (Resa-Infante et al., 2011; Noda et al., 2006). In addition to binding ssRNA, NP is able to form large oligomeric complexes via self-association (Turrell et al., 2013; Elton et al., 1999). Furthermore, NP has been shown to bind to the polymerase subunits PB1 and PB2 and M1 (Gabriel et al., 2008; Biswas et al., 1998). With respect to host factors, NP has demonstrated to interact with at least four cellular polypeptide families: nuclear import receptors of the importin α class, filamentous actin, the nuclear export receptor Crm1 and the DEAD-box helicase BAT1/UAP56 (Gabriel et al., 2008; Elton et al., 2001; Palese et al., 1997; Avalos et al., 1997). It seems logical to reason that NPs multifunctional nature stems from its ability to bind vRNA, associate with numerous viral factors and interact with many host cell factors.
Figure 4. The influenza ribonucleoprotein complex. A) diagram of the structure of a helical vRNP, including the negative-polarity template RNA (red) associated to NP monomers (orange circles). The polymerase subunits are coloured in blue (PB1), green (PB2) and yellow (PA). The promoter is depicted in its corkscrew structure. B) Cryo-EM 3D structure of an influenza mini-RNP complex with a perspective view and two side views and (C), a top view of the same structure with the atomic structure of the NP docked into each monomer (Adapted from: Resa-Infante et al., 2011).
1.2.7 The polymerase proteins: PA, PB1 and PB2

The viral RNA polymerase complex is composed of three subunits, the PB1, PB2 and PA proteins. The majority of transcription and replication of the viral RNA takes place in the infected cell’s nucleus. Once the incoming viral RNAs undergo nuclear translocation, they are transcribed into mRNAs which, in turn, are capped and polyadenylated (Dias et al., 2009; Poon et al., 1999). The cap is derived from host cell pre-mRNAs by a process colloquially referred to as “cap-snatching” and the poly-A-tail is generated by the viral polymerase via a stuttering mechanism (Dias et al., 2009; Poon et al., 1999). Subsequent rounds of infection require newly synthesized polymerase proteins that must be imported into the nucleus to encounter their RNA templates. PB1 and PA form a dimer before entering the nucleus (Fodor & Smith, 2004) and once inside the nucleus, they form a complex with PB2, which is imported independently (Mukaigawa & Nayak, 1991).

The replication of the viral genome can be divided into two processes. The negative-sense vRNA is copied into positive-sense complementary cRNA (1st step of replication) which itself serves as a template to generate more vRNA (2nd step of replication) (Figure 5).

Within the replication complex, PB1 catalyzes the sequential addition of nucleotides to RNA transcripts and initiates replication and transcription by binding to both ends of vRNA or cRNA (Biswas & Nayak, 1994). The PB2 protein binds to the caps of host pre-mRNAs and delivers them to the PA subunit that subsequently cleaves the pre-mRNAs at a position 9-17 nucleotides downstream of the cap (Fechter et al., 2003). The PB2 protein has been identified as being an important determinant of mammalian adaptation and virulence (Subbarao et al., 1993). Specifically, the residue 627 is typically a glutamine in avian viruses and a lysine in human isolates. A/H5N1 viruses possessing a glutamine at position 627 are considered to be less pathogenic in humans; however, isolates that contain a lysine instead of a glutamine have been found to be highly pathogenic (Hatta et al., 2001). Other mutations, for example T271A and D701N, have been implicated in enhancing
avian polymerase activity in human cells (Bussey et al., 2010; De Jong et al., 2006). The PA protein is a component of the polymerase complex that is required for replication and transcription. The N-terminal domain of PA has also been demonstrated to have cap-dependent endonuclease activity with a highly conserved active site (Dias et al., 2009; Yuan et al., 2009; Fodor & Smith, 2004).

Of significant importance to the evolution of the virus is that the viral polymerase does not possess proof-reading capability (Shu et al., 1993; Fitch et al., 1991). The polymerase is thus error-prone and it has been estimated that a mismatch occurs one in every 10,000 nucleotides that is generated (Lauring & Andino, 2010). This implies that in every newly synthesized viral genome, there exists at least one mutation (Lauring & Andino, 2010). This relatively high mutation rate leads to a high number of defective viral particles but also enables the virus to easily escape the pressures exerted on it by the host’s immune response or by treatment with antiviral drugs (Fitch et al., 1991).
Figure 5. Influenza virus replication. The incoming negative sense vRNA(-) is copied in complementary form to cRNA(+) in the first step of replication. This cRNA(+) is subsequently copied back into vRNA(-) during the second step of replication. The vRNA(-) is then transcribed into protein encoding mRNA(+) (Adapted from: Fields Virology, 5th Edition, Chapter of Orthomyxoviridae, Palese and Shaw).
1.2.8 The non-structural protein (NS1) and nuclear export protein (NEP)

Viral segment 8 encodes for both the NS1 and the NEP/NS2 proteins (Robb et al., 2010). NS1 is translated from unspliced mRNA transcripts derived from the full-length vRNA segment, whereas the NEP/NS2 is derived from spliced mRNA transcripts (Chua et al., 2013; Robb et al., 2010). The ratio of unspliced NS1 mRNA and spliced mRNA remains constant during the course of viral infection with NEP/NS2 mRNA being approximately 17% of the total NS mRNA (Robb et al., 2010). Soon after its synthesis, unspliced NS1 mRNA, is rapidly exported out of the cell nucleus in order to minimize the likelihood of the mRNA being spliced (O’Neil, 1998; Alonso-Caplen et al., 1992). The rapidity of this process ensures that the NS1 protein is present at the early stages of the virus replication cycle and is able to counteract the host’s antiviral response (Hale et al., 2008). In addition, this mechanism has the added benefit of preventing early expression of the NEP/NS2 protein, and thus preventing the premature export of vRNPs from the nucleus (Chua et al., 2013; Hutchinson & Fodor, 2013).

1.2.8.1 NS1 – the interferon antagonist

The NS1 protein forms a dimeric complex that prevents recognition of viral RNA by the RNA helicase retinoic acid-inducible gene I (RIG-I) in complex with TRIM25, and by protein kinase R (PKR) by directly binding to dsRNA (Hale et al., 2008; Pichlmair et al., 2006). RIG-I is a cytoplasmic pattern recognition receptor that senses the 5’-triphosphates of viral RNAs and triggers the activation of a signaling cascade which includes both interferon regulatory factor 3 (IRF3) and the nuclear factor-kappa B (NFkB), ultimately leading to the transcription of type I interferon (IFN) (Ruckle et al., 2012; Hiscott et al., 2003). The formation of an NS1-RIG-I/TRIM25 complex leads to the inhibition of IRF-3 activation, thus preventing the transcription and secretion of IFN-β (Kuo et al., 2010). In the absence of NS1, secretion of IFN-β stimulates the IFN α/β receptors in an autocrine and paracrine fashion leading to the up-regulation of interferon stimulated genes (ISGs) including protein kinase R (PKR)
PKR phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF-2α) which results in the suppression of protein translation and thus, viral replication (Bergmann et al., 2000). Importantly, the NS1 protein counteracts the activity of PKR by directly binding to the kinase as well as averting its activation by sequestering dsRNA (Hale et al., 2008; Bergmann et al., 2000). The importance of the NS1 protein to the virus has been demonstrated in studies where genetically engineered viruses lacking the NS1 protein were found to be highly attenuated in cells or hosts with intact interferon systems (Hale et al., 2008; Quinlivan et al., 2005). Although interferon antagonism is the major role for NS1, it is a multifunctional protein and interacts with many different cellular partners (de Chassey et al. 2013).

1.2.8.2 NEP/NS2 – the nuclear export protein

The NEP/NS2 protein functions as an adaptor protein for the nuclear receptor Crm1 and the viral M1 protein (Hutchinson & Fodor, 2013; Iwatsuki-Horimoto et al., 2004; O’Neill et al., 1998). It directs the nuclear export of newly synthesized RNPs that are complexed to M1 (Figure 3). The M1-binding site of NEP/NS2 masks a nuclear localization signal in M1 and that may prevent the re-entry of vRNPs into the nucleus (Akarsu et al., 2011). NEP also regulates the viral polymerase and this may play some role in overcoming the host range restriction (Mänz et al., 2012). The NEP interactome is also reported to be extensive and therefore may have numerous additional roles as yet undescribed (de Chassey et al. 2013).

1.2.9 The PB1-F2 protein

The accessory protein PB1-F2 is expressed from an alternative +1 open reading frame found in segment 2 of some influenza type A virus strains (Chen et al., 2001). PB1-F2 has been shown to be a pathogenicity factor in the pandemic influenza strain of 1918 and in highly virulent strains of A/H5N1 (Conenello et al., 2007; McAuley et al., 2007; Zamarin et al., 2005). It has been proposed that PB1-F2
acts as a virulence factor by modulating the immune response and enhancing the establishment of secondary bacterial infections, a significant cause of increased morbidity and mortality in influenza patients (Conenello et al., 2007; McAuley et al., 2007). Several in vitro studies have demonstrated that upon expression, PB1-F2 localizes to the mitochondria and induces apoptosis by oligomerizing and forming pores in mitochondrial membranes leading to disruption of the membrane potential and the subsequent release of cytochrome C (Zamarin et al., 2005; Gibbs et al., 2003; Chen et al., 2001).

1.3 Influenza virus replication

The replication of the influenza virus is a complex, intricate and elegant process requiring a precise sequence and timing of events (Figure 6). That being said, the entire life cycle of the virus can be simply summarised as a singular drive to infect, replicate and spread. While these processes are taking place, the virus is constantly having to battle and evade the host’s formidable physical and innate immune defences. By better understanding how the virus operates, it has been possible to devise antiviral chemotherapeutic interventions which act by thwarting key steps in this complex and robust life cycle.

1.3.1 Attachment and entry

The initial step in the replication cycle of a viable influenza particle is the attachment to the new host cell. Attachment occurs when HA glycoprotein’s binding site makes contact to the appropriate sialic-acid (primarily, α2,6 – or α2,3-SA) binding receptor (Matrosovich et al., 1997). The cleaving of the HA into its subunit constituents (HA1 and HA2) by a host cell protease enables a low-pH dependent process to expose the fusion peptide region of the HA2 N-terminus which in turn, permits joining of the viral envelope and the cell membrane leading to cell-mediated endocytosis (Skehel & Wiley, 2000; Steinhauer, 1999; Taubenberger, 1998). Once
internalised, the M2 (or B M2 for type B viruses) protein channels permit the inward flow of protons from the endosome to the virion acidifying the core (Ivanovic et al., 2012; Mould et al., 2003). This lowering of the core pH facilitates the disassociation of the vRNPs from the Matrix (M1) proteins and allows the vRNPs to enter the cellular cytosol (Ivanovic et al., 2012; Martin & Helenius, 1991).

Figure 6. A schematic diagram of the life cycle of the influenza virus. Virus particles enter the host cell via receptor-mediated endocytosis. After acidification of the virion, the vRNP complex is released into the cytoplasm. Subsequently, the vRNP is transported to the nucleus, where replication and transcription take place, with mRNAs being exported to the cytoplasm for translation. Early viral proteins, are transported back to the nucleus to aid in the transcription and replication process. Late viral proteins (M1 and NS2) facilitate the nuclear export of newly synthesized vRNPs. The assembly and budding of progeny virions occurs at the plasma membrane at lipid raft microdomains where the glycoproteins HA and NA are in abundance (Adapted from: Neumann et al., 1997).
1.3.2 Nuclear import, transcription and replication of vRNPs

Once the vRNPs have become disassociated from the virion, they localise to the cell’s nucleus due to a nuclear localisation signal (NLS) present on the NP protein (Neumann et al., 1997). This NLS interacts with cellular proteins involved in nuclear import importin α, p10 and Ran (Nieto et al., 1992; Mukaigawa & Nayak, 1991; Nath & Nayak, 1990). The nuclear translocation of the vRNP complexes occurs via nuclear pores in an ATP-dependent manner (Kemler et al., 1994). The virus’ own RNA-dependent RNA polymerase (a component of the vRNP complex) is responsible for both transcription and replication (Hay et al., 1977). The switch from transcription to replication is not completely understood and may depend on the accumulation of viral proteins such as NP, the viral polymerases or NEP. Thus, the first step in the replication process is mRNA transcription to produce the required proteins (Portela & Digard, 2002). Once mRNA transcription has occurred, the mRNAs are transported into the cytosol where the translation step takes place and is mediated by the cellular protein GRSF-1. The proteins of the polymerase complex (PB1, PB2, PA and NP) are translated in the early stages of infection so that the replication process can be further augmented (Garfinkel & Katze, 1993; Katze & Krug, 1990). In addition to the polymerase subunits, NS1 (a potent innate immune antagonist) is also translated early (Hale et al., 2008). The remaining five proteins (HA, NA, NEP, M1 and M2/BM2) are translated later in infection as they are only required during the assembly process of new virions (Garfinkel & Katze, 1993; Katze & Krug, 1990). It is important to note, however, that the evidence for such strict temporal regulation is not robust.

During the transcription process, a critical point is reached and a switch from mRNA transcription to vRNA replication (via a cRNA intermediate) occurs (Figure 5). The de novo vRNA is then encapsulated by NP in a helical manner and associates with the polymerase complex to form new vRNPs which are subsequently transported out of the nucleus via a Crm1-NEP-M1 interaction (Chase et al., 2011; Elton et al., 2001). Once located out of the nucleus, the vRNPs are further
transported on to the cell’s plasma membrane and packaged into newly forming virions (Martin & Helenius, 1991).

### 1.3.3 Virion assembly and release of viral particles

Virion assembly occurs at the apical surface of the cell where new viral particles bud through the plasma membrane at lipid-raft microdomains (Simons & Ikonen, 1997; Naim & Roth, 1993). Why the virus chooses these microdomains as a point for budding is still under examination but it seems likely that the high concentration of viral glycoproteins at these same sites might play a role along with cellular-based factors (Barman et al., 2007; Barman et al., 2004; Barman et al., 2003; Zhang et al., 2000). It is at these lipid-raft locations that the glycoproteins HA and NA accumulate through a transport process involving the addition of N-glycans and the sequence motifs of their trans-membrane domains which potentiate an association with sphingolipid-cholesterol rafts (Simons & Ikonen, 1997; Scheiffele et al., 1995). Under the plasma membrane, a sheet consisting of multiple M1 proteins forms and is thought to interact with the cytoplasmic tails of the HA/NA proteins, aid in genome packaging and shape the morphology of the developing virus particle (Veit & Thaa, 2011). The newly formed vRNPs are then incorporated into the virion via cis-acting signals located with in the vRNA gene segments (Liang et al., 2005; Fujii et al., 2003). Finally, the new virion buds through the plasma membrane while in the process encapsulates itself with part of the membrane (Nayak et al., 2009) (Figure 7). M2, which accumulates at the edge of the rafts, mediates scission of the viral membrane from the host cell (Rossman et al., 2010). The viral NA acts to cleave the terminal sialic acids on surrounding receptors to prevent aggregation on the cell surface of newly released viral particles (Shtyrya et al., 2009). The free-floating virions are thus able to be transmitted to another host or initiate infection in neighbouring cells.
Figure 7. The budding of influenza virions from a host cell membrane. A) Side view of rod-like structure demonstrating the inclusion of vRNA segments into the budding particle. B) ET microscopy of WSN budding from the surface of MDCK cells. C) Scanning EM of spheroid viral buds covering the surface of an influenza-infected cell. (Images adapted from: Nayak et al., 2009; Noda et al., 2006).

1.4 Sialic acid binding and Tropism

The generic term sialic acid (SA) refers to a family composed of nine carbon amino acid sugars (5-amino-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid). SA is typically located at the capping position of glycans which are linked to the glycoproteins and glycolipids that extend from a cell surface (Nicholls et al., 2008; Meng et al., 2010). The hydroxyl groups can be substituted by methyl, sulphate, phosphate, lactoyl, or acetyl residues while the amino group is always substituted with either an N-acetyl or N-glycolyl group producing N-acetylneuraminic (NeuAc) or N-glycolyneuraminic (NeuGc) acid respectively (Suzuki et al., 2000) (Figure 8).
When an SA is added to the hydroxyl group of galactose, it can either be added to the hydroxyl located at the carbon-3 position or the carbon-6 position creating an α2-3 or α2-6 glycosidic linkage respectively (Nicholls et al., 2008). The SA residues are attached to glycolipids or the terminal sugar of a glycoprotein via the sialyltransferase enzymes that are expressed in a species- and cell- specific manner (Angata & Varki, 2002).

Many viral pathogens utilise SA to help them gain entry into host cells (Kumlin et al., 2008). It was Gottschalk in the 1950s who first demonstrated that influenza viruses recognise SA moieties (Schauer, 2000). Since then, a considerable amount of effort has been put into further elucidating and characterising the relationship that exists between SA and the biology of influenza viruses, although the majority of the work concerns the preferences of influenza A virus subtypes while influenza B viruses are poorly characterized in this respect.

Haemagglutinins from various influenza viruses differ in their ability to recognize sialyloligosaccharide structures. The receptor preference of the virus is vital in determining host range, the site of infection (i.e. upper or lower respiratory tract) and general virulence. One of the most conspicuous examples of haemagglutinin receptor specificity lies in the difference between avian and human influenza A virus, described below.
The study of viral tropism made considerable progress after the refinement of staining techniques employing lectin-based fluorescence histochemistry. Lectins are proteins derived from plants, animals or microbial life forms which recognize and bind to specific carbohydrate structures (Sharon, 2007; Loris, 2002). With respect to influenza studies, the plant lectins *Maackia amurenensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA) are used in *in vitro* studies to identify SAα2,3Gal and SAα2,6Gal moieties respectively (Baum & Paulson, 1990). It is important to note, however, that lectin-based assays, while useful in determining the distribution of various SAs in tissue samples, can only act as an indirect measure of virus attachment to such tissues and do not take into account other factors which may influence viral binding (van Riel *et al*., 2007; Ibricevic *et al*., 2006).

With respect to human strains, the influenza virus preferentially targets and binds to SA attached to the sugar chain by an α2-6 glycosidic link (Matrosovich *et al*., 1997; Couceiro *et al*., 1993). In cultured cells from the human respiratory tract, the SAα2,6Gal link is primarily found at the apical surface of the ciliated epithelium whereas basal cells exhibit SAα2,3Gal (Ibricevic *et al*., 2006). It has been reported that mucus-secreting goblet cells possess both the SAα2,3Gal and SAα2,6Gal linkage with a sufficient density to facilitate viral entry and replication of both human and ‘avian-type’ viruses (Baum & Paulson, 1990). More recently however, Matrosovich *et al*., (2004) and Thompson 2006; Shelton *et al*., (2011) have demonstrated that the predominant SA expressed on nonciliated cells is of the α2,6 form.

These receptor types can be found in different regions of the respiratory tract or within the same region but on different cells; the overall picture is that in the human respiratory tract SAα2,6 predominates (Kogure *et al*., 2006; Shinya *et al*., 2006; Matrosovich *et al*., 2004; Thompson *et al*., 2006; Nicholls *et al*., 2007; Dobrovolny *et al*., 2010) (Table 2).
Table 2. Distribution of SAα2,3 or SAα2,6 within the human respiratory tract.  
(Adapted from Kumlin et al., 2008).

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<thead>
<tr>
<th>Tissue</th>
<th>Glycan</th>
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<tr>
<td>M cells in nasopharyngeal</td>
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<td>lymphoid tissue</td>
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<tr>
<td>Nasal mucosa</td>
<td>SAα2,6 &amp; SAα2,3**</td>
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<tr>
<td>Paranasal sinuses</td>
<td>SAα2,6</td>
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<tr>
<td>Nasopharynx</td>
<td>SAα2,6 &amp; SAα2,3</td>
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<td>Pharynx</td>
<td>SAα2,6</td>
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<td>Larynx</td>
<td>SAα2,6</td>
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<tr>
<td>Trachea</td>
<td>SAα2,6 &amp; SAα2,3</td>
</tr>
<tr>
<td>Trachea/Bronchi junction</td>
<td>SAα2,6</td>
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<tr>
<td>Bronchi</td>
<td>SAα2,6** &amp; SAα2,3</td>
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<td>Bronchioles</td>
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<td>Goblet cells</td>
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<td>Alveoli</td>
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** Reported to be in low concentrations

Avian influenza viruses, on the other hand, preferentially bind to SAα2,3Gal, the main receptor type found in the alimentary and respiratory tracts of birds (Hinshaw et al., 1979; Gambaryan et al., 2002; Matrosovich et al., 1997). The widely held view is that for these viruses to adapt for transmission into humans, it is necessary for them to alter their HA binding pattern towards a preference for SAα2,6Gal, the predominant receptor type found in the human respiratory tract (Connor et al., 1994; Matrosovich et al., 2004).

It is important to note that many early studies (pre-mid 1990s) did not routinely employ antigen retrieval techniques (via enzymes or heating) to unmask antigenic sites that may have become cross-linked during the formalin fixation process. Nicholls et al., (2009) have reported that when antigen retrieval techniques...
were used, there was a greater detection of SAα2,3Gal in respiratory epithelium then previously reported. Nevertheless, SAα2,6Gal still predominated.

Primary cultures of human airway epithelium have been generated in the laboratory and can contain a mixture of ciliated and non-ciliated cells, the latter able to produce and secrete mucus. Lectin staining of these cultures has shown a predominance of SAα2,6Gal found at the apical surface of both ciliated and nonciliated cells but that the SAα2,3Gal is exclusively present on ciliated cells (Thompson et al., 2006; Matrosovich et al., 2004).

Matrosovich et al., (2004), and later Thompson et al. (2006) reported that following infection, human adapted influenza viruses spread laterally from cell-to-cell in human tracheobronchial epithelial (HTBE) cultures – infecting both ciliated and non-ciliated cells using the abundant a2,6SA expressed on both cell types. In contrast, avian viruses under the same conditions did not form continuous foci of infection and were found to infect primarily ciliated cells due to their preference for α2-3 linked SA.

Studies have shown that with only a few amino acid substitutions in the gene encoding the viral HA, it is possible to alter the binding specificity of the HA. These amino acid substitutions have been well characterized for H3 HA (for example HA from the H3N2 virus A/Udorn/307/72) and a substitution at position 226 (leucine to glutamine or methionine) of the HA1 protein has been reported to alter the receptor preference from SAα2,6Gal to SAα2,3Gal (Rogers et al., 1983; Ito et al., 2000). In addition, a mutation at position 228 (serine to glycine) in Udorn HA was found to alter the virus’ ability to recognize the SA molecular species Neu5Ac and Neu5Gc. Prior to this mutation, the virus preferentially recognized NeuAca2,6Gal while after the serine to glycine substitution, the virus was able to recognize both Neu5Ac and Neu5Gc equally well (Ito et al., 2000).

Ito and colleagues (1998) found that pig trachea contains both avian and human influenza virus receptors and, as such, could constitute a theoretical “mixing vessel” for the generation of human-avian influenza A virus with pandemic potential. Furthermore, they noticed that with extended replication in pigs, avian viruses
would undergo a total shift in receptor specificity to the SAα2,6Gal form. It was proposed that this receptor switch could arise via two, not mutually exclusive, routes. Firstly, a pig could be infected with both human and avian influenza viruses and that classical reassortment could take place and/or, secondly, an infection with an avian virus alone could take place and through a stepwise fashion the virus could increase its recognition of human cell surface receptors. Either of these routes could potentially yield a virus that readily transmits to humans but for which we are antigenically naive.

While traditional views tend to suggest that influenza virus HAs would only recognize and attach to one specific form of SA, recent studies have demonstrated that receptor preference is not absolute; rather, there exists a degree of plasticity and a range of receptor binding affinity (Meng et al., 2010). In fact, studies have shown that receptor preference can be overcome by employing high concentrations of virus (Nicholls et al., 2008; Meng et al., 2010).

The submucosal glands of the human respiratory tract play two important, albeit conflicting, roles with regard to influenza infection. The mucus produced by these glands has been demonstrated to contain a high concentration of SA – predominantly in the α2,3 form (van Riel et al., 2007). It has been proposed that SAs present in the mucus can act as potential inhibitors of infection by binding the viruses before they are able to reach the cells of the respiratory tract (Gentry et al., 1988). The fact that human respiratory mucin is particularly rich in the α2,3SA would suggest that it would be particularly adept at inhibiting avian influenza viruses and may help to explain the low transmissibility of influenza from avian hosts to humans. In addition, the negative charge held by SAs causes them to repel each other and, when attached to filamentous glycoproteins, help to give mucus its high viscosity (Schauer, 2000). If however, a virus is able to successfully navigate through the mucus layer and establish an infection within the glandular cells, the resultant infection can easily spread to neighbouring cells.

The viral protein neuraminidase, in addition to its role in aiding the detachment of new virions from the SA on the surface of infected cells, also uses the
same enzymatic property to help the virus navigate through the SA-rich mucus. As Wagner et al., (2002) explain, it is important that a functional balance is maintained between haemagglutinin and neuraminidase activity. As such, viruses must be allowed to recognize and attach to the SA present on cell surfaces but their attraction must not be so strong as to inhibit viral release or unduly attach them to decoy receptors in mucus.

1.5 Circulating Strains & Reassortment

The reassortment of viral gene segments has been implicated in the emergence of viruses with novel antigenic properties leading to the pandemics of 1918 (A/H1N1), 1957 (A/H2N2), 1968 (A/H3N2) and recently 2009 (A/H1N1). The pandemic viruses of 1957 and 1968 were found to be reassortants of human and avian viruses (Reid et al., 2004; Schafer et al., 1993; Kawaoka et al., 1989) whereas the 2009 pandemic was a triple reassortant containing the genetic material of human, avian and swine viruses (Trifonov et al., 2009).

After the emergence of the Spanish influenza pandemic virus in 1918, A/H1N1 viruses circulated until they were displaced by an A/H2N2 virus in 1957 which gave rise to the Asian influenza pandemic. The A/H2N2 viruses continued to circulate until 1968 before they, in turn, were replaced by an A/H3N2 virus that caused the Hong Kong influenza pandemic (Figure 9) (Kawaoka et al., 1989; Scholtissek et al., 1978). Since then, A/H3N2 viruses have circulated causing seasonal epidemics. In 1977 A/H1N1 viruses reemerged and have been found co-circulating with A/H3N2 viruses since. These viruses were then displaced by the emergence of the A(H1N1)pdm09 triple reassortment strain. During the 2013-2014 influenza season in the UK, the predominant circulating type A strains continues to be the A(H1N1)pdm09 as well as the A/H3N2 strain (Public Health England, 2014).
Figure 9. Circulating types and strains of influenza from 1900-2000.

1.5.1 Influenza B virus circulation

Antigenically speaking, it is possible to separate Type B viruses into two distinct groups (Figure 9). One set resembling B/Yam/88 circulated in the United States while the other set resembled B/Vic/97 and was found in Asia (Rota et al., 1990). It is from these two proteotypic lineage strains that all subsequent antigenic classification is based. The circulation of genetically similar, yet antigenically distinct lineages with little to no cross reactivity has profound vaccination implications (discussed further in section 1.7).

Multiple strains of the influenza B virus belonging to either lineage frequently arise and, at any one time, variants belonging to the different lineages co-circulate in the human population (Matsuzaki et al., 2004; Rota et al., 1992; Rota et al., 1990; Oxford et al., 1984; Lu et al., 1983). This is hardly surprising as studies have shown that multiple co-circulating Type A viruses belonging to different evolutionary lineages have existed and co-circulated (Cox et al., 1989; Lindstrom et al., 1999 and Xu et al., 1996). This co-circulation last for only short periods of time until a
dominant strain emerges as a result of antigenic selection pressures. The co-
circulation of multiple lineages or subtypes of influenza viruses provides an
opportunity for genetic reassortment to occur during a mixed infection of a single
individual with two viruses belonging to different subtypes in the case of Type A or
two lineages for Type B. Importantly, it should be noted that due to incompatibility
of their polymerase genes, there has been no documented reports of reassortment
between Type A and B occurring in a host (human or otherwise).

1.6 Immune response:

In terms of pathology and clinical presentation, influenza infections are
generally associated with a disease of predominantly the upper, but in some cases
lower, respiratory tract ranging from asymptomatic to fatal. Influenza-related
disease is often most severe in patients/hosts that are either immunocompromised
or have a predisposing co-morbidity including, but not limited to, asthma, chronic
obstructive pulmonary disease (COPD) and neuromuscular disorders (Peiris et al.,
2009; Keren et al., 2005).

As Fukuyama and Kawaoka (2011) re-iterate, the broad spectrum of clinical
presentations described for influenza A and B viruses are quite similar. Over the past
seventy years, influenza B has been responsible for 16 seasonal epidemics and has
been the predominant circulating type every 3-4 years (Thompson et al., 2003).
Influenza B infections have been reported to induce clinical signs and symptoms
similar, if not indistinguishable from, infections caused by influenza type A strains
resulting in morbidity and mortality worldwide (Kim et al., 2009; Wright & Webster,
2001). It has been noted that some influenza-related symptoms occur more
commonly in children after influenza B infection and include leukopenia, myalgia and
myositis, the latter occurring more in males than females (Peltola et al., 2003; Hu et
al., 2004). Typically, the burden of influenza B infections falls mostly on young adults
and children, although in some epidemics, it is the elderly population that is more
adversely affected (Olson et al., 2007; Li et al., 2008; Glezen et al., 1980). On the
other hand, there is a body of opinion in the literature that influenza B viruses cause milder pathogenesis and weaker inflammatory response than influenza A viruses, leading to a much milder clinical presentation (Kim et al., 2009; Kim et al., 1979). Most of these conclusions rely on data derived from animal models (ferrets) or clinical studies conducted from the late 1950’s to the mid 1970’s. It should be noted that these clinical studies took place before the divergence of type B lineages (mid-1980’s, see below) and no recent large-scale clinical-based influenza B study has been conducted examining pathogenesis or immune response. However, serious influenza type B infections are observed in children (Dr. Susan Chiu and Prof. Malik Peiris, personal communications 2011).

Ferret experiments conducted by Kim et al., 2009 demonstrated reduced viral replication and lower induction of inflammatory cytokines in lung tissues in animals infected with influenza B (B/Malaysia/2506/04 and B/Shanghai/361/2002) when compared to those infected with influenza A (A/New Caledonia/20/99 and A/Puerto Rico/8/34). In addition, those ferrets infected with the flu B strains exhibited a reduced level or viral replication and an overall lower induction of inflammatory cytokines. While the viruses selected in this study were representative of the two B lineages, they were not temporally matched with their flu A counterparts. Furthermore, the viruses were amplified in eggs prior to infection and such amplification has been shown to alter the receptor specificity of influenza viruses (Robertson et al., 1985). Furthermore, these studies may simply prove that the influenza B virus, being an exclusively human adapted virus, does not replicate well in the ferret.

The current belief is that much of influenza virus pathogenesis is influenced by the host’s immune response to the virus infection. The vast majority of work in this area is centred around influenza type A viruses. The adaptive immune system plays a vital role in actively protecting a host during an infection and aids in the prevention of repeat infections while the innate immune response serves as a non-specific form providing front line defence against microbial assault. If however, this response is unregulated, it can cause damage to the very organs that the immune system is trying to protect.
During the early stages of infection, the ssRNA of the virus is recognized by toll-like receptor seven (TLR7) in dendritic cells and retinoic acid-inducible gene-I (RIG-I) in epithelial cells (Diebold et al., 2004; Pichlmair et al., 2006). Both of these “ssRNA sensors” independently activate signalling pathways that induce the production of type 1 IFNs and acute antiviral host responses. TLR7 and RIG-I are also responsible for inducing the production of inflammatory proteins (IL-1β, IL-6, IL-8, MCP-1, MTP-1α, TNF-α, IP-10 and RANTES) which is mediated via NFκB activation (Kawai & Akira, 2008). Importantly, the viral protein NS1 can interfere with the RIG-I signalling pathway and the expression of induced mRNAs and thus hinders the host’s ability to mount an effective innate immune response (Hale et al., 2008; Jiao et al., 2007).

Studies have shown that two broad patterns exist related to the kinetics of cytokine gene transcription – an early and late phase. We are primarily interested in the early phase of cytokine gene transcription because this sets up the outcome of infection. To investigate the cell’s early, primary response to the virus, Cheung and colleagues (2002) infected human peripheral-blood monocytes with influenza A (H5N1) strains using a high multiplicity of infection (moi = 2) in order to ensure infection of the vast majority of cells during a single round of viral replication. What they ascertained was that from one hour p.i. onwards, IFN-α, IFN-β, IL-1β and TNF-α were progressively up-regulated (with TNF-α levels peaking at 12-24hrs p.i.) and that at six hours p.i. MCP-1, RANTES and IL-12 were induced. After this period, many of the subsequent responses are the product of feedback loops resulting from this early induction pattern (Mok et al., 2009).

It is currently believed that the most severe presentations of influenza-related hospitalizations result from an unregulated response of pro-inflammatory cytokines and chemokines induced by the TLR signalling cascade, creating a so-called “cytokine storm” (Chung et al., 2002; Fukuyama & Kawaoka, 2011; Osterholm, 2005). The severe pathology and mortality rates associated with 1918 Spanish flu, and in about half the people infected with HPAI H5N1 infection, are believed to be linked to this dysregulation of the immune response (To et al., 2001; Seo et al., 2002; Abdel-Ghafar et al., 2008). Ex vivo studies employing primary human alveolar and
bronchial epithelial cells infected with H5N1 found that cytokine induction was dependent on active viral replication as inactivated viruses did not induce any detectable cytokine up-regulation (Chan et al., 2005). Furthermore, these studies demonstrated that, although high replicating viruses did induce high cytokine levels, the differences in cytokine up-regulation observed between the various strains tested was not only due to differences in replication kinetics (Peiris et al., 2007). Several genotypic variations in influenza A virus have been associated with a highly virulent phenotype. Some of these are listed in Table 3. For influenza B viruses this type of detailed study on the way genetic variation might affect virus virulence has not been undertaken.

Although generally a rare complication, pneumonia can follow an influenza infection and can vary from mild to fatal in consequence. If however, they occur together in the form of a heterologous or secondary infection, the resultant infectious disease is associated with significant morbidity and mortality (Small et al., 2010; Hussell & Williams, 2010). The pneumonia can be primary viral pneumonia or more commonly, be secondary to bacterial infection (Peiris et al., 2009). It has been postulated that the “cytokine storm” associated with certain viruses may create an environment conducive for pneumonia to set in (Small et al., 2010; Madhi et al., 2008; Seki et al., 2007). Interestingly, primary viral pneumonia has been frequently reported as a common manifestation of H5N1 infections in humans (Abdel-Ghafar et al., 2008). In addition, the major findings from autopsy performed on fatal cases of the disease were in line with the pathology observed in Acute Respiratory Distress Syndrome (ARDS) and included diffuse alveolar damage with hyaline membrane formation, patchy interstitial infiltrates (primarily composed of neutrophils, macrophages and activated lymphocytes) and pulmonary congestion with associated haemorrhage (Headley et al., 1997; To et al., 2001; Cheung et al., 2002).
Table 3. Mutations in viral proteins that influence viral pathogenicity. (Adapted from Kumlin et al., 2008).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Virus</th>
<th>Mutation</th>
<th>Pathogenic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>H7N7</td>
<td>A143T</td>
<td>Increased attachment to bronchial epithelial cells and alveolar macrophages in humans</td>
</tr>
<tr>
<td>HA</td>
<td>H1N1 (1918)</td>
<td>D190E, D225G</td>
<td>Switch of receptor specificity (alpha2,6 to alpha2,3)</td>
</tr>
<tr>
<td>HA</td>
<td>H3N2</td>
<td>L226Q</td>
<td>Switch of receptor specificity (alpha2,6 to alpha2,3)</td>
</tr>
<tr>
<td>HA</td>
<td>H1N1 (2009)</td>
<td>D222G</td>
<td>Switch of receptor specificity (alpha2,6 to alpha2,3)</td>
</tr>
<tr>
<td>NA</td>
<td>H3N2</td>
<td>R292K, E119V, N294S</td>
<td>Oseltamivir-resistant (R292K, loss of ability to readily transmit)</td>
</tr>
<tr>
<td>NA</td>
<td>H5N1</td>
<td>H274Y</td>
<td>Oseltamivir-resistant</td>
</tr>
<tr>
<td>PB1-F2</td>
<td>H1N1 (1918)</td>
<td>N66S</td>
<td>Delay of innate immune response and increased cytokine production</td>
</tr>
<tr>
<td>PB2</td>
<td>H5N1</td>
<td>T271A</td>
<td>Increased polymerase activity in mammalian cells</td>
</tr>
<tr>
<td>PB2</td>
<td>H5N1, H7N7</td>
<td>E627K</td>
<td>Increased replication activity in mammalian respiratory tract</td>
</tr>
<tr>
<td>PB2</td>
<td>H5N1</td>
<td>D701N</td>
<td>Increased replication activity</td>
</tr>
<tr>
<td>PA</td>
<td>H5N2</td>
<td>T97I</td>
<td>Adaptation In mice</td>
</tr>
<tr>
<td>NS1</td>
<td>H5N1</td>
<td>P42S</td>
<td>Increase in IFN antagonism</td>
</tr>
<tr>
<td>NS1</td>
<td>H5N1</td>
<td>85-94 inclusive deletion</td>
<td>Impaired inhibition of IFN production</td>
</tr>
<tr>
<td>NS1</td>
<td>H5N1</td>
<td>D92E</td>
<td>Decreased sensitivity to IFN and TNF-α</td>
</tr>
</tbody>
</table>
A large part of the clinical presentation and morbidity associated with influenza-related disease is induced not only by viral factors but also by the host immune response. Mall and colleagues (2011), documented an outbreak in Germany of benign acute myositis in children. During the outbreak, a seasonal influenza B strain (B/Florida/4/06-like) was found to be the causative agent by qRT-PCR in 93% of cases (n=216). As there was no evidence of systemic spread of the virus, the systemic manifestations could be attributed to the actions of cytokines and other inflammatory mediators (To et al., 2001; Hayden et al., 1998; Peiris et al., 2009).

Studies have demonstrated that differences in numerous viral factors including HA antigenicity, expression of virally encoded proteins and RNA (Diebold et al., 2004) contribute to differences observed between type A and B in terms of both the type of cytokines expressed and their relative level of expression by NKT cells and dendritic cells (Minami et al., 2005; Sato et al., 2009; Divanovic & Lai, 2004), but these are not well characterized at the molecular level.

In the late 1980s researchers noticed the emergence of several strains of influenza B that possessed different antigenic properties than the standard reference strains (Rota et al., 1990). It was later determined that a divergence had taken place with two distinct lineages emerging; B/Victoria and B/Yamagata (the former possessing the novel antigenic properties) (Rota et al., 1990; McCullers et al., 2004). Currently, both of these lineages freely circulate within the human population (Li et al., 2008; Belshe, 2010). This has led to speculation that representatives from both lineages should be included in the seasonal vaccine, making it quadrivalent rather than trivalent if full coverage is to be achieved.

During the 2010-2011 influenza season, influenza B virus infections were common and a relatively high number of influenza B cases were hospitalized. Strikingly, there were a number of influenza B cases involving young adults (both male and female) that did not have any underlying comorbidities or risk factors A similar scenario was also reported for influenza A infections and autopsy results of fatal cases found a large accumulation of complement (specifically C4d) cells around the bronchi indicating an abnormal immune response may have contributed to the pathogenesis of these strains (Fukuyama & Kawaoka, 2011).
1.7 Vaccines

The most effective defence we have against insult from the influenza virus is a well-matched vaccine that induces an antigenic response strong enough to protect the individual from the circulating viral strains. Our antiviral arsenal, while generally effective, has limited to no ability to protect an individual against an infection and are only effective at symptom alleviation.

In the UK, influenza vaccines are offered to “at-risk” individuals, influenza researchers, healthcare workers and other frontline occupations deemed a priority by national health and security organisations. The “at-risk” group is typically comprised of anyone over the age of 65, pregnant women, children/adults with underlying conditions (particularly long standing heart or respiratory disease) and children/adults with weakened immune systems. As of the 2013-2014 influenza season, healthy children aged two and three years have the option of receiving the nasal spray flu vaccine (“FluMist”) (http://www.nhs.uk/Conditions/vaccinations/Pages/flu-influenza-vaccine.aspx).

Antibodies raised against one influenza virus type or subtype confer limited, to no protection, against a different type or subtype. Due to the constant antigenic drift and, for type A occasional shift, associated with influenza viruses, influenza vaccines have to be updated annually to account for the new antigenic properties of the predominant circulating stains. The efficacy of a vaccine relies on T<sub>H</sub> cell regulated development of high affinity B cell memory and the consolidation of the response through antigen re-challenge (Wee, 2009).

For example, the influenza vaccine does not induce protective immunity in a large proportion of the elderly population due to what researchers have described as “immunosenescence” (Goronzy & Weyand, 2013). The diminished response in the elderly to a vaccine is believed to be due, at least in part, to a defective T cell memory response. Responses to the vaccine against the influenza virus in the adult rely strongly on somatic mutation of the memory repertoire rather than recruitment of new antibody sequences (Li, 2012; Wrammert, 2011). It is the inability of the T cell memory repertoire to adapt to the new HA/NA present in the vaccine that prevents the induction of the desired immune
response when the individual is challenged by the circulating virus. Numerous attempts to improve vaccine uptake in the elderly have included the use of adjuvant, higher vaccine doses or booster vaccination; however, all have been of limited success (Couch, 2007; Brown, 2010).

A systematic review of reviews concluded that vaccination against the influenza virus provides substantial protection to both healthy and at-risk individuals, children as well as adults. The efficacy of vaccines against laboratory confirmed cases ranges from 26-87% depending on the vaccine match and the age of the recipient individual (Nichol, 2008). Lower protection can be expected in years where there is a suboptimal match between the vaccine strain and the circulating strains. This however, does not mean that a “miss-match” vaccine does not afford the individual any protection since the vaccine generally will reduce disease symptoms even though it may not protect against the primary infection (Nichol, 2007; Herrera, 2007). A more thorough understanding of the concept of vaccine cross-protection could have numerous implications in terms of improving planning for both seasonal and pandemics vaccines.

There are several licensed manufacturers of vaccines around the world, all of which grow their vaccine stocks in eggs, either formulated as a trivalent inactivated virus for intramuscular injection or as a live attenuated virus vaccine for intranasal administration. Studies comparing the protection offered by these two vaccine types have indicated that the live attenuated influenza vaccine offers a significantly better protection than its inactivated counterpart against both well matched strains and strains which have undergone antigenic drift (Belshe, 2007).

Standard trivalent vaccines cover two type A stains and one type B strain. However, since the emergence of two antigenically distinct influenza type B lineages in the 1980’s, one lineage would typically displace the other lineage in circulation for several years only to be almost fully replaced by the other lineage at a later date. However, since 2001 both Type B lineages have been co-circulating with year-to-year variation in the predominant lineage (Glezen, 2013).

As vaccine strains have to be chosen at least 6 to 8 months prior to vaccine distribution, the possibility of a mismatch between the selected vaccine strains and the
predominant circulating strains is a real possibility and has, in fact, occurred numerous times (Schaffner, 2006; Tosh, 2010). From the 2001/02 to the 2010/11 influenza seasons, the Type B component of the vaccine has only matched the predominant circulating strain 5/10 times (Belshe, 2010a). When vaccine mismatch occurs, it can be a serious problem. If the new circulating strains are significantly different, cross-protection afforded through the vaccine may diminish or be absent altogether. Xu et al., (2004) have found that there is only a low level of antigenic cross-reactivity between the hemagglutinins (HAs) of viruses from the two lineages. This lack of cross-reactivity holds serious consequences for seasonal vaccine planning and administration. Indeed, during the 2000-2001 and 2004-2005 seasons in the USA, significant mismatch occurred and there was a higher than normal reported incidence of severe illness (Schaffner, 2006).

Currently, both of these type B lineages freely circulate within the human population (Li, 2008; Belshe, 2010a). This has led to calls for representatives from both lineages to be included in the seasonal vaccine, thus replacing our current trivalent vaccine with a more protective quadrivalent vaccine (Reed, 2012; Belshe, 2010a; Belshe, 2010b). The first FDA licensed quadrivalent vaccine (Sanofi Pasteur’s Fluzone® Quadrivalent) suitable for patients as young as 6 months of age is expected to be commercially available for the 2013/2014 influenza season (http://www.sanofipasteur.com).

There are efforts underway to design a rapid pandemic vaccine production system employing the reverse genetics approach where 6 plasmids would carry attenuating internal genes originated from a stable master donor virus and two additional plasmids encoding for the haemagglutinin and neuraminidase from the potential pandemic strain (Chen & Subbarao, 2009; Hoffmann, 2002). If effective, this system could dramatically cut down vaccine production time and thus offer a much more acute response to an emerging pandemic. Initial vaccine trials employing this approach had yielded disappointing results with the vaccine inducing limited immunogenicity. However, with the development of novel adjuvant strategies that can be used in combination with the reverse genetic vaccines, a more desirable protection level has been achieved (Leroux-Roels, 2007).
1.8 Antivirals

Currently, there are two classes of anti-influenza drugs licensed for the prophylaxis and treatment of influenza infection: 1) neuraminidase inhibitors and 2) M2 channel inhibitors.

National Institute for Health and Care Excellence (NICE) guidelines currently recommend the use of antiviral treatments for influenza only if the patient is deemed to be at-risk. The at-risk group includes individuals aged 65 years and older, those aged 6 months and over with comorbidities which put them at-risk for influenza-related complication, which include diabetes, chronic respiratory, cardiac, renal, neurological and liver disorders and immunosuppression (NICE, 2009).

The licensing of antiviral drugs such as the neuraminidase inhibitors (NAIs) zanamivir and oseltamivir has been largely based on clinical trials in which otherwise healthy adults experiencing seasonal influenza were recruited rather than in the at-risk groups. Here NAIs have been shown to reduce the duration of symptomology resulting from influenza infection, by between half a day and one day (Cayley, 2012). The NICE treatment guidelines do not take the societal perspective into account when evaluating interventions. If, however, a wider view was taken which considered the number of lost working days and the socio-economic impact of influenza infections in a working healthy adult population, more emphasis might be given to treating influenza infections in those not classified as “at-risk.” It should be noted, that if antiviral drugs were made more available and injudiciously used, resistance could spiral out of control.

1.8.1 Neuraminidase inhibitors

The viral neuraminidase facilitates the spread of influenza progeny virions after successful replication by the cleaving of N-acetylneuraminic acid from the cell surface glycoprotein. By inhibiting this cleavage, the release of the newly formed viruses is prevented (Moscona, 2005a). Due to the highly conserved enzyme site of the neuraminidase
glycoprotein and its requirement for successful viral replication, it poses a tempting target for antiviral intervention. The neuraminidase active site is comprised of eight catalytic residues that make direct contact with the sialic acid substrate. A further 11 framework residues stabilize the shape of the active site (Colman, 1983). The modelling of the interactions that occur between these highly conserved residues and the sialic acid eventually led to the development of the first neuraminidase-inhibitor (NAI), the zanamivir, the structure of which is analogous to a sialic acid but also allows for additional interactions that lock this substrate analogue into the active site rendering the neuraminidase inactive (von Itzstein, 1993). Zanamavir has poor bioavailability and is administered as an inhaled formulation. A similar approach was used to design oseltamivir, an oral prodrug formulation (Laver, 1999). Both NAIs have been licensed for use in many countries around the world since 2000 with oseltamivir being the more widely used due to its ease of oral administration (Ikematsu, 2012; Cooper, 2003).

The neuraminidase inhibitors (oseltamivir and zanamivir) have been shown to be effective at reducing the duration of illness from seasonal influenza by approximately one day if the drug is administered within 48 hours after the appearance of symptoms but when used as prophylactics role, when compared to placebo, neuraminidase inhibitors were found to have no effect against influenza-like disease (Jefferson, 2006). This original report was latter re-assessed and augmented in subsequent Cochrane reviews (2007, 2010, 2011, and 2012) where in the clinical efficacy of the neuraminidase inhibitor class of antivirals has been called into question. This new assessment has arisen over a dispute around the Cochrane researchers ability to gain full access to clinical trial data, including non-published results, from studies conducted by the pharmaceutical companies (in this instance, namely GlaxoSmithKline and Roche) (Jefferson & Doshi, 2013). In addition, concerns over study flaws and inappropriate extrapolations from published data by these same companies have become an issue (Kmietowicz, 2013). Despite all these discrepancies, a major meta-analysis conducted by Muthuri et al., (2014) examined the effectiveness of neuraminidase inhibitors in 29,234 hospitalised patients infected with A/H1N1pdm09 from 78 studies conducted between Jan 2, 2009 till March 14, 2011. The results of their analysis indicate that treating A/H1N1pdm09-infected patients at any stage of illness with neuraminidase inhibitors compared to non-treatment patients was associated with a reduction in the risk of
mortality. Additionally, this association in reduced risk of mortality increased the sooner after the onset of symptoms that treatment with a neuraminidase inhibitor was commenced. Furthermore, they reported that their findings were consistent for adults, pregnant women and adults requiring critical care however, the authors were unable to demonstrate any similar associations in paediatric populations.

1.8.1.1 Oseltamivir (Tamiflu)

Oseltamivir ((3R, 4R, 5S)-4-acetylamino-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylic acid ethyl ester) is an orally available and widely-used neuraminidase inhibitors. A prodrug, oseltamivir is metabolised to its active form upon first-pass through the liver (Davies, 2010).

Treanor and colleagues (2000) reported on a 60-center trial where 627 otherwise healthy patients aged 18 to 65 presenting with influenza-like illness of less than 36hrs in duration were randomly assigned to oseltamivir (75 or 150mg, twice daily) or placebo for 5 days. Sixty percent of patients later were determined to have laboratory confirmed influenza infections. Their results indicated that both doses of oseltamivir led to a statistically significant reduction in symptom duration by approximately one day. Furthermore, oseltamivir was found to reduce severity scores and the incidence of secondary complications.

Another study conducted by Nicholson et al., (2000) found comparable results. Their trial consisted of 51-centers with 719 healthy adult patients treated with either oseltamivir or placebo either within 24hrs or 24 to 36hrs post the onset of symptoms. Among the 66% of patients who were found to have laboratory confirmed influenza A infections, oseltamivir significantly reduced the duration of illness by approximately 1.5 to 2 days for the within 24hrs treatment group and 1 day for the 24 to 36hrs treatment group. Muthuri et al., (2014) conducted a meta-analysis in which data from 29,234 patients (across 78 studies) admitted to hospital worldwide with laboratory confirmed or clinically diagnosed pandemic influenza (A/H1N1pdm09) virus infection and assessed the association between NAI treatment and mortality. They found that compared with no treatment, neuraminidase inhibitor treatment
(irrespective of timing of intervention) was associated with a reduction in mortality risk. However, the association of reduced mortality risk associated with NAI treatment was not significant in children.

Within at risk groups, the influenza-related disease processes can be far more severe and antiviral intervention (and the timing of said intervention) is often critical to patient outcome. Indeed, during the 2009 influenza pandemic, the severity of illness was found to increase with increasing interval from onset of symptoms to the start of antiviral therapy (Zarychanski et al., 2010).

In order to exert its effect, oseltamivir requires a re-arrangement in the neuraminidase active site. Thus, a mutation which prevents this re-arrangement of the active site would render the drug inactive. A single nucleotide change can confer resistance such as that encoding the NA amino acid substitution H274Y. The tyrosine causes displacement of glutamine at position 276 and subsequent disruption of the hydrophobic pocket that would otherwise accommodate the pentyloxy section of the Oseltamivir molecule (Figure 10) (Collins, 2008). Several influenza strains have already acquired resistance against oseltamivir including several isolated cases of A/H5N1 (Horimoto & Kawaoka, 2005; Beigel, 2005; Le, 2005 & de Jong, 2005).

A significant rise in the frequency of viruses with mutations conferring oseltamivir resistance became apparent in circulating seasonal influenza A/H1N1 strains in the winter of 2007-2008. The majority of the mutations were characterized by the H274Y switch (Sheu, 2008). The spread of the oseltamivir-resistant virus reached such proportions that by the 2008-2009 influenza season, almost 100% of all tested seasonal A/H1N1 strains in the United States and numerous counties worldwide were found to be oseltamivir-resistant (CDC, 2009; WHO, 2008). Thankfully this seasonal H1N1 virus was displaced by the incoming A(H1N1)pdm09 virus, in 2009. Despite the widespread prophylactic and therapeutic use of oseltamivir during the early waves of the pandemic, oseltamivir-resistant mutations have not thus far become predominant in this strain as it continues to circulate world-wide.
Figure 10. Effect of H274Y mutation on the electrostatic surface potential and hydrogen-bond network of the binding pocket of A/H1N1 neuraminidase. The addition of the H274Y into the NA of A/H1N1, provoked a displacement of the glutamine at position 274 which subsequently results in a disruption of the hydrophobic pocket adversely effecting the ability of Tamiflu (Oseltamivir, yellow structure) to bind to the NA active site. (Adapted from: Vergara-Jaque et al., 2012).

With respect to A/H5N1, the efficacy of oral oseltamivir is limited. In a situation where the overall mortality is reported to be ~60%, the case fatality ratio among patients receiving oseltamivir is approximately 50% compared to nearly 90% of those not receiving oseltamivir therapy (Zhou, 2010; Beigel, 2005). However, the emergence of NAI-resistant strains have been reported in patients treated with oseltamivir (de Jong, 2005). In a case-series study of 8 patients from Vietnam, where a standard course of oseltamivir therapy was initiated a median of 6 days post onset of symptoms, two of the patients had treatment-emergent oseltamivir-resistant viral variants. The mutation in questions was the commonly observed histidine-to-tyrosine at position 274 (de Jong, 2005). This mutation results in oseltamivir having a >400-fold reduction in efficacy against the N1 NA; susceptibility to zanamivir remained unchanged (Ozawa, 2011; McAuley, 2010).
1.8.1.2 Zanamivir (Relenza)

In contrast to oseltamivir, zanamivir (5-acetamido-4-guanidino-6-(1, 2, 3-trihydroxypropyl)-5, 6-dihydro-4H-pyran-2-carboxylic acid) acts on neuraminidase without requiring a conformational change in the glycoprotein’s active site (Moscona, 2005a&b). However, zanamivir is administered via inhalation using a specialized devise and has a poor bioavailability profile (Min, 2007; Cooper, 2003). In special circumstances zanamivir has been given as an iv formulation.

1.8.1.3 NAI resistance

As mutations referring reduced sensitivity and resistance continue to arise and spread, it is essential that detailed surveillance programs track these changes. To date, the inhibitory activity of NAIIs against all 9 of the neuraminidase subtypes found in avian viruses predict that NAIIs will likely be active against any emergent pandemic strain originating from an avian host whether it be A/H5N1 or employing any other of the 9 neuraminidase (Hayden, 2009). Indeed oseltamivir has been used to treat patients infected with the emerging A/H7N9 virus in China, although in some cases resistance in the N9 NA conferred by R292K mutation has been selected (Sleeman et al., 2013). Alarmingly, recent experimental studies in guinea pigs indicates little fitness cost or reduction in transmissibility resulting from this mutation (Hai et al., 2013).

Usually mutations that confer resistance to one of the two licensed NAIIs dose not confer resistance to the other. However cross-resistance can occur, depending on the neuraminidase type and sub-type, and the particular neuraminidase mutation Abed et al., 2006; McKimm-Breschkin et al., 2003). I223R NA mutation is an example of cross-resistance, selected during treatment of a child infected with pH1N109 virus (LeGoff et al., 2012).

While the frequency of resistance-conferring mutations for NAIIs is considered to be less than that for M2-inhibitors, it is still not insignificant. This is especially true in paediatric populations where one study conducted in Japan found resistant-variants in nearly 1 in 5
paediatric patients (Thompson, 2004). While many NAI-resistant viruses usually demonstrate a reduced infectivity and virulence in animal models this is not always the case, for example the seasonal H1N1 viruses of 2007-08 (McAuley, 2007; Zamarin, 2006). As such, the rise and spread of NAI-resistant variants should be considered a key observational target for any surveillance program.

1.8.2 Adamantanes

Amantadine and rimantadine are both members of the adamantanes family. This class of drug exerts its effect by blocking the interior channel within the tetrameric helical bundle of the viral Matrix 2 (M2) protein, thus inhibiting the influx of H+ ions which are crucial for triggering the un-coating stage of the viruses replication cycle (Horimoto & Kawaoka, 2005; Sansom & Kerr, 1993). There is some considerable debate about whether the drug blocks by binding within the channel or on the periphery.

The Cochrane team have analysed the clinical data regarding efficacy of these compounds, and they find little evidence to support their therapeutic use. This is surprising because in vitro these compounds are very good at supressing influenza replication, and in preclinical animal models they are also effective. In terms of prophylaxis, amantadine was found to prevent 61% of confirmed influenza A infections and 25% of cases of influenza-like illness in prevention trials involving human test subjects; both of these results were deemed to be statistically significant (Jefferson, 2006). In contrast rimantidene, a preferred prophylactic due to fewer side effects, was not effective in preventing infection. As a therapeutic treatment, both amantadine and rimantidine significantly shortened the duration of fever when compared to placebo; however, no significant effect was seen in terms of nasal viral shedding or persistence of the virus in the upper airway after 5 days of oral or inhaled treatment (Jefferson, 2006).

The side effect profile of amantadine includes gastrointestinal symptoms (mainly nausea), insomnia and hallucinations. CNS adverse effects and side effects requiring withdrawal from trials were significantly more frequent in subjects receiving amantadine than those given rimantadine (Jefferson, 2006).
Rimantadine was found not to be an effective prophylaxis against either confirmed cases of influenza or influenza-like illness (Jefferson, 2006). In a treatment role for influenza infection, rimantadine was found to significantly shorten the duration of fever when compared to placebo however, no significant effect was seen in terms of nasal viral shedding or persistence of the virus in the upper airway after 5 days of treatment (Jefferson, 2006). However, no statistical difference in efficacy between amantadine and rimantadine was found and both were determined to have mainly symptomatic effect on influenza A, since they do not prevent infection and do not affect viral shedding (Jefferson, 2006).

The clinical usefulness of the adamantanes as a monotherapy has been practically obliterated due to the near complete displacement of A/H3N2 adamantane-sensitive strains by resistant strains since 2005 (Deyde, 2007). Early surveillance studies reported that primary resistance typically occurred at low frequencies among circulating seasonal strains that had a frequency of resistance between 1-3% (Hayden, 2009). However, during the 2003-2004 influenza season, an increasing frequency in M2-inhibitor resistance to A/H3N2 was noted in isolates from China and Hong Kong (Figure 11). This resistance continued to spread and by the 2005-2006 season, had reached global distribution with >90% of isolates in Asia and the United States and ~50% of European isolates being resistant to M2-inhibitors (Hayden, 2009).
Figure 11. The rise of adamantane-resistant A/H3N2 viruses, 1994–2005. Adapted from: Bright et al., 2005.

The M2-inhibitor resistance in recent isolates of A/H3N2 and A(H1N1)pdm09 was identified as being caused by a serine-to-asparagine substitution at position 31 in the M2 protein (Saito, 2003; Holsinger, 1994). As a result of this natural resistance-conferring mutation, in order to work with this virus in the lab, it is necessary to engineer in a N31S backmutation. Unfortunately, resistance to either of the adamantanes confers resistance against both drugs. In laboratory and epidemiology studies, the mutation does not confer a loss of biological activity or fitness and resistant virus is able to spread from human-to-human without any additional hindrance (Hayden, 2009).

Alarmingly, M2-inhibitor resistance has also been described in some A/H5N1 viruses (Tumpey, 2005). The same mutation seen in some A/H3N2 (a serine-to-asparagine substitution at position 31) has been found in clade 1 viruses circulating in Vietnam, Thailand and Cambodia (Cox, 2007). A relatively high frequency of resistance has also been noted in the clade 2.1 viruses circulating in Indonesia due to either a substitution at position 31 or position 27 (WHO, 2005). However, over 90% of clade 2.3 viruses, which circulate
across Eurasia to Europe and Africa, have maintained their sensitivity to the M2-inhibitors (WHO, 2012).

1.8.3 Peramivir

Peramivir, a next generation cyclopentane derivative of a neuraminidase inhibitor, has been shown to demonstrate an equal, if not greater, inhibitory effect against influenza when compared to the first two neuraminidase inhibitors both in vitro and in vivo models (Bantia, 2006; Chand, 2005; Bantia, 2001). Of great interest is that in comparative studies, strains resistant to zanamivir and oseltamivir were susceptible to peramivir (Mishin, 2005). Despite these promising results, the in vivo protection of peramivir is dependent on the route of administration due to low oral bioavailability and is thus more effective if administered as an IV preparation (Barroso, 2005).

1.8.4 Laninamivir

Already approved for clinical use in Japan, laninamivir, is designed to have long retention time in tissues, such that a single inhaled dose (of either 20mg or 40mg) remains present in target tissues and effective for ~5 days (Hayden, 2012; Yamashita, 2010). In contrast, conventional NAIs must be taken twice daily for 5 days to achieve and maintain effective dose levels. Laninamivir, has a similar antiviral spectrum to zanamivir and suitable for use in paediatric patients infected with seasonal oseltamivir-resistant, A/H1N1 (Sugaya & Ohashi, 2010; Yamashita, 2009).

1.8.5 Ribavirin

Well recognised as a broad spectrum antiviral, ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide), is a guanosine analogue that has shown activity against RSV,
hepatitis, herpes and influenza (Eggleston, 1987). Its mode of action is to target a key enzyme required for viral replication, inosine 5’-monophosphate dehydrogenase, which is associated with viral RNA synthesis. This drug has been shown to be highly effective against influenza both in vitro and in vivo models and, to date, there have been no reports of influenza-associated resistance. This lack of resistance may be due to the fact that ribavirin is rarely used in a clinical setting as it performed poorly in clinical trials and has been implicated as having potential teratogenic properties and the ability to cause haemolytic anaemia (Rodriguez, 1994; Smith, 1980; Cohen, 1976). Nonetheless, it has been administered to patients with severe influenza (A(H1N1)pdm09) and infection with avian influenza viruses (A/HSN1). In terms of severe A/H1N1, there are reports of ribavirin being given to ventilated patients as part of combination therapy. However, the results seem to indicate that triple therapy employing ribavirin was no more successful than simply using oseltamivir monotherapy (Kim et al., 2011). These results are further supported by work conducted by Ilyushina et al., (2008) where the effectiveness of employing Oseltamivir-ribavirin combination therapy in mice infected with two different strains of A/H5N1 was evaluated. They found that combination therapy including ribavirin demonstrated a greater or lesser antiviral effect than monotherapy with Oseltamivir, and that the effectiveness depended on the A/H5N1 virus and the concentrations used.

1.8.6 Taribavirin

A carboxamidine analog of ribavirin, taribavirin, has been shown by Sidwell et al., (2005) to have significant antiviral effects on influenza A and B infections both in vitro and in vivo. In Phase IIb testing as a drug candidate against hepatitis C viral infections, it demonstrated equitable activity as ribavirin but with much improved toxicity and bioavailability profiles (Sidwell, 2005). Although there are not currently any clinical trials involving taribavirin and influenza, the improved toxicity and bioavailability profiles could make taribavirin an attractive candidate for further anti-influenza examination.
1.8.7 DAS181 (Fludase)

A recombinant fusion protein, DAS181 incorporates the sialidase from Actinomyces viscosus and acts by targeting and cleaving sialic residues on host cells (Belser, 2007; Malakhov, 2006). It has been shown to cleave both α(2,3)- and α(2,6)-linked sialic acids (Triana-Baltzer et al., 2010). In the mouse model, DAS181 was found to be protective against a lethal challenge of A/H5N1 (Belser, 2007). As this drug targets the host and not the virus, it is likely that it will be effective against all influenza strains and that resistance is unlikely to arise. Moss et al., (2012) recently published a phase II clinical study showing that DAS181 was effective at lowering both viral load and duration of viral shedding. Moreover, despite targeting a host function the drug was reasonably well tolerated. DAS181 is also being used to treat parainfluenza virus infection and in severe cases has been found to improve symptom scores, as well as oxygenation levels and pulmonary function tests. In addition, nasopharyngeal samples have demonstrated that DAS181 in capable of reducing patient viral load (Chen et al., 2011).

1.8.8 T-705 (Favipiravir)

A pyrazinecarboxamide derivative, T-705, has demonstrated broad spectrum antiviral activity against a number of RNA viruses including arenavirus, West Nile virus, Yellow Fever and all three types of influenza (Yuan, 2013; Furuta, 2009). T-705 undergoes ribosylation and then phosphorylation to a nucleoside and thus acts as an RNA polymerase inhibitor (Furuta, 2009). With respect to influenza, T-705 has shown effectiveness against a wide array of different influenza strains both in vitro and in vivo. Sidwell et al., (2007) noted that while T-705’s efficacy was less than that exerted by oseltamivir or zanamivir, it still offered markedly better protection than ribavirin. Furthermore, they found that in mice, even a single dose treatment of T-705, could offer protection up to 60hrs post inoculation with a lethal challenge. Attempts to generate resistance to T-705 in the laboratory showed a high barrier, and led to the suggestion that at least part of the antiviral activity was based
on induction of a higher rate of mutations, perhaps due to base mispairing, adding to error catastrophe (Baranovich et al., 2013).

1.8.9 Nitazoxanide

Already a well-accepted anti-parasitic agent, nitazoxanide (and its active metabolite tizoxanide) have recently received attention for its potential ant-influenza properties. A member of the thiazolide family, nitazoxanide is thought to effect viral replication of seasonal A/H1N1 (PR8) by targeting the viral haemagglutinin at the post-transcriptional level (Rossignol et al., 2009). It is believed that this occurs by blocking a stage preceding resistance to endoglycosidase H digestion and therefore inhibiting intracellular trafficking of the glycoprotein to the cell membrane however, the exact mechanism remains unclear. In vitro experiments have demonstrated that nitazoxanide possesses potent antiviral activity against both human and avian influenza viruses, including Oseltamivir-resistant and amantadine-resistant strains. In addition, nitazoxanide is synergistic in combination with the neuraminidase inhibitors Oseltamivir and zanamivir (Belardo et al., 2011). A Phase 2B-3 clinical trial conducted in the United States looked at treating acute uncomplicated influenza with nitrazoxanide. Approximately half of the influenza-infected patients enrolled in the trial were infected with influenza A/H1N1 with approximately 30% being infected with influenza B and 20% with A/ H3N2. There results indicated that influenza-infected patients treated with NT-300 (nitrazoxanide) administered 600mg twice daily for five days experienced statistically significant reduction in time from beginning treatment to alleviation of flu symptoms when compared to placebo (P=0.008) (Rossignol et al., 2011).

1.8.10 RNA inhibition

In recent years, there have been a growing number of interesting preclinical reports regarding the use of siRNAs and antisense strategies for the treating of influenza and other respiratory viral infections (Devincenzo, 2012; Zhou, 2007). AVI-7100 is a phosphodiamidate
morpholino oligomer which contains three modified linkages and has been designed to interfere with the translation of both the M1 and M2 mRNAs has been highlighted as holding possible clinical application (Hayden, 2012). This oligomer targets the translation initiation start site which, is shared by both the M1 and M2 mRNAs. This molecule has been shown to be effective against oseltamivir-resistant A(H1N1)pdm09 virus infection in the ferret model. Specifically, when administered intranasally or intraperitoneally, AVI-7100 was found to reduce both BAL and nasal virus loads and reduce symptomology (Iversen, 2011).

1.9 Immunomodulation

Directly acting antiviral described above have been used in the clinic but the controversy surrounding their efficacy as measured by reduction of symptoms has led to the idea of controlling the immunopathology associated with influenza infection as a therapeutic strategy. However, this in itself is also controversial for if an over suppression of the immune response was to occur, unchecked viral replication might take place and overwhelm the host. Thus, targeting specific components of the innate immune response linked has been proposed as a possible novel therapeutic route for severe influenza.

1.9.1 Steroids

The transrepression effect of corticosteroids occurs within a few hours after administration, when nuclear transcription factors such as NFkB and AP-1 are sequestered in the cytosol by monomeric glucocorticoid-glucocorticoid receptor alpha (GGRa) complexes (Annane, 2011; Auphan et al., 1995). This in turn, prevents the downstream transcription of genes encoding for the majority, if not all, proinflammatory mediators (Annane, 2011; Ehrchen et al., 2007). In contrast to their transrepressive effects, the transactivation effect of corticosteroids requires several days post administration (Annane, 2011). This process involves the GGRa complex to enter the nucleus where, via glucocorticoid-responsive elements, it up-regulates genes encoding for regulators that are responsible for the
termination of inflammation (Annane, 2011). Following this, other anti-inflammatory processes, including chemokinesis, phagocytosis and antioxidative processes, become activated (Annane, 2011). Thus, one might envision that corticosteroids do not strictly inhibit cellular processes; rather, they divert them to alternative functions that, go against the demands placed on immune cells at that time. Furthermore, corticosteroids induce the migration of anti-inflammatory monocyte subtypes to inflamed tissues and enhance the survival of these same cells by triggering an anti-apoptotic effect via the A3 adenosine receptor (Barczyk, 2010).

Glucocorticoids, a subgroup of corticosteroids, have been demonstrated in clinical trials, using prolonged low-to-moderate doses, to enhance the anti-glucocorticoid-activated GC receptor (Pawliczak, 2005). This receptor acts as a major regulator of inflammation in ALI/ARDS, a prominent feature of severe influenza (Meduri, 2009). In support of the clinical trial observations, glucocorticoids have been demonstrated to suppress cytokine expression and inhibit COX-2 expression, an IFN-induced pro-inflammatory gene, in ex-vivo human bronchial epithelial cell cultures (Pawliczak, 2005; Almawi; 1996).

The efficacy of employing systemic corticosteroids for the treatment of ARDS has been the subject of considerable debate and study. Thus far, their therapeutic value remains unclear with several meta-analysis and reviews offering conflicting evidence and opinions (Agarwal, 2007; Tang, 2009; Steinberg, 2006). Endogenous glucocorticoids play an important part in suppressing local inflammatory responses and low-level systemic inflammation but may not be effective in suppressing the “cytokine storm” driven by unregulated inflammation, even though cortisol levels have been positively correlated with illness severity and mortality (Annane, 2000). With the rise in acceptance of the critical-illness-related-corticosteroid insufficiency (CIRCI) concept and the results of clinical trials demonstrating respiratory immune and hemodynamic benefits of such an approach, corticosteroid therapy has re-emerged as a promising adjunct for the treatment of severe sepsis (Marik, 2008).

Animal studies from the 1950s employing mice infected with influenza A virus and treated with either cortisone and hydrocortisone during the first 4 days of influenza infection lowered the median lethal dose (LD50) of the viral inoculum and increased the viral
titer in the lung (Kass, 1954). More recent studies involving A/H5N1 and glucocorticoid therapy in mice have also found results along similar lines (Salomon, 2007; Xu, 2009). This is further supported by a study conducted by Lee et al., (2009) where patients hospitalized with seasonal A/H1N1 pneumonia and treated with glucocorticoids showed higher viral titers and prolonged viral shedding when compared to non-glucocorticoid-treated patients. This finding was reiterated in recent cases of H7N9 and the higher viral loads may also have allowed development of oseltamivir resistance in these individuals (Hu et al., 2013).

There exists considerable debate regarding the effectiveness and safety of using steroids in patients with severe influenza infections due to the lack of controlled clinical trials. Most of the treatment guidelines for corticosteroid use in influenza patients has been extrapolated from experiences reports relating to the 2002-2003 severe acute respiratory syndrome coronavirus (SARS-CoV) outbreak (Cameron et al., 2008).

Pandemic influenza variant-A/H1N1 was first isolated and described in Mexico in April 2009. Less than 3 months latter on the 11th of June, the World Health Organisation (WHO) declared the first pandemic of the 21st century with mortality rates as high as 38% being reported (Rello, 2009). As of March 2010, more than 214 countries and territories had reported laboratory-confirmed cases of A(H1N1)pdm09 resulting in 17,919 deaths (WHO update 98, 2011). The pulmonary presentation of A(H1N1)pdm09 in infected patients was commonly characterised as a rapidly progressing viral pneumonia with bilateral alveolar infiltrates, visible on chest radiograph, and ARDS (Webb, 2009). Interestingly, Bermejo-Martin et al., (2009) noticed that patients who tended to have a severe presentation (ALI/ARDS with severe refractory hypoxemia) were also found to have an abnormal innate immune response and an exaggerated trafficking of neutrophils and macrophages. Postmortem examination of patients who had succumbed to A(H1N1)pdm09 revealed three distinct abnormalities: classic exudative diffuse alveolar damage, severe necrotizing bronchiolitis with extensive neutrophilic inflammation of bronchiolar walls and diffuse alveolar damage with alveolar hemorrhage often present (Nin et al., 2012). This damage is believed to induce the release of large quantities of pro-inflammatory cytokines, superoxides and reactive oxygen & nitrogen species from the lung parenchyma which go on to induce further “cytokine-mediated” damage (Vlahos, 2011; Kobasa, 2007).
In addition to traditional supportive measures and antiviral therapy, many clinicians administered corticosteroids to patients who developed ALI/ARDS secondary to A(H1N1)pdm09 infection. Two sizable studies of severe respiratory failure associated with A(H1N1)pdm09 reported that between 59 and 69% of patients were given corticosteroids, primarily as an adjuvant – a pharmacological or immunological agent that modifies the effect of another agent(s) (Kumar, 2009; Dominguez-Cherit, 2009). Their rationale was that if the corticosteroids could reduce lung inflammation, clinical outcome could be improved. However, investigators later concluded that corticosteroid administration may actually have deleterious effects in cases of severe influenza pneumonia.

Martin-Loeches et al., (2011) reported the analysis of a large cohort prospective multicenter study which found that, early use of corticosteroids in critically ill patients admitted to the ICU due to influenza A(H1N1)pdm09 virus infection did not result in a reduction of mortality. However, they found that treatment with a corticosteroid was associated with an increased risk of bacterial or fungal super-infection. The authors however, failed to exclude patients with confounding conditions including age, asthma, COPD and chronic use of steroids. Furthermore, the non-influenza ARDS control group contained a substantial number of patients receiving low-to-moderate doses of glucocorticoids (levels associated with reduced mortality in ARDS patients) thus potentially skewing the mortality results.

This work was additionally supported by a cohort study conducted by Brun-Buisson et al., (2011) in which it was reported that corticosteroid therapy administered within 2 days of ARDS presenting was not associated with an improved outcome. Conversely, it was found that commencing treatment with a corticosteroid within the first three days of developing ARDS was actually associated with a significantly poorer outcome. Taken together, their findings suggest that very early administration of corticosteroid therapy might actually be detrimental in cases of severe influenza pneumonia by limiting the host defenses to such a degree that persistent viral replication is allowed to take place (Giannella, 2011).

In a retrospective cohort study conducted by Han et al., (2011), the early use of parenteral corticosteroids for the treatment of fever and pneumonia prevention associated with influenza A(H1N1)pdm09 infection led to an increased risk of developing subsequent
critical illness or death. Alarmingly, this finding was specific to the effects of the glucocorticoids rather than to any other treatment or underlying condition and demonstrated a dose-response effect.

Kim et al., (2011) concluded that the addition of adjuvant corticosteroid therapy among 245 critically ill patients (136 of which met the criteria for ARDS) admitted to ICU or requiring mechanical ventilation with influenza A(H1N1)pdm09 infection was associated with an increased 90-day mortality rate (58% versus 27%, $P < 0.001$) and an increased risk of developing secondary bacterial pneumonia or invasive fungal infection. The short-term mortality rates of the steroid treatment group were similar to the non-treatment group, while the long-term mortality rates were higher for the steroid-treatment group indicating the possible effect of the superinfections. If however, one examines only the subgroup of 136 patients with ARDS, the association between increased risk of death and steroid use is no longer statistically significant, likely due to an issue of power. Furthermore, it is important to point out that, although small in number, this study did not distinguish between patients who received steroids for underlying conditions from those who received steroids for severe A(H1N1)pdm09 infection and refractory shock.

While there is a large body of compelling evidence against the use of steroids in cases of influenza-induced ALI/ARDS due to detrimental outcomes, other investigators have presented positive evidence which leaves the story open to further debate. For instance, a small ($n = 13$) 2010 prospective study of A(H1N1)pdm09 infection in patients demonstrated that low-to-moderate dose corticosteroid treatment was well tolerated and associated with a significant reductions in lung injury, multiple organ dysfunction scores, and hospital mortality (Quispe-Laime, 2010).

A Finnish prospective observational study examined ICU admissions between 11 October and 31 December 2009 across the country during the pandemic. In total, 132 ICU patients tested PCR positive for A(H1N1)pdm09, with 58 patients progressing to ARDS. Oseltamivir was given to 96% of all patients while corticosteroids were given to 55% of all patients and 79% of ARDS patients. Patients who received steroids were significantly more severely ill at presentation than those not given corticosteroids. Both crude hospital
mortality and SAPS II-adjusted mortality indicated that there was no significant difference between patients treated with corticosteroids and those who did not receive this treatment.

A study conducted by Kil et al., (2011) examined the effects of corticosteroid treatment in two paediatric populations in South Korea (n = 32, age range = 4-18, median age = 6). Patients presented on admission, or shortly thereafter, with pneumonia and exhibited respiratory distress with hypoxemia. The therapy regime consisted of oseltamivir in conjunction with a rapid, high-dose of methylprednisolone, 10 mg/kg/day and tapered off within a week. The short duration of the course avoided any complications that normally arise with long-term corticosteroid use and was effective at halting clinical and radiographic exacerbation as well as preventing progression to ARDS. While the study is based on a small patient population, it does raise some interesting points in terms of corticosteroid therapy in a paediatric population.

In a prospective, observational, Spanish multicentre study performed in 148 ICU (covering approximately a third of ICUs in Spain) and involving 372 patients with viral pneumonia, Diaz et al., (2012) found that corticosteroid therapy, when adjusted for case severity and potential confounding factors, was not significantly associated with mortality. Furthermore, it should be noted that there may be other influenza associated complications, such as influenza-associated encephalopathy, where the benefit of corticosteroids may outweigh the risk (Ito, 2011).

Throughout the 2009 pandemic, the World Health Organization did not recommend the use of systemic high-dose corticosteroids for influenza-associated pneumonia. They concluded that prolonged use of systemic high-dose corticosteroids could result in serious adverse events in influenza virus-infected patients, including opportunistic infection and prolonged viral replication. However, physiological replacement doses could be indicated in adrenocortical deficiency states and septic shock when hypotension is poorly responsive to fluid and vasopressor therapy (Dellinger et al., 2013). Despite the WHO guidelines, there was great deal of variability in terms of national and regional protocols for using corticosteroids in patients with severe A(H1N1)pdm09 infections.

It is possible that clinicians were more inclined to give steroids to patients they deemed to be “sicker,” thus biasing the results against the possibility that a steroid therapy
would be beneficial. Furthermore, the timing and doses of steroids were not controlled in the majority of studies; despite some researchers attempting to address this issue by adopting more rigorous inclusion criteria for patients or analyzing mortalities in treatment groups based on the timing of steroid administration. It is important to note that given the acute nature of the A(H1N1)pdm09 influenza pandemic, it was simply not feasible to conduct a prospectively designed, randomized clinical trial to address all these shortcomings. There are numerous examples (dopamine, epinephrine, albumin and synthetic colloids) of treatment regimens that were found to be harmful in cohort studies but not subsequently in randomized trials.

1.9.2 Cyclooxygenase Inhibitors:

In the context of viral infections, the production of exogenous pyrogens (PAMP) stimulates leukocytes resulting in NFkB activation and the up-regulation in the synthesis and release of a variety of endogenous pyrogens (IL-1β, IL-6 and TNF-α). These endogenous pyrogens stimulate the central production of the inducible enzyme cyclooxygenase (COX) and subsequently the production of prostaglandins. These prostaglandins are then able to cross over leaky areas in the blood brain barrier via the circumventricular vascular organs (the oranum vasculosum of the lamina terminalis) and activate the thermoregulatory neurons of the anterior hypothalamic area to elevate body temperature (Watkins et al., 1995). The COX enzyme has been found to exists in two isoforms – COX-1 and COX-2 with both of these isoforms being involved in numerous physiological processes. COX-1 is constitutively expressed and has been implicated in maintaining normal renal function, regulating the gastrointestinal tract’s mucosal protection as well as numerous additional functions (Vane & Blotting, 1998; Rao & Knaus, 2008). COX-2 on the other hand, is believed to play a role in mediating pain, inflammation and fever and the expression of which, been shown to be induced by cytokines and numerous inflammatory mediators (Vane, 2000; Vane & Botting, 1998).

Carey and colleagues (2005), sought to elucidate the impact of inhibiting individual isoforms of the COX enzyme. C57BL/6J x 129/O1a mice that had either their COX-1 or COX-2
genes “knocked out” were infected with A/Hong Kong/8/68 (H3N2) and their response to viral infection was compared to wild type controls. COX-1 (-/-) mice showed enhanced inflammation and up regulation in the production of pro-inflammatory cytokines while the levels of PGE2 in the airways was reduced. In the COX-2 (-/-) there was observed a marked blunting of IFN-γ expression which was linked to milder clinical signs but also markedly elevated viral lung titers and attenuation in the recruitment of inflammatory cells. Interestingly, in a follow-up study by the same group, mice with a C57BL/6J x 129/O1a (COX intact) genetic background were given equivalent doses of a type 1 and type 2 COX inhibitor for two weeks prior to infection with A/Hong Kong/8/68 (H3N2). Lungs were harvested on day 1 and 4 post-infection and the viral titers were examined. While there was no statistical significant difference between the groups – although there is a trend where the COX-2 inhibitor led to higher viral titers in the lung (Carey et al., 2010).

Antipyretic agents, such as NSAIDs, work by lowering the elevated thermal set-point by inhibiting the COX enzyme and by which, inhibit the transformation of arachidonic acid to prostaglandins. The introduction of COX-2-selective agents lead to improved specificity and decreased side-effects for users (most notably improved gastrointestinal safety) and ultimately contributed to a general increase in the use of NSAIDs. Chronic use of NSAIDs has been shown to increase with age and it has been estimated that 10-40% of adults over the age of 65 use a prescribed or over-the-counter NSAID daily (Hawkey et al., 2000; Lanas & Ferrandez, 2007).

The use of antipyretics during a fever attributed to an influenza infection has been a widely debated topic and to date, no clear consensus nor guidance dealing with this issue exists. Shimazu (2009) suggests that the virulence of the 1918 pandemic was enhanced by the use of aspirin and during the early stages of the last pandemic (H1N1) and subsequently, Hama (2009) had suggested that an urgent and critical review be undertaken to assess the use of NSAIDs in Mexico during the start of the most resent pandemic. With conflicting advice originating from numerous organisations and governmental bodies (including the WHO and the HPA), the need for a comprehensive review and evaluation of current data is imperative.
1.9.3 Macrolides

A class of bacteriostatic protein synthesis inhibitors, which includes clarithromycin, azithromycin and erythromycin, that have been shown to have a wide range of anti-inflammatory activity. They have been demonstrated to inhibit the production of a large number of cytokines from monocytes and lung epithelia including IL-1, IL-8, TNF-α, eotaxin and RANTES (Culic, 2001). Possibly contributing further to their anti-inflammatory action, these drugs also are inhibitors of oxidative burst, NO production, degranulation, leucocyte chemotaxis and expression of antigen receptors (Culic, 2001; Schultz et al., 2000). Azithromycin and clarithromycin are notable for accumulation in the cells fluids of the lung of patients – suggesting that they will have strong action in these areas (Hoffman et al., 2003).

It has been suggested that macrolides could prove beneficial in cases of influenza infection by interfering with the viral replication cycle mainly by inhibiting intracellular haemagglutinin HA0 proteolysis (Min & Jang, 2012). Sato et al., (1998) demonstrated that when mice were challenged with a lethal dose of A/H3N2 (A/Kumamoto/Y5/67), erythromycin strongly inhibited inflammatory cellular responses and resulted in a dose-dependent decrease in the level of nitric oxide (NO) in the serum and a reduction of NO-synthase (NOS) in the lungs of the mice. As a result, the administration of erythromycin significantly improved the survival rate of influenza-infected mice. In addition, clarithromycin has been shown to inhibit the activation of NFkB, the migration of neutrophils, and the production of pro-inflammatory cytokines by interfering with extracellular signal-regulated kinases (Min & Jang, 2012; Aoki & Kao, 1999; Labro, 1998).

1.9.4 Sphingosine-1-phosphate receptor agonists

Sphingosine-1-phosphate (S1P) is a blood borne signalling sphingolipid with numerous cardiovascular and circulatory functions (Rosen & Goetl, 2005). In addition to these effects, S1P has been linked to an increase in mast-cell-derived pro-inflammatory
mediators (Rosen & Goetl, 2005). Thus, suppression of S1P could impede this inflammatory activation and prove beneficial in cases of severe influenza infection. Marsolais et al., (2008) published a proof-of-concept paper indicating that modulation of lung S1P receptors during an active influenza infection alters dendritic cell (DC) activation, resulting in blunted T-cell response resulting in a decrease in characteristic immunopathological features of such an infection. This same group have also demonstrated that the S1P4 receptor is an activator of the ERK, MAPK, and PLC cytokine/chemokine signaling pathways and may help explain the anti-inflammatory effect of S1P receptor agonists (discussed further in Section 1.9.7) (Toman & Spiegel, 2002).

Walsh et al., (2011) examined the effects of blocking S1P with a sphingosine analog in mice infected with the influenza virus (A/California.04/2009, A/H1N1). They found that blocking S1P provided significant protection from mortality (82%) over that of Oseltamivir alone (50%). Intriguingly, a combination of the sphingosine analogue along with Oseltamivir provided maximum protection against a lethal challenge of influenza virus (96%). Importantly, the sphingosine analogue inhibited cellular and cytokine/chemokine responses thus limiting immunopathology while at the same time maintaining host control over virus replication. Complementing these results is the work by Teijaro et al., (2014) where S1P receptor agonists were found to be effective at blunting clinical signs and histopathology in ferrets infected with A/H1N1 (A/California.04/2009). Unfortunately, due to the unavailability of reagents, an examination of cytokines/chemokine levels was not possible. However, of great importance, was the observation that (as in the mouse model), inhibition of the inflammatory process did not result in increased viral replication.

1.9.5 HMG-CoA reductase inhibitors

HMG-CoA reductase inhibitors (commonly known as statins) are a widely used class of cholesterol lowering drugs. They exert their anti-cholesterol effect by inhibiting the function of liver HMG-CoA reductase, an enzyme crucial to the synthesis of cholesterol, and by up-regulating low density lipoprotein (LDL) receptors (Ma et al., 1986). These subsequently bind circulating cholesterol facilitating cellular reabsorption (Ma et al., 1986).
In addition to this class of drugs anti-cholesterol activity, statins have also been demonstrated to reduce inflammation (Lahera et al., 2007). Simvastatin, a member of the statin drug family, has been shown to dramatically reduce the induction of proinflammatory cytokines by C-reactive protein in a human umbilical vein endothelial cell inflammatory model and in the mouse model, was found to induced pronounced endothelial barrier protection in times of acute lung injury (Jacobson et al., 2005).

Due to the anti-inflammatory and immunomodulatory effects which could be beneficial in circumstances of acute lung injury, Fedson (2006) has suggested that statins might prove to be beneficial in the treatment influenza infections. This view is echoed by Terblanche et al. (2006) who also suggests that statins might be useful against infectious diseases that are characterized by hyperinflammatory responses, such as influenza. It has been postulated that the anti-inflammatory response elicited by statins occurs via the blocking of influenza -mediated activation of RhoAGTPase protein although the exact mechanism of action is yet to be elucidated (Haidari et al., 2007).

Supporting the potential benefit of statins during an active influenza infection are the results from several retrospective cohort and case-control studies (Kwong et al., 2009; Frost et al., 2007). One such study conducted by Vandermeer et al., (2012) found that when adjusted for age, race, cardiovascular disease, chronic lung disease, renal disease, influenza vaccine receipt, and initiation of antivirals within 48 hours of admission, statins were associated with a decrease in odds of dying among cases hospitalized with laboratory-confirmed cases of influenza. While these results appear quite promising, other studies have reported that statins did not correlate with positive respiratory infection outcomes and as such, there is still considerable debate in this area (Fleming et al., 2010; Smeeth et al., 2009).

1.9.6 Peroxisome proliferator-activated receptor agonists

Another class of immunomodulators are the peroxisome proliferator-activated receptor (PPAR) agonists. Composed of two classes (PPAR-alpha agonists and PPAR-gamma
agonists) these drugs are indicated for alleviating the symptoms associated with various metabolic syndromes (Fievet et al., 2006). Recently, PPARs have been evaluated for their anti-inflammatory abilities in the context of influenza infections. Gemfibrozil, a PPAR-α agonist with a long history of clinical use for lipid reduction, has been noted to exert pleiotropic effects including the reduction of TNF-α and Interferon-γ in serum (Madej et al., 1998). In vivo mice studies have demonstrated a significant mortality reduction from H2N2 challenge virus when animals received gemfibrozil when administered as late as four days post-infection (Budd et al., 2007). While no direct antiviral mechanism has been described for this class of drugs, the in vivo results indicate that the anti-inflammatory effects of PPAR agonists enhance survival in cases of influenza infection.

Taken together, these results providing a proof of principle indicating that immunomodulation might provide a beneficial and complementary arm in the treatment of severe influenza infections. The “silver bullet” would be a treatment regime that enabled one to suppress deleterious over-induction of the innate immune response but did not facilitate an unregulated and un-checked increase in the level of viral replication.

1.9.7 Kinase inhibitors: a brief overview

Protein kinases are a class of enzymes that catalyse the transfer of the gamma phosphate (the phosphate furthest from the ribose and purine ring) of a purine nucleotide triphosphate (ATP or GTP) to the hydroxyl groups of their protein substrates. There are a reported 518 kinases in the human genome that can be divided into eight groups (Kontzias et al., 2012).

While the idea of targeting host kinases is appealing from many aspects, early work was fraught with toxicity, specificity and efficacy complications (Kontzias et al., 2012). Compounding the situation was the fact that all protein kinases bind ATP and that the majority of enzymes use ATP as a substrate; therefore, achieving a limited and targeted effect posed a daunting task as one has to be careful to not effect more than the desired target through structural similarity of the inhibitor to ATP. However, significant progress was
achieved when the differences between subsets of kinases began to be elucidated allowing for a more targeted approach. For example, a significant subset of protein kinases (including Src, p38 MAPKs) were found to possess a threonine residue at a “gate-keeper” site creating a hydrophobic pocket close to the ATP binding site. The addition of a bulky hydrophobic residue on to the end of a kinase inhibitor helps to create specificity for this subset of kinases, leaving the majority of other kinases unaffected, reducing undesirable side-effects while increasing efficacy (Bain et al., 2007).

The original development of kinase inhibitors for application in the treatment of human disease focused on malignancies. One of the first kinase inhibitors developed for clinical application was Imatinib (Kontzias et al., 2012). Imatinib was developed to target the Abl tyrosine kinase in chronic myeloid leukemia (CML). The translocation between chromosome 9 and 22 (specifically designated t(9;22)(q34;q11) commonly know as Philadelphia chromosome) associated with CML results in the generation of an oncogenic BCR-Abl fusion protein (Sawyers, 1999). The application of Imatinib in the clinical setting has revolutionised the treatment of CML by limiting the growth of tumour clones and decreasing the risk of the onset of the final phase of CML the much dreaded “blast crisis” (Gambacorti-Passerini et al., 2011). More recently, the use of kinase inhibitors has expanded beyond malignancies to autoimmune diseases with favourable safety profiles being reported (Kontzias et al., 2012).

With the majority of the major classes of receptors responsible for triggering immune cell activation signalling via protein phosphorylation, the targeting of host regulatory kinases presents an attractive target for drug intervention. Numerous receptors of immune cells (including T cell, NK, B cell and Fc receptors) have been demonstrated to be phosphorylated by, and to physically associate with, kinases with the first step of their respective activations involving the phosphorylation of receptor subunits on tyrosine residues (Janeway et al., 2001). Importantly, cytokine receptors (especially Type I&II) signal directly by activating kinases causing the activation of down stream signalling cascades (Alberts et al., 2002).
1.9.7.1 Kinase-driven signalling cascades and Influenza

The initiation of gene transcription in response to external stimuli occurs as a result of an intricate signalling cascade controlled through phosphorylation and de-phosphorylation of key regulatory subunits by kinases and phosphatases respectively. These signalling systems are key in facilitating an appropriate immune response from cells facing pathogenic insult. In these circumstances, the initiating stimuli often can include cytokines, chemokines, antigen-antibody complexes, reactive oxygen species, and pathogen associated molecules patterns (PAMPs) (Jeffrey et al., 2007).

With respect to infection, the family of mitogen-activated (MAP) kinases mediate a wide variety of cellular functions in response to extracellular stimuli (including viral infection) by acting as focal points in discrete signalling cascades. In mammalian cells, there are four major groups of MAPKs - the ERKs (Extracellular signal-Regulated Kinases, the JNKs (c-Jun NH2-terminal Kinases), the ERK5 (Extracellular signal-Regulated Kinase-5) and the p38MAPKs (Risco & Cuenda, 2012). These MAPKs are activated by dual phosphorylation at the tripeptide motif Thr-Xaa-Tyr, whose sequence is different in each group of MAPKs: ERK (Thr-Glu-Tyr); p38 (Thr-Gly-Tyr); and JNK (Thr-Pro-Tyr) (Roux & Blenis, 2004). Each MAPK pathway contains a three-tiered kinase cascade comprising a MAP3K (MAP Kinase Kinase Kinase), a MAP2K (MAP Kinase Kinase) and the MAPK. This three-tier module mediates ultrasensitive switch-like responses to stimuli (Risco & Cuenda, 2012; Roux & Blenis, 2004). This makes members of the MAPK family and components of their respective cascades attractive targets for novel therapeutic agents that seek to prevent over-induction of the host immune response or “cytokine storm” which has been demonstrated to occur with certain influenza strains (Fukuyama & Kawaoka, 2011; Osterholm, 2005; Chung et al., 2002).

Extensive and ground-breaking work has been conducted by Ludwig & Planz, wherein the potential of supressing part of the MAPK cascade as a treatment for influenza has been evaluated. Specifically, they have examined the potential of targeting the Raf/MEK/ERK pathways – a pathway which has been implicated to contribute to cytokine production and airway inflammation in response to influenza infection, as a novel therapeutic approach (Mizumura et al., 2003). In addition, there is evidence that this
pathway is involved in the efficient replication of the virus itself, suggesting that inhibition could lead to a dual effect of controlling virus as well as controlling immunopathology. The kinase pathway appears to be involved in the process of nuclear release of vRNP complexes for the nucleus in the latter stages of viral replication (Ludwig et al., 2009; Pleschka et al., 2008). Where the pathway is over-stimulated the viral titers recovered from MDCK cell culture are increased compared to the baseline pathway-expression groups (Marjuki et al., 2007; Olschlager et al., 2004; Ludwig et al., 2004). If however, this pathway is blocked by employing specific inhibitors, there is a striking impairment of viral growth for both type A and B viruses (Olschlager et al., 2004; Ludwig et al., 2004; Pleschka et al., 2001). While the exact mechanism is still under investigation, it has been hypothesised the this anti-viral effect may be due in part in the ability of the kinase inhibitors to prevent the phosphorylation of the viral proteins NEP and NP two of the viral proteins in the viral phosphoson (Planz, 2013; Hutchinson et al. 2012).

Another strand of research from the Ludwig and Planz groups has focused on the evaluation and development of nuclear factor-kappa B (NFkB) inhibitors against influenza virus infection. A transcription factor that controls the expression of numerous genes involved in an array of physiological responses to viral attack (including acute phase inflammatory responses, cell adhesion, oxidative stress response, apoptosis and generation of antiviral states), NFkB makes a tempting target for therapeutic intervention (Ludwig & Planz, 2008; Pahl, 1999). A member of the Rel family and composed of five homo- and heterodimer subunits, the NFkB transcription complex bind (as dimers) to DNA regulatory sites referred to as kappaB sites (Gilmore, 2006). The different NFkB transcription complex dimer subunits can act either as transcription activators (RelA, RelB or c-Rel) or suppressors (p50 and p52) (Gilmore, 2006). Although ubiquitously expressed, NFkB complex subunits target specific genes based on the cellular environment and the presence of signalling stimuli where they are located. In their inactive form, NFkB dimers are found in the cytosol bound to the inhibitor-of-kappa-B (IkB). After a stimulation signal is received via the cascade pathway, IkB is phosphorylated by the IkB kinase (IKKα/β), ubiquinated and degraded by the proteasome resulting in the release of the NFkB dimers (Scheidereit, 2006; Hoffmann et al., 2006; Bonizzi & Karin, 2004; Karin & Ben-Neriah, 2000). The unbound, activated NFkB is
then free to translocate to the nucleus to activate and suppress the transcription of specific response genes (Bonizzi & Karin, 2004; Karin & Ben-Neriah, 2000).

Studies conducted in MDCKs, Vero and A549 cell lines have demonstrated that when NFkB signalling is blocked, the production of progeny viruses is impaired (Wurzer et al., 2004; Nimmerjahn et al., 2004). To explain these results, it has been postulated that NFkB must be associated with viral-supportive functions. Namely, the generation of pro-apoptotic factors which help to facilitate the escape of vRNPs from the nucleus, suppression of IFN-induced gene (ISG) expression via cytokine signalling-3 (SOCS-3) and through the regulation of vRNA synthesis have all been proposed (Ruckle et al., 2012; Kumar et al., 2008; Pauli et al., 2008; Mazur et al., 2007; Wei et al., 2006).

Numerous groups have thus conducted screens to see which, if any, compounds known to act as kinase inhibitors found in pharmaceutical collections have anti-influenza activity. Martinez-Gil et al., (2013) report finding that compound ON108110 (a kinase inhibitor) is capable to inhibiting viral replication of not just the influenza A virus but also vesicula stomatitis virus (VSV) and Newcastle disease virus (NDV). The presence of ON108110 in cell cultures reduced expression of a transfected NP-firefly luciferace reporter by the viral polymerase complex in a dose dependent manner. While the levels of the Renilla transfection control were also affected, the fact that the levels of virus-specific transcript were reduced to a much higher degree suggests that ON108110 preferentially inhibits vRNA replication as opposed to a simple nonspecific or cytotoxic effect. As kinase have been shown to be involved in both the induction of the innate immune response and the viral replication process, they represent a tempting target for pharmacological intervention.

1.9.7.2 The kinase inhibitor RV1088

RV1088 is novel narrow spectrum kinase inhibitor that has been developed by the Imperial start-up company RespiVert for the treatment of respiratory disorders linked with a strong inflammatory response (i.e. asthma and COPD). It has been reported that this compound has been able to inhibit human rhinovirus (HRV) -induced IL-8 release and viral
replication in primary airway epithelial cells (Ito et al., 2011; Vuppusetty et al., 2011) and resulted in increased interferon beta and lambda in the presence of virus (Contoli et al., 2011). Personal communication from RespiVert colleagues revealed that RV1088 targets 3 host kinases involved in signaling networks linked to the innate immune response (p38 alpha/p38gamma, c-SRC and Syk). Further information about these kinase targets is discussed below. Unfortunately, due to proprietary restraints, we are unable to provide any further specific information of the detailed structure of this compound or its precise mode of action.

1.9.7.2.1 p38

Discovered in 1994 and originally referred to as cytokine-suppressive anti-inflammatory drug binding protein (CSBP), p38 is a member of the MAPK superfamily (Lee et al., 1994). p38 plays important roles in the signal transduction of a wide array of biological processes including cell maintenance and survival, apoptosis and the regulation of inflammatory cytokine biosynthesis (Lee et al., 1994). Thus far, four splice variants of p38 (α, β, γ, δ) have been described (Chung, 2011). Of these, p38α has been characterised to the furthest extent and is considered to be the most relevant in the inflammatory response (Kumar et al., 2003).

Several studies have linked p38-related cytokine/chemokine expression to the downstream activation and nuclear translocation of IRF3 and NFkB and subsequent IFN-b expression (Hui et al., 2009; Saccani et al., 2002; Uddin et al., 1999). The activation of NFkB via an anti-viral signalling cascade leads to the up-regulation of a variety of antiviral genes (Hiscott et al., 2001). However, a number of studies indicate that the influenza virus have acquired the ability to manipulate and use this pathway to their own advantage; exploiting NFkB for efficient replication (Nimmerjahn et al., 2004; Wurzer et al., 2004). In further support of this observation, Mazur et al., (2007), demonstrated that blocking the activation of NFkB with acetylsalicylic acid (ASA), a known suppressor at sub-toxic doses, resulted in blocking influenza virus replication by several orders of magnitude. It is thought that by blocking NFkB, ASA exerts antiviral effects by decreasing the expression of FasL and TRAIL,
reducing caspase activity and causing the retention of vRNPs in the nucleus as well as suppressing the inflammatory response (Mazur et al., 2007).

Furthermore, p38 MAPK has been shown to phosphorylate a variety of transcription, including some responsible for transcription of genes encoding for inflammatory cytokines (Kumar et al., 2003). In this regard, the p38 kinase has been demonstrated to be essential in the production of virally-induced IL-6, IL-8 and several other cytokines/chemokines (Griego et al., 2000; Hashimoto et al., 1999; Matsumoto et al., 1998). Lee et al., (2007) have reported the co-existence of hyper-activated p38 kinases in the presence of increased cytokine expression in the plasma of patients infected with severe influenza; suggesting a direct link between the level of p38 activity and plasma cytokine levels.

Lee et al., (2005) compared the activation of MAPK signalling pathways and the transcription factor NFκB in A/H5N1- and A/H1N1-infected PBMCs. Their results demonstrated that both of these viruses activate NFκB to comparable levels but that 1hr p.i. A/H5N1 hyperinduces p38 MAPK phosphorylation to a far greater extent than levels observed for A/H1N1. Börgeling et al., (2014) then examined the role of p38 signalling in the context of an A/H5N1 virus infection. They report that in the context of in vitro infection studies employing highly pathogenic avian influenza viruses (HPAIV), p38 controls interferon-stimulated gene expression via co-regulation of STAT1 which is achieved through phosphorylation at serine 727. Interestingly, through a global mRNA profile, they determined that the vast majority of HPAIV-induced genes were either partial (23%) or fully (71%) dependent on the p38 pathway.

Marchant et al., (2010) have demonstrated that prior to host cell entry, PRR recognition of the virus (i.e. recognition of sugars residues on the surface of the virion by TLRs and other such non-receptor host cell proteins) is required to stimulate MyD88 which, in turn, activates p38 and subsequently host cellular internalization machinery. The levels of activation of p38 during the first 10min of infection were found to be approximately 10-fold higher than for other respiratory viruses (RSV and HPIV3, for example) and may represent an effective adaptation mechanism to augment viral uptake by the host cell. Blocking p38 however, did not completely ablate influenza virus entry suggesting that there are also ancillary pathways which facilitate virus entry.
In addition to activating host cell virus internalisation machinery, p38 has been linked to other important stages of the influenza virus life-cycle. The export of vRNPs from nucleus to cytoplasm is a key event in influenza virus life-cycle. The NP protein, one of the component of the vRNP complex, is a phosphoprotein, the phosphorylation status of which, changes during the virus replication cycle and has been reported to affect export of vRNPs from the nucleus. It has been demonstrated that specific intracellular pathways are responsible for phosphorylation of NP (Ludwig, S et al., 2006; Palamara, AT et al., 2005). In particular, Nencioni et al., (2009) have demonstrated the involvement of p38 MAPK in this event. Furthermore, it has been demonstrated that p38 can mediate both apoptosis and cell survival through a variety of mechanisms.

One such mechanism involves the p38-mediated regulation of the apoptosis-controller B-cell lymphoma 2 (Bcl-2) protein. Bcl-2 is widely recognized as a cellular protective mechanism against virally-induced apoptosis and the interaction of p38 with Bcl-2 has been shown to disrupt the protective role of this protein and is accomplished via phosphorylation of Thr-56 and Ser-87 residues of Bcl-2 (De Chiara et al., 2006; Reed, 1994; Hinshaw et al., 1994). Some viruses, such as Herpes simples virus type-1, have developed ways of preventing the phosphorylation of Bcl-2 and thus suppressing virally-induced apoptosis thus freeing p38 to augment viral replication in the nucleus (Zachos et al., 2001).

The quantity of active p38 in the nucleus has been shown to be inversely related to the quantity of Bcl-2 substrate present in the cytosol. This is supported by the observation that in Bcl-2neg cells, the p38-mediated apoptosis is markedly diminished and that this kinase is found almost exclusively in the nucleus where it’s phosphorylation of key targets aids in the efficient export of vRNPs for the nucleus. The expression of Bcl-2 varies between cell types and of great importance is the finding that Bcl-2 is absent in normal, well-differentiated epithelial cells of the respiratory tract thus freeing-up p38 to act in the nucleus and augment influenza viral replication (Tesfaigzi et al., 1998).

Implicated in numerous signalling cascades linked to the over induction of the innate immune response as well as demonstrating pro-viral actions, p38 MAPK represents a unique and tempting target for therapeutic intervention.
1.9.7.2.2 c-Src

The Src family of protein tyrosine kinases (SFK) consists of eight members: Lyn, Hck, Lck, Blk, Fyn, Yes, Fgr and c-Src. These members have all been demonstrated to exert their effect via similar modes of action and are implicated in controlling and regulating a diverse array of signalling networks linked to regulating cellular metabolism, proliferation, viability, differentiation and migration of numerous cell lineages (Frame, 2006; Levin, 2004; Brown & Cooper, 1996). The substrate targeting and activity of the SFK members is highly regulated through intra-molecular interactions of the SH3 and SH2 domains of the kinase and by its association with ancillary molecules including substrates, activators and inhibitors (Sicheri et al., 1997; Xu et al., 1997). Based on biochemical and structural details, the active and inactive configurations of the SFKs have been elucidated as have their common modes of regulation including differential phosphorylation, domain interaction and competitive domain displacement (Xu et al., 1997). SFKs are controlled by receptor protein-tyrosine kinases, antigen- and Fc-coupled receptors, cytokine receptors, steroid hormone receptors and integrin receptors (Thomas & Brugge, 1997).

Proto-oncogene tyrosine-protein kinase Src (c-Src) is a non-receptor protein tyrosine kinase which acts by phosphorylating specific tyrosine residues on other proteins. It has been demonstrated that when TLR3 binds viral dsRNA it, acting via TICAM-1, it activates the transcription factors NFkB, IRF3, and AP-1 leading to cytokine, chemokine and type I IFN (Lowell, 2011; Oshiumi et al., 2003). Intriguingly, TLR3 has also been shown to associate with c-Src on endosomes in response to dsRNA and inhibit the activation of IRF-3; although the exact details of these pathways and their impact on innate immune signalling has yet to be fully elucidated (Lowell, 2011; Kim et al., 2009; Sarker et al., 2004). Thus if over expressed, c-Src could lead to inflammation with out concurrent IFN production. As such, inhibiting c-Src could help prevent this and restore a beneficial balance where IFN is induced but cytokine over induction is prevented. Chu & Yang (2007) reported that when multiple dengue infected cell cultures were treated with dasatinib (a known and specific c-Src inhibitor), this resulted in a dose-dependent inhibition of viral titers. This suggests that Src and its downstream effects may be beneficial for viral replication and that blocking those pathways may prove beneficial in supressing viral replication.
1.9.7.2.3 Syk

In contrast to the SFK, the Syk family (composed of Syk and ZAP-70) are characterised by the presence of two-tandemly arranged Src homology 2 (SH2) domains (Yanagi et al., 1995; Taniguchi et al., 1991). Activation of Syk occurs through the interaction of the two SH2s with immunoreceptor tyrosine-based activation motifs (ITAMs) located on immune response receptors (Turner et al., 2000; Sada et al., 2001). Syk has been implicated in numerous signal transduction pathways linked to the innate immune response (Turner et al., 2000). Studies conducted by Stenton et al., (2000) employed the aerosol delivery of Syk antisense oligodeoxynucleotides to rat lungs causing the suppression of Syk activation. This was in turn linked to the downstream suppression of macrophage activation and decreased pulmonary inflammation. This work was further supported by studies which have demonstrated that Syk antisense oligodeoxynucleotides have also been effective at suppressing Syk expression and chemokine production in human nasal polyps (Yamada et al., 2001). While much work is still needed to further elucidate and map the signalling pathways employed by Syk and it’s downstream effects, there is compelling evidence that this kinase is involved in the activation of the innate immune response in respiratory epithelium and that inhibiting Syk’s actions results in a blunting of this immune response.

1.9.7.2.4 Rational for kinase combination therapy

The concept of using kinase inhibitors, either alone or in combination, has already been examined, and found to be effective, as part of various cancer treatment regimes with promising preliminary results primarily with respect to controlling the immunopathologies associated with certain forms of cancer (Karashima et al., 2014; Donahue et al., 2013; Porta, 2011; Seliger et al., 2010). A similar approach in using a specific combination of narrow spectrum kinase inhibitors might prove far superior than employing just one, to achieve a good balance between inhibition of viral replication and cytokine dampening. Furthermore,
the benefit of employing such kinases may prove even more beneficial when used in combination with currently licenced antivirals.

1.10 Aims of Thesis

1. Determine if the kinase inhibitor RV1088 suppress cytokine/chemokine expression induced by the influenza virus and what, if any, affect RV1088 has on viral replication.

2. Determine if the results observed in cell culture systems holds true in the animal model.

3. Optimize delivery of the influenza virus using a nebuliser system to simulate natural occurring infections and test the usability of such a system to deliver drugs to directly to the respiratory tract.
Chapter 2. Evaluation of RV1088's properties in vitro

2.1 Introduction

The drug compound, RV1088, was provided by RespiVert with preliminary findings that indicated that this compound was able to control the expression of cytokines following viral infection in vitro in immortalized cell lines with RSV (Ito, 2010). In addition the published patent information for this group of compounds (patent WO/2010/067130, June 17, 2010) showed RV1088 to be a p38 alpha and p38 gamma inhibitor.

2.2 Confirmation of the inhibitory effect of RV1088 on p38 MAPK activity

While the aim of this project was not to determine and assess the kinase targets of RV1088 (as this work has previously been conducted by collaborators at RespiVert), before proceeding with more complex models, we wished to confirm an effect in our hands of RV1088 on the reported kinase targets. Of the three kinases reported to be targeted by RV1088 (c-SRC, Syk and p38α/γ) (Ito, 2010), p38 was chosen for analysis due to the large body of evidence supporting its activation following an influenza infection (Börgeling et al., 2013; Ludwig, 2007; Lee et al., 2005) and because of the availability of established commercial, non-radioactive, reagents.

A549 cells were infected with influenza A virus A/England/195/2009 in the presence or absence of RV1088. At 4 hours or 24 hours after infection, cells were lysed and subjected to immunoprecipitation with a p38 specific antibody (Figure 12). The precipitates were tested for their ability to phosphorylate the ATF-2 substrate of p38 MAPK at Thr 71 using a Western Blot. There was no difference in total amounts of p38 immunoprecipitated in presence or absence of drug. In the absence of drug the phosph-ATF-2 band showed an increase in intensity with time after infection indicating activation of the kinase upon infection. Band intensity was decreased at both time points by 1088 and by another drug X...
not discussed further here. The control compound SB203580, a known p38 MAPK inhibitor, decreased the phospho-ATF-2 band intensity at 4 hours post infection but at the effect was not sustained and high levels of phospho-ATF-2 were detected after incubation with p38 from cell lysates obtained by 24 hours pi in the presence of this drug. Control blots showed no difference in protein loading (vinculin) or total p38 MAPK content in lysates under any condition.

Figure 12: Western blot demonstrating RV1088 knock-down of p38 MAPK activity induced by A/England/195/2009. A549 cells were infected with A/England/195/2009 (moi = 3) in the presence or absence of drug. Drug compounds included: RV1088 (1.73nM), DMSO (0.5%) and SB203580 (2.5μM). The compound marked “X” is a new drug compound which is proprietary knowledge and can not be discussed. 24hrs p.i., cells were lysed and p38 MAPK was precipitated out. An in vitro kinase assay was performed using ATF-2 as a substrate. a) Vinculin and, b) Total p38 MAPK acted as loading controls while c) Phosphorylated ATF-2 was then detected by western blotting using Phospho-ATF-2 (Thr71) antibody. Section d) is a diagram demonstrating the major steps involved in determining the phosphorylation activity of p38 MAPK.
The drug also inhibited p38 MAPK activity induced following infection with influenza B virus strain B/Florida/04/2006 (data not shown). Once again, the effects of drugs RV1088 and drug X were sustained until 24 hours post infection whereas the effect of SB control compound had decreased by that time.

2.3 Assessment of RV1088 for cytotoxic properties

In order to demonstrate that the anti-influenza effects observed in presence of RV1088 were not simply due to general cytotoxicity of the compound, a series of tests to elucidate the cytotoxicity profile of RV1088 were undertaken. Three different parameters were used in this assessment and include: examination of membrane integrity (Trypan Blue, Figure 13, a), analysis of apoptosis-inducing cysteine proteases (Figure 13, b) and quantification of cellular reductase activity (MTT, Figure 13, c). Staurosporine, a prototypical ATP-competitive kinase inhibitor that binds to many kinases with high affinity and is a known inducer of apoptosis, acted as a positive control. These experiments were run in two immortalized cell lines commonly employed in influenza research, A549s (adenocarcinomic human alveolar basal epithelial cells) and MDCKs (Madin Darby canine kidney epithelial cells).

The results of all three experiments, each employing a different method for evaluating the viability of cells, indicate that RV1088 when used alone or in combination with a licensed antiviral (Relenza) did not exhibit any detectable toxicity or adversely affect cellular viability.
Figure 13: Results of toxicity assays.

a) Trypan blue exclusion assay. Cells, A549 or MDCK cells after treatment with RV1088 (1.73nM) ± Relenza (3.009nM).

b) Caspase 3/7 activity assay. Cells A549s or MDCKs were assessed for the activity level of the apoptosis-inducing cysteine proteases after treatment with RV1088 (1.73nM) ± Relenza (3.009nM). Staurosporine (1μM) acted as the positive control.

c) MTT assay. The activity of cellular reductase in either A549 or MDCK cells after treatment with RV1088 (1.73nM) ± Relenza (3.009nM). Staurosporine (1μM) acted as the positive control; suppressing cellular reductase activity.  

All results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments.
2.4 Studying the effects of RV1088 on cytokine induction

We used IFN as a model cytokine whose induction pathway is well understood. We first assessed the effect of treatment with RV1088 on the IFN response in immortalised cell cultures to determine at what level in the IFN signalling cascade RV1088 exerted its effects. We employed transfection system in which the particular biological pathway under study could be reconstituted and measured under controlled conditions.

The plasmid vectors employed in such systems are introduced into the target cell, where they commandeer the cell’s own transcription and protein synthesis apparatus to translate mRNA and produce a desired protein. This protein is then free to exert its effect within the cell. By manipulating the promoter sequence on the transfected plasmids it is possible to study the control of transcription of a particular gene. For example, by placing an IFN-β promoter on a plasmid encoding a luciferase gene, the quantifiable luciferase signal will accumulate when the pathway for IFN-β production is triggered (Figure 14).

In addition to using plasmids whose mRNAs are only transcribed under specific stress-induced situations, we also employed a variety of plasmids encoding key components of signalling pathways (TBK-1, NFkB and MAVS for example). These “over-expression” plasmids contained a pcDNA3 promoter which directs efficient transcription during the normal cellular state. By over-expressing a component of the cascade, the pathway is triggered. Thus in the presence of a reporter plasmid and drug compound of interest, it is possible to determine if the drug in question is acting to prevent IFN induction or signalling down-stream from the over-expressed protein.
Figure 14: Schematic representation of IFN-β luciferase assay. A plasmid encoding luciferase with an IFN-β promoter, an over-expression plasmid for a member of the IFN cascade and a pCAGGs renilla expression control plasmid are all co-transfected into human 293T cells. A drug compound can then be added to the cell culture. At specified time points post-transfection, cells are lysed and firefly luciferase activity and renilla expression are measured. Luciferase signal is a measure of activation of the IFN gene while renilla is a vector control containing a pCAGGs “constitutive” promoter.

2.5 Suppression of the interferon response by RV1088

A plasmid possessing an IFN-β promoter upstream of the firefly luciferase reporter gene and a plasmid directing Renilla luciferase from the powerful pCAGGS promotor, used here as transfection control, were co-transfected into 293T (human embryonic kidney) cells. Immediately after transfection the cells were infected with influenza A virus A/England/195/2009 (moi = 3) to stimulate a robust induction of interferon. Immediately following infection, RV1088 was added to the overlaying media at a range of doses (1.73μM...
– 0.0173nM). At 24hrs post transfection/infection, cells were lysed and the luciferase signals measured (Figure 15).

**Figure 15: Determination of an optimal RV1088 dose for suppressing IFN-β.** A plasmid encoding firefly luciferase downstream of an IFN-β promotor and a pCAGGs renilla expression control plasmid were co-transfected into human 293T cells. At the time of transfection, the cell cultures were infected with A/England/195/2009 (moi = 3) and treated with RV1088, at a range of doses (1.73μM to 0.0173nM), which was added to the overlying media. 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity (a) and renilla activity (b) were measured. Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001, ns=non-significant).

Infection with influenza A/England/195/2009, a prototypic strain of the 2009 pandemic, induced a strong interferon response (average 64.3 fold increase in luciferase signal over-mock infected cells, p-value 9.04x10⁻⁵). This response was suppressed by RV1088 treatment in a dose-dependent manner. In addition, it was observed that at higher doses RV1088 also inhibited the expression of the Renilla luciferase gene indicating a possible global effect on host transcription. This effect is addressed further in Section 2.11.
Importantly, at a dose of 0.173nM the firefly luciferase signal indicating interferon gene induction was still suppressed (by 1.5 fold compared to no drug) while Renilla expression indicating basal host gene expression was unaffected.

To further demonstrate the ability of RV1088 to suppress the interferon response and to determine whether this suppression was specific to pathways triggered by infection with influenza virus, a series of experiments employing transfected 293T cells exposed to other IFN inducing stimuli were performed. The first (Figure 16) employed a virus known to be a high-inducer of interferon, the Newcastle Disease Virus (NDV). The second (Figure 17) was void of any viral material and relied instead on treatment with the synthetic double-stranded RNA analogue Polyinosinic:polycytidylic acid (poly I:C) to induce an interferon response.

![Figure 16: Suppression of NDV’s IFN-β response by RV1088.](image)

*Figure 16: Suppression of NDV’s IFN-β response by RV1088.* A plasmid encoding luciferase downstream of an IFN-β promotor and a pCAGGs renilla expression control plasmid were co-transfected into human 293T cells. RV1088 (1.73μM or 0.173nM), was added to the overlying media at the time of transfection. Concurrently, Newcastle Disease Virus (NDV), a potent IFN-β stimulator, was added to the cells in either a 1:80 or a 1:20 dilution. 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity (a) and renilla expression (b) was measured. Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001, ns=non-significant). RLU: relative light units.
**Figure 17: Suppression of IFN-β transcription by RV1088 is not virus-dependent.** A plasmid encoding firefly luciferase downstream of an IFN-β promotor was transfected into human 293T cells. RV1088 (1.73μM and 0.173nM), was added to the overlying media. At the time of transfection, the cell cultures were stimulated with the RNA analogue Poly-I:C (1μg/ml). 24 and 48hrs post-transfection/stimulation, cells were lysed and firefly luciferase activity was measured. Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001). RLU: relative light units.

In both of these experiments, the application of NDV or poly I:C resulted in a strong induction of the interferon response, as indicated by 215.1 or 83.4 fold increase in firefly luciferase signal over mock infected or mock-treated cells respectively. This response was blunted in a dose-dependent manner when in the presence of RV1088. Even at the lowest dose of 0.173nm at which Renilla expression was unaffected, RV1088 inhibited the induced response by 3.46 or 4.6 fold. Thus we concluded that RV1088 targeted a component of the...
interferon induction pathway and that this effect was independent of the manner in which IFN had been induced.

As RV1088 was shown to supress the induction of cytokines and chemokines secondary to an influenza infection, this compound might prove beneficial in the context of a virally-induced cytokine storm. To examine this potential, we induced the IFN-β response in 293T cells by infecting with a virus with a genotype known to induce high levels of IFN-β and other cytokines. This virus carried the internal gene segments from a highly pathogenic avianH5N1 virus (A/Turkey/Turkey/01/2005 combined with the two external antigen genes HA and NA from a laboratory adapted strain, A/PR/8/34. Since the PR8 virus has a long history of safe use, does not infect humans and shares some antigenic properties with the recent pH1N1 viruses to which most people are currently immune, this allowed us to conduct these experiments at containment level 2 or 3 rather than under SAPO 4 conditions as would be required for working with the full H5N1 HPAI virus itself (Health and Safety Executive, 2010). Infection with the Tky/05:PR8 (6:2, HA/NA) virus induced high levels of firefly luciferase expression in the transfected and infected cells. Luciferase expression was induced by more than 2 logs (Figure 18). RV1088 inhibited the induction of the IFN-pathway by the 6:2 virus in a dose-dependent manner. Finally, we examined if RV1088 was exerting its inhibitory effect on a target that was specifically human or whether this compound also inhibited IFN induction in avian cells. Chicken embryo fibroblasts (DF1 cells) were transfected with a luciferase reporter under the control of an avian IFN-β promotor and cultures were infected with A/England/195/2009 influenza virus in the presence or absence of RV1088 (Figure 19). The influenza virus induced expression of luciferase and this was inhibited by co-transfection of a plasmids directing expression of the NS1 protein from influenza A/Victoria/3/75 virus. RV1088 also reduced luciferase expression but not to the same extent as the NS1 protein. The results of these experiments indicated that the target for RV1088 is conserved between avian and human species.
Figure 18: Suppression of H5N1-induced IFN-β by RV1088 occurs in a dose-dependent manner. A plasmid encoding luciferase with an IFN-β promotor and a pCAGGs renilla expression control plasmid were transfected into human 293T cells. RV1088, at a range of doses (0.0173nM to 1.73μM), was added to the overlying media. At the time of transfection, the cell cultures were infected in high containment with A/Turkey/Turkey/01/2005:PR8 (6:2, HA/NA) virus (moi = 3). At 18 and 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity was measured. Levels of significance illustrate difference between the DMSO control and a particular treatment group (Student T-Test). RLU: relative light units.
Figure 19: RV1088 is effective at suppressing the IFN-β response in avian cells. A plasmid encoding luciferase with an avian IFN-β promoter and a pCAGGs renilla expression control plasmid were co-transfected into avian DF1 cells. RV1088 (0.0173nM), was added to the overlying media. At the time of transfection, the cell cultures were infected with either A/England/195/2009 (moi = 3). Transfected plasmids encoding for NS1 (either belonging to A/England/195/2009 or A/Victoria/3/1975) acted as positive controls. At 18 and 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity and renilla expression was measured. Results are expressed as the mean ± standard deviation of triplicate samples from two independent experiments (student t-test, *p<0.5, **p<0.001). RLU: relative light units.

2.6 RV1088 and the IFN induction pathway

We next sought to determine at which level of the IFN pathway RV1088 exerted its effect. Figure 20b, illustrates the various components of this pathway that we were able to examine. Over-expression plasmids for various components of the IFN induction pathway were individually transfected into 293T cells along with pCAGGs Renilla expression control plasmid, and the IFN-β promoter-luciferase reporter plasmid.

Briefly, the IFN induction pathway is activated by the detection of viral PAMP (believed to be viral RNA that possess a 5’ triphosphate naked end) by the pattern
recognition receptors (PRRs) retinoic acid inducible gene-I (RIG-I) or Toll-like receptor-3 (TLR-3) (Rehwinkel et al., 2010; Kawai & Akira, 2008). Following recognition of viral nucleic acids, these PRRs initiate signalling pathways that lead to the synthesis of type I IFNs (a family comprised of several IFN-α subtypes and single IFN-β, IFNω, IFNe, or IFNκ, members) and inflammatory cytokines such as IL-6, TNF-α, and IL-12 (Kimura et al., 2013).

Constitutively expressed at low levels, RIG-I expression is enhanced by activation of IFN-α/β signalling creating a positive feed-back loop (Co et al., 2011). In the absence of stimulation, RIG-I exists in an inactive conformation, preventing access to the N-terminal CARDs and the helicase domain. The recognition and binding of the RNA PAMP by RIG-I’s RNA-binding domain (RBD) facilitates subsequent conformational changes and activation of RIG-I downstream signalling (Beckham et al., 2013). Upon binding the PAMP, RIG-I undergoes the transition from the inactive conformation to an active conformation and this facilitates interactions between the N-terminal caspase activation and recruitment domains (CARDs) and mitochondrial antiviral signalling (MAVS) (Sun et al., 2006).

MAVS activation in turn, results in signalling through the IFN kinases TBK-1/IKKε, which are responsible for the phosphorylation of IFN regulatory factors 3/7 (IRF3/7). IRF3 and 7 are transcription factors that, upon phosphorylation, dimerise and translocate to the nucleus where they act on the IFN promoter to stimulate IFN-α/β production (Grandvaux et al., 2002; Yoneyama et al., 1998).

While the exact activation mechanisms of the transcription factor NFkB are still under investigation, TLR-3 binding of viral PAMP is believed to activate signalling via TRIF and TRAF6 which then are free to activate NFkB directly or via TBK-1 (Abe & Barber, 2014). In another branch of the RIG-I-induced signalling cascade, MAVS has been demonstrated to interact/activate with TBK-1 which in turn is then free to activate NFkB (Liu et al., 2011). Furthermore, the transfer of a phosphate group from IKKβ has also been implicated in NFkB activation by displacing regulatory proteins called inhibitors of κB (IκB) (Batra et al., 2011; Ghosh & Baltimore, 1990). In reality, it is likely that all three of these pathways play a role in NFkB activation to various degrees. Once activated, NFkB translocates from the cytosol to the nucleus where it has been shown to lead to the induction of gene expression including that of IFN-β (Wang et al, 2010).
Plasmids encoding for the NS1 proteins from two different influenza strains were used in these assays as positive controls since they have been previously shown capable of inhibiting interferon induction pathway (Shelton et al., 2013). It is currently believed that all influenza A virus NS1 proteins can inhibit IFN induction by interfering with recognition of viral PAMP by RIG-I. In addition some NS1 proteins, typified by the NS1 protein from A/Victoria/3/75 virus, exert an additional inhibitory effect at the level of IFN gene transcription through its binding of cleavage and polyadenylation specificity factor (CPSF).

Overexpression of the mitochondrial antiviral signaling protein (MAVS) induced an IFN response, with luciferase signal increasing more than 200 fold by 24 hours post transfection (Figure 20a). Cultures transfected with the NS1 protein from A/Victoria/3/75 demonstrated a significant block of MAVS induced IFN response measured by the luciferase reported presumably because the induced mRNA was not properly processed and exported for expression. The IFN response following MAVS overexpression was significantly suppressed at 24hrs post transfection by RV1088 (0.173nM). This result suggests that RV1088 exerts its effect on the IFN signalling pathway at a level below that of MAVS. This result was not surprising as RV1088 was able to block the induction of IFN when stimulated by either Poly-I:C or NDV indicating that this inhibitory effect is not reliant on viral entry, the presence of replicating virus or is limited specifically to the influenza virus.
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Figure 20: Suppression of the MAVS induced IFN-β response by RV1088. An over-expression plasmid for MAVS, a plasmid encoding luciferase with an IFN-β promotor and a pCAGGs renilla expression control plasmid were co-transfected into human 293T cells. Transfected plasmids encoding for NS1 (belonging to A/Victoria/3/1975) acted as positive controls. RV1088 (0.173nM), was added to the overlying media at the time of transfection. 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity (a) was measured. Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001). RLU: relative light units.

Using a TBK-1 over-expression plasmid demonstrated that RV1088 was able to supress IFN stimulation induced by TBK-1 (Figure 21). These results indicated that, in part, RV1088 might be targeting at or below the level of TBK-1. However, as interferon induction by either IRF-3 or IKKβ was unaffected by RV1088 (Figure 22), an examination of drug on NFkB induced signalling was the logical next step.
Figure 21: RV1088 suppresses the IFN-β response by acting at the level of TBK-1. An over-expression plasmid for TBK-1, a plasmid encoding luciferase downstream of an IFN-β promoter and a pCAGGs renilla expression control plasmid were co-transfected into human 293T cells. Transfected plasmids encoding for NS1 (either belonging to A/England/195/2009 or A/Victoria/3/1975) acted as positive controls. RV1088 (0.173nM), was added to the overlying media at the time of transfection. 24hrs post-transfection, cells were lysed and firefly luciferase activity (a) and renilla expression (b) was measured. c) is a diagram of the interferon induction pathway being investigated with over-expression plasmids (TBK-1 is encircled in green). Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001, ns=non-significant). RLU: relative light units.

To more accurately assign the effects of RV1088 to molecules further down the IFN induction cascade, over-expression plasmids for IKKβ, IKKe, IRF-3 and IRF-7 were used. In all of these experiments, overexpression of these molecules induced high activation of the interferon promoter but RV1088 failed to cause any significant suppression of the IFN
response for any of these stimuli (Figure 22). Taken together these results suggest that RV1088 exerts effects on a molecule downstream of MAVS and TBK1 and upstream of NFkB and IRF-3/7.

Figure 22: Inability of RV1088 to suppress the induction of the IFN-β response by over-expression of IKKβ, IKKε, IRF-3 or IRF-7. An over-expression plasmid (either: IKKβ, IKKε, IRF-3 or IRF-7), a plasmid encoding luciferase with an IFN-β promotor and a pCAGGs renilla expression control plasmid were co-transfected into human 293T cells. Transfected plasmids encoding for NS1 (either belonging to A/England/195/2009 or A/ Victoria/3/1975) acted as positive controls. RV1088 (0.173nM), was added to the overlying media at the time of transfection. 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity was measured. Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001, ns=non-significant). RLU: relative light units.
Of interest was the observation relating to the differences in ability of the NS1 proteins from A/England/195/2009 and A/Victoria/3/1975 to block components of the IFN-induction pathway. Specifically, when compared to un-treated mocks, the England-derived NS1 was unable to suppress signalling via IKKe, IRF-3 and IRF-7 but was able to suppress signalling via IKKβ which is surprising as this NS1 has been shown to primarily exert its inhibitory effects by blocking at the level of RIG-I (Wu et al., 2012) suggesting that this NS1 might also inhibit a greater range of targets than previously thought.

In contrast, the Victoria-derived NS1 was able to suppress the IFN-induction pathway at all levels tested except for IKKe. This result is rather surprising as this NS1 has been shown to have strong CPSF binding ability which, should have prevented the IKKe signal from activating the transcription of IFN-β. It is possible that the signal from IKKe is strong enough to “push through” and overwhelm the CPSF-binding inhibitory effect. In both circumstances, the data warrant further examination.

Figure 23: Suppression of NFkB by RV1088 occurs in a dose-dependent manner. A plasmid encoding luciferase with an NFkB-promotor was transfected into human 293T cells. RV1088, at a range of doses (0.0173nM to 1.73µM), was added to the overlying media. At the time of transfection, the cell cultures were infected with A/England/195/2009 (moi = 3). At 8, 16
and 24hrs post-transfection/infection, cells were lysed and firefly luciferase activity was measured. As the stimulation of the NFkB induction pathway was driven by a viral infection and not though the transfection of expression plasmids, renilla was not included. Results are expressed as the mean ± standard deviation of triplicate samples from one experiment that is representative of two independent experiments (student t-test, *p<0.5, **p<0.001). RLU: relative light units.

A reporter plasmid encoding the firefly luciferase gene downstream of an NFkB responsive element was triggered following infection of transfected cells with influenza A virus A/Eng/195/2009. Treatment with RV1088 suppressed induction of this NFkB responsive promoter in a dose-dependent manner at all the time points examined (Figure 23) and to a significant extent at the dose at which no effect of basal host gene transcription was measured (0.173nm). Based on this and other results described above, RV1088 likely exerts its IFN suppressing affects by inhibiting a molecule that activates NFkB, which could be TBK1.

2.7 Polymerase function as measured by the 3-5-8 Luciferase reporter construct

In order to determine if RV1088 has any effect on viral replication, we employed a specialised luciferase reporter. In this experiment, A/England/195/2009 virus (moi = 3) was used to infect 293T cells which have previously been transfected with the 3-5-8 luciferase (3-5-8luc) reporter construct. This luciferase reporter contains a triple mutation in the 3’ non-coding region of the viral promoters that flank the reporter gene. This 3-5-8 mutation was shown by Neumann & Hobon (1995) to increase expression when driven by the influenza virus. The mechanisms for this is not clear but may be by increasing the ability of polymerase proteins produced during virus infection to bind to the altered viral promoter. In this assay carried out in the context of virus infection, the more active a viral polymerase is, the greater the resultant luciferase signal. The luciferase signal was measured after 6 and 24hrs. At 6 hours after infection insufficient replication had occurred for a luciferase signal to accumulate. Figure 24 shows a RV1088 dose-dependent inhibition of the luciferase signal at 24 hours post infection, supporting previous results that this drug exerts an anti-viral action at the level of viral replication. Since in this assay the viral polymerase is not
expressed from plasmids but rather during virus infection, any decrease in luciferase signal is not due to decrease polymerase expression resulting from the nonspecific effects of RV1088 on host gene expression.

**Figure 24: Effect of kinase inhibitors on viral transcription/replication of a 3-5-8 luciferase plasmid in human cells (293T).** A plasmids encoding for a firefly luciferase gene with a 3-5-8 triple mutation in the 3’ non-coding region of the promotor was transfected into human 293T cells. After an over-night incubation, cultures were infected for 1hr with A/England/195/2009 (moi=3) after which, DMSO (0.5%), SB203580 (0.4nM, pan-p38 inhibitor), Dasatinib (100nM, Src inhibitor), BIRB-796 (12nM, pan-p38 inhibitor), Syk R343 (100nM, Syk inhibitor) or RV1088 (0.173nM, targets p38, Src and Syk) were added to the overlying media either singularly or in combination. 24hrs post-infection, cells were lysed and firefly luciferase activity was measured. Results are expressed as the mean ± standard deviation of triplicate samples from three independent experiments (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
2.8 RV1088 does not affect virus HA binding

In order to determine if RV1088 has any effect on other aspects of virus replication, such as virus binding and entry, we tested its effect in a haemagglutinin (HA) assay (Figure 25). This assay examines the ability of a virus to bind red blood cells using HA to attached to sialic acids that are exposed on the red cell surface. Drug was titrated into the assay to see if it could interfere with HA:SA interaction. Even at the high dose of 1.73μm the drug did not affect the ability of virus to crosslink red blood cells.

Figure 25: RV1088 does not affect HA binding. Virus (A/England/195/2009) was diluted in PBS (± RV1088, 1.73μM) from neat to -5 in a 2-fold dilution series. 50μl chicken red blood cells were added to every well and the plate was incubated on ice for 1hr. Positive control, A/England/195/2009+PBS. Negative controls, PBS alone and RV1088 alone.
2.9 The effects of RV1088 on activity of the viral polymerase

The data above suggest that RV1088 is affecting cytokine expression through inhibition of the activation of NFkB. Interestingly NFkB has also been reported to affect virus replication directly. Kumar and colleagues (2008), reported a decrease in viral RNA replication when NFkB was blocked. They and others have postulated that NFkB must be associated with one, or more, viral-supportive functions the suppression of which can negatively impact viral replication. Such supportive functions expoused in the literature have included: the generation of pro-apoptotic factors which help to facilitate the release of vRNPs from the nucleus, suppression of IFN-induced gene (ISG) expression via cytokine signalling-3 (SOCS-3) and the regulation of vRNA synthesis (Rucke et al., 2012; Kumar et al., 2008; Pauli et al., 2008; Mazur et al., 2007; Wei et al., 2006).

Figure 26: Minireplicon assay to measure polymerase activity in human cells (293T). Plasmids encoding the PB1, PB2, PA and NP proteins are co-transfected with a virus-like RNA expression plasmid (either vRNA or cRNA) encoding luciferase and a pCAGGs Renilla expression control plasmid in human 293T cells. Drugs are applied to the transfected cells and at various times post-transfection, cells are lysed and firefly luciferase activity is measured. Adapted from A. Cauldwell (unpublished).
To examine whether RV1088 had an effect on the replicative ability of the influenza virus, a “minigenome” reporter assay was employed using the vRNP complex re-constituted from A/England/195/2009 virus. In this assay vRNP or cRNP complexes are generated from the minimal set of constituents (PB1, PB2, PA and NP) encoded on expression plasmids together with a negative sense viral-like RNA reporter encoding the luciferase gene (termed here the “minigenome”) and transcribed with authentic termini by human polymerase I (Figure 26). The coding sequence of the “minigenome” reporter is flanked by conserved non-coding sequences that are considered to be the minimal viral promoters required for transcription and replication of viral RNA. Viral replication and transcription of the negative-sense “minigenome” RNA take place in the nucleus and occur only in the presence of a reconstituted functional influenza virus polymerase. Upon replication of the “minigenome”, a luciferase signal is readily quantified. The addition of a drug compound, such as RV1088, into this system allows us to determine if the drug has any affect on the functionality of the viral polymerase.

Two different virus-like RNA expression plasmids driving in situ production of either vRNA or cRNA-like RNAs were utilized. Luciferase signals from the vRNA minigenome could result from transcription alone, but may be amplified by rounds of replication whereby the vRNA is replicated to cRNA, back to vRNA and then used a template for more mRNA. Luciferase signal from the cRNA minigenome requires both replication of the cRNA template to a negative sense vRNA and then transcription to generate mRNA. The signal following introduction of the negative reporter/vRNA (Figure 27a) was unaffected by RV1088 at any dose where the Renilla control was unaffected. On the other hand, the signal from the positive reporter/cRNA (Figure 27b) was suppressed in a dose-dependent manner by RV1088. At a dose of 0.173nm the minigenome luciferase signal was significantly suppressed but the Renilla control signal was not. Thus, in addition to effects on host pathways, RV1088 appears to suppress viral genome replication.
Figure 27: Effect of RV1088 on viral transcription/replication of a minigenome by A/England/195/2009 viral polymerase complexes in human cells (293T). Plasmids encoding the PB1, PB2, PA and NP proteins derived from A/England/195/2009 were co-transfected together with a virus-like RNA expression plasmid (either vRNA (a) or cRNA (b)) encoding luciferase and a pCAGGs Renilla expression control plasmid in human 293T cells. c) represents the stages of influenza virus replication. At 24hrs post-transfection, cells were lysed and firefly luciferase activity was measured. Results are expressed as the mean ± standard deviation of triplicate samples from one experiment that is representative of two independent experiments.
2.10 Comparison of suppressive activity of RV1088 against specific kinase inhibitors

After demonstrating the ability of RV1088 to suppress the induction on interferon as well as directly affecting the replicative capability of the viral polymerase, we were interested in comparing RV1088 to other known kinase inhibitors. As previously discussed, RV1088 is a specially designed narrow spectrum kinase inhibitor that has been shown by our RespiVert colleagues to target the kinases c-SRC, Syk and p38α/γ. To evaluate the principle of using a single compound to target a group of kinases versus using single compounds for each specific target, either used alone or in combination, A549 cells stably expressing an IFN-inducible Gaussia luciferase were infected with A/England/195/2009 and treated either with RV1088 or a series of commercially available kinase inhibitors employed at their IC50s. As the results demonstrate (Figure 28), RV1088 was able to achieve a greater level of significant inhibition when compared to the DMSO control (p= 0.007, student t-test) with only Dasatinib (Src inhibitor), BIRB-796 (pan-p38 inhibitor) and the combination of Dasantib+BIRB-796+Syk R334 also obtaining statistical significance when compared to DMSO control (p-values of 0.019, 0.01, 0.033 respectively, student t-test). Of particular note is the observation that RV1088 induced a greater degree of suppression when compared to the tri-compound treatment of Dasantib+BIRB-796+Syk R334 (p=0.026, student t-test), a combination which covers the three main targets of RV1088. This result indicates that RV1088, as a single compound, is either more effective than using individual compounds or that RV1088 suppresses other targets yet to be identified.
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Figure 28: Comparison of the ability of kinase inhibitors to suppress IFN induction. A549 cells stably expressing IFN-inducible gaussia luciferase were infected with A/England/195/2009 (moi =3). DMSO (0.5%), RV1088 (0.173nM, targets p38, Src and Syk), SB203580 (0.4nM, pan-p38 inhibitor), Dasatinib (100nM, Src inhibitor), BIRB-796 (12nM, pan-p38 inhibitor) or Syk R343 (100nM, Syk inhibitor) were added to the overlying media, singularly or in combination, at the time of infection. At 24hrs post-infection, culture supernatant samples were taken and the level of gaussia luciferase was quantified. Treatment groups were compared to DMSO controls (student t-test, *p<0.5, **p<0.001). RLU: relative light units.

2.11 Discussion and Conclusions

This chapter presents results detailing the analysis of the activity of RV1088 in vitro in inhibiting induced cytokine responses and influenza virus replication using a variety of assays. We have attempted to localize the point in the interferon induction pathway where the compound interferes with host cell signalling and viral replication.

Personal communication from RespiVert colleagues revealed that RV1088 targets 3 host kinases (p38, Syk, and c-Src), we wanted to confirm that, in our hands and cell culture systems, that we are able to observe a specific knock-down of one of these named targets. As such, we chose to specifically examine the ability of RV1088 to affect the kinase activity
of p38. This target was chosen due to the general availability of well-established reagents that did not require radio-labelling. As expected, when cell cultures were infected with either A/England/195/2009 or B/Florida/04/06, we observed a strong activation of phosphorylated (i.e. active) p38; however, in the presence of RV1088, the level of activity was suppressed.

Our results demonstrate that RV1088 has the ability to suppress transcription of not only induced IFN-β mRNA but also inhibits the expression of the Renilla control gene expressed from a polymerase II promoter on a transfected plasmid. This phenomenon has been observed by other groups examining the effect of different kinase inhibitors that are known/designed to target other signalling pathways (Martinez-Gil et al., 2013; Ludwig, Personal Communication). Based on discussions with others in the field, and our ability to analyse Renila expression, we decided that the best course of action was to only carry out experiments with a dose of RV1088 that was effective at suppressing IFN-β but not the Renilla control. Using this technique, we found that a dose of 0.173nM fit the profile and is the dose used in the majority of our further experiments (Figure 15). The basis for Renilla inhibition at higher concentrations remains unknown.

The suppressive effects of RV1088 on interferon induction were not specific for influenza virus and did not require a replicating virus to be the inducer for the effect to be observed. Since the compound also inhibited IFN induction by Newcastle Disease Virus (NDV) and Polyinosinic:polycytidylic acid (poly I:C) (both well-known, stimulators of the IFN pathway). We can therefore conclude that there should be an effect of RV1088 on interferon induction that is separate from any effect if this compound in inhibiting virus replication, and in this way the drug likely has dual activity against this pathway. As RV1088 is believed to act by inhibiting the phosphorylation of key kinases involved in cytokine/chemokine signaling cascades normally up-regulated during productive influenza infections, we sought to examine its potential to counteract the cytokine response induced by a highly pathogenic virus.

One such virus that has been implicated in the generation of a cytokine storm within the host is A/H5N1. This particular strain is a highly pathogenic virus infecting aquatic birds and poultry. Sporadic incidences of species-barrier crossings of the virus into humans have
been documented and are associated with significant morbidity and mortality; causing an estimated mortality rate between 2003 and 2014 of ~60% for the 600 or more confirmed cases (WHO, 2014).

Examinations of plasma taken from A/H5N1-infected patients demonstrated significantly increased levels of macrophage and neutrophil-attractant chemokines as well as a range of pro-inflammatory and anti-inflammatory cytokines (Abdel-Ghafar et al., 2008). While post-mortem examinations of several individuals revealed: organizing diffuse alveolar damage with interstitial fibrosis, extensive hepatic central lobular necrosis, acute renal tubular necrosis and lymphoid depletion (To et al., 2001).

Whilst the exact pathogenic mechanism(s) employed by the virus are not completely understood, hypercytokinemia is thought to play an important role (Sakabe et al., 2011). Initially, it was suggested that this hyperinduction of cytokines/chemokines was directly related to the replicative ability of the virus but more recent work has shown this does not entirely explain the situation (Abdel-Ghafar et al., 2008; Sakabe et al., 2011).

In our experiments, we chose to use the A/Turkey/Turkey/01/2005 virus strain which had its HA and NA gene segments swapped for those of A/Puerto Rico/8/1934 (PR8, A/H1N1) to serve as our prototypic high cytokine-inducing virus. The swapping of the viral HA and NA gene segments was performed in order to facilitate easy viral uptake by cells in culture and to make the virus more safe for laboratory work as our adaptive immune systems have already been primed to recognize the PR8 HA/NA via vaccination with the pH1N1 2009 strain that shares antigenicity with PR8 (Skountzou et al., 2010). All A/Turkey/Turkey/01/2005:PR8 experiments were conducted in Cat 3 containment facilities.

Our results (Figure 18) demonstrate that, in 293T cells transfected with a luciferase plasmid encoding an IFN-b promotor, the A/Turkey/Turkey/01/2005:PR8 (6:2, HA/NA) virus induces a strong IFN response which is partially suppressed by the NS1s of A/Victoria/3/1975 and A/England/195/2009 (a reduction of 15.5- and 6.4-fold respectively when compared to DMSO treatment group). With respect to the RV1088 treatment groups, treatment with 0.0173nM failed to inhibit interferon induction while treatment with 0.173nM was able to significantly inhibit this response (2.6 fold decrease, p value = 0.0003,
student t-test, at 18hrs p.i). These results indicate that RV1088 may prove beneficial in controlling the over-induction of the IFN response in cases of A/H5N1.

In order to examine if RV1088’s inhibitory activity relies on targeting a species-specific factor or whether this compound’s target is common across birds and humans, we examined the ability of RV1088 to suppress the induction of IFN-β in avian cells. Chicken embryonic fibroblasts (DF1 cells) were transfected with a luciferase reporter containing an avian IFN-β promoter. Cultures were infected with A/England/195/2009 both in the presence and absence of RV1088 and after a set incubation time, the luciferase signal was quantified. The results demonstrated that RV1088, at least in part, exerts its inhibitory actions on targets/pathways that are conserved between the avian and human species. Unfortunately, further examinations were considered to be beyond the scope of this project however, a more detailed examination may be warranted at a later date.

By employing a series of plasmids possessing a pCMV5 promoter upstream of a sequence which encode for a key components of the IFN-β signalling pathway, we have mapped RV1088’s activity to a level at or below that of MAVS with some of its potential effects occurring via TBK-1 resulting in the suppression of NFkB activation and IFN induction. Furthermore, we have shown that his interference occurs in a dose-dependent manner.

These results are in line with already published information that has demonstrated that p38 is up-regulated during influenza infections (Ludwig, 2007; Lee et al., 2005). Furthermore, this kinase has been implicated in the induction of the IFN response via the NFkB signalling pathway (Hui et al., 2009; Saccani et al., 2002; Uddin et al., 1999). Additional sources have shown that NFkB, a transcription factor, has been shown to control the expression of numerous genes and is involved in an array of physiological responses to viral attack (including acute phase inflammatory responses, cell adhesion, oxidative stress response, apoptosis and generation of the antiviral state (Ludwig & Planz, 2008; Pahl, 1999). However, despite this, some studies have demonstrated that when NFkB signalling is blocked, the production of progeny viruses is impaired, indiciating that the influenza virus may exploit NFkB signalling to its own advantage (Wurzer et al., 2004; Nimmerjahn et al., 2004). Specifically, NFkB has been shown to be involved in the generation of pro-apoptotic factors which help to facilitate the escape of vRNPs from the nucleus, suppression of IFN-
induced gene (ISG) expression via cytokine signalling-3 (SOCS-3) and through the regulation of vRNA synthesis have all been proposed (Ruckle et al., 2012; Kumar et al., 2008; Pauli et al., 2008; Mazur et al., 2007; Wei et al., 2006).

In addition to exerting effects on host cell signalling, we have also demonstrated that in our “minigenome” assay (discussed in detail in Section 2.9) RV1088 is able to suppress the transcription of the vRNA (+) reporter but not the cRNA (-) reporter. This indicates that RV1088 does indeed affect the viral polymerase at the level of viral replication. It is important to note that this system relies on the transcription of transfected plasmids each of which encode for a component of the viral polymerase and it is possibly that the inhibitory effect observed in the mini-replicon assay results from a general suppression by RV1088 of transcription activity in the transfected cells rather than a specific anti-polymerase activity. This conundrum has been previously described in the literature by Martinez-Gil and colleagues (2013) in which, the authors ascribed the effect to possible and specific inhibition of host RNA synthesis or to a nonspecific cytotoxicity effect and simply looked at the relative difference in the suppression levels seen between the Renilla transfection control and their polymerase reporter assay. They reported observing inhibition in the replication of viral RNA as well as suppression of the transfection control however, the suppression of the former was significantly greater than the later leading them to conclude that the suppression of viral RNA was not simply a result of general transcription suppression. While this interpretation is open to debate, no consensus yet exists in the field regarding how reliable such results may be and what is the best way to interpret such results.

Our observation that RV1088 does have an effect on viral RNA replication was further confirmed through the suppression by RV1088 of the transcription of a 3-5-8 luciferase reporter plasmid. The 3-5-8 reporter plasmid, when actively transcribed by the viral polymerase, produces a quantifiable luciferase signal. Unlike the mini-genome assay, the 3-5-8 reporter system is driven directly by replicating virus and not by the expression of polymerase-encoding plasmids. In this assay system, RV1088 also was observed to significantly suppress the replicative ability of the viral polymerase. Our findings are consistent with the results reported by another group where the kinase inhibitors ON108110 was found to suppress influenza viral replication in a dose-dependent manner.
The inhibitory action of this kinase inhibitor was also mapped to preferentially inhibit vRNA replication indicating that this stage in viral replication may prove to be an effective target for intervention.

The mechanism by which RV1088 is able to exert an inhibitory effect on the viral polymerase has yet to be elucidated. However, other groups in the field have suggested that kinases are involved in the process of nuclear release of vRNP complexes during the latter stages of viral replication (Ludwig et al., 2009; Pleschka et al., 2008). Supporting this hypothesis is the observation that when certain kinase pathways (for example, p38) are over-stimulated in cell culture, recovered viral titers are generally higher when compared to non over-stimulated cultures (Marjuki et al., 2007; Olschlager et al., 2004; Ludwig et al., 2004). Furthermore, if such a pathway is blocked via specific kinase inhibitors, there is a striking impairment of viral growth for both types (A and B) of influenza (Olschlager et al., 2004; Ludwig et al., 2004; Pleschka et al., 2001). It has been hypothesised that this anti-viral effect may be due, in part, to the ability of the kinase inhibitors to prevent the phosphorylation of the viral proteins NEP and NP, two of the viral proteins in the viral phosphoson. A detailed examination of this postulated mechanism has yet to take place (Planz, 2013; Hutchinson et al. 2012).

Furthermore, we have demonstrated that the suppression of IFN induction and vRNA synthesis by RV1088 is not due to a general cellular toxicity effect. This is concluded based on the observations that at the concentrations we have used in our work, RV1088 does not adversely affect cellular reductase activity, cell wall integrity, or increase the expression/activity of the known apoptosis-inducer Caspase 3/7 (Figure 13).

Finally, we examined whether RV1088 has any effect on the viruses ability to bind to host cell surface receptors. Previous studies have demonstrated that prior to host cell entry, PRR recognition of the residue on the surface of the influenza virion stimulates MyD88, which, in turn, activates p38 and subsequently host cellular internalization machinery (Marchant et al., 2010). As RV1088 has been shown to inhibit the activity of p38, we felt that an evaluation of viral-host receptor binding was warranted. The levels of activation of p38 during the first 10min of infection were found to be approximately 10-fold higher than for other respiratory viruses and may represent an effective adaptation
mechanism to augment viral uptake by the host cell. Thus, by supressing p38 activation, RV1088 may be having an indirect suppressive effect on viral entry.

To examine any effect that RV1088 might have on receptor binding, we employed the haemagglutination assay (HA assay), a universally used technique in the field of influenza research that measures the ability of a virus to agglutinate red blood cells (RBCs) via sialic acid receptor binding. This assay was performed in the presence or absence of RV1088 and it was discerned that RV1088 dose not effect the ability of A/England/195/2009 to bind RBCs (Figure 25).

Finally, we compared the IFN suppressive activity of RV1088 against other specific kinase inhibitors (employing previously reported IC50 values) and determined that RV1088 was able to achieve a greater level of significant inhibition when compared to the DMSO control (p= 0.007, student t-test) (Figure 28). In contrast, only Dasatinib (Src inhibitor), BIRB-796 (pan-p38 inhibitor) and the combination of Dasantib+BIRB-796+Syk R334 also obtaining statistical significance in terms of supressing IFN-induction when compared to DMSO control (p-values of 0.019, 0.01, 0.033 respectively, student t-test).

In addition, it was noted that RV1088 induces a greater degree of suppression in terms of IFN-induction when compared to the tri-compound treatment of Dasantib+BIRB-796+Syk R334 (p=0.026, student t-test) - a combination which covers the three main targets of RV1088. This result indicates that RV1088, as a single compound, is either more effective than using individual compounds or that RV1088 supresses other targets yet to be identified and warrants further investigation beyond the scope of this project.

Taken together, this chapter’s results, indicate that RV1088 does not directly effect viral attachment to host cells and that the suppressive effects we have previously observed are not due to inhibition of the virus’ ability to infect target cells rather, RV1088 must be exerting its effect directly at the level of cell signalling and/or viral replication.
Chapter 3. Evaluating the activity of RV1088 against the influenza virus

3.1 Treatment of influenza-infected CALU-3 with RV1088

To examine the potential of RV1088 to suppress the replication of influenza and/or affect the cytokine response, we first performed assays in CALU-3 cells (an immortalised human-epithelial, lung adenocarcinoma line). Since the HAE 3D cell cultures are very costly we wished to ask whether the drug had any effect at all in a simpler culture system. The virus chosen for these experiments was A/England/195/2009 (A(H1N1)pdm09) as it is representative of currently circulating virus. Monolayer cultures were infected in the presence or the absence of RV1088 (1.73nM) and, at specified time points (24 and 48hrs p.i.), culture supernatants were harvested to determine viral titers by plaque assay on MDCK cells (Figure 29a). At either time point, no significant difference was observed between the RV1088 and non-treatment groups. At 24 hours, the titres of virus released were the same in presence or absence of drug. At 48hrs, there was an average 2.57-fold decrease in viral titres as measured by plaque assay. On the other hand, qRT-PCR analysis on cell lysates collected at 48 hours p.i. revealed that RV1088 significantly suppressed cytokine/chemokine mRNA levels induced by A/England/195/2009 (A(H1N1)pdm09 (p≤ 0.05 for both IFN-β and RANTES) (Figure 29b-d). There was an average 1.58- and 4.76-fold decrease in IFN-β and RANTES mRNAs respectively. In addition qRT-PCR for viral M gene mRNA (normalized to 18S) indicated that treatment with RV1088 also suppressed levels of M-gene message by an average 3.78-fold, which may account for the same decrease in viral titres measured in supernatant at that time point.

Unfortunately, mRNAs for viral or host genes were not measured at 24hrs p.i. and as such, it was not possible to extrapolate if the observed cytokine mRNA decrease at 48hrs resulted from a decrease in viral titers or was a direct effect of RV1088 on the cytokine induction pathway.
Figure 29: Growth curve results of RV1088 in CALU-3s. CALU-3 cells were infected with A/England/195/2009 (moi = 0.0001) in the presence or absence of RV1088 (1.73nM). Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and at 48hrs p.i. total RNA was extracted for quantification of cytokine/chemokine mRNA expression via qRT-PCR (b-d). Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
3.2 Air-liquid interface primary cell cultures

We wished to study the effect of the kinase inhibitor RV1088 on influenza virus replication and cytokine induction in a cell system that most closely represented the target cells in the human respiratory tract.

Traditional cell culture, typically used for the study of influenza virus replication, comprises a single type of cell, usually immortalized, grown in a two dimensional plastic dish with complete submersion of cell in liquid media. There is an increasing demand for in vitro models that capture the relevant in vivo tissue complexity which the traditional 2-D monocultures are unable to achieve. In vivo, the response of individual cells to stimuli is regulated by spatio-temporal cues that reside in the local environment or the extracellular matrix and from signals from neighbouring and distant cells of a variety of cell types, soluble factors and physical forces, lacking in the 2-D systems but available in the 3-D primary cell cultures (BeruBe, 2010).

Cells of the respiratory tract have been cultured with an air liquid interface (ALI) and these contain ciliated surface cells in the same or even greater numbers as basal and goblet cells, whereas submerged cultures contain significantly fewer-to-no cilia on their apical surface (Figure 30). Furthermore, submerged cultures can exhibit different permeabilities to substances (such as drugs) when compared with cultures grown at the ALI (BeruBe, 2010).

One worry in employing primary cells for basic science studies would be that variation may exist between cells obtained from different donors, and also that even using these primary cultures creates a different cell environment than in vivo. However, these misgivings appear to be unfounded: In a study conducted by Pezzulo and colleagues (2011), cells harvested from tracheal and bronchial brushings of 16 individuals were isolated and each sample was divided into two lots. Immediate transcriptional analysis via microarray was performed on one sample lot while the second sample lot was used to grow separate tracheal and bronchial air-liquid interface (ALI) cultures for each donor and subjected to the same microarray analysis as the first lot two weeks later. The transcriptional profiles of the brushing samples were subsequently compared to that of the ALI cultures for each donor. Within each donor, a comparison was made between the profile of their tracheal cells and
bronchial cells, and the differences between donor-to-donor were also examined for both cell types (i.e. tracheal and bronchial). The profiles of Calu-3 cultures grown at both the ALI and submerged manner were also examined and compared to the profiles to both primary cell lots.

Figure 30: Schematic representation of the Human Airway Epithelial (HAE) cultures. Primary human airway donor biopsies reconstituted in a transwell are commercially available (Epithelix, CH). Cultures are maintained at an air-liquid interface and contain basal cells, non-ciliated columnar cells (often called pre-ciliated) active ciliated columnar cells, and mucus secreting goblet cells.

Their results demonstrated that primary cultures of human airway epithelia maintained a very high degree of similarity of gene expression patterns as in vivo airway epithelial samples and that these similarities relied on both culture of primary non-immortalised cells and the presence of an ALI.

They also found a very high degree of similarity between the transcriptional profiles of the airway epithelium from different donors and further, that the airway epithelia from trachea and bronchi samples had very similar expression profiles (both within each donor and between all donors). Thus normal/healthy human airway epithelium showed an exceptionally constant transcriptional profile that sometimes prevailed despite inter-donor and environmental variability. This suggests that using a small number of biological replicates to study the transcriptional profile of the airway is an acceptable approach.
The above conclusions are further supported by the work of Dvorak and colleagues (2010) who, using a similar approach as Pezzulo, compared the transcriptome of epithelial brushings from 12 donors to that of primary cell cultures grown from the original samples. They determined that 81% of the gene sets expressed in both cell types showed similar expression profiles between cultured and brushed cells. The majority of those genes with differential expression were again linked to the different proportion of cell types expressed as well as the lack of certain effector immune cells (primarily macrophages). Signalling pathways were also examined and while differences did exist between the brushing samples and the primary cell cultures, overall a remarkable level of similarity exists.

In recent years, a wealth of studies using primary cell-derived, highly differentiated airway cultures have been undertaken to examine viral replication and virally-induced responses. In studies conducted by Wan and Perez (2007), HAE cell cultures were effectively employed to demonstrate how specific viral molecular traits (e.g. L-226 in the HA protein mediating strong binding for α26SA) can have strong implications in terms of viral host range and viral growth. By employing these culture systems, it was possible to identify the specific cell type targeted by the virus and that are necessary for the initiation of infection. Furthermore, investigations into how specific molecular changes can alter the binding specificity (i.e. tropism) of the virus have also been examined.

In work conducted by Matrosovich et al., (2004) and others the HAE culture system has been instrumental in demonstrating the difference in binding patterns that exist between human and avian viruses in the human respiratory tract and thus have greatly aided in studies wishing to examine components of the host-range barrier. Interestingly, influenza A viruses which have the ability to transmit between people, appear to target primarily the non-ciliated cells in these cultures of human airway epithelium (Matrosovich et al., 2004; Thompson et al., 2006). This is in contrast to some other respiratory viruses such as SARS, coronavirus or RSV that seem to preferentially infect ciliated cells (Pickles, 2004; Zhang et al., 2002; Bartlett et al., 2008).

With respect to examinations of the virally-induced cytokine/chemokine response, the HAE culture system has proved to be a instrumental and reliable tool. Schaap-Nutt et al., (2012) demonstrated in HAE cultures that human parainfluenza virus serotypes (PIV1 and 2)
differ in their replication kinetics and cytokine secretion helping to shed light on some of the serotype-specific differences in PIV pathogenesis and epidemiology. Additional work conducted by Zhang et al., (2011) found that PIV5 infection of HAE occurs preferentially at the apical surface and that in comparison to RSV and PIV3, PIV5 was unexpectedly more cytopathic, as characterized by enhanced shedding of infected cells and extensive formation of syncytia. Work along similar lines has also been conducted in the HAE culture system for human Coronavirus (Dijkman et al., 2013; Dominguez et al., 2013), Respiratory Syncytial Virus (Wright et al., 2005), Human Rhinovirus (Hao et al., 2012) and Human Bocavirus 1 (Deng et al., 2013; Dijkman et al., 2009).

HAE cultures are also proving to be a useful system for the testing of efficacy and delivery of various therapeutic agents. DAS181, a sialidase fusion protein that acts by a host-targeting entry-blocking mechanism, was successfully evaluated against influenza in HAEs by Hedlund et al., (2010) and Triana-Baltzer et al., (2009). In these studies, DAS181 was found to effectively desialylate HAE cultures and ex-vivo bronchi tissues indicating its potential to inhibit replication of different influenza strains. Furthermore, Frieman et al., (2011) employed HAE cultures to assess and differentiate the in vitro efficacy of NSC158362 (a small molecule identified to inhibit the activity of the papain like protease) against SARS-CoV and influenza.

Finally, the delivery of therapeutic genes to the diseased airway epithelium represents a promising avenue for the treatment of numerous genetic lung diseases (e.g. cystic fibrosis, α-1-antitrypsin). The ability of adeno-associated virus (AAV) serotypes 1–9 as well as numerous other vectors have been evaluated for genetic cargo delivery in HAE cultures (Limberis et al., 2009). All these studies exemplify the utility and versatility of the HAE culture system for evaluating viral pathogenesis and the ability of pharmacological compounds to combat such infections.
3.2.1 Treatment of influenza-infected HAEs with the corticosteroid Fluticasone

Before examining the effects of RV1088 on viral replication and cytokine/chemokine suppression, we sought to first examine how a corticosteroid (Fluticasone) would act in the human airway epithelium culture system in the context of an influenza infection as this steroid is specifically indicated for treating inflammation associated with the upper respiratory tract. As the results in Figure 31 demonstrate, steroid treatment resulted in significant increases in viral replication levels with cytokine/chemokine levels either unchanged or increased when compared to DMSO controls.
Figure 31: Results of pre-infection treatment with Fluticasone in HAEs infected with A/England/195/2009. HAE cell cultures were pre-treated for three days both apically and basally with Fluticasone (0.1 μg/ml) or DMSO (0.5%). Cultures were infected with A/England/195/2009 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-e). Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
3.2.2 Treatment of HAE cultures with RV1088 prior to infection with A(H1N1)pdm09 and B/Florida/04/2006

To evaluate the effect of RV1088 on viral replication and the innate immune response in the primary HAE cultures, cells were pre-treated (apically for 2hrs each day and basally) with RV1088 in DMSO (0.5%) for three days then infected with either A/England/195/2009 (Figure 32) or B/Florida/04/2006 (Figure 33) at an moi of 0.0001 to allow multiple replication cycles to occur. At the time of infection, the drug containing basal media was replaced with drug-free media which remained in place until the end of the experiment. Samples collected from the apical surface were titrated for infectious virus by plaque assay in MDCK cells. When compared to DMSO control, RV1088 significantly decreased the titre of virus released at the apical surface only in the case of B/Florida/04/2006 at 24hrs p.i. (p≤0.05). For all other time points any inhibitory effect was not statistically significant (with fold decreases ranging from 0 to 4.3) (Figure 33).

The same apical washings were used to quantify cytokine/chemokine protein level using a Luminex™ platform. The results demonstrated that RV1088 significantly suppressed cytokine/chemokine protein levels induced by A/England/195/2009 (A(H1N1)pdm09) (Figure 23). Indeed under these conditions no induced protein responses were detected at all. RV1088 predominantly no significant effect on replication of the influenza B virus strain B/Florida/04/2006 in HAE cultures (save for the 24hr time point, p≤0.05) (Figure 33), but did significantly suppress cytokine/chemokine protein levels induced by B/Florida/04/2006, mirroring the results previously described for the type A virus.
Figure 32: Results of Pre-infection treatment with RV1088 in HAEs infected with A/England/195/2009. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) or DMSO (0.5%). Cultures were infected with A/England/195/2009 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-e). Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
Figure 33: Results of pre-infection treatment with RV1088 in HAEs infected with B/Florida/04/2006. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) or DMSO (0.5%). Cultures were infected with B/Florida/04/2006 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-e). Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
3.2.3 Treatment of HAE cultures with RV1088 delays the onset of A(H1N1)pdm09-related CPE

Cytopathic effects (CPE) are observable, structural changes in cell morphology resulting from virally induced changes in the cells physiology and perturbation of cellular biosynthesis. Common morphological changes include: rounding of infected cells, the formation of syncytia (polykaryocytes) and disruption of confluent monolayers.

In order to determine if RV1088 had any effect on the development of CPE caused by A/England/195/2009 (A(H1N1)pdm09) in the HAE culture system, cells were examined under 20x microscopy each day for the duration of the experiment. Representative micrographs (Figure 34) demonstrate that RV1088 treatment delayed the onset of observable CPE to day 2 p.i, one day later than for the DMSO treatment group. Furthermore, the extent of CPE in infected cells treated with RV1088 at day 2 p.i. was less than the CPE seen for the DMSO treatment group on the same day.
Figure 34: RV1088 delays the onset of viral-induced cytopathology when added after the initiation of infection. HAE cell cultures were infected with A/England/195/2009 (moi = 0.0001). One hour post infection, RV1088 (1.73nM) or DMSO (0.5%) was applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. At set time point, cultures were examined and representative samples photographed at 20x magnification. Arrows mark sites of evident CPE. Results presented are from one representative well from a set of three.
3.2.4 Treatment of HAE cultures with RV1088 during an active A(H1N1)pdm09 infection

The previous experiments employed pre-treatment of the HAE cultures with RV1088 for 3 days before virus infection to give the compound every possible chance of modifying the cellular environment. We next performed an assay in which the drug was added after influenza virus infection. As before, a slight decrease in viral titre released at the apical surface was noted after RV1088 treatment but the small 3.22 and 4.54 (corresponding to 48 and 72hrs p.i. respectively) fold decrease was not statistically significant (Figure 35, a). The cytokine/chemokine protein analysis of the apical washings (Figure 35, b-d) demonstrated that post-infection treatment of HAE cultures with RV1088 resulted in suppression of cytokine/chemokine proteins when compared to the DMSO control group. However, it should be noted that this suppression, while significant, was not the same extent (i.e. it did not flatline) as that seen in the pre-infection treatment experiments.

3.2.5 Treatment of HAE cultures with RV1088 after infection with B/Florida/04/2006

As seen for influenza A virus, treatment with RV1088 at one hour post infection of HAE cells had little effect on virus yields (1.2 fold decrease in titre at 24 hours p.i.) but did suppress the cytokine response elicited by a type B virus (B/Florida/04.2006), although not to the same extent as in cells that had been pre-treated with drug for 3 days before infection (Figure 36).
Figure 35: Results of post-infection treatment with RV1088 in HAEs infected with A/England/195/2009. HAE cell cultures were infected with A/England/195/2009 (moi = 0.0001). One hour post infection, RV1088 (1.73nM) or DMSO (0.5%) was applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-d). Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
Figure 36: Results of post-infection treatment with RV1088 in HAEs infected with B/Florida/04/2006. HAE cell cultures were infected with B/Florida/04/2006 (moi = 0.0001). One hour post infection, RV1088 (1.73nM) or DMSO (0.5%) was applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-d). Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001). RLU: relative light units.
3.2.6 Cytokine responses in human PBMCs infected with A(H1N1)pdm09 and treated with RV1088

In vivo the cytokine response induced by influenza virus will be contributed to by cells of the respiratory epithelium and also by specialist immune cells that are exposed to virus in the lung.

The pathways that lead to the cytokine response in immune cells are different than those in epithelial cells and so it was important to assess the effects of RV1088 on immune cell responses to influenza. Since knowledge regarding the specific role certain immune cells might play during the initial stages of an influenza infection is as yet unclear, we opted to isolate and employ a heterogeneous mixture of immune cells (PBMCs) in order to ensure that we were able to observe the effects of RV1088 across the full spectrum. Cytokine responses were measure by specific qRT-PCR for cytokine genes.

In order to evaluate the effect of RV1088 on the immune response, peripheral blood mononuclear cells (PBMCs) were isolated from donor human whole blood and subsequently infected with A(H1N1)pdm09. One hour post-infection, cultures were treated with RV1088 (either 1.73μM (high dose) or 0.0173nM (low dose)). Serum free media (SFM) with DMSO (0.5%) acted as a no-treatment control while another set of cells were left uninfected to determine baseline levels of cytokine gene expression. At 24 and 48hrs p.i., total RNA was extracted from PBMC cultures and qRT-PCR was performed for cytokines (Figure 37, parts a-c) and viral M-gene (Figure 37, part d). At 24hrs p.i., both doses of RV1088 suppressed cytokine/chemokine mRNA transcription (with 1.73μM being more effective than 0.0173nM). However, by 48hrs p.i., in the presence of 0.0173nm RV1088, the cytokine/chemokine levels reached levels seen in untreated cells. In contrast, the higher dose 1.73μM RV1088 suppressed the cytokine response throughout the experiment. RV1088 also suppressed viral M-gene mRNA transcription in a dose dependent manner at 24hrs p.i. and continued to suppress viral gene accumulation at 48hrs p.i.
Figure 37: qPCR results of human PBMCs infected with A/England/195/2009 and treated with RV1088. Human PBMCs isolated from donors were infected with A/England/195/2009 (moi = 3). One hour post infection, RV1088 (1.73μM or 0.0173nM) was added to the cell cultures. At 24 and 48hrs p.i., total RNA was extracted from cultures and qRT-PCR was performed for cytokines (a-c) and viral M-gene (d). Results are expressed as the mean ± standard deviation of triplicate samples.

3.3 Discussion and Conclusions

There exists considerable debate as to the efficacy and wisdom of employing steroids in cases of influenza-induced ALI/ARDS with evidence existing on both sides of the argument (Agarwal, 2007; Tang, 2009; Steinberg, 2006; Salomon, 2007; Xu, 2009; Kumar, 2009; Dominguez-Cherit, 2009). One of the major concerns regarding using
imunomodulators in cases of influenza infection is that by inhibiting the natural host response, mechanisms which restrict viral replication could be inactivated leading to an augmented rate of viral replication to the host’s detriment. Before examining the effects of RV1088 on viral replication and cytokine/chemokine suppression, we sought to first examine how a corticosteroid (Fluticasone) would act in our culture system in the context of an influenza infection. As the results in Figure 31 demonstrate, steroid treatment resulted in significant increases in viral replication levels with cytokine/chemokine levels either unchanged or increased when compared to DMSO controls.

There exists surprisingly little information in the literature with respect to the in vitro effects of the corticosteroid fluticasone against influenza. What virological studies have been conducted have primarily employed either RSV or rhinovirus. Several groups have reported that in primary human cells, fluticasone was able to suppress RSV-induced cytokines (IL-6, IL-8, RANTES) without any reported effects on viral titers (Van Ly et al., 2011; Noah et al., 1998). However, in one paper a strong, yet non-significant trend can be seen with fluticasone-treated cells producing higher viral titers than their non-treated counterparts (Noah et al., 1998). Further supporting this result is the observation by Puhakka et al., (1998) that in patients, treatment of rhinovirus infections with fluticasone resulted in a significant decrease in some symptoms (e.g. reduced nasal congestion and cough) but also had the undesired effect of resulting in prolonged viral shedding. An additional study conducted by Ruohola et al., (2000) found that in children infected with rhinovirus, significantly more subjects developed acute otitis media when treated with fluticasone when compared to placebo group. In summary, there is divided opinion as to the efficacy of using fluticasone in the context of an upper respiratory infection initiated by either RSV or rhinovirus. Unfortunately, we were unable to locate any peer-reviewed studies that looked at the effects of fluticasone when used during an influenza infection. However, the use of other steroids in cases of influenza infection have been published with Hu et al., (2013), for example, describing how employing corticosteroids led to antiviral treatment failure, the emergence of Oseltamivir resistance and adverse clinical outcome. It is possible that our results indicate that this steroid is ineffective against this virus and that a more targeted approach might prove more beneficial. RV1088’s targeting of certain key innate immune
response regulatory pathways may prove to be superior strategy, when compared to global suppression tactics, leading to a more favourable outcome for the host.

In this chapter we examined the effects of RV1088 in cell cultures infected with influenza viruses. In the first instance, CALU-3 cells were infected at a low moi with A/England/195/2009 and it was determined that, while RV1088 used alone did not have a significant effect on viral titres, cytokine expression induced by virus infection was suppressed at the level of transcription (1.58- and 4.76-fold decrease in IFN-β and RANTES respectively). M-gene, a marker of viral replication, was also suppressed (3.78-fold decrease) but this suppression did not seem to be enough to significantly affect viral titers as measured by plaque assay (2.57-fold decrease). The qRT-PCR results suggest that a several fold difference in RNA levels have little effect on viral titres as measured by infectious unit on a log scale. This discrepancy could simply be explained by the innate difference in sensitivity that exists between these two techniques and the fact that qRT-PCR is a method for determining mRNA levels with no distinction made between replicative and non-replicative viruses.

CALU-3 cells are an immortalised lung epithelial cell line and may have alterations in their signalling pathways which could alter their ability to respond to pathogenic insult. Therefore we further assessed the effect of RV1088 on virus replication and cytokine induction in primary airway cells. We employed commercially available highly differentiated human airway epithelial cells (HAEs) as the viral target cells. These cultures are maintained at an air-liquid-interface, recapitulate the cellular 3-D morphology of the airways, with active ciliated cells and mucus secreting goblet cells. Our lab and others have previously shown that there exists remarkably little differences between these cultures and airway biopsies in terms of there transcriptional profiles and gene expression (Pezzulo et al., 2011; Dvorak et al., 2010). In terms of evaluating both virus replication kinetics and virally-induced cytokine/chemokine responses produced by the airway epithelium, highly-differentiated culture systems has proven to be an important and reliable tool (Schaap-Nutt et al., 2012; Crotta et al., 2013; Zhang et al., 2011).

When RV1088 was applied to HAE cultures, either pre- or post-infection with low moi of influenza A or B virus, A/England/195/2009 or B/Florida/04/2006, there was no
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overall significant suppression of viral replication as measured by viral titres harvested from
the apical surface (save for the case of B/Florida/04/2006 at 24hrs p.i. when cultures pre-
treated with RV1088 resulted in a significant decrease in viral titers, p≤0.05), although
consistently a slight trend in decreased replication was seen for the RV1088 treatment
groups when compared to the DMSO controls. The most striking results were with respect
to the suppression of cytokine/chemokines released from the infected cultures in apical
washings. In all cases, RV1088 significantly suppressed chemokine/cytokine induction. This
is in line with work conducted by Salomon et al., (2007) where corticosterone treatments
were found to have cytokine/chemokine suppressive properties but also had little-to-no
effect on viral replication or survival outcome in H5N1 infected mice.

Although RV1088 inhibited the innate immune response to the virus, we did not
observe any increase in viral replication in this in vitro system. This might be taken to
suggest that viral titre in not greatly regulated by the innate response induced under natural
conditions. However, in studies where patients hospitalized with seasonal A/H1N1 and
treated with corticosteroids, higher viral titers and prolonged viral shedding were found
when compared to non-glucocorticoid-treated patients Lee et al., (2009). These finding are
further reiterated in patients infected with A/H7N9 (Hu et al., 2013) and in mice infected
with A/H5N1 (Xu, 2009) where treatment with corticosteroids resulted in an increase in viral
titers. Therefore an alternative explanation for our findings is that, unlike corticosteroids
which acts by preventing the downstream transcription of genes encoding for the majority,
if not all, proinflammatory mediators (Annane, 2011; Ehrchen et al., 2007), RV1088 is a
narrow spectrum kinase inhibitor; targeting only a cluster of key regulatory kinases believed
to play a role in the over-induction of the host’s innate immune response. By only targeting
this “cluster” an over-induction of cytokines/chemokines may be prevented while leaving a
sufficient section of the immune response untouched and thus allowing for a proportionate
response to viral insult. However, RV1088 did decrease the secretion of all
cytokines/chemokines assessed (save for the protein levels of the chemokine CXCL1 which
were unaltered, data not shown). In order to determine if any additional protein(s) are also
unaffected, it might be necessary to perform a microarray of proteomic analysis to discover
their identity. The other explanation is that the virus has been inhibited in addition to the
cytokines so that on the one hand the drug removes the inhibitory cytokine that would
cause rebound of virus titre (Cyclosporine A in the context of encephalomyocarditis, for example (Miyamoto et al., 2001)) but at the same time the drug affects virus itself and inhibits virus titres.

Upon microscopic examination of the cultures, it was apparent that RV1088 was able to protect infected airway cells from the development of virus induced cytopathic effects (CPE). In all instances, the application of drug delayed the appearance of observable CPE by a minimum of one day. Moreover the extent of CPE was greatly reduced when compared to the DMSO controls. Several groups have explored the interaction of p38 MAPK (a target of RV1088) with Bcl-2, a molecule with a widely recognized cellular protective mechanism against virally-induced apoptosis (De Chiara et al., 2006; Reed, 1994; Hinshaw et al., 1994). p38 MAPK-directed phosphorylation of the Thr-56 and Ser-87 residues of Bcl-2 have been shown to adversely affect the ability of this factor to regulate apoptosis. As reported in Chapter 2 employing three different methods, we did not observe any negative effects of RV1088 on cell viability or metabolic state potentially indicating that RV1088 has been able to prevent an over-induction of the apoptotic pathway by blocking the p38 MAPK-directed phosphorylation of Bcl-2.

In addition, the inhibitory effect of RV1088 on virus replication, albeit mild, may also have contributed to delayed CPE by staving accumulation of damaging viral products. p38 MAPK has been show to be beneficial to the virus in terms of viral entry (Marchant et al., 2010) and viral replication (Nencioni et al., 2009). The decreased appearance of CPE in HAE cultures could represent the combined effects of all of these factors.

There is mounting evidence that innate immune cells (such as lymphocytes, monocytes and macrophages), play a significant role in the pathogenesis of severe cases of influenza. Lee et al., (2009) have reported that ex vivo human PBMCs infected with seasonal influenza actively express cytokines and chemokines and that these levels were significantly correlated to plasma levels from infected individuals. Moreover, these levels dynamically changed throughout the course of infection. Their results indicate that immune cells may, in part, contribute to the host’s overall cytokine/chemokine response to an influenza infection.

Despite the importance of cytokines and chemokines in the development of influenza-related pathogenesis, remarkably little is know about the cellular origin of many of
these vital small signalling proteins. While it is generally accepted that numerous cell types, including pneumocytes, macrophages, and dendritic cells, produce interferon in response to an influenza infection, the timing and their relative contributions were unknown (Hogner et al., 2013; Kaminski et al., 2012; Cheung et al., 2002). Kalffass et al., (2013) have sought to address the cellular origin of interferons and have reported that during the course of an influenza infection, vigorous interferon production is undertaken by a surprisingly small number of cells. The specific cell types responsible for the virally-induced synthesis of interferon depends on the infection time-course with various subsets of macrophages being the primary synthesisers at early time points (peaking at 24-48hrs p.i.) after which time, epithelial cells were found to take over as the major IFN-synthesis cell type. The authors hypothesize that the synthesis of other cytokines/chemokines in response to an influenza infection may follow a similar cell/time response pattern.

This work is further supported by studies conducted by Song et al., (2013) where PBMCs were isolated from severely ill patients infected with A(H1N1)pdm09 and an analysis of their microRNA levels was performed. Their results indicate that, when compared to healthy controls, the microRNA levels found within PBMCs can be markedly different in severely ill patients. Of specific interest, was their reported finding that several miRNAs (miR-769-5p, miR-146b-5p, let-7g, miR-30b, miR-361-3p, and miR-362-3p) implicated in supressing p38 MAPK expression were themselves suppressed in severe influenza cases, creating a situation where p38 might be overexpressed in those severely ill. The authors suggest that supressing p38 could thus be of benefit in inhibiting viral replication and over-induction of the innate immune response in this subset of severely ill patients.

Given these findings, we investigated if RV1088 was able to suppress cytokine induction in immune cell types infected with pandemic H1N1. Primary peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood acquired from human donors. We purposely did not further sort between cell types as there is still considerable debate in the field as to what role each cell type actually has during the course of an influenza infection (Iwasaki & Pillai, 2014; Levy et al., 2011; Thomas et al., 2006). The isolated PBMCs from several different donors were pooled in order to achieve a representative response. RV1088 suppressed cytokine/chemokine mRNA transcription and viral M-gene mRNA
transcription in a dose-dependent fashion. These results mirror those seen in the HAE culture system and thus we can propose that in both epithelial and immune cells, RV1088 maybe targeting the virus directly as well as targeting key components of the cytokine/chemokine signaling cascades. Determining the exact immune cell subtype responsible for the production of the cytokines/chemokines that are in turn suppressed by RV1088 could prove to be an interesting avenue for future investigation.
Chapter 4. RV1088 as a component of combination therapy

4.1 Introduction

4.1.1 Combination therapy

RV1088 is capable of suppressing the transcription of cytokines and chemokines induced by the influenza virus in an immortalised cell line, and also in primary human airway cultures, without rebound in viral titre. Any novel agent for influenza therapy is unlikely to be employed in a treatment setting on its own, but will rather be employed as a component of combination therapy in conjunction with a currently licensed antiviral. In the context of influenza, it is unlikely that a novel drug would be given in the absence of Tamiflu today. As such, we next examined how employing RV1088 in conjunction with a licensed antiviral might affect both viral replication and cytokine/chemokine expression.

One approach to help combat the rise of antiviral resistance in a patient is to employ combination chemotherapy consisting of two or more drugs that target different viral components and/or host factors for which the mechanism by which resistance can arise differs. Using combination therapy may reduce the likelihood that resistance to a single drug will emerge and also lessen the potential clinical effect of that single drug resistant strain in the patient. The principle behind multi-drug combination therapy is that two or more drugs are combined to produce interactions defined as “synergistic,” “additive,” or “antagonistic” whereby their combined effect either exceeds, equals or is less than the sum of their individual effects, respectively (Suhnel, 1990).

An additional benefit to the patient of a combination therapy approach is the potential for potentiation of antiviral activity and enhancement of clinical outcome by allowing for the doses of individual drugs to be reduced which, in turn, reduces dose-related toxicities and side-effects. The use of multidrug therapy for the treatment of viral diseases is not novel, and the pharmacological rationale for such an approach is typified by the
combination treatment regime used in cases of HIV. This strategy known as HAART (highly active antiretroviral therapy) keeps plasma viral loads below the level of detection, prevents the emergence of resistance and allows for stabilization of the patient’s immune system (Raboud et al., 2002; Gulick, 1998).

The aim of any combination therapy is to inhibit the selection and outgrowth of resistant viruses by reducing the number of overall replication cycles and by reducing the proportion of virions carrying the resistance mutations. Using a mathematical model, Perelson et al., (2012) estimated the probability of producing drug-resistant variants during infection. Their results indicate that all possible single mutants are produced hundreds of thousands of times during the infection of a single host. While the maintenance of such variants within the host depends on numerous factors (drug sensitivity, fitness costs of the mutation, state of the host’s immune response, etc.), they predict that if antiviral therapy is given to enough infected individuals and the selective pressures are strong enough, drug-resistant mutants are inevitable. If, however, one uses a dual-combination therapy, such that the single point mutations confer resistance to only one of the two compounds the odds of resistance arising is decreased and is likewise is further decreased when a triple therapy is employed.

Several studies have been conducted to assess the effects of both double and triple drug combinations on influenza infection in vitro using drugs previously shown to have anti-influenza properties (Table 4).

Currently, the mouse model is the most accepted animal model for the evaluation of antiviral drug combinations (Govorkova & Webster, 2010). Leneva et al., (2000) conducted one study where Oseltamivir was used in conjunction with either amantadine or Rimantadine in mice inoculated with either A/H5N1 or A/H9N2. Their results demonstrated that combination therapy was more effective than monotherapy in preventing death and further support similar results seen by other groups (Ilyushina et al., 2008; Wilson et al., 1980).
Table 4. Effect of double drug combinations on different influenza subtype infections \textit{in vitro}. Adapted from: Govorkova & Webster, 2010.

<table>
<thead>
<tr>
<th>Drug Combinations</th>
<th>Influenza subtypes tested</th>
<th>Methods of analysis used</th>
<th>Drug interactions</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oseltamivir + Amantadine</td>
<td>H1N1, H3N2, H5N1</td>
<td>PFU, NR staining, TCID\textsubscript{50}</td>
<td>Enhanced/Additive-synergistic</td>
<td>Ilyushina et al., 2006; Smee et al., 2009; Nguyen et al., 2010</td>
</tr>
<tr>
<td>Oseltamivir + Rimantadine</td>
<td>H1N1, H3N2</td>
<td>ELISA, TCID\textsubscript{50}</td>
<td>Additive-synergistic</td>
<td>Govorkova et al., 2004</td>
</tr>
<tr>
<td>Oseltamivir + Ribavirin</td>
<td>H1N1</td>
<td>Neutral Red (NR) staining</td>
<td>Additive</td>
<td>Nguyen et al., 2010</td>
</tr>
<tr>
<td>Zanamivir + Rimantadine</td>
<td>H1N1, H3N2</td>
<td>ELISA, TCID\textsubscript{50}</td>
<td>Additive-synergistic</td>
<td>Govorkova et al., 2004</td>
</tr>
<tr>
<td>Zanamivir + Ribavirin</td>
<td>H1N1, H3N2</td>
<td>ELISA</td>
<td>Additive</td>
<td>Madren et al., 1995</td>
</tr>
<tr>
<td>Peramivir + Rimantadine</td>
<td>H1N1, H3N2</td>
<td>ELISA, TCID\textsubscript{50}</td>
<td>Additive-synergistic</td>
<td>Govorkova et al., 2004</td>
</tr>
<tr>
<td>Peramivir + Ribavirin</td>
<td>H1N1</td>
<td>TCID\textsubscript{50}</td>
<td>Synergistic</td>
<td>Smee et al., 2002</td>
</tr>
<tr>
<td>Rimantadine + Ribavirin</td>
<td>H1N1, H3N2, H7N7, B/Lee/40</td>
<td>PFU, ELISA</td>
<td>Additive-synergistic</td>
<td>Galegov et al., 1977; Hayden et al., 1980; Hayden et al., 1984; Madren et al., 1995</td>
</tr>
<tr>
<td>Amantadine + Ribavirin</td>
<td>H1N1, H3N2, H5N1</td>
<td>PFU, NR staining, TCID\textsubscript{50}</td>
<td>No added benefit to additive-synergistic</td>
<td>Hayden et al., 1980; Smee et al., 2009; Nguyen et al., 2010</td>
</tr>
</tbody>
</table>

A different and relatively new approach has been to combine directly acting antiviral agents with use of an immune modulating agent with the rational being that such an approach could simultaneously tackle excessive viral replication and excessive cytokine induction that leads to the cytokine storm driven immunopathology.

In a paper by Zheng \textit{et al.}, (2008), mice were inoculated with a high titre of A/H5N1 and treated with either triple therapy, including a combination of Relenza and two inhibitors of inflammation (Celecoxib and Mesalazine), or just Relenza alone. The triple therapy group had a significantly better survival rate, survival time and levels of inflammatory markers than the group receiving only the monotherapy. This result is further supported by a study.
conducted in the cotton rat model where a neuraminidase inhibitor was used in conjunction with a range of doses of corticosteroids. The effect was the inhibition of inflammatory cell recruitment to the lung and suppression of proinflammatory mediators in a dose dependent manner without an effect on viral clearance (Ottolini, 2003). Importantly for the current study, Haasbach et al., (2013) have reported a synergistic inhibition of virus replication when A(H1N1)pdm09 was treated with a combination of Oseltamivir plus a MEK (mitogen-activated protein kinase kinase)-inhibitor in vitro although a mechanism has yet to be proposed. While preliminary, this result indicates that conventional treatments in combination with kinase inhibitors specific for host targets could have a role in influenza chemotherapy strategies.

It is important to note however, that most drug studies involving 2 or more drugs have employed viruses that are sensitive to all drugs administered; rarely has a resistant strain been employed (Govorkova & Webster, 2010). One study conducted by Smee et al., (2009) found that using amantadine and Oseltamivir carboxylate, in combination against the amantadine-resistant strain of A/H5N1 (A/Duck/MN/1525/81), had no additive or synergistic effect on viral replication in MDCK cells.

A few clinical trials have also sought to examine the potential of using combination therapy in patients infected with the flu virus. Ison and colleagues (2003) conducted a double-blind, randomized, placebo-controlled trial to access the efficacy of using Rimantadine plus either nebulized Relenza or nebulized saline to treat patients infected with non-resistant A/H3N2. The results of this study demonstrated that patients treated with the Rimantadine plus Relenza combination therapy had a non-significant trend towards fewer days of virus shedding and improved symptomology scores when compared to the monotherapy plus saline group. Furthermore, two out of eleven patients in the monotherapy group developed resistance to Rimantadine while no resistance arose in the combination therapy group. Another study conducted by Morrison et al., (2007) importantly indicates that the pharmacokinetics of Oseltamivir (75mg po bd) are not affected by concomitant use of amantadine (75mg po bd) and vice versa. Furthermore, there was no evidence of an increase in reports of adverse side-effects when these two classes of drugs were used in combination at normal dosing levels.
4.2 Results

4.2.1 Identification of synergistic activity

There are numerous examples of anti-influenza agents working either additively or synergistically when used in combination. To examine the potential of RV1088 to work in combination with known and proven anti-influenza drugs, CALU-3 cells (human epithelial, lung adenocarcinoma line) and HAE cultures (primary airway cell culture were employed.

A method commonly used to analyse the synergistic effect of a drug when used in combination with other drugs is the combination index (CI) theorem of Chou-Talalay. This test used the quantitative definitions of antagonism (CI >1), additive effect (CI=1), and synergistic effect (CI < 1) in drug combination (Chou, 2010). The median-effect equation (MEE) works by combining enzyme kinetics and specific mechanisms from hundreds of individual equations and reduces them to a single general equation. Both left and right sides of the MEE are dimensionless quantities. When the m (slope) and Dm (half-affected dose) are determined, the full dose-effect curve is defined. As the median-effect plot yields straight lines, the theoretical minimum of only two data points allow the drawing of the full dose-effect curve. Using a specific software program which employs the Chou-Talalay theorem equations (Calcusyn, Biosoft - Cambridge, UK), we examined the relationship produced by RV1088 when used in combination with Relenza.

The presence of synergy against A/England/195/2009 (A(H1N1)pdm09) (moi = 0.0001) was determined from the results of 9 experimental drug combination each in triplicate performed in airway cells. In terms of drug doses, the Relenza dose was held constant at 3.009nM while the concentration of RV1088 were varied until the ED50, ED75 and ED90 was determined (0.75nM, 1.0nM and 1.73nM respectively – data not shown). These predetermined drug combinations were applied to the airway cell cultures for three days prior to infection. 48hrs post infection, culture supernatants were taken and used to determine viral titer levels via plaque assay in MDCK cells. The results from these plaque assays was then in turn inputted into the Chou-Talalay theorem software. As indicated in Table 5, a combination of RV1088 and Relenza led to very low CI values indicating that a very strong synergistic anti-viral effect exists when these two drugs are used in combination. This synergistic effect is also represented visually by the median-effect plot (Figure 38).
Table 5: Combination index for RV1088 and Relenza when used against influenza virus replication.

<table>
<thead>
<tr>
<th>Drug</th>
<th>ED50</th>
<th>ED75</th>
<th>ED90</th>
<th>Dm</th>
<th>m</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV1088</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>3.83E+08</td>
<td>0.597</td>
<td>0.918</td>
</tr>
<tr>
<td>Relenza</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>8.62E+05</td>
<td>0.414</td>
<td>0.995</td>
</tr>
<tr>
<td>RV1088+Relenza</td>
<td>1.76E-06</td>
<td>1.48E-07</td>
<td>1.25E-08</td>
<td>7.94E-01</td>
<td>6.289</td>
<td>0.917</td>
</tr>
</tbody>
</table>

For each drug, and combination there of, the Chou-Talalay method for drug combination was used to calculate the median effect equation. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for antagonism (CI >1), additive effect (CI=1), and synergistic effect (CI < 1) in drug combination. ED50, 75 and 90 are the dose at which 0.75nM, 1.0nM and 1.73nM of RV1088 in the presence of a constant concentration of Relenza (3.009nM) respectively inhibited viral growth, Dm = the median-effect dose signifying potency, m = an exponent signifying the sigmoidicity (shape) of the dose-effect curve and r = linear correlation coefficient of the goodness of fit for the data to the median-effect equation (a value of r>0.90 is typically expected when tissue/cell cultures have been employed).

Figure 38: The median-effect plot of RV1088 when used in combination with Relenza. The transformation of data using the median-effect equation allows for the calculation of the level of growth inhibition at a given dose. The dose-response relationship is displayed as a linear regression line (for RV1088+Relenza, r = 0.917). The x-intercept of the median effect-plot represents the median effect dose (Dm, ED50) at which a 50% growth inhibition (Fa = 0.5) occurs. Fa, affected fraction; Fu, unaffected fraction; D, dose.
Further combination experiments were undertaken employing RV1088 in combination with several other licenced antivirals. The known antivirals chosen for these experiments were Relenza, Rimantadine and Oseltamivir that are representative of the two major classes of anti-influenza agents (the NA-inhibitors and the M2-inhibitors). The virus chosen for these experiments was a mutated variant of A/England/195/2009 (A(H1N1)pdm09) known as M2S31N. During 2009 H1N1 pandemic, a mutation in the M2 gene (N31S) naturally arose conferring sensitivity to Rimantidine while at the same time not adversely effecting viral replication. We back engineered this mutation into our prototypic A(H1N1)pdm09 virus (A/England/195/2009) to render it susceptible to Rimantidine for the purposes of being able conduct our synergy studies with the same virus that was sensitive to Relenza, Rimantadine and Oseltamivir. As expected, the virus carrying this mutation did not display any adverse effects in terms of replication (data not shown).

In our synergy experiments, the concentrations for each licensed drug employed was based on previously reported IC\textsubscript{50} values and the concentration of RV1088 was chosen through consultation with our collaborators at RespiVert as 1.73nM. In Calu-3 cells, all three licensed drugs inhibited viral replication. At time point where maximal inhibition was observed, Relenza, Oseltamivir and Rimantadine treatments resulted in an average log reduction in titers of 3.8, 4 and 4.22-fold respectively (Figure 39). On the other hand, the maximum fold reduction from RV1088 when used on its own was 1.23, thus reinforcing the results in Section 3.2 that the drug has a small but insignificant antiviral effect. In contrast there was a significant suppressive effect of RV1088 when used in combination with a licenced anti-viral. In each instance, the use of combination treatment resulted in a dramatic suppression of viral replication with the combinations of Relenza+RV1088, Oseltamivir+RV1088 and Rimantadine+1088 resulting in average maximum log fold reductions in titers of 5.44-, 1.85- and 5.65-fold respectively.
Figure 39: Inhibition of influenza virus replication by combinations of drugs. CALU-3 cells were infected with A/England/195/2009 ± N31S mutation to confer sensitivity to Rimantadine (moi = 0.0001) in the presence or absence of various drug combinations: (a) RV1088 (1.73nM) ± Relenza (3.009nM), (b) RV1088 (1.73nM) ± Rimantadine (40uM) and, (c) RV1088 (1.73nM) ± Oseltamivir (12nM). Culture supernatant was harvested at 24 and 48hrs p.i. and plaqueed on MDCKs. Results represent the average of triplicate samples from two independent experiments.
4.2.2 Studying the effects of combination treatment on cytokine/chemokine induction in CALU3s

To examine the effect of the drug combination therapies on cytokine/chemokine expression, total RNA was extracted from cultures at 24hrs p.i and qRT-PCR was performed (Figure 40). What is evident from these results is that the strong induction of transcription of mRNAs for cytokines and chemokines by virus alone was significantly reduced in the presence of RV1088, or Rimantadine, when used on their own, but not by the NA inhibitors. Specifically, RV1088 reduced the transcription of IFN-β by 1.43-fold and RANTES by 3.4-fold while Rimantidine was able to inhibit IFN-β and RANTES transcription by 1.1- and 1.87-fold respectively. Oseltamivir when used alone, in fact, had the opposite effect and was responsible for an increase in cytokines/chemokine mRNA levels (a 0.8-fold increase with respect to IFN-β transcription). However, regardless of the antiviral used, the suppressive action of RV1088 plus a licensed anti-viral against the transcription of cytokine/chemokine mRNA was either greater than, or equal to, the suppressive effect generated by using two antivirals (an M2-inhibitor + and an NA-inhibitor) in combination (Figure 40, part b). Specifically, the combination of Rimantadine+Oseltamivir resulted in a 3.38-fold decrease in the transcription of IFN-β mRNA and a 1.38-fold decrease for RANTES mRNA. In contrast the combinations of RV1088+Rimantadine resulted in a 2.88-fold reduction for IFN-β and a 2.22-fold reduction for RANTES mRNA while the RV0188+Oseltamivir combination resulted in a 2- and 10.6-fold reduction for IFN-β and RANTES mRNAs respectively.
Figure 40: Quantitation of induced mRNAs for representative cytokine and chemokine following infection of CALU-3 with influenza virus in presence of drug combinations. Total RNA was extracted from CALU-3 cells 24hrs p.i. with A/England/195/2009 M2 N31S mutant at moi = 0.0001 and qRT-PCR was performed using standardised primers and probes. At the time of infection, cells were treated with either (a) RV1088 (1.73nM) ± Oseltamivir (12nM) or (b) RV1088 (1.73nM) ± Rimantadine (40μM). Results are expressed as the mean ± standard deviation of triplicate samples.
4.2.3 Treatment of HAE cultures with RV1088 in combination with licenced antivirals prior to infection with A(H1N1)pdm09.

To further examine the potential of using RV1088 as a component of a combination therapy, HAE cell cultures were employed. A/England/195/2009 (A(H1N1)pdm09, N31S or wild-type depending on the specific drug being tested) was chosen to act as the representative type A virus. As seen previously, RV1088 had a small inhibitory effect on viral replication that was not significant when used alone while Relenza was able to significantly reduce the level of viral replication by 1000-fold at 48 hours post infection. When these two drug compounds were used in conjunction, there was a significant suppression of viral replication which was greater than the level observed for either individually resulting in a $1.55 \times 10^5$-fold reduction in titers at 48hrs p.i. (Figure 41, part a).

The same apical washings were used to quantify cytokine/chemokine protein levels quantification using a Luminex™ platform (Figure 41, parts b-d). The results demonstrated that RV1088, when used alone, significantly suppressed cytokine/chemokine protein levels. In comparison, Relenza when used on its own suppressed protein expression for IP-10 and IL-6 to varying degrees but had no significant effect on the expression levels of IL-8. Combination treatment, on the other hand, resulted in near complete suppression of the expression of IP-10 and IL-6 while IL-8 expressions levels remained around those seen in the RV1088 alone treatment group.
Figure 41: Virus replication after pre-infection treatment with RV1088±Relenza in HAEs infected with A/England/195/2009. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with A/England/195/2009 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaque on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-d). Results are expressed as the mean ± standard deviation of triplicate samples.
4.2.3.1 Treatment of HAE cultures with combination therapy delays the onset of A(H1N1)pdm09-related CPE

In order to determine if the combination of RV1088 with Relenza had any effect on the development of CPE in the HAE culture system caused by A/England/195/2009 (A(H1N1)pdm09), cells were examined under 20x microscopy each day for the duration of the experiment. Representative micrographs (Figure 42) demonstrate that in this culture code RV1088 treatment delayed the onset of observable CPE to day 3 p.i, one day later than for the Relenza alone treatment group. Furthermore, the extent of CPE in infected cells treated with RV1088 at day 5 p.i. was less than the CPE seen for the Relenza group on day 2 p.i. Quite remarkably, in the RV1088/Relenza combined treatment group, the emergence of observable CPE was prevented until day 4 p.i and even then was quite limited in nature.
Figure 42: RV1088 delays the onset of viral-induced cytopathology in HAE cultures infected with A/England/195/2009. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with A/England/195/2009 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then emoved) and added to the basal media for the duration of the experiment. At set time point, cultures were examined and representative samples photographed at 20x magnification. Arrows mark sites of evident CPE. Results presented are from one representative well from a set of three.
4.2.4 Treatment of HAE cultures with RV1088 in combination with licenced antivirals prior to infection with B/Florida/04/2006.

The effectiveness of combination therapy employing RV1088 was examined against a type B influenza virus. B/Florida/04/2006 was chosen as a representative type B virus of the currently circulating B Yamagata lineage. RV1088 had no significant effect on type B viral replication when used alone. Interestingly, Relenza when used alone, did not decrease replication at the earliest time point. However, when these two drug compounds were used in conjunction, there was a significant suppression of viral replication (Figure 43, part a).

The same apical washings were used to quantify cytokine/chemokine protein levels quantification using a Luminex™ platform (Figure 43, parts b-d). The results demonstrated that RV1088 alone significantly suppressed cytokine/chemokine protein levels. In comparison, Relenza used on its own, suppressed protein expression for IP-10 and IL-6 to varying degrees but had no significant effect on the expression levels of IL-8. Combination treatment, on the other hand nearly completely suppressed the expression of IP-10 and IL-6 while IL-8 expressions levels remained around those seen in the RV1088 alone treatment group.
Figure 43: Virus replication after pre-infection treatment with RV1088±Relenza in HAEs infected with B/Florida/04/2006. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with B/Florida/04/2006 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a) and for quantification of cytokine/chemokine protein expression via Luminex (b-d). Results are expressed as the mean ± standard deviation of triplicate samples.
4.2.4.1 Treatment of HAE cultures with combination therapy delays the onset of B/Florida/04/2006-related CPE

To assess the ability of RV1088±Relenza to delay the development of CPE in the HAE culture system caused by B/Florida/04/2006, cells were examined under 20x microscopy each day for the duration of the experiment. Representative micrographs (Figure 44) demonstrated that, RV1088 treatment delayed the onset of observable CPE to day 4 p.i, 2 days later than for the Relenza treatment group. Furthermore, the extent of CPE in infected cells treated with RV1088 at day 5 p.i. was far less than the CPE seen for the Relenza group on day 2 p.i. For the RV1088/Relenza combined treatment group, the emergence of observable CPE was prevented until day 2 p.i. and even then, was quite limited in nature and its progression, indeed until day 4 p.i. was greatly restricted.
Figure 44: RV1088 delays the onset of viral-induced cytopathology in HAE cultures infected with B/Florida/04/2006. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with B/Florida/04/2006 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. At set time point, cultures were examined and representative samples photographed at 20x magnification. Arrows mark sites of evident CPE. Results presented are from one representative well from a set of three.
4.2.5 Treatment of HAE cultures with RV1088 in combination with licenced antivirals prior to infection with A/Sydney/5/1997 (A/H3N2).

To further augment the evaluation of combination therapy treatments employing RV1088 against influenza, we expanded the viral panel to include another type A virus, A/H3N2 (A/Sydney/5/1997). Using HAE cultures (reconstituted from a different donor due to supply issues), we conducted the experiment in an identical manner as previously described. RV1088 and Relenza displayed similar patterns as before (Sections 4.2.3 and 4.2.4) having no effect and a minor suppressive effect on virus replication respectively (Figure 45). While not as pronounced as seen for either A/England/195/2009 (A(H1N1)pdm09) or B/Florida/04/2006, the combination treatment group (RV1088+Relenza) followed a similar trend, suppressing viral replication to an extent greater than either drug when used alone.

Figure 45: Viral replication after pre-infection treatment with RV1088 in HAEs infected with H3N2. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with A/Sydney/5/1997 (H3N2, moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs. Results are expressed as the mean ± standard deviation of triplicate samples.
In addition to viral quantification, the same apical washings were also used for cytokine/chemokine protein level quantification using a Luminex™ platform (Figure 46). The results demonstrated that RV1088 significantly suppressed cytokine/chemokine protein levels induced by A/Sydney/5/1997. Relenza when used to treat cells on its own, suppressed protein expression of RANTES and of IL-6, and IP-10 at early time points. However, Relenza was found to have no significant effect on the expression levels of IL-8 and at later time point, actually resulted in increased levels of IL-6, IL-8, and IP-10 production when compared to the untreated infected group. Combination treatment, on the other hand, nearly completely suppressed the expression of IL-6, IL-8, IP-10 and RANTES to the level secreted from uninfected cells.
Figure 46: Cytokine and chemokine protein levels after pre-infection treatment with RV1088 in HAEs infected with H3N2. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with A/Sydney/5/1997 (H3N2, moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used for quantification of cytokine/chemokine protein expression via Luminex. Results are expressed as the mean ± standard deviation of triplicate samples.
4.2.6 Treatment of HAE cultures with RV1088 in combination with licenced antivirals prior to infection with B/Malaysia/2506/2004.

Expanding the viral panel even further, an additional type B virus, B/Malaysia/2506/2004 that is representative of the other circulating lineage B Victoria, was employed. Using HAE cultures reconstituted from the same donor as those used in Section 4.2.5, the experiment was conducted following previously described protocols. RV1088 and Relenza displayed similar patterns demonstrated previously (Sections 4.2.3 and 4.2.4) having no effect and only a minor suppressive effect on virus replication respectively. The combination treatment group (RV1088+Relenza) suppressed viral replication to a greater extent than either drug when used alone to a similar proportion seen for A/H3N2 (A/Sydney/5/1997) (Figure 47). Intriguingly for both viruses (A/Sydney/5/1997 and B/Malaysia/2506/2004) the complete suppression of viral replication observed for other viruses in the RV1088+Relenza treatment groups was not seen. As both of these former viruses were tested in HAE wells derived from the same donor, it is possible that these results might represent donor-to-donor variation. In the future, it might be advisable for these viruses to be tested in a mixed donor set.

Apical washings were also used for cytokine/chemokine protein level quantification using a Luminex™ platform (Figure 48, parts b-d). The results demonstrated that RV1088, significantly suppressed cytokine/chemokine protein levels induced by B/Malaysia/2506/2004. Relenza, when used to treat cells on its own, suppressed protein expression for RANTES but had no significant effect on the expression levels of IP-10 at the majority of time points examined. Strikingly and as noted above, Relenza actually resulted in increased levels of IL-6, and IL-8 protein production when compared to the untreated infected group. Combination treatment, on the other hand, suppressed the expression of IL-6, IL-8, IP-10 and RANTES almost to the level of the uninfected cells.
Figure 47: Viral replication after pre-infection treatment with RV1088 in HAEs infected with B/Malaysia/2506/2004. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with B/Malaysia/2506/2004 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs. Results are expressed as the mean ± standard deviation of triplicate samples.
Figure 48: Cytokine and chemokine protein levels after pre-infection treatment with RV1088 in HAEs infected with B/Malaysia/2506/2004. HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with B/Malaysia/2506/2004 (moi = 0.0001, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used for quantification of cytokine/chemokine protein expression via Luminex. Results are expressed as the mean ± standard deviation of triplicate samples.
4.2.7 Treatment of HAE cultures with RV1088 in combination with licenced antivirals prior to infection with A/Turkey/2005:PR8 (6:2) (A/H5N1).

The final virus assessed in HAE for combination treatment was an A/H5N1 (A/Turkey/05:PR8-6:2 reassortment) virus. This particular virus was chosen due to its reported ability to be a high inducer of the human innate immune response. Using HAE cultures reconstituted from the same donor as those used in Section 4.2.3, the experiment was conducted following previously described protocols under a high containment setting. RV1088 and Relenza had no effect and a minor suppressive effect respectively on virus replication (Figure 49). Importantly, when RV1088 and Relenza were used in combination, they suppressed viral replication to a significant extent and were far superior than the effect observed for either drug when used alone. Due to high containment (CAT-3) requirements and regulations, an examination of cytokine/chemokine protein levels via Luminex™ was not possible.

Figure 49: Viral replication after pre-infection treatment with RV1088 in HAEs infected with A/Turkey/05:PR8 (6:2). HAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) ± Relenza (3.009nM) or DMSO (0.5%). Cultures were infected with A/Turkey/Turkey/05/2005 (moi = 0.01, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the
duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs. Results are expressed as the mean ± standard deviation of triplicate samples.

4.3 Discussion and Conclusions.

Within this chapter, we sought to examine the potential of using RV1088 in conjunction with licenced influenza antivirals, evaluating this potential in both terms of cytokine/chemokine suppression quantified via qRT-PCR and Luminex and effect on viral replication as measured by plaque assay.

The use of combination therapy might potentiate antiviral activity, decreasing the likelihood of resistant strains emerging and enhancing clinical outcome (Raboud et al., 2002; Gulick, 1998; Suhnel, 1990). Furthermore, combination therapy often allows for lower doses of the individual constituents in order to obtain an effect superior to either constituent used alone at a higher dose thus decreasing dose-related toxicities and side effects. As discussed in the chapter introduction, numerous studies have been performed examining the potential of employing combination therapy in cases of influenza. Thus far, the published reports have been very favourable but surprisingly this practise is rare in the clinic, with antivirals often being used singly and one after the other in severe cases. Most studies have combined directly acting antivirals. To our knowledge, however, no clinical study has yet been undertaken which has examined the potential effects on cytokine/chemokine expression and viral replication by a kinase inhibitor used in conjunction with a licenced antiviral.

Our first set of experiments were based in CALU-3 cells and employed RV1088 used in combination with Relenza, Rimantadine or Oseltamivir. The doses chosen were based on the specific drugs’ reported IC_{50}. In these experiments, we opted to employ A(H1N1)pdm09 (A/England/195/2009) into which we engineered a mutation in the M2 gene (N31S). The S31N mutation in the M2 protein arose naturally during in swine viruses prior to the emergence of 2009 influenza pandemic and confers resistance to Rimantadine. By reverse
Chapter 4 | Results

genetics we generated a back mutant that restored sensitivity to the M2 inhibitors. The rational behind this approach was to allow synergy testing against a currently circulating virus that was sensitive to both classes of antivirals (M2- and NA-inhibitors). Importantly this mutation does not adversely effecting viral replication (Elderfield and Cocking personal communication).

All three licenced drugs suppressed viral replication by several logs as expected. RV1088, when used alone, resulted in very little virus suppression, in line with previous results. However, when a conventional antiviral and RV1088 were used jointly, a striking and almost complete suppression of viral replication was noted. A qRT-PCR analysis of cytokines/chemokines mRNA levels demonstrated that infection of the CALU-3 cultures with A/England/195/2009 resulted in a strong induction of cytokines and chemokines that was suppressed by RV1088 and, to an even greater extent, when RV1088 was used in conjunction with another antiviral. Furthermore, this level of inhibition observed in the RV1088+antiviral group was greater than that observed in the group where two antivirals were used in combination (Oseltamivir and Rimantadine). Interestingly, in some cases where NA inhibitors were used alone, the level of cytokines/chemokines was either unchanged or greater than the virus alone groups. This lack of change or increase in cytokines/chemokines in the antiviral treated conditions occurred despite viral replication concurrently being decreased. This phenomenon, while en face may be viewed as being counterintuitive, has previously been documented in the literature (Zheng et al., 2008; Ilyushina et al., 2008). This phenomenon may hold significant implications in terms of treating severe cases influenza with this class of antiviral which, could potentially potentiate an over-induction of the innate immune response resulting in a “cytokine storm”. This progression to an unregulated innate immune response has been linked to detrimental clinical prognosis and outcomes especially when bacterial co-infection occurs (Annane, 2011; Small et al., 2010; Madhi et al., 2008; Seki et al., 2007).

While puzzling at first, this phenomenon, may be explained by examining how this drug class (NA-inhibitors) is believed to work. The neuraminidase inhibitor class of antivirals acts by inhibiting the activity of the NA glycoprotein preventing the release of progeny viruses from the replicative cell. This results in the accumulation of numerous virions both within the cell and on the cellular surface (Lee et al., 2012; Gubareva, 2004). Each
“trapped” virion might then stimulate an immune response either by binding to some extracellular receptor, or by re-entering the same cells and being detected as a PAMP. The sum of these individual immunological-stimulating effects might overwhelm cellular suppressive effects resulting in an increased transcription of cytokine/chemokine mRNAs.

Contradicting this explanation are results from a recent Cochrane review (Jefferson et al., 2014) which has suggested that Oseltamivir carboxylate, may reduce the symptoms of influenza via a manner believed to be unrelated to the inhibition of influenza virus replication and thus contrary to commonly held beliefs. Citing Hayden et al., (1999), the authors put forward evidence that the main symptomatic relief of Oseltamivir stems from the drugs ability to suppress components of the host’s innate immune response. This suppression including certain pro-inflammatory cytokines (including: IL-6, TNF-α and IFN-γ) that, when Oseltamivir is administered 28 hours after experimental inoculation of influenza virus, complete suppression occurs while the reduction of viral titre in nasal lavages is only partial. In further support of this is work conducted by Ono and colleagues in which they postulated that the potential hypothermic or antipyretic effect observed in in mice treated with Oseltamivir may be due to the ability of this drug to act as a central nervous system depressant; and in this way contribute to the apparent reduction of host symptomology (Ono et al., 2013; Ono et al., 2008).

Despite this mounting evidence, the Cochrane reviewers admit that the majority of their postulates and conclusions about interactions with Oseltamivir and the immune system are based on data collected from animal experiments (primarily mice). Evidence from human trials is sparse and data for influenza negative population conducted by the pharmaceutical companies during initial testing of Oseltamivir has not been released. Until a full review of all data can be conducted, the questions whether NAIs do affect the immune response and how remain.

To date, we have been unable to find any reports in the literature that have examined the effect of Oseltamivir on cytokine/chemokine levels in HAEs infected with influenza. As the HAE system does not contain immune cells (e.g. macrophages) which, in response to influenza infections, are believed to be the major producers of cytokines/chemokines (Kallfass et al., 2013) it is possible that the immunsospressive
effects reported in the Cochrane review (inferred from \textit{in vivo} models) do not apply to epithelial cells but rather target these immune effector cells. As such, any immunomodulation would be missed in this particular \textit{in vitro} model. An examination of potential immunomodulatory effect of Oseltamivir in PBMC cultures infected with influenza virus could help address this issue and represents a potential avenue for future study. An additional area of investigation would employ using Oseltamivir (either alone or in combination with RV1088) in the presence of an Oseltamivir-resistant influenza virus and quantify cytokine/chemokine expression as well as viral replication kinetics.

In order to determine if the inhibitory effect on virus replication observed when RV1088 and Relenza was used in combination was additive or synergistic, we employed the well-established principles of the combination index (CI) theorem of Chou-Talalay (Chou, 2010). This test uses the median-effect equation (MEE) and works by combining enzyme kinetics and specific mechanisms from hundreds of individual equations and reduces them to a single general equation. Both left and right sides of the MEE are dimensionless quantities. When the m (slope) and Dm (half-affected dose) are determined, the full dose-effect curve is defined. By using a specific software program which employs the Chou-Talalay theorem equations (CalcuSyn, Biosoft - Cambridge, UK), we were able to determine that the relationship produced by RV1088 when used in combination with Relenza is very strongly synergistic in nature (Table 5 and Figure 38).

After ascertaining the effectiveness of combination therapy in the CALU-3 system, we progressed to test the efficacy of combinations of RV1088 and the neuraminidase inhibitor drug Relenza to inhibit replication and induced cytokines in highly differentiated human airway epithelial cells (HAEs). We have previously demonstrated that in this system RV1088, when used alone, in cells infected with A/England/195/2009 of B/Florida/04/2006 suppressed the induction of cytokines/chemokines but had no significant effect on viral replication.

For both A/England/195/2009 and B/Florida/04/2006 we observed significant suppression of cytokine/chemokine induction at, or below, the level seen for RV1088 or the antiviral when used alone. In addition, and mirroring the results seen in the CALU-3 culture system, combination therapy resulted in significant suppression of viral replication. Upon
examination of the cultures under magnification, a remarkable level of cellular protection by RV1088 was observed in comparison to the Relenza treatment group. The level of cellular protection was greatly augmented in the combination treatment cells. This result may indicate much of the cell damage during infection of the HAE cells is mediated by the cytokine response induced rather than directly by replicating virus. The moderation of cellular damage could play an important role in vivo by decreasing recruitment signals for extra immune cells that otherwise may mediate immunopathology.
Chapter 5. RV1088 in the animal model

5.1 Introduction

5.1.1 The animal model for influenza

After demonstrating that RV1088 was capable of suppressing cytokines and chemokines as well as virus replication in an immortalised cell line and primary human airway cultures, we wanted to examine how this compound might affect viral replication and cytokine/chemokine expression in vivo.

Animal models have been employed by researchers to explore normal physiology states, the pathophysiology of diseases, both infectious and inborn, and the safety and efficacy of novel therapies in preclinical studies. In terms of lung-related biomedical studies, animal models have been developed for an array of human diseases including asthma, pneumonia, cystic fibrosis, acute lung injury and respiratory infections including influenza. All animal models have inherent limitations as the normal anatomy and physiology of the lung differs among species as does the innate and adaptive immune responses to pathogen insult.

Animal models for influenza are vital to understanding the pathogenicity of the influenza virus and therapeutic treatments. Despite the diverse array of mammalian species susceptible to the influenza virus, only a few species are considered amenable for study in the laboratory. These species, which includes the ferret, the mouse, the cotton rat and the guinea pig, have their own inherent advantages and limitations that must be carefully considered before use.

The first human influenza virus was isolated from ferrets in 1933 by Smith, Andrews and Laidlaw after the ferrets were intranasally inoculated with filtered throat washings from patients with influenza (Smith, 1933). Since then, the ferret model has been used extensively for studying the transmission and pathogenesis of influenza viruses (O’Donnell & Subbarao, 2011). Ferrets are susceptible to a wide array of human influenza viruses that do not require prior adaptation to the species (in contrast to mouse models). Like humans,
ferrets demonstrate primarily an upper respiratory tract infection when infected with seasonal strains. This is most likely due to the ferret having the similar distribution as humans do of sialic acids along their respiratory tract (Jia et al., 2014). The distribution of SAα2,6 and SAα2,3 within the respiratory tract varies from species to species and is an important factor in the modelling of influenza infection and transmission. While the ferret is widely accepted as the most suitable model for influenza study, its large size, specialized handling techniques, required facilities and general high cost cause many researchers to opt to use murine models instead (Table 6).

Table 6. Animal models commonly employed in the study of influenza. Adapted from: O’Donnell & Subbarao, 2011

<table>
<thead>
<tr>
<th>Animal Model</th>
<th>Parameters</th>
<th>Positive attributes</th>
<th>Negative attributes</th>
</tr>
</thead>
</table>
| Mice         | -Weightloss  
-Levels of viral replication  
-Lethality  
-Seroconversion  
-Overt signs of illness | -Inexpensive  
-Well-characterized genetic profiles  
-Abundance of reagents  
-Infected strains | -Not natural host for influenza  
-Lack of clinical symptoms  
-Lack of fever response to influenza  
-Do not transmit virus  
-Ability to infect can vary between strains |
| Ferrets      | -Weightloss  
-Fever  
-Levels of viral replication  
-Levels of viral shedding  
-Lethality (MLD50) - rare  
-Seroconversion  
-Overt signs of illness | -Similar SA distribution to humans  
-Similar pathological changes in respiratory tract as humans | -Lack of specific immunological reagents  
-Expensive  
-Special housing required  
-Large size  
-Outbred  
-Supply problems |

5.1.2 The mouse model for influenza

Laboratory mice strains have been used extensively as human surrogates in health research for understanding the pathological progression of numerous diseases and for studying the therapeutic efficacy and toxicity of drug treatments.

Their small size, low maintenance costs, and short generation times, make mice an ideal candidate for controlled laboratory studies. Adding to their value is the fact that mice have the highest level of genetic and immunological characterization of any laboratory animal. The availability of in-bred, transgenic and knockout strains has been a significant factor in advancing our understanding of the immunological response of the host to pathogenic insult as well as vaccine development and therapeutic evaluations (Davidson &
Rolfe, 2001; Brodie & Deng, 2001; Hock & Lamb, 2001). However, in interpreting mouse experiments one should bear in mind the innate differences between the mouse and human immune systems (Shay et al., 2013) and in particular for influenza studies the fact that most inbred mouse lab strains lack a functional Mx protein, one of the most potent antiviral genes induced by the interferon response (Verhelst, et al., 2012).

The intranasal method of instilling a liquid directly into the nose of the test subject is another method frequently employed to either inoculate with a virus or administer a pulmonary drug. This method is performed on an anaesthetised mouse kept in either a supine or vertical position (Southam, 2002). With the aid of a micropipette, the solution of interest is slowly administered onto the opening of the nostrils and is taken in by normal respiration.

With respect to influenza, commonly employed laboratory mouse strains express both SAα2,6 and SAα2,3 receptors along their respiratory tracts, making them an appropriate model for both human and avian influenza viruses. The exact distribution and relative quantities of the two different SA-receptor types is still being evaluated although some differences have already been noted (Table 7).

<table>
<thead>
<tr>
<th>Table 7. Influenza-specific host factors in animal models.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adapted from: O’Donnell &amp; Subbarao, 2011.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Host factor</th>
<th>BALB/c Mice</th>
<th>B6 Mice</th>
<th>Ferrets</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAα2,3 receptor distribution</td>
<td>Trachea, lungs, spleen, liver, kidney, cerebellum</td>
<td>Ciliated cells in the airways, type II alveolar cells</td>
<td>Low levels of expression in trachea, bronchus, and lung alveoli</td>
</tr>
<tr>
<td>SAα2,6 receptor distribution</td>
<td>Trachea, lungs, spleen, liver, kidney, cerebellum</td>
<td>None in the lungs</td>
<td>Predominant type of SA in the respiratory tract</td>
</tr>
<tr>
<td>Mx proteins</td>
<td>Absent</td>
<td>Absent</td>
<td>Present (Mx 1)</td>
</tr>
</tbody>
</table>

The predominance of SAα2,3 in murine respiratory tract may explain why numerous humanized influenza A virus subtypes often have to undergo passage in eggs to adapt them to the mouse host while several avian viruses are able to replicate efficiently in the mouse
model without having to undergo adaptation first (Belser, 2009; Joseph, 2007; van Riel, 2006). Specifically, it has been found that mouse adapted strains are needed to model seasonal A/H3N2 and A/H1N1 virus infections while the 1918 A/H1N1 pandemic strain (Tumpey, 2005), highly pathogenic avian influenza (HPAI) A/H5N1 strains (Belser, 2009; Lu, 1999; Gao, 1999), some H7 subtypes (Belser, 2009; Joseph, 2007) and A(H1N1)pdm09 (Besler, 2010; Maines, 2009) strains (all strains that retain some avian like virus binding) do not require adaptation.

A virus’ pathogenicity and the severity of its associated clinical signs in mice, depends upon the specific virus employed and the challenge dose administered. In most cases, when a lethal dose is administered, the resultant severe disease is characterised by huddling, ruffled fur, lethargy, weight loss and ultimately death. It is important to note, however, that unlike humans, mice do not develop a fever following an influenza infection; rather, hypothermia has been reported (Yang, 1961). Thus, the parameters most commonly used to access and monitor the pathogenesis in the mouse model are body weight loss and mortality and on necropsy, viral titers, pathology scores and cytokine/chemokine levels.

Whilst studies performed in animal models are fundamental for the evaluation of disease processes and therapeutic regimes, extrapolation of the results to the human condition is not straightforward due to the significant anatomical and physiological differences that exist between our species. These differences must be kept in mind when attempting to make cross-species comparisons.

5.2 Results

5.2.1 Evaluation of RV1088 in lone & combination use in the mouse model

In a first experiment in vivo, drugs and virus were administered by the intranasal route. The challenge virus chosen for the in vivo work was the prototypic strain of A(H1N1)pdm09, A/England/195/2009. Being a recently introduced pandemic strain, this virus was already shown by us and others to infect mice, ferrets and pigs without prior
adaptation (van Doremalen et al., 2011). Moreover, this virus is clinically relevant as related strains are still in circulation to date.

Female BALB/c, 6-8 week-old mice divided into five treatment groups composed for 15 mice each were employed in this study. RV1088+Relenza acted as the drug combination group, groups employing each drug individually were also tested, and a vehicle alone group of infected mice acted as a negative control for drug treatment whilst a mock-infected group of mice were the negative control for virus and cytokine induction. Each group of 15 mice was pre-treated with a specified drug combination or vehicle for three days prior to infection (days -3 to -1). This pre-treatment was conducted in order to give the compound every possible chance of modifying the cellular environment. On the day of infection (day 0), the mice were once again given specified treatments and infected intranasally under anaesthesia with 40μl of a 2x10^5 pfu solution (thus, 8x10^3 pfu actually instilled) of A/England/195/2009 (A(H1N1)pdm09 [an inoculation dose of 1x10^4 pfu for A/England/195/2009 has previously been demonstrated by our lab to induce a robust and measurable infection in the mouse model (Shelton et al., 2012)]. Daily weights were recorded. A severity limit of 20% weight loss was established and any mice dropping below this limit were culled as specified in the Home Office License for this work. On days +3, +7 and +11, five mice from each treatment were culled and their lungs harvested. Lungs were subsequently homogenised and used for determining viral lung load via plaque assay and IFNα levels via ELISA (Figure 50).

The mice infected with the A/England/195/2009 virus in the absence of antiviral treatment lost weight in two distinct phases, between days 1 and 3 post infection and then again between days 5 and 7 (Figure 50 & 51). The mean weight loss in the infected group was 10%. After day 7 infected and untreated mice gained weight as they recovered. Interestingly, while all groups gained a demonstrable amount of weight from day 7 p.i., the vehicle alone (i.e. non-infected group) did not display this weight gain and infact kept a near constant weight for the entirety of the experiment. While the reason for this late-stage weight gain by the infected groups is not know, it is possible that once the infection had been cleared, these mice attempted to recuperate the caloric deficit aquired during their illness and as food and water was provided ad-libitum, simply gorged themselves. Treatment with Relenza did not significantly change the weight loss in infected mice under
these conditions. In contrast, infected mice treated with RV1088 either alone or in combination with Relenza lost substantially less weight than the vehicle treatment group. This analysis was conducted by means of examining the area under the curve (AUC) for each treatment group which was then in turn normalised to the vehicle alone group (no virus) - with higher numbers indicating less weight loss and visa versa. The RV1088 treatment group experienced far less weight loss than the Relenza only treatment group yielding AUC values of 94.85 and 92.74, respectively and possibly indicating that RV1088 might represent an improvement to the current standard antiviral practise in terms of symptomology. The combination of RV1088+Relenza resulted in less weight loss than observed for any of the single treatment groups (Vehicle, RV1088 or Relenza alone; AUC = 99.70 for combination treatment compared to 90.58, 94.85 and 92.74, respectively).

Supporting these results are those of the survival curve. While no mice succumbed to their infection, if they reached the 20% weight loss mark, individuals were culled on humanitarian grounds and this is what is represented in Figure 50. The vehicle+virus group experienced a 46% loss of individuals due to this cut off point. In comparison, the Relenza group resulted in a 6.67% reduction starting on day 4 p.i. while both the RV1088 and the RV1088+Relenza groups required no culls due to severity limits.

With respect to viral lung titres, virus was recovered from the lung on days 3 and 7 for all infected mice, but virus was cleared by day 11 p.i. (Figure 50). Viral loads were highest on day 3 and there were no differences between the groups at this time point. On day 7, although statistical significance was not reached mice within the RV1088 alone and the RV1088+Relenza groups had less virus in their lungs than the other groups. Both the vehicle alone and Relenza alone groups had roughly equivalent levels of viral load in the lungs at this time.

IFN-α levels in homogenised lungs was quantified using a specific commercial ELISA. IFN-α was highest in all groups on day 3 p.i. and declined over the course of the experiment but was still higher in any of the infected mice lungs than in uninfected mice lungs at day 11 p.i. even though all virus was cleared by this time. RV1088 treatment alone resulted in a significant reduction in the levels of IFN-α levels on all days examined compared to any other group of infected mice. Surprisingly on day 3 p.i. the Relenza group experienced a
significant increase in lung IFN-α when compared to the vehicle+virus treatment group ($p = 4.8 \times 10^{-5}$). The RV1088+Relenza group displayed mean IFN-α levels lower than this, at a similar level as the vehicle group, possibly indicating a moderating effect of RV1088 on the unexpected increase after Relenza treatment. Furthermore, the combination treatment group resulted in a significant suppression of IFN-α on day 11 p.i. ($p = 9.2 \times 10^{-7}$) when compared to the infected (vehicle treated) group.

Homogenised lung samples were analysed on a multiplex plate for further cytokine/chemokine analysis (Figure 52 & 53). Specifically, FGF, IL-1β, IL-12, MCP1α, MCP1, IL-5, VEGF, IL-1α, IFNg, TNF-α, IP-10, MIG and KC were examined. Infection with influenza, when compared to non-infected controls, produced detectable increases in all cytokines/chemokines examined save for VEGF and FGF. With respect to drug treatments, only IL-1β and IP-10 demonstrated any significant difference in protein levels of infected compared to mock infected mice. No significant differences were noted except in the case of IL-1β and IP-10 where, RV1088 appeared to increase IL-1β on day 1 p.i., reaching statistical significance for the combination RV1088+Relenza treatment group ($p = 9.0 \times 10^{-4}$) while the RV1088 only and Relenza+RV1088 groups showed significantly decreased expression of IP-10 on day 5 p.i ($p = 4.3 \times 10^{-2}$ for both condition groups) when compared to the infected (vehicle treated) group. IP-10 was undetectable after RV1088 treatment on day 5 p.i in contrast to the un-treated or Relenza group.
Figure 50: Effects of RV1088 in the mouse model. Female BALB/c (6-8 weeks) were pre-treated with RV1088 (2µg/mouse) ± Relenza (3µg/mouse) suspended in a 5% DMSO/Saline vehicle and infected intranasally with H1N1 virus (A/England/195/2009). a) Daily weights were taken at the same time during each trial day. b) Mean group weight loss was determined and area under the curve calculated. Any subjects which dropped below the 20% weight loss limit was culled (c). On set days (3, 7 and 11 p.i.), mice from each group were culled and viral titers (d) and IFN-α (e) in the lungs was assessed. Results are expressed as the mean ± standard deviation of each sample set (student t-test, *p<0.5, **p<0.001). Figure continued onto the next page.
Figure 50: Effects of RV1088 in the mouse model. Female BALB/c (6-8 weeks) were pre-treated with RV1088 (2μg/mouse) ± Relenza (3μg/mouse) suspended in a 5% DMSO/Saline vehicle and infected intranasally with H1N1 virus (A/England/195/2009). a) Daily weights were taken at the same time during each trial day. b) Mean group weight loss was determined and area under the curve calculated. Any subjects which dropped below the 20% weight loss limit was culled (c). On set days (3, 7 and 11 p.i.), mice from each group were culled and viral titers (d) and IFN-α (e) in the lungs was assessed. Results are expressed as the mean ± standard deviation of each sample set (student t-test, *p<0.5, **p<0.001). Continued from previous page.
**Figure 50: Effects of RV1088 in the mouse model.** Female BALB/c (6-8 weeks) were pre-treated with RV1088 (2μg/mouse) ± Relenza (3μg/mouse) suspended in a 5% DMSO/Saline vehicle and infected intranasally with H1N1 virus (A/England/195/2009). a) Daily weights were taken at the same time during each trial day. b) Mean group weight loss was determined and area under the curve calculated. Any subjects which dropped below the 20% weight loss limit was culled (c). On set days (3, 7 and 11 p.i.), mice from each group were culled and viral titers (d) and IFN-α (e) in the lungs was assessed. Results are expressed as the mean ± standard deviation of each sample set (student t-test, *p<0.5, **p<0.001).

*Continued from previous page.*
Figure 51: Separated out weight loss curves of mice dosed intranasally with RV1088±Relenza. Female BALB/c (6-8 weeks) were pretreated with RV1088 (2μg/mouse) ± Relenza (3μg/mouse) suspended in a 5% DMSO/Saline vehicle and infected intranasally with H1N1 virus (A/England/195/2009). Daily weights were taken at the same time during each trial day and a group survival cure was created. Results are expressed as the mean ± standard deviation of each sample set (student t-test, *p<0.5, **p<0.001).
Figure 52: Multiplex analysis of homogenised mouse lung. Female BALB/c (6-8 weeks) were intranasally inoculated with H1N1 virus (A/England/195/2009). On set days, mice from each group were culled and the lungs harvested. Lungs were subsequently homogenised and a multiplex analysis for cytokines/chemokines was conducted. All results were adjusted for lung weight. Results are expressed as the mean ± standard deviation of five samples (student t-test, *p<0.5, **p<0.001).
Figure continued on the next page.

Figure 5.3: Additional results adjusted for lung weight. All results were conducted. All results were cytokines/chemokines was and a multiplex analysis for subgroups. The homogenized lungs were harvested. Lungs were were cultured and the lungs were cultured each group (A/England/195/2009). On set H1N1 virus intranasally inoculated with BALB/c (6-8 weeks) were infected test subjects. Female homogenized mouse lung tissue multiplex analysis of.
Figure 53: Additional multiplex analysis of homogenised mouse lung from intranasally treated and infected test subjects. Female BALB/c (6-8 weeks) were intranasally inoculated with H1N1 virus (A/England/195/2009). On set days, mice from each group were culled and the lungs harvested. Lungs were subsequently homogenised and a multiplex analysis for cytokines/chemokines was conducted. All results were adjusted for lung weight.

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5.2.2 Evaluation of RV1088 in the ferret model

In a study primarily conducted by Kim Roberts and Rebecca Cocking (both members of the Barclay lab group), female ferrets (26-30 weeks) were pre-treated with RV1088 (2mg/kg) or placebo for one day prior to infection, and for four days p.i. – by intranasal administration conducted under isoflurane. On day 0, the ferrets were infected under anaesthesia intranasally with A/England/195/2009 (1x10⁴ pfu). Daily weights were recorded (Figure 54, a), and viral loads were determined via the plaquing daily nasal wash samples on MDCK cells (Figure 54, b). The results demonstrate that in this model, RV1088 did not significantly affect either weight loss or viral load when compared to the placebo control. Unfortunately, at the time of this experiment, the lack of ferret-based immunological reagents made cytokine/chemokine analysis unfeasible. However, with modern advances in this area, such work could fruitfully be undertaken in the near future.

Figure 54: Effects of RV1088 in the ferret model. Female ferrets (26-30 weeks) were pre-treated with RV1088 or placebo for one day prior to infection (and for four days p.i. - all under isoflurane). On day 0, the ferrets were infected under anaesthesia intranasally with A/England/195/2009 (1x10⁴ pfu). Daily weights (a) were taken at the same time during each
trial day. No significant difference in weight loss was observed between the drug treatment groups and the placebo group (right-hand weigh loss figure contains std dev bars). In addition, each day, the ferrets were nasal washed and these washings were plaqued on MDCKs (b) to determine viral shedding levels. No significant differences between the RV1088 and the placebo groups in terms of viral shedding was observed.

Subsequent to this in vivo experiment in which we observed little-to-no demonstrable effect of RV1088 on either weight loss or viral shedding, we wanted to examine if RV1088 has any effect in a simpler in vitro model in ferret cells. In order to conduct these experiments, ferret airway (tracheal) primary cells were collected post mortem and used to create highly-differentiated, air-liquid interface ferret airway epithelial (FAE) culture. These cultures were established by Peter Stillwell under the auspices of an NC3R grant and closely resembled commercially acquired HAE cultures.

FAE cultures were pre-treated in a fashion similar to the previously described HAE experiments with either RV1088 (1.73nM) or DMSO (0.5%) added to the basal media and to the apical surface of each well for three days prior to infection (day -3 to -1) with virus. On the day of infection (day 0), wells were once again treated both apically (removed after 2hrs) and basally (left in place for the remained for the experiment) and then infected with A/England/195/2009 (moi = 0.1). Culture supernatants were harvested at set time points and plaqued to quantify viral shedding (Figure 55, part a). Additionally, at 48hrs p.i. total RNA was harvested from three wells from each treatment condition and used for qRT-PCR analysis for viral M gene and for ferret cytokine genes using primers based on available ferret genome sequence (Figure 55, part b). A qRT-PCR analysis revealed that RV1088 significantly suppressed cytokine/chemokine mRNA levels induced by A/England/195/2009 (A(H1N1)pdm09 (p≤ 0.05 for IP-10). There was an average 2.01-fold decrease in IP-10 mRNA levels for the RV1088 treatment group when compared to the DSMO treatment group. In addition qRT-PCR for viral M gene mRNA (normalized to GAPDH) indicated that treatment with RV1088 also suppressed levels of M-gene message by an average 5.2-fold (compared to DSMO treatment group), however, this decrease was not reflected in viral titres measured in supernatant by plaque assay at that time point (an occurrence previously observed and discussed in Chapter 2).
Figure 55: Results of Pre-infection treatment with RV1088 in FAEs infected with A/England/195/2009. FAE cell cultures were pre-treated for three days both apically and basally with RV1088 (1.73nM) or DMSO (0.5%). Cultures were infected with A/England/195/2009 (moi = 0.1, 1hr 37°C). After infection, fresh drugs were applied apically (2hrs, 37°C then removed) and added to the basal media for the duration of the experiment. Culture supernatant was harvested at specified time points and used in the determination of viral replication by being plaqued on MDCKs (a). At 48hrs p.i. total RNA was extracted and qRT-PCR analysis for IP-10 (b) and viral M-gene (c) was performed. Results are expressed as the mean ± standard deviation of triplicate samples (student t-test, *p<0.5, **p<0.001).

5.3 Discussion and Conclusions

Within this chapter, we sought to examine if the synergistic potential for RV1088 and a directly acting antiviral translated to an in vivo model. Several animal models for studying influenza currently exist with the most common animals including the mouse, ferret and guinea pig. As discussed above, each model has its own particular pros and cons with some animals considered to be more suitable for specific areas of investigation (the ferret model for transmission studies, for example). In our preliminary investigation, due to the number of animals required as well as the availability of standardised protocols and reagents, we employed the mouse model.
The parameters utilised to monitor the pathogenesis of the virus (A/England/195/2009) in this model were total body weight loss and mortality and on necropsy, viral titers, and cytokine/chemokine levels. The results demonstrated that RV1088 (when used alone or as part of a combination therapy with a directly acting antiviral, Relenza) significantly reduced the amount of weight the mice lost following influenza infection. Furthermore, the combination treatment group (RV1088+Relenza) had the lowest amount of weight loss of any of the infected groups and this reduction was statistically significant when compared to either RV1088 or Relenza alone treatment groups, possibly indicative of a synergistic mechanism similar to that observed in vitro (detailed in Chapter 4).

Interestingly the weight loss in mice infected with A/England/195/2009 occurred in 2 rather distinct phases, the first between days 1 and 4 and then the second between days 6 and 8. The two cull days that allowed analysis of lung viral loads and lung cytokine levels were fortuitously spaced to be in each of these two temporal phases. This revealed that the decreased weight loss in the early phase seen in mice treated singly with RV1088 or receiving dual RV1088 plus Relenza treatment was not associated with less viral load but rather with a reduction in IFN-α levels.

Despite the decrease in IFN levels induced in infected mouse lung in presence of RV1088 on day 7, the mice still cleared the virus more efficiently than untreated mice or mice treated with Relenza alone since viral loads by day 7 were lowest in RV1088 treated groups. As there was no difference in viral load in the mouse lungs at day 3 p.i. we infer that the reduced weight loss in RV1088 treated group(s) must be due to its immune modulating activities. However, the higher IFN levels in lungs of Relenza treated mice on day 3 did not drive greater weight loss.

In the later part of the weight loss curve, the two RV1088 treated groups again showed less weight loss than the other mice. In this instance day 7 lung homogenates revealed that these groups also had lower lung viral loads as well as less IFN and less IP-10. It is not clear whether the lower cytokine levels here are a result of increased virus control and also why RV1088 appeared able to control viral load at late time points but not at early time points.
Multiplex data obtained from homogenised lung samples demonstrated that RV1088+Relenza treatment group resulted in significantly more IL-1β production on day 3 p.i. These results are interesting as the cytokine IL-1β (also known as catabolin) is produced by activated macrophages and is considered to be an important mediator of the inflammatory response (Dinarello, 2010). Activation of the inflammasome results in the cleavage of pro-IL-1β by caspase-1 and produced the mature form of IL-1β (Hornung et al., 2009). During an influenza virus infection, IL-1β has been demonstrated to mediate acute pulmonary inflammatory pathology (Schmitz et al., 2005). In addition, IL-1β (in conjunction with IL-1α) induces the expression of a variety of inflammatory mediators, which are believed in turn to initiate part of the inflammatory response cascade and to induce the activation of T cells (Acosta-Rodriguez et al., 2007; Luft et al., 2002). Further supporting the pivotal role of IL-1β in the context of an influenza infection is the work conducted by Liu et al., (2013) where they report that a single-nucleotide polymorphism (SNP) of IL-1β (rs1143627, allele C) increased the carriers’ susceptibility to infection with A(H1N1)pdm09. While more work in this area is required, it is postulated that the expression of IL-1β is lower in people who carry allele C, leading to weaker immune response in the face of viral insult. In terms of epidemiology, the frequency of the rs1143627 allele C is high in ethnic Han Chinese (minor allele frequency = 0.476) when compared to other ethnic groups (Liu et al., 2013). However, the detailed significance of this polymorphism and its contribution to disease burden has yet to be fully established.

IP-10 (also known as CXCL10) on the other hand, is an IFN-induced chemokine; attracting monocytes/macrophages, T cells, NK cells and dendritic cells to sites of inflammation. The lower levels of IP10 at day 11 associated with RV1088 treatment might result in less immune infiltration in to the lung and less immunopathology.

Research conducted by Wang et al., (2013) has demonstrated that in mice, a monoclonal antibody specific to IP-10 ameliorates influenza A (A/Beijing/501/2009, 10^5.5 TCID50) virus induced acute lung injury and enhance survival. Taken together, these results exemplify the strategy behind employing drugs that specifically target key pathways of the innate immune response rather than a simply pan-suppressive approach. The ability of RV1088 to supress pathology-inducing IP-10 while increasing the protective IL-1β resulted in a marked protective effect in infected mice.
One potentially worrying set of results demonstrated that, under our experimental condition, Relenza did not prove to be greatly protective against an influenza infection with A/England/195/2009. Despite this group receiving 4 doses at 3μg/mouse/dose, little-to-no protective effect was seen in terms of weight loss and lung viral titers compared to the vehicle alone treatment group. Although fewer mice in the Relenza group lost more than 20% body weight requiring cull, compared to the vehicle alone group, with respect to the cytokine/chemokine response, treatment with Relenza resulted in a significant increase in IFN-α levels on day 3 p.i. which may have contributed to an increased immunopathology driving weight loss, compared with the RV1088 treated groups.

Recently, there has been a great deal of debate and controversy over the efficacy of employing neuraminidase inhibitors in the treatment of influenza infections. This debate has been spurred on by a new Cochrane Group Review released on April 10th, 2014 in which they concluded that oseltamivir (Tamiflu) and zanamivir (Relenza) have small, non-specific effects on reducing the time to alleviation of influenza symptoms in adults and that using neuraminidase inhibitors as prophylaxis can reduces the risk of developing symptomatic influenza (Jefferson et al., 2014). Complicating the debate has been the call from the Cochrane Group and the British Medical Journal for pharmaceutical companies to fully disclose all results of their clinical trials (both published and unpublished) as it has been alleged that the virus-specific mechanism of action proposed by these companies does not fit the currently available clinical evidence (Belluz, 2014). Our results have demonstrated that Relenza, when used on its own, has only a modest effect but the synergistic effect created by using Relenza in combination with RV1088 is markedly protective. The results presented in Chapter 4 indicate that this synergistic effect is not limited to Relenza alone but that it was also observed for oseltamivir and rimantadine and it is quite possible that these combinations might prove even more protective then that seen with Relenza and warrant further investigation at a later date.

Unfortunately, in the ferret model, RV1088 failed to result in any significant reduction of weight loss or viral loads. This may be due to a number of factors including the quantity of drug in each dose and the adequacy of delivery of the drug to the animals’ respiratory tract and warrants further investigation. Complicating any additional analysis, specifically in terms of cytokine/chemokine levels, is the general lack of reliable reagents.
However, an increasing amount of work is currently being conducted in the field to address this problem and the hope is to have many of the key reagents available in the future.

Interestingly, some preliminary work testing ferret nasal washes from infected or uninfected animals suggests that some proteins in ferret nasal wash cross-react with reagents in the canine commercially available kits. This option may provide an interim solution although the extent of cross-reactivity and the specificity of these reactions will be difficult to confirm.

While we were not able to detect any appreciable effect of RV1088 in the ferret in vivo model, we were successfully able to measure an effect of the drug in highly-differentiated, air-liquid interface ferret airway epithelial (FAE) cultures. We showed that IP10 was induced by infection with A/England/195 and the level of IP-10 induction was significantly suppressed in the presence of RV1088 (p = 2.8x10^{-4}). However, in accordance with our previously discussed HAE results, RV1088 failed to have any significant effect on viral replication. However, qRT-PCR analysis for viral M gene mRNA (normalized to GAPDH) indicated that treatment with RV1088 suppressed levels of M-gene message by an average 5.2-fold when compared to DMSO treatment group. A hypothesis of why this decrease was not reflected in viral titres measured in supernatant by plaque assay is discussed further in Chapter 3 where the M-gene, was also suppressed (3.78-fold decrease) but this suppression did not seem to be enough to significantly affect vital titers as measured by plaque assay (2.57-fold decrease). The qRT-PCR results suggest that a several fold difference in RNA levels have little effect on viral titres as measured by infectious unit on a log scale. This discrepancy could simply be explained by the innate difference in sensitivity that exists between these two techniques and the fact that qRT-PCR is a method for determining mRNA levels with no distinction made between replicative and non-replicative viruses.

Due to a limited amount of verified and published information pertaining to ferret cytokine/chemokine gene sequences at the time of the experiment, we were unable to perform as detailed an examination of cytokine/chemokine effects as with the HAE culture system. In this particular experiment, we examined mRNA level of ferret IP-10 and observed that RV1088 treatment significantly suppressed (p = 2.8x10^{-4}) induction of this chemokine in comparison to the DMSO vehicle control group. These results indicate that in ferret airway
cells, RV1088 was able to suppress the induction of innate signalling cascades but this did not result in increased viral shedding.

As we have established that RV1088 is indeed effective at targeting a key chemokine in ferret-derived airway cultures, any future experiments should focus on the potential alleviation of symptoms in this model. As the generation of fever in response to the virus is one of the hallmarks of an influenza infection, we suggest that any future studies employ intraperitoneal implanted telemetry devices for the monitoring of changes in body temperature secondary to viral insult and to determine what, if any, effect RV1088 might have in reducing this symptom (see for example Roberts et al, 2012). This approach has the added benefit of producing non-biased, and quantifiable results that are directly relevant to the disease process. In addition, plethysmography to measure the ferret respiratory capacity during infection may be possible.

Further complicating any analysis is the necessity to keep any species-specific responses in mind. The generation of an excessive cytokine/chemokine response in humans (and by extension in human-derived cell cultures) is often viewed as being detrimental and a key factor in the development of a pathological state. In mice, however, the picture might be more complicated. In work conducted by Connaris et al., (2014) the efficacy of using engineered carbohydrate-binding, sialic acid-specific, molecules as a means of preventing influenza from gaining access to its cell surface receptors and thus preventing infection was assessed. Intriguingly, despite their molecules having little-to-no effect on viral replication until day 9 p.i., mice dosed with these molecules were afforded protection against a lethal challenge with A/California/04/2009 (H1N1). A possible explanation for this lies in the cytokine/chemokine levels observed in mouse lung homogenates. Specifically, their molecules, rather than suppressing the cytokine/chemokine responses, actually induced significantly stronger levels of certain cytokines such as IL-1β, MIP-2 (mouse homolog of IL-8), IFN-γ, and TNF-α. It is possible that in this model, the higher levels of cytokines/chemokines afford greater protection to pathogenic insult resulting directly from virus replication in the lung.

One disadvantage of the animal model in testing the efficacy of drugs and combination therapy is the inability to accurately score reductions in symptomology. While
we can make attempts to determine this through indirect measurements such as weight loss and cytokine levels, any effect on this parameter can only be inferred. In the case of RV1088, a drug compound that acts primarily by reducing/preventing an over-induction of the innate immune response, any alleviation of symptoms could prove of particular benefit to a patient with severe influenza. Unfortunately, only clinical trials in human subjects will answer these key questions definitively but the animal model does play a significant and crucial role in determining safety profiles, side-effect profiles and early stage efficacy assessments.
Chapter 6. The nebulisation of A(H1N1)pdm09 virus for inoculation of mice treated with nebulised RV1088

6.1 Introduction

6.1.1 The lung

After demonstrating the effectiveness of RV1088 in the mouse model using intranasal instillation of both treatment compounds and virus, we were interested in seeing if RV1088 could be delivered successfully in vivo via the nebulised route and be effective against a virus delivered via the same route. This is partly driven by the knowledge that the RV compounds were designed to be bioavailable in the lung from a local delivery similar nebulization.

In human infection studies using strains of either influenza A (PR8) or Influenza B (Lee) conducted by Henle et al., (1946) involving over 200 volunteer subjects, the route of inoculation (either intranasal drops or via aerosol) influenced the likelihood of developing fever. Eighty nine percent of 65 volunteers infected by inhalation developed fever while only 12% of 16 exposed to nasal drop instillation did. In their analysis of this data, the authors speculated that the differences in symptomology with respect to method of viral inoculation indicate that the influenza virus may preferentially enter cells in the lower respiratory tract over those of the nasal epithelium. While more recent experiments have demonstrated that a number of factors including virus strain, dose and immune response (to name a few) are important determining factors for the influenza virus to initiate and develop a productive infection, it has also been shown that the estimated human infectious dose (HID50) for influenza is at least 5-10 fold lower when virus is delivered by aerosol when compared to intranasal inoculation (Hayden, 1996; Alford, 1966; Knight, 1965). This suggests that deep lung deposition of virus may be much more efficient at establishing an infection.
The delivery of drugs directly to pulmonary tissue allows for local drug targeting and thus, decreases systemic drug concentration levels, resulting in reduced side effects. The specific physiological and anatomical features of the lungs make them attractive targets for drug delivery. These specific features include a large epithelial surface area, thin alveolar epithelium and a high vascularization rate (Patton & Byron, 2007).

During the course of its normal daily function, the human lung is continually assaulted by pathogens and particles that can harm its tissues. Indeed, it has been estimated that our airways are exposed to >7kg of pollutants each year (McWilliam & Holt, 1998). Thankfully, our lungs have in place a series of protective mechanisms to help remove such offending particles. These mechanisms include a successive bifurcation of the airways encouraging impaction, a rich overlay of mucus and the mucociliary clearance system (Figure 56).

**Figure 56:** Schematic representation of the lateral view of cells and surface fluids that comprise the human respiratory tract from the bronchus to alveoli (Figure from: Fernandes & Vanbever, 2009).
Produced by goblet cells lining the airways, the mucus is composed of mucins which are high molecular-mass glycoproteins with a protein backbone upon which oligosaccharide side chain are attached, producing a fibre-like structure which give mucus its gel-like consistency. This viscoelastic gel is composed of 95% water, 2% mucins, 1% albumin, 1% salts, enzymes and immunoglobulins and <1% lipids (Houtmeyers, 1999). The mucus forms a bilayer ~5-55μm thick and lies directly on top of the thin periciliary fluid layer which is responsible for lubricating the cilia (Lai, 2009; Rogers, 2007). As the cilia beat rhythmically upward, the mucus layer and its entrapped particles are pushed further and further up the airways to the oropharynx where it is either expectorated or swallowed (Chilvers & O’Callaghan, 2000).

Further down the respiratory tract is the alveoli region. The epithelial lining is protected by a thin (~20-80nm thick) overlay of surfactant fluid (Perez-Gil, 2008; Patton, 1996). Lung surfactant is composed of ~80% phospholipids, 5-10% neutral lipids and 8-10% proteins (Perez-Gil, 2008). While surfactant plays an important role in reducing pulmonary surface tension, it also has been implicated in having an innate immune function (Wright, 2005). Specifically, some researchers have described the phenomenon whereby small insoluble proteins and particles aggregate in the surfactant that is then enveloped by alveolar macrophages (Nag, 2007; Patton 1990). Large proteins (>40kDa) and soluble compounds can also be taken up by macrophages through the process of pinocytosis or “cell drinking” (Lombry, 2004; Matsukawa, 1997). In contrast, these same alveolar macrophages have no effect on small proteins and molecules (<25kDa), which diffuse across the mucus layer from the airspace within minutes (Lombry, 2004). Thus, the environment in which influenza viruses replicates in the lung might be quite different than in the upper airways. Since lung replication is generally associated with more severe disease, that would be more requiring of therapeutic intervention, it seems important to try to reproduce that site of infection in the animal models in which novel therapeutics are tested.

It may also be that an aerosolised virus might gain access to endothelial cells of the respiratory tract more readily by establishing an infection in the deeper lung/alveolar regions than in the trachea or bronchi. This difference may, in part, be
due to the decreased thickness of the airway mucus layer (secondary to a decrease in mucus-producing goblet cells) further down the respiratory tree. For example, it has been suggested that the thickness of airway surface liquid (ALS) between the trachea and bronchioles can differ by as much as 20- to 50-fold (Widdicombe, 1997). Endothelial cells have been identified as the site of induction of the sphingosine-1-phosphosphate (S1P₁) signalling system that leads to the robust activation of cytokine expression (Teijaro et al., 2011). As such, it is plausible that a virus in the lung (as opposed to a virus in the conducting airways) would have greater access to epithelial cells, the subsequent infection of which, might result in a profound triggering of numerous cytokine/chemokine signalling cascades to such a strong degree that the conditions for the generation of a cytokine storm are created. Supporting this supposition is the observation that viruses (such as the D225G mutants isolated in Norway) that have a greater ability to infect cells of the lungs by possessing an enhanced ability to bind α₂-3 SA have been implicated in more severe cases during the pH1N1 2009 pandemic (Kilander et al., 2010).

6.1.2 An alternative route of inoculation in the mouse model

The most common method to either inoculate with a virus or administer a pulmonary drug remains the intranasal method. This method involves instilling a liquid directly into the nose of the test subject and is performed on an anaesthetised mouse kept in either a supine or vertical position (Southam, 2002). With the aid of a micropipette, the solution of interest is slowly administered onto the opening of the nostrils and is taken in by normal respiration. Despite its wide use, this method has inherent limitations and problems which can be addressed by alternative methods.

Ideally since influenza viruses are acquired by transmission through the air, this route of inoculation for an animal model might be preferred to reproduce the correct site of infection. Delivery of an aerosol to the murine pulmonary system can be quite challenging because of their relatively small tidal volumes. In addition, while the position of the lungs in humans is vertical, it is horizontal in mice during
inhalation. Furthermore, mice, as well as rats, guinea pigs and hamsters, are obligate nose breathers due to the close apposition of their epiglottis to the soft palate (Rao & Verkman, 2000). The smaller tidal volumes result in a smaller volume of aerosol reaching the lower respiratory tract which, in turn, results in lower aerosol deposition rates (Rao & Verkman, 2000). In general terms, the lung deposition of an aerosol in any host depends on the aerosol particle size which is dependent on a number of factors including the method of aerosol generation, formulation parameters and the subjects breathing pattern (McCallion, 1996; Finlay, 2001). Taking all this into account, it has been proposed that for tidal breathing, the optimal aerosol particle size for deposition in the alveolar region is 3μm for both humans and mice and that for tracheobronchial deposition, the optimal particle size is 6μm for humans and 4μm for mice (Hsieh, 1999; Stahlhofen, 1989).

There are two widely-used methods for administering an aerosol to the murine model: the whole-body approach and the more targeted nose only approach. For the whole-body approach, the mice are placed in an exposure chamber into which an aerosol is delivered and the mice are allowed to inhale with their entire bodies, including eyes, being exposed to the aerosol cloud. This method has the benefits of being simple to employ, not requiring the use of either restraints or anaesthesia and numerous animals can be exposed to the same cloud at the same time. However, the disadvantages to this system are the large deposition of aerosol onto the animals fur, leading to over-exposure and subsequently skewing results, and the possibilities for inequitable distribution of the aerosol between all the test subjects due to the common practice of their huddling within the chamber (Phalen, 1984). Alternatively, the nose-only exposure method avoids many of these issues and ensures an equal distribution of the aerosol cloud to all subjects. However, the high cost specialized equipment-and use of restraint and technical skill involved are distinct disadvantages (Lore, 2011).
6.1.3 Nebulisation

The delivery of drugs directly to pulmonary tissue allows for local drug targeting and thus, decreases systemic drug concentration levels, resulting in reduced side effects. The specific physiological and anatomical features of the lungs make them attractive targets for drug delivery. These specific features include a large epithelial surface area, avoidance of “first-pass metabolism” associated with parenteral medications, thin alveolar epithelium and a high vascularization rate (Patton & Byron, 2007; Nadithe, 2003). The direct pulmonary route might also prove to be an attractive method for vaccine delivery due to the large number of dendritic cells and macrophages localized in the mucosal surface (Nadithe, 2003).

Ultrasonic nebulizers generate an aerosol using vibrations from a crystal that are transmitted to the surface of the overlaying liquid where standing waves are formed. Droplets subsequently break free from the tops of these waves creating a fine aerosol mist. The size of the aerosol particle generated is inversely proportional to two thirds of the power of the acoustic frequency. In a method similar to jet-style nebulizers, baffles within the system remove the largest of the droplets as well as a significant proportion of the aerosol, returning them to the liquid reservoir – only the smallest particles are allowed to escape (O’Callaghan & Barry, 1997).

In delivering aerosolized virus, particle size must be considered. Scott & Sydiskis (1976) have shown that in the mouse model, large-particle aerosols of 10μm median diameter or greater, primarily localized to the upper respiratory tract and require a large dose to initiate infection. Particles of 2μm or less primarily localised in the lower respiratory tract and required less virus to establish an infection than virus delivered in large particles. Reinforcing this is the observation that mice immunized by infection with small particles containing virus (A/Aichi/2/68) and later challenged with a lethal dose of the same virus were protected, while the group receiving immunization by infection with a large particle virus aerosol were not afforded protection – protection being afforded only when the initial large particle exposure dose was increased by several fold (Scott & Sydiskis, 1976). A similar study
showed that serum neutralizing and haemagglutination inhibition (HAI) antibodies reached detectable levels in mice 28 days after exposure to small particle aerosols (2um) containing A/H3N2 virus while the large particle (8um) exposed group did not demonstrate any detectable antibody response (Jemski & Walker, 1976). Taken together, these studies suggest that a smaller quantity of virus is required to stimulate a robust immune response when the virus is delivered to the mouse lungs in a small particle form when compared to its large particle counterpart – the latter requiring a higher titre to initiate the same degree of response.

Smith and colleagues (2011) demonstrated that aerosol inoculation with the X31 strain of influenza virus caused a larger degree of lung pathology and enhanced morbidity and mortality compared to intranasal inoculation. Upon histopathological examination, the lungs of the aerosol-inoculated mice exhibited severe bronchointerstitial pneumonia across all lung lobes with evidence of acute alveolar injury and necrosis. Interestingly, lung pathology in the intranasal inoculated group was often confined to a single lung lobe with substantial pathology not being seen until 3 days post infection (much later than with the aerosol inoculated group). Their results also suggest that an aerosol infection induces predominately a Th-2 type cytokine response. The authors attributed the difference in pathology between the two inoculation groups to the levels of activation of granular cell infiltrates and IL-6 levels, both of which were found to be higher (6-fold higher in the case of IL-6) in BAL fluid in the aerosol inoculated mice group. In other studies employing other animal models (Svitek, 2008; Kobasa, 2004) and humans (Hayden et al., 1996), the pathological effect of severe influenza infections has been associated with increased levels of IL-6 in nasal wash fluids and sera, especially at day 3 post infection.

In a study conducted by Nadithe et al., (2003) where C57BL/6 mice were exposed in a nose-only fashion to jet aerosolized $^{99m}$Tc-labeled human serum albumin (HSA), they calculated that no more than $0.27 \pm 0.67\%$ of the aerosol delivered to the chamber was deposited in the mice and that only $0.0216 \pm 0.005\%$ reached the lungs. This study demonstrates that only a small proportion of an aerosolised virus or drug may reach the lungs and that an adequate and equal air flow through an exposure chamber must be maintained. Both of these concepts...
should be taken into account when trying to achieve adequate virus or drug levels in nebulisation experiments.

6.1.4 Development of a working nebulisation system

The generation of an aerosol was performed with the *Aeroneb* nebulizer (EMMS, Hants, UK). This device includes a concave aperture plate that is surrounded by a vibrational element. Receiving an electronic signal of 128 kHz from a control unit, the aerosol generator vibrates at a rate of ~120,000 vibrations per second. These rapid vibrations result in the formation of an aerosol cloud within the nebulisation chamber with particle sizes ranging from 4 to 6 μm.

The even distribution of the aerosol cloud throughout the entire system (Figure 57 and 58) is achieved by the generation of a constant airflow. This is carried out by a bias airflow generator (EMMS, Hants, UK) that transports compressed air with a flow rate of 10 L/min from the nebulisation chamber (Figure 59) to the exposure chamber. Additionally, 5 L/min of aerosol cloud is also sucked out of the exposure chamber and further transported through virkon cleaning traps, filters and silica traps before being released through the class II biological safety cabinet’s air-extraction system (undergoing further HEPA filtering in the process).

The nose only exposure of aerosols to mice was carried out with a 6-port inhalation tower. The functional design of the tower consists of an inner and outer tower. The inner tower conveys the aerosol through the system while the outer tower connects the inner tower with restrained mice in the Perspex tubes (Figure 60) (EMMS, Hants, UK). This internested two-tower design ensures equal distribution of the aerosol cloud to all exposure ports.
Figure 57: Schematic representation of the nebuliser system employed in RV1088 mouse experiments.
Sampling of the aerosol cloud was made through one of the exposure chamber ports using a modified bung that permitted the passage of a small diameter tube. This tube was place roughly in the same location that a mouse’s nose would occupy. The tubing was then connected to a midget impinger (Figure 61) containing PBS through which the pulled air would flow, entrapping any viral particles in the PBS. To pull the aerosol cloud through the impinger, we employed a small variable pump (Air sampling pump 2223-229(230)A, SKC, Dorset, UK) which was programmed to simulate the breathing patterns of a female BALB/c mouse. We devised this set up to try to ascertain how many infectious particles a mouse might inspire during an exposure period (Results section 6.2.1).
6.2 Results

6.2.1 Determining aerosolisation rates using the midget impinger

In order to determine the quantity of replicating virus that the nebulisation system was capable of depositing in a mouse lung during normal respiration, we used a series of well-established mathematical equations (Bowen et al., 2012) and a midget impinger to sample the aerosol.
Determination of the virus particle (here PFUs) concentration in an aerosol cloud as measured by midget impinge:

Employing the equation directly below, we were able to calculate the number of infectious viral particles (PFU) suspended in the aerosol cloud with the aid of a midget impinger. The impinger contained 10ml of PBS and was connected directly to the nebuliser’s exposure chamber (i.e. where an animal subject would be exposed to the aerosol cloud). Downstream of the impinger was a small programmable pump that was set to mimic the normal respiratory rate of an average female BALB/c mouse (volumetric flow set to = 0.6L/min) drawing the aerosol cloud through the PBS for 10min thus trapping any aerosolised virus in the PBS. The trapped virus was later titred via plaque assay and gave an impinger concentration of 300 pfu/ml. Inserting this value into the equation below we estimate that the concentration of infectious virus achieved in the aerosol was 500pfu/L.

\[
Ac = \frac{\left( Ci \times Vi \right)}{\left( Qi \times Ts \right)}
\]

\( Ac = \) Aerosol concentration (pfu/L)
\( Ci = \) Impinger concentration (pfu/ml)
\( Vi = \) Final Vol of PBS in impinge (ml)
\( Qi = \) Impinger volumetric flow (L/min)
\( Ts = \) Sample collection duration (min)

\[
Ac = \frac{\left( 300 \text{pfu} / \text{ml} \right) \times \left( 10 \text{mls} \right)}{\left( 0.6 \text{L/min} \right) \times \left( 10 \text{min} \right)}
\]

\( Ac = 500 \text{ pfu} / \text{L} \)
Determination of virus deposition into mouse lungs based on known aerosol concentration:

After determining the concentration of active viral particles in the aerosol cloud, we next sought to calculate what concentration of virus might be deposited into the lungs of a normal and healthy female BALB/c mouse. We achieved this via the equation below and employed our previously calculated virus aerosol concentration (Ac) of 500pfu/L, the normal ventilation rate for the test subjects (0.6L/min) and the experimental exposure time (10min). Thus, from these calculations, we can expect a typical BALB/c female mouse to inhale approximately 3000pfu over the course of 10min of normal respiration.

\[ ID = [Ac \times Vm \times Tc] \]

ID = Inhaled lung deposition (pfu)
Ac = Aerosol concentration (pfu/L)
Vm = Ventilation rate (L/min)
Tc = Challenge duration (min)

\[ ID = [(500pfu / L) \times (0.6L / min) \times (10min)] \]

\[ ID = 3000pfu \]

Determination of the percentage of liquid virus solution (i.e. nebuliser input) that becomes deposited into a mouse lung:
Building on the results above, we were able to determine what percentage of the nebuliser input actually reaches the test subject's lungs using the equation directly below.

\[ Ip = \left( \frac{ID}{In} \right) \times 100 \]

**ID** = Inhaled lung deposition (pfu)
**In** = Nebuliser virus input (pfu)
**Ip** = Inhaled percentage of input (%)

\[ Ip = \left( \frac{1.154 \times 10^4 \text{ pfu}}{4.50 \times 10^7 \text{ pfu}} \right) \times (100) \]

**Ip** = 0.026%

Averaging the results of three separate experiments, we calculated that approximately 0.026% (std dev 0.0006%) of the liquid virus concentration added to the nebuliser system was inhaled by a test subject.

### 6.2.2 The nebulisation of A(H1N1)pdm09 virus

The first step in validating the nebuliser system was the determination of what input viral dose/titer was required to create quantifiable clinical signs (weight loss) consistent with an influenza infection. In order to achieve this, groups of 5 mice were exposed to a range of nebulised input virus amounts (ranging from: 2x10^4 to 1x10^8 pfu) via the nose-only exposure method (a full description of this can be found in section 8.2.6.4). In addition, an intranasally instilled viral dose of 2x10^5 pfu was employed to act as the comparator (Figure 62). The first experiment (Figure 62, part a) did not result in any weight loss in mice given virus by the nebulized route. Two
possible reasons may account for this, the first being that the input amount of virus was simply too low, bearing in mind the losses of virus titre in the system and the level of 0.026% that reached the mouse lung and the second was that, due to the trapping of infections particles in condensing droplets within the system’s tubing very little virus was actually delivered to the mice (Figure 63). The latter of these issues was addressed in subsequent experiments by employing new and shorter tubing for each nebulising event. Ultimately, it was determined that in the system, an input amount of $1\times10^8$ with a calculated lung deposition of $2.6\times10^4$ pfu was capable of inducing a weight loss curve of similar magnitude as the $2\times10^5$ intranasal instillation (Figure 62, part b). The weight loss in the animals who received virus through the nebulized route also occurred in two distinct stages, as observed previously for the intranasal inoculation route. This level of input virus was chosen to serve as baseline for pharmacological intervention.

Figure 62: Determination of nebulant input required for creation of desired weightloss curve. Female BALB/c (6-8 weeks) were exposed to nebulised H1N1 virus (A/England/195/2009). Two experiments (a&b) were conducted in which each treatment group was exposed to different quantity of input virus a) $10^4$, $10^5$ and $10^6$ and b) $10^6$, $10^7$ and $10^8$ pfu. In addition, each group also contained an intranasal instillation group (input = $2\times10^5$ pfu) acting at the control. Daily weights were taken at the same time during each trial day. Weight loss curves were generated and compared to the intranasal control group. It was determined that an input of $10^8$ pfu in the nebulised system produces a weight loss curve similar to that seen in the intranasal instillation group.
6.2.3 The nebulisation of RV1088

Next, the feasibility of delivering nebulised RV1088 to mice infected by the same route was assessed. Groups of 15 mice were infected with A/England/195/2009 (A(H1N1)pdm09) with a calculated viral lung deposition of 2.6x10^4 pfu. Three days prior to infection, a treatment group were exposed daily to nebulised RV1088 (1.5mg/ml in 5 mls of vehicle) or drug vehicle alone [Tween20 (1mg/ml), Span20 (0.2mg/ml), Monosodium phosphate dihydrate (9.4mg/ml), Dibasic sodium phosphate anhydrous (1.75mg/ml), Sodium chloride (4.2mg/ml), 500ml H2O, 5ml/exposure]. An additional group received only nebulised drug vehicle but remained uninfected. The quantity of drug used was determined after discussions with our colleagues at RespiVert and are partially based on previously unpublished work involving Guinea Pigs conducted at the University of Cardiff (Personal communication with RespiVert). Post infection, daily weights were recorded and at days 1, 3 and 5 p.i., 5 mice from each group were culled and their lungs removed (Figure 64, part a). Lungs were subsequently homogenised and used to quantify viral loads via plaque assay on MDCKs, as well as lung IFNα levels by ELISA (Figure 64, parts b&c). Finally, whole lung specimens from day 3 p.i. were fixed in formalin, sectioned and stained with H&E (Figure 64, part d).
All the infected mice lost weight over the course of the experiment. There was no significant difference in timing or extent of weight loss for any treated group from the vehicle only infected animals. In addition, on days 1, 3 and 5 p.i., there was no difference in viral loads in the lungs in any group. On the other hand evidence that the course of infection was modified by drug treatment was supported from monitoring IFN-α levels in the lungs.

Importantly, on day three post-infection, the infected untreated mice had a significant IFN response, and the RV1088 treatment group had significantly lower IFN-α levels than the vehicle/virus group indicating that nebulised RV1088 did reach the lungs and that it was able to exert an effect on the innate immune response of the mouse. Surprisingly, the IFN-α levels in lungs of uninfected but vehicle-treated mice were very high on day 1. This suggested that the delivery by nebulization to the lungs had in itself induced an innate response. How this affected the outcome of infection is not clear but overall the levels of IFN in infected animals were not very different from the levels seen in previous experiments where virus and drug were delivered by the intranasal route suggesting that the effects of virus infection overrode the effect of nebulization of vehicle.

Blinded histological examination of lung tissue sections also indicated that RV1088 was modifying the infection outcome. H&E stained sections examined at both 10x and 20x revealed that infection with A/England/195/2009 (A(H1N1)pdm09) by nebulisation, resulted in distinct histological changes when compared to the control uninfected group; specifically, increased polymorphonuclear neutrophil (PMN) infiltration and airway plugging can be found (Figure 64, part d). The absence of these pathological markers in the RV1088 treatment group suggests that nebulised RV1088 did indeed reach the target tissues and that, at this location, it exerted an effect on the innate host response, modifying cellular recruitment to the site of infection.
Figure 6.4: Effects of RV1088 in the mouse model. Female BALB/c (6-8 weeks) were exposed to nebulised RV1088 (A/England/195/2009) and were randomly assigned to drug treatment groups. On set days, mice from each group were culled and viral titres (a), IFN-α levels (b), and lung tissue sections were processed (c). Histology of lung tissue sections was performed (d). Histology with H&E staining of lung tissue sections was performed (d). Results are expressed as the mean ± standard deviation of five samples (Student t-test, *p<0.05, **p<0.001).
In addition to analysing the lung samples for mouse IFN-α, homogenised lung samples from all subjects were analysed on a multiplex plate for further cytokine/chemokine expression (Figures 65 & 66). Specifically, IL-1β, IL-12, IL-13, KC, VEGF, IP-10, IFN-γ, MIG and IL-6 were examined. Of these, only IL-6 demonstrated any significant suppression with the RV1088 demonstrating significant suppression on day 5 p.i. when compared to the vehicle+virus group. In contrast, there was a marked high level of IL-6 in the lungs of mice infected and treated with Relenza on day 1 when compared to all other treatment groups.

**Figure 65: Multiplex analysis of homogenised mouse lung.** Female BALB/c (6-8 weeks) were exposed to nebulised drug treatment and nebulised H1N1 virus (A/England/195/2009). On set days, mice from each group were culled and the lungs harvested. Lungs were subsequently homogenised and a multiplex analysis for cytokines/chemokines was conducted. All results were adjusted for lung weight. Results are expressed as the mean ± standard deviation of five samples (student t-test, *p<0.5, **p<0.001).
Figure 66: Additional multiplex analysis of homogenised mouse lung. Female BALB/c (6-8 weeks) were exposed to nebulised drug treatment and nebulised H1N1 virus (A/England/195/2009). On set days, mice from each group were culled and the lungs harvested. Lungs were subsequently homogenised and a multiplex analysis for cytokines/chemokines was conducted. Results are expressed as the mean ± standard deviation of five samples (student t-test, *p<0.5, **p<0.001).

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Figure 66: Additional multiplex analysis of homogenised mouse lung. Female BALB/c (6-8 weeks) were exposed to nebulised drug treatment and nebulised H1N1 virus (A/England/195/2009). On set days, mice from each group were culled and the lungs harvested. Lungs were subsequently homogenised and a multiplex analysis for cytokines/chemokines was conducted. Results are expressed as the mean ± standard deviation of five samples (student t-test, *p<0.5, **p<0.001).

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To further illustrate that the nebuliser system had deliver aerosolised pandemic influenza virus to the lung, lung tissue slides were stained using fluorescent antibodies specific to viral antigen (Figure 67). Using this technique, areas of infectious foci became visible with all foci of infection being found adjacent to an airway space. In addition, all foci observable per tissue section were counted and no significant difference in the number of foci with respect to treatment group was noted. These results supported the viral lung loads as determined by plaque assay employing homogenised lung tissue in which no significant difference in lung viral load between treatment groups was found (Figure 67).

**Figure 67: Immunofluorescence staining of mouse lung tissue slides.** a) tissue sections were stained with anti-NP (type A) and anti-Actin (alpha). Images of infectious foci were then either merged with the anti-Actin or unstained cell images. b) A table of the average number of infectious foci (similar to the one shown in part a) per tissue section of lung.
6.3 Discussion and Conclusions

In the previous chapter we showed that RV1088 administered alone or in combination with Relenza was able to reduce weight loss in mice induced by influenza infection. However intranasal instillation of both virus and RV1088 in the ferret model failed to result in any significant reduction of weight loss or viral loads. It is possible that reducing cytokine/chemokine levels (the proposed primary mode of action of RV1088) in the ferret does not prevent them losing weight. Furthermore, in our ferret model, the virus alone group lost very little weight secondary to their infections and it is possible that any effect on weight loss of RV1088 might not be evident. We hypothesized that a number of factors might explain the lack of effect in the ferret including: the quantity of drug in each dose, possibly inadequate delivery of the drug to the animal’s respiratory tract and, as of yet, unknown differences in their innate response to viral infection when compared to mice.

Within this chapter, we sought to establish a working model for the delivery of aerosolised virus and drug compounds to an animal model. The aim of this approach was to simulate as closely as possible a “natural” infection by allowing aerosolised virus to penetrate deep into the lower airways of the lung, resulting in a proportionate stimulation of the innate immune response. As discussed above, intranasal instillation can result in skewed infections, wherein pathology can be isolated to a single lung lobe and the onset of histopathological changes can be delayed (Smith et al., 2011). In contrast, aerosol inoculation has been demonstrated to cause a larger degree of lung pathology and enhanced morbidity and mortality compared to intranasal inoculation in both animal models and in humans. Supporting the hypothesis that aerosol inoculation leads to a more severe clinical outcome are human studies conducted by Cowling et al., (2013) which found that that aerosol transmission accounted for approximately half of all transmission events and carried a higher risk of developing fever (55% compared with 23 and 22% risk for droplet and contact routes respectively). In addition, by nebulising a drug formulation, particles of the same diameter as those created during the
aerosolisation of a virus suspension could theoretically be distributed to multiple areas of the lung resulting in a more targeted drug delivery approach.

The nebulisation of drugs for the treatment and alleviation of numerous human conditions (corticosteroids and bronchodilators for asthmatics, for example) is well established in the medical world. Normally administered via simple inhalation, nebulised Relenza, was employed during the 2009 pandemic under a global compassionate use programme in seriously ill, ventilated patients as a second line treatment however with inconclusive results (HPA, 2012). Unfortunately, while data was collected and returned to GlaxoSmithKline, this data has not yet been made available for public scrutiny and thus, the efficacy of this route of administration has not been determined (UKCRN ID 14218). It has been reported that nebulised Relenza blocked ventilation tubing resulting in failed delivery and even the death of a patient (Duff, 2009; FDA, 2009). As a result IV zanamivir became the preferred second line treatment under compassionate use (Thacker et al., 2009).

The efficacy of a nebulised kinase inhibitor to control influenza infection in the mouse model has been demonstrated in experiments conducted by Droebner and colleagues (2011) in which mice were treated with a MEK inhibitor (U0126) via the aerosol route. This treatment was shown to reduce progeny virus titers and provided protection of influenza-infected mice against a 100X lethal viral challenge (intranasal administration) when compared to untreated controls. However, before we could test RV1088 in such a manner, we first needed to develop a working nebulizer system.

The initial stages of this project involved the design and validation of a new nebuliser system for our laboratory in consultation with EMMS (Hants, UK). The system we devised differed from many of those described previously in the literature (Masihi et al., 2007; Donovan et al., 2000; Utsunomiya et al., 1997) by several important ways. Firstly, our system allows for nose-only exposure thus eliminating over exposure through deposition on the subjects fur and eyes. Secondly, the use of a “nebulant chamber” allows for the generation of a uniform aerosol cloud before subjects are exposed thereby allowing for a greater accuracy in our exposure
calculations. Finally, by employing a small miniature pump that we programmed to mimic the normal respiratory rate of a standard test subject, we are able to calculate the expected inhaled viral dose by a test subject to a great degree of accuracy.

Additional positive features of our system include the ability to dose and/or infect up to 6 test subjects simultaneously while, due to the inherent design of both the “nebulant chamber” and “exposure chamber,” that all subjects received a uniform and equal level of exposure. In addition, the use of “crush bars” in the “exposure tubes” provides a harmless and well-tolerated means of restraining subjects and negates the need for anesthetic–based restraint. The latter has been previously described (for both parenteral and inhaled anesthesia) to adversely affect the respiratory functions of a test subject during the course of sedation resulting in different levels of infection severity (Cesarovic et al., 2010; Miller et al., 2012).

In terms of drawbacks, our system does contain a few that are worth noting. Firstly, due to the inherent design, a large quantity of tubing is required to conduct air/nebulant cloud throughout the system. The temperature differential, albeit minor, is enough to permit the formation of condensation along large sections of this tubing. While this was addressed by changing-out tubing between each run, and shortening the overall tubing length this remains an area for potential improvement. Secondly, the nebulizer unit can experience difficulty in creating a suspended-drug aerosol cloud if the substance or compound in question does not dissolve or remains poorly suspended in the vehicle solution [a problem previously reported for powdered Relenza formations (Duff, 2009; FDA, 2009)]. Finally, the system’s relative large size and cost-prohibitive nature may preclude the use of this particular set-up in certain situations.

Before we exposed any animals to our system we first sought to determine what quantity of viral input might actually reach a mouse during a set exposure period. To achieve this, air sampling was conducted via a midget impinger connected to one of the exposure ports that was in turn connected to a miniature pump. The miniature pump settings allowed for air to be drawn through the impinger at a volume and rate consistent with the normal breathing patterns of the type and sex
of mice we planned to employ in further experiments (i.e. female, BALB/c). The resultant impinger fluid was then plaqued out on MDCK cells to determine viable virus yield (Figure 62). From these results we were able to determine what percentage of viral input titer would potentially reach the lungs of a mouse (refer to calculations in section 6.2.1).

The next step in validating the model was to move to the in vivo phase which involved exposing groups of mice to different calculated viral inputs to determine which specific input resulted in clear quantifiable clinical signs related to an active influenza infection (i.e. weight loss). Using this approach, we determined that an input titer of 1x10^8 (equating to approximately 2.6x10^4 inhaled active virus particles as calculated using the same equations detailed above) created a weight loss curve similar to that seen with a nasal instillation of 2x10^5 (Figure 62). Thus, for the drug trial study it was this viral input titer that we opted to employ. It is important to note, that we have only used one virus [A/England/195/2009 (A(H1N1)pdm09] in this system and that different viral inputs might be required if other strains are used.

These initial viral deposition calculations/results compare well with those of Nadithe et al., (2003). They found that when mice were exposed to a nose-only aerosolized 99mTc-labeled human serum albumin (HSA), no more than 0.27 ± 0.67% of the aerosol delivered to the chamber was deposited in the mice and that only 0.0216 ± 0.005% reached the lungs. In our system, we calculated (detailed above) that, on average, approximately 0.026% (std dev 0.0006%) of the liquid virus concentration added to the nebuliser system was inhaled by each mouse. This result is very satisfying as it corresponds nicely to the 99mTc-labeled HAS results reported by Nadithe and colleagues (2003) while at the same time displaying a remarkably low standard deviation – indicating the reproducibility and robustness of our system. Supporting this is the observation that for all treatment groups, the nebulised delivery of virus±drug compound(s) resulted in markedly less variation in terms of group weight loss when compared to its intranasal counterparts (for example, the RV1088 treatment groups have an average standard deviation of 3.41 and 2.40 for intranasal and nebulised delivery respectively). Unfortunately, we did not assess the level of cytokine/chemokine induction in the animals used during these preliminary
experiments caused by the different viral input levels. Such data would have been useful in comparing the innate immune responses elicited by differing viral input levels in both the intranasal and nebulised systems.

We proceeded to test the effect of treating an influenza infection resulting from nebulised virus delivery, with nebulised RV1088, with or without Relenza. Results showed that RV1088 reduced weight loss compared with vehicle treated group but the difference was not significant. In addition there was no significant different in lung viral load in drug treated or vehicle treated groups. However there was a significant decrease \((p = 3.18 \times 10^{-5})\) in lung IL-6 after RV1088 treatment on day 5 p.i. and in lung IFN-\(\alpha\) on day 3.

These results may suggest that RV1088 might act to suppress IL-6 from a particular type of cell that is only present at day 5 as the day 3 levels of this cytokine were not affected. In a study conducted by Matheu et al., (2013) immune cell recruitment and activity in the lungs of mice in response to H1N1 infection \((1 \times 10^5\) pfu, intratracheal inoculation) was assessed in the mouse model. Overlaying our cytokine results onto Matheu and colleagues’s immune cell response timeline, we can postulate that RV1088 might be exerting its IL-6 suppressive effect by acting against macrophages and/or T cells. However, further investigation is warranted. In contrast, and to our surprise, there was a marked increase in IL-6 levels in the lungs of mice infected and treated with Relenza on day 1 that remained elevated on days 3 and 5 p.i.

The rather small effect of drug in this study suggests that either the delivery of aerosolised virus produced such a severe infection that RV1088 was unable to control the immune response elicited by the virus, or that the amount of RV1088 delivered was simply an insufficient dose. Although we were able to monitor delivery of virus in the nebulized system we did not have any assay to quantify drug in lung tissue, to confirm sufficient dosing. It is possible that the drug adhered to the plastic tubing in the system- indeed this type of problem has also been noted with Relenza given in a hospital setting (Duff, 2009; FDA, 2009). While the experiments differed in both length and sampling days, there are some general observations that can be
made. The weight loss curves, and cytokine profiles did not differ markedly between mice who received virus via nebulizer or intranasal. For example, peak weight loss for the RV1088 treatment groups was 91.3 and 95.09 (% of group starting weight) for nebuliser and intranasal respectively. In terms of IFN kinetics, levels detected in homogenised lung corresponded, and were proportional, to detected viral levels – both peaking on day 3 p.i. However, the RV1088 nebulised group produced almost three times the quantity of IFNα when compared to that detected for the intranasal group (476.8 compared to 159.4 pg/gram lung tissue for nebulised and intranasal respectively) presumable reflecting a difference in the type of cell infected (discussed previously above) or higher viral load in the lungs of the former.

However, the virus delivered to the lower lung fields via nebulisation did replicate to significantly higher levels for all treatment groups when compared to the intranasal instillation route. For example, on day +3, the vehicle-alone infected group had an average pfu/gram of lung tissue of $4.14 \times 10^9$ after nebulized delivery and only $5.34 \times 10^6$ after the intranasal delivery. As such, it is possible that this increased viral replication resulted in an overwhelming pathological state against which, the dose of RV1088 employed was unable to compensate for. It is also possible the system input dose was correct but that an adequate concentration of this input failed to reach the target tissue due to several factors including the build-up of condensation in the tube or poor suspension of the drug in the delivery vehicle.

While RV1088 was not delivered at a dose high enough to significantly affect weight loss or lung viral load, enough was deposited to significantly suppress IFN-α & IL-6 levels ($p = 1.014 \times 10^{-6}$ and $3.18 \times 10^{-5}$, respectively) and to prevent dramatic histological changes related to pathological insult. It is possible that if a higher dose of RV1088 were added to the system, even greater effects could be observed, providing future avenue of investigation.

Immunofluorescence staining (Figure 67) of mouse lung tissue sections for viral antigen (anti-influenza Type A NP) demonstrated that the delivery of nebulised A/England/195/2009 (A(H1N1)pdm09) virus via our system resulted in the deposition of a small number of virions which created finite foci of infection. These
foci may correspond to the small, non-extensive foci of acute inflammation in the airspaces and interstitium seen on histological examination of lungs taken from fatal cases of A(H1N1)pdm09 (Mukhopadhyay et al., 2010). Further supporting this observation are results reported by Harms et al., (2010) where, out of eight autopsies, small microscopic foci of diffuse alveolar damage-associated necrosis was present in 6 cases. Taken together, these reports indicate that a large degree of the pathology associated with an A(H1N1)pdm09 virus infection results not from an en masse infection of the lung but rather from a small number of distinct foci of infection.

Taken together, the results in this chapter indicate that we can successfully infect mice with aerosolized A(H1N1)pdm09 virus, producing observable and quantifiable pathological changes. Furthermore, we have shown that RV1088 can be delivered to the mouse lung and modulate the cytokine/chemokine response of the host; however, further dosing trials are required to determine the optimal dose for these effects to reach their maximum potential.
Chapter 7. Discussion

Respiratory infections caused by influenza viruses are responsible for significant morbidity and mortality world-wide. Seasonal and pandemic influenza outbreaks have a huge impact on both the individual and society at large. The economic cost of influenza-like illness each year is difficult to directly calculate due to the large number of variables involved. Smith and colleagues (2009) constructed a general equilibrium-based computer model to estimate the “economy-wide impact” of the 2009 H1N1 pandemic in the UK. Using several assumptions based on previous influenza pandemics and other respiratory viral outbreaks and economic data from 2004, they predicted a reduction in the UK GDP of between 0.5-4.3%, translating to between £8.4-72.3bn. In a report released by the Health Protection Agency (Pitman et al., 2007), that examined the burden of influenza-related disease in the UK between 1996 and 2004, it was found that a total of 1,430,121 GP consultations took place during this period with an estimated GP consultation cost per episode of influenza-like illness of £30 which added up to over £42.9 million. During this same period, an average of 18,327 deaths per year have been directly attributed to influenza (both Type A and B) infections in the UK alone.

In recent years, considerable effort and progress have been made in the development and application of new and enhanced influenza vaccines. These efforts range from multi-national licencing and subsequent role out of quadrivalent vaccines (due to premier in the UK in the autumn of 2014), to introduction of live attenuated vaccines for children and promising results yielded in the quest for a “universal” vaccine (Yewdell, 2013). However, despite all these important and promising advances, it is important to note that currently vaccines are only intended for a relatively small segment of the population (i.e. those with pre-existing/pre-disposing medical conditions, certain child age groups and the elderly). While protecting these vulnerable groups should not be underestimated it is important to note that vaccinating at risk groups only against influenza offers little-to-no direct benefit to the majority of the healthy UK population. And even if a person does receive the
vaccine, strain miss-match can render the vaccine useless or significantly decrease its protective value.

With such potentially significant economic cost, effective treatment regimes are an imperative. The currently licenced classes of anti-influenza agents, the neuraminidase inhibitors (Oseltamivir and Zanamivir) and the M2 inhibitors (Rimantadine and Amantadine), represent the established approach to combating viral infections by targeting the virus directly. One of these drug classes the adamantanes is limited to the treatment of type A viruses as it is ineffective against type B viruses. However, given the virus’ innate ability to mutate at astonishing rates [estimated mismatch rate 1:10,000 nucleotides (Lauring & Andino, 2010)] and reassort its gene segments with other circulating strains, the emergence of resistant strains is an ever-present concern - limiting available treatment options.

Exemplifying the growing problem of resistance and the significant and rapid rise in the frequency of viruses with antiviral-resistant mutations is the story of the seasonal HN1 viruses that acquired oseltamivir resistance and spread community wide. The H274Y mutation, conferring Oseltamivir resistance, emerged during the 2007-2008 influenza season and by the following season (2008-2009) almost 100% of all tested seasonal A/H1N1 strains in the United States and numerous counties worldwide were found to possess this mutation (CDC, 2009; WHO, 2008; Sheu, 2008).

New treatments targeting both the virus and its corresponding receptor targets on host cells have been proposed and investigated but with mixed results (Hayden, 2009). A relatively new approach, in the context of an influenza infection, is specific targeting of the host’s immune response. The principle behind this approach stems from the current belief that severity of influenza virus pathogenesis is influenced by the host’s immune response to virus infection with the most severe presentations of influenza-related hospitalizations resulting from an unregulated response of pro-inflammatory cytokines and chemokines induced by the TLR and RIG-I signalling cascade, creating a so called “cytokine storm” (Chung et al., 2002; Osterholm, 2005; Fukuyama & Kawaoka, 2011).
Suppression of the over induction of the host’s immune response to infectious insult by the influenza virus has been an area of intense research and debate. Studies aimed at suppressing the immunopathology have examined a number of potential pharmacological interventions including: COX-2 inhibitors, anti-TNF agents, Statins and Glucocorticoids but have yielded varying degrees of success and clinical potential (Darwish et al., 2011). This approach has been subject to debate as many researchers have questioned the wisdom of suppressing the immune response during an active infection. The contention lies with the possibility that if one inhibits the host’s defences during the course of infection, the virus could replicate at an increased rate or lead to increase incidences of secondary infections. This exact concern has been born out in several studies where the use of steroids had been employed (Martin-Loeches et al., 2011).

Intriguingly, a recent Cochrane meta-analysis and review of the use and efficacy of employing neuraminidase inhibitors for preventing and treating influenza in healthy adults and children reported evidence that Oseltamivir may indirectly exerts its symptom alleviation effect via suppression of the immune system (including decreasing IL-6, TNF-α, IFN-γ and other chemokines) and not only through suppression of viral replication as previously thought. Furthermore, Oseltamivir’s anti-pyretic effect has also been postulated not to be a direct result of decreased viral titers but rather via a general depression of the central nervous system (Ono et al., 2013).

The concept of a employing a more targeted approach with respect to suppressing the host’s innate immune response to viral assault represents a new and exciting avenue for research. Protein kinases, a class of enzyme responsible for catalysing the transfer of phosphate to specific substrates, are key regulators in numerous cellular processes and are notoriously druggable. In terms of an influenza infection, kinases have been shown to play pivotal roles in the generation of the innate immune response through their role as members of numerous cytokine/chemokine signaling cascades (Jeffrey et al., 2007; Alberts et al., 2002). As such, members of this class of enzyme represent tempting targets for immunomodulatory interventions.
RV1088, a compound developed by RespiVert (London, UK), is a synthesised compound specifically designed to target and inhibit certain key kinases (p38 MAPK, c-Src and Syk). This compound was originally intended for use in the treatment of respiratory disorders linked with a strong inflammatory response (i.e. asthma and COPD). However, this PhD project has been concerned with evaluating the potential of using this compound to modulate the hyper-activation of the innate immune response associated with influenza infection.

We firstly conducted a series of examinations to demonstrate that in cell culture systems RV1088 was non-cytotoxic and that a representative kinase was measurably suppressed in the presence of this compound. Once having satisfied these two points, we progressed to examining how this compound might exert its reported ant-inflammatory action.

By employing a series of plasmids which encode key components of the IFN-β signalling pathway, we mapped RV1088’s activity to a level at or below that of MAVS with some of its potential effects occurring via TBK-1 resulting in the suppression of NFkB activation and IFN induction. Furthermore, we have shown that this interference occurs in a dose-dependent manner. These results tie in nicely with work previously conducted by several groups where p38 MAPK (one of the targets of RV1088) has been implicated in the induction of the IFN response via the NFkB signalling pathway (Hui et al., 2009; Saccani et al., 2002; Uddin et al., 1999). Despite being a transcription factor associated with the up-regulation of numerous genes in responses to viral attack [including acute phase inflammatory responses, cell adhesion, oxidative stress response, apoptosis and generation of the antiviral state (Ludwig & Planz, 2008; Pahl, 1999)] the up-regulation of NFkB has been implicated in potentiating influenza virus replication (Wurzer et al., 2004; Nimmerjahn et al., 2004). As such, the suppression of NFkB has been suggested as a potential antiviral target (Ludwig & Planz, 2008; Pahl, 1999).

An examination of potential interactions between RV1088 and the viral polymerase revealed that this compound was able to suppress transcription of the vRNA (+) reporter but not the cRNA (-) reporter in a minigenome assay indicating
that RV1088 does indeed affect the viral polymerase and inhibit viral replication. Further RV1088 also suppressed expression of a viral-like RNA driven directly by replicating virus. While the exact mechanism of inhibition of polymerase has yet to be determined, other groups in the field have suggested that kinases are involved in the process of nuclear release of vRNP complexes during the latter stages of viral replication (Ludwig et al., 2009; Pleschka et al., 2008). This could be investigated in the future using immunofluorescence to monitor cellular location of the vRNPs during infection. In addition, a kinome analysis examining the phosphorylation status of viral proteins both in the presence and absence of RV1088 could establish if this compound has any direct effect on viral proteins.

In cultured CALU-3 cells (immortalised lung epithelial cell line) infected at a low moi with A/H1N1 RV1088 used alone did not have a significant effect on viral titres, but cytokine expression induced by virus infection was supressed at the level of transcription (1.58- and 4.76-fold decrease in IFN-β and RANTES respectively). In highly differentiated human airway epithelial cells (HAEs) which are maintained at an air-liquid interface and faithfully recapitulate the human respiratory tract RV1088 used alone again did not exert any significant suppression of viral replication But did lead to striking suppression of cytokine/chemokines released from the infected cultures in apical washings These results were also mirrored in primary peripheral blood mononuclear cells (PBMCs). Furthermore, upon microscopic examination of the cultures, it was apparent that RV1088 was able to protect infected airway cells from the development of virus induced cytopathic effects (CPE) and in all instances, the application of drug delayed the appearance of observable CPE by a minimum of one day. Importantly, in either culture system, although RV1088 inhibited the innate immune response to the virus, we did not observe any increase in viral replication or virally-induced, observable pathology.

This result is especially noteworthy and highlights the potential of employing a targeted approach towards immunomodulation during an active influenza infection. In contrast to a target approach, the use of corticosteroids in patients hospitalized with seasonal A/H1N1 resulted in higher viral titers and prolonged viral shedding when compared to non-glucocorticoid-treated patients Lee et al., (2009).
One explanation for this could be that RV1088 had a dual action, both inhibiting the innate immune response and at the same time inhibiting virus replication such that the two effects cancelled each other out in terms of virus titre when RV1088 was used alone.

The use of combination therapy has been employed in the treatment of countless diseases (including influenza) as a means of potentiating antiviral activity and decreasing the likelihood of resistant strain emergence thereby enhancing clinical outcome. However, to our knowledge, no clinical study has yet been undertaken which has examined the potential effects on cytokine/chemokine expression and viral replication by a kinase inhibitor used in conjunction with a licenced antiviral. To address the question if there existed any additive or synergistic effect if RV1088 was used in conjunction with a licenced antiviral, we again employed both the CALU-3 and HAE cell culture systems. In our initial experiments, RV1088 was used in combination with Relenza, Rimantadine or Oseltamivir (with doses chosen being based on the specific drugs’ reported IC₅₀). All three licenced drugs suppressed viral replication by several logs as expected. RV1088, when used alone, resulted in very little virus suppression, in line with previous results. However, when a conventional antiviral and RV1088 were used jointly, a striking and almost complete suppression of viral replication and cytokine/chemokine induction was noted.

In order to determine if the inhibitory effect on virus replication observed when RV1088 and Relenza was used in combination was additive or synergistic, we employed the well-established principles of the combination index (CI) theorem of Chou-Talalay (Chou, 2010). By using a specialised software program which employs this theorem (CalcuSyn, Biosoft - Cambridge, UK), we were able to determine that the relationship produced by RV1088 when used in combination with Relenza is very strongly synergistic in nature.

After obtaining robust in vitro data demonstrating the immune-modulating effect of RV1088 and the existence of a strongly synergistic relationship between RV1088 and Relenza, we progressed to in vivo models. Intranasal infection and drug
dosing with RV1088 administered alone or in combination with Relenza was able to reduce weight loss in mice induced by influenza infection. However intranasal instillation of both virus and RV1088 in the ferret model failed to result in any significant reduction of weight loss or viral loads. It is possible that reducing cytokine/chemokine levels (the proposed primary mode of action of RV1088) in the ferret does not prevent them loosing weight. Furthermore, in our ferret model, the virus alone group lost very little weight secondary to their infections and it is possible that any effect on weight loss of RV1088 might not be strictly evident. Unfortunately, limitations in reliable and proven ferret cytokine/chemokine reagents restricted detailed analysis. We believed that the lack of a strong response in our drug treatment groups resulted from inadequate distribution of the compounds to the sites of infection. As such, we sought an alternative route of administration.

The nebulisation of drugs for the treatment and alleviation of numerous human conditions (corticosteroids and bronchodilators for asthmatics, for example) is well established in the medical world. During the 2009 pandemic, under a global compassionate use programme, Relenza (normally administered via simple inhalation) was given to seriously ill/ventilated patients via a nebulised route however with inconclusive results due to lack of reporting (HPA, 2012). To model this route of administration for our kinase inhibitor, we worked to establish a model for the delivery of aerosolised virus and drug compounds to an animal model. The aim of this approach was to simulate as closely as possible a “natural” infection by allowing aerosolised virus to penetrate deep into the lower airways of the lung, resulting in a proportionate stimulation of the innate immune response. The efficacy of a nebulised kinase inhibitor to control influenza infection in the mouse model has been demonstrated in experiments conducted by Droebner and colleagues (2011) with favorable outcomes reported.

After numerous “dry runs” and calculations aimed at determining the virus concentration in the aerosol cloud produced by the nebulizer unit, the inhaled lung deposition concentration and the determining the theoretical percentage of liquid virus solution (i.e. nebuliser input) that is deposited into a mouse lung we were ready to proceed with the animal model work. Further experiments employing the
live animal models were conducted to determine what viral titer input was required to initiate an infection with a weight loss curve that would approximate that observed in our standard intranasal inoculations. Only after all these parameters had been taken into account did we proceed to test the effect of treating an influenza infection resulting from nebulised virus delivery, with nebulised RV1088, Relenza or a combination of the two. Results showed that RV1088 reduced weight loss compared with vehicle treated group but the difference not significant. In addition there was no significant different in lung viral load in drug treated or vehicle treated groups. However, a significant decrease in IL-6 was observed for the RV1088 treatment group. Alarmingly, Relenza did not prove to be greatly protective against an influenza infection with A/England/195/2009. Despite this group receiving 4 doses at 3μg/mouse/dose, little-to-no protective effect was seen in terms of weight loss and lung viral titers compared to the vehicle alone treatment group. This lack of response ties in with the growing debate on the efficacy and wisdom of employing neuraminidase inhibitors in the treatment of influenza infections (highlighted by the recent Cochrane Group Review released on April 10th, 2014). Unfortunately, further analysis is greatly hindered by that lack of disclosure of vital drug trials by the manufacturers of these compounds.

The rather small effect of drug in this study suggests that either the delivery of aerosolised virus produced such a severe infection that RV1088/Relenza was unable to control the immune response elicited by the virus, or that the amount of RV1088 delivered was simply an insufficient dose. Although we were able to monitor delivery of virus in the nebulized system we did not have any assay to quantify drug for example in lung tissue, to confirm sufficient dosing. It is also probably that the “sticky” nature of the drug formulations (leading the drugs to adhere to the plastic tubing in the system) might have impaired adequate delivery to the lung fields. This particular problem has been previously noted for Relenza when given previously in a hospital setting (Duff, 2009; FDA, 2009).

We are however, confident that our nebuliser system effectively allowed for the delivery of aerosolised virus to our in vivo models. Immunofluorescence staining of mouse lung tissue sections for viral antigen (anti-influenza Type A NP)
demonstrated that the delivery of nebulised A/England/195/2009 (A(H1N1)pdm09) virus resulted in the deposition of a small number of virions which, in turn, lead to the formation of finite foci of infection. This result was particularly exciting as it mirrored autopsy findings of necrotic human lung tissue reported during the A/H1N1 2009 pandemic (Mukhopadhyay et al., 2010; Harms et al., 2010). This result indicates that with our nebulizer system, we can closely recapitulate a “natural” infection with a pandemic virus resulting in an observable clinical sign (i.e. weight loss) and the generation of observable and quantifiable pathology.

As with any research undertaking, every question addressed and answered will invariably result in more questions arising. This project is no exception and during our efforts to adequately address the aims of this thesis, a number of intriguing areas of potential future investigation have arisen. These new questions can roughly be divided into two broad categories: *in vitro* and *in vivo*.

In our synergy examinations, all viruses employed were naturally sensitive to the chosen antiviral or possessed an engineered mutation to afford them sensitivity. The immunosuppressive and antiviral effects of RV1088 plus an antiviral to which the test virus is resistant would be of interest and would help to elucidate if RV1088 directly augments the antiviral properties of a licenced drug or whether replication suppression occurs via some other mechanism. Furthermore, we believe that the nebulised mouse experiment indicates that RV1088 exerts its effect, at least in part, on immune cells recruited to sites of infection. To adequately address this postulation, we would recommend isolating specific immune cells via Fluorescence Assisted Cell Sorting (FACS) making use of cell-specific markers and conducting examinations along the same lines that we performed in human PBMCs. We have demonstrated through a series of experiments employing various expression plasmids encoding for key members of the IFN pathway that RV1088 exerts its effect at the level of MAVS and TBK1 (Chapter 2). However, to further understand the mechanism behind RV1088’s activity, we propose employing a series of Western blots and specifically probing for the phosphorylation status of TBK-1 in infected and treated cells and in cells overexpressing MAVS and treated with RV1088. In addition, we suggest analysing virus replication and cytokine induction under condition of
siRNA knockdown for the known targets of RV1088. Finally, we would recommend examining if the activity observed when RV1088 is used (either alone or in combination with an antiviral) in the context of an influenza infection is conserved during other respiratory virus infections.

There also exists potential for further in vivo experiments. Specifically, for the mouse model, it would be of considerable interest to conduct an experiment where RV1088±Relenza (or other antiviral) is given as a treatment course after the animal(s) develop signs/symptoms of disease (i.e. post infection). While we have examined this in HAEs (Chapter 3), such an undertaking was not performed in vivo. In addition, with respect to the nebuliser system, it is imperative that the quantity of drug deposited in the lungs is established so that an accurate dosing regimen could be established. Finally, a further investigation of RV1088s activity in the ferret model would be invaluable to understanding how this compound might act in humans. Recent advances in cytokine/chemokine detection and understanding of viral pathogenesis in this model could make a new examination profitable. Furthermore, as we believe that RV1088 acts by modulating the immune response, a detailed observation and examination of ferret symptomology would prove interesting. We specifically recommend employing both qualitative and quantitative parameters (i.e. weight loss, sneezing, activity levels, and core body temperature via implanted telemetry devices). While the nebuliser system addresses many of the shortcomings of the intranasal instillation route of infection, it still is not fully representative of a natural infection. However, by employing a chain of transmission system, where in an intranasally infected animal would be used to naturally infect a series of other animals via both the direct contact and aerosol routes of transmission, one could determine how RV1088 acts in the context of naturally acquired infections.

With the continued rise of novel reassortment viruses and the emergence new subtypes in the human population coupled with the spread of resistance to our already limited anti-viral arsenal, the identification of new and innovative drug targets and compounds should be of paramount concern. By targeting the host and not the virus, kinase inhibitors prove to be an attractive class of compounds for potential therapeutic intervention. Already, kinases have been demonstrated to play
pivotal roles in potentiating virus replication while at the same time, have also been strongly implicated in the generation of hypercytokinemia leading to the dreaded "cytokine storm." Compounds, such as RV1088, which exert their effects by targeting a key cluster of kinases, represent a novel paradigm in the treatment of influenza. Importantly, this approach indicates that the targeting of these kinases, while suppressing the cytokine/chemokine response, did not lead to increased viral replication. Furthermore, the significant synergistic effect observed when such a compound is used in conjunction with a licensed anti-viral provides exciting new avenues for further investigation.
Chapter 8. Materials and Methods

8.1 Materials

Table 8. Cell lines used during the course of this project.

<table>
<thead>
<tr>
<th>Name</th>
<th>Cell Type</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>293-T</td>
<td>Human (embryonic) kidney cell line</td>
<td>Glaxo Smithkline</td>
</tr>
<tr>
<td>A-549</td>
<td>Human (lungcarcinoma) epithelial cells</td>
<td>ATCC</td>
</tr>
<tr>
<td>DF-1</td>
<td>Chicken Fibroblast line</td>
<td>ATCC</td>
</tr>
<tr>
<td>Madin Darby Canine Kidney (MDCK)</td>
<td>Dog kidney epithelial cells</td>
<td>Glaxo Smithkline</td>
</tr>
<tr>
<td>CALU-3</td>
<td>Human (adenocarcinoma) epithelial cells</td>
<td>ATCC</td>
</tr>
<tr>
<td>Human Airway Epithelial (HAE)</td>
<td>Biopsy-derived, primary airway cultures, ALI</td>
<td>Epithelix, CH</td>
</tr>
<tr>
<td>Ferret Airway Epithelial (FAE)</td>
<td>\textit{Ex vivo}-derived, primary airway cultures, ALI</td>
<td>Barclay Lab</td>
</tr>
</tbody>
</table>

Table 9. Animal species used in this, together with age and experimental use.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex/Age</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c Mice</td>
<td>Female, 6-8 weeks</td>
<td>Viral infection (intranasal &amp; nebulisation) ± Treatment</td>
</tr>
<tr>
<td>Ferrets</td>
<td>Female, 16-21 weeks</td>
<td>Viral infection (intranasal) ± Treatment</td>
</tr>
</tbody>
</table>
### Table 10. Viruses used during the course of this project.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Florida/04/2006</td>
<td>Wild type virus, human clinical isolate, Yamagata lineage</td>
<td>Barclay lab stock</td>
</tr>
<tr>
<td>B/Florida/04/2006 - RG</td>
<td>Reverse genetics (RG) virus</td>
<td>This project</td>
</tr>
<tr>
<td>B/Malaysia/2506/2004</td>
<td>Wild type virus, human clinical isolate, Victoria lineage</td>
<td>Barclay lab stock</td>
</tr>
<tr>
<td>A/England/195/2009 - RG</td>
<td>Reverse genetics (RG) virus</td>
<td>Barclay lab stock</td>
</tr>
<tr>
<td>A/Sydney/5/1997</td>
<td>A/H3N2 influenza virus</td>
<td>Dr. Maria Zambon, HPA</td>
</tr>
<tr>
<td>A/Turkey/05/05:PR8 (6:2)</td>
<td>A/H5N1:PR8, 6:2 (HA/NA) reassortment virus. Biocontainment level 3 virus.</td>
<td>Barclay lab stock</td>
</tr>
<tr>
<td>Newcastle Disease Virus (NDV)</td>
<td>Epizootic avian virus – transmissible to humans. Potent IFN inducer.</td>
<td>Barclay lab stock</td>
</tr>
</tbody>
</table>
Table 11. Plasmid Constructs used during the course of this project*.

<table>
<thead>
<tr>
<th>Plasmid Constructs</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV-Victoria-NS1</td>
<td>Expression plasmid encoding for human influenza Victoria NS1 protein</td>
<td>Barclay Lab Stock</td>
</tr>
<tr>
<td>pCMV5-HA-MAVS</td>
<td>Expression plasmid encoding for human MAVS protein</td>
<td>Prof. Andrew Macdonald (University of Leeds)</td>
</tr>
<tr>
<td>pCMV5-HA-IKKb</td>
<td>Expression plasmid encoding for human IKKb protein</td>
<td>Prof. Andrew Macdonald (University of Leeds)</td>
</tr>
<tr>
<td>pCMV5-HA-IKKe</td>
<td>Expression plasmid encoding for human IKKe protein</td>
<td>Prof. Andrew Macdonald (University of Leeds)</td>
</tr>
<tr>
<td>pCMV5-HA-IRF-3</td>
<td>Expression plasmid encoding for human IRF-3 protein</td>
<td>Prof. Andrew Macdonald (University of Leeds)</td>
</tr>
<tr>
<td>pCMV5-HA-IRF-7</td>
<td>Expression plasmid encoding for human IRF-7 protein</td>
<td>Prof. Andrew Macdonald (University of Leeds)</td>
</tr>
<tr>
<td>pCMV5-HA-TBK-1</td>
<td>Expression plasmid encoding for human TBK-1 protein</td>
<td>Prof. Andrew Macdonald (University of Leeds)</td>
</tr>
<tr>
<td>pCMV5-HA-NFkB</td>
<td>Expression plasmid encoding for human NFkB protein</td>
<td>Barclay Lab Stock</td>
</tr>
</tbody>
</table>

* For plasmids used to generate recombinant viruses, refer to Table 18.
Table 12. Reporter Constructs used during the course of this project.

<table>
<thead>
<tr>
<th>Plasmid Constructs</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-luc (human)</td>
<td>Firefly luciferase reporter gene under the control of a human IFN-promoter</td>
<td>Prof. Steve Goodbourn, St. Georges, London</td>
</tr>
<tr>
<td>IFN-luc (avian)</td>
<td>Firefly luciferase reporter gene under the control of a avian IFN-promoter</td>
<td>Prof. Steve Goodbourn, St. Georges, London</td>
</tr>
<tr>
<td>ISG54-Luc</td>
<td>Firefly luciferase reporter gene under the control of a human ISG54 promoter</td>
<td>Barclay Lab Stock</td>
</tr>
<tr>
<td>Renilla Luciferase</td>
<td>Transfection control – constitutive expression of the Renilla Luciferase</td>
<td>Barclay Lab Stock</td>
</tr>
<tr>
<td>cRNA reporter</td>
<td>Firefly luciferase and non-coding ends of an influenza segment in positive orientation (cRNA) flanked by a RNA polymerase I promoter and Pol I terminator sequence</td>
<td>Olivier Moncorge &amp; Anna Cauldwell, Barclay Lab</td>
</tr>
<tr>
<td>vRNA reporter</td>
<td>Firefly luciferase and non-coding ends of an influenza segment in negative orientation (vRNA) flanked by a RNA polymerase I promoter and Pol I terminator sequence</td>
<td>Olivier Moncorge &amp; Anna Cauldwell, Barclay Lab</td>
</tr>
</tbody>
</table>
Table 13. Antibodies used during the course of this project.

<table>
<thead>
<tr>
<th>Antibody/Stain</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-influenza A NP monoclonal</td>
<td>Mouse monoclonal antibody raised against the influenza A virus NP</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>Anti-β tubulin monoclonal</td>
<td>Mouse monoclonal antibody raised against β-tubulin</td>
<td>Sigma</td>
</tr>
<tr>
<td>Goat anti-mouse IgG:BGAL</td>
<td>Goat monoclonal antibody raised against the mouse antibody</td>
<td>AbD Serotec</td>
</tr>
<tr>
<td>Rabbit anti-Tubulin alpha</td>
<td>Rabbit polyclonal antibody raised against tubulin</td>
<td>Spring Bioscience</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>Alexa Fluor 488 anti-mouse IgG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Alexa Fluor 568</td>
<td>Alexa Fluor 568 anti-mouse IgG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>Alexa Fluor 647 donkey anti-rabbit IgG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Anti-IFNα</td>
<td>Mouse IFN-α</td>
<td>Verikine</td>
</tr>
<tr>
<td>DAPI</td>
<td>Targets the nucleus</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Anti-Cytokeratin</td>
<td>Targets cell membrane</td>
<td>bdBiosciences</td>
</tr>
<tr>
<td>Anti-Phospho-ATF-2 (Thr71)</td>
<td>Rabbit polyclonal - detects ATF-2 only when phosphorylated at Thr71</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Immobilised Phospho-p38 MAPK</td>
<td>Mouse mAb immobilized by crosslinking to agarose hydrazide beads</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>(Thr180/Tyr182)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>HRP-linked antibody</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-biotin</td>
<td>HRP-linked antibody</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>Anti-Vinculin</td>
<td>Goat polyclonal antibody raised against vinculin</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Anti-p38 (total)</td>
<td>Detects all isoforms of p38 regardless of phosphorylation status</td>
<td>Cell Signaling</td>
</tr>
</tbody>
</table>
Table 14. qRT-PCR Primer and Probe Sequences used during the course of this project.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP-10 (CXCL10) Fw</td>
<td>CGATTCTGATTTGCTGCCTTATC</td>
</tr>
<tr>
<td>IP-10 (CXCL10) Rev</td>
<td>GCAGGTACAGCGTACGGTTCT</td>
</tr>
<tr>
<td>IP-10 (CXCL10) Probe</td>
<td>CTGACTCTAAAGTGCAATTCAAGGAGTACCCTCTCTC</td>
</tr>
<tr>
<td>IL-6 Fw</td>
<td>CCCAGGAGGAAGCAGCTATGAAC</td>
</tr>
<tr>
<td>IL-6 Rev</td>
<td>CCCAGGAGGAAGCAGCTATGAAC</td>
</tr>
<tr>
<td>IL-6 Probe</td>
<td>CTTTCTCCACAAGCGCCTTCGGT</td>
</tr>
<tr>
<td>IFN-β Fw</td>
<td>CGCCGCATTGACCATCTA</td>
</tr>
<tr>
<td>IFN-β Rev</td>
<td>TTAGCCAGGAGGTTCTCAACAATAGTCTCA</td>
</tr>
<tr>
<td>IFN-β Probe</td>
<td>TCAGACAAGATTCATCTAGCAGCTGGCTGGA</td>
</tr>
<tr>
<td>RANTES (CCL5) Fw</td>
<td>GCATCTGCGTCCCCATATTC</td>
</tr>
<tr>
<td>RANTES (CCL5) Rev</td>
<td>CAGTGGGCGGGCAATG</td>
</tr>
<tr>
<td>RANTES (CCL5) Probe</td>
<td>TCGGACACCACCCTGCTGCT</td>
</tr>
<tr>
<td>IL-8 Fw</td>
<td>CTGGCCGTGGCTCTCTTG</td>
</tr>
<tr>
<td>IL-8 Rev</td>
<td>CTTTGCGAAACTGCACCTT</td>
</tr>
<tr>
<td>IL-8 Probe</td>
<td>CAGCCTTCTGATTCTGCAGCTCTGTTG</td>
</tr>
<tr>
<td>18S Fw</td>
<td>CGCCGCTAGAGGGTAAATTCT</td>
</tr>
<tr>
<td>18S Rev</td>
<td>CATCCTTGGCAAAATGCCTTTGC</td>
</tr>
<tr>
<td>18S Probe</td>
<td>ACCGGCGCAAGACGGACAGA</td>
</tr>
<tr>
<td>M-gene Fw</td>
<td>AAGACAAAGACAAATGTCACCTCT</td>
</tr>
<tr>
<td>M-gene Rev</td>
<td>TCTACGYTGCACTCCYCGCT</td>
</tr>
<tr>
<td>M-gene Probe</td>
<td>TYACGCTCACCGTGCCAGTG</td>
</tr>
</tbody>
</table>
Table 15. Compounds and Chemicals used during the course of this project.

<table>
<thead>
<tr>
<th>Compound/Chemical</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>RV1088</td>
<td>Novel, narrow-spectrum kinase inhibitor</td>
<td>RespiVert</td>
</tr>
<tr>
<td>Oseltamivir phosphate (Tamiflu)</td>
<td>Licensed Neuraminidase inhibitor</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>1-(1-Adamantyl)ethylamine hydrochloride (Rimantadine)</td>
<td>Licensed M2 inhibitor</td>
<td>Maybridge</td>
</tr>
<tr>
<td>Relenza (Zanamivir)</td>
<td>Licensed Neuraminidase inhibitor</td>
<td>RespiVert</td>
</tr>
<tr>
<td>Fluticasone</td>
<td>Synthetic glucocorticoid</td>
<td>RespiVert</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Polar aprotic solvent</td>
<td>Sigma</td>
</tr>
<tr>
<td>SB203580</td>
<td>Specific inhibitor of p38α and p38β</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Polyinosinic:polycytidylic acid (Poly-I:C)</td>
<td>dsRNA analogue</td>
<td>Sigma</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Potent, broad spectrum inhibitor of protein kinase</td>
<td>Abcam</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Src family tyrosine kinase inhibitor</td>
<td>RespiVert</td>
</tr>
<tr>
<td>BIRB-796</td>
<td>Inhibitor of all p38 MAPK isoforms</td>
<td>RespiVert</td>
</tr>
<tr>
<td>Syk R343</td>
<td>Syk kinase inhibitor</td>
<td>RespiVert</td>
</tr>
<tr>
<td>Lipofectamine 2000</td>
<td>Transfection reagent</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>
Table 16. Solutions used during the course of this project.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Recipe</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP A Buffer</td>
<td>100mM NaCl (50mM iodoacetamide) 1% Nonidet-P40 0.1% SDS 0.5% Sodium deoxycholate 20mM Tris-HCl, pH 7.5</td>
<td>Lysis of cells for western blot analysis/SDS-PAGE</td>
</tr>
<tr>
<td>SDS loading buffer (2x)</td>
<td>45% Glycerol 6.5% SDS 15% 1M Tris pH 6.8 dH$_2$O</td>
<td>Western blot/SDS-PAGE sample loading buffer</td>
</tr>
<tr>
<td>SDS Running buffer</td>
<td>25 mM Tris base 250 mM Glycine 0.1% SDS pH 8.3</td>
<td>Buffer for gel electrophoresis in western blot</td>
</tr>
<tr>
<td>Western Blot Transfer Buffer</td>
<td>0.037 % (w/v) SDS 48 mM Tris 39 mM Glycine 20% (v/v) Ethanol added just before use</td>
<td>Buffer for transfer of proteins to PVDF membrane in western blot (semi-dry system)</td>
</tr>
<tr>
<td>Western Blot Wash Buffer</td>
<td>PBS 0.1% Tween 20</td>
<td>Buffer for washing membrane during antibody staining of proteins in western blots analysis</td>
</tr>
<tr>
<td>DNA-Loading Buffer (6x)</td>
<td>0.25% Bromophenol blue 40% (w/v) sucrose in TAE</td>
<td>Buffer used to load DNA samples in gel electrophoresis</td>
</tr>
<tr>
<td>TAE Buffer</td>
<td>40mM Tris acetate pH 8.0 1mM EDTA</td>
<td>Buffer for making and running agarose gel for DNA separation</td>
</tr>
<tr>
<td>PBN</td>
<td>PBS 0.5% BSA 0.02% Na azide</td>
<td>Blocking buffer used in immunofluorescence experiments</td>
</tr>
<tr>
<td>Virus diluent</td>
<td>3% FCS (Bio Sera)</td>
<td>Resuspension of virus from</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th><strong>Poly-L-Lysine</strong></th>
<th>International) 0.1% Pen/Strep (Gibco) PBS</th>
<th>plaque picks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:20 dilution poly-L-Lysine in dH2O</td>
<td>Attach 293T cell to base of dish/slide for virus infection/immunofluorescence</td>
</tr>
</tbody>
</table>

#### Nebulisation Vehicle

| Drug delivery vehicle | 500mg Tween 20 100mg Span 20 4700mg Monosodium phosphate dihydrate 875mg Dibasic sodium phosphate anhydrous 2100mg Sodium chloride 500mL dH2O | Vehicle used to suspend drug compounds for nebulized delivery |

#### Culture Media

<table>
<thead>
<tr>
<th>LB (Luria-Bertani Broth)</th>
<th>1% Tryptone 0.5% Yeast extract 0.5% NaCl 0.1% Glucose dH2O</th>
<th>Growth of <em>E. Coli</em> for plasmid production</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOC</td>
<td>2% Tryptone 0.5% Yeast extract 10mM NaCl 2.5mM KCl 10mM MgCl₂ 10mM MgSO₄·7H₂O 20mM Glucose</td>
<td>Growth of <em>E. Coli</em> for plasmid selection</td>
</tr>
<tr>
<td>Serum-Free DMEM</td>
<td>Dulbecco’s modified Eagle’s medium (Life Technologies) 1% glutamine (200mM) 2% non-essential amino acids 1% penicillin-streptomycin (5000 IU/ml; 5000μl) Sodium Pyruvate</td>
<td>Cell culture media for virus infection/propagation.</td>
</tr>
<tr>
<td>10% DMEM</td>
<td>Dulbecco’s modified Eagle’s medium (Life)</td>
<td>Cell culture media for MDCK, 293T, A549 cell growth</td>
</tr>
<tr>
<td>Technology</td>
<td>Materials and Methods</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td>1% glutamine (200mM)</td>
<td>2% non-essential amino acids 1% penicillin-streptomycin (5000 IU/ml; 5000μl) Sodium Pyruvate 10% Foetal calf serum (FCS)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plaque assay overlay media</th>
<th>Media for MDCK cells for titration of viruses by plaque assay.</th>
</tr>
</thead>
<tbody>
<tr>
<td>50ml 10x EMEM 14ml 7.5% fraction V BSA 5ml 100x L-glutamine 10ml 7.5% NaHCO3 5ml 1M HEPES 2.5ml 1% dextran 5ml 10x penicillin/streptomycin dH2O up to 350ml Store in 17.5ml aliquots, add 7.5ml 2% oxoid agar/aliquot</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MucilAir culture media</th>
<th>Cell culture media for HAE cell growth.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum free, contains growth factors and Phenol Red.</td>
<td></td>
</tr>
</tbody>
</table>
8.2 Methods

8.2.1 Molecular Biology

8.2.1.1 Standard polymerase chain reaction conditions

PCR was performed with K.O.D. Taq polymerase (Novagen) or GoTaq® green master mix (Promega). For K.O.D. Taq polymerase PCR a 50μl PCR mix was made consisting of 5μl 10X Buffer and 3μl MgSO4 for KOD DNA Polymerase, 0.15μl (100pmol/μl) forward and reverse primers, 5μl dNTPs 2 mM, 1μl KOD polymerase (2.5U/μl), 10-100ng of DNA template and nuclease free water to 50μl. For Go-Taq polymerase PCR of bacterial colony screening, the following was used: 7.5μl 2X GoTaq® Green Master Mix, 0.25μM forward and reverse primers, bacteria swab and nuclease free water to 15μl. Cycling conditions were performed to the manufacturer’s guidelines and varied according to the construct length.

Table 17. Standard PCR thermal cycling conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>96°C</td>
<td>3 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>2</td>
<td>96°C</td>
<td>30 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>55°C</td>
<td>30 sec</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72°C</td>
<td>1 min/Kb</td>
<td>Elongation</td>
</tr>
<tr>
<td>5</td>
<td>Go to step 2, repeat 25 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>72°C</td>
<td>4 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>7</td>
<td>4°C</td>
<td>hold</td>
<td></td>
</tr>
</tbody>
</table>

8.2.1.2 Agarose gel electrophoresis

DNA fragments were separated in 0.2% agarose gels diluted with 0.5x TAE buffer and supplemented with 1μg/ml Gel Red (Cambridge Bioscience). Gels were submerged in TAE buffer (1X). 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.
8.2.1.3 DNA product purification

DNA fragments were excised from the agarose gel, and extracted using the SV Gel and PCR Clean-Up system (Promega) following manufacturer’s instructions. Similarly, PCR product or digestion products were purified using the same kit. DNA fragments were eluted with 35µl of warm (37°C) sterile water.

8.2.1.4 Reconstituting plasmids

Expression plasmids encoding for members of the IFN induction cascade and whose transcription was under the control of a pcDNA3 promotor were a kind gift from Professor Andrew Macdonald (University of Leeds). Plasmids were shipped embedded in filter paper and were reconstituted by soaking plasmid rich areas in 50µl TE buffer for 5min min at 37°C. Stocks were created by transforming competent E. coli cells (detailed below).

8.2.1.5 Transformation of competent bacterial cells

Approximately 50ng of plasmid were transformed into 50µl OneShot®TOP10 chemically competent E. coli (Invitrogen) and incubated on ice for 30 minutes. Cells were heat shocked for 30 seconds at 42°C and returned to ice for 2 minutes. 250µl of pre-warmed S.O.C. media (Invitrogen) was added to the mix and incubated for 1 hour at 37°C in a shaking incubator. After which, a suitable volume was spread on to pre- warmed LB agar plates containing 1% Ampicillin (or kanamycin if specified) and incubated overnight at 37°C to allow for colony formation. Colonies were screened by PCR, and grown overnight in 5ml LB supplemented with appropriate antibiotic (100µg/ml). For large-scale bacterial growth, 54ml of LB media containing appropriate antibiotic (50µg/ml final) were inoculated with 1ml of the bacterial suspension and incubated over night at 37°C at 225-250 rpm. An aliquot of the bacteria suspension (500ul) was mixed with sterile 80% glycerol and stored at -80°C. The remaining bacterial culture was centrifuged and the pellet stored at -80°C for later plasmid extraction and purification.
8.2.1.6 Small scale plasmid purification

Single bacterial colonies were picked from agar plates containing a selection antibiotic (ampicillin or kanamycin) and grown overnight in 5ml Lysogeny Broth (LB) containing the same antibiotic at 37°C, with shaking. 250µl of the bacteria suspension was mixed with 850µl of sterile glycerol 80% and stored at -80°C as a glycerol stock. The remaining bacteria culture was pelleted using a centrifuge at 3000xg for 5 minutes. The supernatant was discarded. 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 with 50µL of warm (37°C) sterile water in a 1.5ml Eppendorf tube. Plasmids were stored at -20°C to prevent DNA degradation.

8.2.1.7 Large scale plasmid purification

A 100µl sample from a glycerol stock or a single colony was inoculated into 250ml 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 were pelleted by centrifugation at 3000xg for 30 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 genomic DNA. The suspension was neutralised, precipitating contaminants, centrifugated at 3000xg for 5 minutes and the supernatant passed through a resin-containing column that bound 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.
8.2.1.8 DNA concentration determination

DNA concentrations were determined using a spectrophotometer (BioPhotometer, Eppendorf), with optical density measurements at 260nm with the assumption that 1 OD260 unit corresponds to 50μg/ml of double stranded (ds) DNA.

8.2.1.9 Sequencing of constructs

Constructs were sequenced using the MRC sequencing (MRC Clinical Sciences Centre, Hammersmith Campus, Du Cane Road, London, W12 0NN). Samples were subjected to automated fluorescent DNA sequencing using the ABI3730xl instrument. The reaction mixture was dispatched as a 10μl solution containing 3.2pmol primer, 400 – 600ng DNA and water.

8.2.1.10 Virus gene sequencing

All virus mutations were verified by sequencing the mutated region of the viral gene of successfully generated RG virus. qRT-PCR was carried out on viral RNA using QiAgen OneStep qRT-PCR kit (QiAGEN) according to the manufacturer’s instructions. The product was isolated using QiAquick Gel extraction kit (Qiagen). Chain termination PCR was performed using the BigDye Terminator x3.1 Cycle Sequencing Kit (Applied Biosystems).
8.2.2. Cell lines and transfection

8.2.2.1 Cell lines, media and maintenance

293T, DF-1, CALU-3, A599 and MDCK cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) with pyruvate and L-Glutamine (Invitrogen) supplemented with 1% penicillin and streptomycin (Gibco), and 10% (v/v) heat inactivated foetal Calf serum (Biosera). Cells were kept at 37°C in a 5% CO\textsubscript{2} atmosphere except DF-1 which were maintained at 39°C in a 5% CO\textsubscript{2} atmosphere. Cells were trypsinised using a PBS, 2X EDTA and cell trypsin mixture.

8.2.2.2 Freezing and thawing

Long-term frozen stocks of all immortalised cell lines were kept in liquid nitrogen. Frozen stocks were prepared from cells grown to a semi-confluent degree in T75 cm\textsuperscript{2} flasks. Cells were pelleted by centrifugation at 1,200 rpm for 5 minutes. Cell pellets were then re-suspended in freezing medium containing 30% foetal calf serum plus 20% DMSO in DMEM. Cells were immediately slow cooled at -80°C before being transferred to liquid nitrogen the following day.

Frozen vials of cells were thawed at 37°C for approximately 1 minute before being rapidly transferred to a T25 cm\textsuperscript{2} flask containing 5mls of pre-warmed media containing 1% penicillin and streptomycin, and 10% (v/v) heat inactivated foetal Calf serum and kept at 37°C in a 5% CO\textsubscript{2} atmosphere.

8.2.2.3 Human Airway Epithelium (HAE) Cultures

Highly differentiated, specific-pathogen free, human airway epithelium (HAE) cultures re-constituted from healthy donors are commercially available from Epithelix-Sàrl (Switzerland). Upon arrival culture well inserts, containing cells grown onto of a permeable mesh, were removed from their gelled shipping media. The apical surface of the cultures was washed 3X (100µl) with warmed SFM to remove excess culture-produced mucus. Cultures were maintained at 37°C, 5% CO\textsubscript{2}
subsequently washed with 300µl SFM once a week and maintained with 700µl MucilAir (Epithelix) which was refreshed on a bi-weekly basis. The liquid/air interface was preserved throughout both the maintenance and experimental phases.

8.2.2.3.1 Treatment of HAE Cultures with drug compounds

HAE cultures maintained at an ALI (as described above) were treated both apically and basally. Treatment was either initiated 3 days prior to infection or only on the day of infection (Figure 68). In either case, prior to apical application of the compound in question, the surface of the HAEs was washed with SFM (300µl) which was left on for 20min at 37°C and then removed. At this time, the well insert supporting the HAE culture was transferred to a new 24-well plate containing fresh, pre-warmed, MucilAir media (700µl) supplemented with the compound in question or vehicle control. If treatment occurred 3 days prior to infection, the basal media would be replaced with fresh media and drug on the day of infection (and left in place for the remainder of the experiment). SFM (200µl) containing the same compound or vehicle control was then added to the apical portion of the HAE culture and left in place for 2hrs at 37°C. After such time, the apical media was removed returning the culture to an ALI. If treatment of the HAEs commenced 3 days prior to infection, this process of apical treatment was repeated each day (including the day of infection after which time, apical treatment was stopped). If however, infection preceded infection, the apical section was treated with either the compound of interest or vehicle control one time, 1hr p.i. for 2hrs before being removed.
Figure 68: HAE experimental design. a) Timeline diagram of a typical HAE experiment involving pre-infection treatments. Indicated are the timings of apical and basal treatments as well as infection and sample harvesting. b) Schematic representation of an HAE cross-section indicating the location of the apical and basal media in relation to the cellular layer.

8.2.2.3.2 Infection of HAE Cultures with influenza virus

HAE cultures were infected via apical exposure to a virus diluted in SFM. Briefly, the apical surface of the culture was washed 1X, 300µl SFM, 20min incubation at 37°C in order to remove the mucus overlay. After washing, 200ul of diluted virus was added drop-wise to the surface of the culture and incubated at 37°C for 1hr. After which time, the inoculum was removed and the apical surface washed 1X with SFM and returned to 37°C, 5% CO₂. Cultures treated with drug compound or vehicle controls were either pre- or post-infection treated as described above.
8.2.2.3 Harvesting apical washes from HAE cultures

At desired time points, cytokines, chemokines and viral progeny were harvested from the surface of HAE cultures via apical washings. Briefly, 400µl of SFM was added to the apical section of the HAE cultures. After a 5min incubation at 37°C, the apical wash was collected, aliquoted and stored promptly at -80°C to await further analysis.

8.2.2.4 Isolation of PBMCs

Whole blood was collected from healthy donors and spun down (2000rpm, 30min) and washed with HI-FCS wash media. Peripheral-blood leucocytes were isolated via Ficoll-Paque density gradient (GE Healthcare). Monocytes were seeded onto 96-well round-bottom tissue culture plates (2x10^5 cells per well). Cultures were infected at a moi of 3 unless otherwise indicated. After 1hr of virus adsorption at 37°C, the virus inoculum was removed, and the cells were incubated in RPMI1640 supplemented with L-glutamine, HEPES 10ml, Na bicarbonate 5ml, penicillin/streptomycin 5ml and 10% foetal bovine serum 50ml). After 1hr of incubation, compounds of interest were added to the culture media and left in place for the duration of the experiment. At 24hr and 48hr post infection, cultures were centrifuged for 3min at 2000rpm, 4°C, overlying media was removed and total RNA extracted from the cellular pellet and used for cytokine/chemokine quantification via qRT-PCR.

**Wash Medium (HI-FCS)**

Add 0.4ml of Heat Inactivated Foetal Calf Serum (HI-FCS) and 0.2ml of L Glutamine to 20mls RPMI in a universal. Invert to mix. This mixture will last 7 calendar days if stored in the fridge (inclusive of the day it was made). This buffer must be brought to room temp before use.

**Media control** (for stimulation):

RPMI1640 with L-glutamine, HEPES 10ml, Na bicarbonate 5ml, penicillin/streptomycin 5ml and 10% foetal bovine serum 50ml)
8.2.2.5 Transfections

Transfections were performed either on sub-confluent monolayers of cells plated 24 hours beforehand or conducted in suspension.

**Monolayer Transfections**

Conducted in 24-well cell culture plate, 1.5μl of Lipofectamine 2000 transfection reagent (Invitrogen) was mixed with 250μl of Optimem (Invitrogen). Separately, plasmid DNA (typically 0.5μg) was mixed with 250μl of Optimem, before the two mixtures were combined and incubated at room temperature for 20 minutes. Renilla (0.04μg/well) acted as a transfection control. The transfection mixture was then added drop-wise to the monolayer. Cell culture media was replaced 3hrs post transfection. Quantities were increased or decreased as appropriate for larger or smaller scale transfections.

**Transfection in Suspension**

An alternative to monolayer transfections is the transfection of cell cultures while in suspension. 24-well culture plates were pre-treated for 15min with Poly-L-Lysine (Sigma) diluted 1:20 in dH2O and then washed with PBS. During the Poly-L-Lysine treatment, a confluent T75 flask of 293T cells was trypsinised until all the cells had disassociated from the flask. Trypsin was removed from the cells via centrifugation at 1000rpm for 5min. The cellular pellet was re-suspended in 25mls with 5% DMEM (without the addition of PenStrep). Of this suspension, 3.75ml of were set-aside for each treatment group to be run in triplicate wells.

Expression and/or reporter plasmids (0.375μg each/set of 3-wells) and 0.125μg of Renilla transfection control were added to 31.25μl Optimem and incubated at room temperature for 5min. To a second aliquot of 31.25μl Optimem, 1.5μl of lipofectamine was added and also incubated at room temperature for 5min. Post incubation, the two aliquots containing the plasmids and lipofectamine were combined, gently mixed and incubated at room temperature for 15min.
Post incubation, the combined transfection mix was added to the 3.75ml cell suspension aliquots and any compound(s) of interest could be added to the cell/transfection mix at this point. The cell/compound/transfection mix was gently vortexed to ensure adequate distribution of all components and aliquoted out (0.5ml/well). Cultures were incubated at 37°C, 5% CO₂.

8.2.2.6 Minigenome assay

To assess any effect of the drug compounds on viral polymerase activity, cells were transfected in 12-well plates with pCAGGS expression plasmids encoding PB1 (0.16μg), PB2 (0.16μg), PA (0.04μg) and NP (0.32μg) proteins per well, together with 0.16μg of the mini-genome firefly reporter plasmid (pHuman-Poll-Firefly or pChicken-Poll-Firefly were employed in confluent human 293T or avian DF-1 cells, respectively). When DF1 cells were used, twice the normal amount of DNA was transfected. Transfected were conducted with the aid of Lipofectamine 3000 (Invitrogen) and OptiMEM media (Gibco) and cultures were incubated at 37°C for three hours. After the initial three hour incubation, overlaying culture media was removed and replaced with 1ml 10% DMEM (Gibco) supplemented with either drug or vehicle and returned to 37°C. 24hrs post-incubation, media was removed and cells lysed with 300μl passive lysis buffer (Promega) per well. Luciferase signal measured using a FLUOstar Omega plate reader (BMG Labtech).

8.2.2.7 Firefly luciferase activity assay

To measure firefly activity, 24 hours after transfection, cells were lysed with 300μl (12-well) or 200μl (24-well) of passive lysis buffer (Promega), and firefly luciferase activity measured using Luciferase Assay System reagent (Promega) on a FLUOstar Omega plate reader (BMG Labtech).
8.2.2.8 IFN Luciferase assays

Cells transiently or stably expressing IFN luc reporter construct were infected with influenza virus at MOI 2 (unless otherwise stated) in SF DMEM. After 1 hour incubation at 34°C or 37°C the media was removed, cells were washed with PBS, and the media replaced with 2% DMEM. After 8 hours or appropriate incubation cells were lysed in CCLR for 5 minutes, centrifuged 1,000 RPM for 1 min, supernatants were transferred to clean Eppendorf and stored -20°C. Luciferase was measured using a luminometer (BMG Labtech).

8.2.2.9 IFN-β luciferase reporter assay

To measure the ability of NS1 protein to inhibit IFN-β induction, 0.375μg pCAGGS-NS1, 0.25μg human-IFN-β luciferase reporter and 0.04μg/well Renilla plasmid DNA was transfected into 293T cells in 12-well format. After 24 hours, stimulation of IFN induction by influenza virus infection (MOI 3.0), Poly(I:C) or NDV was conducted. Cells were incubated at 37°C and harvested after a further 24 hours.

8.2.2.10 BCA Assay for protein content

The Thermo Scientific Pierce BCA (bicinchoninic acid) Protein Assay was used as per manufactures instructions to quantify the protein levels in the cell lysates generated with RIPA buffer prior to western blot analysis. The microtitre plate methodology was used, briefly the lysates were microfuged; the samples were then prepared in PBS (1:50 and 1:100 dilutions) alongside protein standards provided by the kit. 1:50 ration of A & B solutions were made up, added to the plate and incubated at 37°C for 30 minutes. Then the absorbance was read at 562nm on a FLUOstar Omega plate reader (BMG Labtech).
8.2.2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Commercially available SDS-PAGE gels were used to separate proteins according to size (Ready gel Tris HCl 4-15% linear gradient, BioRad). Samples were diluted in 6X gel loading dye (375 mM Tris-HCl pH 6.8, 6% SDS, 30% glycerol, 9% 2-Mercaptoethanol, and 0.03% bromophenol blue). Before loading, samples were heated at 95°C for 10 minutes. Gels were run at 100V for approximately 2 hours in running buffer (0.1% SDS, 27.6 mM Tris base, 0.2 M glycine, pH 8.8). Protein sizes were determined by comparison to the Novex sharp pre-stained protein standard (Invitrogen).

8.2.2.12 Immunoblot analysis

Proteins were electrically transferred onto polyvinylidene difluoride (PVDF, GE Healthcare Life Sciences) membranes. Transfers were carried out via the semi-dry method (~30min at 15V). Following transfer, membranes were blocked in a 5% milk solution (milk powder, 0.1% tween, dissolved in PBS) on a shaker for 1 hour at room temperature and then washed with three times for five min each with wash buffer (0.1% tween in PBS). Membranes were then incubated with the primary antibody, diluted in milk solution, overnight at 4°C. The next day, the antibody solution was removed and the membrane was washed with wash buffer twice for 5 minutes each. Following the washes, membranes were then incubated for 1 hour at room temperature with horseradish peroxidise (HRP) conjugated secondary antibodies for detection by enhanced chemiluminescence (ECL). Subsequently, blots were washed 4 times for 10 minutes in wash buffer, as described above. HRP-conjugated antibodies were detected by incubation of the membrane for 1 minute with LumiGLO Western Blotting Detection System (Cell Signaling) as manufacturer’s instructions. Films (GE Healthcare Life Sciences) were exposed for required times and developed on a RGII Fuji x-ray film processor.
8.2.3 Toxicity Assays

8.2.3.1 Cell viability assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is based on an enzymatic reaction of the mitochondrial succinic dehydrogenase. In viable and proliferating cells these enzyme cleaves the tetrazolium rings of the pale yellow MTT which results in formation of dark blue formazan that is largely impermeable to cell membranes and therefore accumulates in healthy cells. The amount of formed formazan can be measured in a colorimetric assay at OD 562 nm and is directly proportional to the number of viable cells.

8.2.3.2 Measurement of caspase activity

Activities of caspase 3/7, caspase 8 and caspase 9 were measured using commercial available Caspase-Glo kits (Promega), according to the manufacturer’s protocol. Influenza A/FPV/ Bratislava/79 (H7N7) infected cells were incubated for 30 min with reaction buffer (Promega) and luminescence was measured using the FLUOstar Omega plate reader (BMG Labtech). For each sample two biological replicates were analysed.

8.2.3.3 Trypan blue exclusion test of cell viability

MDCK or A549 cells were grown on 96 well plates to a confluency of ~80% at which point, the drug compound or the vehicle control were added to the overlying media and the cultures incubated for a further 24hrs. Following this incubation, the media was removed and the cells trypsinised. An aliquot of this cell suspension containing 5x10^5 cells/ml was taken and centrifuged for 5min at 100xg and the supernatant discarded. The cellular pellet was resuspended in 1ml SFM. The resuspended cells were then mixed 1:1 with a 0.4% trypan blue solution and incubated for 3min at room temperature. Cells were counted within 5min of the addition of the trypan blue solution with the aid of a haemacytometer.
8.2.4 Infectious studies

8.2.4.1 In vitro

8.2.4.1.1 Safety & Biosecurity considerations

All infectious experiments involving A/H5N1:PR8 6:2 reassortment virus were performed in Biosafety Level 3 facilities at Imperial College London, St Mary’s Campus. All other infectious experiments were conducted at BSL-2.

8.2.4.1.2 Growth of virus stocks

MDCK cells grown to confluency in 75 cm$^2$ flasks were infected with virus at a low multiplicity of infection (MOI) 0.001 in a low volume of serum free media for one hour at 37°C. On removal of the initial inoculum, 12mls of serum free DMEM and 1μg/ml of trypsin (Worthington) were added to the flask which was then incubated until virus cytopathic effect (CPE) was observed. The virus was harvested by removal and centrifugation of the supernatant at 2500 RPM for 10 minutes to pellet cell debris. After confirmation of viral presence by haemagglutination assay and assessment of quantity through plaque assay, the virus was stored in aliquots at -80°C.

8.2.4.1.3 Co-culture virus rescue

Reassortment or wild-type viruses were “rescued” by means of a reverse genetics system. Briefly, 12 plasmids (8 pPol1 plasmids and 4 polymerase protein expression plasmids) were transfected into 293T cells (confluency ~70-80%) seeded in 12-well plates. 20μl Fugene HD (Roche) was added to 200μl Serum Free (SF) DMEM + NEAAs, lacking antibiotics and incubated for 5 minutes at room temperature. The DNA mix (Table 18) was then added and incubated with the Fugene mix for a further 15 minutes at room temperature, before being added, drop-wise, to the 293T monolayer. The transfections were incubated overnight at 37°C. The next day, a confluent layer of MDCK cells were trypsinised and
resuspended in 20ml DMEM + 10% FCS + pyruvate/75cm2 flask. The transfected 293T cells were washed gently in PBS and detached by forcefully pipetting 1 ml of the MDCK cell suspension into the 293T well. After the cells were dislodged 4 additional mls of the MDCK suspension was added and this mix was transferred to a 25cm2 flask. These were allowed to adhere for 6 hours at 37°C. Cells were gently washed in serum-free DMEM, to remove any FCS, and then 5 ml serum-free DMEM + NEAA’s, with 2.5μg/ml porcine trypsin was added. Cultures were incubated at 37°C for at least 3-6 days until cytopathic effect was observed, or haemagglutination activity was observed by a Haemagglutination assay. Virus rescues were harvested from the supernatant and cell debris was removed by centrifugation at 3000rpm for 10 minutes. The resulting aliquots were stored at -80°C.

<table>
<thead>
<tr>
<th>vRNA Plasmids</th>
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<tbody>
<tr>
<td>pPolI PB1</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI PB2</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI PA</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI HA</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI NP</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI NA</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI M</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pPolI NS</td>
<td>0.5 μg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Helper Plasmids</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>pCMV PB1</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pCMV PB2</td>
<td>0.5 μg</td>
</tr>
<tr>
<td>pCMV PA</td>
<td>&lt;0.05μg</td>
</tr>
<tr>
<td>pCMV NP</td>
<td>1 μg</td>
</tr>
</tbody>
</table>

**Table 18. Quantities of plasmids used in the rescue of influenza viruses.** The eight pPolI plasmids correspond to the eight vRNA virus segments and the four helper plasmids are added to provide the polymerase complex to initiate virus replication. Using this system, pPol’s with genetic modifications could be switched in to create a new virus possessing the desired characteristic.
8.2.4.1.4 Haemagglutination (HA) assay

Viruses were diluted in a two-fold serial dilution using PBS in 96 well V-bottomed plates to a total volume of 50μl (PBS alone acted as a negative control). Next, an equal volume of 0.5% chicken blood cell suspension (diluted in PBS) was added to each well. Plates were then incubated on ice for a minimum of 1 hours time. The resulting HA titre was defined in this case as the well prior to the first well displaying a blood pellet.

8.2.4.1.5 Influenza virus titration by plaque assay

12-well plates were seeded with MDCKs split 1:4 in order to form a confluent monolayer to infect the next day. Media was removed, and the cells washed in PBS. Viruses were diluted in SF DMEM in a 10-fold dilution series. 200μl of each diluted virus was added to each well and the plate incubated for 1 hour at 37°C. After incubation, 1ml of 2% agarose plus flu overlay (0.7x EMEM, 0.303 % BSA (fraction V), L-Glutamine, 0.2% NaHCO2, 0.7M HEPES, 0.7x Pen/Strep (Gibco), 0.007%, dextran DEAE (Sigma), containing 0.4μl/ml of flu trypsin, was added to each well and allowed to solidify. After 4-5 days incubation at 37°C, the overlay was removed and the cells fixed and stained with Crystal Violet (CV) stain for at least 20 minutes. The excess CV stain was then removed and cells washed with water 3X before counting the plaques.

8.2.4.1.6 In vitro growth kinetics

6-well plates were seeded with cells of interest split 1:4 in order to form a confluent monolayer to infect the next day. Media was removed, and the cells washed in PBS. Virus was diluted to an moi of 0.001 in 1mL of SFM. The virus/SFM mix was added to the cell monolayer and incubated for 1hr at 37°C, 5% CO2. After incubation, the inoculum was removed and the cells washed with PBS and then 2mL SFM was added to each well. A baseline sample (1mL) was then taken and placed in -80°C. Subsequent time points were taken as required and samples stored in -80°C until needed. Titers were determined via plaque assay.
8.2.4.1.7 *In Vitro* p38 Kinase Assay

The effect of RV1088 on the activity of p38 MAP kinase was analysed by a commercially available kit (p38 MAP Kinase Assay Kit, New England Biolabs Inc.). The kit employs two different antibodies, anti-p38 MAP kinase antibody which is specific for p38 MAP kinase and does not cross-react with ERK1/2 or JNK, and antiphosphospecific ATF-2 antibody to detect p38 MAP kinase-induced phosphorylation of ATF-2. Analysis of activity of p38 MAP kinase was performed according to the manufacturer’s instruction.

Briefly, HAEs that had been previously treated with RV1088 (or DMSO control) and infected with influenza virus were washed with ice-cold PBS and lysed with 1X cell lysis buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF) for 5 min on ice. The cellular solution was then transferred to an eppendorf for further disruption via sonication. After sonication, the cell lysate was microcentrifuged for 10 min at 4°C. 200 μL of cell lysate was incubated with anti-p38 MAP kinase antibody (1:100 dilution) to selectively immunoprecipitate p38 MAP kinase from cell lysates at 4°C overnight with gentle shaking. The resulting immunoprecipitate was then mixed with protein A sepharose beads. After microcentrifugation, the pellet was washed twice with lysis buffer and then washed twice with kinase buffer (25 mmol/L TRIS pH 7.5, 5 mmol/L β-glycerolphosphate, 2 mmol/L DTT, 0.1 mmol/L Na3VO4, 10 mmol/L MgCl2). The pellet was suspended in 50 μL of kinase buffer with 100 μmol/L ATP and 2 μg of ATF-2 fusion protein, and then incubated for 30 min at 30°C. The pellet was mixed with the sample buffer consisting of 62.5 mmol/L TRIS base, 10% glycerol, 50 mmol/L DTT and 2% SDS to terminate the reaction, and then heated 95°C for 5 min to fully denature the protein prior to Western Immunoblotting.

8.2.4.2 *In vivo* infection studies

8.2.4.2.1 Ethics statement

The animal experiments conducted under the remit of this study were evaluated and approved by the Imperial College’s ethics committee for animal studies and was carried out in accordance with the UK 1986 Animal Scientific Procedure Act. In all *in vivo* experiments, the animals health and wellbeing were routinely monitored. Any animal falling below preset cut-off points (e.g. falling below our established 20% weight loss cut off point) were
killed humanely.

8.2.4.2.2 Experiments Involving Mice

All in vivo mouse experiments employed BALB/c females aged between 6-8 weeks and were certified as specific pathogen free. Subjects were housed in Isocages in groups of 5. Individuals within each group were distinguished on the basis of ear markings. Treatment with a compound of interest or infection with the influenza virus was conducted via either intranasal instalation or exposure to an aerosol cloud (for all nebuliser-related protocols, please refer to section 8.2.6).

8.2.4.2.2.1 Pre-infection Treatment

The pre-infection treatment of subjects with either a compound of interest or DMSO vehicle control was undertaken three days (inclusive) prior to infection. If conducted via the intranasal route of administration, subjects were first anesthetized via inhaled isoflurane. As mice are obligate nose breathers, small volumes (adding up to a total volume of 40μl/day) of the treatment solution placed on the nares is readily taken up by without any complications.

8.2.4.2.2.2 Viral Inoculation

Intranasal infection was achieved in a manner similar to that described for the administration of pre-infection treatments. Briefly, subjects were anesthetized with inhaled isoflurane and inoculated by means of drop-wise placement of the viral containing solution on the subject’s nares. For the experiments described in this work, virus employed was A/England/195/2009 (A/H1N1) at a titer of 2x10^5 pfu/ml in a total volume of 40μl/subject.

8.2.4.2.2.3 Health Monitoring

Subjects were monitored daily for any signs of deterioration in their health status. In addition, daily weights were obtained and compared to their individual starting (i.e. pre-
experimental) weight. If a subject lost 20% or more of their starting weight, they were determined to have reached an experimental severity limit and culled via Schedule one on the basis of humanitarian grounds.

8.2.4.2.2.4 Necropsy and Tissue Processing

On set days post-infection, individual subjects from each treatment group were culled via Schedule one. Within minutes of death, the subjects lungs were removed and either frozen down (-80°C) for cytokine/chemokine and viral quantification or placed in formalin for later histopathological analysis.

8.2.4.2.2.5 Lung Homogenisation

In order to quantify the cytokine/chemokine and viral load found within a subjects lungs, whole lungs were weighed, placed in 500μl PBS and homogenised (“Minilys”, Bertin Tech). From this homogenate, ELISAs, LUMINEX and plaque assays were conducted. All results were subsequently normalised to the subject’s lung weight.

8.2.4.2.2.6 Immunoflouresence & Imaging

Paraffinised tissue sections mounted on glass slides (processed by ProPath, UK) were deparaffinised by immersion in Neoclear for 5 min followed by immersion is 100% ethanol for 3 min (X2), 90% ethanol for 3min (X2) and 70% ethanol for 10min. Slides were then placed under slow flowing cool tap water for 10 min. Blocking was conducted at room temperature in a humidity chamber with 3% BSA in PBS plus 0.05% Tween 20 (PBST) overnight. The next day, slides were rinsed in briefly with PBST, followed by the addition of the primary antibody (in 1% BSA in PBST) and incubated in the humidity chamber overnight at 4°C. Slides were then washed 3X with PBST for 15 min each. The secondary antibody antibody (in 1% BSA in PBST) was added and the slides incubated in the humidity chamber for 1hr at room temperature. Washing was repeated as before and slides permealised with movicol and viewed after allowing time to set.
8.2.4.2.3 Experiments involving ferrets

Weight matched female ferrets aged between 16 and 21 weeks were employed in this study and were housed in a specially equipped ferret room to ensure adequate comfort with environment enrichments.

8.2.4.2.3.1 Pre-infection Treatment

Treatment with RV1088 began one day before infection and continued daily throughout the infection until day 10 p.i. Daily doses of RV1088 (2mg/kg body weight) suspended in the vehicle or placebo (vehicle alone) was instilled into the nose under light anaesthesia (isofluorane). Total installation volume was 50μl per day with dosing occurring at the same time each day.

8.2.4.2.3.2 Viral Inoculation

Subjects were inoculated intranasally in duplicate with 4x10^5 pfu of virus in PBS. Inoculations were performed under general anesthesia (ketamine 22 mg/kg/xylazine 0.9 mg/kg). Post-inoculation, subjects were returned to their cages and placed in the recovery position and monitored until full consciousness returned.

8.2.4.2.3.3 Health Monitoring

Subjects were monitored daily for any adverse signs or symptoms resulting from either treatment or infection. In addition, daily weights were recorded and any animal which lost 20% or more of its pre-experimental weight was culled via a Schedule one method on humanitarian grounds.
8.2.4.2.3.4 Nasal Washing

Daily nasal washes (1ml PBS in total divided equally between nares) was performed on non-anesthetised subjects. The recovered PBS (~800μl) was kept on ice and tested daily for vial proteins using a BD directigen™ xA&B kits (BD Diagnostic systems) and subjected to a plaque assay to assess viral titre.

8.2.5 Cytokine and chemokine quantification

8.2.5.1 Cytokine Assay/Luminex System

Culture supernatants or lung homogenate, collected at a designated time points, were assayed for an array of cytokines/chemokines by using a Luminex xMAP multiplexing technology employing a magnetic bead panel according to the manufacturer’s instructions (Milliplex Map Kit - Millipore). The assay plate was analyzed with the MAGPIX instrument and the results complied using xPONENT software (both by Luminex Corporation).

8.2.5.2 ELISA

Samples of viral culture supernatant or lung homogenate were irradiated with ultraviolet light (CL-100 Ultra Violet Cross linker) for 15min to inactivate any infectious virus before the ELISA assays were performed. The assays themselves, were conducted with R&D System’s “Quantikine” kit and conducted following manufacturer’s instructions.

8.2.5.3 RNA extraction and qRT-PCR

Total cellular RNA from cell cultures or lung homogenate was extracted using TRIzol (Invitrogen) or via RNeasy Mini kit (Qiagen) according to their respective manufacturers instructions. In terms of the TRIzol method, cells were washed with PBS, lysed in 1 ml TRIzol, transferred to an RNase free Eppendorf. Following addition of 200μl of chloroform, the protocol is the same as for TRIzol LS RNA extraction. The pellet was air dried for 10 minutes and dissolved in 50-100μl nuclease free water. RNA was quantified by spectrophotometry by measurement of absorbance at 260nm. Concentration (μg/ml) = A260 X e where for RNA
e = 44.19. mRNA was combined with poly(dT) primers and Superscript II reverse transcriptase (Life Technologies) in order to synthesis cDNA. The process was quantified by real-time PCR analysis with a LightCycler (Roche). The mRNA for various cytokines/chemokines, viral M-gene and 18s were quantitated using real-time RT-PCR.

8.2.6 Nebuliser

8.2.6.1 System overview

Our nebuliser system is a closed system which operates based on a push/pull technique where in the aerosol could is simultaneously pushed through the system by a current of air (10L/min) and drawn out by a separate pump (Bias Flow Generator, Info Disp) at the same flow rate. The aerosol cloud (whether viral or pharmacological) is produced by the Aroneb nebuliser (Aerogen). The nubulant is first introduced in to the nebuliser chamber where the cloud is allowed to form a uniform density before flowing via tubing into the exposure chamber. All tubing was replaced between each aerosolisation event to reduce incidences of condensation. Designed by EMMS (Haunts, UK), the exposure chamber is composed of an inner chamber that ensures an equal flow and distribution of the cloud to an outer chamber. This outer chamber contains a series of six equally spaced ports to which sampling equipment (discussed further below) can be attached or to which animal exposure tubes (EMMS) can be affixed. Leading out of the exposure chamber was tubing which conducted any non-inhaled nebulant cloud through a series of Virkon disinfectant traps and filters before finally passing through a series of large capacity indicating silica drying columns (EMMS) columns to dry the air prior to entering the “pulling” pump.

8.2.6.2 Nebulisation of virus and drug compounds

Viral stocks were diluted to the required concentration in PBS, vortexed for 2min on medium speed and added directly to the nebuliser unit. On the day that nebulisation was due to occur, powdered drug suspensions were freshly prepared. Compounds were diluted/suspended by vortexing (4min, max speed) in a specialised vehicle (Table 16) to enable efficient nebulisation and distribution throughout the system. After each
nebulisation event, the nebuliser unit was thoroughly washed three times with PBS in accordance with manufacturer’s specifications.

8.2.6.3 Sampling of the nebulant cloud

Employing the equation discussed in Chapter 6, we calculated the number of active viral particles (PFU) suspended in the aerosol cloud with the aid of a midget impinger (Sigma). The impinger contained 10mls of PBS and was connected directly to the nebulisers exposure chamber (i.e. where an animal subject would be exposed to the aerosol cloud). Downstream of the impinger was a small programmable pump (model 210-1002MTX, SKC, Dorset) that was set to mimic the normal respiratory rate of an average female BALB/c mouse (volumetric flow set to = 0.6L/min) drawing the aerosol cloud through the PBS for 10min thus trapping any aerosolised virus in the PBS. The trapped virus was later titered via plaque assay.

8.2.6.4 Subject exposure to nebulant cloud

Each individual exposure tube is capable of holding and securing a single mouse in a nose-first orientation via a lockable crush-bar. This design ensures that only the test subject’s nose is directly exposed to the nebulant cloud; thus avoiding unwanted secondary exposure to the subjects eyes and fur. Animals were placed in the tubes easily without the use of anaesthetics and were exposed to a specific nebulant cloud for a period of 10min. After which time, subjects were returned to their cages with no apparent ill effects.

8.2.6.5 Biohazard & Safety considerations

Prior to employing any infectious agents, the systems seals were tested at maximum pressure and flow with PBS. In all experiments, any part of the system considered to be potentially “dirty” was kept in a biological safety cabinet to minimise the risk of accidental exposure or leakage. All air flowing from “dirty” to “clean” components was first passed through a series of Vikon traps and filters. Once experiments were complete and while still in the hood, the system was disassembled and soaked in Virkon for 45min prior to removal.
The virus employed in these experiments was susceptible to Oseltamivir treatment and was covered by that year’s seasonal vaccine (user was vaccinated).

8.2.7 Bioinformatics analysis

8.2.7.1 Sequence analysis

All influenza sequences were downloaded from the NCBI Influenza Virus Resource and aligned using the Clustal Omega EBI resource. Sequence alignments were analysed using Geneious R6 software.

8.2.7.2 Synergistic analysis

The presence or absence of synergy was determined by means of the combination index (CI) theorem of Chou-Talalay. This test used the quantitative definitions of antagonism (CI > 1), additive effect (CI=1), and synergistic effect (CI < 1) in drug combination. Calculations were made with the aid of the CalcuSyn software program (Biosoft - Cambridge, UK) which employs the Chou-Talalay theorem equations.

8.2.7.3 Statistical analysis

All statistical analyses were performed using Prism 6 software (GraphPad). Statistical calculations included: two-tailed paired t-test, two-way ANOVA with Bonferroni’s post-test, one-way ANOVA and Area Under the Curve (AUC) analysis. P-values less than 0.05 were considered significant.
Chapter 9. References


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