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CHARACTERISATION OF A NOVEL FAMILY OF PEPTIDES WITH POTENT ANTI-VIRAL ACTIVITY AGAINST INFLUENZA A VIRUSES

Seema Naseralla Jasim

Dissertation submitted for the degree of Doctor of Philosophy
ACKNOWLEDGEMENTS

This work was not possible without the guidance of my primary supervisors: Professors Mark Stevens and Paul Digard. I would like to thank them for providing me with the opportunity to learn and for their continued encouragement and support throughout my project and as I begin the next scientific adventure. I have been lucky in my studies to have had two additional supervisors; Professor Anthony Nash and Dr Lonneke Vervelde.

A huge thank you is owed to Professor Colin Farquharson, Dr Jo Stevens and Dr Tali Pechenick Jowers for mopping up my tears and sending me back to the lab.

I would like to thank members of the Flu lab, Drs Helen Wise, Saira Hussain Nikki Smith, Lita Murphy, Liliane Chung and for their continued guidance, support and friendship. A particular thank you to the PhD students Matty Turntable and Anabel Clements. During my studies I was lucky to learn tricks from three research groups, the Stevens’ lab: Dr Robin Cassady-Cain, Dr Prerna Vohra, Christina Vrettou and members of ‘MMBP’: Chas Vander-Broek, Hamimah Zainal-Abidin and Nina Jiprasutwit. As Brett wrote in his acknowledgements ‘All accomplishments are in some way shared and none more than the work presented here’.

‘A good predictor of workplace productivity is quality of friendship in the workplace’ – I read that somewhere. The article showed (with some stats) that employees with a best friend at work tended to be more focused, passionate, loyal, pulled fewer sickies and…had fewer accidents (?). In the case of my PhD studies this was true. Thank you, Becca Dewar, Kate Harrison and Cosmin Chintoan-Uta, for bringing joy to my workday and my sister Alaa for her endless supply of puns, tea and support.

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Finally my family, mum Iman, dad Naser and brother Ahmad, their continued support has given me the confidence to complete this project.
DECLARATION

I declare that this thesis entitled ‘Characterisation of a novel family of peptides with potent anti-viral activity against influenza viruses’ represents my work. This work has been generated solely by myself as the result of my original research and has not been submitted for any other degree or professional qualification. All the sources of information have been acknowledged.

Seema Naseralla Jasim
Avian influenza viruses (bird flu) can cause infectious disease in domesticated poultry and persist naturally in wild birds. Domesticated poultry with influenza display a wide spectrum of clinical signs that range from asymptomatic carriage or mild illness (low-pathogenicity avian influenza) to fatal disease, with rapid spread of virus and high mortality (high-pathogenicity avian influenza). Often influenza leads to large outbreaks in domesticated poultry, which places pressure on global food supplies and economies. There are a number of measures used to control influenza that include disinfection, surveillance, vaccination, closure of live markets and mass culling in the event of an outbreak. However, despite implementing these measures outbreaks continue. Considering the uncertainty over whether strains of avian influenza will adapt to infect and transmit in humans and the limitations of how their spread may be controlled, this study investigated the use of two families of anti-viral peptides (small proteins), ‘FluPep’ and ‘Entry Blocker’ for protection against low-pathogenicity avian influenza viruses.

Assays quantifying influenza replication showed that both peptide families were active in a dose-dependent manner against a number of different virus strains. Furthermore, different strains of influenza displayed different sensitivities to FluPep but not Entry Blocker. Influenza viruses contain two surface proteins called haemagglutinin (HA) and neuraminidase (NA), which allow the virus to attach to cells and allow new virus progeny to leave the cell and spread. In order to examine whether the peptides stopped viral replication by interacting with one of these proteins, the HA and NA proteins of a relatively resistant virus were swapped with those of a relatively sensitive strain. Resistant strains were made more susceptible by the swap suggesting that the peptides acted on these proteins. To further dissect their mode of action a number of experiments were designed to investigate the stage(s) within the viral life cycle at which the peptides are active. Results indicated that the peptides acted early during the virus replication cycle causing the virus to clump together in aggregates on the surface of the cells and as a result interfere with their entry.

Further work evaluated how the peptides may be delivered to the avian intestine, a key site of viral replication and shedding. Attempts were made to engineer beneficial gut bacteria (Lactobacillus) to produce and secrete these peptides, with the aim of designing a cheap and easy to administer protective agent. However, combining the elements required to drive the production of the peptides in the bacteria proved challenging, possibly due to toxicity. Nonetheless, this study identified FluPep as a potent inhibitor influenza replication that acts early in the virus life cycle.
ABSTRACT

Avian influenza viruses can cause devastating outbreaks in domesticated poultry, with rapid transmission of virus between birds and high mortality. Current measures for control of influenza involve surveillance, closure of live poultry markets and mass culling. However, despite implementing these measures, outbreaks continue. Considering the uncertainty over whether certain strains of avian influenza viruses will adapt to human transmission and limitations over how their spread may be controlled, this study considers the use of anti-viral peptides as protective agents against low pathogenicity avian influenza (LPAI) virus.

This study investigated the activity and mode of action of two cell-penetrating anti-viral peptide families against influenza A virus infection: ‘FluPep’ (a family of short, hydrophobic peptides related to suppressor of cytokine signaling-1 proteins) and ‘Entry Blocker’ (derived from the signal sequence of fibroblast growth factor-4). Plaque reduction assays demonstrated dose-dependent anti-viral activity of both peptide families against a panel of influenza viruses with diverse haemagglutinin subtypes. Determination of IC₅₀ values showed strain-specific differences in sensitivity to FluPep but not Entry Blocker. The IC₅₀ of FluPep 4 for A/PR/8/34 was reduced by reassorting in the HA and NA from a relatively sensitive avian strain using a reverse genetics approach, suggesting inhibitory effects on the viral glycoproteins. Accordingly, viral entry assays focusing on binding, internalisation, fusion and import were designed and optimised to dissect the mechanism(s) of action of the peptides. Results indicated that the peptides acted upstream of nuclear import of viral ribonucleoprotein complexes but did not reduce overall virus binding to cells. However, the peptides caused aggregation of the virus particles on the surface of the host cells and reduced their internalisation.

Further work evaluated how the peptides may be delivered to the site(s) of viral replication in poultry. A screen of the current literature was completed to allow for the design of an expression cassette for poultry-derived *Lactobacillus* to express FluPep and Entry Blocker. Though the cassette has been reported to be suitable for expression of heterologous proteins in *Lactobacilli*, rescue of recombinants for expression of anti-viral peptides or a reporter protein proved challenging, possibly owing to toxicity. A stable construct for the expression of FluPep 4 in *Lactobacillus* was obtained but culture supernatant did not inhibit virus replication.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CC50</td>
<td>50% cytotoxicity concentration</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HA0</td>
<td>Precursor uncleaved haemagglutinin</td>
</tr>
<tr>
<td>HA1 and HA2</td>
<td>Cleaved haemagglutinin</td>
</tr>
<tr>
<td>HPAI</td>
<td>High pathogenicity avian influenza</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>IC50</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenicity avian influenza</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix protein</td>
</tr>
<tr>
<td>M2</td>
<td>M2 ion channel</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-structural protein 1</td>
</tr>
<tr>
<td>NS2</td>
<td>Non-structural protein 2</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase basic protein 1</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase basic protein 2</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pfu</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>p.i</td>
<td>Post infection</td>
</tr>
<tr>
<td>PRA</td>
<td>Plaque reduction assay</td>
</tr>
<tr>
<td>RG</td>
<td>Reverse genetics</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>SA</td>
<td>Sialic acid</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free media</td>
</tr>
<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
Influenza viruses belong to the *Orthomyxoviridae* family and possess a segmented single-stranded negative sense RNA genome, which is contained within a lipid envelope (Figure 1.1a). Influenza A virus is often described as pleomorphic; as viruses isolated from fresh clinical samples can contain both spherical particles (c.100nm) and filamentous virus that can reach over 20 μM in size. Egg-grown influenza viruses however are typically spherical in shape (Chu et al., 1949; Roberts and Compans, 1998) (Figure 1.1 d&e). The eight genomic segments of influenza A virus encode up to 18 known proteins (listed in Table 1.1). Influenza A viruses obtain a lipid envelope from the host cell plasma membrane and contained within this are the viral glycoproteins haemagglutinin (HA) and neuraminidase (NA) in a ratio of approximately 4:1. Currently, 16 haemagglutinin and 9 neuraminidase antigenically distinct subtypes have been isolated from within the avian reservoir, mostly from waterfowl species. In addition, H17N10 (Tong et al., 2012) and H18N11 viruses (Tong et al., 2013) have recently been identified in bats. Despite the nomenclature these haemagglutinin subtypes do not haemagglutinate and the corresponding neuraminidase subtypes have no neuraminidase activity (Wu et al., 2014).

HA forms a homotrimer and mediates viral attachment to host cell sialic acid receptors and promotes viral fusion within the endosomal membrane during entry. HA is synthesised as an HA0 precursor. Outside the cell, prior to entry, HA0 undergoes post-translational cleavage by host proteases into HA1 and HA2. Exposure of the hydrophobic amino acid terminus of HA2 then mediates fusion between the viral envelope and the cell membrane. HAs of most mammalian and low pathogenic avian influenza (LPAI) strains have a single arginine residue at the
cleavage site, limiting their proteolytic cleavage to respiratory and intestinal organs. This results in relatively localised infections which are generally mild. However, the HA of more virulent highly pathogenic avian influenza (HPAI) viruses contain a series of basic amino acids at the protease cleavage site which can be cleaved by ubiquitous Golgi proteases (reviewed in 1.6.1). Such viruses have the capacity to infect a greater number of tissues leading to systemic infection and increased pathology (Steinhauer, 1999). NA is a homotetramer and catalyses the cleavage of sialic acids allowing for the release of new viral progeny from the host cell. Anchored within the viral envelope is the M2 ion channel but it is found in low abundance (Pinto et al., 1992). Underlying the lipid membrane is the Matrix 1 protein (M1), which is the major structural protein of the virus. Also found within the structure, though in much lower quantities, are the viral nuclear export protein/non-structural protein (NEP/NS2), which is associated with M1, as well as NS1 (Hutchinson et al., 2014; Yasuda et al., 1993).

The eight RNA genomic segments reside within the core of the virion and the RNAs are encapsulated into ribonucleoprotein complexes (vRNP) by viral proteins (Figure 1.2) (Noda et al., 2006). Within the vRNP complex, single-stranded RNA is bound to nucleoprotein (NP) monomers with high affinity but without sequence specificity at approximately 24 nucleotides per NP molecule. Studies using cryo-electron microscopy among other techniques (Jennings et al., 1983; Moeller et al., 2012; Ye et al., 2006) show a double helical arrangement of RNP with the highly conserved 5’ and 3’ ends of the vRNA bound by a single trimeric RNA-dependent RNA polymerase (composed of PB1, PB2 and PA). The RNA genome is compact, totalling ~13.6 kilobases (Kb) with individual segments ranging from 2.3 to 0.89 Kb (Table 1.2). A number of studies have shown that virions selectively incorporate one of each of the vRNP complexes in a 7+1 circular arrangement at the edge of viral budding (as seen in Figure 1.1c). How the virus packages its genome is reviewed in Hutchinson et al. (2010).
Figure 1.1 Structure of the influenza virus The host-derived lipid envelope contains the (a) haemagglutinin (green), neuraminidase (blue), and M2 proteins (grey) and overlies a protein layer comprised of the matrix protein M1 (red). A nuclear export protein (NEP/NS2 green and yellow) is also associated with the virus. (Figure courtesy of Professor P. Digard) (b) A high magnification transmission electron micrograph of a single virion; the ‘dots’ represent the position of the individual RNPs. (c) Transmission electron micrograph of a thin section of infected cells showing influenza virus release. Images b&c have been obtained from Noda et al. (2006) (d) Scanning electron micrograph showing spherical virus particles on the surface of an A/PR/8/34 infected cell and (e) filamentous virus emerging from cells that have been infected with a A/PR/8/34 reassortant virus with M segment from A/Udorn/1972. Images courtesy of P.Digard.
Figure 1.2 Structure of the influenza vRNP complex. (a) Each viral ribonucleoprotein complex (vRNP) consists of one single-stranded, negative sense genomic RNA that is associated with nucleoprotein (NP) monomers at approximately 24 nucleotides per NP molecule. The genomic ends of the vRNA do not associate with NP but fold-back and base pair to form a partially double stranded structure that is bound by the viral polymerase complex (composed of PB1, PB2 and PA). Internal RNA regions are organised into an antiparallel double helix, which are driven by interactions between NP monomers (known as the minor grooves). Schematic edited from figure provided by P. Digard. (b) Transmission electron micrograph showing a negatively stained RNP (Jennings et al., 1983) (c) three-dimensional cryo-EM reconstructions of the central portion of a virion determined by Arranz et al., (2012) (d) Reconstruction of the influenza A polymerase complex bound to the vRNA promoter. Figure displays two surface views at 0˚ and 180˚ relative to each other. PA, PB1 and PB2 have been colour coded green, cyan and red, respectively. The major subdomains are also labelled. The vRNA 5' (pink) and 3' (yellow) ends are also displayed. Polymerase representations are obtained from (Pflug et al., 2014)
Table 1.1 Overview of influenza A viral proteins and known function (adapted from Fields Virology, 4th edition).

<table>
<thead>
<tr>
<th>Segment</th>
<th>Protein/Protein/Protein</th>
<th>Amino acid length</th>
<th>Protein Molecular Weight (kDa)</th>
<th>Known function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polymerase basic protein 2 (PB2)</td>
<td>759</td>
<td>85.7</td>
<td>Component of the viral polymerase that recognises the 5' cap from host mRNA</td>
</tr>
<tr>
<td></td>
<td>PB2-S1¹</td>
<td>508</td>
<td>55</td>
<td>Expressed by alternative splicing of segment 1. Protein is localised to mitochondria and inhibits the RIG-I-dependent signalling pathway</td>
</tr>
<tr>
<td>2</td>
<td>Polymerase basic protein 1 (PB1)</td>
<td>757</td>
<td>86.5</td>
<td>Component of the viral polymerase and is involved in elongation of mRNA</td>
</tr>
<tr>
<td></td>
<td>PB1-F2²</td>
<td>87</td>
<td>10.5</td>
<td>Expressed from an alternative translation initiation site within PB1. Interferon antagonist that has pro-apoptotic function</td>
</tr>
<tr>
<td></td>
<td>PB1-N40³</td>
<td>718</td>
<td>82</td>
<td>Expressed from alternative translation initiation site in PB1. Function unknown but thought to interact with viral polymerase</td>
</tr>
<tr>
<td>3</td>
<td>Polymerase acidic protein (PA)</td>
<td>716</td>
<td>84.2</td>
<td>Component of the viral polymerase and has endonuclease activity</td>
</tr>
<tr>
<td></td>
<td>PA-X⁴</td>
<td>252</td>
<td>29</td>
<td>Expressed via ribosomal frameshifting of segment 3. Modulates host immune responses to IAV infection by its host shut-off activity</td>
</tr>
<tr>
<td></td>
<td>PA-N155⁵</td>
<td>562</td>
<td>62</td>
<td>Proteins are expressed from an alternative translation initiation site in PA. The functions of these N-terminal truncated forms of PA remain unclear, but they have no polymerase activity. Viruses deficient of PA-N155 replicate more slowly in vitro and show reduced pathogenicity in vivo</td>
</tr>
<tr>
<td></td>
<td>PA-N182⁶</td>
<td>535</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Haemagglutinin (HA)</td>
<td>566</td>
<td>61.5</td>
<td>Surface glycoprotein that mediates receptor binding and fusion activity</td>
</tr>
<tr>
<td>5</td>
<td>Nucleoprotein (NP)</td>
<td>498</td>
<td>56.1</td>
<td>RNA binding protein; involved in RNA synthesis and vRNP nuclear export</td>
</tr>
<tr>
<td>6</td>
<td>Neuraminidase (NA)</td>
<td>454</td>
<td>50.1</td>
<td>Surface glycoprotein with sialidase activity; allows release of virus progeny from the cell surface</td>
</tr>
<tr>
<td>7</td>
<td>M1</td>
<td>252</td>
<td>27.8</td>
<td>Involved in viral budding</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>97</td>
<td>11</td>
<td>M2 is expressed by alternative splicing of segment 7 and acts as a protein ion channel</td>
</tr>
<tr>
<td></td>
<td>M42²</td>
<td>99</td>
<td>~13</td>
<td>Variant of M2 expressed by alternative splicing</td>
</tr>
<tr>
<td>8</td>
<td>Non-structural protein 1</td>
<td>230</td>
<td>26.8</td>
<td>Variety of functions have been described, acts as an interferon antagonist</td>
</tr>
<tr>
<td></td>
<td>Nuclear export protein (NS2)</td>
<td>121</td>
<td>14.2</td>
<td>Expressed following alternative splicing of segment 8 and is involved in nuclear export of vRNP</td>
</tr>
<tr>
<td></td>
<td>NS3³</td>
<td>187</td>
<td>~21</td>
<td>Alternatively spliced variant of NS1 and is thought to play a role in host adaptation</td>
</tr>
</tbody>
</table>

¹ (Yamayoshi et al., 2015) ² (Chen et al., 2001) ³ (Wise et al., 2009) ⁴ (Jagger et al., 2012) ⁵ (Muramoto et al., 2013) ⁶ (Wise et al., 2012) ⁷ (Selman et al., 2012). Each genomic segment encodes a core protein with some segments encoding additional/accessory proteins, which are described and referenced within the table.
1.2 Influenza virus replication cycle

A schematic presentation of the influenza A virus replication cycle is presented in Figure 1.3.

1.2.1 Attachment and internalisation

Viral entry into the host cell begins with the binding of HA to cell surface sialic acid receptors (SA) (Chu and Whittaker, 2004; Gottschalk, 1959). Crystal structures of HA were first resolved by Wilson et al., (1981) and reveal the glycoprotein to have a trimeric rod-like structure. The carboxyl terminus of HA is inserted into the viral membrane and the hydrophilic end projects away from the viral surface (Figure 1.1a). Interactions between host cell SA and the viral HA are believed to be low affinity, but the binding of several HA molecules allows attachment with increased avidity. Much published work proposes that the HA receptor-binding specificity determines host restriction. Human and classical H1N1 swine viruses preferentially bind to host cell receptors containing sialyoligosaccharides terminated by N-acetyl sialic acid linked to galactose (gal) with an α2,6 linkage (Connor et al., 1994; Shinya et al., 2006; Suzuki et al., 2000). In contrast, avian viruses have higher binding affinities for α2,3 gal sialic acid receptors that are expressed within the intestine and respiratory tract of wild birds. Sialic acid moieties with α2,6 gal linkages are expressed abundantly on epithelial cells of the human upper respiratory tract, nasal mucosa, pharynx, trachea and bronchi, while α2,3 gal sialic acids are often found in the lower respiratory tract. Consequently viruses with α2,6 gal specificity bind to the epithelial cells lining the trachea, and are able to replicate in the upper airway and transmit effectively. Upon receptor binding, virus particles are internalised into clathrin-coated pits by receptor-mediated endocytosis (Matlin et al., 1981) as well as by other non-clathrin and non-caveolae-mediated processes (Sieczkarski and Whittaker, 2002).
Figure 1.3 Schematic representation of the influenza virus replication cycle; adapted from Fields Virology, 5th edition, chapter 47. Influenza virus entry is initiated by attachment of haemagglutinin (HA) to sialic acid receptors on the plasma membrane. The virus then enters cellular endosomes by receptor-mediated endocytosis. Acidification of the early endosomes triggers a conformational change of HA which activates viral fusion between the virion and endosomal membranes. This creates a pore between the two membranes where the vRNPs are able to deposit into the cytoplasm. The vRNPs are then imported through the nuclear pore complex and into the nucleus. Primary transcription allows for the generation of mRNAs, which are exported into the cytoplasm for protein production. Following the translation and nuclear import of progeny polymerase proteins and NP, genome replication is initiated for the generation of vRNP. New vRNP are exported out of the nucleus and trafficked to the apical plasma membrane on Rab11 positive vesicles. vRNPs, HA, NA, M2 and M1 are then assembled into progeny virus and the virus buds off.
1.2.2 Fusion, uncoating and nuclear import

Once within the endosome the virus requires the release of the vRNP complexes into the cytoplasm. To accomplish this, the precursor HA molecule (HA0) must first have been cleaved by host cell proteases into HA1 and HA2 subunits to expose the hydrophobic amino acid terminus of HA2. HA2 is required to mediate fusion between the viral envelope and endosomal membrane (reviewed by Palese and Shaw, 2007). The low pH environment of the late endosomes (~pH5) triggers a conformational change of HA exposing the fusion peptide at the N-terminus of HA2. The fusion peptide is then inserted into the endosomal membrane to bring the viral and endosomal membranes close where they begin to fuse (Durrer et al., 1996; Takahashi et al., 2001). Several HA trimers are required to form a pore between the two membranes through which the vRNPs can enter the cytoplasm (Danieli et al., 1996). Additionally, the M2 ion channel lowers the pH of the interior of the virion by allowing H+ ions to pass from the acidified endosome into the virion (Stegmann, 2000). Acidification of the virion weakens the protein protein interactions between the M1 protein capsid and the vRNP, which then allows them to exit the virion and move into the cytoplasm. Studies have shown that different influenza HAs display varying pH requirements for initiating fusion; for example, HA from human isolates require a lower pH than avian isolates of the same subtype (Galloway et al., 2013).

Upon release, vRNP complexes translocate to the host cell nucleus for transcription of viral messenger RNA (mRNA) and replication into full-length complementary RNA (cRNA), which is used as the template for generation of full-length negative sense segments for packaging (vRNA) (reviewed by Eisfeld et al., 2015). While proteins of less than 40kDa are able to passively cross the nuclear pore complex (NPC), larger molecules such as the vRNP complexes must gain entry by an energy-dependent process known as the classical importin-α-importin-β1 (IMPα-IMPβ1)-dependent nuclear import pathway (Martin and Helenius, 1991). This
pathway is initiated by recognition of nuclear localisation signals (NLSs) located within the cargo proteins by IMPα adaptor proteins (which are typically arginine and lysine rich). IMPα is in turn recognised by the IMPβ transport receptor. This ternary IMPα-IMPβ-cargo (vRNPs) complex shuttles through the NPC, and once inside the nucleus IMPβ dissociates from the complex by the activated form of RAN GTPase, allowing the release of IMPα-cargo(vRNP) into the nucleus (O'Neill et al., 1995). A further dissociation step involving cellular CSE1L protein frees IMPα from the cargo molecules into the cell nucleoplasm (Kutay et al., 1997). Following release, IMPα and IMPβ are individually recycled back to the cytoplasm. The classical nuclear import pathway shuttles cargo by recognition of NLS motifs, which have been identified in all four proteins of the vRNP complex. PB2 and NP have been shown to enter the nucleus either alone and PB1 and PA are imported into the nucleus in complex (Fodor and Smith, 2004).

1.2.3 Viral protein expression

Unlike most RNA viruses, influenza A virus transcription occurs within the nucleus of the cell and is performed by the vRNPs (reviewed by Eisfeld et al., 2015; Fodor, 2013; Fodor and Brownlee, 2002; Portela and Digard, 2002). Transcription of viral messenger RNA (mRNA) from vRNA is initiated by cap snatching from cellular RNA polymerase II (polII) pre-mRNAs. Briefly, PB1 recognises and binds to the 5'-vRNA promoter, while PB2 binds 7-methylguanosine (m(7)GpppNm) capped pre-mRNA. PA cleaves host cell pre-mRNAs along with 10-14 associated nucleotides in a process called cap-snatching (Blaas et al., 1982a, b; Bouloy et al., 1978; Braam et al., 1983; Fechter et al., 2003; Plotch et al., 1979; Plotch et al., 1981). The 3’ end of the capped RNA fragment is then positioned at the active site of PB1 along with the 3’ end of the vRNA template where it is used to prime transcription. Elongation continues until a sequence of around 5-7 uracil residues is reached. This poly-U-sequence is located 16 nucleotides from the 5’ end of the vRNA segment and
acts as a signal for polyadenlyation (addition of a poly-A-tail to the mRNA) by the viral RNA polymerase. During the process of elongation and while the vRNA template is threaded through the active site (in a 3′-5′ direction), the 5′ end of the vRNA is thought to remain associated with the viral polymerase. As the poly-U sequence reaches the active site of the polymerase it cannot proceed because of steric hindrance caused by the 5′ end being bound to the polymerase. This leads to stuttering of the viral polymerase on the poly-U-stretch, resulting in the addition of a poly-A-tail to the viral mRNA. This action mimics polyadenlyation of cellular pre-mRNAs and aids the export of newly synthesised mRNA into the cell cytoplasm, where they are now translated by cellular ribosomes. Once translated, the polymerase subunits, M1, NEP and NP viral proteins import back into the nucleus.

Influenza viruses take advantage of various mechanisms to increase protein-coding capacity. While segments 4, 5 and 6, HA, NP and NA respectively, are believed to encode a single protein, segments 1, 2, 3, 7 and 8, (encoding PB2, PB1, PA, M, and NS respectively), have the ability to encode additional proteins, which are described in Table 1.1. For example, segment 7 mRNA encodes matrix protein (M1) (Inglis and Brown, 1981) and an additional two spliced variants encoding the M2 ion channel (Lamb and Choppin, 1981), and (in some viral strains) a form of M2 known as M42 (Wise et al., 2012). Viral genes are not expressed at the same time or in equal proportions. During the early stages of infection high levels of NP and NS1 (mRNA and protein) are detected (Hatada et al., 1989; Hay et al., 1977), while the polymerase proteins are found in low abundance. Additionally synthesis of HA, NA and M1 proteins are also delayed (Inglis et al., 1976; Skehel, 1973). The mechanisms governing the temporal regulation of influenza A virus (IAV) protein expression are poorly understood but may involve differential vRNA synthesis, mRNA nuclear export and/or sequence polymorphisms of the vRNA promoters.
1.2.4 Genome replication and nuclear export

Genomic vRNA is negative sense and transcription into mRNA does not produce a full-length copy of the vRNA, it lacks the terminal non-coding region downstream of the poly(A) ligand. As a result a second positive sense transcription step is required for genome replication. First, genomic vRNA is replicated into complementary RNA (cRNA), which complements the full vRNA transcript and acts as replicative intermediate (vRNA→cRNA). Next, a second replication step copies the cRNA into vRNA (cRNA→vRNA). Unlike viral transcription, viral replication is primer independent and does not require cap snatching or polyadenylation (Hay et al., 1982; Young and Content, 1971). Both cRNA and vRNA have a triphosphorylated nucleotide at the 5’ terminus; this suggests that initiation of synthesis is primer independent. Replication is mediated by the viral RNA-dependent RNA polymerase. The viral replication cycle consists of an early transcriptive and late replicative phase (Hay et al., 1977). Studies have shown that treatment of infected cells with cycloheximide, an inhibitor of protein biosynthesis, prevents the switch from transcription to replication suggesting that de novo protein synthesis governs replication (Taylor et al., 1977). NP is believed to direct this switch as its accumulation has been shown to protect viral replication products from degradation, and stabilise replicative intermediates (Medcalf et al., 1999; Noton et al., 2009; Vreede et al., 2004). Additional studies have also shown several temperature sensitive NP mutants to be defective in RNA binding and replication (cRNA synthesis).

Following replication, the matrix protein M1 binds to the newly synthesised RNPs through interactions with NP (Huang et al., 2001). The vRNP complexes then require export out of the nucleus and towards the plasma membrane and the site of virus assembly. The first step in this process uses the exportin 1 (CRM1) pathway. Treatment of infected cells with leptomycin B (an irreversible inhibitor of CRM1)
leads to nuclear retention of vRNP, supporting the importance of the CRM1 pathway for vRNP nuclear export (Elton et al., 2001; Ma et al., 2001; Watanabe et al., 2001). Cellular CRM1 is a nuclear export receptor that recognises and binds cargo containing leucine-rich nuclear export signals (NES). On replication and assembly, vRNP complexes are localised to chromatin at the nuclear periphery. Here, they associate with CRM1 and are released from the chromatin following a phosphorylation signal, the nature of which is poorly understood but thought to involve HA accumulation at the plasma membrane (Marjuki et al., 2006). Following this, the vRNP complexes are exported by incorporation into a ‘daisy-chain’ complex that is composed of M1 and NEP/NS2. NEP is believed to act as an adapter between the vRNP-M1 and CRM1-RAN-GTP complexes (Akarsu et al., 2003; Neumann et al., 2000; O’Neill et al., 1998; Yasuda et al., 1993). Once the ‘daisy-chain’ complex has shuttled across the NPC, RAN-GTP hydrolyses via an RNP-GTPase activating protein and releases the export factors into the cytoplasm (reviewed in Paterson and Fodor, 2012). Here, the vRNA remain associated with M1. vRNP complexes (comprising of the cRNA, viral polymerase and NP) are selectively exported out of the cell nucleus over cRNP; the molecular mechanisms behind this selectivity are not understood but differences in promoter structures (Tchatalbacachev et al., 2001) and preferential access of vRNP complexes to cellular export machinery (over cRNP) by chromatin targeting have been suggested (Chase et al., 2011). The ‘daisy-chain’ complex is the current model for vRNP export from the nucleus but direct evidence supporting complex formation is limited.

Following nuclear export, the vRNP complexes begin to accumulate by the pericentriolar recycling endosomal compartment through interaction of PB2 with Rab11. They subsequently traffic towards the apical cell membrane along the microtubule network on Rab-11-positive cargo vesicles (Amorim et al., 2011; Momose et al., 2011). Studies have shown cellular Y-box-protein-1 (YB-1) interacts
with vRNPs and it is believed to act as a porter that eases association of progeny vRNPs with the microtubules (Kawaguchi et al., 2012).

1.2.5 Assembly and budding

Influenza viruses assemble and are released from the apical membrane of polarised cells (Rodriguez Boulan and Sabatini, 1978). The trans-membrane proteins, HA, NA and M2 are translated by membrane-bound ribosomes and enter the endoplasmic reticulum, where they are folded and glycosylated (Doms et al., 1993). The proteins are then directed through the Golgi network by apical sorting signals (for HA and NA the signals are located within their trans-membrane domains), to sites of viral assembly at the apical plasma membrane (reviewed in Barman et al., 2001). Here, the viral proteins begin to accumulate and assemble in lipid rafts. Lipid rafts are regions of plasma membranes rich in sphingolipids and cholesterol (Scheiffele et al., 1999). The remaining viral proteins traffic to the site of viral assembly by the actin cytoskeleton and microtubule network (Avalos et al., 1997; Digard et al., 1999). Here the lipid rafts act as micro-domains where the viral glycoproteins accumulate and serve as a platform for budding.

Viral budding from the plasma membrane is initiated by HA and NA, which target lipid rafts and are able to alter membrane curvature (reviewed by Nayak et al., 2004; Rossman and Lamb, 2011). Here, M1 protein is believed to bridge together the surface membrane proteins by interaction with the cytoplasmic tails of the viral surface glycolproteins and the vRNP (Ali et al., 2000). Membrane bending at the budding site is then required to allow the virus ‘bud’ to form. A combination of factors, which include interactions between host and viral components lead to an outward curvature of the plasma membrane and the formation of a bud with the vRNP at its head. Once the bud has formed the opposing membranes can then begin to close and fuse. M2 protein mediates membrane scission, pinching off the virus at the base of the bud to allow for release of virus from the plasma membrane.
(Rossman et al., 2010). Following membrane scission, NA is required to release the virus particle from the sialic acid receptors (Palese et al., 1974).

1.3 Influenza virus epidemiology and disease

Influenza A viruses cause seasonal epidemics and the World Health Organisation (WHO) estimates 2-5 million cases of severe infection and up to 250,000-500,000 deaths each year. In adults, infection rates are estimated to be between 5-10% per year, which are slightly increased in children to 20-30% (WHO, 2016). Influenza viruses present a major public health concern because of their ability to unpredictably and rapidly evolve new antigenic variants that can escape pre-existing immunity and transmit efficiently. Emerging pathogenic strains can arise as a consequence of antigenic ‘drift’ which describes the accumulation of mutations within surface glycoproteins, or by the process of antigenic ‘shift’ where genome segments of animal and human influenza viruses undergo reassortment within a reservoir host (Nelson and Holmes, 2007). In the UK live attenuated and inactivated vaccines are approved for use in humans. Most influenza vaccines are produced in embryonated hen’s eggs. However, not all circulating wild-type viruses grow efficiently in eggs and as such high-growth reassortant (HGRs) viruses are generated for vaccine manufacture. HGRs are typically synthesised from two parental viruses, a wild-type virus representing the current circulating strain (and that has been recommended for inclusion in the vaccine) and a strain that is able to grow efficiently in eggs. Traditionally the high-yield virus has been A/PR/8/34. HGRs usually contain a mixture of genome segments from both virus strains and most contain six segments from A/PR/8/34 and the HA and NA from the wild-type virus Vaccine formulations contain two influenza A viruses of H1N1 and H3N2 subtype and one or two influenza B viruses. However, due to antigenic drift, vaccines require seasonal updating, which is costly and time-consuming (the latest
developments in vaccine design/production are reviewed by Krammer and Palese, 2015).

1.4 Current prevention and therapy

Though vaccination is the primary strategy for control and prevention of influenza, antigenic drift during the influenza season may render the vaccine ineffective and outbreaks can occur particularly among high-risk populations. The production of an effective vaccine from initial identification of a novel pandemic strain requires a minimum of six months and would therefore not be available during the first wave of viral spread. For this reason pandemic preparedness relies on the use of anti-viral agents (as discussed by Hayden, 2004).

1.4.1 Adamantanes

Amantadine was the first compound shown to inhibit influenza A viral replication (Davies et al., 1964) and was approved for prophylactic and therapeutic use in 1976. Amantadine derivatives prevent viral uncoating by blocking the M2 ion channel and inhibiting proton migration to the interior of the virion (Hay and Zambon, 1984). Amantadine and Rimantidine (Koff and Knight, 1979) have been licenced for use in a number of countries, but their use is limited by rapid emergence of drug resistance and association with side effects related to the central nervous system. Resistance to M2 inhibitors is widespread and circulating human pandemic swine H1N1 and H3N2 (Bright et al., 2005; Bright et al., 2006; Ziegler et al., 1999), highly pathogenic avian H5N1 (Govorkova et al., 2013) and swine-origin H1N1 pandemic viruses (Dawood et al., 2009) have all acquired resistance. In fact, only one nucleotide substitution is required at any of 4-5 sites within the transmembrane region of M2 to establish resistance (resistance markers include: Val27Ala, Ala30Thr, Glu34Lys, Leu26Phe and the most prevalent M2 mutation, Ser31Asn, which is located alongside the rim of the pore) (Dong et al., 2015) and some of these substitutions do not confer a fitness cost to the virus (Hayden and
Hay, 1992). As a result, the WHO does not recommend the use of adamantane derivatives as a mono-therapy for influenza treatment. A recent study reported the development of novel adamantane agents that are able to block Ser31Asn resistant strains and these agents may be used to overcome current circulating resistant virus strains (Wang et al., 2013).

1.4.2 Neuraminidase inhibitors (NAI)

The crystal structure for neuraminidase was first resolved by Colman et al., (1983), and paved the way for the design of neuraminidase inhibitors (NAI) (reviewed by von Itzstein et al., 1993). These include Zanamivir (not bioavailable orally but delivered by inhalation) and Oseltamivir (delivered orally), which entered clinical practice in 1999. The sialidase activity of NA (removal of sialic acid from sialyoligosaccharides) has two essential roles in the viral replication cycle. First, removal of sialic acid from HA and the host cell surface facilitates viral release and second, removal of sialic acid from the mucin layer assists the movement of the virus though the respiratory tract. Neuraminidase inhibitors act as transition state mimics of sialic acids and prevent virus spread by blocking NA. The efficacy of NAI have been extensively investigated and reviewed. The Cochrane review recently claimed that there was insufficient evidence to support the use of oseltamivir for reducing the risk of transmission and preventing complications of infection (such as pneumonia, sinusitis and bronchitis). In this review, Jefferson et al. (2014) showed that compared to placebo, oseltamivir and zanamivir were found to reduce the duration of symptoms by less than a day when administered therapeutically (within the 48 hour of the onset of symptoms) in adults. However considering the Cochrane review findings, it is important to note that the therapeutic treatment of IAV is challenging because of the timing of peak viral replication and then onset of clinical symptoms. IAV replication occurs predominantly within the respiratory tract and peaks within the first couple of days of infection. At this stage however, the patient
is unlikely to have presented symptoms. It is therefore very difficult to administer
the anti-viral to a patient ‘on-time’ because patients present at a late stage relative to
virus replication. It also explains why these drugs perform better when
administered prophylactically (as described in the Cochrane review). This suggests
that NI inhibitors are in fact effective against the virus but that they are not
administered correctly. The report highlights that the drugs were able to reduce the
time in which adults (16 hours) and children (29 hours) were sick, given that the
disease only lasts six days, a day is very good. Pandemic H1N1 and some H3N2
subtype viruses have developed resistance against oseltamivir primarily through a
His274Tyr substitution, but so far resistance is not as widespread as observed for
adamantanes.

1.4.3 Arbidol hydrochloride

Arbidol hydrochloride is a broad-spectrum inhibitor of influenza A, B and C
viruses (this includes H5N1 and H9N2 subtype viruses) (Leneva et al., 2005). The
compound is licenced for treatment in Russia and China. Arbidol functions by
binding to the HA2 subunit of haemagglutinin, stabilising HA and preventing the
conformational change required to bring about membrane fusion. Arbidol-resistant
viruses have been generated and resistance markers mapped to HA, all of which
impacted the acid stability of HA (Leneva et al., 2009). However, the WHO does not
recommend Arbidol for treatment of influenza as there is insufficient clinical
evidence supporting the efficacy and safety of the compound. At present, all
licenced agents for prophylaxis of influenza are either limited by the emergence of
resistance or lack evidence supporting their efficacy. Thus, highlighting the need for
development of novel anti-viral drug targets and treatments of influenza.

1.4.4 Drugs with therapeutic potential

Drugs currently in development target the early and late stages of the
influenza replication cycle. This study focuses on anti-viral peptides believed to
target virus entry into the cells. As such, inhibitors of influenza entry have been reviewed and are described in Table 1.2. Novel approaches for inhibition of influenza include inhibitors of viral attachment by removal of surface sialic acid receptors using sialidases. DAS181 (NexBio Fludase) is a compound consisting of a bacterial sialidase derived from Actinomyces viscosus conjugated to amphiregulin and is currently in phase II clinical trials. DAS181 is effective against a broad panel of influenza subtypes (Nicholls et al., 2013). Monoclonal antibodies targeting conserved epitopes within the globular head or stem region of HA have also been developed (Dreyfus et al., 2012; Ekiert et al., 2011; Sui et al., 2009; Wu et al., 2015). Anti-viral agents acting downstream of virus entry include nucleoside analogues such as ribavirin, viramidine and favipiravir (T-705) that target the influenza virus RNA-dependent-RNA polymerase and are active against H1N1, H2N2 and H3N2 viruses.

1.4.5 Anti-viral drug development – considerations prior to clinical trials.

For progression of an antiviral agent to phase I clinical trials drug standards agencies such as The Food and Drug administration (FDA) require in vitro (nonclinical) and clinical data that look to: define the in vitro activity of the agent (cytotoxicity should be investigated and determination of therapeutic indexes), identify the mechanism of drug action and assess the effects of administrating the agent in combination with other antiviral agents. Further experimental considerations should include resistance studies and potential for cross-resistance when administered with other approved antiviral compounds.
Table 1.2 Inhibitors of IAV entry. Table adapted from Edinger et al. (2014)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Entry Step</th>
<th>Potential use as drug</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb (to HA binding site)</td>
<td>Attachment</td>
<td>Yes</td>
<td>(Clementi et al., 2012)</td>
</tr>
<tr>
<td>SA mimics</td>
<td>Attachment</td>
<td>Yes</td>
<td>(reviewed by Vanderlinden and Naesens, 2014)</td>
</tr>
<tr>
<td>SA binders</td>
<td>Attachment</td>
<td>Yes</td>
<td>(reviewed by Vanderlinden and Naesens, 2014)</td>
</tr>
<tr>
<td>Sialidases e.g. DAS181</td>
<td>Attachment</td>
<td>Yes</td>
<td>(Nicholls et al., 2013)</td>
</tr>
<tr>
<td>Carbohydrate-binding module</td>
<td>Attachment</td>
<td>Yes</td>
<td>(reviewed by Vanderlinden and Naesens, 2014)</td>
</tr>
<tr>
<td>Dynasore</td>
<td>Internalisation</td>
<td>Laboratory use</td>
<td>(de Vries et al., 2011)</td>
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<td>Lj001</td>
<td>Internalisation</td>
<td>Yes</td>
<td>(Wolf et al., 2010)</td>
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<td>Bafilomycin A1</td>
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<td>Laboratory use</td>
<td>(Bowman et al., 1988; Ochiai et al., 1995)</td>
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<td>mAb (HA stalk)</td>
<td>Fusion</td>
<td>Yes</td>
<td>(Corti and Lanzavecchia, 2013)</td>
</tr>
<tr>
<td>Small molecule inhibitors (HA stalk)</td>
<td>Fusion</td>
<td>Yes</td>
<td>(Vanderlinden and Naesens, 2014)</td>
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<td>S20</td>
<td>Fusion</td>
<td>Laboratory use</td>
<td>(White et al., 2015)</td>
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<td>TBHQ</td>
<td>Fusion</td>
<td>Yes</td>
<td>(Bodian et al., 1993)</td>
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<td>Arbidol</td>
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<td>Approved in Russia in 2005</td>
<td>(Leneva et al., 2005; Liu et al., 2013)</td>
</tr>
<tr>
<td>Amantadine</td>
<td>Uncoating</td>
<td>Approved in US in 1976</td>
<td>(Davies et al., 1964)</td>
</tr>
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<td>Rimantadine</td>
<td>Uncoating</td>
<td>Approved in US in 1993</td>
<td>(Koff and Knight, 1979)</td>
</tr>
<tr>
<td>Benzyl-substituted amantadine derivatives</td>
<td>Uncoating</td>
<td>Yes</td>
<td>(Wang et al., 2013)</td>
</tr>
<tr>
<td>Importazole</td>
<td>RNP nuclear import&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Laboratory use</td>
<td>(Chou et al., 2013; Soderholm et al., 2011)</td>
</tr>
</tbody>
</table>

1. Table describes inhibitors that target the early stages of influenza virus entry up to the translocation and import of genetic material to the nucleus.
2. Importazole has been considered as an inhibitor of virus entry as it prevents vRNP import into the host cell.
1.5 Peptides as anti-viral agents

Following the success of Enfuvirtide, a human immunodeficiency virus entry blocker (Matthews et al., 2004), there has been renewed interest in peptides as anti-viral agents. The focus of this study are two cell-penetrating peptide families, the first are the tyrosine kinase inhibitory proteins (Tkip) and the second are ‘Entry Blocker’ peptides derived from a fibroblast growth factor-4 signal sequence.

1.5.1 Tyrosine kinase inhibitory proteins (Tkip)

Innate immune responses to influenza infection are characterised by an influx of macrophages, neutrophils and monocytes into the lung and alveolar spaces, with increased levels of inflammatory cytokines and chemokines (reviewed by Iwasaki and Pillai, 2014). Critically ill patients present with severe inflammation (T-cell lymphopenia and haemophagocytosis) caused by an overproduction of cytokines and chemokines. This is sometimes referred to as a ‘cytokine storm’ and often occurs in patients even in the absence of high viral load. The immune system is made up of positive and negative regulators that act together to maintain immune homeostasis. An intracellular family of proteins known as the suppressor of cytokine signalling (SOCS) proteins negatively regulate receptor-associated tyrosine kinases for example, JAKs which play a role in cytokine signalling and receptor kinases. On binding to target receptors, cytokines trigger an intracellular cascade involving the JAK/STAT pathway that leads to activation of interferon and other pro-inflammatory mediators (described in Figure 1.4).

The suppressor of cytokine signalling (SOCS) proteins are negative feedback regulators of the JAK/STAT pathway, and are required for regulation of cytokine networks. The SOCS family comprises of eight members: SOCS1-7 and cytokine-inducible Src homology 2 protein (CIS). These proteins contain a conserved C-terminal SOCS box and central Src Homology 2 (SH2) domain but have variable amino termini. SOCS 1 and 3 are able to bind directly to Janus kinase (JAK) by their
kinase inhibitory region (KIR) and SH2 domain to inhibit JAK phosphorylation and the downstream signalling cascades leading to activation of interferon and transcription of nuclear factor kappa-light-chain-enhancer (the role of SOCS in immune regulation is reviewed by Yoshimura et al., 2007). A small hydrophobic SOCS1 protein mimetic called Tyrosine kinase inhibitory peptide (Tkip) was designed for the treatment of autoimmune and inflammatory disorders (Flowers et al., 2004). Tkip is a 12 amino acid peptide that mimics the negative regulatory activity of SOCS-1 (reviewed by Ahmed et al., 2015). Tkip is able to bind to the autophosphorylation site of Janus kinase 2 (JAK2), inhibiting the JAK/STAT signalling pathway and blocking IFNγ activity. Tkip has been previously shown to protect mice from experimental allergic encephalomyelitis, an animal model for multiple sclerosis (Mujtaba et al., 2005) and inhibit proliferation of prostate cancer cells by blocking JAK2 autophosphorylation and subsequent activation of SOCS3 (Flowers et al., 2005). The protective activity of Tkip has not been limited to autoimmune disease. To promote survival, some viruses such as poxvirus are able to activate JAK2 during infection. Recognising this Ahmed et al. (2009) tested Tkip activity against vaccinia infection and found that Tkip inhibited vaccinia virus replication in vitro and protected C57BL/6 mice against otherwise lethal doses of vaccinia virus. The mechanism of Tkip action against these viruses has not yet been described it is thought that the mechanism differs for each virus as it varying effects on the virus life cycle.

Following the work of Ahmed et al. (2009) and on recognising that SOCS (SOCS-1 and SOCS-3) proteins are up-regulated during influenza A virus infection (Pothlichet et al., 2008), Professor Anthony Nash and his research laboratory determined the activity of Tkip (also known as FluPep 1) against influenza A virus infection. Tkip displayed potent anti-viral activity against A/PR/8/34 in plaque reduction assays with a recorded IC₅₀ of 0.09 µM (Nicol et al., 2012). Nicol et al.
(2012) also formulated a number of Tkip derivatives (FluPep 2-9), which are listed in Table 1.3. FluPep 4 (containing an additional RRKK tetrapeptide for improved solubility) was active at lower concentrations than Tkip (FluPep 1). FluPep 4 was then tested for anti-viral activity against A/PR/8/34 recombinant viruses containing the HA and NAs of A/England/195/09 (H1N1), A/Victoria/3/75 (H3N2) and A/Vietnam/1194/04 (H5N1) and found to be active in the low micromolar range. The protective effects of FluPep 4 were also evident in vivo where intranasal administration of peptide at the time of infection (prophylactic treatment) protected BALB/c mice from lethal doses of A/WSN/33 (Nicol et al., 2012). Mice receiving peptide maintained their body weight and showed reduced viral lung titres relative to vehicle treated mice.

The anti-viral mechanism(s) of FluPep 1 (Tkip) appeared to be independent from the anti-inflammatory effects. FluPep 2 contains amino acid substitutions of the phenylalanines at positions 8 and 11 of Tkip (which mimic the phenylalanines at positions 56 and 59 of SOCS 1 and are critical for preventing JAK2 autophosphorylation) and retains activity against A/PR/8/34 relative to FluPep 1. Nicol et al. (2012) proposed that FluPep prevents viral attachment by a mechanism which involves an interaction with HA. Using a virus adsorption assay Nicol et al. (2012) provided evidence that viral attachment to cells was reduced in the presence of FluPep 3.
Figure 1.4 Cross talk between the suppressor of cytokine signalling proteins (SOCS) and nuclear factor kappa-light chain-enhancer pathways. a) The binding of interferon-γ to the type 1 interferon receptor triggers the activation of the Janus kinase-signal transducer and activation of transcription pathway (JAK-STAT1) pathway. Ultimately, a sequence of phosphorylation events leads to the up regulation of IFN-inducible genes, CD40 and nitric oxide synthase. This results in an enhanced pro-inflammatory response, which can be negatively regulated by the SOCS1 protein. b) SOCS1 inhibits the catalytic activity of JAKs by binding to the activation loop of the catalytic domain through its kinase inhibitory region and SH2 domain. Tkip is a small SOCS1 protein mimetic that was developed by Ahmed et al. (2009). The small protein was designed to mimic the tyrosine kinase inhibitory region of SOCS1, which is known to complement the activation loop of JAKs. Binding of Tkip to the activation loop of JAK has been shown to inhibit the antiviral actions of IFN-γ as well as up regulate MHC class molecules on fibroblasts.
Table 1.3 Properties of FluPep and derivatives. Table adapted from (Nicol et al., 2012)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MW</th>
<th>Reason for modification</th>
<th>Activity (IC&lt;sub&gt;50&lt;/sub&gt; µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FluPep 1 (Tkip)</td>
<td>WLVFFVIFYFFR</td>
<td>1684</td>
<td>Substitution of phenylalanines to separate anti-inflammatory and anti-viral properties</td>
<td>A/WSN/33 (0.09)</td>
</tr>
<tr>
<td>FluPep 2</td>
<td>WLVFFVIAYFAR</td>
<td>1532</td>
<td></td>
<td>A/WSN/33 (0.0009)</td>
</tr>
<tr>
<td>FluPep 3</td>
<td>WLVFFVIFYFFRRKK</td>
<td>2253</td>
<td>Addition of RRKK tetrapeptide to C-terminus in order to improve solubility</td>
<td>A/WSN/33 (0.0003)</td>
</tr>
<tr>
<td>FluPep 4</td>
<td>RRKKWLVFFVIFYFFR</td>
<td>2253</td>
<td>Addition of RRKK tetrapeptide to N-terminus in order to improve solubility</td>
<td>A/WSN/33 (0.0004) PR/8/Eng09 (0.08)&lt;sup&gt;1&lt;/sup&gt; PR/8/Vic (0.05)&lt;sup&gt;1&lt;/sup&gt; PR/8/Viet (0.1)&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>FluPep 7</td>
<td>RRKKIFYFFR</td>
<td>1460</td>
<td>N-terminus truncation to identify active residues</td>
<td>A/WSN/33 (0.15)</td>
</tr>
<tr>
<td>FluPep 8</td>
<td>WLVFFVRRKK</td>
<td>1378</td>
<td>C-terminus truncation to identify active residues</td>
<td>A/WSN/33 (0.64)</td>
</tr>
<tr>
<td>FluPep 9</td>
<td>FFVIFYRRKK</td>
<td>1404</td>
<td>Truncations of the C and N-terminal amino acids. Here the ‘trunk’ of the peptide was tested</td>
<td>A/WSN/33 (1.48)</td>
</tr>
</tbody>
</table>


1.5.2 Anti-viral activity of a modified fibroblast growth factor-4 signal sequence

In a study by Bultmann and Brandt (2002), a number of cell-penetrating peptides were screened for anti-viral activity against herpes simplex virus type-1 (HSV-1). The authors found that a peptide they called ‘Entry Blocker’ (EB), in which the sequence is derived from the fibroblast growth factor 4 signal peptide (FGF-4) exhibited broad-spectrum activity against HSV-1 (Bultmann et al., 2001; Bultmann et al., 2010), vaccinia virus (Altmann et al., 2012; Altmann et al., 2009), and influenza
A virus (Jones et al., 2006). Full length EB prevented virus-induced agglutination of chicken red blood cells by a panel of influenza A virus strains (these included H1N1, H3N2 and H5N1 sub-types) and influenza B virus. Activity was detected in the micromolar range with reported IC₅₀ values of between 3-20 µM against all the influenza virus strains tested. A scrambled EB peptide (EBX) was synthesized and found not to affect agglutination. Furthermore, EB prevented attachment of a fluorescently labelled A/Turkey/Oregon/71 (H3N7) virus to cells at concentrations higher than 10 µM. The anti-viral effects of the peptides were translated from *in vitro* to *in vivo*. BALB/c mice treated intranasally with 25 µL of PBS containing 2 mM EB prior to infection with A/Hong Kong/156/97 (H5N1) displayed fewer clinical signs and lower viral lung titres. Prophylactic treatment with peptide proved to be more effective than therapeutic treatment (Jones et al., 2006). EB is composed of 16 amino acids derived from FGF-4 and an N-terminal RRKK tetrapeptide for maintaining solubility. Unlike FluPep, removal or modification of the RRKK motif resulted in a loss of anti-viral activity which was therefore deemed essential for maintaining peptide activity (Jones et al., 2011a). A library of EB peptides with serial deletions of single residues from either the N or C terminus were synthesised and a minimal peptide sequence of 13 amino acids (termed B10iso) which retained the same levels of anti-viral activity relative to the full-length peptide was found (Jones et al., 2011a). The authors have proposed that EB acts by a mechanism involving HA because of its ability to inhibit virus-mediated agglutination of red blood cells. Later studies showed that EB caused aggregation of influenza virus by treating gradient purified A/PR/8/34 with 30 µM EB and assessing particle size by sucrose gradient ultracentrifugation and electron microscopy (Jones et al., 2011b).
1.6 Influenza A virus ecology and the avian reservoir

1.6.1 Molecular determinants of pathogenicity for avian influenza viruses

Avian influenza viruses may be classified into low pathogenic avian influenza (LPAI) or highly pathogenic avian influenza (HPAI) on the basis of virulence in chickens. Poultry infected with LPAI generally present with mild respiratory disease, a decrease in egg production, ruffling of the feathers and depression (MacLachlan and Dubovi, 2011). However, viruses of subtypes H5 and H7 can evolve into highly virulent strains of influenza (HPAI) on introduction to poultry. HPAI strains can cause sudden death without apparent clinical signs, however in cases where birds survive, clinical signs include respiratory distress, diarrhoea, thrombosis and multifocal haemorrhages (Horimoto and Kawaoka, 2005). For instance, in December 2014, the US Department of Agriculture began reporting outbreaks of HPAI H5 subtype infections in domestic poultry within the northern states of America. By mid-June 2015 outbreaks were documented in 15 states, leading to the destruction of 50 million birds and an estimated loss of $1.57 billion. In order to contain and eradicate infection, the US Department of Agriculture committed a further $500 million and paid $190 billion to farmers as compensation for lost birds (USDA, Accessed 01/01/2016). The UK was not clear of infection either in 2014-2015; an outbreak of HPAI H5N8 was reported in North Yorkshire, which led to the destruction of 6,000 birds. H7N7 was detected in Hampshire and Lancashire where 170,000 birds were culled to contain the outbreak. Such outbreaks have impacts on the global food supply and economy and reinforce the need to devise control strategies.

This difference in virulence of avian influenza viruses can be partly attributed to a series of basic amino acids at the proteolytic cleavage site within HA. Surface HA glycoproteins of LPAI have a single arginine at the cleavage site, limiting proteolytic cleavage to respiratory and intestinal organs resulting in
localised infections that are usually asymptomatic or mild. However, the HA of HPAI viruses often contain a series of basic amino acids at the protease cleavage site which are susceptible to cleavage by a number of ubiquitous proteases found within a wide range of organs leading to systemic infections and increased pathology (Horimoto and Kawaoka, 1994).

However, the possession of a multibasic cleavage site does not always lead to a high pathogenicity phenotype. For example, A/Turkey/Texas/04 (H5N2) was sequenced following an outbreak in the United States in 2004 and found to have a polybasic cleavage site. But, the genotype of the virus did not match the LP phenotype observed, as determined using the standard intravenous chicken pathotype test (Lee et al., 2005; Londt et al., 2007), suggesting that pathogenicity can be modulated by other factors. Further determinants of pathogenicity and virulence involving glycosylation sites along the HA molecule have been shown to affect virus phenotype or mediate adaptation to a different host (Klenk et al., 2002). Neuraminidase is also thought to contribute to pathogenicity as studies of H5N1 subtype viruses have identified strains with deletions of 19-20 amino acids within the stalk portion of NA (Blumenkrantz et al., 2013). This type of deletion is thought to be associated with adaptation of viruses from wild waterfowl to domestic poultry (Baigent and McCauley, 2001; Hoffmann et al., 2012). As with HA, glycosylation sites near to the globular head of NA are also associated with increased virulence (Hulse et al., 2004). There are also a number of studies discussing the role of the non-structural proteins in pathogenicity. One study showed that increased virulence in chicken embryos were linked to two amino acid substitutions in the NS2 protein (Perdue, 1992). Another study pinpointed a single amino acid substitution at position 149 of the NS1 protein that affected the virulence of an H5N1 Asian chicken strain (Li et al., 2006). Additionally, the polymerase complex is thought to determine pathogenicity even though it is highly conserved. Polymerases from avian strains do not function well in the mammalian host.
and amino acids in PB1 and PB2 are thought to be associated with enhancing polymerase activity and pathogenicity in H5N1 viruses (Schmolke et al., 2011; Subbarao et al., 1993; Taubenberger et al., 2005). Determinants of host range pathogenicity have been reviewed by Cauldwell et al. (2014) and Long et al. (2015).

1.6.2 Virus replication sites

The nasal cavity is thought to be the primary site of LPAI replication in domesticated poultry, where the virus is then released and spreads to cells of the respiratory and intestinal tract. Recent studies determining LPAI replication sites detected viral RNA in multiple organs including the lung, brain, intestine, peripheral blood, heart liver and spleen. Studies also note differences in systemic distribution of virus between different stains (Post et al., 2012; Post et al., 2013). HPAI viruses initially replicate within the nasal epithelium. However, 16 hours following exposure viral antigens are detected in the respiratory epithelium. By 24 hours, the nasal epithelium is often ulcerated and inflamed with sub-mucosal macrophages and heterophils. It is believed that the virus first replicates within endothelial cells and spreads by the vascular and lymphatic systems to replicate within multiple organs including the brain and skin (Swayne, 2008).

1.7 Current strategies for control of avian influenza viruses in poultry

1.7.1 Biosecurity in poultry

Biosecurity is the first line of defence against LPAI and HPAI. Broadly speaking, biosecurity measures to fall into two categories: ‘inclusion biosecurity’ and ‘exclusion biosecurity’ (Swayne, 2008). Inclusion biosecurity describes the measures required to contain an outbreak on an affected premises; often practises include quarantine, improved hygiene, disinfection of fomites and removal of infected animals. Exclusion biosecurity describe practises that keep AI out of ‘clean’
reservoirs. Where poultry are raised in containment, or if raised with outdoor access, birds are confined or separated during periods that correspond to wild bird migration. This measure was successfully implemented in Minnesota where the numbers of reported AI outbreaks in turkeys reared outdoors (in the 1970s to mid-1990) were eliminated by moving the birds indoors (Swayne, 2008). The highest risk of infection comes from direct exposure of naïve birds to infected birds that shed high levels of virus from the respiratory and digestive systems.

1.7.2 Diagnosis and surveillance

Successful control of infection requires accurate and rapid diagnosis. There are a number of tests used to detect both virus and antibody in poultry, which are described in Table 1.4. Serological surveillance is performed on wild birds globally (119 countries between 2008 and 2013, CDC (2016)) and over 95% of domesticated poultry in the US. Surveillance provides information on the diversity, evolution, and spread of the virus within the avian host. This information can serve as a predictor of outbreaks and is used to guide selection of vaccine strains (Hoye et al., 2010; Verhagen et al., 2015).

1.7.3 Elimination of infected poultry

In the event of an AI outbreak infected birds would be euthanized, and carcasses, eggs and manure disposed of to prevent spread and eradicate infection. Elimination methods vary depending on viral strain, numbers of affected poultry, local conditions, available finances and levels of biosecurity practised within the farm. Though depopulation is effective in eradicating infection there are economical and ethical implications.

1.7.4 Use of anti-viral agents in domestic poultry

In the west, anti-viral compounds that have been licenced for use in human are not used to control influenza A viruses in poultry because of their expense and
the rapid speed with which resistant mutants arise during experimental challenge (Beard et al., 1987; Webster et al., 1985). Reports have suggested that Chinese farmers have routinely used amantadine and rimantadine in the animal reservoir to combat H9 subtype viruses (Cyranoski, 2005; Parry, 2005). The Chinese ministry of agriculture have denied approving or permitting their use. However, their use in poultry would explain how H5N1 in the region has acquired resistance.

1.7.5 Vaccination

Avian influenza vaccines serve to increase resistance to infection, prevent clinical disease and limit viral shedding from the cloaca and oropharynx (vaccine use in domesticated poultry has been reviewed by Capua and Alexander, 2008; Capua et al., 2004; Suarez, 2005; Swayne, 2009, 2012). Currently, the majority of AIV vaccines available include inactivated vaccines, which include monovalent vaccines containing either the H5 or H7 strains, bivalent that include both strains. These require subcutaneous or intramuscular administration, which can be labour intensive, costly and to ensure a good antigenic match, must be re-evaluated every 2-3 years (Swayne et al., 2014). Vaccine use varies across the world with some countries documenting routine use whereas others vaccinate infrequently. It is estimated that 99% of vaccine use is in China, Egypt, Indonesia and Vietnam where HPAI H5N1 is endemic (Swayne et al., 2011). In the developing world vaccination is considered one of the main methods of control as domestic birds are often farmed in flooded rice paddies, open fields, or bodies of water where biosecurity measures are impractical. There are a number of recombinant live-vectored vaccines for H5N1 reported in the literature. Where fowl poxvirus, Newcastle disease viruses, herpes virus of turkeys that vector H5 HA have been licenced for use. They have several advantages over inactivated vaccines as they may be administered by aerosol or in the drinking water and in the case of herpes virus the vaccine may be given in ovo this means they are economical and much easier to administer. Additionally these
vaccines have been shown to elicit a good immune response. However, they are not recommended for use because they have been associated with respiratory disease, a reduction in egg production and have potential to reassort/mutate into pathogenic subtypes where they can easily be spread between birds (Lee and Suarez, 2005).

Table 1.4 Novel experimental technologies for poultry vaccines for protection against highly pathogenic influenza. Table adapted from (Spackman and Swayne, 2013).

<table>
<thead>
<tr>
<th>Technology</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Live vaccines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vectored Vaccines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fowl Pox virus</td>
<td>DIVA compatible</td>
<td>Requires handling of individual birds</td>
<td>(Liljebjelke et al., 2010; Steel et al., 2008; Taylor et al., 1988)</td>
</tr>
<tr>
<td>Newcastle disease virus</td>
<td>May be administered by mass application</td>
<td>Unknown long-term safety</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Maybe administered with other vaccines</td>
<td>Maternal antibodies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Elicits effective immunity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DIVA compatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herpes virus of turkeys</td>
<td>Administered in mass</td>
<td>Requires handling of individual birds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>May be administered in ovo</td>
<td>Needs cold storage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(and escape interference from maternal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>antibodies)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>Induction of mucosal immunity</td>
<td>Long-term safety unknown</td>
<td></td>
</tr>
<tr>
<td></td>
<td>May be administered in mass</td>
<td>Challenge data suggests incomplete</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DIVA compatible</td>
<td>protection</td>
<td></td>
</tr>
<tr>
<td><strong>Live attenuated vaccines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold adapted</td>
<td>Induces immunity</td>
<td>Safety unknown</td>
<td>(Steel et al., 2009)</td>
</tr>
<tr>
<td>Inactivated vaccines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole virus</td>
<td>Safe</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Can be used with adjuvants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Easily updated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DIVA compatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvants</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mineral oil</td>
<td>Inexpensive</td>
<td>Requires handling of individual</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Efficacious</td>
<td>birds</td>
<td></td>
</tr>
<tr>
<td>CD154</td>
<td>Induces good immunity</td>
<td>No challenge data</td>
<td></td>
</tr>
<tr>
<td>Liposomes</td>
<td>Effective as adjuvant</td>
<td>No challenge data</td>
<td></td>
</tr>
</tbody>
</table>
1.7.6 Genetic resistance

There is relatively little research focused on naturally occurring resistance to AIV in poultry or the genetic basis thereof. One recent approach however has been to genetically modify chickens to shed less virus and lower the rate of transmission. Lyall et al. (2011) engineered an RNA expression cassette that allows for the production of a short-hairpin RNA that acts as a competitive inhibitor of the polymerase complex and this interferes with viral replication. This ‘decoy RNA’ is expressed in all the cells of the chicken and the authors reported that following exposure to virus the transgenic birds succumbed to infection but were less able to transmit virus to both non-transgenic and transgenic cagemates. These studies provide proof-of-principle for the use of genetic modification for controlling AI in the field and this research now focuses on the identification of further potential decoys with the aim of introducing chickens that are resistant to infection. Such technology limits scope for the evolution of resistance as the decoy match short conserved regions within the eight viral genome segments, therefore mutations of all eight vRNAs would be required to acquire resistance. Moreover, mutations that escape the decoy strategy may reduce viral fitness as the terminal untranslated regions of the RNA segments are necessary for transcription and translation of the genome. There are a number of different stages within the influenza A viral life cycle that may be targeted by GM approaches for example, innate immune responses may be boosted to fight infection or it may be possible to alter the sialic acid receptor profile to weaken viral binding. However, public perception of the safety of GM products and hesitation on the part of industry to fund and commit to GM technologies may limit the field applications of transgenic birds, at least in the short-term. In addition to this work, studies investigating the genetic differences of AIV pathogenicity between domestic birds (such as ducks and chickens) have been
performed and include the work of Smith et al. (2015). Such studies hope to identify the contributing factors involved in the differing pathogenicity phenotypes between bird species and that may be bred into domestic poultry for resistance against infection.

1.8 Aims

This study aimed to evaluate the use of two anti-viral peptide families as protective agents against LPAI. First, work aimed to define the anti-viral activity of FluPep and Entry Blocker peptides against a panel of influenza A viruses with diverse HA subtypes, with focus on avian influenza strains. Second, peptide mode of action studies were performed through optimisation of a series of viral entry assays that singled out the individual stages of the virus replication cycle. Finally, this study evaluated a novel approach of delivering the peptides to poultry. This work aimed to engineer avian Lactobacillus intestinal isolates to express the peptides in a secreted form, thus providing a cheap and easy to administer prophylactic agent against LPAI.
2.1 Introduction

Wild birds are the natural reservoir hosts of influenza A viruses and infection within bird species can produce a variety of outcomes. The ability of IAV to cause disease is both subtype and avian species dependent. In domestic birds such as chickens or turkeys, avian influenza may be classified based on pathogenesis; low pathogenic avian influenza (LPAI) tends to circulate silently between birds with clinical signs limited to ruffled feathers and a reduction in egg production. However, high pathogenicity avian influenza (HPAI) outbreaks cause systemic infection with rapid transmission of virus and high mortality. The variable pathogenicity of influenza in avian species means that it is difficult to predict the cost of the next outbreak. Outbreaks therefore pose major threats to the poultry industry and the national food supply, and are currently controlled by implementing measures that involve surveillance, closure of live poultry markets and mass culling. Considering the uncertainty over whether certain strains will adapt to human transmission and limitations over how their spread may be controlled, this chapter considers the use of two classes of anti-viral peptides as broad-spectrum protective agents against LPAI.

Research initiated by Professor Anthony Nash focused on a family of short (around 12 amino acids in length) hydrophobic, anti-viral peptides collectively called FluPep (Table 2.1). The design of FluPep and its derivatives originated from the work of Flowers et al. (2004) who synthesised peptide mimetics of the tyrosine kinase inhibitory region (Tkip) located at the N-terminus of suppressor of cytokine signalling-1 (SOCS-1) proteins (section 1.5.1). Their design and use are reviewed by Ahmed et al. (2015). Studies within the Nash laboratory evaluated the activity of TKip and FluPep derivatives against influenza A and B viruses (Nicol et al., 2012).
FluPep was shown to protect against A/WSN/33 and A/PR/8/34 recombinant viruses containing the HA and NA glycoproteins from human pandemic H1N1 and H3N2 viruses. Anti-viral activity was dose-dependent and observed within the micromolar range. Furthermore, the anti-viral properties of FluPep were successfully translated in vivo, where intranasal instillation of the peptide in the murine model reduced viral replication in the lungs, and decreased weight loss and clinical signs upon experimental infection (Nicol et al., 2012).

A second family of peptides termed Entry Blocker (EB) were found to be protective against influenza A virus following a screen of cell-penetrating peptides (Jones et al., 2006); section 1.5.2. The authors found that EB, derived from a fibroblast growth factor-4 signal peptide, was able to inhibit a number of different influenza subtypes, which included: A/PR/8/34 (H1N1), A/Singapore/1/57 (H2N2), A/Turkey/England/69 (H3N2), and A/Turkey/Oregon/71 (H7N3), suggesting that EB possesses broad-spectrum anti-influenza virus activity. The authors proposed EB to act via a mechanism involving the blocking of HA function as they observed a significant reduction in fluorescently-labelled virus attachment to MDCK cells following treatment relative to experimental controls (Jones et al., 2006). In vivo experimental work showed a reduction of viral lung titres of BALB/c mice treated intranasally with EB prior to infection compared to control (Jones et al., 2006). In later studies, Jones et al. (2011a) were able to pinpoint the residues of EB critical for maintaining anti-viral activity by systematically introducing truncations of the peptide. They identified a peptide named B10\textsuperscript{NP}, which was seven amino acids shorter than the original EB peptide that retained full anti-viral activity; this B10\textsuperscript{NP} peptide is included in this present study.

This study expands our current understanding of these peptide families by determining their anti-viral activity against a panel of influenza A strains with diverse HA subtypes. This panel includes LPAI and A/PR/8/34 a lab adapted influenza strain, which may be purified easily in containment level two laboratories
for the later studies of peptide action by optimisation of virus entry assays. Where viruses with different HA/NA subtypes differ in sensitivity to peptide. This study sought to determine the contribution of HA/NA to this phenotype by using reverse genetics to obtain PR/8 recombinant viruses with different HA/NA segments.

Table 2.1 Peptides used in the present study. Name, primary sequence, size, source and description of FluPep and Entry blocker peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Abbreviation</th>
<th>Sequence</th>
<th>Mw (Da)</th>
<th>Reference</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tkip (FluPep 1)</td>
<td>FP1</td>
<td>WLVFFVIFYFFR</td>
<td>1684</td>
<td>(Flowers et al., 2004)</td>
<td>Mimetic of SOCS-1 kinase inhibitory region</td>
</tr>
<tr>
<td>FluPep 4</td>
<td>FP4</td>
<td>RRKKWLVFFVIFYFFR</td>
<td>2252.7</td>
<td>(Nicol et al., 2012)</td>
<td>Tkip with additional charged residues to improve solubility</td>
</tr>
<tr>
<td>Entry Blocker</td>
<td>EB</td>
<td>RRKKAAVALLPAVLLALLAP</td>
<td>2084.7</td>
<td>(Jones et al., 2006)</td>
<td>Fibroblast growth factor-4 signal peptide derivative</td>
</tr>
<tr>
<td>B10&quot;&quot;&quot;</td>
<td>B10</td>
<td>RRKKAAVALLPAVLLALLA</td>
<td>1351.8</td>
<td>(Jones et al., 2011)</td>
<td>Minimum active sequence of EB</td>
</tr>
<tr>
<td>FluPep 2</td>
<td>FP2</td>
<td>WLVFFVIYFAR</td>
<td>1531.8</td>
<td>(Nicol et al., 2012)</td>
<td>Substitution of phenylalanine to alanine to allow JAK2 activation thus, separating the anti-viral and anti-inflammatory properties of the peptide</td>
</tr>
<tr>
<td>RRKK-FluPep 2</td>
<td>RRKK-FP2</td>
<td>RRKKAAVALLPAVLLALLAP</td>
<td>2100.9</td>
<td>(Bacon, 2013)</td>
<td>Addition of ‘RRKK’ residues to N-terminus of FP2</td>
</tr>
<tr>
<td>FluPep 3</td>
<td>FP3</td>
<td>WLVFFVIYFRRRRKK</td>
<td>2252.7</td>
<td>(Nicol et al., 2012)</td>
<td>Addition of ‘RRKK’ residues to N-terminus of FP1</td>
</tr>
<tr>
<td>FluPep 7</td>
<td>FP7</td>
<td>RRKKFYFFR</td>
<td>1460.7</td>
<td>(Nicol et al., 2012)</td>
<td>Truncated Tkip derivative</td>
</tr>
</tbody>
</table>

Table 2.2 Influenza A viruses used in this study (source and reference listed in 5.1.6) The haemagglutinin virus subtypes are further classified according to structural differences with the region involved in influenza virus fusion.

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Subtype</th>
<th>Phylogenetic grouping</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td>H1N1</td>
<td>Group 1</td>
<td>A/WSN</td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>H1N1</td>
<td>Group 1</td>
<td>PR/8</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10</td>
<td>H1N1</td>
<td>Group 1</td>
<td>A/Mall</td>
</tr>
<tr>
<td>A/Turkey/Canada/65</td>
<td>H3N2</td>
<td>Group 2</td>
<td>A/Turk/Can/65</td>
</tr>
</tbody>
</table>
2.2 Results

2.2.1 Cytotoxicity of peptides on MDCK and A549 cells

In order to exclude any indirect anti-viral effects resulting from cytotoxicity, the toxic effect of peptides on MDCK and A549 cells was determined. Increased cell death following peptide treatment could lead to reductions in virus titre simply because of a loss of viable cells. The effect of peptide treatment on cell viability was evaluated using a commercially available CellTitre-Glo® Luminescent Cell Viability kit. This kit estimates cultured cell viability and/or numbers by quantifying the amount of adenosine triphosphate (ATP) present in a culture dish. The protocol requires the addition of a reagent composed of a detergent and luciferase-based enzyme to treated cells. This reagent lyses the cells to release ATP, inhibits endogenous ATPases and provides luciferin and luciferase to measure ATP using a luminescent output.

First, because the inhibitory peptides are typically dissolved in DMSO (Nicol et al., 2012), the effect of the solvent on cell viability was tested. MDCK and A549 cells were treated for 48 hrs with increasing amounts of DMSO before lysis and measurement of ATP content. Treatment with DMSO at concentrations of up to 1 % (v/v) had little effect on cell viability for either cell line (Figure 2.1a). A DMSO concentration that caused a decrease in cell viability of 50 % (CC50) was not reached, but 2.2 % of the solvent decreased ATP content by ~ 40 % compared to an untreated control (Figure 2.1a). Using the previously published conditions for in vitro analyses of peptide activity and mode of action experiments (Nicol et al., 2012) requires a concentration of 1.5 % (v/v) DMSO for peptide delivery. Although 48 hrs of DMSO treatment at this concentration would be expected to reduce MDCK and A549 cell “viability” by around 20 % compared to untreated controls, this minor reduction was viewed as acceptable for the sake of consistency with previous studies.
Figure 2.1 Cytotoxicity of cell penetrating peptides on MDCK and A549 cells. MDCK (black) and A549 (blue) cells were treated with increasing concentrations of (a) DMSO (b) FP4 (c) FP1 (d) EB or (e) B10NP in serum free media (SFM). 48 hrs post treatment cell viability was determined using the CellTitre-Glo® viability assay. (a); DMSO control data are plotted as percentage viability relative to untreated control. (b-e); Peptide data are plotted as the percentage viability related to 1.5 % (v/v) DMSO control cells. The mean percentage ±SEM of two independent experiments each performed in duplicate are shown. Where possible, 50% cytotoxicity concentration (CC50) values are indicated by linear interpolation from the graph.
Next, the effect of peptide treatment on MDCK and A549 cell viability was determined. Cells were treated for 48 hrs with increasing quantities of peptide with a constant final concentration of 1.5% (v/v) DMSO before ATP content was measured as above. Both cell lines tolerated FluPep (1 and 4) treatment at concentrations of up to 100 µM without reductions in luminescence, relative to controls (Figs 2.1b and c respectively). However, at the highest concentration tested (200 µM), decreases in metabolic activity were observed. A CC$_{50}$ value of 200 µM was obtained for FP4 treatment of A549 cells, but the CC$_{50}$ values for FP4 treatment of MDCK cells and FPI treatment of both cell lines exceeded 200 µM. Similarly to FluPep treatment, both cell lines tolerated Entry Blocker (EB and B10NP) treatment at concentrations of up to 100 µM without reductions in luminescence relative to untreated controls. However, at the highest concentration of 200 µM, decreases in metabolic activity were observed. While the CC$_{50}$ values exceeded 200 µM, declines in apparent cell viability of up to 43% were recorded following peptide treatment. Therefore, the peptides could be tested in vitro at concentrations up to at least 100 µM without major effects on cell viability.

2.2.2 Anti-viral activity of peptides against LPAI

Previous studies have shown FluPep to be active against A/WSN/33 and PR/8 recombinant viruses with the surface glycoproteins of human outbreak virus strains A/England/195/09 (H1N1), A/Victoria/3/75 (H3N2) and A/Vietnam/1194/04 (H5N1) (Nicol et al., 2012). In order to test whether the peptides were active against a panel of non-reassorted influenza A virus with more diverse HA subtypes including LPAI strains, plaque reduction assays (PRA) were performed. Here, the effect of peptide treatment on viral replication was quantified using the ability of the virus to form lytic plaques within an MDCK cell monolayer.
Figure 2.2. Plaque reduction assay (PRA) of FP4 activity against A/Mallard/Netherlands/10. MDCK cell monolayers were mock or virus infected with 250 pfu/mL in the presence of vehicle DMSO (1.5 % v/v final concentration) or increasing quantities of peptide (0.001-10 µM). Virus replication was assayed using the ability of the virus to form lytic plaques within the cell monolayer. 48 hrs post infection (p.i.) plates were fixed and plaques visualized by toluidine blue staining.
PRA were performed according to previously published conditions (Nicol et al., 2012). Briefly, cell monolayers were infected with 250 pfu/well of virus in the presence of increasing quantities of peptide (0.0001-10 µM) dissolved in a constant (final) concentration of 1.5% (v/v) DMSO. Forty-eight hours post-infection (p.i), the cells were fixed and the plaques visualised using toluidine blue staining. A representative assay with A/Mall infected cells in the presence of increasing quantities of FluPep 4 is presented (Figure 2.2). In this experiment, infection of cells with 250 pfu/mL of virus resulted in the formation of around 80-120 plaques (Figure 2.2, virus control) that were unaffected by 1.5% (v/v) DMSO treatment (vehicle control). Typically, no reductions in plaque number were observed following peptide treatment at the lower concentrations (0.0001-0.01 µM) and the peptides had no effect on plaque size. However, treatment with more than 0.1 µM peptide resulted in reductions in the number of plaques with complete inhibition of plaque formation following 10 µM of peptide.

The IC₅₀ values for FluPep 1 and 4 against A/WSN/33 have been previously reported to be 0.09 µM and 0.00004 µM, respectively (Nicol et al., 2012). In the first instance these experiments were repeated as controls. Data for peptide activity against A/WSN/33 was plotted as percentage titre (pfu) relative to DMSO treated control cells, and fitted to a dose response curve by non-linear regression using Prism 6.0 (Figure 2.3a). The concentration required to cause a 50% decrease in viral replication was then determined based on the curve fitting (Table 2.3). At the lower concentrations of FluPep (0.0001 µM) plaque formation was reduced by around 10-20% relative to DMSO treated cells with near complete inhibition at 10 µM. The curve showed a gradual reduction of plaque formation with increasing quantities of FluPep. In these experiments FluPep 4 was a more potent inhibitor of A/WSN/33 than FluPep 1, as an IC₅₀ of 0.0078 µM was recorded for FluPep 4 and 0.02 µM for
Figure 2.3. Activity of peptides against influenza A virus infection as assessed by PRA. Plaque reduction activity of FP1 (gold), FP4 (black) EB (red) and B10NP (blue) was assayed against (a) A/WSN/33 (H1N1) (b) A/PR/8/34 (H1N1) (c) A/Mallard/Netherlands (H1N1) (d) A/Duck England/62 (H4N6) and (e) A/Turkey/Canada/65 (H3N2). Data are plotted as percentage plaque forming units relative to DMSO treated control samples and are fitted to a dose response curve by non-linear regression using Prism 6.0. The mean percentage ± SEM is shown of two independent experiments each performed in duplicate.
FluPep 1 against A/WSN/33. Overall, it was possible to detect the anti-viral activity of FluPep against A/WSN/33 at concentrations with the same relative potencies as Nicol et al. (2012). FluPep 4 was found to be more active than FluPep 1, which was in line with previously published work but the values reported in these experiments were generally higher. For example the IC$_{50}$ for FluPep 1 here was approximately 4.5 fold higher, the reasons for the increased IC$_{50}$ values are not clear but may be related to the different batches of synthetic peptide or different DMSO. This PRA system did however provide a quantifiable assay, which could be plotted as dose response curves.

The effects of Entry Blocker peptides (EB & B10$^\text{NP}$) on A/WSN/33 replication were then tested. EB treatment of A/WSN/33 resulted in minimal reduction of plaque formation at the higher quantities of peptide. While the truncated EB derivative (B10$^\text{NP}$) was not active at lower concentrations (Figure 2.3a), the peptide exhibited dose dependent anti-viral activity at concentrations greater than 0.1 µM. All the active peptides were able to substantially inhibit A/WSN/33 replication at 10 µM.

Table 2.3 Anti-viral activity of peptides against influenza A virus with different HA subtypes. IC$_{50}$ data was determined from PRA data and is displayed ± 95% confidence interval.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>IC$_{50}$ (µM) ±95% confidence interval (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FluPep 4</td>
</tr>
<tr>
<td>A/WSN/33</td>
<td>H1N1</td>
<td>0.008 (0.004-0.02)</td>
</tr>
<tr>
<td>A/PR8/34</td>
<td>H1N1</td>
<td>0.2 (0.1-0.4)</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10</td>
<td>H1N1</td>
<td>0.09 (0.04-0.2)</td>
</tr>
<tr>
<td>A/Duck/England/62</td>
<td>H4N6</td>
<td>0.5 (0.3-0.7)</td>
</tr>
<tr>
<td>A/Turkey/Canada/65</td>
<td>H3N2</td>
<td>0.6 (0.4-0.8)</td>
</tr>
</tbody>
</table>

Next, the peptides were tested for anti-viral activity against a number of influenza virus subtypes. These included (H1N1) PR/8 and various LPAI viruses: (H1N1) A/Mall, (H4N6) A/Duck/England/62, and (H3N2) A/Turkey/Canada/65.
As with A/WSN/33, EB did not reduce the replication of any of the viruses tested. FluPep and B10\textsuperscript{NP} did not affect plaque formation at the lower concentrations of peptide (0.0001-0.001 \(\mu\text{M}\)) but at concentrations higher than 0.001 \(\mu\text{M}\) they inhibited viral replication in a dose-dependent manner (Figure 2.3 b-e).

Analysis of peptide activity was largely based on the curve fittings of the dose response curves and therefore to better understand how accurately the curve fit about the data, a regression analysis was performed (Table 2.4). The R-squared values for each of the curves were determined (Table 2.4). Values of above 0.9 were observed for all the active peptides against A/Turkey/Canada/65 and FluPep 1 and B10\textsuperscript{NP} against PR/8. Values of above 0.9 indicated that the dose response curves that fitted closely to the data, which in turn suggested that the values (such as IC\textsubscript{50}) extrapolated from the curves were accurate relative to values of below 0.9. However, lower R\textsuperscript{2} values (0.7-0.9) were observed for FluPep 1 against A/Mall and A/Duck/England/62, since the data were relatively scattered around the curve, perhaps because of variability in the data. Against this panel of viruses, the IC\textsubscript{50} values for FluPep 1 were lower relative to the other peptides tested, suggesting that the peptide was a more potent inhibitor of viral replication than FluPep 4 and B10\textsuperscript{NP} (Table 2.3).

**Table 2.4 Regression analysis.** The R\textsuperscript{2} value is a statistical measure of how well the data fits the regression line the values were calculated using Prism 6.0.

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Subtype</th>
<th>FluPep 4</th>
<th>FluPep 1</th>
<th>B10NP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td>H1N1</td>
<td>0.85</td>
<td>0.82</td>
<td>0.97</td>
</tr>
<tr>
<td>A/PR/8/34</td>
<td>H1N1</td>
<td>0.8</td>
<td>0.96</td>
<td>0.9</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10</td>
<td>H1N1</td>
<td>0.89</td>
<td>0.7</td>
<td>0.81</td>
</tr>
<tr>
<td>A/Duck/England/62</td>
<td>H4N6</td>
<td>0.91</td>
<td>0.78</td>
<td>0.75</td>
</tr>
<tr>
<td>A/Turkey/Canada/65</td>
<td>H3N2</td>
<td>0.94</td>
<td>0.97</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Differences between the steepness of the dose-response curves for some of the active peptides were noticeable (for example the dose response curve of B10\textsuperscript{NP} versus FluPep 4 in Figure 2.3a). As a means of quantifying these differences, the Hill coefficient, usually defined as a measure of cooperativity was calculated using Prism 6.0 based on the curve fittings (Table 2.5). For these experiments, the Hill coefficient could be used to describe the cooperativity of peptide binding to the site of action. Generally, the Hill coefficient values were around one, suggesting that peptide affinity for binding was not dependent on other ligands/peptides already bound. However, the Hill coefficient values recorded for FluPep 4 and B10\textsuperscript{NP} (with the exception of FluPep 4 against A/WSN/33) were above one and perhaps suggestive of cooperative binding (Table 2.5), where the binding of one peptide increases the affinity of other peptides to bind to the site of ‘inhibition’. On the other hand, Hill coefficient values for FluPep 1 were all below one, suggesting that repeated binding may have decreased the affinity of further peptides from binding.

### Table 2.5 The Hill coefficient analysis of the steepness of the dose response curves.

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Subtype</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td>H1N1</td>
<td>0.5</td>
</tr>
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<td>A/PR/8/34</td>
<td>H1N1</td>
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</tr>
<tr>
<td>A/Mallard/Netherlands/10</td>
<td>H1N1</td>
<td>2.4</td>
</tr>
<tr>
<td>A/Duck/England/62</td>
<td>H4N6</td>
<td>1.5</td>
</tr>
<tr>
<td>A/Turkey/Canada/65</td>
<td>H3N6</td>
<td>1.5</td>
</tr>
</tbody>
</table>

To facilitate comparison of the relative IC\textsubscript{50} values (Table 2.3), data were plotted in bar graph form (Figure 2.4). The data showed little variation in the sensitivity of each viral subtype to B10\textsuperscript{NP} treatment, where a maximum IC\textsubscript{50} of 3.82 µM was recorded against A/Turkey/Canada (H3N2) and a minimum of 0.62 µM
Figure 2.4 The 50% inhibitory concentration (IC\textsubscript{50}) for antiviral peptides. The IC\textsubscript{50} values for each peptide were calculated from the dose response curves and are plotted ±95% confidence interval. Phylogenetic group I (grey) and II (blue) of HA are indicated. Data are from at least two independent experiments performed in duplicate.
for A/WSN/33 (H1N1), giving an approximate 3-fold difference in sensitivity. In contrast however, the IC<sub>50</sub> data revealed strain specific differences in sensitivity to FluPep, with an approximately 100-fold difference between the relatively ‘sensitive’ and ‘resistant’ virus subtypes. FluPep 4 was a potent inhibitor of A/WSN/33 with a recorded IC<sub>50</sub> of 0.0078 μM; this was lower than all other viruses tested. FluPep 1 was most efficacious against A/Mall (0.0067 μM) but approximately 100 times more peptide was required to reduce A/Turkey/Canada/65 replication (IC<sub>50</sub>0.62 μM).

Overall, these plaque reduction assays revealed that FluPep and B10<sup>NP</sup> were able to inhibit viral replication in a dose-dependent manner within the micromolar range. Both peptide families displayed broad-spectrum activity with some strain-dependent variation, but generally FluPep peptides were more potent inhibitors of LPAI virus replication than B10<sup>NP</sup>. Data also revealed that group I subtype viruses (H1, grey) to be more sensitive to FluPep treatment relative to group II HA subtypes (blue). These differences may perhaps be explained by the structural differences of HA between the two phylogenetic groupings, though analysis of a wider sample of viruses of each HA phylogenetic group will be required to establish robust conclusions.

### 2.2.3 Action of FluPep on viral surface glycoproteins

The strain-specific differences in sensitivity to FluPep (observed in Figure 2.4b) were then used to test whether the anti-viral effects of the peptides were a result of a direct interaction with viral surface glycoprotein. As A/Mall was observed to be approximately 2.5-fold more sensitive to FluPep 4 treatment and 10-fold more sensitive to FluPep 1 than PR/8, the effect of FluPep on the surface glycoprotein was tested by reassorting in the HA and NA of A/Mall into the less sensitive PR/8 backbone using reverse genetics. It is therefore expected that if the peptides targeted one of the glycoproteins then the introduction of ‘susceptible’ HA and/or NA would therefore be predicted to reduce the IC<sub>50</sub> of PR/8.
2.2.3.1 Recovery of recombinant virus by reverse genetics

Recombinant viruses were rescued using the eight-bidirectional promoter plasmid system previously described by de Wit et al. (2004). Briefly, sets of pDual vector constructs encoding the eight gene segments of A/Mall and PR/8 kindly received from L. Tiley (Bourret et al., 2012) and R. Fouchier (de Wit et al., 2004) respectively, were transfected into 293-T cells. The constructs contained cDNA from each virus segment inserted between a human RNA polymerase I promoter (pol I) and terminator sequence. The pol I transcription unit is flanked in the opposite orientation by an RNA polymerase II promoter and polyadenylation site. The orientation of the two transcription units allows for the synthesis of negative-sense viral RNA required for production of progeny vRNPs and positive-sense mRNA for translation of viral proteins (Hoffmann et al., 2000). Transfection of 293-T cells resulted in the generation of a passage zero (P0) stock, which were of low titre and therefore were further propagated in MDCK cells to generate passage one (P1) stocks (Figure 2.5). For these experiments wildtype parental viruses were rescued alongside reassortant viruses that have a PR/8 backbone containing either segment 4 or 6 from A/Mall. A 6:2 reassortant virus containing both HA and NA from A/Mall was also generated for these studies (Table 2.6a).

2.2.3.2 Plaque and HA titres of recombinant viruses

Following virus rescue, the HA and infectious titres of each recombinant virus were obtained (Table 2.6b). Wild-type (WT) PR/8 grew to 6x10⁷ pfu/ml in MDCK cells, while A/Mall grew to 7x10⁶ pfu/ml, which was approximately one log₁₀ lower than PR/8. All the reassortant viruses grew to high titres; PR/8 viruses containing A/Mall HA grew similarly to WT PR/8, while PR/8-A/Mall NA grew
Figure 2.5 Eight-plasmid reverse genetics system (a) pDual vector constructs encoding the eight gene segments of influenza virus are transfected into 293-T cells. Each viral vector contain human polI and pol II promoters that allow for synthesis of viral mRNA for eventual translation of virus protein and vRNA for generation of progeny vRNPs (b) 293-T transfection resulted in the generation of a ‘passage zero’ stock which is generally of low titre and therefore propagated further by (c) amplification in either MDCK cells or embryonated hen’s eggs to make a passage one stock (P1).
to 3X10⁶ pfu/ml. The haemagglutination (HA) titres were also determined using standard methods with 1% chicken red blood cell suspension. HA assays showed that WT PR/8 (HA titre, 64) grew better than WT A/Mall (HA titre, 16). Compared to the WT PR/8, the HA titres were reduced with the introduction of HA with the 6:2 reassortant having the same titre as WT A/Mall. Conversely however, PR/8-A/Mall NA had a titre of 128. An HA to plaque forming unit (pfu) ratio (HA:titre) was then calculated relative to PR/8 in order to give a rough estimate of virus particle:pfu ratio.

Table 2.6 Description of the gene segments contained within each recombinant virus. (a) the origin of each gene segment for the recombinant viruses are shown with PR/8 segments labelled in black and A/Mall in red (b) infectivity titres (pfu), haemagglutination titres (HA) and values for the ratio of particle:pfu relative to PR/8 are also included

<table>
<thead>
<tr>
<th>A</th>
<th>PR/8</th>
<th>PR/8 7:1 Mal</th>
<th>PR/8 7:1 Mal</th>
<th>PR/8 6:2 Mal</th>
<th>Mal</th>
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<tbody>
<tr>
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<td>PR/8</td>
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<td>PR/8</td>
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<tr>
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<td>PR/8</td>
<td>PR/8</td>
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<table>
<thead>
<tr>
<th>B</th>
<th>HA titer</th>
<th>64</th>
<th>32</th>
<th>128</th>
<th>16</th>
<th>16</th>
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<tr>
<td>PFU</td>
<td>6x10⁷</td>
<td>3.79x10⁷</td>
<td>3.1x10⁶</td>
<td>2.04x10⁷</td>
<td>7.17x10⁶</td>
<td></td>
</tr>
<tr>
<td>HA titer normalised to PR/8</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>PFU titer normalised to PR/8</td>
<td>1</td>
<td>0.631</td>
<td>0.051</td>
<td>0.34</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>relative particle:pfu</td>
<td>1</td>
<td>0.8</td>
<td>40</td>
<td>0.7</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>
Swapping in A/Mall HA into a PR/8 backbone did not affect the virus particle to pfu ratio as both the PR/8 7:1 A/Mall HA and PR/8 6:2 A/Mall HANA had a particle:pfu that was comparable to WT PR/8. However, reassortment of A/Mall NA resulted in an increase in the particle:pfu ratio of around 40-fold relative to WT PR/8.

2.2.3.3 PR/8-Mall recombinant virus plaque morphology

Plaque morphology as one method of confirming successful reassortment was determined by plaque assay as described in section 2.2.3.3 (Figure 2.6). MDCK cell monolayers were infected with 10-fold serial dilutions of virus and fixed 48 hrs p.i. Plaques were visualised with toluidine blue staining (Figure 2.6a), and by immunostaining of NP (Figure 2.6b). Wild-type parental viruses formed large plaques but introduction of A/Mall segment 4 (HA) to PR/8 resulted in a marked reduction of plaque size. Conversely, PR/8 plaques were marginally increased by the introduction of A/Mall segment 6 (NA). Altered plaque morphology was consistent with the generation of the reassortant viruses.

2.2.3.4 Validation of recombinant viruses by haemagglutination inhibition assay

Validation of the recombinant viruses initially began by using haemagglutination inhibition assays (HAI) with anti-PR/8 serum (Figure 2.7). HAI assays were performed to determine whether segments 4 and 6 were successfully reassorted into the desired backbone (as per Table 2.6). Briefly, 8 HA units of each virus were added to serial dilutions of anti-PR/8 serum, and incubated for thirty minutes before the plates were overlaid with 1% chicken red blood cells (cRBC). Negative PBS and anti-PR/8 serum controls lacking virus confirmed that the 1% cRBC used did not non-specifically agglutinate. WT PR/8 antibodies present in the sera neutralised virus surface HA and prevented cRBC agglutination up to sera dilutions of 1:80. However, A/Mall HA was not reactive to the PR/8 sera and addition of A/Mall virus to the assay led to cRBC agglutination. Next, the
Figure 2.6 PR/8-Mall recombinant virus plaque morphologies. Representative images of PR/8-Mall recombinant virus plaques on MDCK cell monolayers using conditions described in 5.4.4. MDCK cells were fixed 48 hrs p.i. and visualised using either (a) toluidine blue staining or (b) immuno-staining for viral NP.
Figure 2.7 Validation of recombinant viruses by haemagglutination inhibition assay. Eight HA units of each virus were added to serial dilutions of anti-PR/8 serum incubated for 30 mins and overlaid with 50 µL of 1% chicken red blood cells, the plates were incubated for a further 30 mins prior to scanning. Influenza A virus HA binds to sialic acid receptors on the surface of red blood cells (RBCs) but the viruses are not able to enter the cells. RBCs not bound to virus sink to the bottom of a v-bottom microtitre plate but where HA binds the RBCs, the cells form a lattice.
remaining reassortant viruses were tested and as with the WT PR/8 the anti-PR/8/34 serum prevented PR/8 7:1 A/Mall NA virus mediated agglutination of cRBC, whilst the viruses containing A/Mall HA agglutinated the cRBC. Overall, results from the HAI assay were consistent with successful reassortment of segment 4 and 6 into each virus.

2.2.3.5 Sequence validation of recombinant viruses

To confirm that each recombinant virus carried the desired HA and NA, vRNA was extracted from the virus stock and amplified by RT-PCR using terminal Uni12 primers. Forward and reverse oligonucleotide primers were then designed to complement regions specific to PR/8 and A/Mall segments 4 and 6 to allow amplification of a fragment from each gene. Additionally, segment 7 primers that anneal to non-coding viral regions were used to validate the virus backbone (Figure 2.8a). PCR was performed using both the PR/8 and A/Mall primer sets and the resulting PCR products were visualised by agarose gel electrophoresis (Figure 2.8b). DNA products were not observed in the ‘no template’ control reactions confirming that PCR amplification was a direct result of viral cDNA, (lanes 30-34). Amplification of PR/8 and A/Mall segment 7 resulted in DNA fragments of 1030bp for both viruses (Figure 2.8b, in the lanes denoted ‘7’) confirming vRNA extension and cDNA synthesis.

Considering the wild-type virus controls first, A/Mall specific HA and NA primers gave products of the expected size with no products visible when amplification was completed using PR/8 segment 4 and 6 primers (Figure 2.6b, lanes 24-28). As with A/Mall, the PR/8 specific HA and NA primers gave products of the expected size (Figure 2.8b). No products were visible when amplification was attempted using the A/Mall segment 6 primers (lane 4). However, a small DNA fragment was observed in lane 3, where RT-PCR reactions contained ‘supposed’ PR/8 cDNA transcript only. These PCR products were sent for sequencing and
were identified as PR/8 HA using BLASTn (data not shown). This suggested that the primers designed to anneal to A/Mall HA were also able to amplify a smaller fragment of PR/8 HA, which is a result of primer mismatch. Then, RT-PCR was performed on the reassortant viruses using both the A/Mall and PR/8 segment 4 and 6 primers (Figure 2.8b). The presence of product of the expected size within each sample demonstrated successful reassortment of each glycoprotein gene into the recombinant virus. Additionally, the PCR products from Figure 2.8 were sent for DNA sequencing and the identity of each fragment was determined using BLASTn (data not shown). Sequencing analysis showed that all the recovered viruses contained the required glycoprotein segments.

### 2.2.4 Anti-viral activity of FluPep against PR/8-A/Mall recombinant viruses

In order to test whether the observed anti-viral activity of FluPep was a consequence of an interaction with viral surface glycoproteins, the activity of FluPep against PR/8-A/Mall recombinant viruses was determined. Peptide activity was tested by plaque reduction assay, as described in section 2.2.2. MDCK cell monolayers were infected with 250 pfu/mL of virus in the presence of increasing quantities of FluPep 4 (0.0001-10 µM). 250 pfu/mL of virus was used in order to maintain consistency with previous work (Nicol et al., 2014). Cell monolayers were fixed 48 hrs p.i and plaques were visualised using either toluidine blue or immunostaining (as per 2.2.4.3). Data were plotted relative to the DMSO treated controls and a dose-response curve was fitted as described earlier (section 2.2.2). The IC$_{50}$ values were calculated based on the curve fit (Table 2.7). Because the IC$_{50}$ for FluPep against PR/8 and A/Mall had already been determined (Table 2.3), the experiment was repeated as a control for the assay. The IC$_{50}$ values for FluPep 4 against PR/8 (0.32 µM) and A/Mall (0.13 µM) were found to be marginally increased compared to the values reported in earlier experiments (Table 2.3). But nonetheless, the IC$_{50}$ for PR/8 remained higher than that for A/Mall (Figure 2.9) so
the effect of FluPep 4 on the remainder of the viruses was determined. The introduction of either HA or NA glycoprotein reduced the IC$_{50}$ of FluPep 4 for PR/8 and generally, the remaining reassortant viruses had lower IC$_{50}$ values than the parental PR/8, with the 6:2 reassortant virus having the lowest value (0.09 µM) albeit with substantial variability. In order to perform statistical tests for significance the experiment would need to be repeated. Overall, the data showed that peptide susceptibility was increased with the introduction of A/Mall glycoproteins and suggests that the peptide is acting via a mechanism involving the surface glycoproteins. However these observed differences may also be explained by a potential shift in the HA/NA balance between each of the viruses. The proportion of each glycoprotein incorporated into each recombinant virus may be investigated by quantification of protein using silver stain. Or this experiment may be repeated using virus-like particles where virus fitness or differences in protein can be overcome.

**Table 2.7** Activity of FluPep 4 against recombinant viruses. Data represents two independent experiments performed in duplicate ± 95 % confidence interval.

<table>
<thead>
<tr>
<th>Virus name</th>
<th>IC$_{50}$ (µM) ±95% confidence interval</th>
<th>R$^2$</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34</td>
<td>0.321 ± 0.201 to 0.513</td>
<td>0.956</td>
<td>4</td>
</tr>
<tr>
<td>PR/8 7:1 A/Mall HA</td>
<td>0.124 ± 0.0957 to 0.161</td>
<td>0.962</td>
<td>4</td>
</tr>
<tr>
<td>PR/8 7:1 A/Mall NA</td>
<td>0.138 ± 0.0945 to 0.201</td>
<td>0.943</td>
<td>4</td>
</tr>
<tr>
<td>PR/8 6:2 A/Mall HANA</td>
<td>0.0878 ± 0.0382 to 0.202</td>
<td>0.809</td>
<td>4</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10</td>
<td>0.131 ± 0.0793 to 0.216</td>
<td>0.923</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 2.8 Validation of recombinant viruses by RT-PCR (a) Forward and reverse oligonucleotide primers were designed to complement A/PR/8/34 or A/Mallard/Netherlands/10 specific regions within segment 4 and 6 of the influenza viral genome. Primers designed to complement non-coding regions of segment 7 of each virus were also included. The expected PCR fragment sizes are indicated in the schematic. (b) Virus genomic segments were partially amplified by RT-PCR using both primer sets. DNA fragments were then separated by 1% (w/v) agarose gel electrophoresis and visualised by SYBERsafe staining. M segment primers (7) that anneal to non-coding viral regions were included as positive controls. ‘P’ denote primers used for detection of A/PR/8/34 segment 4 (P4), and 6 (P6). ‘M’ for detection of A/Mallard/Netherlands segments 4 (M4) and 6 (M6). (*) denotes unexpected DNA amplification.
Figure 2.10 Activity of FP4 against PR/8-Mall recombinant viruses (a) Plaque reduction activity of FP4 against A/PR/8/34 (black), PR/8 7:1 A/Mall HA (gold), PR/8 7:1 A/Mall NA (blue), PR/8 6:2 A/Mall HANA (purple) and A/Mallard/Netherlands (red). Data are plotted as percentage plaque forming units relative to DMSO treated control cells and fitted to a dose response curve by non-linear regression using Prism 6.0. (b) The IC\textsubscript{50} values of FP4 for each of the PR/8-A/Mall recombinant viruses were calculated from the dose response curves and are plotted ± 95% confidence interval. Data are from two independent experiments performed in duplicate.
2.3 Discussion

Previous studies identified two peptide families with anti-viral activity against influenza A virus (Nicol et al. 2012, Jones et al. 2006). This study aimed to extend this work by testing the peptides against a larger set of influenza viruses. The anti-viral activity of the peptides were determined against influenza A viruses with different haemagglutinin subtypes. In this study, Entry Blocker peptide was not active against any of the viruses tested. However, 20 µM of EB had been previously reported to inhibit PR/8-mediated agglutination of cRBC (Jones et al. 2006). The reasons for a lack of activity remain unclear but higher concentrations of peptide may be required to see an effect. This study was limited to testing EB at low concentrations because of time constraints. Future experiments may consider testing this peptide at higher concentrations and confirming purity. Nonetheless, both FluPep derivatives and B10NP displayed dose-dependent activity, within the micromolar range, against all the viruses tested. Generally, FluPep1 was a more potent inhibitor of viral replication than FluPep 4 or B10NP. This study is the first to show the anti-viral activity of FluPep against avian influenza A virus. Previously, Nicol et al. (2012) reported improved FluPep 1 (Tkip) peptide activity against A/WSN/33 following modifications to FluPep 1 to include an RRKK motif at the N-terminus, to give FluPep 4.

To aid comparison of peptide efficacy the IC₅₀ values were calculated from the dose-response curves using Prism 6.0. These values revealed strain-specific differences in sensitivity to FluPep but not B10NP. FluPep tended to be more potent inhibitors of the H1 subtype viruses tested than H3 and H4. These observed differences may be related to the target region of HA, if peptide interaction was occurring at HA₂ then strain-specific sensitivities may correlate with the phylogenetic groupings of HA. The 16 non-chiropteran HA virus subtypes are classified according to structural differences within the residues involved in membrane fusion events (Thoennes et al., 2008). Previous work has identified
influenza virus fusion inhibitors that are active or display more potent activity against one phylogenetic class of HA but not the other. One example is tertiary butylhydroquinone (TBHQ), a small molecule inhibitor that binds to the stem region of HA and obstructs the conformational changes required to bring about membrane fusion (Bodian et al., 1993). The anti-viral activity of TBHQ is limited to phylogenetic group II virus sub-types. This current work is limited to the study of FluPep activity against influenza H1, H3 and H4 subtype viruses and future studies are needed to test FluPep against H5, H7 (group I) and H9 (group II) subtype viruses.

As a means of determining whether the anti-viral effects of FluPep were a consequence of a direct interaction with viral surface glycoproteins, the HA and NA of the relatively sensitive A/Mall were reassorted into the ‘less’ sensitive PR/8 backbone. Once the viruses were successfully recovered and validated, peptide efficacy was determined by PRA. Here the IC$_{50}$ values of PR/8 were reduced by changing both the HA and NA. However, this reduction was greater with a HA substitution even when similar magnitudes of effects were observed for both the glycoproteins. The addition of A/Mall NA to PR/8 altered the particle to pfu ratio as it was found increased relative to the remaining viruses. This change may have caused increased variability in the PRA data obtained for PR/8 7:1 A/Mall NA. It is possible that the PRA performed using the 7:1 NA reassortant had a 40-fold higher number of virus particle than the remaining PRA, therefore, sequestering and/or ‘diluting’ the effect of FluPep 4. Alternatively, swapping the NA may have affected other biological properties of the virus that indirectly affected peptide activity. To better understand and quantify the effects of swapping the glycoprotein, particle to PFU ratios may be measured using PCR methods. Additionally, the HA content of each virus may be examined by purifying the viruses and quantifying HA by SDS-PAGE as performed by Harvey et al. (2008). Future experiments could also test other peptides where differences in susceptibility are detected between PR/8 and
A/Mall. Alternatively, different recombinants may be generated (for example PR/8 which is relatively resistant to FluPep relative to WSN) to strengthen the association of susceptibility between segments. Overall, FluPep and the truncated form of Entry blocker have been shown to be active against avian influenza viruses perhaps through a mechanism involving the virus surface glycoproteins.
3.1 Introduction

The previous chapter described the anti-viral activity of FluPep and Entry blocker peptides against influenza A viruses of various HA subtypes. Determination of IC$_{50}$ values revealed strain-specific differences in sensitivity to FluPep but not Entry Blocker. Accordingly, a reverse genetics approach was used to investigate whether the anti-viral activity of FluPep was a result of an interaction with surface HA. The IC$_{50}$ of FluPep 4 for PR/8 was reduced by reassorting in the HA and/or NA from a relatively sensitive strain, suggesting effects on the surface glycoproteins. However, the data did not provide strong evidence for this interaction or describe how the peptide may be acting on the glycoproteins to bring about an anti-viral effect.

Work focusing on how FluPep exerts its anti-viral activity on influenza A virus is limited. Previous work describes two ELISA assays performed to test the effects of the peptides on viral attachment and surface haemagglutinin. In these studies, chilled MDCK cells were incubated with A/WSN/33 (MOI 6), which had been pre-treated with increasing concentrations of FluPep 3 (0.0006-6 µg/mL) for one hour on ice. Following virus binding, the cells were washed to remove unbound virus, fixed and stained with an anti-influenza antibody to quantify virus binding in the absence of uptake owing to chilling. Nicol et al. (2012) reported a dose-dependent reduction in virus attachment with complete inhibition following 6 µg (3.6 µM) of FluPep 3 treatment. However, inhibition of viral attachment was observed at concentrations greater than the reported IC$_{50}$ values, which is suggestive of an additional function of the peptide. Nicol et al. (2012) also tested whether FluPep bound to haemagglutinin. Microtitre plates were coated with 100 µg/mL of peptide and incubated with baculovirus-derived recombinant HA obtained from
A/WSN/33. This ELISA test showed the peptide did indeed bind to influenza HA. However, no further mechanistic studies of FluPep function have been reported.

Studies of Entry Blocker (EB) activity against influenza A virus initially led Jones et al. (2006) to propose a mechanism of action involving blocking of HA function. As the peptides were able to inhibit influenza A-mediated agglutination of chicken red blood cells and prevented the attachment of a fluorescently labelled virus to MDCK cells. Additionally, the group found that EB peptides had no effect on NA enzymatic activity, which was assessed by cleavage of MUNANA [2’-(4-methylumbelliferyl)-α-d-N-acetyleneuraminic acid]. Later studies showed EB led to agglutination of influenza A virus and subsequent prevention of attachment. Gradient purified PR/8 was treated with 30 µM EB and the resulting virus particle size was assessed by sucrose gradient ultracentrifugation. Jones et al. (2011b) found that PR/8 treated with peptide was eluted within higher density fractions (41%-50% sucrose) relative to vehicle treated PR/8 (around 30%), suggesting that EB induced influenza virus aggregation. These aggregations were then observed by electron microscopy. As a result Jones et al. 2011 suggested that the peptides could be used as vaccine adjuvants because aggregation may promote virus uptake by antigen presenting cells.

This chapter describes optimisation of viral entry assays focusing on binding, internalisation and import to further elucidate the peptide mechanism of action.

3.2 Results

3.2.1 Time of drug addition assay

To examine where the peptides acted within the viral replication cycle, time of drug addition experiments (TODA) were performed. A549 cells were infected with PR/8 at an MOI of 3 and treated with peptide (FluPep 4 and B10NP at 10 µM) an
hour prior to infection (-1), at the time of infection (0), or 1, 3 and 6 hrs post-infection (p.i). Eight hrs p.i the cells were fixed, permeabilised, and stained with DAPI (blue) and anti-NP (green) (Figure 3.1). The total number of infected cells were determined by counting NP expressing cells, and data were plotted relative to the 1.5% (v/v) DMSO vehicle treated controls (Figure 3.2). FluPep treatment 3 and 6 hours p.i did not affect viral replication as the NP positive cell counts were similar to the vehicle treated cells. However, FluPep demonstrated activity early in the replication cycle because administration of the peptide one-hour prior to infection (-1) and post-infection (1) resulted in reductions of 30-40% in infected cells. FluPep was most efficacious when administered at the time of infection (0), as the total number of infected cells dropped to 10% relative to DMSO treated cells. Like FluPep, B10\textsuperscript{NP} treatment 3 and 6 hours post-infection (p.i) did not reduce the number of NP positive cells relative to the controls, suggesting the peptide was not active late in infection. B10\textsuperscript{NP} however, was most efficacious when administered an hour prior to and up to an hour p.i as NP positive cells were reduced by around 50% relative to controls. In this experiment, FluPep 4 treatment at the time of infection led to greater reductions of NP positive cells compared to B10\textsuperscript{NP} suggesting that it was a more potent inhibitor of PR/8 infection, consistent with earlier data (section 2.2.2).

As an independent method of quantifying peptide activity during the different stages of infection, NP expression was determined by western blot analysis. The time of drug addition experiments were repeated following the same conditions described above but cell lysates were collected for western blot analysis. Probing with an anti-NP antibody revealed that FluPep had little effect on NP
Figure 3.1 Immunofluorescent microscopy analysis of NP expression following peptide treatment at different stages of influenza infection. A549 cells were infected with A/PR/8/34 at an MOI of 3 and treated with (a) FP4 or (b) B10NP (10 µM) either one hour prior to infection (-1), at the time of infection (0), or (1), (3), or (6) hours post infection (p.i). Cells were fixed 8 hrs p.i, permeabilised and stained with anti-NP (green) and DAPI (blue) prior to imaging. Images were collected using the Leica DLMB microscope at a magnification of X20 and are representative of two independent experiments.
Figure 3.2 NP-positive cell count and detection of NP by Western blot analysis. MDCK cells were infected with A/PR/8/34 at an MOI of 3 and treated with (a) FP4 or (b) B10NP (10 µM) either one hour prior to infection (-1), at the time of infection (0), or (1), (3), or (6) hours post infection (i) total number of virus-infected cells were quantified by counting NP positive cells and are presented relative to the vehicle controls ±SEM. For each experimental group 50-150 cells were counted (ii) Lysates from MDCK cells following TODA were analysed by SDS-PAGE followed by western blotting. The membrane was probed with mouse anti-NP and rabbit anti-tubulin. An extra band visible between NP and tubulin was considered non-specific as it is also observed in the mock control. Images are representative of two independent experimental repeats.
expression when administered 3 and 6 hrs p.i, but expression was reduced when the peptide was given during the early stages of infection (-1, 0 and 1). This was also true for B10\textsuperscript{NP} but a slight reduction in NP expression at 3 hrs was also detected. Whilst absolute quantification of infection is difficult by the methods used. Overall, the TODA experiments indicated that the peptides were active early in the viral replication cycle.

3.3 Viral entry assays

3.3.1 Nuclear Import

To test whether the peptides acted at or before the point of viral ribonucleoprotein (vRNP) import into the nucleus, a nuclear import assay was performed following conditions previously described by Martin and Helenius (1991). A549 cells were infected with gradient-purified PR/8 (nominal MOI of 3, MOI was assigned and not calculated from data analysis), in the presence and absence of cycloheximide (CHX, 100 µg/mL). Three hours p.i the cells were fixed, permeabised, and stained with anti-NP (green) and DAPI (blue), prior to imaging by confocal microscopy. Maximum intensity projections representative of two independent experiments are presented (Figure 3.3). Within three hours, the virus is expected to have internalised into cellular endosomes where acidification of the endosomal compartments lead to conformational changes in HA thus, activating the fusion of the endosomal and viral membranes. Fusion provides the vRNPs a pore by which vRNP complexes access the cell cytoplasm. Here, the vRNPs then begin to translocate to the cell nucleus where they are imported by the nuclear pore complex (reviewed in section 1.2 and Eisfeld et al., 2015). Normally therefore, input vRNP should be accumulated within the nucleus by 3 hrs p.i.

CHX is an inhibitor of protein biosynthesis and in its absence infection proceeded naturally resulting in the appearance of abundant amounts of new NP in
Figure 3.3 vRNP nuclear import assay in the presence and absence of peptide. A549 cells were infected with A/PR/8/34 at an MOI 3, in the presence of cycloheximide (100 µg/mL) an inhibitor of protein biosynthesis and either importazole (10 µM) or peptide (FP4, FP1, EB and B10NP at 10 µM). Cells were fixed 3 hrs p.i, permeabilised and stained with anti-NP (green) and DAPI (blue) before imaging by confocal microscopy at a magnification of x63. Maximum intensity projections of a series of optical sections are presented as merged two-colour images or the green channel alone. Images are representative of two independent experiments.
the cytoplasm (-CHX in Figure 3.3). The addition of CHX was therefore necessary to track the movement of input vRNP complexes into the nucleoplasm, as observed in the vehicle (with CHX) control sample. Here, clear accumulation of NP in the nucleus was observed. As a control for the assay, infected cells were treated with importazole (10 \( \mu \)M), a known inhibitor of influenza A virus nuclear import. Importazole interferes with interactions between importin-\( \beta \) and RAN-GTP preventing the release of imported cargo. Treatment with importazole therefore blocks import and leads to a retention of cargo at the rim of the nuclei as was observed in the importazole treated cells (Chou et al., 2013; Soderholm et al., 2011).

Next, the virus was treated with peptide at 10 \( \mu \)M and their affects on vRNP were tested. NP of EB treated virus was imported into the nucleus indistinguishably from the vehicle control, which suggested no activity of EB at this stage (Figure 3.3). However, in the FluPep treated groups NP was observed within aggregates away from the cell nucleus. Greater quantities of NP aggregates were seen following FluPep 1 treatment relative to FluPep 4. Like FluPep, B10\( ^{NP} \) also led to extracellular aggregations of virus, but these were smaller and less pronounced than those observed following FluPep treatment. It is most likely that NP observed in the importazole treated cells remains enclosed within endosomes. Co-staining with endosomal or extracellular markers was not attempted here however, dual labelling of 488-PR8 in section 3.3.3.3 suggested that FluPep treated virus remained outside the cell. Overall, the data indicated that FluPep and B10\( ^{NP} \) acted either at the level of or upstream of RNP nuclear import, consistent with an early-acting inhibitory mechanism.

### 3.3.2 Virus binding to cells

Results so far indicated that the peptides acted early in the virus life cycle and upstream of nuclear import. As a first step, the influence of the peptides on
virus attachment was tested by haemagglutination inhibition assay (HAI). Influenza A virus HA is able to bind to sialic acid receptors on the surface of red blood cells (RBCs) but the viruses are not internalised. RBCs that are not bound to virus typically sink to the bottom of a v-bottom microtitre plate. However, when HA binds the RBCs, the cells form a lattice and this agglutination may be quantified. One HA unit is defined as the minimum dilution of virus required to agglutinate a set amount of standardised red blood cell suspension. If the peptides acted on surface HA and prevented viral binding to host cells, it would be expected that following treatment the peptides would neutralise RBC agglutination and this may be then be measured.

3.3.2.1 Anti-viral peptides agglutinate red blood cells

First, to eliminate any effect of the peptides on the assay, their ability to agglutinate RBCs was determined. The peptides were diluted 2-fold across a 96-v-bottomed well plate (at a starting concentration of 100 µM) and overlaid with either 1% chicken red blood cells (cRBCs) or 0.75% turkey red blood cells (tRBCs) (Figure 3.4). Blood from two different species was used to test for differences in agglutination between species. Negative PBS controls were included which showed that in the absence of peptide the cRBCs and tRBCs did not spontaneously agglutinate (Figure 3.4). High concentrations of both FluPep and Entry Blocker peptides agglutinated RBCs. As a result, a ‘safe’ concentration of peptide that did not agglutinate RBCs, and could therefore be used without interfering with the assay, was determined. FluPep 4 and B10NP had an ‘HA titre’ of 12.5 µM, but greater quantities of FluPep 1 and EB were required to agglutinate the RBCs, around 50 µM. Since the peptides did not agglutinate RBCs within the IC₅₀ range for virus replication (Table 3.1), it was reasoned possible to test the effects of the peptides on virus binding by HAI. No substantial differences were seen between the behaviour
of chicken or turkey RBCs and therefore subsequent assays were performed only with cRBCs.

**Table 3.1** Agglutination concentrations of red blood cells by anti-viral peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Agglutination concentration cRBC (µM)</th>
<th>Agglutination concentration tRBC (µM)</th>
<th>IC50 A/WSN/33 (relatively sensitive virus)</th>
<th>IC50 A/Turk/Can (relatively resistant virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FluPep 4</td>
<td>12.5</td>
<td>12.5</td>
<td>0.02</td>
<td>0.6</td>
</tr>
<tr>
<td>FluPep 1</td>
<td>50</td>
<td>50</td>
<td>0.04</td>
<td>0.6</td>
</tr>
<tr>
<td>Entry Blocker</td>
<td>50</td>
<td>50</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>B10NP</td>
<td>12.5</td>
<td>12.5</td>
<td>1</td>
<td>3.6</td>
</tr>
</tbody>
</table>
Figure 3.4 Red blood cell agglutination by anti-viral peptides. The peptides (FP1, FP4, B10NP and EB) were diluted 2-fold across a 96-well dish from a starting concentration of 100 µM and overlaid with either (a) 1% chicken red blood cells (RBC) or (b) 0.75% Turkey RBC. Peptide treatment at the high concentrations lead to agglutination of RBC. Plates are representative of two independent experiments each performed in duplicate.
3.3.2.2 Effect of anti-viral peptides on viral adsorption by HAI

Next, the influence of the peptides on viral binding to RBCs were tested. The peptides were first diluted to the ‘safe’ concentrations as determined in section 3.3.2.1, and peptide agglutination of cRBCs at the ‘safe’ concentration was checked (peptide only control, Figure 3.5). Most of the peptides did not agglutinate cRBC at the ‘safe’ concentrations apart from FluPep 4, which in this experiment agglutinated the cRBC at 6.25 µM. Data for FluPep 4 at this concentration was therefore not considered. The peptides were then diluted two-fold across a 96-v-bottomed dish (from the safe starting concentration), overlaid with 8 HA units of PR/8 and incubated at room temperature for 30 mins. Following incubation, the plates were overlaid with 1% cRBC, incubated for a further 30 mins and the HAI titres obtained. A PR/8 virus control was included and showed the virus mediated-agglutination of cRBCs at the standardised concentration. As expected, addition of an anti-PR/8 serum prevented viral agglutination at dilutions of up to 1:160. HA antibodies present in the serum masked the HA epitopes on the viral surface and neutralised agglutination. However none of the peptides inhibited PR/8-mediated agglutination of cRBC even at concentrations substantially higher than the IC₅₀ values previously reported (Nicol et al. 2012) or described previously in this thesis.

Plaque reduction assays demonstrated differing sensitivities of the various virus strains to peptide (Figure 2.4) and therefore, it is possible that the peptides were acting differently on some of the viruses. To test this, HAI assays were performed on all the remaining virus sub-types. The viruses considered included: A/Duck/England/62, and A/Turkey/Canada/65 (Figure 3.6), as well as A/WSN/33 and A/Mall (data not shown). However, as with PR/8, the anti-viral peptides did not prevent virus-mediated agglutination of cRBC even at concentrations higher than the IC₅₀(Figure 2.4). This suggested that the peptides
Figure 3.5 Haemagglutination inhibition assay. Representative HAI evaluating the effects of peptide treatment on PR/8 binding. First, A/PR/8/34 was standardised to 8 HA units per 50 µL of cRBC and the peptides diluted to a concentration that did not agglutinate cRBCs (peptide only control). The antiviral peptides were then serially diluted two-fold in PBS across a 96-V-bottomed plate, overlaid with standardised A/PR/8/34 and incubated for 30 mins. Following incubation the plate was overlaid with 1% cRBC, incubated a further 30 min and visualised. (*) denote the IC₅₀ values for each peptide. Plates are representative of two independent experiments each performed in duplicate.
Figure 3.6 Haemagglutination inhibition assay with A/Duck/England/62 and A/Turkey/Canada/65. Representative haemagglutination inhibition assays (HAI) investigating the effects of peptide treatment on influenza virus binding. First the peptides were diluted to a concentration that did not agglutinate cRBCs (peptide only control). The anti-viral peptides were then serially diluted two-fold in PBS across a 96-V-bottomed plate, overlaid with standardised virus (at 8 HA units) and incubated for 30 mins. Following incubation the plates were overlaid with 1% cRBC incubated a further 30min and visualised. The plates are representative of two independent experiments each performed in duplicate.
were not affecting viral binding. However, this assay was an indirect measure of peptide action on virus binding and a more sensitive measure was now sought.

3.3.3 Fluorophore-labelling of gradient purified PR/8

Towards the development of a more sensitive assay for assessment of virus binding to cells, a purified fluorophore-labelled-PR/8 was produced. PR/8 was first purified by ultracentrifugation through a 15-60% sucrose gradient. Following this, total viral protein was quantified and a maximum of 1 mg of PR/8 was labelled with a green Alexa-fluorophore dye using a protein labelling kit (Life Technologies). Residual dye was separated from labelled virus using a second ultracentrifugation step. Viral proteins of purified unlabelled PR/8 and fluorophor-labelled virus were separated using SDS-PAGE and unlabelled protein was visualised using coomassie blue staining, while fluorophore-labelled protein was visualised using a UV transilluminator (Figure 3.7). Coomassie staining showed that the viral structural proteins were running at the predicted size with no major signs of contamination. Additionally, both the unlabelled and labelled samples had similar protein profiles suggesting there was no degradation of virus resulting from the labelling process. Additionally, dye was conjugated predominantly to surface HA suggesting that the dye had no access to the interior of the virion and therefore the virus remained structurally intact. This labelling method did not compromise the integrity of the virus. Therefore, using these protocols it was possible to generate a fluorophore-labelled virus, which was then used to optimise a virus entry assay where binding could be measured directly.
Figure 3.7 SDS-PAGE analysis of fluorophore-labelled-A/PR/8/34 (a) A/PR/8/34 was first purified through a 15-60% sucrose gradient and viral proteins were separated by SDS-PAGE and visualized by 1% (w/v) coomassie brilliant blue R-250. A precision plus protein marker (Bio-Rad) was run in the first lane and the corresponding protein sizes are displayed (b) surface glycoproteins were labelled with a green Alexa Fluor 488 dye using a protein labelling kit (Life Technologies). To determine which proteins were conjugated to dye the gel was also imaged on a UV transilluminator. The protein standard used was pre-stained red and blue, the red standards were auto-fluorescent under the UV light and were visualised as yellow bands (running at 75 and 25 kDa).
3.3.3.1 Fluorescence analysis of virus binding in the presence of peptide

The effects of the anti-viral peptides on influenza A viral binding were tested using the fluorophore labelled PR/8. First, 488-PR/8 generated in section 3.3.3 was used to visualise attachment by fluorescence microscopy. A549 cells were seeded onto coverslips and incubated at 4°C with purified 488-PR/8 in the presence of either vehicle (1.5% (v/v) DMSO) or peptide (1 and 10 µM), for one hour to allow viral binding. Performing the assay at 4°C allowed the virus to bind but not internalise into the cells. Following binding, the cells were washed three times with PBS to remove unbound virus, fixed, and stained with DAPI to provide a means of quantifying virus binding. The cells were then visualised by confocal microscopy and maximum intensity projections were generated from a series of optical sections taken through the depth of the cells. In the absence of virus, mock-infected samples displayed no 488-fluorescence, however in the 488-PR/8-virus treated sample, virus was visualised as fluorescent ‘spots’ distributed evenly over the surface of the cells.

As a control for assay specificity, A549 cells were treated with 2U of bacterial neuraminidase from Vibrio cholera (bacterial NA) to remove cell surface sialic acid receptors. Successful removal of sialic acid receptors was confirmed by lectin staining (data not shown), and resulted in a marked reduction of virus binding relative to the vehicle control cells (Figure 3.8). Within the EB treatment group, the virus bound to the surface of the cells in a manner similar to the vehicle control cells, suggesting no effect of the peptide on binding. FluPep and B10\textsuperscript{NP} treatment at the higher concentration (10 µM), did not result in an overall reduction in virus binding but perhaps led to viral aggregations over the cell surface. FluPep treatment perhaps led to an apparent increase in total fluorescence as the aggregates appeared to be increased in number and more pronounced relative to B10\textsuperscript{NP} treated cells. These aggregations were less obvious following peptide treatment at the lower
Figure 3.8 Fluorescence imaging of virus binding in the presence of antiviral peptide A549 cells were infected with 488-A/PR/8/34 for one hour at 4°C, in the presence of 1.5% DMSO vehicle control or peptide (FP4, FP1, EB and B10NP at 10 or 1 µM). Following binding, unbound virus was removed by excess washing using PBS, the cells were then fixed, permeabilised and stained with DAPI (blue) before imaging using the Zeiss LSM710 confocal microscope at a magnification of X63. Maximum intensity projections of a series of optical sections through the depth of the cells are presented. Data are representative of three independent experiments.
concentration (1 µM). Overall, the 488-PR/8 binding assays suggested that the peptides were not reducing overall binding to cells, but were causing virus to form aggregates on the surface of the cells, at least at the high peptide concentrations.

3.3.3.2 Flow cytometry analysis of viral binding in the presence of peptide

To quantify the effects of peptide treatment on virus binding, analysis by flow cytometry was performed. The conditions used for the binding assay were similar to those described for the fluorescence imaging experiments, except that A549 cells (2x10⁶ cells) were incubated with 488-PR/8 in suspension either in the presence of peptide (1 and 10 µM) or 1.5% (v/v) DMSO. Following a number of PBS washes to remove unbound virus, cells were fixed with 1% (w/v) PFA and virus binding was determined by flow cytometry. Single cell populations were gated based on the forward and side scatter, and 10,000 single cell events from each experimental group were recorded. The individual scatter plots were analysed using FlowJo and a set from one experimental repeat are presented (Figure 3.9). Mock-infected samples (uninfected) displayed low levels of fluorescence that was scarcely detectable. However, in the vehicle control samples the cell population shifted to the right and at the concentration of virus used more than 98 % of A549 cells now displayed surface attachment of 488-PR/8. Removal of cell surface sialic acid receptors by treatment of A549 cells with bacterial NA, resulted in a reduction in fluorescence relative to the vehicle control. In fact, ~70 % of the cells displayed fluorescence levels comparable to mock samples. ‘Peptide only’ treated cells (no virus) showed no effect of the peptides on the fluorescence and therefore did not interfere with the assay. Next, the peptides were tested for their effects on viral binding. Peptide treatment at both concentrations had no effect on the fluorescence intensity relative to the vehicle treated cells. Suggesting that the peptides did not affect overall virus binding. Furthermore, peptide treatment had no effect on the shape of the histograms (data not shown), which was somewhat surprising.
Figure 3.9 Flow cytometric analysis of binding of fluorescently-labelled influenza A/PR/8/34 to A549 cells in the absence and presence antiviral peptides. A549 cells in suspension were incubated in the presence of either vehicle or peptide (1 and 10 µM) for one hour at 4°C to allow binding of virus. Immediately after binding the cells were washed extensively with PBS to remove unbound virus and fixed. Virus binding was determined by flow cytometry using a FACScalibur. Gating was determined based on forward and side scatter determinants and 10,000 single cell events were recorded for each experimental group. The y-axis represents the size and internal complexity of the cells and x-axis presents the 488-positive and negative cells. Dot plots are a representation of data collected from three independent experiments.
considering the detection of peptide-mediated agglutination of virus in the previous microscopy experiment.

The mean fluorescence intensity (MFI) was calculated from the scatter plots and the data displayed relative to the vehicle control (Figure 3.10). A one-way ANOVA was performed using Prism 6.0 to test for statistical significances of the experimental groups relative to the vehicle control. Statistical significance was only achieved by removal of the cell surface sialic acid receptors and no differences in the MFI were detected following treatment with any of the peptides tested. Overall, relative to the vehicle control samples, no significant reductions in viral binding were observed following peptide treatment.

3.3.3.3 Immunofluorescence microscopy analysis of internalisation

The next question was whether the peptides influenced virus internalisation. This was investigated by performing a virus-binding assay using the 488-PR/8, followed by staining of extracellular virus with an anti-PR/8 antibody (in red). Therefore allowing differentiation between internalised and external virus. To synchronise infection, A549 cells were first incubated with purified 488-PR/8 for one hour at 4°C in the presence of peptide (1-10 µM) or 1.5% (v/v) DMSO vehicle. Following incubation, the cells were washed three times with PBS to remove unbound virus and warmed to 37°C for one hour to allow endocytosis. Cells were then fixed and extracellular virus (i.e unpermeabilised cells) was stained with an anti-PR/8 antibody, followed by a 594-Alexa Fluor conjugated secondary (red) and DAPI (blue). Following staining extracellular virus appeared ‘yellow’ as there was a co-localisation of the green and red fluorescence however internalised virus appeared green. The cells were visualised by confocal microscopy and maximum intensity projections are presented (Figure 3.11). No green or red fluorescence was detected in the mock-infected samples. Control cells that were incubated with 488-
Figure 3.10 Flow cytometric analysis of virus binding in the presence or absence of antiviral peptides. Viral binding to cells was quantified by flow cytometry using the FACSCalibur. Single cell populations were gated based on forward and size scatter and 10,000 cells were counted for each experimental group. The mean fluorescence intensity was determined using FlowJo and are presented ±SEM relative to the vehicle treated samples. Data are from three independent experiments. A one-way ANOVA was performed using prism 6.0 (**denotes a P-value of between 0.001-0.01 and *** a P-value <0.001)
PR/8 at 4°C for an hour and fixed immediately following incubation were included as ‘binding controls’. Extracellular staining of the 488-PR/8 virus with an anti-PR/8 antibody (red) showed most of the virus in the binding control was co-stained with red and green fluorescence, and thus likely remained bound to the cell surface. Cells that were incubated for an additional hour at 37°C were also included as internalised controls. In this sample the majority of the staining was green suggesting that the virus had begun to internalise into the cells (Figure 3.11a).

As with the previous binding experiment, EB treatment had similar staining to the internalised control and suggested no effect of the peptide on the virus at this stage of infection (Figure 3.11b). However, treatment with FluPep and B10NP (10 µM) led to the aggregation of virus on the cell surface (Figure 3.11c). These appeared larger and in greater quantity following FluPep 4 treatment relative to FluPep 1 and B10NP. Large areas of co-localisation (yellow) staining were observed suggesting that the virus aggregates were bound to the cell surface and were unable to internalise into the cells. However, at the lower concentration of peptide, the virus aggregations appeared reduced in magnitude and number.

In order to quantify the effect of the peptides on the internalisation of ‘non-agglutinated’ virus, the immunofluorescence images were manually inspected. Green and red virus particles that were the same size as virus in the vehicle control samples were quantified. Percentage extracellular virus within each treatment group was quantified and presented in Figure 3.12. In the binding control samples, over 85% of the virus was extracellular and on increasing the temperature (to 37°C) the number of virus outside the cells was reduced to 20%. Generally relative to the internalised control the percentage of extracellular virus remained unchanged following FluPep 1 and Entry Blocker treatment. However, FluPep 4 treatment did have some effect as the percentage extracellular virus was increased by 2.5-fold at 10 µM and just under 2-fold 1 µM relative to the internalised control.
Figure 3.11a Internalisation assay - Control cells A549 cells were either mock infected (mock) or infected with 488-A/PR/8/34 for one hour at 4°C in the presence of 1.5% DMSO vehicle control and fixed following the removal of unbound virus (binding control) or incubated for a further hour at 37°C (internalisation control). Following the incubation steps the cells were fixed and extracellular virus stained with aPR/8 (red) and DAPI (blue) before imaging by confocal microscopy using the Zeiss LSM710 at a magnification of x63. Maximum intensity projections of a series of optical sections through the depth of the cells are presented. Images are representative of two independent experiments.
Figure 3.11b Internalisation assay – Entry Blocker treated cells. A549 cells were infected with 488-A/PR/8/34 for one hour at 4°C in the presence of the Entry Blocker peptides at 10 or 1 µM. Following binding, unbound virus was removed by extensive washing with PBS and the cells were warmed to 37°C for a further hour. Following incubation the cells were fixed and extracellular virus stained with αPR/8 (red) and DAPI (blue). Images presented are maximum intensity projections of a series of optical sections through the depth of the cells taken using the Zeiss LSM710. Images are representative of two independent experiments.
Figure 3.11c Internalisation assay – FluPep treated cells A549 cells were infected with 488-A/PR/8/34 for one hour at 4°C in the presence of FluPep at 10 or 1 μM. Following binding, unbound virus was removed by washing three times in PBS and the cells were warmed to 37°C for a further hour, fixed and extracellular virus stained with αPR/8 (red) and DAPI (blue). Images presented are maximum intensity projections of a series of optical sections through the depth of the cells taken using the Zeiss LSM710 at a magnification of x63. Images are representative of two independent experiments.
Figure 3.12 Percentage extracellular virus following peptide treatment (µM). Extracellular virus was quantified from the maximum intensity projections and presented ±SEM. The dotted line represents the percentage extracellular virus of the internalised control. Data represents two independent experiments performed in duplicate. For each experiment two maximum intensity projections were obtained and manually inspected counts are representative of around 300 to 400 virus particles per section. Two sections were inspected for each treatment group per experiment.
suggesting some effect of FluPep 4 on non-agglutinated virus.

3.3.3.4 Flow cytometry analysis of internalisation

In the previous assay the effect of peptide treatment on internalisation was quantified manually and to gain a more accurate quantification of peptide influence on virus internalisation a flow cytometric analysis was performed. In this experiment, A549 cells were incubated for 1 hr at 4°C with 488-PR/8 in the presence of either vehicle or FluPep 4 (10 µM) in order to synchronise infection. Then the temperature was raised to 37°C to allow the virus to internalise and the MFI was recorded every ten minutes. A binding control sample was included in the assay and represented cells that were fixed immediately following the 4°C incubation. External vs. internalised virus was distinguished by extracellular quenching of the 488 signal using 4% (w/v) trypan blue. Single cell populations were gated according to the forward and side scatter and 10,000 single cell populations were recorded for each experimental group. The MFI was calculated from the individual scatter plots using FlowJo, and data are presented relative to the 488-PR/8 time zero control (Figure 3.13). Considering the unquenched 488-PR/8 sample groups first (without Trypan blue treatment), over the one-hour (37°C) incubation the MFI decreased by approximately 20% relative to the time zero control. This loss in fluorescence intensity may be explained either by gradual internalisation of the virus into the cells or a loss of surface bound 488-virus that may have not been removed by the PBS washes. At time zero, the MFI of the FluPep 4 treated sample was reduced to a little over 80% relative to the vehicle control sample. The magnitude of this reduction was consistent with the data obtained in the binding assay. In the FluPep 4 treated group the MFI was reduced over time as with the vehicle sample group. The magnitude of this reduction however was less pronounced. The treatment of samples with trypan blue resulted in a total reduction of fluorescence by
approximately 90% relative to the 488-PR/8 control sample. Over the one-hour incubation, extracellular virus became resistant to trypan blue quenching and the MFI increased. This increase was detected as the virus began to internalise into the cells. In the FluPep 4 treatment group however, the MFI remained constant or static. This suggests that the peptide was preventing virus internalisation.

3.3 Discussion

To determine where the peptides acted within the influenza virus replication cycle, time of drug addition studies were performed. Cells were infected with virus at an MOI of 3 and treated with peptide at -1, 0, 1, 3 and 6 hours relative to infection. Marked reductions in virus replication were only observed when the peptides were administered at the time of, one-hour prior or one-hour post infection suggesting an early action of the peptide within the virus replication cycle. These observations fell in line with the nuclear import assay which showed that the peptides acted at/or upstream of RNP nuclear import. Virus entry assays focussing on binding were then performed to test whether the peptides reduced replication by preventing viral attachment to host cells. Both HAI assays and entry assays directly measuring 488-PR/8 binding showed that the peptides did not reduce overall binding at times a reduction in binding was observed following FluPep 4 treatment (10 μM) but this was not consistent between experimental repeats. However, FluPep and B10^NP^ treatment at high concentrations (10 μM) led to virus aggregation on the surface of the cells. These aggregations appeared larger following FluPep 4 treatment compared to FluPep 1 and B10^NP^. Furthermore, the size and quantity of the aggregations appeared reduced following treatment at the lower concentrations (1 μM), at concentrations known to reduce infectious titre therefore suggesting an additional or alternative mechanism of action. As with the binding assays, a virus entry assay evaluating the effects of the peptides on virus internalisation also showed the formation of aggregates which were again more pronounced following
Figure 3.13 Analysis of virus internalisation in the presence of FluPep 4 by flow cytometry. Virus internalisation was quantified by flow cytometry using a FACSCalibur. A549 cells were infected with 488-PR/8 for 1 h at 4°C in the presence of vehicle or FluPep 4 (10 µM). The temperature was then raised to 37°C and the mean fluorescence intensity was determined every ten minutes over a one-hour incubation. To quantify internalisation, extracellular 488-virus was quenched using 4% (w/v) Trypan blue. The mean fluorescence intensity (MFI) is plotted ±SEM relative to the vehicle treated samples at time zero. A linear regression line has been fitted to the data points using Prism 6.0. Data are representative of four independent experiments for the 488-PR8 and 488-PR8 trypan blue treated groups and two independent experiments for the FluPep4 treated groups.
FluPep 4 treatment (10 µM). The aggregation effect of the peptides may have also contributed to the outcome of the plaque reduction assays, it may be that virus particles capable of forming single new foci of infection are aggregating in the presence of peptide and as such it may be that there are more particles per infected cell, but that are still counted as one plaque.

The observation that FluPep 3 reduced cell-associated virus using an ELISA binding assay led Nicol et al., (2012) to propose that FluPep inhibited influenza virus attachment. In this study, no affect of the peptides was observed at point of binding and perhaps FluPep 4 had some effect at the point of internalisation. Discrepancies between the proposed mechanism(s) of action here and by Nicol et al (2012) may be because of differences in the methods used in the studies. For example, Nicol et al. (2012) used FluPep 3 while this study focuses on FluPep 4, results have shown different behaviours in the peptides and it may be that FluPep 3 and 4 act via different mechanisms. These sets of entry assays were performed with A549 cells, whereas Nicol et al. (2012) focussed work on MDCK cells. Additionally Nicol et al. (2012) performed experiments using A/WSN/33, while purified PR/8 is used here. Plaque reduction assays performed in chapter 2 showed both viruses to have differing sensitivities to peptide. Using two independent methods, this study has shown that FluPep and Entry Blocker do not act at the point of virus attachment.

Table: 3.2 Hypothesis for peptide mechanisms of action

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Act at early stage of infection</th>
<th>Binding to HA</th>
<th>Aggregations</th>
<th>Internalisation</th>
<th>Fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>FluPep 4</td>
<td>Yes</td>
<td>Some (N.S)</td>
<td>Yes</td>
<td>Yes</td>
<td>N.D</td>
</tr>
<tr>
<td>FluPep 1</td>
<td>Yes</td>
<td>N.D</td>
<td>No</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>B10NP</td>
<td>Yes</td>
<td>N.D</td>
<td>Yes</td>
<td>N.D</td>
<td>N.D</td>
</tr>
</tbody>
</table>

*No data (N.D), Not significant (N.S)
Future work could test the extent of the virus aggregation/clumping by the peptide by density ultracentrifugation of purified virus through a sucrose gradient. Changes to particle size may be then determined by testing individual gradient fractions for the presence of HA by dot immunoblotting or HA assay. Previous studies by Jones et al. (2011) used these methods to show that EB treatment lead to changes in the elution profile of PR/8. Electron microscopy was then used to confirm the formation of viral aggregates in peptide treated samples. In this study, strain related differences in virus sensitivity to FluPep have been observed and it may be possible to compare changes to the elution profile of a relatively sensitive virus such as A/WSN/33 with a relatively resistant PR/8. It may be possible that peptide-HA interactions lead to premature conformational changes of HA decreasing HA affinity for sialic acid binding to the cell surface or preventing virus fusion events. Alternatively the peptides are interacting at regions close to the receptor binding pocket of the HA₁ subunit and preventing the glycoprotein from docking or perhaps binding to glycans present on HA₁. Future work would need to perform an acidification and/or fusion assay either by using antibodies that can detect the different conformational states of HA for example, the Vn04 series used by Mair et al. (2014) or the A1 antibodies described by Banerjee et al. (2013) other methods include lipophilic labelling of either the virus or endosome to single out fusion events.
CHAPTER 4 RATIONAL DESIGN AND EVALUATION OF BIOSHIELDS FOR CONTROL OF AVIAN INFLUENZA VIRUS

4.1 Introduction

Wild birds are the natural reservoir hosts of avian influenza where infection does not always cause disease. Since 1996, Asian-origin A/goose/Guangdong/1/1996 (H5N1) has been circulating relatively silently within wild birds and zoonotic spread of the virus has resulted in high morbidity and mortality in human (~60%) (Claas et al., 1998) and domesticated poultry (Lee et al., 2015; Yoo et al., 2011) populations. The spread of influenza into domestic bird species and its continued circulation can lead to the generation of a HPAI phenotype. Within the last year, H5 viruses originating from A/goose/Guangdong/1/1996 have been detected in Asia, Europe (H5N8) and North America, where collectively outbreaks have led to the systematic destruction of around 14 million poultry in Asia (Keun Bon et al., 2014), 6,000 ducks in North Yorkshire, 180,000 laying hens in the Netherlands with continued spread of H5 subtype viruses in America. Outbreaks of this nature place pressure on global food supplies and economies.

A novel approach to protect poultry from HPAI would be to deliver broad-spectrum anti-viral peptides to the sites of influenza virus replication with the view of preventing LPAI circulation. Prophylactic treatment of poultry with peptide requires a method of delivery, which is inexpensive to manufacture, stable within the host, safe, and effective at reducing the transmission of influenza. In order to meet these requirements work in this chapter aimed to develop a system whereby Lactobacillus isolates from the chicken intestine act as self-renewing delivery systems for cell-permeable anti-influenza peptides. The aim was to design and evaluate the activity of an expression cassette capable of delivering the peptides to the avian intestines.
4.1.1 Lactic acid bacteria as delivery vectors for viral antigens and therapeutic peptides

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating bacteria that produce lactic acid; a primary product of fermentation. LAB genera include *Streptococcus, Lactobacillus, Peidococcus* and *Leuconostoc*. For centuries, LAB have been used for the production of yoghurts and cheeses from milk (Tannock, 2004). LAB have been proposed as delivery vehicles for diverse therapeutic and prophylactic substances (Wells and Mercenier, 2008). For example, *Lactococcus* has been engineered to secrete the anti-inflammatory cytokine IL-10 to treat inflammatory bowel disease; such agents are currently in clinical trials for use as therapeutic agents for disease in human patients (Braat et al., 2006; Steidler et al., 2000). Moreover, *Lactobacillus plantarum* and *L. gasseri* from the human vaginal microflora and rectum have been engineered to constitutively express peptide inhibitors of HIV-1 entry and fusion (Pusch et al., 2006). It has long been established that the levels of *Lactobacillus* within the flora at these sites play a significant role in maintaining vaginal health via the production of lactic acid, hydrogen peroxide and bacteriocins, which act as antimicrobial agents. Indeed, it has been reported that absence of *Lactobacillus* is associated with increased susceptibility to HIV infection (Martin et al., 1999). Pusch *et al.* (2006) fused the Usp45 secretion signal and pro-peptide DTNSD (both taken from a lactococcal secretion peptide) to an HIV C protein fusion inhibitor under the control of the phage promoter LPS2 and transferred the cassette to *L. plantarum*. Levels of peptide expression and secretion were determined by western blot analysis and found to be approximately 4 mg/litre of culture medium (Pusch et al., 2006). The authors demonstrated that the supernatant of an *L. plantarum* culture engineered to express the fusion inhibitor was able to suppress HIV-1 infection by up to 98% of TZM-bl cells (engineered HeLa cells that express CD4, CCR5 and CXCR4 and contain reporter genes for firefly luciferase and beta-galactosidase under the control of an HIV-1 promotor)
Further studies genetically manipulated *Lactobacillus* vaginal isolates to express high affinity HIV-binding proteins. The HIV envelope contains the glycoprotein gp160, which is cleaved to form stable gp120 and gp41. gp120 interacts with CD4 receptors on host cell membranes and triggers a conformational change in gp41 that allows the virus envelope to fuse with the host cell membrane. Chang et al., (2003) were able to engineer *Lactobacillus* to express soluble CD4, which is able to bind gp120 and block subsequent entry of HIV into the host cells. However, in the case of this study and other bioshields engineered for control of HIV transmission (Chang et al., 2003; Pusch et al., 2005; Vangelista et al., 2010), proof-of-potential *in vivo* is lacking owing to the host-restricted nature of the pathogen and challenge of obtaining ethical and regulatory consent.

In addition to delivery of anti-viral peptides, LAB have been engineered to express viral antigens. For example, Lei et al. (2010) constructed vectors for expression of antigens of H5N1 influenza virus in *Lactococcus lactis*. The authors expressed a fragment of the H5 HA1 in cytoplasmic and secreted forms. In subsequent studies they also produced a surface-anchored form of HA1 by fusion to the PgsA protein from *Bacillus subtilis*. The authors showed that *L. lactis* expressing an HA1 fragment were able to induce antigen-specific serum IgG and faecal IgA antibody following oral administration in a mouse model (Lei et al., 2011; Lei et al., 2010). However, *in vivo* induction of IgG and IgA was only possible for engineered bacteria enclosed within polymer capsules as loss of *L. lactis* cell viability in the gastric environment affected the expressed antigens. Oral administration of the HA1 antigen tethered to the surface of a *L. lactis* strain combined with the cholera toxin subunit B protected the mice from a lethal dose of H5N1 virus. It is possible that such live vaccines could be engineered to simultaneously express anti-viral peptide(s) and/or be engineered to express multiple HA subtypes in order to provide broad-spectrum protection. Furthermore, current studies indicate that prophylactic administration of native *Lactobacillus* either orally or intranasally...
protects against lethal doses of influenza in the murine model (Hori et al., 2002; Kechaou et al., 2013; Kiso et al., 2013). Studies describe reductions in viral lung titre following treatment with either live (Youn et al., 2012), or heat inactivated strains of Lactobacillus (Kobayashi et al., 2011). Prior or concurrent infection with Lactobacillus is thought to protect against infection by activation of local innate immunity, though the precise mechanisms are unknown.

There are several advantages for proposing Lactobacillus as delivery vectors for anti-viral peptides. First, lactobacilli are generally regarded as safe (GRAS) and are already in use in poultry probiotics owing to their ability to competitively exclude pathogens for example, Aviguard® and Primalac® poultry probiotics are used as competitive exclusion agents of pathogens such as Salmonella (Kerr et al., 2013). Probiotics are recommended for use in birds that have been treated with antibiotics, where the balance of beneficial commensal gut microflora can be disrupted. Aviguard is effective in reducing gross lesions and performance loss caused by Clostridium perfringiens, it has been shown to reduce establishment and shedding and multi-resistant E-coli. Second, they are capable of forming biofilm-like communities on epithelia in the avian intestinal tract (Lebeer et al., 2011), a key site of influenza replication (Swayne, 2008), suggesting that they may present a barrier to entry and/or shedding. Third, they are able to persist in the GI tract of poultry that are fed different diets even in the presence of competitive strains (Stephenson et al., 2010). Fourth, they are proven to be suitable for transformation and constitutive expression of heterologous peptides. The cell penetrating nature of FluPep may mean that anti-viral peptide expressed in the gut could spread to distal sites of viral replication.

In this chapter, a survey of the current literature was completed to allow for the design of an expression cassette for Lactobacilli to express and secrete FluPep and Entry Blocker peptides. Following this, tests were performed to determine whether elements of the engineered cassette had deleterious effects on the efficacy of the
peptides and to determine if the active peptides were effectively expressed and secreted.

4.2 Results

4.2.1 Design of a cassette for the expression and secretion of anti-influenza peptides

A literature survey was conducted to identify elements that have been previously used to direct efficient transcription, translation and protein secretion in *Lactobacillus*. The elements contained within the expression cassette are presented together with descriptions of their function, location within the cassette and rationale for selection (Figure 4.1). In order to direct secretion of anti-viral peptides in *Lactobacillus*, it is necessary to drive transcription of the cognate gene, initiate translation of the mRNA, use a signal peptide to direct the peptide for secretion, ensure the signal peptide is efficiently cleaved to release active peptide with the addition of a pro-peptide C-terminal to the cleavage site and to place such a cassette in the chromosome or a stable plasmid such that it is maintained. A further requirement for efficient expression of the protein is that codon use is optimised to that of the host strain.

The promoter and ribosome binding site were selected based on a study by Stephenson et al. (2011) using production of green fluorescent protein as a reporter (Stephenson et al., 2011). The signal peptide and pro-peptide were selected owing to their use in secretion of HIV-1 fusion inhibitor (Pusch et al., 2005; Pusch et al., 2006). The regions cloned and sequenced were precisely as described in these studies.

4.2.2 The effect of fusing a pro-peptide to FluPep on anti-viral activity

A predicted requirement for efficient secretion of anti-viral peptides using this system is the inclusion of a ‘pro-peptide’, which is encoded immediately C-terminal to the cleavage site for the signal peptidase. Pusch et al. (2006) who engineered
*Lactococcus lactis* to secrete cyanovirin (CV-N) as a potential means to prevent HIV-1 infection reported that the introduction of a DTNSD pro-peptide (normally found C-terminal to the signal peptidase cleavage site in the Usp45 protein) increased the translocation efficiency of CV-N across the bacterial cytoplasmic membrane by approximately 6-8-fold (Pusch et al., 2005). However, when extending these studies to expression of C-protein fusion inhibitors the team reported that while secretion was more efficient, the potency of the anti-viral peptide was decreased as a result of the addition of DTNSD (Pusch et al., 2006).

To determine whether the addition of DTNSD had deleterious effects on the anti-viral activity of FluPep, synthetic FP3 and DTNSD-FP3 were purchased with the pro-peptide located at the N-terminus (Cambridge peptides) and plaque reduction experiments at a range of peptide concentrations performed using A/WSN/33 (H1N1), following previously published conditions (Nicol et al., 2012). Analysis of the predicted structure of the peptides using PEP-FOLD software indicated that the addition of DTNSD to FP3 is not anticipated to disrupt the predicted α-helical structure of FP3 (data not shown). Plaque reduction activity was detected for FP3 against A/WSN/33 relative to DMSO controls and at comparable final concentrations to earlier work (Figure 2.3) and the addition of the DTNSD pro-peptide at the N-terminus did not substantially alter its activity (Figure 4.2), at least at the concentrations tested. We therefore elected to include a pro-peptide in the design of the expression cassette with the aim of enhancing peptide secretion efficiency.
Figure 4.1 Design of a cassette for the expression and secretion of anti-viral peptides. (a) Schematic representation of the cassette designed for the expression and secretion of antiviral peptides (b) elements of the cassette were colour coded as follows; promoter sequence (blue), ribosome binding site (purple), signal peptide (green), pro-peptide (pink) and anti-viral peptide (red) (c) Location, source, and function of the elements required within the cassette driving the expression and secretion of the anti-viral peptides.

<table>
<thead>
<tr>
<th>Element</th>
<th>Source and Genebank accession No</th>
<th>Nucleotides</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction site NcoI</td>
<td></td>
<td>6-12</td>
<td>Allows movement of cassette in and out of plasmid vectors</td>
</tr>
<tr>
<td>Restriction site BglII NotI</td>
<td></td>
<td>12-18, 238-246</td>
<td>Provides cassette with a modular structure allowing for exchange of different promoters in and out of the cassette, for future optimisation</td>
</tr>
<tr>
<td>Promoter for (ahl, lactate dehydrogenase) gene</td>
<td>Lactobacillus casei M76708</td>
<td>18-238</td>
<td>Allows for the initiation of transcription. pldhL has previously been shown to be the most active of a number of screened promoters in driving the transcription of CFP in Lactobacillus (Stephenson et al., 2011)</td>
</tr>
<tr>
<td>Ribosome-binding site (RBS) from the amyA (amylase) gene</td>
<td>Lactobacillus amylovorus X80271</td>
<td>246-265</td>
<td>Required to direct efficient ribosomal entry and translation (Stephenson et al., 2011)</td>
</tr>
<tr>
<td>N-terminal signal peptide Usp45</td>
<td>Lactococcal secretion peptide</td>
<td>265-346</td>
<td>The hydrophobic signal peptide allows for the passage of DTNSD-Flupep across the cell membrane through the Sec-dependent system. Usp45 reported to direct efficient secretion of HIV-C-peptide fusion inhibitors in Lactobacillus (Pusch et al., 2006)</td>
</tr>
<tr>
<td>Pro-peptide DTNSD</td>
<td>This peptide represents first 5 amino acids of secreted Usp45</td>
<td>346-361</td>
<td>Reported to enhance secretion efficiency by preventing non-specific association with the cell wall and degradation of peptide of interest by host cell/environmental proteases (Pusch et al., 2005)</td>
</tr>
<tr>
<td>Anti-viral peptide</td>
<td>(Ahmed et al., 2009; Jones et al., 2006; Nicol et al., 2012)</td>
<td>361-409</td>
<td>Anti-viral peptide or reporter</td>
</tr>
<tr>
<td>Stop codon</td>
<td></td>
<td>409-412</td>
<td>Terminates translation</td>
</tr>
</tbody>
</table>
4.2.3 *De novo* synthesis and cloning of an expression and secretion cassette for FP3

Following its design, the sequence of a cassette for expression and secretion of FP3 was sent to Dundee Cell Products for *de novo* synthesis using codons optimised for *Lactobacillus*. However, Dundee Cell Products were unable to clone the full FluPep expression cassette described in Figure 4.1. Several attempts were made to clone the expression cassette into pBluescript but the team reported a low frequency of recombinants despite several attempts to optimise protocols.

Measures such as recovery of transformants at 30°C and culture in the absence of the inducer used for blue-white screening for recombinants were taken without success. On the rare occasions recombinants were obtained all the sequenced inserts had truncations in the predicted C-terminus of the fusion protein in the region corresponding to FluPep. In an attempt to overcome potential instability during *de novo* DNA synthesis, Dundee Cell Products designed a GC-rich variant of the expression cassette to test whether a 74% AT-rich region which encodes the C-terminus of FP3 was causing the insert to be unstable at the site of the truncations (appendix Figure 7.1). However, these changes did not overcome the problems experienced during cloning and recombinants again possessed truncations or stop codons in the FluPep-encoding region (Appendix Figure 7.1). At this stage, it was unclear if the issue was due to DNA instability or toxicity of the FluPep region.

The manufacturers were able to supply a construct with the desired sequence in the promoter, RBS, signal peptide and pro-peptide regions, but which was truncated by 47 base pairs at the 3’ end (Appendix Figure 7.1). On receiving the truncated DNA, we sought to fuse the missing FluPep region by PCR as shown schematically (Figure 4.3). Plasmid DNA of the pBluescript construct containing *pldhL, amyA* RBS, Usp45 signal peptide and DTNSD pro-peptide and truncated FP3 (pBluescript:Ec:FP3-47) was purified, as described (5.2.12) and used as a template
for PCR with a long 3’ primer containing the missing FP3 sequence and which overlaps with the pro-peptide sequence. The PCR amplicon was then ligated to pCR 4-Blunt-TOPO plasmid using a Zero Blunt TOPO PCR cloning kit and transformed into *E. coli* TOP10 K-12 cells. Putative recombinants were screened by colony PCR using M13 forward and reverse primers that span the site of insertion of the blunt-ended PCR product. Clones which were positive for inserts of the expected size were sent for sequencing. However, sequencing revealed that all clones arising from attempts to fuse FP3 to the signals for expression and secretion from pBluescript:Ec:FP3-47 contained insertions affecting the predicted C-terminus of FP3. An example is shown in the appendix Figure 7.2; other clones contained truncations and the position and span of these truncations varied.

Taken together with the inability of Dundee Cell Products to rescue a full-length clone after *de novo* synthesis of the cassette, we reasoned that FP3 may be toxic to *E. coli*. The effect of exogenous FluPep on growth and viability of *E. coli* was investigated by incubating *E. coli* strain DH5α in the presence of varying concentrations of synthetic peptide. This did not reveal any effect of exogenous FP3 on growth or viability of *E. coli* up to quantities of 4 µg of peptide per well (data not shown), but the data do not exclude the possibility that FP3 is toxic only when produced inside the bacterial cell.
Figure 4.2 Effect of fusing a DTNSD pro-peptide to FluPep on anti-viral activity. The effect of fusing a pro-peptide to the anti-viral activity of FP3 was tested by plaque reduction assay on MDCK cells. MDCK cell monolayers were infected with 250 pfu/well of virus in the presence of vehicle 1.5% (v/v) DMSO or increasing quantities of peptide (0.001-10 µM). Plaque reduction activity of FP3 (blue) and DTNSD-FP3 (green) were assayed against A/WSN/33 (H1N1) (a) Data are plotted as percentage titres (pfu) relative to 1.5 % (v/v) DMSO treated cells. (b) Representative image of a plaque reduction assay with A/WSN/33 virus infected cells in the presence of increasing quantities of peptide. Differences in the molecular weight of the peptides have been accounted for in the calculation of concentration. The mean percentage ± SEM are shown for two independent experiments performed in duplicate.
Figure 4.3 PCR-mediated repair of a truncated construct for expression and secretion of anti-viral peptides. Missing peptide nucleotide sequences were fused onto the cassette encoded by pBluescript:EcFP3-47 by PCR using a long 3’ primer. (a) Representative schematic of pBluescript:EcFP3-47 construct synthesised by Dundee Cell Biologicals. (b) PCR reaction allowed for the amplification of the region containing PldhL, amyA RBS, Usp45 signal peptide and DTNSD pro-peptide and addition of the missing peptide sequence using a long 3’ primer which includes overlapping sequence homologous the pro-peptide sequence. (c) This PCR amplicon was then ligated into pCR4Blunt-TOPO and the resulting plasmids transformed into chemically-competent TOP10 cells.
4.2.4 Generation of other constructs for expression and secretion of FluPep peptides

Owing to complications relating to the cloning of the FP3 expression cassette, attempts were made to fuse other FluPep derivatives to the elements for expression and secretion using the PCR strategy described in Figure 4.3. Nicol et al. (2012) have developed a library of FluPep derivatives, some of which exhibit improved potency and solubility. The peptides selected for cloning were those that displayed the greatest efficacy in vivo and/or in plaque-reduction tests (Table 4.1). No recombinants were recovered that contained the cassette fused to FP1, FP2, FP7 or truncated FP8, despite repeated experiments using different conditions and in the presence of control reactions which gave good transformation efficiencies. A number of putative RRKK-FP2 and FP4 clones were obtained and screened by colony PCR. All the RRKK-FP2 clones sequenced were either contained truncations or insertions at the C-terminus of FluPep, an example of which is displayed in the appendix (Figure 7.3). For FP4, deletion mutations and frameshift mutations that abolish FluPep expression were observed (appendix, figure 7.4). However, a clone containing the full DNA sequence for FP4 was retrieved and validated by sequencing (appendix, Figure 7.4). This recombinant was subsequently tested for stability and following five passages by batch culture in E. coli in LB broth the clone was found to be stable with no change in sequence (data not shown).
Table 4.1 Properties of FluPep derivatives studied in this chapter and outcome of attempts to clone in cassette for expression and secretion in *Lactobacillus*.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MW (Da)</th>
<th>Cloning outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>(DTNSD) FP1</td>
<td>DTNSDWLVFFVIFYFFR</td>
<td>2216.52</td>
<td>No recombinants</td>
</tr>
<tr>
<td>(DTNSD) FP2</td>
<td>DTNSDWLVFFVIAYFAR</td>
<td>2064.33</td>
<td>No recombinants</td>
</tr>
<tr>
<td>(DTNSD) FP3</td>
<td>DTNSDWLVFFVIFYFFRRKK</td>
<td>2785.25</td>
<td>Truncated/mutated clones</td>
</tr>
<tr>
<td>(DTNSD) FP4</td>
<td>DTNSDRKKWLVFFVIFYFFR</td>
<td>2785.25</td>
<td>Truncated/mutated clones but one with the expected sequence</td>
</tr>
<tr>
<td>(DTNSD) FP8</td>
<td>DTNSDWLVFFVRRKK</td>
<td>1911.19</td>
<td>No recombinants</td>
</tr>
<tr>
<td>RRKK-FP2</td>
<td>DTNSDRKKWLVFFVIAYFAR</td>
<td>2633.05</td>
<td>Truncated/mutated clones</td>
</tr>
</tbody>
</table>

Schematic detailing the different types of mutations observed in *E.coli*. Recombinant clones were sequenced but the majority were either truncated within the C-terminal region of FluPep or had frame-shift and deletion mutations. Only one complete clone was generated and encoded the full expression cassette. This vector was first amplified in *E.coli* then transferred to *Lactobacillus*. 

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4.2.5 Generation of constructs for expression and secretion of Entry Blocker peptides.

Further to the apparent toxicity of selected FluPep derivatives in *E. coli*, Entry Blocker peptide and a truncated derivative of EB (B10\(^{NP}\)) which retains broad spectrum activity against LPAI (Figure 2.4a), were separately fused to the expression cassette (with or without the DTNSD pro-peptide, Table 4.2) by PCR using long 3’ primers following the same cloning strategy outlined for FluPep (Figure 4.3). The amplicons were then cloned into the PCR® 4Blunt-TOPO® pTOPO vector using a Zero Blunt TOPO PCR cloning kit. Plasmids pTopo:Ec:EB, pTopo:Ec:B10\(^{NP}\) and pTopo:Ec:DTNSDB10\(^{NP}\) were successfully generated and validated by sequencing. The sequence of the insert of pTopo:Ec:B10\(^{NP}\) is shown as an example (appendix, Figure 7.5).

Table 4.2 Properties of Entry Blocker derivatives studied in this chapter and outcome of attempts to clone in cassette for expression and secretion in *Lactobacillus*.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>MW (Da)</th>
<th>Cloning outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10NP</td>
<td>RRKKLAVLLALLA</td>
<td>1464.90</td>
<td>Multiple positive stable clones</td>
</tr>
<tr>
<td>(DTNSD) B10NP</td>
<td>DTNSDRRKKLAVLLALLA</td>
<td>1997.37</td>
<td>Multiple positive stable clones</td>
</tr>
<tr>
<td>EB</td>
<td>AAVALLPAVLLALLAP</td>
<td>1515.94</td>
<td>Multiple positive stable clones</td>
</tr>
</tbody>
</table>

4.2.6 Cloning of pTopo:Ec:peptide constructs into the pUK200 shuttle vector

Following the successful generation of pTopo:Ec:FP4 and pTopo:Ec:B10\(^{NP}\) stable clones, the next step was to sub-clone the peptide amplicons into the *Lactobacillus* shuttle vector pUK200. pUK200 was selected based on previous studies
that characterise pUK200 as a plasmid that is stable within the host (Horn et al., 2005), contains a Nisin inducible promoter and may be shuttled between *E. coli* and *Lactobacillus*. Initial experimentation attempted cloning using JM109 *E. coli* (in which pUK200 has been reported to replicate), however the transformation efficiencies remained low despite several attempts at optimisation. Typically, transformation efficiencies of $7.0 \times 10^3$ per microgram of DNA were observed. An alternative *E. coli* host (MC1061) was identified based on the work of Wegmann et al. (1998). MC1061 cells were made electrocompetent and found to have higher transformation efficiencies for cloning empty pUK200 (typically around $4.0 \times 10^4$). The cassettes for expression and secretion of FP4 and B10$^{NP}$ were excised from the corresponding pTopo vectors by digestion with *NcoI* and *XbaI* and cloned into the pUK200 vector cut with the same enzymes. Recombinants were recovered and sequence validated (data not shown). The question then was whether anti-viral activity could be detected within the bacterial supernatant.

For this present study three *Lactobacillus* strains were selected based on available literature. *Lactobacillus vaginalis* (Lv5) and *Lactobacillus agilis* (La3) were received from D. Stephenson and selected based on data, which identifies them as robust colonisers of the chicken gut (Stephenson et al., 2010). These strains persisted within the chicken gut for periods of up to 42 days post-inoculation and their colonisation in the gut was not affected by diet. Furthermore *L. vaginalis* Lv5 and *L. agilis* La3 have also been shown to express GFP using the same promoter and signal sequence selected in this study (Stephenson et al., 2011). A third strain, *Lactobacillus johnsonii* FI9785, was also selected for anti-viral peptide delivery. It is a poultry-derived isolate reported to competitively exclude the necrotic enteritis pathogen *Clostridium perfringens* (Horn et al., 2005; Wegmann et al., 2009) and reduces intestinal carriage of the zoonotic pathogen *Campylobacter* (M. Stevens, unpublished). FI9785 has also been fully sequenced (Wegmann et al., 2009) allowing for codon-optimisation and selection of appropriate promoters, ribosome-binding
sites and signal peptides for secretion. However, on receiving *L. johnsonii* the bacterium did not exhibit the expected phenotypes of chloramphenicol sensitivity as required for the lactobacillus plasmid. Therefore it was decided to proceed with Lv5 and La3.

4.2.7 Testing bacterial supernatants from *Lactobacillus* containing pUK200:Ec:peptide constructs

As a first step towards testing whether the recombinant clones were able to produce anti-viral peptide, bacterial supernatants from the recombinant clones were collected and tested for anti-viral activity by plaque reduction assay (PRA). *Lactobacillus* bacterial cultures were prepared by growing the bacteria for 48 hrs in de Man, Regosa and Sharpe (MRS) broth under anaerobic conditions (37°C). Then to test whether the supernatant could be used in a PRA, the supernatant was harvested by spinning down the bacteria twice (at 4° at 62000 x g), diluted two-fold in serum free media (SFM) and used to overlay the MDCK cell monolayer (bacterial supernatant, Figure 4.4a). Treatment of uninfected MDCK cells (mock, no virus) with the supernatant led to a loss of cell viability relative to the cells incubated within SFM. This loss may be explained by the low pH of the bacterial supernatants, as when *Lactobacillus* grow they produce acids and the pH of the supernatant was routinely recorded to be pH 3.5-4. Buffering the supernatant to pH 7 by addition of sodium hydroxide (before treating the MDCK cell monolayer) somewhat restored the integrity of the monolayer. From this point the PRA were performed using the buffered supernatant.

First, as a control for the assay MDCK cells were infected with A/WSN/33 in serum free media (SFM) as described in Chapter 2 (Figure 4.4b). In line with previous experiments, infection with 250pfu/mL (450µL in SFM) of A/WSN/33 resulted in the formation of around 80-120 lytic plaques per well. Bacterial supernatant was collected from overnight cultures of recombinant Lv5 lacking plasmid (Figure 4.4c,
no pUK200), or clones containing either empty vectors or vectors expected to drive peptide expression (Figure 4.4d). They were then buffered and used to infect MDCK cell monolayers with A/WSN/33. Generally, infection with the bacterial supernatants led to reductions in plaque numbers compared to infection with SFM. Additionally, there were no differences between the number of plaques observed within supernatant taken from empty or ‘peptide expressing’ bacteria. Suggesting an effect of the supernatant on cell health and/or virus. To test whether plaque formation could be inhibited whilst performing the infection within the bacterial supernatant, the supernatant was spiked with FluPep 4 at concentrations shown to prevent plaque formation (0.1-10 µg/well, 10µg equivalent of 10μM) (Figure 4.4e). In the spiked samples, no reductions in plaque number were observed. It became clear that using this system it would be difficult to determine whether the bacteria were expressing/secretion peptide or whether they were active. The results also cast doubt on whether the peptides are able to function in lactobacillus due to their unknown toxic effects. Therefore, the aim now was to fuse a gene for a reporter protein to the elements for expression and secretion to gain a more accurate quantification of expression.
Figure 4.4 Testing bacterial supernatants from *Lactobacillus vaginalis* containing pUK200:Ec:Peptide for anti-viral activity by plaque reduction assay. a) MDCK cells were treated with bacterial supernatant (supernatant buffered to pH 7) harvested from *Lactobacillus* containing empty vector or serum free D-MEM b) serum free media virus and vehicle control samples c) buffered supernatant virus infected and vehicle control samples d) bacterial supernatant obtained from bacteria containing pUK200:Ec:peptide anti-viral peptide expression and secretion constructs e) bacterial supernatant was ‘spiked’ with FP4 at increasing quantities (0.1-10 μg/well).
4.2.8 Attempts to generate a cassette for expression and secretion of enhanced green fluorescent protein (EGFP) by PCR-ligation-PCR.

In order to confirm that the elements for transcription, translation and secretion of peptides within the expression cassette were active (because no activity was detected in the bacterial supernatant, Figure 4.4), attempts were made to fuse the enhanced green fluorescent protein (EGFP) to the expression cassette from pBluescript:Ec:FP3-47. The expression cassette was designed to be modular to allow the various elements of the cassette to be substituted in and out (for example, introduction of different chicken promoters). An EGFP reporter system would allow for a direct measure of the protein expression driven by the elements of the cassette and could be used as a means of optimising the system. This system may also be used to evaluate the activity of the cassette within different lactobacillus species and in vivo to identify the location of bacterial colonisation by detection of the reporter. Initially, PCR-ligation-PCR mutagenesis was used (Figure 4.5) (Ali and Steinkasserer, 1995). Briefly, an initial PCR reaction allowed for the amplification of the two gene fragments; EGFP from pEGFP-C1 and the expression cassette from pBluescript:Ec:FP3-47 ending with the DTNSD motif. To join the expression cassette and EGFP amplicons in frame, a phosphorylation reaction followed by ligation in the same buffer was performed. The fused amplicons were then amplified by a second round of PCR using the 5’ primer for the expression cassette and the 3’ primer for EGFP. An amplicon of the expected size was resolved by agarose gel electrophoresis, excised, purified and ligated into pCR-4 Blunt TOPO to generate pTopo:Ec:EGFP. Next, the expression cassette fused to EGFP was excised from pTopo:Ec:EGFP by digestion with NcoI and XbaI and cloned into the Lactobacillis shuttle vector pUK200 cut with the same enzymes. Putative recombinant clones were difficult to obtain however, restriction analysis of plasmid DNA recovered from four of these indicated that the expected 1146bp NcoI-XbaI fragment was released (data not shown). On transformation into Lactobacillus no green
flourescence was detected. Two pUK200:Ec:EGFP plasmids were recovered and sequenced. Both clones contained deletion mutations one of which was at the junction between the expression cassette and EGFP coding sequence (Appendix, Figure 7.7).

Figure 4.5 Generation a construct in which EGFP is fused to signals for expression and secretion in Lactobacillus by PCR-Ligation-PCR. The fusion of two gene fragments was attempted by PCR-Ligation-PCR (a) The first PCR reaction allowed for the amplification of the two required gene fragments; the expression cassette from pBluescript:Ec:FP3-47 ending with the DTNSD motif (oligonucleotides a & b) and EGFP from pEGFP-C1 (oligonucleotides c & d), (b) a phosphorylation reaction followed by a (c) ligation step were performed and allowed for the fusion of the two gene fragments (d) a second PCR step was then performed using primers ‘a & d’. This PCR step allowed for the amplification of the fused fragment. The required amplicon was then excised, purified and ligated into pCR-4 Blunt TOPO, this plasmid vector is covalently bound with vaccinia virus DNA topoisomerase I.
4.2.9 Attempts to generate pTopo:Ec:EGFP by gene splicing overlap extension PCR.

Following the failure to clone pTOPO:Ec:EGFP by PCR-ligation-PCR, an alternative cloning strategy known as overlapping extension was attempted. This cloning strategy was first described by Horton et al. (1989), and the conditions used in this study were essentially as described by Heckman and Pease (2007). An initial PCR reaction was required to amplify two gene fragments (in this case the expression cassette and EGFP) with extensions at the ends of the primers b + c (Figure 4.6) such that they overlap by twenty nucleotides. During a second PCR reaction the two DNA fragments were mixed in equimolar amounts, denatured and annealed in order to serve as a chimeric template for the generation of a hybrid product using flanking primers a + d (Figure 4.6). As with PCR-ligation-PCR, an amplicon of the expected size was resolved by gel electrophoresis, excised, purified and ligated into pCR-4 Blunt-TOPO. Again, it was difficult to rescue putative recombinant clones but where a clone could be rescued restriction analysis of plasmid DNA indicated the release of the expected 1146bp fragment (data not shown). These recombinants were sent for sequencing however, as with previous attempts to recover pTopo:Ec:EGFP following PCR-ligation-PCR, deletion mutations where again detected within all the clones rescued. Appendix Figure 7.7, shows the sequence of a clone with a frameshift mutation at the junction of the expression cassette and the EGFP coding sequence, despite this sequence being determined by the overlapping oligonucleotides used for PCR to generate the chimeric template. As expected on transformation of *lactobacillus* with the mutated construct, green fluorescence was absent. The presence of deletion mutations about the fusion site of Ec:EGFP and the difficulties rescuing recombinant clones, despite using two independent cloning strategies, are suggestive of toxicity for unknown reasons.
Figure 4.6. Generation of a construct in which EGFP is fused to the signals for expression and secretion in *Lactobacillus* by over-lapping extension PCR. The fusion of two gene fragments was achieved by an initial PCR reaction using two oligonucleotides (b&c) and flanking primers (a&d) to generate intermediate PCR products AB and CD that are overlapping fragments of product AD. (b) In a second PCR reaction products AB and CD were used as templates, which were denatured and strands of each product join at the overlapping, complementary region. Extension of the free 3’ ends then completes the molecule, which is amplified by PCR with primers a and d. Schematic adapted from (Heckman and Pease, 2007).
4.3 Discussion

Toward the development of a bioshield to control avian influenza virus in farmed poultry, a cassette was designed to direct transcription, translation and secretion of anti-viral peptides in *Lactobacillus*. A predicted requirement for efficient secretion of anti-viral peptides in this system (a pro-peptide downstream of the signal peptidase cleavage site in the secretion signal) was confirmed not to dramatically affect the activity of a model anti-influenza peptide (FluPep derivative FP3) in PRA with the A/WSN/33 virus on MDCK cells. Unexpectedly, significant problems were encountered in *de novo* synthesis and cloning of the cassette for expression and secretion of FP3. No full-length clones could be recovered and the issue could not be solved by altering the GC composition of the cassette or reducing insert expression by growth of transformants at 30°C or in the absence of the inducer used to screen for recombinants. Where it was feasible to clone cassettes synthesised *de novo* these always contained insertions or point mutations resulting in frameshifts in the region encoding FP3. The same issue was encountered when we attempted to add back the FP3 region by PCR with a long 3' primer encoding FP3 using the expression and secretion cassette as a template. The basis of such toxicity to *E. coli* is not understood, but presumably reflects interference in processes essential for bacterial replication or damage to the bacterial cell. To obviate the toxicity issues with FP3, attempts were made to attach other FluPep derivatives to the expression and secretion signals by PCR but the same problems were encountered in all cases except a single FP4 clone. Stable clones containing FP4 and B10NP were recovered and transferred to a shuttle vector for production into *Lactobacillus*.

Future work may consider investigating alternative cloning methods which may overcome the complications relating to toxicity observed in *E. coli*, as it is feasible that FluPep or expression cassette toxicity is peculiar to *E. coli* and would not be observed in lactic acid bacteria. Previous studies describe the use of
*Lactococcus lactis* as a cloning host which may be considered in this case (Horn et al., 2005). Cloning directly into *Lactobacillus* was not considered because of the low transformation efficiency, which is expected to limit the scope to recover rare recombinant plasmids in cloning reactions. *E. coli* are attractive intermediate hosts because they offer far higher transformation efficiencies and it may be worth using strains with genotypes that reduce the potential for recombination events such as SURE (Stop Unwanted Rearrangement Events) cells.

Despite facing difficulties during cloning, FP4 and B10<sup>NP</sup> stable clones were rescued in both *L. agilis* and *L. vaginalis* strains reported to be capable of efficient colonisation of the chicken gastrointestinal tract. The question then was whether anti-viral activity in the supernatants of such bacteria could be detected by plaque reduction assay. Initially, the conditions for the plaque reduction tests required optimisation to eliminate the toxic effects of the acids produced by *Lactobacillus* during growth. In these experiments, bacterial supernatants were first buffered in order to maintain the integrity of the MDCK cell monolayer and prevent a premature conformational change of HA. Subsequent testing showed no reduction in the number of plaques of A/WSN/33 infected cells by *L. vaginalis* and *L. agilis* strains containing the anti-viral peptide constructs relative to empty vector controls. However, whether the elements chosen for expression and secretion of peptide were active remained unknown. It may be that the strains are producing quantities of peptide, which are too low to see an effect and therefore would be undetectable using this test. Furthermore, no plaque reduction activity was detected when the peptide was ‘spiked’ into media used to culture *Lactobacillus* at concentrations known to inhibit plaque formation. It may be that components contained within the bacterial supernatant were prematurely breaking down or interacting with the peptides. For this reason, we also aimed to fuse signals for expression and secretion in *Lactobacillus* to a reporter protein that can be readily detected by fluorescence microscopy, fluorimetry and western blotting (EGFP). However, despite a number
attempts at cloning and using two independent methods of recovery, all Ec:EGFP recombinants contained deletions at or proximal to the site of fusion of the expression cassette and the EGFP coding sequence. Again the basis of toxicity when EGFP is fused to the expression cassette is unknown, especially when considering that the Usp45 signal peptide and DTNSD pro-peptide has been reported to be stable for the secretion of HIV-I fusion inhibitor (Pusch et al., 2005; Pusch et al., 2006). Moreover, Usp45 is widely described as stable for the secretion of heterologous proteins. This study aimed to design an expression cassette allowing for secretion of peptides from *Lactobacillus* isolates. However, despite several attempts to determine whether the elements chosen were active it remains unclear. Future studies may consider fusing smaller epitope tags (such as c-myc) to the end of the FP4 or B10\textsuperscript{NP} expression and secretion cassettes. It may then be possible to test whether the elements were active by western blot or ELISA using epitope-specific antibody.
This study aimed to evaluate the activity and mode of action of two antiviral peptide families as protective agents against influenza A virus infection in poultry and to devise a novel delivery system. Previous studies have shown that FluPep inhibits replication of A/WSN/33 and A/PR/8/34 recombinant viruses containing the glycoproteins of H1N1, H3N2 and H5N1 human outbreak strains (Nicol et al., 2012). A second family of peptides studied by the Shultz-Cherry laboratory and termed ‘Entry Blocker’ (EB) has been reported to prevent virus-mediated agglutination of cRBCs by a number of influenza A strains, however the in vitro activity of such EB and derivatives by plaque reduction assay has not been described. Work presented in this thesis showed that both FluPep and an Entry Blocker derivative had low cytotoxicity in MDCK and A549 cell culture and inhibited the replication of avian A/Mallard/Netherlands/10 (H1N1), A/Duck/England/62 (H3N2) and A/Turkey/Canada/65 (H4N6) viruses, within the micromolar range. FluPep derivatives were generally found to be active at lower concentrations than the EB family and appeared to inhibit H1 viruses better than H3 and H4 subtypes. These differences may be related to the phylogenetic grouping of HA but for a more robust analysis a wider panel of viruses from each HA phylogenetic group would need to be tested. Further experiments sought to determine whether the surface glycoproteins of the viruses contributed to their sensitivity phenotype by using reverse genetics techniques. While data suggested that the peptides may act on the surface HA, a more direct measure of peptide activity on HA was required to understand peptide mechanisms of action.

Viral entry assays were therefore optimised and performed to gain a better understanding of peptide action. Overall, the active peptides were found to act early within the virus replication cycle and by a mechanism either at/or upstream of nuclear viral ribonucleoprotein import into the cell nucleus. This was consistent with
previously published work by Jones et al. (2006) and Nicol et al. (2012). However, our virus entry assays showed that the peptides did not reduce overall virus binding to cells. Instead, FluPep 4 and B10NP treatment led to virus aggregation about the surface of the host cells. FluPep 4 also reduced virus internalisation however, because of the limited time, analysis of internalisation by flow cytometry was performed only at the higher concentration of peptide (10µM). Future studies would need to investigate the effects of the peptides at the lower concentrations of peptide, with particular focus on concentrations previously shown to decrease infectious titre. Earlier studies have shown EB to agglutinate influenza A virus on the cell surface (Jones et al., 2011b). For this reason the group has investigated its use as a vaccine adjuvant with the hypothesis that EB-mediated aggregation of virus would lead to increased virus uptake by antigen-presenting cells. In our studies, full length EB was not active and did not cause virus aggregation on the cell surface. These studies may be extended for study of other FluPep peptides.

The question now is whether FluPep targets HA and/or other viral proteins, which may be answered using a number of additional approaches. The effects of the peptides on virus fusion may be investigated by labelling of viral and endosomal membranes with lipophilic dyes and tracking fusion events by either microscopy or flow cytometry as previously employed (Banerjee et al., 2013; Sakai et al., 2006). Another approach may be to use monoclonal antibodies that preferentially bind different conformational states of surface expressed HA as used by Mair et al. (2014). Alternatively, sequence comparisons of the surface glycoproteins from viruses with differing sensitivities to FluPep may be performed to identify amino acids associated with these differences. It may also be possible to select for a peptide resistant influenza A virus. This could be achieved by passaging virus (at a low MOI) in the presence of sub-optimal concentrations of peptide, which would yield enough virus for subsequent passage. Drug escape mutants would then be sequenced to determine amino acid changes contributing to resistance. The location
of the escape mutations could then be mapped to give an indication of where the peptides act. In a recent study White et al. (2015) used this approach so show that compound S20 inhibited viral replication by binding to HA. Most of the escape mutants contained mutations surrounding or within the large ‘B’ loop structure that connects the large and small α-helices of the HA2 subunit. Alternatively, sequence comparisons of the surface glycoproteins from viruses with differing sensitivities to FluPep may be performed to identify amino acids associated with these differences. For the progression of these agents it would be necessary to fully understand the peptide mechanisms of action for example, if the peptides are causing aggregation of virus outside the cell then would this promote uptake of virus by antigen presenting cells and have implications for adverse effects (for example, antibody dependent enhancement during Dengue virus infection). Furthermore, if the peptides do result lead to non-specific agglutination of viruses, it may not be possible to generate escape mutants. This study did not consider the effects of the peptides on cellular immune responses; future work could also consider microarray studies to test whether the peptides are influencing immune responses. This study identified FluPep as a potent inhibitor of avian influenza A viruses with low cytotoxicity. The question now is how these peptide(s) could be delivered cost effectively and efficiently to poultry?

Current measures for the control of influenza primarily involve vaccination, surveillance and implementation of biosecurity measures that rely on mass culling in the event of an outbreak. One approach to protect domestic poultry from influenza A virus infection would be to selectively breed resistance traits into the lines used in farming. However, this technology is currently unavailable and is still within the early stages of research. An alternative approach for prevention of influenza A virus transmission between birds has been described by Lyall et al. (2011) where chickens have been genetically engineered so that they are less able to transmit avian influenza owing to constitutive expression of a decoy RNA
resembling a vRNA segment that replicated in preference to the viral genome. This approach provides a number of advantages: first, broad-spectrum protection preventing the need to seasonally update vaccines. Secondly, the technology does not rely on egg-based vaccines, which are costly and require updating. Therefore this approach would be cheaper than current vaccines. But, while the WHO regards genetically-modified food/crops (GMOs) as safe to consume, GMOs have not yet gained public acceptance and there is hesitance towards the use of this technology in food production.

Probiotics have however gained acceptance and are currently used in poultry in formulations such as Aviguard® and Broilact®. These are commonly administered in drinking water and are inexpensive to culture. Probiotic bacteria pose little threat to animal and human health and therefore are generally regarded as safe (GRAS). During the time of this study, a scientific group in China have engineered variants of indigenous flora to express beneficial molecules. In the context of influenza A virus Lei et al., have presented a number of studies that genetically engineer Lactococcus Lactis to display virus surface glycoproteins HA1 (Lei et al., 2011), HA2 (Lei et al., 2015b), neuraminidase (Lei et al., 2015c) and nucleoprotein (Lei et al., 2015a). Oral administration of recombinant L. lactis has been shown to protect from lethal challenge of influenza A virus in both the murine and galliform models. However, in most cases convincing protection was only achieved with the administration of a cholera B toxin adjuvant. None the less, the group have produced proof-of-potential data that shows L. lactis expressing either HA or NA fragments to induce antigen-specific induction of IgA from the upper respiratory tract and serum IgG. This method of delivery is less controversial than the use of live-attenuated pathogens or transgenic animals. Using this rationale, our study attempted to engineer Lactobacillus Lv5 and La3 to constitutively express and secrete FluPep for delivery to the intestinal tract of poultry. The aim was to design a cheap and easy approach to administer a prophylactic agent. However attempts to
clone an expression cassette with the elements required to drive expression of heterologous proteins in *Lactobacilli* proved challenging owing to toxicity, despite using elements described in published studies. A stable FluPep 4 construct was obtained however, but no antiviral activity could be detected by plaque reduction assay. This work was limited to testing the constructs using plaque reduction assay, alternative approaches may include investigating activity in gut organoid explants. Due to the difficulties faced in manipulating *Lactobacillus* future studies may consider using alternative delivery vectors such as *Lactococcus lactis* where directed cloning may be possible and can be used for intranasal vaccines. Vectored vaccines approved for poultry use include Newcastle virus, fowl poxvirus and herpes virus. It may be possible to explore viral vectors for delivery of FluPep because these provide a strategy for administering FluPep to the respiratory tract, which is the route of IAV entry. The disadvantage however would be reduced efficacy as a consequence of active immunity to the virus used to vector the peptide. This study opens up many questions for using lactic acid bacteria for delivery of therapeutic agents, for example once administered what is the fate of *lactobacillus* in the mucosal cavities? How long would the bacteria survive or persist in the gut? How long would the peptide persist before it is broken down? Would the *in vivo* replication of vectored bacteria intensify the induced immune response? How does the bacterial dose influence immunogenicity? Some of these questions may be answered either by *in vivo* challenge or by the use of explants.
## Chapter 6 Materials and Methods

### 6.1 Materials

#### 6.1.1 Oligonucleotides and plasmids

All primers were purchased from Sigma Aldrich.

i) Oligonucleotides used for sequencing of plasmid constructs and viruses

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Description</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Virus Cloning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uni-12</td>
<td>cDNA synthesis</td>
<td>AGCAAAAGCAGG</td>
</tr>
<tr>
<td>MF1</td>
<td>Amplification of M segment</td>
<td>AGCAAAAGCAGGTAGATATTGAAAGA</td>
</tr>
<tr>
<td>MR1027</td>
<td>Amplification of M segment</td>
<td>AGTAGAAACAAGGTAGTTTTTACTC</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10 HA fwd</td>
<td>Partial amplification of HA</td>
<td>TGCTTGGGGAGTGCATCAT</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10 HA rev</td>
<td>Partial amplification of HA</td>
<td>GTTGAACTCCTGCTCAGTAC</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10 NA fwd</td>
<td>Partial amplification of NA</td>
<td>GGTTCAGTGACATTAGCGGG</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10 NA rev</td>
<td>Partial amplification of NA</td>
<td>GTCAGTTCTGGATGCTGGACA</td>
</tr>
<tr>
<td>A/PR/8/34 HA fwd</td>
<td>Partial amplification of HA</td>
<td>ACGGAGAAGGGAGGCTCATA</td>
</tr>
<tr>
<td>A/PR/8/34 HA rev</td>
<td>Partial amplification of HA</td>
<td>TCCAGAGTCCTTCTTTTCAGT</td>
</tr>
<tr>
<td>A/PR/8/34 NA fwd</td>
<td>Partial amplification of NA</td>
<td>TCTTTGTCCCATCCGTTGGT</td>
</tr>
<tr>
<td>A/PR/8/34 NA rev</td>
<td>Partial amplification of NA</td>
<td>AAGCACGGCCCTCATACAGTC</td>
</tr>
<tr>
<td><strong>Molecular cloning</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13_Fwd</td>
<td>TOPO vector cloning</td>
<td>GTAAAACGACGCGCAG</td>
</tr>
<tr>
<td>M13_Rev</td>
<td>TOPO vector cloning</td>
<td>CAGGAAACAGCTATGAC</td>
</tr>
<tr>
<td>PUk200_p54_Fwd</td>
<td>Flanking multi-cloning site</td>
<td>CGGCCTGATTAATTCTGAAG</td>
</tr>
<tr>
<td>PUk200_p181_rev</td>
<td>Flanking multi-cloning site</td>
<td>GCGAAGATAACAGTGACTCTA</td>
</tr>
</tbody>
</table>
ii) Oligonucleotides used for addition of anti-viral peptides within a cassette for the expression and secretion of peptides

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Description</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC_Fwd</td>
<td>Expression cassette (EC) Fwd</td>
<td>ATATATGGATCCCCCATGGAGATCTCCT</td>
</tr>
<tr>
<td>EC_DTNSD-FP1_rev</td>
<td>Addition of DTNSD-FP1 rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTAAGAAGTAGAA AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-FP2_rev</td>
<td>Addition of DTNSD-FP2 rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-RRKKFP2_rev</td>
<td>Addition of DTNSD-RRKKFP2 rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-FP3_rev</td>
<td>Addition of DTNSD-FP3 rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-FP4_rev</td>
<td>Addition of DTNSD-FP4 rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-FP7_rev</td>
<td>Addition of DTNSD-FP7 (6’mer FluPep) rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-FP8_rev</td>
<td>Addition of DTNSD-FP8 (6’mer FluPep) rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-EB_rev</td>
<td>Addition of DTNSD-EB rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_B10NP_rev</td>
<td>Addition of DTNSD-B10NP rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
<tr>
<td>EC_DTNSD-B10NP_rev</td>
<td>Addition of B10NP rev</td>
<td>ATATATCTGCAAGTCTAGACTATCTCGCAAGAATAGAC AATAACGAAGAATATAACCAATCGAGAATTAGTGTC AGCATATAACCAAGATAATG</td>
</tr>
</tbody>
</table>

For fusion of EGFP to expression cassette by PCR-Ligation PCR-PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Description</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEGFP-C1_fwd</td>
<td>Amplification of EGFP from pEGFP-C1 fwd</td>
<td>GTGAGCAAGGGCGAGGAGCT</td>
</tr>
<tr>
<td>pEGFP-C1_rev</td>
<td>Amplification of EGFP from pEGFP-C1 plus Xba</td>
<td>ATATATCTGAGATTACCTAGATC</td>
</tr>
<tr>
<td>Cassette fwd+Ncol</td>
<td>Amplification of EC and introduction of Ncol</td>
<td>ATATATCCATGGAGATCTCC</td>
</tr>
<tr>
<td>Cassette rev primers</td>
<td>Amplification of EC</td>
<td>ATCAGAATTAGTGTCAGCAT</td>
</tr>
</tbody>
</table>
For fusion of EGFP to expression cassette

<table>
<thead>
<tr>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_No_3_EGFP_O/L Fusion of EGFP to cassette</td>
<td>GACACTAATTCTGTAGTGAGCAAGGCGAGGAG</td>
</tr>
<tr>
<td>p_No_2_EGFP_O/L Fusion of EGFP to expression cassette</td>
<td>CTGCCCCTTGCTCACATCAGAATTAGTGTAGGAGC</td>
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</table>

6.1.2 Sequence validated plasmids used to generate recombinant viruses.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR 4Blunt-TOPO</td>
<td>Used for initial topoisomerase 1-mediated cloning of PCR amplified expression cassettes in <em>E. coli</em> Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUK200</td>
<td><em>Lactobacillus</em> shuttle vector, Chl&lt;sup&gt;r&lt;/sup&gt;, <em>PnisA</em>, terminator of <em>brnQ</em>, pSH71 replicon</td>
<td>Arjan Narbad institute for food research</td>
</tr>
<tr>
<td>pEGFP-C1</td>
<td>Source of the EGFP gene</td>
<td>Clontech</td>
</tr>
<tr>
<td>pBluescript:Ec:FP3-47</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt; Contains fragment coding for pLdhL, Usp45, DTNSD and a truncated FP3 region</td>
<td>Dundee Cell Products</td>
</tr>
<tr>
<td>pTopo:Ec:FP4</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt; contains the expression cassette from pBluescript:Ec:FP3-47 fused in-frame to FP4</td>
<td>This study</td>
</tr>
<tr>
<td>pTopo:Ec:EB</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt; contains the expression cassette from pBluescript:Ec:FP3-47 fused in-frame to EB</td>
<td>This study</td>
</tr>
<tr>
<td>pTopo:Ec:B10&lt;sup&gt;NP&lt;/sup&gt;</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;, contains the expression cassette from pBluescript:Ec:FP3-47 fused in-frame to B&lt;sup&gt;10&lt;/sup&gt;NP,</td>
<td>This study</td>
</tr>
<tr>
<td>pTopo:Ec:DTNSDB10&lt;sup&gt;NP&lt;/sup&gt;</td>
<td>Kan&lt;sup&gt;r&lt;/sup&gt;, Amp&lt;sup&gt;r&lt;/sup&gt;, contains the expression cassette from pBluescript:Ec:FP3-47 fused in-frame to DTB&lt;sup&gt;10&lt;/sup&gt;NP,</td>
<td>This study</td>
</tr>
</tbody>
</table>

**pDual virus vectors**

<table>
<thead>
<tr>
<th>pDualA/PR/8/34 Segment 1</th>
<th>EF467818</th>
<th>(de Wit et al., 2004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDualA/PR/8/34 Segment 2</td>
<td>EF467819</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/PR/8/34 Segment 3</td>
<td>EF467820</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/PR/8/34 Segment 4</td>
<td>EF467821</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/PR/8/34 Segment 5</td>
<td>EF467822</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/PR/8/34 Segment 6</td>
<td>EF467823</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/PR/8/34 Segment 7</td>
<td>EF467824</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/PR/8/34 Segment 8</td>
<td>EF467817</td>
<td>(de Wit et al., 2004)</td>
</tr>
<tr>
<td>pDualA/Mall/Segment 1</td>
<td>KC209512</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>Segment</td>
<td>Accession Number</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>2</td>
<td>KC209513</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>3</td>
<td>KC209514</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>4</td>
<td>KC209515</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>5</td>
<td>KC209516</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>6</td>
<td>KC209517</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>7</td>
<td>KC209518</td>
<td>(Bourret et al., 2012)</td>
</tr>
<tr>
<td>8</td>
<td>KC209519</td>
<td>(Bourret et al., 2012)</td>
</tr>
</tbody>
</table>

### 6.1.3 Antibodies

(i) Antibodies to influenza A virus components

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse-monocolonal anti-NP (AA5H)</td>
<td>Abcam (20343)</td>
<td>Immunofluorescence (IF) (used at 1:1000)</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-PR8</td>
<td>(Amorim et al., 2007)</td>
<td>Immunofluorescence and western blot (WB) (used at 1:500)</td>
</tr>
</tbody>
</table>

(ii) Other primary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat monoclonal anti-tubulin</td>
<td>Serotec (MCA77G)</td>
<td>Western blot (1:1000)</td>
</tr>
</tbody>
</table>

(iii) Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Reference</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>AlexaFluor 488 conjugated goat anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>IF (1:1,000)</td>
</tr>
<tr>
<td>Alexa 594 conjugated goat anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>IF (1:1,000)</td>
</tr>
<tr>
<td>IRD680 conjugated donkey anti-mouse IgG</td>
<td>Licor</td>
<td>WB (1:10,000)</td>
</tr>
<tr>
<td>IRD800 conjugated goat</td>
<td>Licor</td>
<td>WB (1:10,000)</td>
</tr>
</tbody>
</table>
### 6.1.4 Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli JM109</td>
<td>e14-((mcrA)–) recA1 endA1 gyrA96 thi-1 hsdR17 (rK– mK+) supE44 relA1 (\Delta) (lac-proAB) ([F' traD36 proAB lac_Z_AM15])</td>
<td>Promega</td>
</tr>
<tr>
<td>E. coli MC1061</td>
<td>recA(^+) araD139 (ara, leu)7697 (\Delta) lacX74 galU galK hsr strA</td>
<td>MOBI tech</td>
</tr>
<tr>
<td>E. coli Top10</td>
<td>F- mcrA (\Delta)(mrr-hsdRMS-mcrBC) (\phi)80lac_Z_AM15 (\Delta) lacX74 recA1 araD139 (\Delta)(ara+leu)7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E. coli DH5(\alpha)</td>
<td>F- (\phi)80lac_Z_AM15 (\Delta)(lacZYA-argF) U169 recA1 endA1 hsdR17(_)rk-, mk(_) supE44 thi-1 gyrA96 relA1 A</td>
<td>Promega</td>
</tr>
<tr>
<td>L. agilis (La3)</td>
<td>Isolated from chicken jejunum, persists within gastrointestinal tract</td>
<td>(Stephenson et al., 2011)</td>
</tr>
<tr>
<td>L. vaginalis (Lv5)</td>
<td>Isolated from chicken jejunum, persists within gastrointestinal tract</td>
<td>(Stephenson et al., 2011)</td>
</tr>
</tbody>
</table>

### 6.1.5 Eukaryotic cells

<table>
<thead>
<tr>
<th>Eukaryotic cell</th>
<th>Source/Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>293T</td>
<td>Human embryonic kidney cells expressing SV40 large T antigen</td>
<td>(DuBridge et al., 1987)</td>
</tr>
<tr>
<td>A549</td>
<td>Human lung epithelial cells</td>
<td>(Giard et al., 1973)</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney carcinoma cells</td>
<td>(Gaush et al., 1966)</td>
</tr>
</tbody>
</table>

### 6.1.6 Virus strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subtype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/PR/8/34</td>
<td>H1N1</td>
<td>R. Fouchier (de Wit et al., 2004)</td>
</tr>
<tr>
<td>A/WSN/33</td>
<td>H1N1</td>
<td>W. Barclay</td>
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<tr>
<td>Reverse genetics (RG)</td>
<td></td>
<td>L. Tiley</td>
</tr>
<tr>
<td>A/Mallard/Netherlands/10/99</td>
<td>H1N1</td>
<td></td>
</tr>
<tr>
<td>A/Duck/England/62</td>
<td>H4N6</td>
<td>W. Barclay</td>
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<tr>
<td>A/Turkey/Canada/65</td>
<td>H4N6</td>
<td>W. Barclay</td>
</tr>
<tr>
<td>Strain</td>
<td>Antigenic Type</td>
<td>Study</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>RG A/PR/8 7:1 A/Mall HA</td>
<td>H1N1</td>
<td>This study</td>
</tr>
<tr>
<td>RG A/PR/8 7:1 A/Mall NA</td>
<td>H1N1</td>
<td>This study</td>
</tr>
<tr>
<td>RG A/PR/8 6:2 A/Mall HANA</td>
<td>H1N1</td>
<td>This study</td>
</tr>
</tbody>
</table>

### 6.1.7 Peptides

HPLC-purified peptides were purchased from Cambridge Peptides. Mass spectrometry QC data was supplied to confirm purity in all cases the peptides were 98% pure. On arrival, lyophilised peptide was dissolved in DMSO to 2mg/mL before use. Unless stated otherwise the peptides were mixed with virus immediately prior to administration to cells. For this study only one batch of peptide was used.

### 6.2 Methods

#### 6.2 Molecular cloning techniques and nucleic acid preparations

##### 6.2.1 Preparation of plasmid DNA

For small preparations, plasmid DNA was isolated from an overnight bacterial (*E. coli* and *Lactobacillus*) culture using a QIAprep miniprep kit (Qiagen). Larger preparations required the use of a QIAprep midiprep kit (Qiagen). Both were used as per the manufacture instructions. DNA was reconstituted in nuclease-free water (Qiagen) and the final concentration was determined by an absorbance reading at 260 nm using a NanoDrop spectrophotometer.

##### 6.2.2 Isolation of total viral RNA

Purification of viral RNA (vRNA) was performed using a QIAamp viral RNA mini kit (Qiagen), as per manufacture recommendations. RNA was
reconstituted in 50 µL of nuclease-free water and quantified using a NanoDrop spectrophotometer.

6.2.3 Reverse Transcription (RT)

Single-stranded DNA (cDNA) synthesis from viral RNA was carried out using a Verso cDNA synthesis kit (Thermo Scientific) and the Uni12 universal primer (Table 5.1.1), which binds to 12 conserved nucleotides at the 3’ end of all influenza RNA segments. Each RT reaction was prepared as follows: 1 ng of vRNA (1-5 µL) and 0.5 µM of Uni12 primer were made up in nuclease free water to a reaction volume of 10 µL and incubated at 65°C for 10 min. Following incubation, the rest of the Verso reaction mix was added and was composed of 20% (v/v) cDNA synthesis buffer, 0.5 mM of each dNTP and 1 µL Verso enzyme mix to give a final reaction volume of 20 µL. This was further incubated for 1 hr at 65°C. No template controls were included separately.

6.2.4 Polymerase Chain Reaction (PCR)

A) Amplification of influenza A virus segments was completed using Taq DNA polymerase (Invitrogen). Forward and reverse oligonucleotide primers were designed to complement regions of segment 4 and 6 of A/PR/8/34 and A/Mallard/Netherlands (Table 6.1.2). Each reaction contained: 1 mM of each dNTP, 1.5 mM MgCl₂, 0.2 µM of each primer and 0.05% buffer W1. PCR reactions were cycled in a Hybaid PCR cycler (Thermo Scientific) using the following programme: 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 3 min with a final incubation at 72°C for 10 min.

B) Bacterial colony screening and diagnostic PCRs were carried out using Taq DNA polymerase (Invitrogen) in a reaction volume of 25 µL. Each reaction contained 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.5 µM of each primer and 0.05% buffer W1. PCR
reactions were cycled in a Hybaid PCR cycler (Thermo Scientific) using the following programme: 98°C for 3 min, followed by 30 cycles at 94°C for 30s, 48°C for 30s and 72°C for 90s with a final incubation at 72°C for 10 min.

C) High fidelity PCR reactions were performed using Phusion polymerase (Thermo Scientific). Reactions containing 0.02 U/µL Phusion polymerase, 0.5 µM of each primer, 200 µM dNTPs and 200-300 ng of template DNA to a reaction volume of 20µL were prepared. PCR was performed using an initial cycle at 98°C for 30s, followed by 30 cycles at 98°C for 10s and annealing cycles at 65°C for 30s with a final extension cycle 72°C for 10 min.

6.2.5 Restriction enzyme digests

Restriction endonucleases (New England Biolabs or Invitrogen) were typically used with 2 µL of recommended 10X REact buffer, 0.2 µL acetylated bovine serum albumin (BSA), up to 1 µM DNA in nuclease free water to a total of 20 µL. Reactions were incubated at room temperature for 2 hrs.

6.2.6 Ligation of DNA fragments

DNA ligation reactions were performed using 1U of T4 DNA ligase (New England Biolabs), 1X Ligation buffer and 100-300 ng of template DNA in a reaction volume of 20 µL. Reactions were prepared as per manufacturer’s recommendations and incubated overnight at 4°C.

6.2.7 Glycogen precipitation reaction

Glycogen precipitation was required for purification and concentration of DNA from ligation reactions. Glycogen precipitation reactions contained: 5 µL of 20 mg/mL glycogen, 300 µL ethanol (EtOH), 10 µL of 3 M sodium acetate NaAc (pH5.2), 65 µL nuclease-free H₂O and the 20 µL ligation mix from 6.2.6. Reactions were incubated at -20°C for 30 min. DNA pelleted was by centrifugation at 17,900 x
g, washed three times in 70% EtOH and resuspended back into 5 µL nuclease free H₂O. Concentrated DNA was stored at -20°C until required.

6.2.8 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a 0.8-2% (w/v) agarose gel (Sigma Aldrich) containing a 1:10,000 dilution of SYBRsafe DNA gel stain (Invitrogen). Loading buffer was added to each sample prior to loading the gel and separation was carried out at 80-90 V for 45-60 min in TAE loading buffer made using 0.04 M Tris-acetate, 1mM EDTA.

6.2.9 DNA purification

A) Extraction of DNA fragments from an agarose gel

Following restriction endonuclease digestion, mixtures of insert and vector DNA fragments where separated by agarose gel electrophoresis and purified. Briefly, DNA fragments were separated using a 1% (w/v) agarose gel, visualized using a UV transilluminator and excised from the gel using a clean scalpel. DNA within the agarose gel slices was purified using a QIAquick gel extraction kit according to the manufacturer’s instructions.

B) Column purification of DNA fragments

Purification of DNA from samples containing primers, nucleotides, salts, enzymes, agarose etc. was achieved using a QIAquick PCR purification kit (Qiagen) according to manufacturer’s recommendations. The kit contains a column containing a silica membrane, which binds the DNA in the presence of a high salt buffer and elutes DNA in the presence of a low salt buffer. DNA samples were eluted into 30 µL of nuclease free water.
6.2.10 Sanger sequencing

GATC Biotech preformed DNA sequencing of purified plasmid DNA or purified PCR amplicons. A reaction mix of 5 µL template DNA (100 ng/mL plasmid DNA and 20-80 ng/mL PCR amplicons) and 5µM (5µL) of appropriate primer were prepared and sent for sequencing. Sequencing results were analysed using DNASTAR Lasergene 9 core suite, BLAST, ClustalW and the protein translation tool ExPASy.

6.2.11 Preparation of competent bacterial cells and their transformation

A) E.coli

Chemically-competent E. coli JM109 and Top10 (Stratagene) were transformed according to manufacturer’s recommendations. Electrocompetent MC1061 E. coli were prepared as previously described by Sambrook and Russell, (2001). Briefly, overnight cultures were sub-cultured in 100 mL fresh LB broth and grown to an OD₆₀₀ of 0.4-0.45, chilled on ice for 20 min and harvested by centrifugation (6800 x g) at 4 °C for 10 min. Cells were then washed twice in 5 mL ice-cold ultrapure (Milli-Q) water followed by two washes in 5 mL 10% (v/v) glycerol. 100 µL aliquots of cells in 16.7% (v/v) glycerol were snap frozen using a dry-ice ethanol bath and stored at -80°C until required. Electroporation was carried out using a GenePulser machine (BioRad) in 0.1 cm cuvettes (BioRad) using the following parameters; 1.8 kV, 25 µF and 200 Ω. 1 µg of DNA was added to 100 µL of electrocompetent E. coli MC1061. Following electroporation, cells were recovered in SOC media shaking at 37°C. The cells were then spread onto LB agar supplemented with appropriate antibiotic selection.
B) *Lactobacillus vaginalis* and *Lactobacillus agilis*

Electro-competent *L. vaginalis* and *L. agilis* were prepared as previously described by Luchansky *et al.* (1988). Overnight cultures were sub-cultured (1:100) in 100 mL of pre-warmed fresh MRS broth supplemented with 0.5% glycine (v/v) and grown to an OD$_{590}$ of 0.6. Cells were then harvested by centrifugation (6800 x g) at 4°C for 5 min and washed four times in electroporation buffer (composed of 1 M sucrose and 25 mM magnesium chloride). Following this, cells were re-suspended in 2 mL of electroporation buffer and 100 µL aliquots of cells were snap frozen and stored at -80°C until required. Electroporation was achieved using 1-2 µg of DNA per 100 µL of thawed electrocompetent cells in 0.2 mm cuvettes and under the following parameters; 2.0 kV, 25 µF and 200 Ω. Following electroporation, cells were recovered under anaerobic conditions for 3 hrs at 37°C in fresh pre-warmed MRS broth prior to plating to MRS agar with antibiotic selection as appropriate.

6.3 Eukaryotic cell culture

6.3.1 Bacterial culture

*E. coli* strains were cultured in Luria-Bertani (LB) broth agar containing appropriate selection at 37°C with shaking at 200 rpm, unless stated otherwise. *Lactobacillus* strains were cultured in de Man, Rogosa and Sharpe (MRS) broth or agar (Difco) containing appropriate selection (37°C for 48 hrs), under anaerobic conditions using a BD GasPAK EZ container with an AnaeroGen sachet (Oxoid). Antibiotics were used at the following concentrations: ampicillin (amp) 50 µg/mL, kanamycin (kan) 50 µg/mL and chloramphenicol (chl) 15 µg/mL.

6.3.2 Cell passage

All the cells used in this study were routinely grown and maintained in complete media, Dulbecco’s modified Eagle’s Medium (DMEM, GIBCO)
supplemented with heat inactivated (10% v/v) fetal calf serum (FCS), 200mM L-glutamine, penicillin and streptomycin both at 100 U/mL (GIBCO), in appropriately sized tissue culture flasks (Corning, USA). When the cells reached 90% confluency, the medium was removed and the cell monolayer washed twice in PBS (138 mM NaCl, 2.7 mM KCl, 8 mM Na$_2$HPO$_4$, pH 6.7). 0.25% (w/v) Trypsin-EDTA solution (GIBCO) was added to the flask and incubated at 37°C until the cells detached. The cells were then resuspended in complete medium, where required cells were counted and used to seed fresh flasks, multi-well plates or coverslips at the required density.

6.3.3 Cell viability assay

Cytotoxicity studies were performed using the CellTitre-Glo® viability assay (Promega). This test determines cell viability in a culture dish by quantifying the production of ATP. In the presence of metabolically active cells, the Cell-Titre-Glo® reagent lyses the cell membranes to release ATP inhibits endogenous ATPases and provides luciferin and luciferase to measure ATP using a bioluminescent reaction. Prior to peptide treatment cells were seeded at 1x10$^4$ per well (to a total of volume 100 µL) in 96-well plates and incubated overnight (37°C; 5% CO$_2$). Following incubation, cell monolayers were washed once in SFM, and treated for 48 hrs with increasing concentrations (0.0001-200 µM) of peptide in SFM. The protocol used reflected the concentrations of peptide typically used in plaque reduction assays. Dimethyl sulphoxide (DMSO, Sigma) was used as the solvent for dissolving the peptides and so cells were also treated with increasing concentrations of DMSO (to a final concentration of 0.0001-2%). 48 hrs post-treatment cells were lysed with an equivalent volume of Cell-Titre-Glo reagent (100 µL), gently mixed and incubated at room temperature for 10 min. Luminescence was recorded from each well using the GloMax® 96 microplate luminometer (Promega) and data was presented as percentage cell viability relative to untreated controls.
6.4 Virus rescue, growth and titration

6.4.1 Reverse genetics rescue of recombinant viruses and generation of passage zero virus stock

Recombinant viruses were rescued using eight-plasmid transfection into 293T cells (de Wit et al., 2004; Hoffmann et al., 2000). To recover recombinant virus, 1x10^6 cells/mL in complete medium were transfected with 250 ng of each segment plasmid (Table 6.1.2) and 4 µL of lipofectamine 2000 (Invitrogen) in 96 µL Opti-MEM (Invitrogen) to a final volume of 1.1 mL. Following an overnight incubation the medium was changed to viral growth medium (2 mL) and the cells were incubated for a further 48 hrs. The supernatant containing virus was harvested by low-speed centrifugation (5min at 10,000 x g) this is termed the passage ‘zero’ (P0) viral stock.

6.4.2 Propagation of passage one viral stocks in MDCK cells

For the production of working virus stocks, a P0 prepared as described 6.4.1 was used to infect a fresh confluent T25 flask of MDCK cells. Briefly, confluent MDCK cells were washed twice with SFM and infected with 100 µL of P0 stock to a total volume of 1 mL in SFM. Following a one-hour incubation (at 37°C; 5% CO₂), the infection media was overlaid with 5 mL of virus growth medium (DMEM supplemented with 1 µg./ml N-acetylated trypsin (Sigma Aldrich) and 0.14% (w/v) BSA) and incubated for a further 48 hrs. Cell supernatant containing virus, known as passage one (P1) was harvested, clarified by centrifugation (spun twice for 10 min at 2100 x g), aliquoted and stored at -80°C until required.

6.4.3 Generation of egg grown viral stocks

In some cases viral stocks were amplified in embryonated hen’s eggs. Hen’s eggs were preferred for the generation of high titre viral stocks required for virus purification or high MOI infections. Prior to infection, 10-12 day-old eggs were
candled to confirm viability and to mark the location of the air sac. Concurrently viral stocks were diluted to 1x10^4 pfu/mL (or 100 µL of P0 per egg) in pre-warmed SFM. A hole was drilled at the border of the air sac and virus inoculated into the allantoic cavity using a 25G syringe needle. Following inoculation the hole was resealed using wax and incubated at 37°C for a further 48 hrs (with rotation at 40-50% humidity). Prior to harvest, eggs were chilled at 4°C overnight to euthanize the embryo and forceps were used to break away the eggshell above the air sac. The shell membrane was then peeled back to allow for the collection of the allantoic fluid, which was clarified by centrifugation (10 min at 2100 x g). HA and infectious virus titres were determined to confirm viral growth.

6.4.4 Quantification of infective virus titre by plaque assay

MDCK cells were seeded onto six-well plates at 1 x 10^6 per well and incubated for 2 days until confluent. Cell monolayers were first washed twice in SFM and incubated (37°C; 5% CO₂) with 0.4 mL of 10-fold-serial dilutions (typically 10^2-10^8) of virus stock in SFM. Following a 1h incubation (with shaking every 10min to prevent the cells from drying out) the virus inoculum was removed and the cells were overlaid with 1% (w/v) sterile agarose in 2X DMEM containing 1 µg/ml N-acetylated trypsin (Sigma Aldrich). 48 hrs p.i. the cell monolayers were fixed with 10% (v/v) neutral buffered formalin and stained with either 0.1% (w/v) toluidine blue or immunostained for influenza NP. Plaque assays were performed in duplicate using independent virus dilutions.

6.4.5 Plaque reduction assay

Influenza A virus replication was assayed by plaque reduction assay (PRA) following conditions previously published by Nicol et al. (2012). First, MDCK cells were seeded onto six-well plates at 1 x 10^6 cells/well and incubated for 2 days until confluent. MDCK monolayers were then washed twice in SFM and infected with
250 PFU/well of virus in the presence of either 1.5 \((v/v)\) DMSO or increasing quantities of peptide (to a final concentration of 0.0001-10 \(\mu M\)). Next, cells were incubated for 1h \((37^\circ C; 5\% CO_2)\) to allow for viral adsorption. Following incubation, the infection medium was removed and the cells overlaid with 1\% \((w/v)\) sterile agarose in 2X DMEM containing 1 \(\mu g/ml\) N-acetylated trypsin (Sigma Aldrich) and incubated for a further 48hrs. Cell monolayers were fixed with 10\% \((v/v)\) neutral buffered formalin and stained with either blue 0.1\% \((w/v)\) toluidine or plaques immunostained for NP. Plaque reduction assays were performed in duplicate using independent virus and peptide dilution series. Data was plotted as percentage titre (plaque forming unit) relative to DMSO treated control cells and fitted to a dose response curve by non-linear regression using Prism 6.0.

6.4.6 Immunostaining for viral NP

Following infection with or without peptide present, MDCK cell monolayers were fixed with 10\% \((v/v)\) neutral buffered formalin for at least 3 hrs before the agar overlay was removed. The cell monolayer was then washed three times with PBS, permeabilised with 0.2\% \((v/v)\) Triton-X 100 diluted in PBS for 5 min at room temperature and washed a further three times in PBS. Following wash steps the cells were incubated with mouse-monoclonal anti-NP (AA5H) diluted to 1:500 in 5\% PBS(BSA) for 1h at room temperature. The cells were then washed three times with PBS and immunostained with anti-mouse horse radish peroxidase (HRP) conjugate diluted to 1:1000 in 5\% PBS(BSA) for 1h at room temperature. The cells washed a further three times with PBS and the plaques were visualised by the action of HRP on a tetra methyl benzidine (TMB) substrate with TrueBlue™ (KPL) for 10 min at room temperature. Cells were washed twice with deionised H\(_2\)O and plates dried before scanning.
6.4.7 Haemagglutination assay and haemagglutination inhibition assay

A) Haemagglutination assays (HA) were performed in 96-well v-bottom microtitre plates (Greiner). 50 µL of virus was added to 50 µL PBS and serially diluted two-fold across the 96-well plate. Next, 50µL of 1% (v/v) chicken red blood cells in PBS were overlaid into each well and the plate was incubated at room temperature for 30 min. The plates were imaged and an HA titre, the reciprocal of the last dilution of which agglutination was obtained.

(B) Haemagglutination inhibition assays (HAI) were performed in a v-bottom microtitre plate, anti-A/PR/8/34 serum (diluted 1:5) was added to 50 µL of PBS and serially diluted two-fold across the microtitre plate. The virus samples were standardized to 8 HA units per 50 µL of 1% (v/v) chicken blood (or in some cases 0.75% (v/v) turkey blood cells) as described above then 50 µL of the standardized virus was added to each well. Plates where shaken gently and incubated at room temperature for 30 min. Following incubation plates where imaged. The haemagglutination inhibition titre is the reciprocal of the last dilution of antiserum that inhibits agglutination.

6.4.8 Influenza virus purification by ultracentrifugation

Purification of A/PR/8/34 from allantoic fluid was achieved following two ultracentrifugation steps. First, 100 ml of allantoic fluid was clarified (spun twice for 10 min at 2100 x g), loaded onto a 30% sucrose/PBS cushion and spun at 30,000 rpm using an SW28Ti Beckman rotor for 1 hour and 30 min at 4°C. The resulting pellet was gently washed once with 500 µL of PBS to remove residual sucrose and re-suspended back in 50 µL of PBS overnight. The virus was further purified A/PR/8/34, by a second ultracentrifugation step. First, a 15- 60% sucrose/PBS gradient was prepared using a gradient pourer (GE Healthcare, Life sciences) and
200 µL of virus sample was layered on top of the gradient. The gradients were then spun at 38,000rpm for 40 min using a Beckman SA41Ti rotor at 4°C without the brake. The band was extracted from the gradient using a 1 mL syringe with a 19G needle. To remove the remaining sucrose, the purified virus was pelleted by centrifugation at 30,000 rpm for 90 min at 4°C, the resulting pellet was resuspended overnight at 4°C in 100 µL of PBS and used within 10 days of purification or snap frozen and stored at -80°C until required.

6.4.9 Virus infection of eukaryotic cells

For infection, complete medium was removed and the cells were washed once in SFM to remove remaining fetal calf serum. Infections were typically carried out in small volumes (200 µL/well in a 24-well dish and 400 µL/well in in a 6-well dish. To obtain the required multiplicity of infection (MOI), viral stocks were diluted in SFM; the MOI was determined by dividing infectious titre by the total cell number. Cells were incubated with virus at 37°C for 1h before overlaying with 2 mL 1% (w/v) agarose in virus growth MEM. Plates were fixed 48 hours p.i.

6.5 Protein quantification, purification and detection

6.5.1 SDS-PAGE and immunoblotting

Proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, equal volumes of protein samples were mixed with Laemmli’s sample buffer (composed of 20% (v/v) Glycerol, 2% (w/v) SDS 62.5mM Tris-HCl, 100mM DTT, 0.01%(w/v) bromophenol blue) and heated to 95°C for 5 min. Samples were loaded and separated on either 10% or Any kDa Mini-PROTEAN TGX gels (Bio-Rad) at 150V for 45 min in Tris-Glycine running buffer (SLS). Gels were then used to visualise proteins by Coomassie Blue Staining (see below) or identify proteins of interest by western blot analysis. For western blot
analysis, proteins where transferred onto a nitrocellulose membrane using the Trans-Blot Turbo system (Bio-Rad) at 1.3A for 7 min. Following transfer, the nitrocellulose membrane was blocked with 5% (w/v) dried non-fat milk in PBS containing 1% (v/v) Tween-20 (PBS-T) for 1h. Following this, the membrane was washed twice in PBS-T (5 min) and incubated with the appropriate primary antibody diluted in PBS-T either for 1h at room temperature or at 4°C overnight. Blots where then washed a further three times with PBS-T and for detection by infrared fluorescence, incubated with appropriate secondary antibodies conjugated to DyLight 800 or DyLight 700 at a dilution of 1:10,000 (45 min). Blots where imaged using the Odyssey Infrared Imaging System (LI-COR) according to the manufacturer’s instructions. Western blots were captured and analysed using Image Studio (LI-COR).

6.5.2 Staining of viral proteins

Proteins were first separated by SDS-PAGE and fixed by washing three times for 15 min in fixing solution made up of 50% (v/v) methanol, 10% (v/v) acetic acid in deionised water. The gels were stained using 0.2% (w/v) Coomassie Brilliant Blue R-250 in fixing solution for 30 min. Gels were then de-stained using a solution made up of 25% (v/v) methanol, 5% (v/v) acetic acid in deionised water before scanning.

6.5.3 Quantification of total protein by DC protein assay

Viral proteins from gradient purified preparations were quantified using the DC protein assay (Bio-Rad) following the manufacture recommendations. First, protein standards of 2.5-0.125 mg/mL bovine serum albumin (BSA) were prepared and used to generate a standard curve, which was then used to calculate total protein. The DC protein assay was measured at 750 nm with a FLUOStar Omega microplate reader.
6.6 Entry assay techniques

6.6.1 Fluorophore-labelling of A/PR/8/34

The surface proteins of gradient purified A/PR/8/34 were labelled using the Alexa Fluor® 488 protein labelling kit (Life Technologies). The Alexa Fluor® 488 dye contains a TFP ester, bis-(trithylammonium salt, MW 885) moiety which is stable and reacts with the primary amines of proteins to form a dye-protein conjugate. Prior to labelling, purified virus was first visualized by Coomassie Blue Staining to confirm purity and total virus protein was quantified by DC assay (6.5.3). 0.5 mL of 2 mg/mL of protein was then labelled by addition of 50 µL of 1 M sodium bicarbonate and transfer into the vial containing the reactive dye provided in the kit. The vial was inverted a few times to allow the dye to dissolve and the mixture incubated at room temperature for 1h with stirring. Following incubation, to remove the unbound dye from 488-A/PR/8/34, the protein-dye mixture was ultracentrifuged at 125,000Xg (48,000 rpm) in a TLA 100.3 rotor for 90 min. The resulting pellet was re-suspended overnight in 20 µL of clean PBS. The 488-labeled proteins were then visualized by placing an SDS-PAGE gel of the resolved labelled proteins on a UV transilluminator. The gel was later stained with Coomassie to ensure purity and recovery of virus. The 488-labelled virus was used within 10 days of labelling but was also snap frozen for storage at -80°C.

6.6.2 Virus binding assay by fluorescence microscopy

Viral binding assays were performed at 4°C to allow viral attachment but not internalisation into the cells. First to reduce the likelihood of internalisation, equipment and reagents were chilled and the assays were performed in the cold room (4°C). A day prior to infection, A549 cells were seeded onto round 13 mm coverslips at a density of 1X10⁵ per slide. As a control for the assay, A549 cells were first stripped of cell surface sialic acid receptors by treating them with 2U per 200 µL
of bacterial neuraminidase from *Vibrio cholerae* (Sigma) at 37°C for 30 min. A459 cells were washed once in SFM and incubated with 488-A/PR/8/34 in SFM at 4°C for 1h in the presence of peptide contained in 1.5% (v/v) DMSO (1 and 10 μM) or DMSO as vehicle control. Following viral adsorption, the cells were washed three times with ice-cold PBS to remove any unbound virus and fixed with 4% (v/v) formaldehyde in PBS at room temperature. Coverslips contained in 24-well plates were then stained with 4’,6-Diamidino-2-phenylindole (DAPI, Life Technologies) and mounted onto inverted glass slides with ProLong Gold (Life Technologies) before imaging using the Zeiss LSM710 confocal microscope.

6.6.3 Analysis of virus binding to cells by flow cytometry

For quantification and analysis of peptide action on viral binding, flow cytometry was performed. As with the fluorescence microscopy experiment (6.6.2), all equipment and reagents were chilled at 4°C and the assay was performed in the cold room to prevent internalisation of the virus. A549 cells seeded in a T-175 flask (Nunc) were detached using 0.25% (w/v) Trypsin-EDTA (Life Technologies) and resuspended in 5 mL of complete media. The cells were then counted to give 5x10⁴ cells per 100μL SFM for each treatment group and chilled at 4°C for 15 min prior to the assay. The cells were then incubated with 488-A/PR/8/34 in the presence of either peptide (1 and 10 μM) or 1.5% (v/v) DMSO at 4°C for 1h to allow viral adsorption. Bacterial neuraminidase treated cells were included in the assay as controls (treated as described in section 6.6.2). Following binding, the cells were washed three times with ice-cold PBS to remove any unbound virus and fixed with 1% (w/v) PFA in PBS at room temperature (for a minimum time of 20 min). The flow cytometry was performed on the FACs Calibur (BD biosciences). Single cell populations of mock-infected cells were gated based on their forward and side
scatter, and 10,000 events of each experimental group were recorded. Data was processed using FlowJo.

6.6.4 Virus internalisation assay by fluorescence microscopy

A549 cells were first seeded onto 13mm round coverslips at a density of 1X10^5 and to synchronise viral infection a binding assay was performed at 4°C (as per 6.6.2). Briefly the A549 cells were washed once with ice-cold SFM and incubated with 488-PR/8 in SFM at 4°C for 1h. Binding was performed in the presence of peptide (at 1 and 10 µM) or 1.5% (v/v) DMSO. Following binding the cells were washed three times in PBS to remove unbound virus and overlaid with ice-cold SFM before warming to 37°C for 1h. A shift in temperature allowed bound virus to endocytose into the cell. Following internalisation, the cells were washed three times in warm PBS and fixed with 4% (v/v) formaldehyde in PBS. Extracellular virus (unpermeabilised cells) was stained using a with a primary PR/8 primary antibody followed by 594-conjugated goat anti-rabbit IgG secondary antibody (as per 6.7.1). As with the virus-binding assay, the coverslips contained in 24-well plates were stained with DAPI (Life Technologies) and mounted onto glass slides with ProLong Gold (Life Technologies) before imaging using the Zeiss LSM710 confocal microscope.

6.6.5 Flow cytometry analysis of internalisation

As a method of quantifying the effects of the antiviral peptides on viral internalisation, flow cytometric analysis was performed. As with the virus binding experiment (6.6.2), equipment and reagents were chilled at 4°C and a binding assay was performed to synchronise infection. A549 cells seeded onto T-175 flasks (Nunc) were detached using 0.25% (w/v) Trypsin-EDTA (Life Technologies) and resuspended back into complete media. The cells were counted and resuspended back in SFM to give 5x10^4 cells per 100µL as needed for each treatment group and
were then chilled at 4°C for 15 min prior to the assay. The cells were then incubated with 488-A/PR/8/34 in the presence of either FluPep 4 (10 µM) or 1.5% (v/v) DMSO at 4°C for one hour. Following the 4°C binding incubation the temperature was raised to 37°C to allow the virus to endocytose into the host cells. Samples were taken starting from time ‘zero’ where the cells were fixed with 1% PFA (w/v) in PBS immediately following the 4°C binding incubation and every ten minutes following the temperature increase. To differentiate between internal and external viral particles, extracellular 488-PR/8 was ‘quenched’ using 0.4% (w/v) trypan blue solution (Life Technologies). Flow cytometry was carried out on a FACSCalibur (BD biosciences). Single cell populations of mock-infected cells were gated based on their forward and side scatter and 10,000 events of each experimental group were recorded. Data was processed using FlowJo.

6.6.6 RNP nuclear import assay

First, A459 cells seeded onto coverslips (6.6.2) were washed once in SFM and incubated with gradient purified A/PR/8/34 at an MOI 3 for 1h (37°C). Infection of A549 cells was completed in the presence or absence of (100 µg/mL) cycloheximide (CHX, Sigma Aldrich), which is an inhibitor or protein biosynthesis and allowed for visualisation of input viral ribonucleoprotein complexes (vRNPs) (Martin and Helenius, 1991). At the time of infection, A549 cells were treated with either 1.5% (v/v) DMSO vehicle control or peptide (10 µM). Following incubation the infection media was removed and the cells were overlaid with complete media. Three hrs p.i the cells were washed three times with pre-warmed PBS before fixing with 4% (v/v) formaldehyde in PBS. At this point, the virus will have internalised within cellular endosomes, undergone a conformational change of HA and activated membrane fusion activity resulting in vRNP import to the nucleus of the cells. Following fix, cells were permeablised with 0.2% (v/v) Triton-X 100 and stained with a mouse-
monoclonal anti-NP (AA5H) applied at a dilution of 1:1000 in PBS containing 2% (v/v) foetal calf serum (PBS(FCS)) and a mouse anti-rabbit IgG conjugated to Alexa-Fluor-488 secondary (Abcam) used at a dilution of 1:1000. Cells were also stained with DAPI (blue) before imaging by confocal microscopy using the Zeiss LSM710. Maximum intensity projections of a series of optical sections through the cells were taken and merged.

6.6.7 Time of drug addition assay

To examine the stage(s) within the viral life cycle at which peptides are active, time of drug addition experiments were performed. Here, A549 cells seeded onto 13mm round coverslips (6.2.6) were incubated with A/PR/8/34 at an MOI of 3 for 1h. Cells were either treated with peptide (10 µM) an hour prior to virus adsorption (time 0), at the time of infection (during the 1h incubation) or peptide was added to the cells, three, five and six hours p.i. Following adsorption the infection media was removed and the cells were overlaid with 500 µL of complete media. Eight hours p.i the complete media was removed and the cells were fixed with 4% (v/v) formaldehyde in PBS. Following fix, cells were permeablisied with 0.2% (v/v) Triton-X 100 and stained first with a mouse-monoclonal anti-NP (AA5H) applied at a dilution of 1:1000 in PBS(FCS) and a mouse anti-rabbit IgG conjugated to Alexa-Fluor-488 secondary (Abcam) used at a dilution of 1:1000 in PBS(FCS). The cells were also stained with DAPI (blue) before imaging by fluorescence microscopy using the fluorescence Leica DLMB microscope.

6.7 Fluorescence imaging

6.7.1 Staining protocol for immunofluorescence microscopy

Infected cells seeded onto coverslips were washed three times in pre-warmed PBS and fixed in 4% (w/v) PBS-formaldehyde in PBS for 20 min at room temperature. Cells were then washed twice with PBS containing 2% (v/v) FCS
(PBS(FCS)) and permeabilised where appropriate with 0.2% (v/v) Triton X-100 in PBS (for detection of intracellular antigens) for 5 min at room temperature. Cells were washed three times with PBS(FCS) and incubated for 1h with the appropriate primary antibody diluted in PBS(FCS) at room temperature. Following a further three washes with PBS(FCS) the cells were incubated with secondary antibody conjugated to the selected fluorophore. For visualising the nuclei the cells were stained with 4′,6-Diamidino-2-phenylindole (DAPI) at 100 ng/mL for 2 min at room temperature. Following staining the coverslips were mounted onto glass slides with ProlongGold (Life Technologies) and imaged using a Leica DLMB fluorescence. Samples were stored at 4°C in the dark to prevent bleaching of the fluorophore.

6.7.2 Confocal microscopy

Images were captured on a Zeiss LSM 710 confocal microscope using x63 objective lens. Samples that were stained with more than one fluorophore were acquired sequentially to avoid bleed through and confocal settings, such as the fluorescence intensities of regions of interest, were kept consistent throughout each experiment.

6.8 Statistical analysis

All statistical analyses were performed using Prism 6 (GraphPad, USA). Continuous data with a normal distribution was tested for significance using ANOVA. For the virus binding assays, each peptide was tested at two concentrations for this experiment a 2-way ANOVA was performed (Figure 3.10).
Figure 7.1 Expected nucleotide sequence of expression cassette and ClustalW alignments with sequences obtained

(a) Nucleotide sequence of expression cassette designed for the secretion of FP3. The expression cassette was codon optimised for peptide expression in *Lactobacillus*

(b) ClustalW alignment of desired sequence of a GC-rich variant designed by Dundee Cell Biologicals to overcome apparent DNA instability during synthesis

(c) ClustalW alignment of redesigned expression cassette and cassette provided by Dundee Cell Biologicals which was truncated by 47 base pairs at the 3' end. The expression cassette was colour coded to highlight the individual elements of the cassette; promoter sequence (blue), ribosome-binding site (purple), signal peptide (green), pro-peptide (pink) and anti-viral peptide (red).
ClustalW alignment of the sequence of a cassette for expression and secretion of FP3 and a representative truncated clone following attempted PCR-mediated repair. a) Nucleotide sequences are accompanied by the amino acid sequence to highlight the location of the C-terminal insertions in the desired sequence introducing a premature stop. The expression cassette has been colour coded to indicate the individual elements of the cassette, promoter sequence (blue), ribosome-binding site (purple), signal peptide (green), pro-peptide (pink) and anti-viral peptide (red). b) Translated peptide sequence highlights changes to the peptide resulting from the C-terminal nucleotide insertions.

Figure 7.2
Figure 7.3 ClustalW alignment of the expected sequence of a cassette for expression and secretion of RRKK-FP2 and a clone with frameshift mutation. Recombinants recovered following attempts to fuse RRKK-FP2 to the signals for expression and secretion from pBluescript:Ec:FP3-47 also contained frameshift mutations affecting the predicted C-terminus of the peptide. Here, the nucleotide sequences are accompanied by the amino acid sequence to highlight the location of the frameshift mutation (yellow). The expression cassette has been colour coded to indicate the individual elements of the cassette, promoter sequence (blue), ribosome binding site (purple), signal peptide (green), pro-peptide (pink) and anti-viral peptide (red). b) Translated peptide sequences revealed that the nucleic acid insertions resulted in one amino acid substitution and insertion of two amino acids at the 3’ end of the peptide.
Figure 7.4 ClustalW alignment of the expected and obtained sequences of a cassette for expression and secretion of FP4. Attempts to fuse FP4 to the signals to the signals for expression and secretion from pBluescript:Ec:FP3-47 by PCR using the strategy described in Figure 4.4 resulted in a single clone containing the expected full DNA sequence for FP4. Here, the nucleotide sequence is accompanied by the amino acid sequence. The expression cassette was colour coded to highlight the individual elements of the cassette, promoter sequence (blue), ribosome binding site (purple), signal peptide (green), pro-peptide (pink) and anti-viral peptide (red).
Figure 7.5 ClustalW alignment of the expected and obtained sequence of a cassette for expression and secretion of B10NP fused to DTNSD. Attempts to fuse B10NP to the signals for expression and secretion from pBluescript:EcFP3-47 resulted in a clone containing the full DNA sequence for B10NP. Here, the nucleotide sequence is accompanied by the amino acid sequence. The expression cassette was colour coded to highlight the individual elements of the cassette, promoter sequence (blue), ribosome binding site (purple), signal peptide (green), pro-peptide (pink) and anti-viral peptide (red).
Figure 7.6 ClustalW alignment of the expected and obtained nucleotide sequence of the expression cassette fused to the gene encoding EGFP following PCR-Ligation-PCR cloning. Two candidate pTopo:Ec:EGFP clones were sequenced and found to contain deletion mutations, one of which was about the Ec:EGFP fusion site and is presented here. Deleted nucleotide is highlighted in yellow and resulted in a frameshift mutation and EGFP truncation. Expression cassette (black), EGFP (green)
Figure 7.7. ClustalW alignment of the desired nucleotide sequence of Ec:EGFP and a representative clone recovered following overlapping extension. Candidate pUK200:Ec:EGFP recombinant clones were sequenced and found to contain deletion mutations about the Ec:EGFP fusion site. This deletion lead to a stop codon immediately after the DTNSD An example of a deletion mutation is highlighted in yellow.


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