This is the accepted version of the following article: H. Patel and A. Kukol, 'Recent discoveries of influenza A drug target sites to combat virus replication', *Biochemical Society Transactions*, 44(3): 932-936, Portland Press, which has been published in final form at doi: 10.1042/BST20160002

Recent discoveries of influenza A drug target sites to combat virus replication

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Abstract

Sequence variations in the binding sites of influenza A proteins are known to limit the effectiveness of current antiviral drugs. Clinically this leads to increased rates of virus transmission and pathogenicity. Potential influenza A inhibitors are continually being discovered as a result of high-throughput cell based screening studies, while the application of computational tools to aid drug discovery has further increased the number of predicted inhibitors reported. This review brings together the aspects that relate to the identification of influenza A drug target sites and the findings from recent antiviral drug discovery strategies.

Keywords: Influenza A, viral proteins, antiviral discovery, resistance, inhibitors

Introduction

Every year the Influenza A virus infects humans worldwide with varying levels of severity. Since the worst human outbreak known as the H1N1 Spanish flu was reported in 1918 which killed approximately 50 million people [1], major pandemics with significant mortality rates have continued to occur [2,3]. Besides human to human transmission, this zoonotic virus has the potential to be transmitted between a range of hosts such as birds and pigs, amongst other animals. Such cross-species transmission, in particular of avian origin can lead to the formation of re-assortant viruses through mixing of genetic segments and has shown to confer an increase in pathogenicity to the human population [4]. The virus particle consists of eight RNA segments in complex with proteins required for the initial stages of replication that are surrounded by a protein shell and a lipid bilayer. Various proteins traverse the lipid bilayer, such as haemagglutinin (HA), neuraminidase (NA) and the matrix protein 2 (M2) proton channel. The complex replication cycle requires several functional proteins to enable attachment, genome replication and release of the virus from infected cells [5]. Currently up to 17 proteins have been discovered; many of which are produced from a single RNA segment. However, not all of these proteins contribute to infection, nor are they found to be present in all virus subtypes [6]. Subtypes are classified according to antigenic properties of the HA and NA surface proteins as HxNy. The HA glycoprotein is also the major virulence determinant and stimulates production of neutralising antibodies at the start of the infectious cycle. A schematic overview of the virus life cycle is illustrated in figure 1.

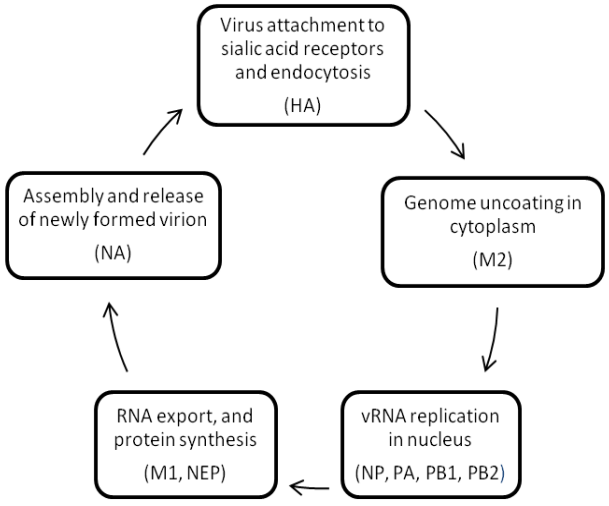

Figure 1. A brief overview of the Influenza A infectious virus life cycle including the major proteins involved.

In addition to genome re-assortments, replication errors by the polymerase also leads to genomic diversity and rapid evolution of the genome which can impair constituent protein functions, or alternatively enable proteins with additional functions [5,7]. Furthermore, the annual trivalent vaccine formulated against the expected circulating strains does not provide extensive protection against the virus. In February 2015, the high rate of evolution of the virus was exemplified by a mutation in the circulating H3N2 vaccine strain, which severely impacted the effectiveness of the vaccine [8]. Consequently, once infection is established in a host, antiviral drugs are the only treatment options available.

Current influenza antivirals and documented resistance

In the United Kingdom there are currently two classes of antiviral drugs licensed for treatment and prophylaxis of influenza A based on their method of action. The neuraminidase inhibitors (NAIs) Oseltamivir and Zanamivir developed in the 1990's function to block the neuraminidase active site, preventing enzymatic cleavage of sialic acid residues on the surface of infected cells to stop the virus from spreading [9]. The drugs Laninamivir and Peramivir also act as NAIs and have recently been approved for use in certain countries only. Each of these drugs has different routes of administration; with Oseltamivir taken orally, Zanamivir taken through inhalation and Laninamivir and Peramivir taken intravenously. The M2 inhibitors (adamantanes) are a much older class of orally administered drugs developed in the 1960's and function to obstruct the M2 proton channel, preventing the uncoating and entry of viral particles into cells [10]. Additionally, a compound known as Arbidol that inhibits haemaglutinin membrane fusion *in vitro* is licensed in Russia and China [11], and the novel antiviral compound Favipiravir which acts as a purine analog to target the RNA polymerase is currently in late stage clinical trials in the USA [12].

However, since most of these drugs were approved, there has been increasing reports of drug resistance against seasonal and pandemic strains [13]. The mechanism of resistance relates to amino acid changes (single point mutations) near or within the binding sites of functional regions in viral proteins. These mutations may confer high levels of antiviral resistance and can emerge in all influenza strains worldwide. Such examples include pandemic H1N1 strains harbouring the Histidine to Tyrosine substitution at position 274 (H274Y). This change alters the NA catalytic site conformation resulting in reduced drug binding affinity corresponding to antiviral treatment failure [13,14]. Similarly, the affinity of drug binding is reduced by the S31N substitution in the M2 transmembrane domain [15], and due to widespread resistance, adamantanes are no longer recommended for antiviral treatment by the Centres for Disease Control and Prevention. Several other amino acid mutations in functional or framework residues of the neuraminidase and M2 proteins have been found which also confer a resistant phenotype [16,17]. The clinical use and choice of influenza antivirals is therefore complicated by resistance issues and drug induced selective pressures, which highlights the requirement to discover new targets and novel antiviral agents to reduce replication and combat infection. Additionally, due to lower rates of evolution based on sequence and structure analysis, there is increasing focus on investigating internal proteins as target sites for antiviral drugs [18]. In order to avoid a fruitless arms race between drug discovery and virus evolution, it has been suggested that antiviral drug discovery efforts should be focussed on the most evolutionary conserved binding sites [19], even if this places restrictions on the drug target sites available.

The essential internal proteins required for virus replication

The RNA dependent RNA polymerase enzyme involving the acid polymerase (PA), basic polymerase 1 (PB1) and basic polymerase 2 (PB2) are the largest proteins which form a heterotrimeric complex in the virion to synthesize mRNA templates for protein synthesis and cRNA for further genome transcription. Each subunit has a distinct function; the PA contains a N-terminal endonuclease domain to cleave capped host mRNA, the N-terminus of PB1 binds to PA to regulate transcriptase activity and mRNA chain elongation, and PB2 binds to the 5' methylated cap of host cell mRNA to generate primers for transcription [20,21]. Sequence analysis have proven that the polymerase is highly

conserved amongst functional regions of different strains and contains multiple sites for potential drug discovery [7,18,22]. Together with viral RNA, the polymerase subunits also associate with the nucleoprotein (NP) to form ribonucleoprotein (RNP) complexes, which act as a mediator between the virus and host cell. The NP also facilitates RNA synthesis, controls RNP trafficking and regulates polymerase activity and packaging of the genome, as well as performing many other functions [23]. The NP is also the most abundant internal protein, consisting of a body (N-terminal), head, and tail (C-terminal) loop domain and is also considered to be an attractive antiviral target [19,24]. Structures of some of the internal proteins are shown in figure 2.

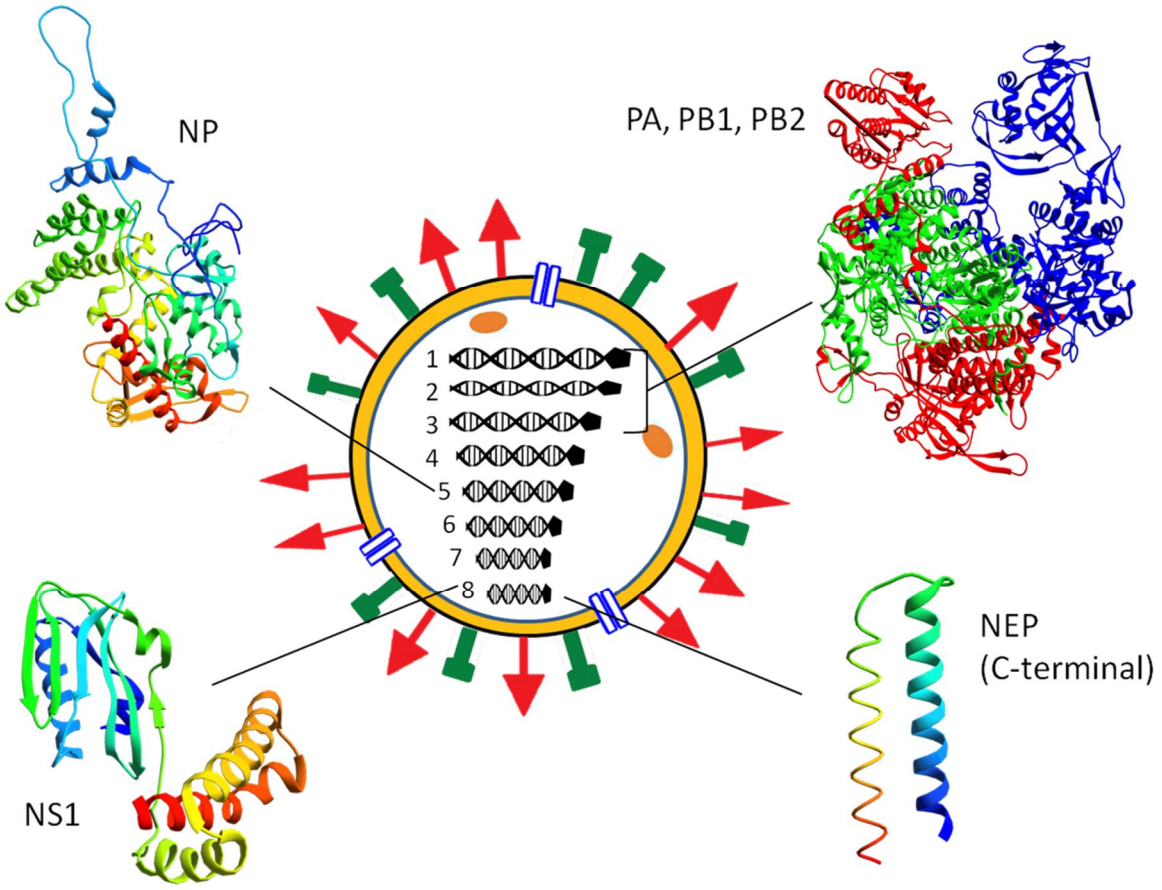

Figure 2. Structures of the internal influenza A proteins encoded by the RNA genome segments. The NP (PDB 2Q06), NS1 (PDB 3F5T) and NEP (PDB 1PD3) are coloured with reverse rainbow gradient from the N-terminal in red to C-terminal in blue. The polymerase subunits are shown as a trimer complex (PA, red; PB1, green; PB2, blue (PDB 4WSB)). This figure was made using EzMol [25].

Modelling of protein structures

Unlike the surface proteins HA, NA and M2 for which there are a number of solved structures in the Protein Data Bank (PDB), there is currently limited availability of full length crystal structures for some of the internal influenza A proteins. Various modelling tools for reliable protein structure prediction have been developed [26–28] and can be used to account for the lack of 3D structural information available. However, there is a low template coverage for some of the influenza proteins such as NS2, PB1 and PB2 using homology modelling methods [18]. Accurately constructed models and experimentally determined structures can aid with characterisation of binding sites, understanding the effects of mutations as well as permit *in silico* drug discovery studies. One example is a receptor based virtual screening study which specifically targeted the CPSF30 binding pocket on the non-structural protein (NS1) effector domain. From a library of ~200,000 compounds, the docking results

revealed compounds with predicted binding affinities up to -10.0kcal/mol, but whether these compounds can reduce virus replication is yet to be elucidated [29]. Computational methods combining sequence and structural information have also been used to identify new ligand binding sites in highly conserved regions of internal proteins such as NS1, NEP and NP [30,31]. Furthermore, proteins which are flexible and can adopt different conformations to influence the function and ligand binding activity can also be analysed with molecular dynamics simulation as exemplified by studies of the NP [32], M2 [17] and NA [33,34] proteins.

Recent approaches to discover virus replication inhibitors

In recent years, a number of high throughput cell based screening assays have been performed, which have lead to discoveries of potential inhibitors of various subtypes. Several NS1 antagonists that reduce virus replication have been identified in different studies reviewed for example by Engel [35]. This includes the inhibitor molecule known as JJ3297 identified in 2010, which has also been tested in virus quantification assays. Results showed that JJ3297 was able to increase production of IFN mRNA; a mechanism which NS1 is well known to antagonise, and reduced influenza A replication by at least three orders of magnitude. However, the precise mechanism of action and binding regions of JJ3297 to NS1 have not been identified [36]. The molecule was synthesized based on a previously identified NS1 inhibitor NSC125044 [37]. Similarly, a novel small molecule inhibitor ASN2 that targets the viral polymerase and inhibits influenza A replication has been discovered from a high-throughput cell screening assay. The suggested target of ASN2 is the Y499 residue of the PB1 protein resulting in impaired polymerase function and consequently reduced expression of NS1 [38]. In 2011, the inhibitor Nucleozin was identified from a virtual screening study and has been found to target the nucleoprotein [39,40] or the viral RNP complex. Inhibitory effects on virus replication have been shown *in vitro* as a result of NP aggregation [38]. In 2013, the small molecule Naproxen was also initially identified by virtual screening, followed by molecular dynamics, and verified by *in vitro* antiviral tests to show reduced viral titres. The binding region of Naproxen is reported to be at the RNA binding groove of NP consisting of the aromatic residues, Y148 and F489 [41]. Most recently, the compound RK424 identified in July 2015 from a compound library screening was also found to target the NP/RNP complex and reduced virus replication of several strains including H1N1, H5N1 and H7N9 in cell based replication assays. Molecular docking against a H1N1 NP crystal structure was used for further analysis to determine how RK424 inhibits NP function, and results showed that the molecule occupied a small pocket near the residues R162, S165, L264, and Y487 [42].

Concluding Summary

The influenza A virus undoubtedly remains a threat to public health. The ongoing reports of antiviral drug resistance and circulation of resistant subtypes have emphasised the fact that alternative antivirals with long term effectiveness should be developed. Finding inhibitors to accommodate the diversity of the virus genome and unpredictable rate of evolution continues to present challenges in drug discovery. However, with increased understanding of virus biology and the application of computational methods together with experimental investigations, the field of influenza drug discovery is rapidly progressing. Molecules that could inhibit virus replication at stages other than entry and release by targeting internal proteins such as the polymerase, nucleoprotein and non-structural protein are being explored, and may serve as an approach to overcome antiviral resistance.

Funding
Hershna Patel is supported by a studentship from the University of Hertfordshire.

Abbreviations:
HA, Haemaglutinin; NA, Neuraminidase; M2, matrix protein 2; PB1, Basic Polymerase 1; PB2, Basic Polymerase 2; PA, Acid Polymerase; NS1, Non-Structural; NP, Nucleoprotein; NAI, Neuraminidase Inhibitor; RNP, Ribonucleoprotein; PDB, Protein Data Bank

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