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MK2206 inhibits influenza A(H1N1)pdm2009 virus infection

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Preface

First of all, I would like to thank Dr. Denis Kainov for offering me a place in his research group at the Institute for Molecular Medicine Finland (FIMM). I would also like to thank my coworkers at FIMM for the invaluable help. It has been a great time here and it's good to have a better understanding of viruses, their host cell interactions and how these host cell interactions are targeted by the next generation of antivirals. I will not forget the trip we had in the Balkan, because of the good lectures and awesome moments. I also want to mention Sandra Söderholm, because she has been such a great help in various experiments and taught me several new techniques. Also special thanks to Hilde Van Maele, Christophe Wille and Tiina Soininen for offering me the chance to have this Erasmus experience and guiding me through the paperwork. Also big thanks to Jonita Martelius for checking English language. Finally I would like to thank my family for giving me the opportunity to study abroad.

Helsinki, December 2013

Jens Desloovere

Abstract

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In 2009, influenza A(H1N1)pdm09 virus caused a global pandemic and is now around as a seasonal epidemic peaking from December to March. It was confirmed that influenza A(H1N1)pdm09 is resistant to amantadine treatment. To treat people with severe illness, the World Health Organisation (WHO) recommended using oseltamivir or alternatively zanamivir if there were signs of resistance to the former. Because of the risk for emerging resistance, a next generation of antivirals against the virus is needed.

Recently, studies of influenza virus-host interactions revealed a number of host proteins that are potential targets for antivirals. Special interest goes to Raf/MEK/ERK, NF-κB, PI3K/Akt and PKC signaling pathways (Planz, 2013). It is proposed that inhibition of host factors is less likely to cause resistance and more importantly, a lot of these inhibitors are in development or already approved for other diseases such as cancer . Repurposing of these drugs is facilitated, because much is known about their toxicity and working mechanism.

The present study suggests an important role for Akt in influenza A(H1N1)pdm09 virus infections and that MK-2206 could be further developed as an antiviral against influenza A(H1N1)pdm09 viruses. MK-2206 accumulates in endocytic compartments and blocks the release of the virus in the cytoplasm.

Keywords	Influenza A, innate immunity, antivirals, MK-2206, PI3K/Akt

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List of symbols

5′ppp	5′-triphosphate
APAF-1	apoptotic protease-activating factor 1
APC	antigen-presenting cells
ASC	apoptosis associated speck-like protein containing a CARD
ATP	adenosine triphosphate
ATS	quantitative activity and toxicity score
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BCR	B cell receptors (BCR)
BH3	Bcl-2 homology 3
BID	BH3 interacting-domain death agonist
BSA	bovine serum albumin
CAK	CDK-activating-kinase
CARD	Caspase activation and recruitment domain
CDK	Cyclin-dependent kinase
CPSF	cleavage and polyadenylation specificity protein
Crm1	Chromosome region maintenance 1 protein
CTD	carboxy-terminal domain
CTG	Cell Titer Glo
DISC	death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DSIF	5,6-dichloro-1-β-d-ribofuranosylbenzimidazole sensitivity-inducing factor
eIF2α	Eukaryotic initiation factor 2 alpha
eIF3(e)	Eukaryotic initiation factor 3(e)
FADD	Fas-Associated protein with Death Domain
FBS	fetal bovine serum
HA	haemagglutinin
HIV-1	human immunodeficiency virus type 1
hpi	hours post infection
HSV	herpes simplex virus
GAS	IFN-γ-activated site

GSK-3β	glycogen synthase kinase-3β
GTP	guanosine triphosphate
IAV	influenza A virus
IFN	interferon
IFNAR1	interferon (alpha, beta and omega) receptor 1
IFNAR2	interferon (alpha, beta and omega) receptor 2
IL	interleukin
IPS-1	β-interferon promoter stimulator 1
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory transcription factor
ISG	IFN stimulated genes
ISRE	sensitive response elements
JAK	Janus activated kinase
LGP2	Laboratory of genetics and physiology 2
LRR	Leucine rich repeats
M	matrix proteins
m^7G	7-methylguanosine
M1	matrix protein 1
M2	matrix protein 2
MAPK	mitogen-activated protein kinases
MDA5	melonoma differentiation associated antigen 5
MDCK	Madin-Darby canine kidney
MHC	major histocompatibility complex
moi	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
MyD88	myeloid differentiation primary response gene 88
NA	neuraminidase
NELF	negative elongation factor
NEP	nuclear export protein
NK cells	natural killer cells
NLR	nucleotide-binding domain, leucine-rich repeat-containing proteins
NP	nucleocapsid protein
NPC	nuclear pore complexes
NS	non-structural proteins

NS1	non-structural protein 1
PA	polymerase acidic protein
PABII	poly(A)-binding protein II
PACT	protein activator of the interferon-induced protein kinase
PAMPs	pathogen associated molecular patterns
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PBS	phosphate buffered saline
PCI	Pharmacological CDK inhibitors
pDC	plasmacytoid dendritic cell
PFU	plaque forming units
PKR	protein kinase RNA regulated
PRR	pattern recognition receptor
RanBP5	β-importin Ran binding protein 5
Ran	Ras-related nuclear protein
RdRP	RNA dependent RNA polymerase
RIG-I	retinoic acid-inducible gene-I
RIP-1	receptor-interacting protein 1
NLR	NOD-like receptor
RNP	ribonucleoprotein
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SaliPhe	saliphenylhalamide
STAT	signal transducer and activator of transcription
TBK1	TRAF family member-associated NF-κB activator binding kinase 1
TIR	Toll/IL-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
TRAF	TNF receptor associated factor
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TRIM25	tripartite motif-containing protein 25
vATPase	cellular vacuolar ATPase
VGM	virus growth medium
WHO	World Health Organisation

1 Introduction

In 2009, Influenza A(H1N1)pdm09 virus caused a global pandemic and is now around as a seasonal epidemic peaking from December to March. It was confirmed that influenza A(H1N1)pdm09 is resistant to amantadine treatment. To treat people with severe illness, the World Health Organisation (WHO) recommended using oseltamivir or alternatively zanamivir if there were signs of resistance to the former. Because of the risk for emerging resistance, a next generation of antivirals against the virus is needed (Schang et al., 2001).

Recently, studies of influenza virus-host interactions revealed a number of host proteins that are potential targets for antivirals. Special interest goes to Raf/MEK/ERK, NF-κB, PI3K/Akt and PKC signaling pathways (Planz, 2013). It is proposed that inhibition of host factors is less likely to cause resistance and more importantly, a lot of these inhibitors are in development or already approved for other diseases such as cancer (www.clinicaltrials.gov). Repurposing of these drugs is facilitated, because much is known about their toxicity and working mechanism.

In this study, the role of four Akt-inhibitors were tested for their antiviral activities against influenza A(H1N1)pdm09.

First, this study briefly discusses flu and the emergence of pandemics. Next, the properties of the influenza A virus are introduced. Then the role of the immune system in the recognition of the virus is explained. After showing how the virus tries to evade the immune system, antiviral drugs are discussed. Finally the results of the testing with Akt-inhibitors are discussed.

2 Theoretical Background

In this part of the study flu, the virus that causes it and how the immune system tries to clear the infection are discussed. Next the fact that influenza A viruses are able to regulate some of the immune reactions to ensure successful replication are discussed. Finally, the mechanisms of antivirals against influenza A are explained.

2.1 Flu

Infuenza A viruses (IAV) cause influenza, commonly known as flu. It is a highly contagious disease of the respiratory tract which causes a feverish illness that can lead to death. Influenza A viruses are responsible for periodic global pandemics with high mortality rates. In 1918 a pandemic caused about 50 million deaths all over the world, which makes it the most devastating pandemic known until today (Taubenberger & Kash, 2010).

Normally flu is a self-limiting disease that infects all age groups. Young children, elderly people and people with immunodeficiency are more susceptible to pneumonia and lethal infections.

The disease can be transmitted from human to human through mucus produced in the airways. Sneezing and coughing creates aerosols which can be inhaled by persons in the surrounding area. Also direct contact with mucus can infect others. It is therefore very important to apply a good hand and respiratory hygiene. When feeling sick, it is advised to stay at home to prevent transmission to other persons. IAV spreads very fast in younger population, because they are lacking antibodies to the virus (WHO).

Flu is characterized by fever, headaches, coughing, sore throat, muscle pain, stuffy nose, loss of appetite and fatigue. It is hard to identify if someone has flu or a common cold which is caused by rhinoviruses and coronaviruses. A study by Monto et al. (2000) mainly performed on adults, created a model to distinguish between the diseases. It was found that the main predictors were fever and coughing (Table 1).

Table 1. Modeling the influenza symptoms (Monto et al., 2000)

Stepwise Logistic Regression Analysis of Predictors of Influenza Infection

Symptom	Stepwise Analysis Odds Ratio	95% Confidence Intervals	*P*
Fever (body temperature ≥37.8°C)	3.26	3.87-2.75	<.001
Cough	2.85	3.68-2.21	<.001
Nasal congestion	1.98	2.54-1.54	<.001
Age (≥55 y)	1.60	2.16-1.18	.003
Weakness	1.54	2.22-1.07	.008
Onset (>36 h)	1.53	1.90-1.24	<.001
Loss of appetite	1.43	1.86-1.10	.008
Sex (male)	1.27	1.50-1.08	.004
Sore throat	0.72	0.91-0.57	.01
Feverishness	. . .*	. . .	. . .
Headache	. . .	. . .	. . .
Myalgia	. . .	. . .	. . .
High-risk	. . .	. . .	. . .

Ellipses indicate symptom was not selected in stepwise procedure.

Multivariate Predictors of Influenza Infection With Sensitivity and Specificity Analyses*

Symptoms	PPV	NPV	Sensitivity, %	Specificity, %
Fever	76.85	49.14	67.79	60.38
Cough	69.43	60.89	93.24	20.41
Fever + cough	79.04	48.91	63.81	67.19
Fever + cough when onset ≤36 h	77.28	51.35	63.32	67.54
Fever + cough when onset >36 h	85.37	42.33	50.30	80.89
Fever + cough + nasal congestion	81.45	48.21	59.03	73.94
Fever + cough + weakness	80.27	47.85	59.80	71.51
Fever + cough + myalgia	79.11	47.86	61.50	68.52
Fever + cough + loss of appetite	79.04	47.75	61.38	68.45
Fever + cough + sore throat	79.02	45.30	55.51	71.43
Fever + cough + headache	78.69	46.81	59.80	68.60

**PPV indicates positive predictive value, the probability of having laboratory-confirmed influenza when the symptom is present; NPV, negative predictive value, the probability of not having laboratory-confirmed influenza when the symptom is not present; sensitivity, the probability of having the symptom when the patient has laboratory-confirmed influenza; and specificity, the probability of not having the symptom when the patient does not have laboratory-confirmed influenza (ie, when the test result for influenza is negative).*

Multivariate analysis showed that the combination of both fever and cough (onset after 36h) is the best combination to predict whether the patient is infected with influenza or has a common cold. According to the positive predicted value, people who have both symptoms have 77 – 85 percent chance of having laboratory-confirmed influenza (Monto et al., 2000).

2.2 Pandemics

In the last 500 years, there have been at least 14 IAV pandemics. An overview of the evolution of the pandemic viruses can be found in Figure 1. The worst pandemic known occurred in 1918 and was responsible for approximately 675,000 deaths in the U.S. and about 50 million people worldwide (Johnson & Mueller, 2002). The "Spanish flu", as it was called, had an extremely high mortality owing to the result of bacterial infection of the lungs (pneumonia) and an unexplained peak in child deaths. It is a H1N1 type of IAV and continued to circulate for nearly 40 years. Later, in 1977 it reappeared and is still around at this moment (Taubenberger & Kash, 2010).

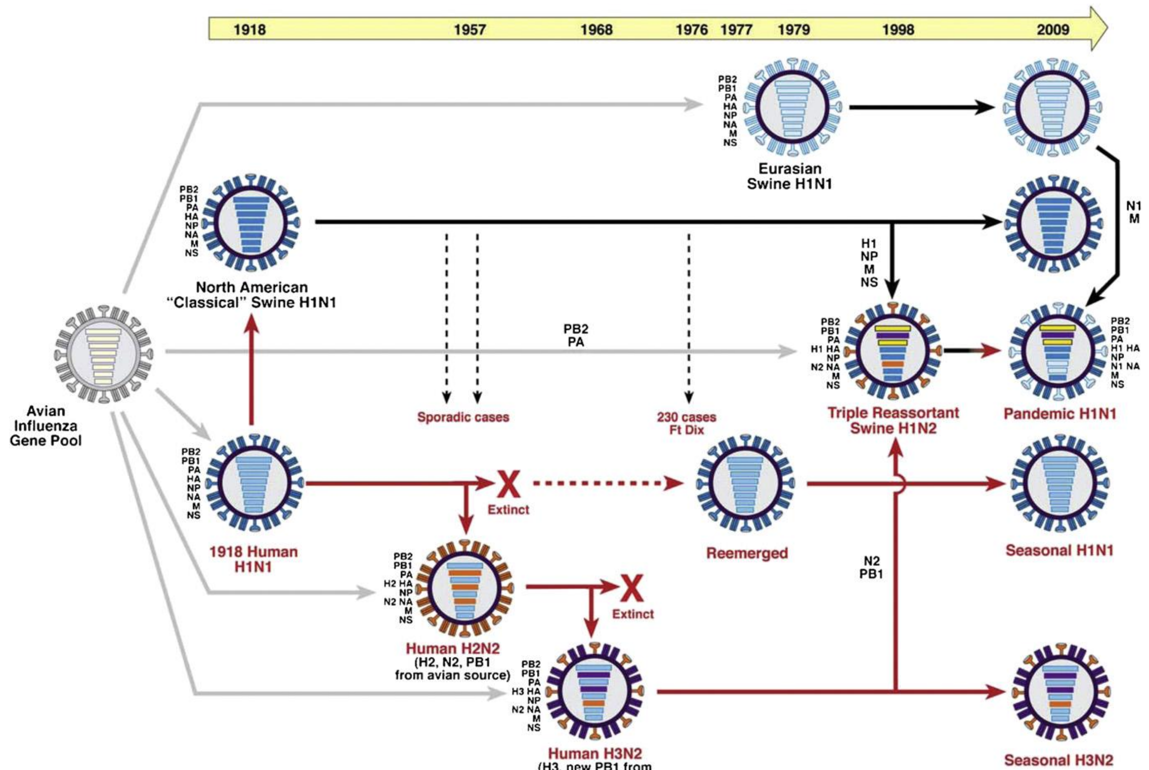


Figure 1. Emerge of new IAV strains (Taubenberger & Kash, 2010)

In 1957 the "Asian flu" pandemic occurred through reassortment. The haemagglutinin (HA) and neuraminidase (NA) genes of the 1918 H1N1 virus got substituted by avian-like H2 and N2 subtypes and also PB1 was changed. The H2N2 IAV remained active for about 11 years and killed about 4 million people (Oxford, 2000). It has not returned up to the present (Taubenberger & Kash, 2010).

The 1968 "Hong Kong" pandemic is subtyped H3N2 and was formed by reassortment between the human H2N2 virus and an avian variant. It was responsible for 2 million deaths (Oxford, 2000). HA and polymerase basic protein 1 (PB1) segments were changed. The virus still had the N2 type NA of the previously circulating Asian flu and because a lot of people already had antibodies for this NA subtype, the virus was quite mild. It didn't take much time before the virus turned endemic and began circulating as a seasonal flu (Taubenberger & Kash, 2010).

In 1977, a descendant of the 1918 H1N1 virus that disappeared in 1957 started to recirculate. It is very unlikely that the virus was able to 'survive' in a population with high natural immunity and vaccines. Because genetic analyses proved that all eight

segments were nearly identical to the 1918 variant, it has been suggested that a frozen strain from early 1950s was released. The virus is still around today (Taubenberger & Kash, 2010).

More recently, in 2009, a new H1N1 type virus arose. As seen in Figure 1, first a triple reassortment H1N2 swine flu was formed by combination of avian-like influenza, North American swine flu and the H3N2 strain. It became pandemic when segments were exchanged with the Eurasian Swine H1N1. The result is a virus containing NA and matrix proteins (M) segments from the European avian like H1N1 lineage. HA, nucleocapsid protein (NP) and non-structural proteins (NS) originated from the classical swine H1N1. Avian IAV provided polymerase basic protein 1 (PB2) and polymerase acidic protein (PA) segments and the origin of the PB1 segment lies in the human seasonal H3N2 virus (Taubenberger & Kash, 2010).

In the 2009 influenza season, 12.564 cases of Influenza A were reported in Finland. Of those cases, 7.646 were the pandemic swine flu, also called influenza A(H1N1)pdm09. Swine flu was first found in Finland in May and was responsible for 44 deaths in 2009 (Hulkko et al., 2010). In 2010, swine flu became a seasonal epidemic and there were much less (418) cases of influenza A reported. Only 228 of swine flu cases were confirmed. This is probably because of the high coverage (51%) of pandemic vaccinations in 2009 or resistance due to an earlier infection (Hulkko et al., 2011). In 2011, 1.900 influenza A cases were reported, of which 666 were pandemic (Jaakola et al., 2012). Swine flu was shown to be resistant to amantadine and research at FIMM found the first isolate in Finland that is resistant to both amantadine and oseltamivir. Worldwide, over 40 oseltamivir resistant isolates have been reported (WHO, 2009).

2.3 Influenza A Virus

Influenza A is a genus located in the family *Orthomyxoviridae*, which is characterized by an 8 segmented, negative-strand RNA genome. The viruses evolve continuously and can infect people multiple times in a lifetime. This evolution is due to mutations (antigenic drift) and reassortment (antigenic shift). Even though the different genera have a common ancestry, reassortment only occurs within the genera. Influenza A has different subtypes which are characterized by both haemagglutinin (HA) and

neuraminidase (NA), which are glycoproteins located in the lipid envelope of the virus. There are 16 variations of HA and 9 of NA. Beneath the surface, there are matrix proteins (matrix protein 1, M1) which support the lipid envelope. Ion channels (matrix protein 2, M2) are present in the virion as tetramers and are responsible for transport through the membrane. In the interior of the virion, there is RNA which is surrounded by nucleocapsid protein (NP) and joined with the RNA dependent RNA polymerase (PB1, PB2, PA). These structures are also called ribonucleoproteins (RNPs) (Bouvier & Palese, 2008). An overview of IAV's structure can be found in Figure 2 (Racaniello, 2009).

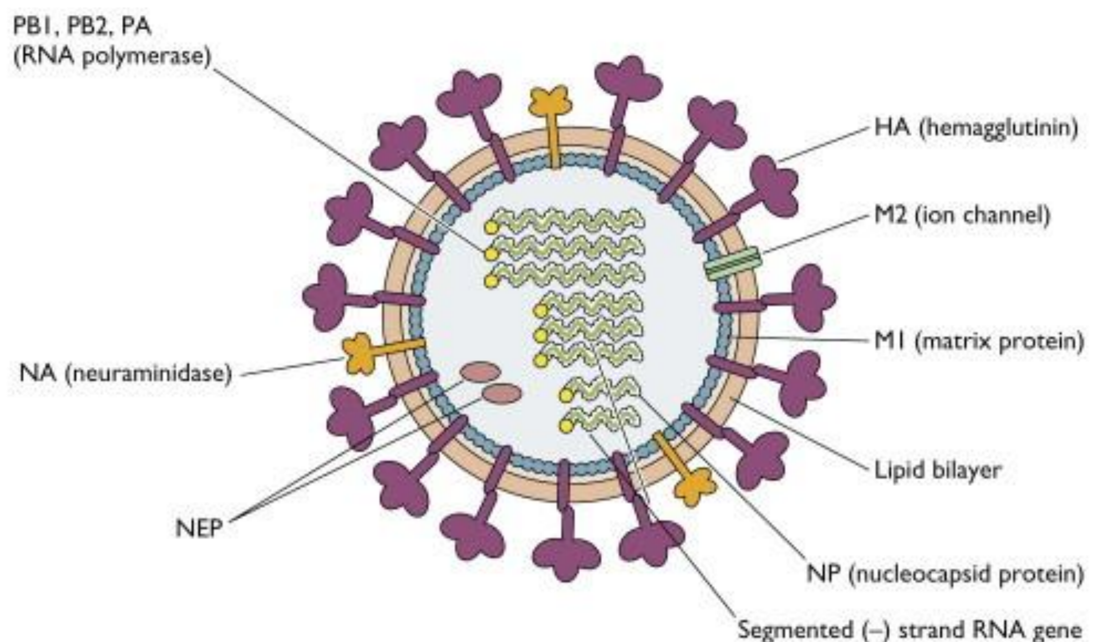


Figure 2. Virion structure of influenza A virus (Racaniello, 2009)

Also some nuclear export proteins (NEP) can be found inside the virion as it is used to export viral RNPs out of the cell nucleus (Samji, 2009).

2.3.1 Genome

As seen in Figure 3, the genome of IAV consists out of 8 single negative RNA segments and is about 13,500 bases long. Each segment encodes for one or two proteins by alternative splicing. PB1-F2 is somewhat different as it is established by a +1 shift of the open reading frame in the PB1 segment. The protein is typical for IAV and has no

analogs in influenza B and C. After infection it resides in the mitochondria and is able to overcome immune responses and increase apoptosis in immune cells (Dermody & Chappell, 2011).

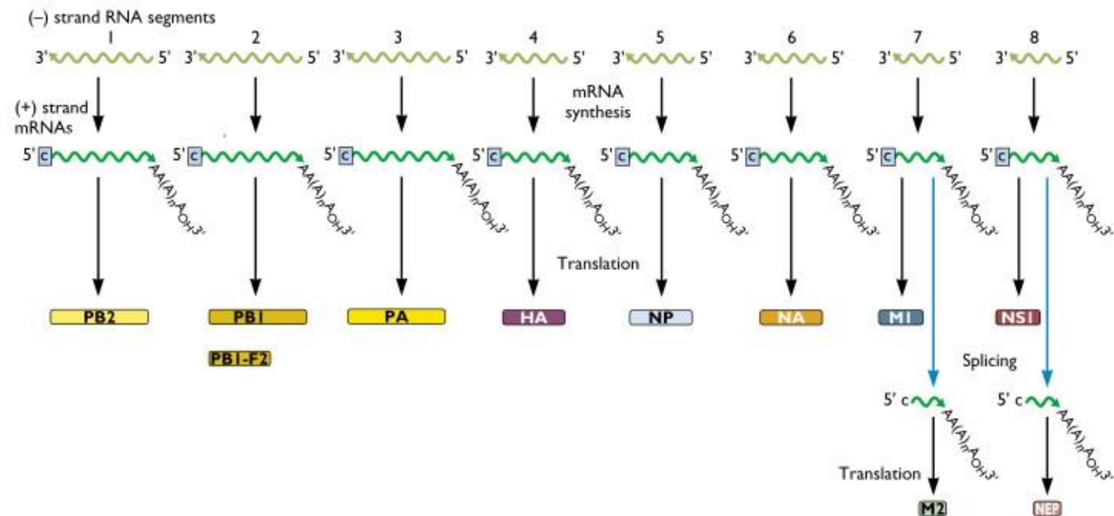


Figure 3. Genome of influenza A virus (Racaniello, 2009)

The 8 stranded genome codes for 11 different proteins. Three proteins PB1, PB2 and PA form the RNA dependent RNA polymerase complex, which means that there are only 8 proteins left for other mechanisms. How the virus is able to replicate with this limited amount of proteins will be explained in next chapter.

2.3.2 Replication

Here the replication mechanism of influenza A viruses will be explained. Because of their limited amount of proteins, viruses are heavily dependent on host cells and use many host cellular pathways in their own advantage. Understanding these complex mechanisms is very important to understand and develop antiviral drugs.

Binding of the virion

IAV attach to sialic acid glycoproteins with haemaggluttinin protein. HA on influenza is a trimer and needs to be cleaved to be able to fuse the viral and cell endosomal membranes. Normally this is done by human proteases (such as Clara) in the respiratory tract, but when different cell lines are used in-vitro, trypsin has to be

added. HA is responsible for the selectivity of IAV as it decides which type of sialic acid-galactose linkage the virus can bind. Figure 4 illustrates two important configurations of the linkage. Human IAV prefer to bind sialic acid attached to galactose in an α(2,6)-configuration, while avian IAV prefer an α(2,3)-configuration (De Clerq, 2006; Bouvier & Palese, 2008).

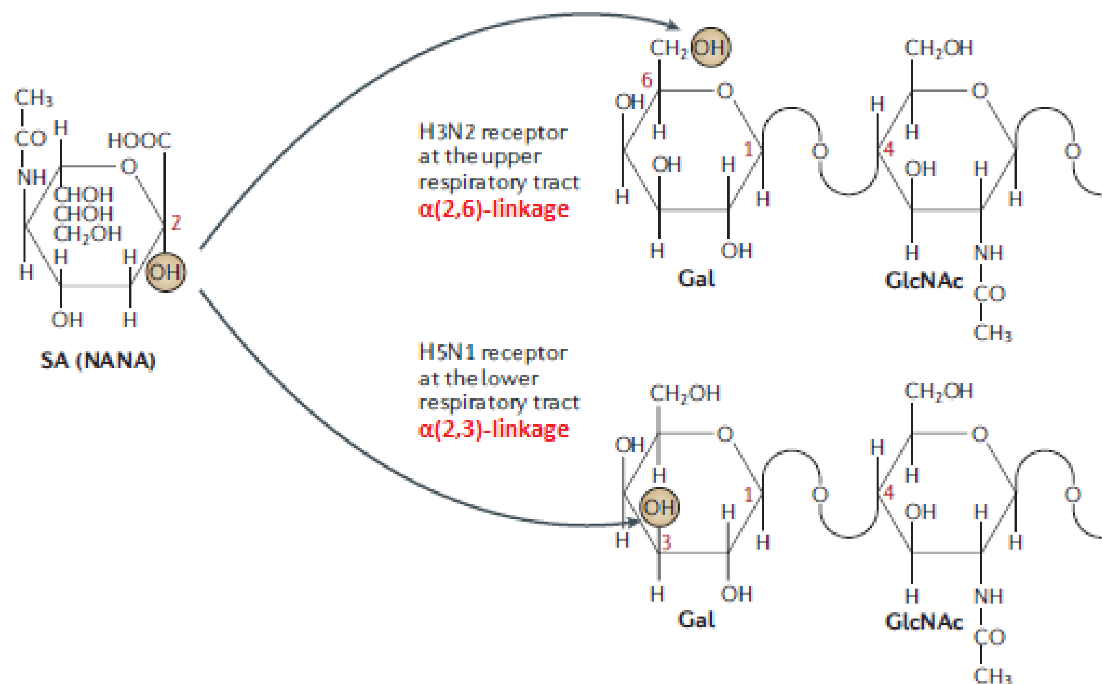


Figure 4. Types of sialic acid – galactose linkages (De Clerq, 2006)

Although it is rare, humans can still get infected by an avian variant of IAV because human also possess a minor population of the α(2,3)-configuration in the lower respiratory tract (Shinya et al., 2006).

Entering the cytoplasm

The virus is taken into the cells by endocytosis. The process is illustrated in Figure 5. When getting closer to the cell nucleus, protons are pumped into the vesicle by cellular channels until pH 5 is reached. At this stage, the haemagglutinin undergoes a conformational change which allows the endosomal membrane and viral envelope to fuse (Stegmann, 2000).

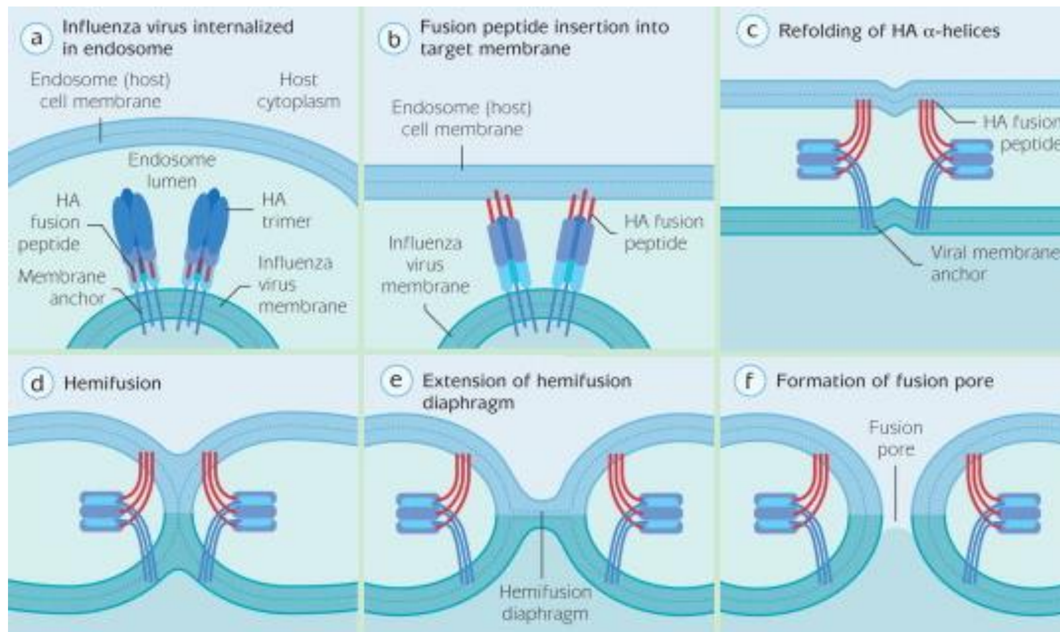


Figure 5. Fusion of viral and endosomal membrane (Cross et al., 2004)

At the same time, viral M2 protein tetramers transfer protons to the virion's interior to weaken the interaction of the M1 protein layer with the viral RNPs (see Figure 6). M2 has been a popular target for antiviral drugs such as amantadine. Amantadine binds to several amino acids which are located inside the M2 channel and blocks the entrance of protons. By preventing the acidification of the virion, M1-RNA cannot be split.

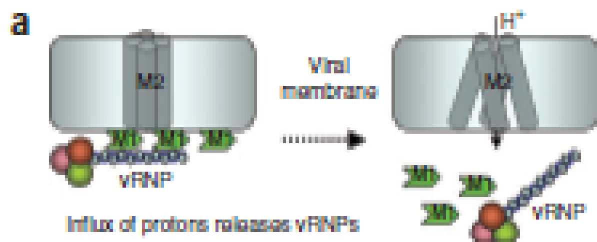


Figure 6. M2 proton pump (Das et al, 2010)

However, the M2 protein is subjected to many mutations, which prevents the operation of the antiviral drug. After release in the cytosol, the viral RNPs can enter the cell nucleus due to nuclear localization signals located on NP (Das et al, 2010).

Entering the nucleus

Transcription and replication are done in the cell nucleus. The cell nucleus is enclosed by a double membrane which contains nuclear pore complexes (NPC) for traffic

between the cytoplasm and nucleoplasm. Small molecules (<9 nm or 20-30 kDa) are able to pass through the NPCs by passive diffusion. Because vRNPs are bigger, they need the help of transport receptors that are able to pass through the NPC (Hutchinson et al., 2012). The process is illustrated in Figure 7.

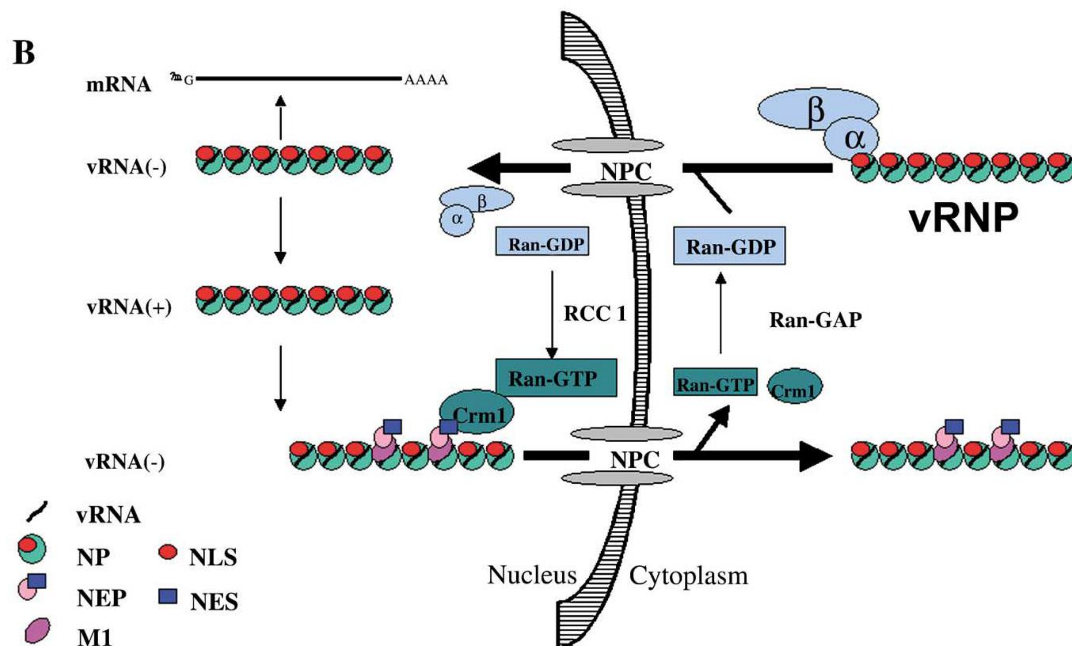


Figure 7. Nuclear import of vRNPs (Cros & Palese, 2003)

vRNPs enter the nucleus via the 'classical import pathway' . They bind karyopherin α1 with the nuclear localization signal (NLS) which can be found on NP. Then karyopherin β binds the complex and allows the vRNP to be transported by Ras-related nuclear protein (Ran). Ran is a small protein driven by guanosine triphosphate (GTP) that shuttles between the cytoplasm and nucleus (Cros & Palese, 2003).

Transcription and replication of viral RNA

As soon as the vRNPs enter the nucleus, they start producing (+)mRNA that will be used for protein synthesis. The (-)vRNA doesn't contain the sequence to produce a 7-methylguanosine (m^7G) cap that is required for functional mRNA and protect the mRNA from degradation by RNAse. Therefore the viral RNA dependent RNA polymerase (RdRP) needs to find an active host RNA polymerase II and steal the cap. This process is called 'cap snatching' and is illustrated in Figure 8 (Zhang et al., 2001).

First the vRNP binds a host cellular mRNA cap that is recognized by PB2. After binding with PB2, the RNA is cleaved approximately 10-13 bases from the cap-1 structure by PA. This cap forms the 5′-end of the new viral mRNA. After the addition of a guanosine nucleoside, a primer is formed and transcription starts. The template strand is read from 3′ to 5′ and transcription is done by PB1 (Zhang et al., 2001). When the polymerase hits the 5′ end of the template strain, they remain bound. Due to steric hinder, the polymerase begins to stutter-read and produces a poly(A)-tail (Pritlove et al., 1998; Poon et al., 1999; Zheng et al., 1999).

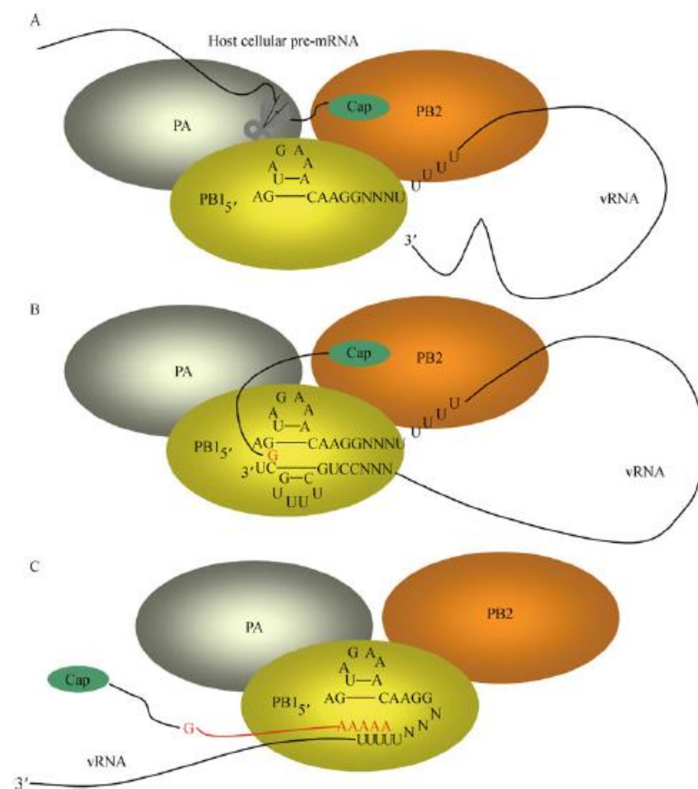


Figure 8. Transcription of vRNA to viral mRNA (Zhang, 2001)

To amplify the viral genome, (+)cRNA is formed. It is a complete copy of the vRNA and in contrary to mRNA, cRNA is not capped or polyadenylated. After transcription, cRNA becomes encapsidated by NP and the polymerase complex. The cRNA serves as a template for vRNP amplification (Zhang et al, 2010).

Multiplication of vRNA does not require cap-snatching. The mechanism is shown in Figure 9.

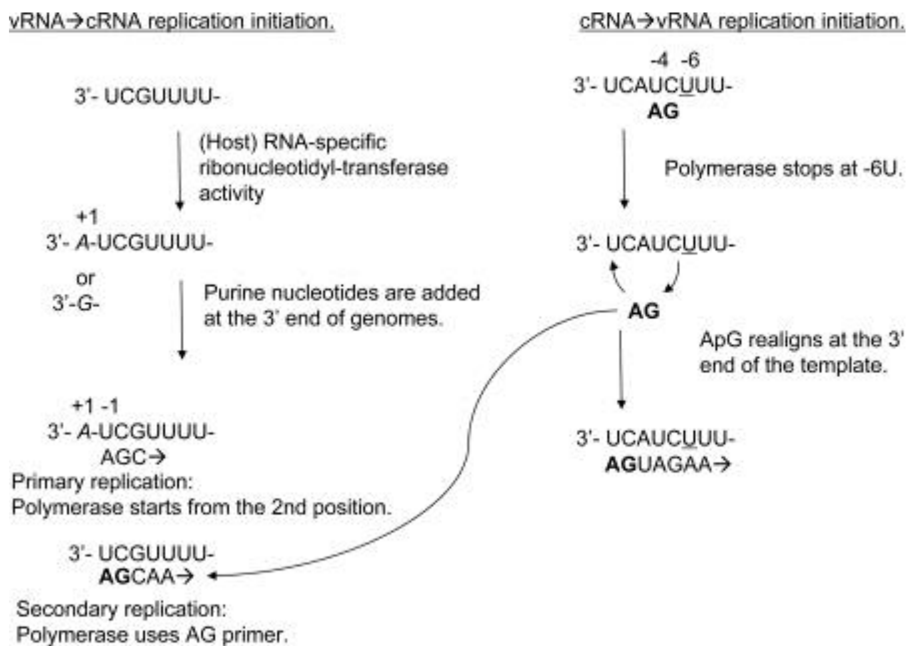


Figure 9. Transcription of vRNA to cRNA and back (Zhang et al, 2010)

The transcription from vRNA to cRNA is primer independent and is believed to be initiated through the addition of an adenine or guanine by host ribonucleotidyltransferase to the 3'-end of vRNA. Then the viral RdRP starts transcription from the uracil until the 5' end to form the cRNA (Zhang et al, 2010)

The transcription back to vRNA is started at uracil(-4). After the RdRP gets stuck at the uracil(-6), the AG dinucleotide is relocated to the 3'-end and permits the RdRP to read till the end (Zhang et al, 2010 and 2011).

Translation into proteins

Here non-structural protein 1 (NS1) plays an important role. It minimizes host translation by inhibiting cleavage and polyadenylation specificity protein (CPSF) and poly (A)-binding protein II (PABII). Without these proteins pre-mRNA cannot be spliced and polyadenylated, a requirement for translation. The translation of viral proteins is not affected, because an alternative mechanism to add a poly-A tail is used (Nemeroff & Qian, 1995).

When the (+)mRNA is ready for translation, it travels to the ribosomes where the proteins are produced. At the same time, unmodified (+)RNA serves as a template (cRNA) to produce more (-)RNA strands for the assembly of new viruses. The envelope proteins HA, NA and M2 are transported through the golgi-apparatus to the cell membrane. To prevent a conformational change of HA induced by the low pH in the trans-golgi network, virus protein M2 also functions to neutralize the acidic pH in the vesicles. The core proteins of the virus are made entirely in the cytosol. M1 proteins interact with the C-terminal domains of HA and NA and form parts with high HA and NA density on the membrane. Newly synthesized RNPs can then interact with the M1 to form a new virus particle.

PB1, PB2, PA, NP, NS1 and NEP are transported back into the cell nucleus after translation (Figure 10). PB2 and NP make use of the 'classical import pathway' while PB1 and PA form a complex that is recognized by β-importin Ran binding protein 5 (RanBP5) (Hutchinson & Fodor, 2012).

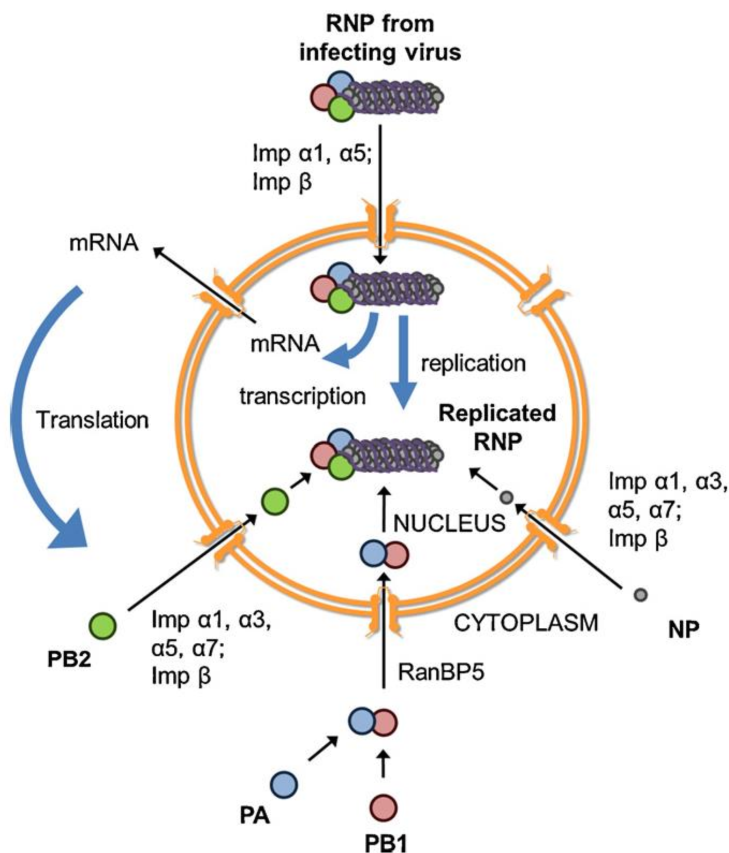


Figure 10. Import of newly made proteins into nucleus (Hutchinson & Fodor, 2012)

Back in the nucleus PB1, PB2 and PA form new polymerases resulting in more transcription. NP binds vRNA to produce vRNPs so they can leave the nucleus with the help of M1 and NEP. M1 is thought to interact with the vRNP, allowing the formation of a vRNP-M1-NEP complex. This complex can be bound by chromosome region maintenance 1 protein (Crm1) and is subsequently recognized by Ran which traffics the vRNP through the nuclear membrane (cf. Figure 7). In the cytosol M1 remains bound and prevents the vRNP from re-entering the nucleus (Cros & Palese, 2003).

Assembly of new particles

The budding of new particles only happens at the apical side of polarized epithelial cells. This way the virus is released back into the respiratory tract and can easily infect new cells. This also causes the high transmission from person to person. When a person is infected with influenza, it is important to cover nose and mound when coughing or sneezing, wash hands regularly and keep more distance from other people to prevent transmission (WHO).

Release from host cell

After budding, the virions are still attached to the cell membrane through the binding of the HA with sialic acid bound to glycoproteins. To release the virion, the virus possesses NA which is able to cleave this binding. NA is also a target for antiviral drugs that block the release of new virions. This way they prevent the virus from spreading, but they don't affect its synthesis (Bouvier & Palese, 2008; Samji, 2009)

Cell death

Infection with IAV triggers apoptosis, which is an immune reaction to prevent the virus from replicating in infected cells. However the virus is still able to make thousands of new virions before cell lysis occurs.

Despite the small amount of viral proteins, influenza A viruses are able to replicate very successfully in mammalian cells. Two surface proteins, HA and NA, are very important for binding to and releasing from target cells. The most important role of M2 is the acidification of the interior of the virion which leads to the dissociation of the M1-vRNP complexes. To get into the nucleus, the virus is able to trick the host cell and travel through the nuclear pore complexes. Once arrived in the nucleus, transcription is done

by PB1, PB2 and PA and requires cap-snatching. The translation is done by host cellular proteins. This means that the virus is very dependent on host cells for their replication cycle.

2.3.3 Genetic Variation

Genetic variation in IAV is driven by two different processes: antigenic drift and antigenic shift. A direct consequence of those processes is that IAV can become resistant to antiviral drugs and bypass human immunity.

Antigenic drift

Influenza A RdRP lacks exonuclease proofreading capability and thereby IAV are subject to high mutation rates. Point mutations occur at rates from $1x10^{-3}$ to $8x10^{-3}$ per site per year (Taubenberger & Cash, 2010). Substitutions at antigenic sites of structural proteins can lead to poor binding of antibodies by steric interference, energetically unfavorable binding or obscuration of the antibody binding by the introduction of new oligosaccharide attachment sites (Rappuoli et al, 2010). The same problem occurs with drugs that target specific structures determined by amino acid sequences. Mutations can change the affinity of the drugs by altering hydrophobic interactions, hydrogen bonds, *etc*. Viruses that are poorly recognized by the immune system are more favorable to spread throughout the population.

Antigenic shift

When a host cell is coinfected by two different types of IAV, a more drastic change can occur. The viruses can exchange segments which cause a new hybrid virus to be formed. It is called antigenic shift when the reassortment involves HA and/or NA gene segments. Antigenic shift is important for the evolution of IAV. Pigs can be infected by both avian and human IAV leading to reassortant IAVs that have potential to infect humans and are therefore labeled as “mixing vessels” (Ma et al., 2009). The introduction of new subtypes of HA and/or NA can lead to epidemics if there are no antibodies present in the human population (Taubenberger & Cash, 2010).

2.4 Immune System

IAV trigger the immune system when entering the body. This results in the excretion of interferons and chemokines/cytokines. A few important cytokines/chemokines and their functions are shown in Table 2. Chemokines attract cells of the innate immune system to fight the infection. They also induce proliferation of lymphocytes which are part of the adaptive immune system. The innate immune system acts immediately after infection, while it takes a few days for the adaptive immune system to be fully activated (Madigan, 2010).

Cytokine (chemokine)	*Major producer cells*	*Major target cells*	*Major effect*
IL-4[a]	T_H2	B cells	Activation, proliferation, differentiation, IgG1 and IgE synthesis
IL-5	T_H2	B cells	Activation, proliferation, differentiation, IgA synthesis
IL-2	Naive T cells, T_H1, and T_C	T cells	Proliferation (often autocrine)
IFN-γ[b]	T_H1	Macrophages	Activation
GM-CSF[c]	T_H1	Macrophages	Growth and differentiation
TNF-α[d]	T_H1 Macrophages	Macrophages Vascular epithelium	Activation, production of pro-inflammatory cytokines Activation, inflammation
IL-1β	Macrophages	Vascular epithelium, lymphocytes	Activation, inflammation
IL-6	Macrophages, dendritic cells	Lymphocytes	Activation
IL-12	Macrophage	NK cells, naive T cells	Activation, enhances differentiation to T_H1
IL-17	T_H17	Neutrophils	Activation
CXCL8 (chemokine)	Macrophages	Neutrophils, basophils, T cells	Chemotactic factor
CCL2 (MCP-1[e]) (chemokine)	Macrophages	Macrophages, T cells	Chemotactic factor, activator

[a]IL, interleukin; [b]IFN, interferon; [c]GM-CSF, granulocyte, monocyte-colony stimulating factor; [d]TNF, tumor necrosis factor, [e]MCP, macrophage chemoattractant protein.

Table 2. Examples of major immune cytokines and chemokines (Madigan, 2010)

As seen in Figure 11, immune cells are divided into two important groups: myeloid cells and lymphoid cells. The group of myeloid cells are active in the innate immune response and don't need previous contact with pathogens to be efficiently activated. There are 2 distinct groups of phagocytes which differentiate from myeloid cells. Macrophages and dendritic cells are phagocytes which have antigen-presenting capabilities. A second group is granulocytes (neutrophils and mast cells) which contain granules with toxins or enzymes that are used to kill target cells (Madigan, 2010).

The second group, lymphoid cells are activated by the innate immune cells and need some time before they are ready to fight the infection. They mainly consist out of T cells and B cells which are active in the adaptive immune response. B cell receptors (BCR) on the surface of B cells can bind the corresponding antigen and digest it. The antigen is cleaved and presented to helper T cells by a histocompatibility molecule

(MHC). When a T helper cell binds the B cell, it starts producing lymphokines that stimulate mitosis of B cells. Two types of B cells will be made, B plasma cells that produce high quantities of soluble antibodies and B memory cells that bear copies of the activated BCR on their surface. Antibodies can neutralize antigens and enhance their recognition by phagocytes. If there was contact with a pathogen before, the adaptive immune response will occur faster thanks to memory B-cells (Madigan, 2010). A third group of lymphocytes, natural killer cells (NK cells), are lymphocytes that are active in the innate immune system. They constantly scan MHC for foreign protein fragments and kill target cells with perforins and granzymes that induce apoptosis. They can also recognize virus infected cells as the expression of MHC is often disrupted. Cells that have no or few MHC on their membranes are also killed by NK cells.

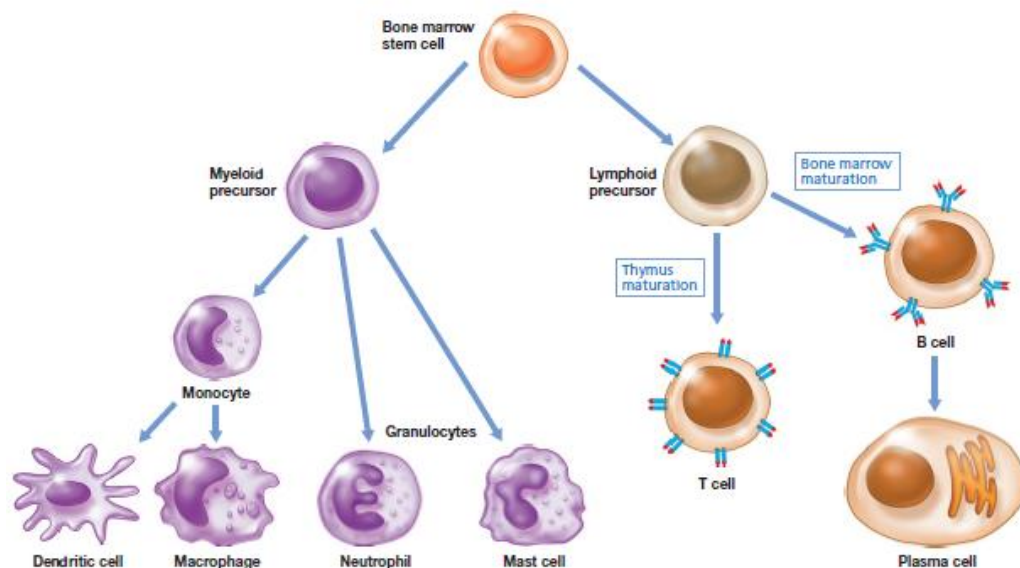


Figure 11. Classification of immune cells (Madigan, 2010)

IAV target the human lung epithelial cells. When cells are infected with IAV, they only show a weak cytokine and chemokine response. The main attributors to the antiviral response are the macrophages and dendritic cells. They are located very close to the epithelium and can produce high quantities of Tumor necrosis factor (TNF)α and type I interferons (IFNs). As seen in Figure 12, these IFNs consequently trigger a stronger second cytokine/chemokine response at a later stage of the infection (Veckman et al., 2006).

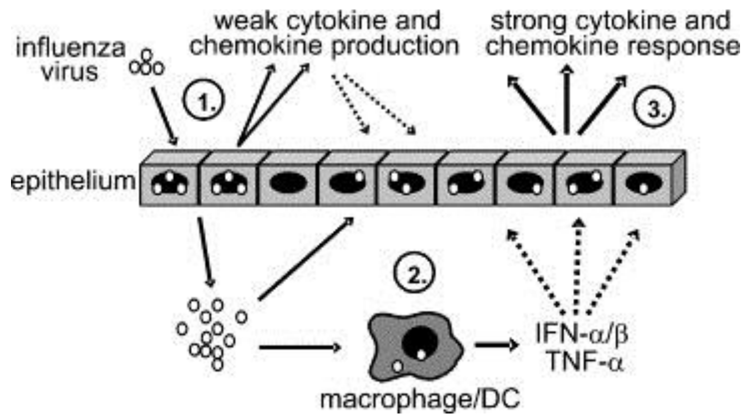


Figure 12. Interaction between epithelium and macrophages/dendritic cells (Veckman et al, 2010)

After digestion of the pathogen, they also serve as antigen-presenting cells (APC) that initiate the adaptive immune system. Dendritic cells have the same function as macrophages, but are present in body tissues instead of in the blood. After infection, these APC migrate to lymph nodes and activate T- and B-lymphocytes by presenting the antigen.

2.4.1 Recognition by Innate Immune System

Influenza viruses are detected by several pattern recognition receptors (PRRs). The most investigated ones are Toll-like receptors (TLRs), retinoic acid inducible gene-I-like receptors (RLRs) and oligomerization domain NOD-like receptors (NLRs). The responses of the innate immune systems are complex and often redundant, which means that the same outcome can be achieved through different routes.

Toll-like receptors

Toll-like receptors are transmembrane proteins. Humans have 10 types of TLRs that are located at the cell surface or in case of TLR3, -7, -8 and -9 at the membrane of intracellular compartments. They recognize pathogen associated molecular patterns (PAMPs) such as lipopolysaccharides (TLR4), flagellin (TLR5) and nucleic acid motifs (TLR3, -7, -8 and -9) from fungi, parasites, micro-organisms and viruses. The horseshoe shaped ectodomain of the receptors contains leucine rich repeats (LRR) and is responsible for PAMP recognition. A transmembrane domain connects the

ectodomain with the cytoplasmic domain which is called the Toll/IL-1 receptor homology (TIR) domain. The recognition of a PAMP induces the dimerization of TLRs and their TIR domains. All TLRs except TLR3, which is using TIR domain-containing adaptor protein inducing IFN-β (TRIF) as an adaptor molecule, use a pathway that is very similar to the IL-1 receptor and includes the recruitment of myeloid differentiation primary response gene 88 (MyD88) to initiate signal transduction. TLR4 uses both MyD88 and TRIF pathways (Jensen & Thomsen, 2012).

The types of TLR that are involved in IAV recognition are Toll-like receptor 3 (TLR3) and Toll-like receptor 7 (TLR7). They are located in the endosomal membrane and sense the presence of respectively dsRNA and ssRNA. Although IAV is an ssRNA virus, dsRNA is formed as intermediate during replication and thus IAV can be detected by TLR3. The signalling pathways are illustrated in Figure 13 (Jensen & Thomsen, 2012).

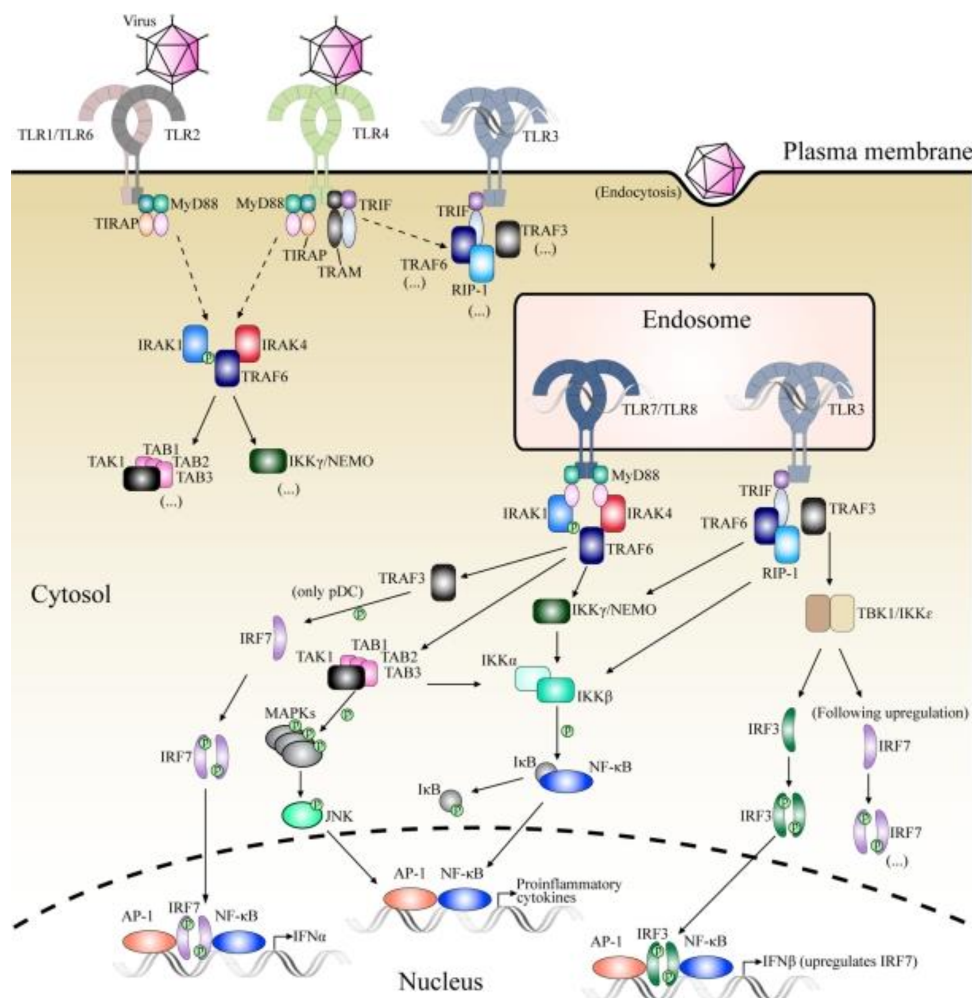


Figure 13. TLR pathways (Jensen & Thomsen, 2012)

TLR3 uses TRIF as adaptor molecule and is able to recruit TNF receptor associated factors (TRAF) members TRAF6 and TRAF3. TRAF6 leads to the activation of transcription factor NF-κB via the TAK1-dependent IKKγ-NEMO pathway and a TAK1-independent pathway initiated by Receptor-interacting protein 1 (RIP-1). TRAF6 also induces the mitogen-activated protein kinases (MAPK) pathway that activates the transcription factor AP-1. Together NF-κB and AP-1 induce the expression of proinflammatory genes (interleukins: (pro-) IL-1β, IL-6, (pro-)IL-18) and genes contributing to adaptive immune response (TNF). TRAF3 associates with TRAF family member-associated NF-κB activator binding kinase 1 (TBK1), and IKKε. The complex phosphorylates interferon regulatory transcription factor (IRF) 3 which leads to dimerization and transcription of IFN-α4 and IFN-β genes. Those IFNs cause the upregulation of IRF7 via a positive feedback system (see later). IRF7 is also phosphorylated by the TBK1-IKKε complex and regulates the transcription of IFN-β and IFN-α – not limited to IFN-α4 (Jensen & Thomsen, 2012).

TLR7 on the other hand uses the MyD88 adaptor molecule. TRAF6 is bound via interleukin-1 receptor-associated kinases 1 and 4 (IRAKs). Then IRAK1-TRAF6 dissociates and the TAK1-dependent IKKγ/NEMO pathway is activated, leading to the translocation of AP-1 and NF-κB to the nucleus where transcription starts. In plasmacytoid dendritic cells (pDCs) IRF7 is also phosphorylated by IRAK1. Phosphorylated IRF7 then translocates to the nucleus where it regulates IFN-α and IFN-β expression (Jensen & Thomsen, 2012).

Retinoic acid inducible gene-I-like receptors

Retinoic acid inducible gene-I-like receptors are proteins that are located in the cytoplasm and sense RNA. They are aspartate-glutamate-any amino acid-aspartate/histidine (DExD/H)-box helicases which are present in most cell types except pDCs. The member of interest for IAV infections is Retinoic acid-inducible gene-I (RIG-I) as it senses cytoplasmic both ssRNA and dsRNA containing a 5′-triphosphate end (5′ppp) (Jensen & Thomsen, 2012). Recently it has been shown that RIG-I is able to recognize RNA in vRNPs. Viruses, including IAV, that contain a 5′ppp dsRNA panhandle structure were detected even when viral RNA synthesis was blocked. This suggests that the 5′ppp panhandle structure is a PAMP for RIG-I and RIG-I triggers an immune response at the early stage of entry (Weber et al., 2013).

Other members are melanoma differentiation-associated antigen 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). Although MDA5 nearly shares the same domains and structure, it has a different type of ligand: MDA5 binds long dsRNA fragments with a length of at least 2 kb. LGP2 lacks a caspase activation and recruitment domain (CARD) and thus can't bind to β-interferon promoter stimulator 1 (IPS-1) which is needed for further signal transduction. LGP2 is thought to be a modulator of the innate immune system as studies showed that LGP2 binds dsRNA and thereby inhibits RIG-I signalling. They also suggest that LGP2 can form a complex with MDA5 to enhance the ability to recognize long dsRNA. RLR signalling pathways are found in Figure 14 (Jensen & Thomsen, 2012).

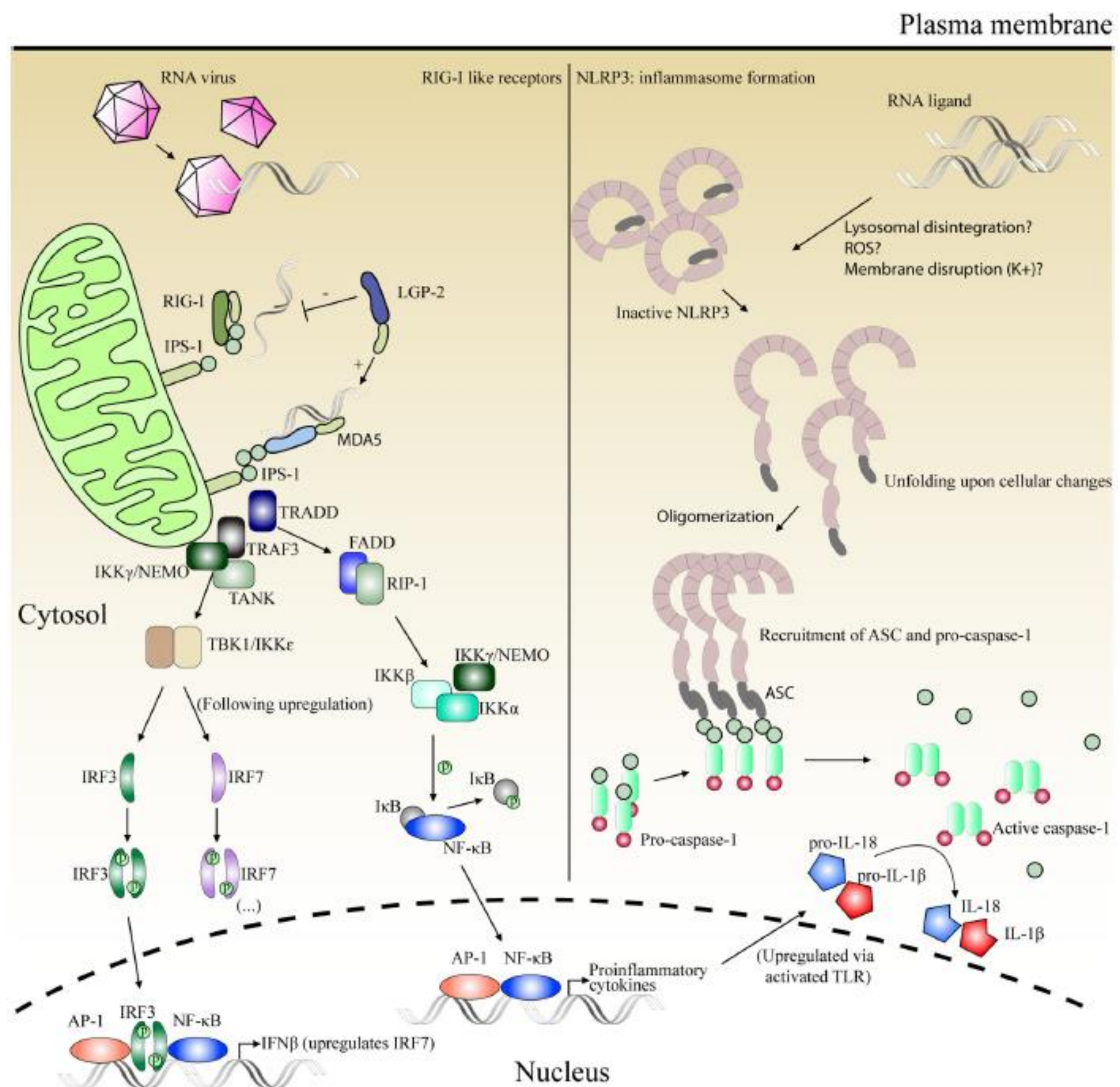


Figure 14. RLR and inflammasome pathway (Jensen & Thomsen, 2012)

RIG-I and MDA5 both trigger the same pathway via the adaptor protein IPS-1 that is located in the mitochondrial membrane. After binding with RNA and ATP, there is a structural change that causes CARD to be available to IPS-1. Then IPS-1 activates RIP-1 and IKKγ-NEMO mediated pathways, which are also activated by TRIF in the signalling from TLR3. As previously described, the recruitment RIP-1 ultimately leads to the transcription of proinflammatory cytokine genes, while IKKγ-NEMO leads to the production of IFN-β and upregulation of IRF7 (Jensen & Thomsen, 2012).

Nucleotide-binding domain, leucine-rich repeat-containing proteins

Nucleotide-binding domain, leucine-rich repeat-containing proteins (NLR) or previously named nucleotide-binding oligomerization domain receptors are intracellular proteins that have an important function in antiviral defence. They are responsible for inflammatory reaction and regulate cell death. Activation of NLRP3 results in the formation of the NLRP3 inflammasome as illustrated in Figure 15 (Jensen & Thomsen, 2012).

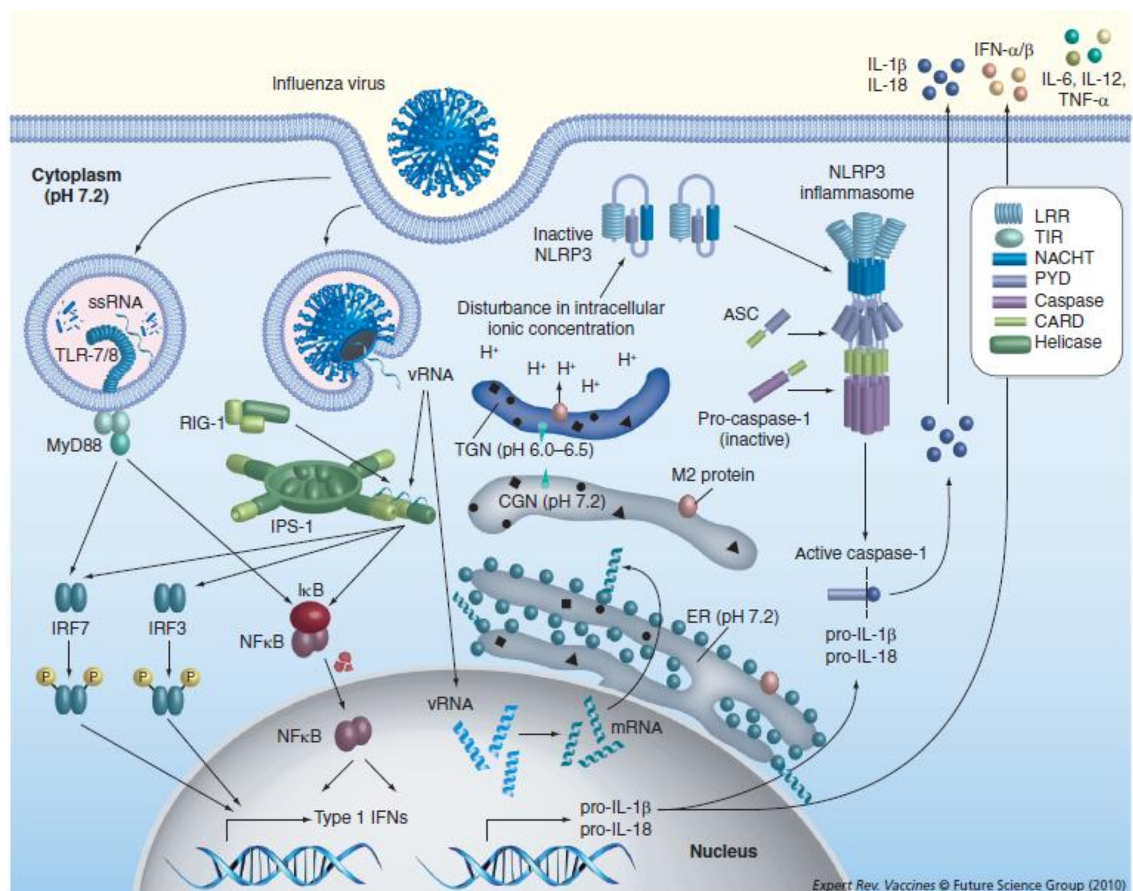


Figure 15. NLRP3 inflammasome and its relation to TLR and RLR pathways (Ichinoche, 2010)

The mechanism of activation is still unclear and it is most likely an indirect sensor of viral infection induced by imbalances of ionic concentrations in intracellular vesicles (Ichinoche, 2010). The inflammasome consists out of oligomerized NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1. When bound, pro-caspase-1 is autocatalytically cleaved and is able to turn pro-IL-1 and pro-IL-18 in their active forms (Jensen & Thomsen, 2012).

2.4.2 Further Activation by IFN-α/β

Type-I Interferons or IFN-α/β are secreted after recognizing the virus. There are 13 IFN-α and one IFN-β species that all bind to the same receptor. They induce the transcription of IFN stimulated genes (ISG) by activating their promoters, termed IFN sensitive response elements (ISRE) through the Janus activated kinase-signal transducer and activator of transcription (JAK-STAT) pathway which is shown in Figure 16 (Shaw & Palese, 2005).

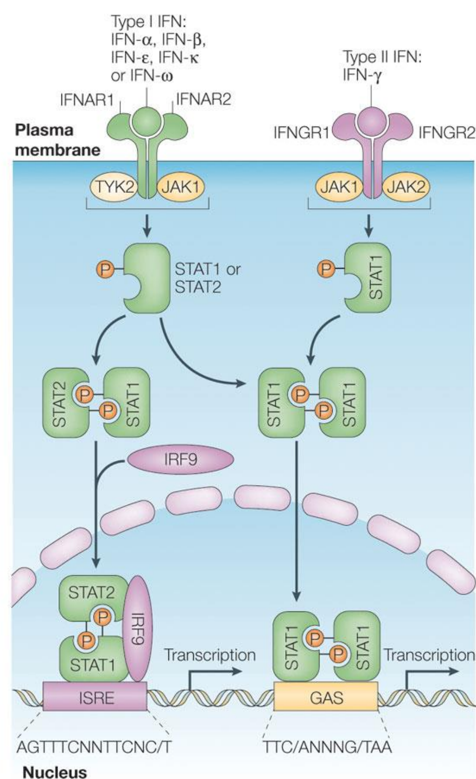


Figure 16. Activation of JAK-STAT pathway (Ichinoche, 2010)

Type I IFN receptors consist out of two subunits interferon alpha, beta and omega receptor 1 (IFNAR1) and interferon alpha, beta and omega receptor 2 (IFNAR2). After binding to the receptor, the JAK-STAT pathway is activated. The formation of the STAT1-STAT2-IRF9 or STAT1-STAT1 complex, activate the ISRE and IFN-γ-activated site (GAS) respectively to initiate the transcription of the ISGs (Ichinoche, 2010).

There are hundreds of ISGs with diverse functions, including IRF-7 which is upregulated for the transcription of IFN-α genes and allows a second wave with a much higher IFN production. Most of the ISGs inhibit cellular and viral protein synthesis (Shaw & Palese, 2005).

Protein kinase RNA regulated (PKR) for example, is stimulated by dsRNA. It has an important role in a broad array of cellular processes, but it most importantly phosphorylates Eukaryotic Initiation Factor 2 α (eIF2α). This leads to an inhibition of the cellular protein synthesis and the activity of the immune system. It can also be activated by Protein Activator of the Interferon-induced Protein Kinase (PACT) which is phosphorylated as result of cellular stress (Sarkar et al., 2005).

Also 2′-5′ oligoadenylate synthetases (OAS) are induced by type I IFN production. They are activated in presence of dsRNA and ATP and activate RNase L through dimerization to a ribonuclease that is able to degrade RNA (Sarkar et al., 2005).

P56 is highly induced by IFN and is activated by viral stress. With the help of p48, a structural analogue of the Eukaryotic Initiation Factor 3e (eIF-3e), P56 can bind to Eukaryotic Initiation Factor 3 (eIF3) and block translation initiation by preventing the enhancement of the eIF2.GTP.tRNA Met_i ternary complex (Sarkar et al., 2005).

2.4.3 Programmed Cell Death

Programmed cell death or apoptosis is an innate immune response to limit viral replication and a reaction to cell stress. Two major pathways that cause the cell to degrade are well known. Both pathways involve caspase activity. Caspases are a group of cysteine proteases that cleave their substrates at aspartic acid residues. Caspases

are present in all animal cells, but they need to be activated before they can start cleaving and cause apoptosis (Reed, 2000). They can be classified in two groups (Figure 17): initiator caspases and effector caspases. Initiator caspases require dimerization to be activated and have a small number of targets, including self-cleavage of the pro-domain, the protein Bcl-2 homology 3 (BH3)-interacting domain death agonist (BID) and effector caspases. Effector caspases are present as inactive dimers and are activated by initiator caspases. Effector caspases have a large range of substrates and are the main attributors to apoptosis. The cell fragments are ultimately cleared by phagocytosis (Tait & Green, 2010).

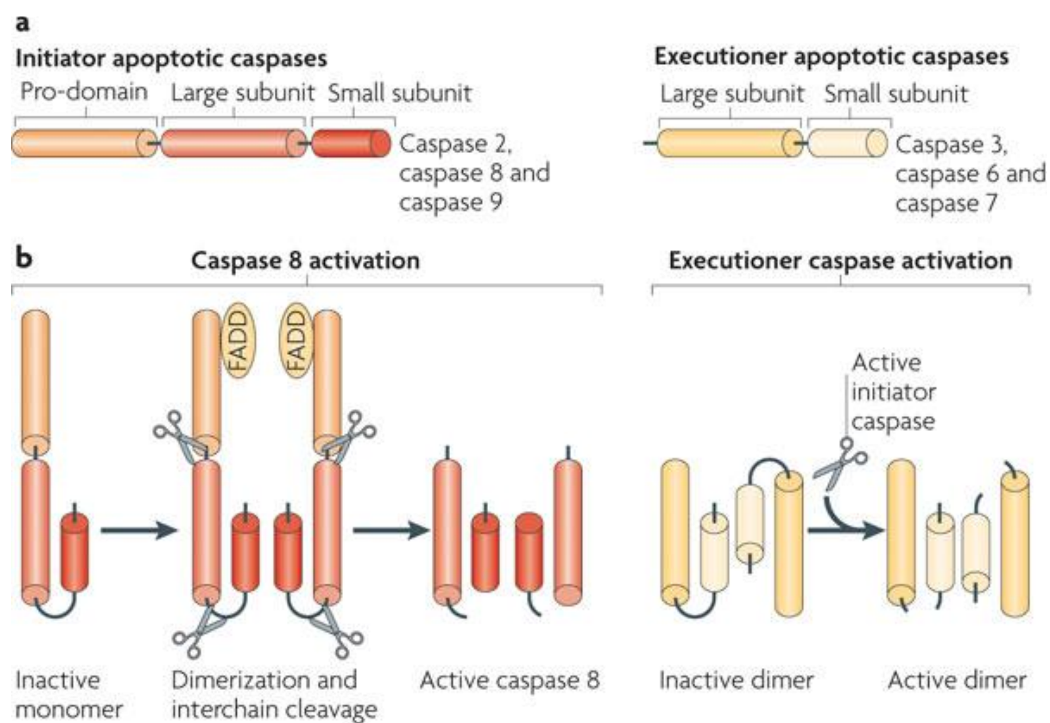


Figure 17. Activation of caspases (Tait & Green, 2010)

Two important pathways of programmed cell death have been extensively studied and are shown in Figure 18.

A first pathway is the extrinsic death receptor pathway. The pathway is activated when ligands bind to a tumor necrosis factor (TNF) family receptor. Through adaptor proteins like Fas-Associated protein with Death Domain (FADD), the death-inducing

signaling complex (DISC) that activates caspase 8 is formed (Reed, 2000; Haupt et al., 2003)

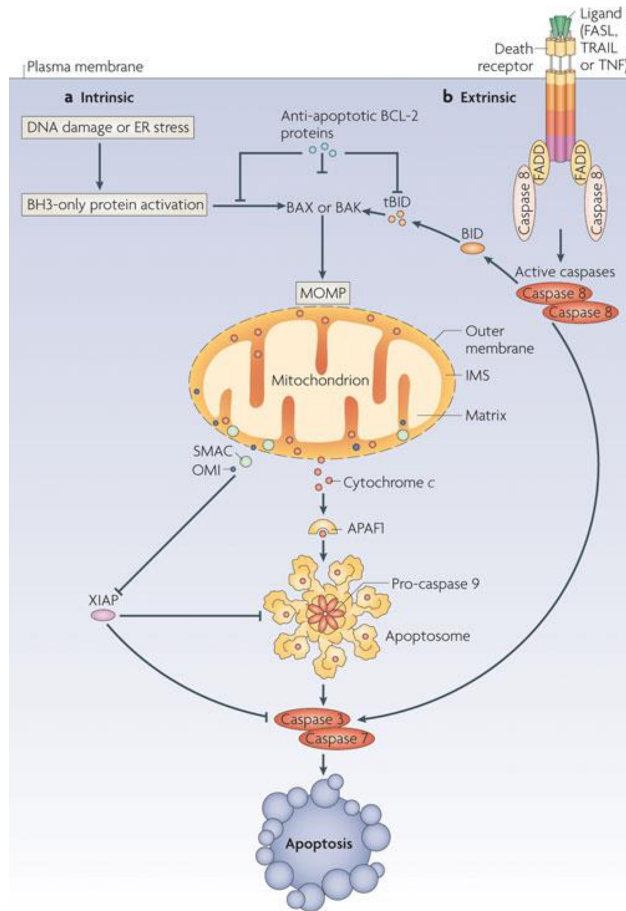


Figure 18. Apoptosis pathways (Tait & Green, 2010)

A second pathway is the intrinsic pathway. It is triggered by the release of cytochrome c from the mitochondria by a process that is called mitochondrial outer membrane permeabilization (MOMP). MOMP is controlled by relative amounts of pro-apoptopic and anti-apoptopic proteins of the BCL-2 family. BH3-only proteins are thought to inhibit anti-apoptotic BCL-2 proteins that are bound to Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK) to inactivate them. Activation of BH3-only proteins will thus result in the activation of BAX and BAK. A second model suggests that there are two types of BH3-only proteins: one that can directly activate BAX/BAK, but is inhibited by anti-apoptotic proteins (direct activators) and another group that neutralize anti-apoptotic proteins which are inhibiting the activators (sensitizers). Both models are illustrated in Figure 19 (Tait & Green, 2010).

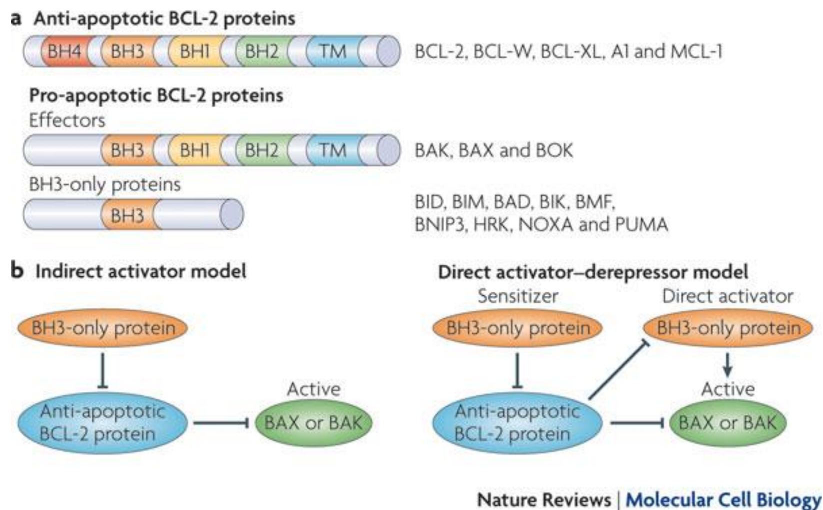


Figure 19. BCL-2 family of proteins and BAX/BAK activation model (Tait & Green, 2010)

When BAX or BAK proteins are activated by BH3-only proteins, they undergo a conformational change which allows them to form symmetrical dimers (Figure 20). Along with this, a dimer-dimer interaction interface is exposed, which allows higher-order oligomerization. Binding of the BAK/BAX oligomers to the outer mitochondrial membrane causes the release of cytochrome c. It is still unclear if this happens due to the formation of protein channels or lipidic pores (Tait & Green, 2010).

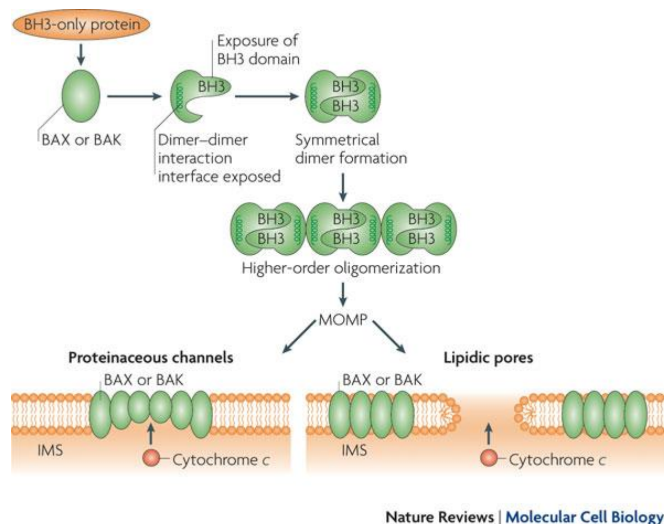


Figure 20. Mitochondrial outer membrane permeabilization (Tait & Green, 2010)

Together with apoptotic protease-activating factor 1 (APAF-1) and procaspase-9, cytochrome c forms a complex called the apoptosome that causes the activation of caspase-9. Both caspase 8 and 9 promote the activation of the effector caspases

caspase-3 and caspase-7. This process is often referred to as the caspase cascade (Haupt et al., 2003; Tait & Green, 2010).

Both pathways are connected to each other by BID. Capase-8 which is activated by the extrinsic pathway cleaves BID in a way that a new N-terminal glycine residue is formed. A myristoyl group is then added to this terminal in a process called myristoylation. The myristoylated BID is now able to insert into the mitochondrial membrane to cause MOMP by BAX and BAK (Tait & Green, 2010).

2.4.4 Evasion of Immune System by IAV

To be able to successfully replicate in human cells, IAV inhibits various pathways that are involved in the formation of immune responses. Tests with both wild type influenza A and mutant delNS1 show that infection with wild type IAV results in a low production of IFN-α and IFN-β, while delNS1 results in much higher concentrations. It is concluded that NS1 is able to inhibit the activation of transcription factors which results in IFN production (Talon et al., 2000).

NS1 is shown to interact with tripartite motif-containing protein 25 (TRIM25), a protein that is needed to ubiquinate and activate RIG-I. By preventing TRIM25 oligomerization, the RIG-I pathway is blocked (Gack et al., 2009). It also prevents the inhibition of cellular translation by PKR by binding it (Li et al., 2006). Because NS1 binds and masks dsRNA, it prevents the activation of the OAS/RNase L-pathway which in normal circumstances would lead to the degradation of RNA (Min & Krug, 2006).

Not only NS1 has immune repressing properties, M2 and NP also regulate PKR by activating $P58^{IPK}$ which is a cellular inhibitor of the PKR phosphorylation. Later in the infection, M2 is produced and disables $P58^{IPK}$ again, which leads to the inhibition of protein synthesis and cell apoptosis. This helps the virus to release from the cell membrane (Kreijtz et al., 2011).

PB2 also plays an important role in regulating host anti-viral responses as it is able to inhibit IPS-1, the adaptor molecule used in RIG-I signaling. PB2 consequently limits the transcription of IFN-β (Iwai et al, 2010).

2.4.5 PI3K/Akt Pathway

NS1 also activates the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. Figure 21 shows ten proteins that are regulated by Akt. The PI3K/Akt pathway has functions ranging from cell survival, proliferation, growth, angiogenesis and glucose uptake to the cell metabolism (Manning & Cantley, 2007).

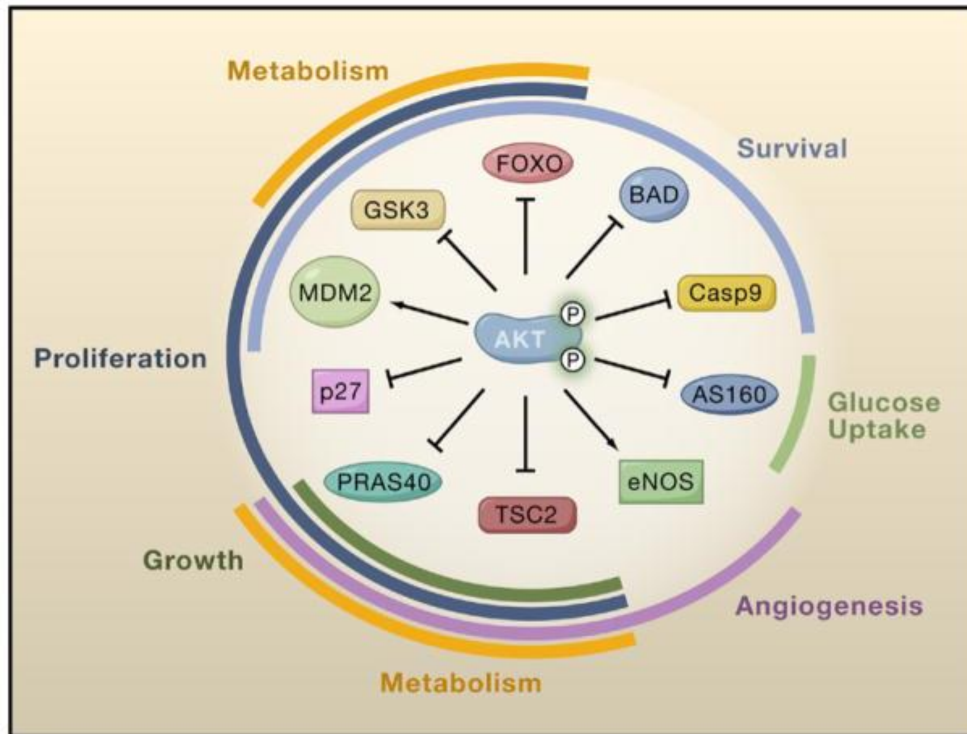


Figure 21. Ten proteins regulated by Akt (Manning & Cantley, 2007)

It is shown that PI3K is needed in a very early step of the viral replication cycle as inhibition of PI3K leads to accumulation of virus particles at the cell surface. It is thought that IAV attachment induces an early and short peak of PI3K activation during viral attachment, but the mechanism it not known yet (Ehrhardt & Ludwig, 2009).

At later stages PI3K is activated for a longer time by viral NS1 protein. NS1 can bind the p85 regulatory subunit PI3K and activate it. This leads to the suppression of apoptosis due to inactivation of proapoptotic proteins such as BAD, procaspase-9 and glycogen synthase kinase-3β (GSK-3β) by phosphorylation. By suppressing premature apoptosis, the virus ensures successful replication (Ehrhardt et al., 2007; Ehrhardt & Ludwig, 2009).

2.5 Antiviral Drugs

Drugs against influenza traditionally target viral genes to inhibit the replication within the human cells. By using this approach the risk of cell toxicity is low, because of their specificity. On the other side viral proteins are few in number and viruses can develop resistance against drugs due to antigenic drift/shift (Schang et al., 2001).

To overcome this problem an alternative view can be used. Viruses often use cellular mechanisms to replicate. Influenza A for example, needs the help of cellular RNA-polymerase II to provide the m^7G-cap of the mRNA. IAV also target some pathways to inhibit the cytokine/chemokine production of cells, whereby adjacent cells don't get warned and replication can proceed unrestricted. By targeting these mechanisms, viral propagation can be reduced or even stopped. An overview of the advantages/disadvantages of both approaches is shown in Table 3 (Schang et al., 2001).

Table 3. Comparison between targeting viral proteins and cellular proteins (Schang et al., 2001)

Target	Advantages	Disadvantages
Viral protein	• Specificity (-> safety)	• Selection for drug-resistant mutants • Limited number of targets • Narrow specificity (that is, one virus or one virus family) • Viral pathogen must be isolated and its proteins must be characterized
Cellular protein	• Selection for drug-resistant mutants less likely to occur • Large number of potential targets • Broad specificity (that is, all viruses that require the targeted cellular proteins) • Viral proteins need not be characterized	• Potential for toxicity

The main advantages of targeting cellular proteins is that viral proteins are less subject to mutations and the drugs can cover a big range of viruses that require the targeted pathway. When developing these drugs, cytotoxicity has to be monitored closely as the targeted protein can be essential for the organism (Schang et al., 2001).

2.5.1 Virus Directed Drugs

The earliest approach to develop antivirals was targeting the viral genes. These antivirals have saved many lives, but unfortunately many virus isolates have developed resistance against them. In this chapter, two groups of virus directed antivirals will be discussed.

Adamantan(amin)e derivates

Amantadine is the first drug found to inhibit IAV replication. When endosomes are travelling towards the cell nucleus, the pH starts to drop. Matrix 2 (M2) proteins are transmembrane proteins that transport protons to the interior of the virion. This acidification helps the release of vRNPs from M1. Amantadine stops this process by blocking the interior channel of the tetrameric M2 helix bundle (Sansom & Kerr, 1993). The use of the drugs has been limited because of the rapid emerge of drug resistance and the transmissibility of drug-resistant viruses. In the 2005-2006 U.S. influenza season for example, 90.5% of the H3N2 and 15.5% of the H1N1 were reported resistant to the drug (Deyde et al., 2007) and the substitution with asparagine at the serine 31 was predominantly found in those isolates. Along with S31N, mutations at the 26, 27, 30, and 34 positions were also responsible for drug resistance. The locations of these mutations and the binding sites of rimantadine and amantadine are shown in Figure 22. Also side effects on the central nervous system were reported (Pielak et al., 2009).

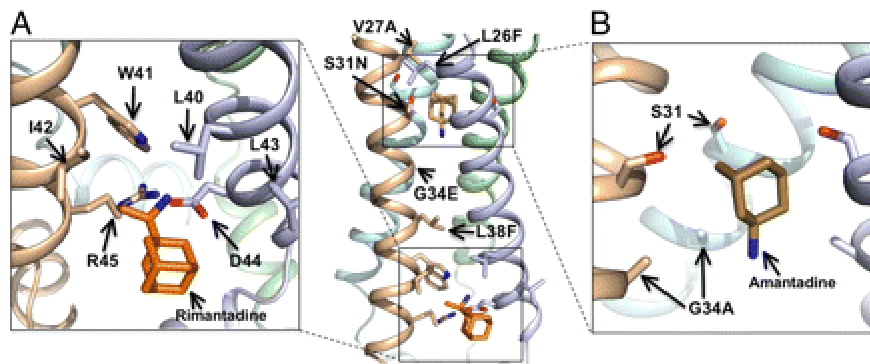


Figure 22. Proposed binding sites of rimantadine and amantadine (Pielak et al., 2009)

Rimantadine also acts on the M2 channels and is thought to bind to four equivalent lipid-facing pockets near to the Trp41 gate. It locks the channel to a closed state ignoring the pH drop. It is shown that rimantadine resistance occurs when serine 31 is mutated (Figure 23). The mutation destabilizes the helix-helix assembly and disrupts the drug pocket, whereby the drug affinity is dramatically reduced (Pielak et al., 2009).

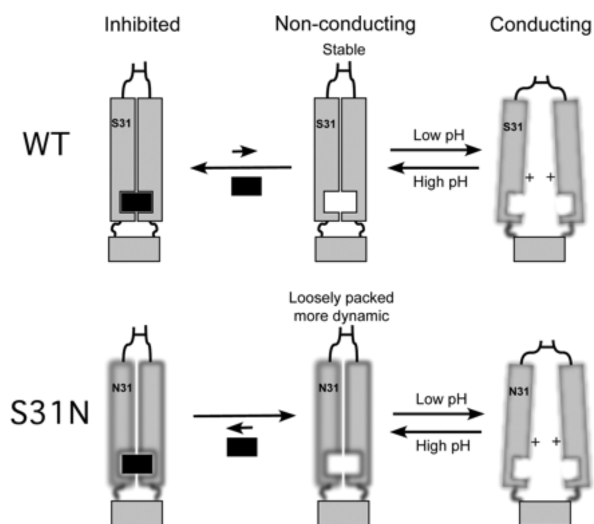


Figure 23. Disruption of the binding pocket used by rimantadine (Pielak et al, 2009)

The amino acid residues in the pockets are highly conserved. They are important for function and mutations such as D44A show much lower proton conductance compared to the wild type (Pielak et al., 2009).

Neuraminidase inhibitors

Another class of antiviral drugs against influenza is neuraminidase inhibitors (zanamivir and oseltamivir). They are also effective against influenza B and interfere with the

release of progeny influenza viruses from the infected host cells. This stops the virus from spreading in the respiratory tract (Moscona, 2005). The drugs are also found to be well tolerated and both prophylactically and therapeutically active against influenza. (De Clerq, 2006).

NA inhibitors were designed on the basis of X-ray crystallography of N2-type NAs (N2 and N9). Zanamivir is a drug based on sialic acid, but has a guanidino group instead of the 4-hydroxyl group. The drug binds to the active sites of the neuraminidase proteins and prevents interaction with sialic acid. The active site contains 19 conserved amino residues, 8 of which are catalytic (R118, D151, R152, R224, E276, R292, R371 and Y406) and 11 structural residues (E119, R156, W178, S179, D/N198, I222, E227, H274, E277, N294 and E425) (Colman et al., 1993). Because the NA has a higher affinity for the drug than for sialic acid, cleavage of HA is effectively inhibited (Das et al., 2010). Zanamivir has a low bioactivity and has to be administered by inhalation in order to be effective (Hurt et al., 2006).

Later oseltamivir, which contains a lipophilic pentyl ether chain, was developed as a prodrug. It can be administered orally, which makes it favorable over zanamivir. Oseltamivir is converted to its active form by the liver. It takes about 3-4 hours before the maximum drug levels are present in the respiratory tract (Hurt et al., 2006).

The virus has developed two distinct mechanisms to gain resistance against neuraminidase inhibitors. First, mutations in NA can lead to a reduced affinity of the drug to the active site, which allows the virus to cleave the sialic acid. A second mechanism is caused by mutations in HA. The mutations alters the affinity between HA and its receptor. If the affinity is low enough, NA can still cleave the bond although its activity is strongly reduced by the drug (Govorkova et al., 2013)

2.5.2 Host Cell Targeting Drugs

To overcome the problem of drug resistance, a lot of research is now focused on modulating cellular pathways to fight viral infections. This chapter contains two examples on how this can be achieved.

vATPase inhibitors

Cellular vacuolar ATPases (vATPase) are multisubunit proteins with an important function in the acidification of the endosomes. They pump protons across the endosomal membrane and the pH change causes the viral and endosomal membrane to fuse. By targeting vATPase, the virus can be stopped before membrane fusion occurs. Saliphenylhalamide (SaliPhe) is an attractive drug, because its cytotoxicity is much lower than other known vATPase inhibitors. SaliPhe has an IC_{50} of 1.74 ± 0.2 µM, while other drugs have IC_{50}'s at (sub)nanomolar scale which make them unattractive for antiviral use. Antiviral activity of SaliPhe ranges from 28 nM to 206 nM, which is significantly lower than the IC_{50} value in non-infected cells (Müller et al., 2011).

Table 4. Compound testing (Müller et al., 2011)

Compound	IC_{50} (MDCK)	IC_{50} (A549)
Saliphenylhalamide	1.74 ± 0.2 µM	1 ± 0.19 µM
Bafilimycin A1	2.9 ± 0.1 nM	1.3 ± 0.2 nM
Concanamycin A	0.56 ± 0.09 nM	0.2 ± 0.02 nM
Archalozid B	1.4 ± 0.1 nM	0.16 ± 0.02 nM

Further testing on different IAV strains has shown that SaliPhe was the most effective drug, but it is about 6 times less effective against H5N1 viruses. This effect has been described before and may rely in the fact that H5N1 viruses are less dependent to HA activation through endosomal pH changes (Reed et al., 2010).

SaliPhe is also shown to rescue mice from a lethal IAV, while Bafilimycin A1 did not show any protection at concentrations that were toxic for spleen and liver (Müller et al., 2011).

CDK inhibitors

Cyclin-dependent Kinases (CDKs) are required for replication of many viruses which replicate in dividing cells *e.g.* adeno- and papillomaviruses. It has been shown CDKs are also required for the replication of viruses that can replicate in non-dividing cells *e.g.* herpes simplex virus (HSV), human immunodeficiency virus type 1 (HIV-1) and influenza (Schang, 2002 & Zhang et al., 2010). They are small (30-40 kD)

heterodimeric protein kinases which have a function in regulating the cell cycle, transcription and mRNA processing. Kinases change the activity of other proteins by adding phosphate groups. Because the concentrations of CDKs are constant during cell cycle, CDKs require interaction with cyclin subunits in order to get phosphorylated and activated by CDK-activating-kinase (CAK). Most CDKs can bind different cyclins which determine the activity and substrate specificity. As seen in Figure 24, cyclins are only present during a particular stage in the cell cycle where they promote/inhibit the CDK's. To prevent the cells from going 'backwards' in the cell cycle, cyclins get marked for degradation by proteasomes. This is done by ubiquitin-protein ligases SCF or anaphase-promoting complex (APF/C) which polyubiquitinylate the cyclins (Lodish, 2012).

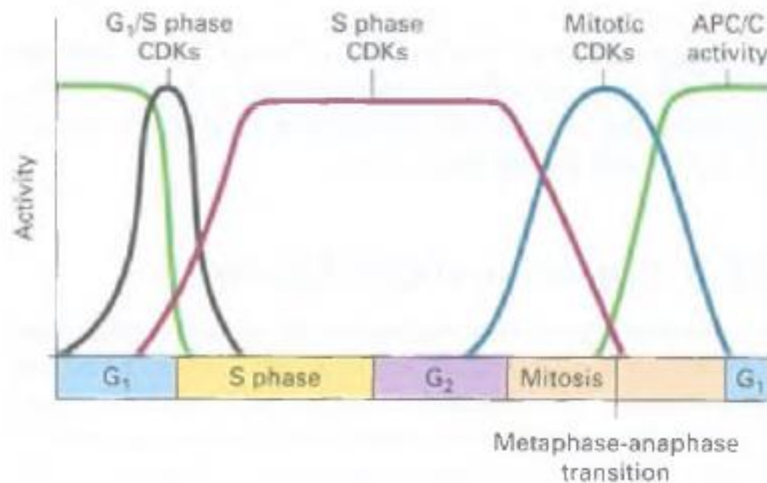


Figure 24. Activity of different CDKs regulated by cylins (Lodish, 2012)

Pharmacological CDK inhibitors (PCI) compete with ATP and bind to the ATP binding pockets of CDK's. Flavopiridol is classified as a 'wide spectrum' PCI, because it inhibits CDKs 1, 2, 4, 9 and possibly 7. It is thought that the inhibition of cellular transcription by flavopiridol is a direct consequence of inhibition of CDK9 (Zhang et al., 2010).

The viral polymerase can bind to hyperphosphorylated cellular RNA polymerase II (Figure 25). In normal cells, hyperphosphorylation of serine 2 located at the carboxy-terminal domain (CTD) is done by cyclin T/CDK9 (P-TEFb) and stimulates the transcription elongation which is stalled by negative elongation factors 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole sensitivity-inducing factor (DSIF) and negative

elongation factor (NELF). Elongation is resumed after phosphorylation of DSIF and dissociation of NELF (Zhang et al., 2010).

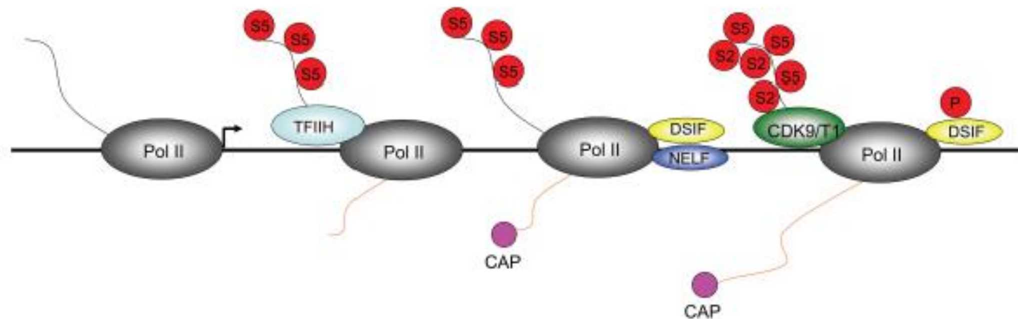


Figure 25. CDK9 function in normal transcription (Zhang, 2010)

A study by Zhang et al. (2010) reported that depletion of cyclin T1 greatly inhibited viral transcription and replication and that overexpression of cyclin T1 expedited the formation of new viruses. They suggested that it is not likely that the kinase activity of CDK9 is involved in the virus transcription process, but that the ability of CDK9 to associate with vRNP is responsible (Figure 26). Free CDK9 can bind with vRNP in the absence of cyclin T1, but in order to guide the vRNP to host RNA polymerase II, CDK9 needs its presence (Zhang et al., 2010).

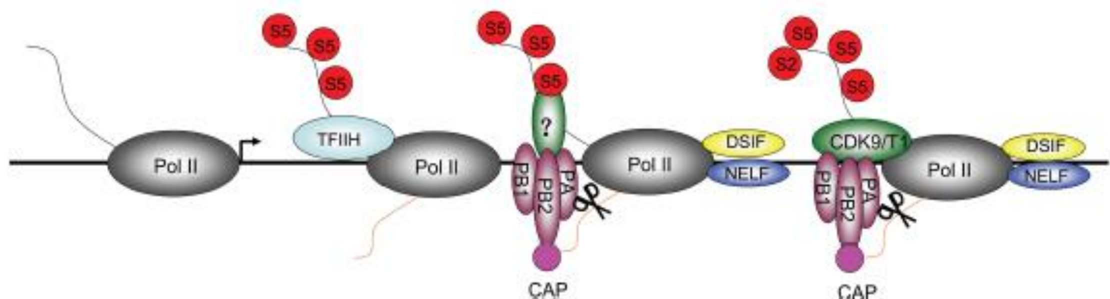


Figure 26. Proposed mechanism of CDK9 for viral transcription (Zhang, 2010)

The mechanism that Zhang et al. (2010) proposed greatly helps RdRP to find active RNA polymerases and steal the cap and thus enhances replication of the virus (Zhang et al., 2010).

3 Materials and Methods

3.1 Compounds, Cells and Viruses

An overview of the compounds, cells and viruses used in the study at hand is shown in Table 5.

Table 5. Compounds, cells and viruses

Compounds			
MK-2206 (Selleck)	ABT-263 (Selleck)	Perifosine (Selleck)	Obatoclax (Selleck)
Akt inhibitor VIII (Sigma-Aldrich)	GDC-0068 (ChemieTek)	SaliPhe (Jef De Brabander)	
Cells from ATCC			
MDCK	A549	NCI-H1666	NCI-H1703
NCI-1437	Calu-1	NCI-460	NCI-H2126
Influenza Virus strains			
A/Helsinki/P14/2009	A/Helsinki/552/2013	A/Helsinki/629/2013	A/Helsinki/551/2013
A/Helsinki/628/2013	A/Helsinki/P15/2009	A/Helsinki/568/2013	A/Helsinki/678/2013
A/Helsinki/526/2013	A/Helsinki/598/2013	A/Helsinki/Vi1/2009	A/Helsinki/668/2013
A/Helsinki/604/2013	A/Helsinki/Vi2/2009	A/Helsinki/P18/2009	A/Helsinki/Vi3/2009
A/Helsinki/543/2013	A/WSN/33	A/PR/8/34	A/Sydney/5/1997
A/Udorn/72	A/Chicken/Nigeria/BA211/2006	A/Anhui/01/2013*	
* provided by the WHO Collaborating Centre for Reference and Research on Influenza at the National Institute for Medical Research, London, UK			

All compounds were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to 10 mM stock solutions and stored at -80°C until use. Madin-Darby canine kidney (MDCK) and A549 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM , Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine (Lonza), and 50 U/mL penicillin–streptomycin mix (PenStrep; Lonza). Human non-small lung cancer lines (NCI-H1666, NCI-H1703, NCI-1437, Calu-1, NCI-460, NCI-H2126 from ATCC) were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS, 2 mM L-glutamine, and 50 U/mL PenStrep. All cells were grown at 37°C and 5% CO2.

Influenza A virus strains were propagated and titered in MDCK cells. Sequence of HA of A/Helsinki/552/2013 was verified by Sanger sequencing.

3.2 Compound Efficacy Testing

The compound efficacy testing was performed in 96-well plates in MDCK, A549 and NCI-H1666 cells. Typically, cells were seeded in 100 µl of appropriate cell growth medium and grown for 24 h to reach 95% confluence. The growth medium was changed to appropriate virus growth medium (VGM) containing 0.2 % bovine serum albumin (BSA, Sigma-Aldrich), 2 mM L-glutamine, and 1 µg/ml (MDCK) or 0.4 µg/ml (A549 and NCI-H1666) L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin (TPCK)-trypsin (Sigma-Aldrich) in DMEM (A549 and MDCK) or RPMI 1640 (NCI-H1666). The compounds were added into the medium, DMSO was added to the control wells. The cells were infected with viruses at multiplicity of infection (moi) 0.04-5 or non-infected (mock). Cell viability was measured using Cell Titer Glo assay (CTG; Promega) at 24, 48 or 72 h post infection. Luminescence was read with PHERAstar FS plate reader (BMG Labtech). For each virus-compound pair the quantitative activity and toxicity score (ATS) was calculated as described in Denisova et al. (2012). ATS varies between -100 and +100, where negative values indicate excessive toxicity and highest positive values indicate most potent compounds.

3.3 Virus Titration

The cells were non-treated or treated with the MK2206 at noncytotoxic concentrations and infected with A/Helsinki/P14/2009 at moi 0.1. Supernatants were collected at 48 h post infection, 10-fold diluted in DMEM-based VGM and added to MDCK cells on 6-well plates. After 1 h the cells were overlaid with medium containing 1.2% Avicel (FMC Biopolymer), 0.2% BSA, 50 units/mL PenStrep, 2 mM L-glutamine, and 1 µg/mL TPCK-trypsin in minimal essential medium and incubated for 2 days. The cells were fixed with 4% formaldehyde in phosphate buffer solution (PBS) for 3 h and stained with 0.1% crystal violet in 1% methanol, 20% pure ethanol, and 3.6% formaldehyde. Virus titers were determined by calculating plaque forming units (PFU) (clear spots) for sample dilutions and expressed as PFU/mL for the sample.

3.4 Quantitative Real-time PCR

Total cellular RNA was isolated from NCI-H1666 cells non-infected or infected with A/Helsinki/p14/2009 strain using the RNeasy Plus Mini Kit (Qiagen). The RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. RT-qPCR was performed with an ABI PRISM 7500 Sequence Detection System. The TaqMan primers and probes (18S: Hs99999901_s1, IFN-β: Hs01077958_s1, and IL-29: Hs00601677_g1) and the TaqMan® Fast Advanced Master Mix from Applied Biosystems were used. The SYBR Green Master Mix (Applied Biosystems) was applied for the SYBRGreen runs. A mix of the following GAPDH oligomers (Oligomer Oy) was used: huGAPDH_r with sequence 5'-TGA CCT TGG CCA GGG GTG CT-3' and huGAPDH_f with sequence 5'-GGC TGG GGC TCA TTT GCA GGG-3'. The sequences of the NS1 oligomers (Sigma Aldrich) were: 5'-AGA AAG CTC TTA TCT CTT G (the reverse sequence) and 5'-GAA ATG TCA MGA GAC T (the forward sequence). The RT-qPCR data was processed and quantified as described by Ohman et al. (2010) and the results are represented as relative units (RU).

3.5 Fluorimetry

The fluorescence spectra were recorded using a Carry Eclipse fluorescence spectrophotometer (Varian/Agilent). A Hitachi U-3900 spectrophotometer was used for the absorbance measurements. The absorbance and fluorescence spectra of MK2206 were measured in PBS. The pH titrations were done using a series of buffers based on the Hydrion Buffer Chemvelopes (Micro Essential Laboratory, USA).

3.6 Automated Image Acquisition and Image Analysis

The MDCK cells were grown in 96-well plates and treated with different concentrations of MK2206. In another experiment MDCK cells were treated with MK2206 (10 μM), and its combinations with saliphenylhalamide (0.4 μM), NH4Cl (20mM) or obatoclax (0.4 μM), or non-treated. Cells were fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature and imaged with modular epifluorescence microscope ScanR

(Olympus), which is designed for fully automated image acquisition. Long working distance 40x (NA 0.6) and 20x (NA 0.45) objectives were used for image acquisition. A standard filter set for DAPI (ex. 377/50 nm) was used for visualizing the autofluorescent MK2206 within the cells. Every sample was made in two replicates and for each well, 9 different fields of view were analysed using the ScanR Analysis software (Olympus). An automated background correction was applied for the DAPI channel. The autofluorescent vesicles (size between 1-20 pixels) visible in the DAPI channel were defined as the main objects, using edge detection. These objects were counted, and their area, circularity and mean intensity were measured. The images were manually controlled for quality and for potential errors. The average and standard deviation, for the number of objects as well as for the mean intensity of the objects, was calculated for each sample.

3.7 Immunofluorescence

NCI-H1666 cells were grown on cover glasses in 6-well plates. Cells were non- or MK2206-treated (10 µM) and mock- or A/Helsinki/P14/2009 -infected (moi 30) on ice for 1h. Cells were washed twice with ice-cold VGM, overlaid with pre-wormed media with or without compound, and incubated at 37°C. At 1, 2, and 4 h post infection cells were fixed with 4% paraformaldehyde (in PBS) for 20 min at room temperature. PBS with 1% BSA and 0.1% Triton X-100 was used for blocking and permeabilization of the fixed cells and for dilution of antibodies. Viral NP and M1 were detected with rabbit polyclonal antibodies (1:1000; from Ilkka Julkunen, Finland); the secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:2000; Invitrogen). Nuclei were counterstained with DAPI. Images were captured with a Nikon 90i microscope and processed with NIS Elements AR software.

3.8 Phospho-protein, Transcription and Cytokine Profiling

NCI-H1666 cells were non- or MK2206-treated (10 µM) and mock- or virus-infected with A/Helsinki/P14/2009 at moi 3. For phospho-protein profiling media was discarded after 4 and 12 h of infection, cells were lysed and phosphorylation profiles of 43 kinases and 2 related substrates were analysed using human phospho-kinase array

according to the manufacturer's instructions (R&D Systems). For cytokine profiling the supernatants were collected after 24 h and 26 different cytokines were assayed using the human cytokine array panel A according to the manufacturer's instructions (R&D Systems). The membranes were exposed to X-ray films and the films were scanned. Each image was analysed with ImageJ software (NIH, USA). For the whole genome gene expression analysis cells were collected after 8 h and total RNA was isolated using RNAeasy plus mini kit (Qiagene). Illumina TotalPrep 96 RNA Amplification Kit was used to amplify and label the RNA. Human HT-12_V3_expression arrays were processed using Limma and Beadarray packages from Bioconductor suit. The raw data was exported from GenomeStudio and log2 transformed prior to quantile normalization. We applied IlluminaHumanv3.db annotation data freely available from Bioconductor in order to map Illumina probes to known genes and annotate the probes. To rank the genes in order of evidence for differential expression we computed moderated t-statistics and log-odds of differential expression by Empirical Bayes estimator.

3.9 Multi-passage Experiment

NCI-H1666 cells were infected with A/Helsinki/P14/2009 at moi 0.1 in presence or absence of MK2206. At 48 h post infection 20 µl of media was used for the next passage in presence or absence of the inhibitor. During passaging we increased MK2206 concentration from 0.1 to 10 µM. Fifteen passages were performed and the virus titers were determined by plaque assay.

3.10 Ethics

Virus experiments were carried out under BSL-2 conditions and in compliance with regulations of the University of Helsinki (permit No 21/M/09) and under BSL-3 conditions and in compliance with regulations of the Centre de Recherche Public de la Sante/Laboratoire National de Santé.

4 Results and Discussion

Four Akt inhibitors were tested on their ability to rescue MDCK cells from infection with A/Helsinki/p14/2009 (moi 3). The cell viability testing (Figure 27) done at 48 hpi showed that only MK-2206 was successfully inhibiting virus-mediated cell death. The positive ATS value indicated that the drug rescues cells at concentrations without excessive cytotoxicity.

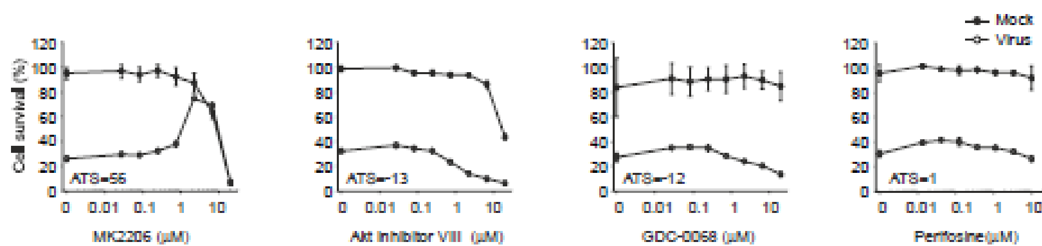


Figure 27. Compound efficacy testing of four Akt-inhibitors shows only MK-2206 was active against A/Helsinki/p14/2009 infection.

Cell viability of mock and MK-2206 treated (3 μM) MDCK cells showed that the inhibition of the virus was dependent on the moi at 48 hpi. At moi 1 and higher the drug wasn't able to rescue cells anymore (Figure 28).

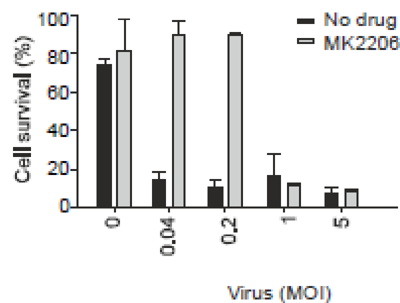


Figure 28. Inhibition of the virus by MK-2206 was dependent on the moi.

These results both suggested that MK-2206 could be used as an antiviral against influenza A/Helsinki/p14/2009 at non-cytotoxic concentrations.

As seen in Figure 29, the plaque assay of mock and MK-2206 treated (3 μm) MDCK cells clearly showed that the drug was able to reduce virus production at moi 0.2. After treating the cells with MK-2206, the virus titer dropped from 1.1×10^7 plaque forming units PFU to zero.

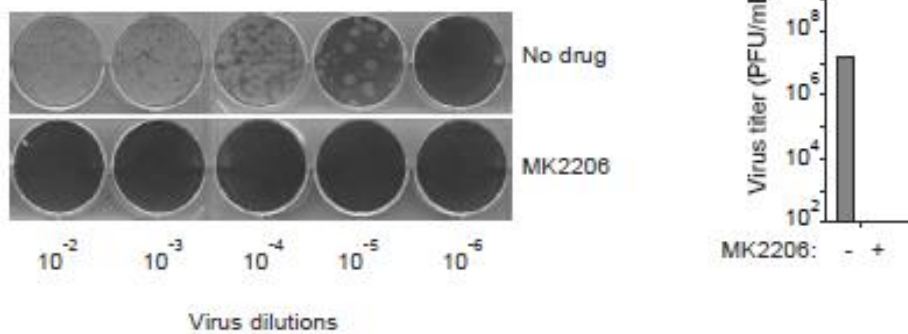


Figure 29. Treatment with MK-2206 brings down the virus titer to zero.

The effect of MK-2206 was also tested on other influenza A(H1N1)pdm09 isolates to check if the antiviral effect is not limited to A/Helsinki/p14/2009. Cell viability results (Figure 30A) showed that 17 out of 18 isolates were sensitive to the drug, whereas one, influenza A/Helsinki/552/2013, was resistant to the treatment.

After comparing full-genome sequences of the A(H1N1)pdm09 viruses that were used in the experiment, an unique A26T mutation was found in the haemagglutinin (HA) of influenza A/Helsinki/552/2013. This mutation can probably be associated with MK-2206 resistance (Figure 30B).

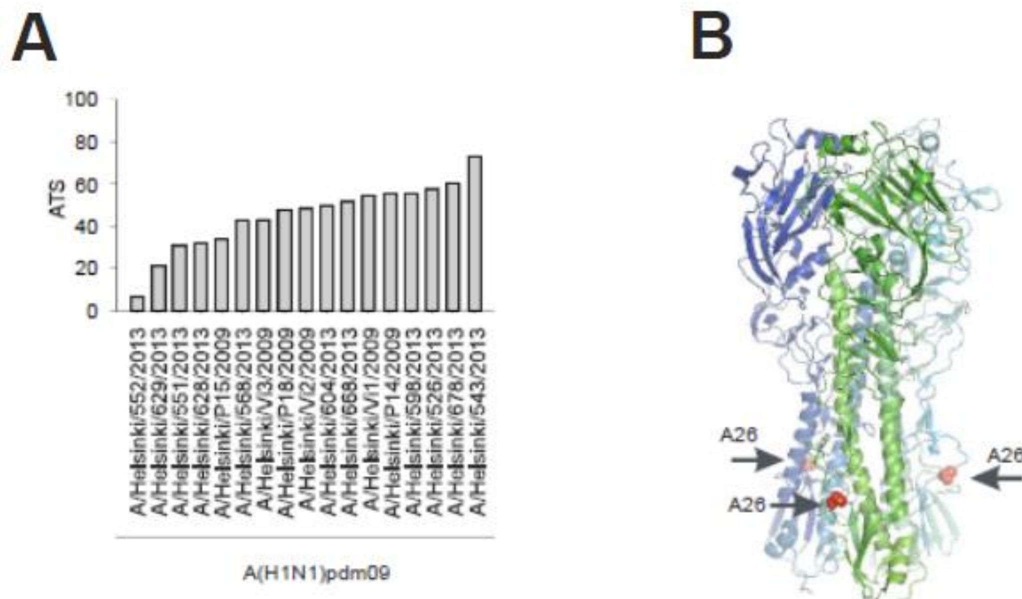


Figure 30. **A.** Susceptibility of different influenza A(H1N1)pdm09 isolates was high, except for influenza A/Helsinki/552/2013. **B.** A26T mutation in HA that was probably responsible for resistance of A/Helsinki/552/2013 to MK-2206.

Also seasonal (H1N1 and H3N2) and potentially pandemic (H5N1 and H7N) IAV strains were tested on their sensitivity regarding MK-2206, but as shown in Figure 31 they were resistant to the treatment. H5N1 and H7N9 were tested by our collaborators, because BSL-3 conditions are needed to be allowed to work with them.

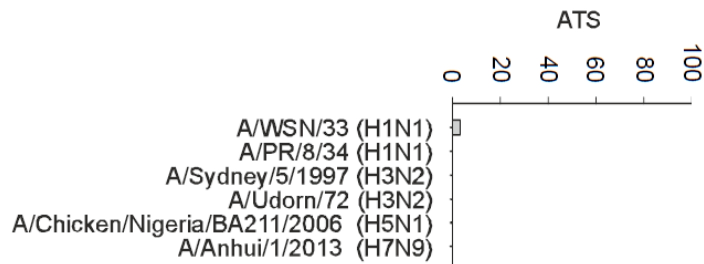


Figure 31. Seasonal and potentially pandemic influenza A viruses were resistant to MK-2206.

These viruses have a different structure of HA compared to A(H1N1)pdm09 viruses. This also suggests that resistance to MK-2206 might be due to a different HA structure and activity.

To find a suitable human cell line to use with our assays, different untreated human lung cancer cell lines were tested for their susceptibility to influenza A/Helsinki/p14/2009 (at moi 2). Results of cell survival at 48 hpi are shown in Figure 32A. NCI-H1666 was found to be very permissive for the virus and had a similar response to MK-2206 treatment as MDCK cells (Figure 32B). A plaque assay (Figure 32C) also showed a significant drop of the virus titer (10 μM was used). NCI-H1666 will be used for assays that require human cell lines.

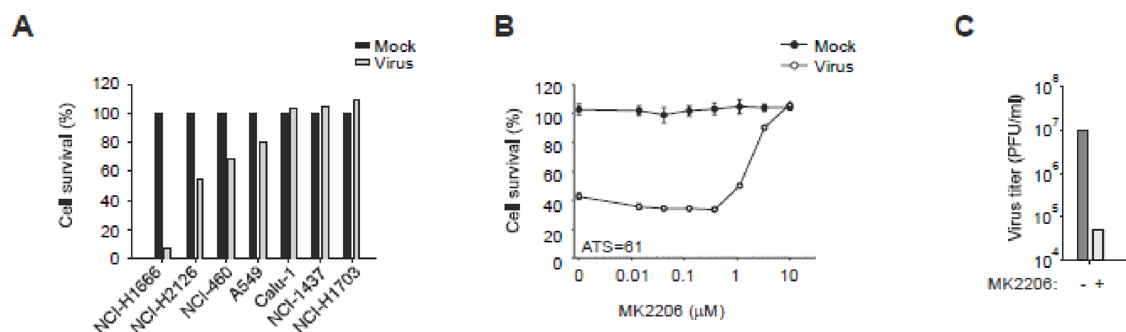


Figure 32. **A.** NCI-H1666 was most permissive to influenza A/Helsinki/p14/2009. **B.** NCI-H1666 had a similar response to AIV compared to MDCK cells **C.** A large drop in virus titer was seen after treatment with MK-2206.

A time-of-compound addition was performed to see when the drug has the largest effect and at what stage of replication the virus is stopped. When the drug was added within 2 hpi, the cells were protected against cell death measured at 24hpi (Figure 33). MK-2206 is most likely inhibiting early steps of replication.

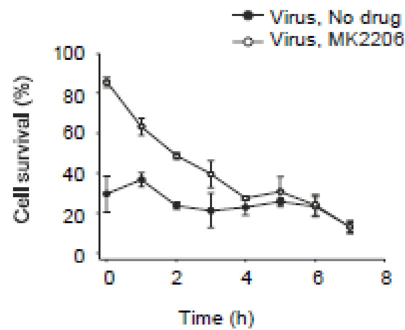


Figure 33. Time-of-compound addition showed that the virus was blocked in an early stage.

To further analyse where the virus was stopped, ABT-263 was used. ABT-263 accelerates virus mediated cell death and is triggered by the release of viral RNA into the cytosol (Kakkola et al., 2013).

Figure 34A shows that more cells are rescued at higher concentrations of MK-2206. MK-2206 is thus inhibiting the virus before it enters the cytoplasm. Figure 34B illustrates that the concentration of ABT-263 had no effect on the efficacy of MK-2206. The drop at high concentrations was caused by cytotoxic effects of ABT-263.

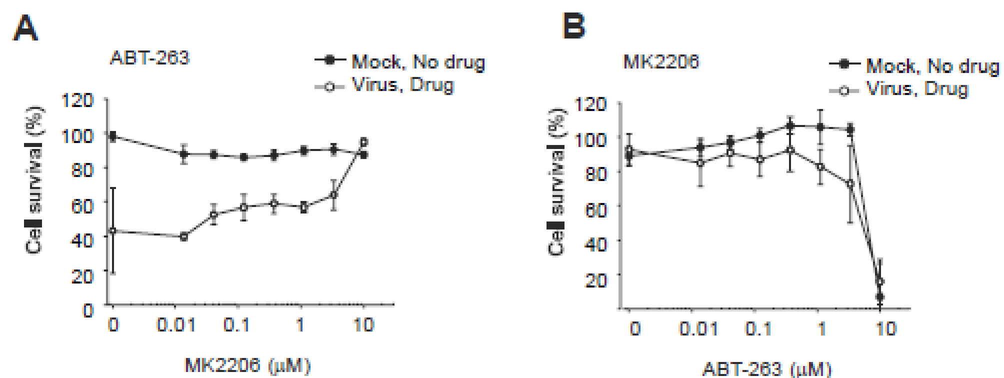


Figure 34. **A.** MK saves infected cells from ABT accelerated virus mediated cell death. **B.** Concentration of ABT-263 has no effect on the efficacy of MK-2206.

To confirm that MK-2206 inhibits viral replication, RT-qPCR and immunofluorescence microscopy were performed. The results of RT-qPCR showed (Figure 35) that MK-2206 treated cells clearly had a lowered transcription of viral RNA (like NS1), while there was no change in the amount of transcription of the house-keeping genes (like GAPDH). Again, this confirmed that the virus is inhibited at early steps of replication.

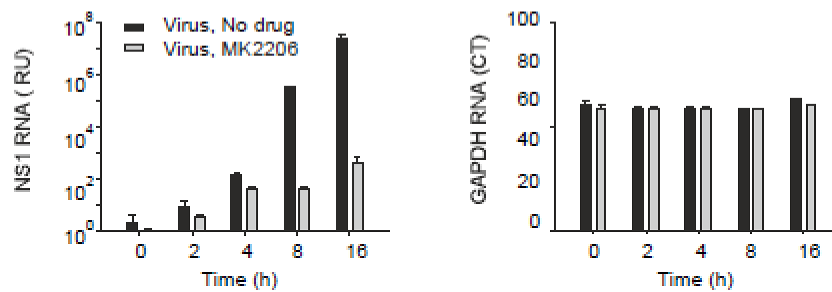


Figure 35. Transcription of viral protein (NS1) is inhibited, while the transcription of housekeeping protein (GAPDH) is unaffected.

Immunofluorescence confirmed that viruses do not enter the nucleus of the cells. M1 and NP are viral proteins that are colored green in the combined images (Figure 36). As they were not visible inside the nuclei (blue color), they must have been trapped in the endosomes.

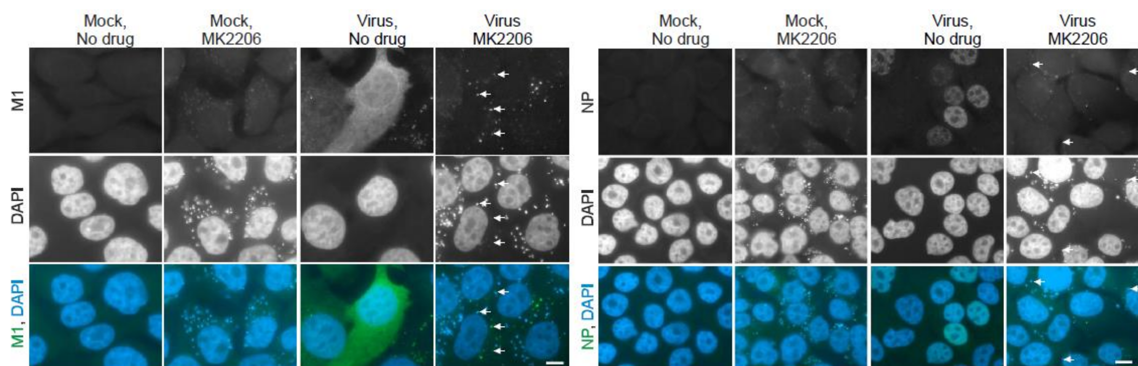


Figure 36. Immunofluorescence with M1 and NP antibodies showed that viral proteins were trapped in the endosomes after treatment with MK-2206. Scale bars are 10 µm.

Another interesting fact was that MK-2206 is autofluorescent. In the pictures, small fluorescent particles were visible when MK-2206 is added, independent of the used antibody.

Our collaborators further investigated the fluorescent properties of the drug by scanning the absorbance and fluorescence spectra. As seen in Figure 37B, fluorescence peaked at 456 nm and was highly dependent on pH. Maximal fluorescence was reached between pH 5.7-6.0.

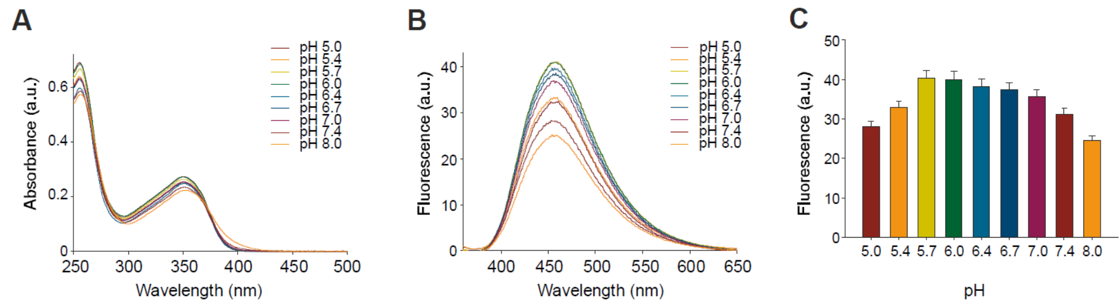


Figure 37. Fluorometric data of MK-2206 showed that **A**. absorbance peaks at 259 and 354 nm. **B**. fluorescence peaks at 456 nm. **C**. Fluorescence is dependent on pH and peaks at pH 5.7-6.0.

Fluorescence microscopy (Figure 38) showed that MK-2206 (10 µM) accumulated in vesicles in the cytoplasm of non-infected cells. The fixing and imaging was done at 24 hpi.

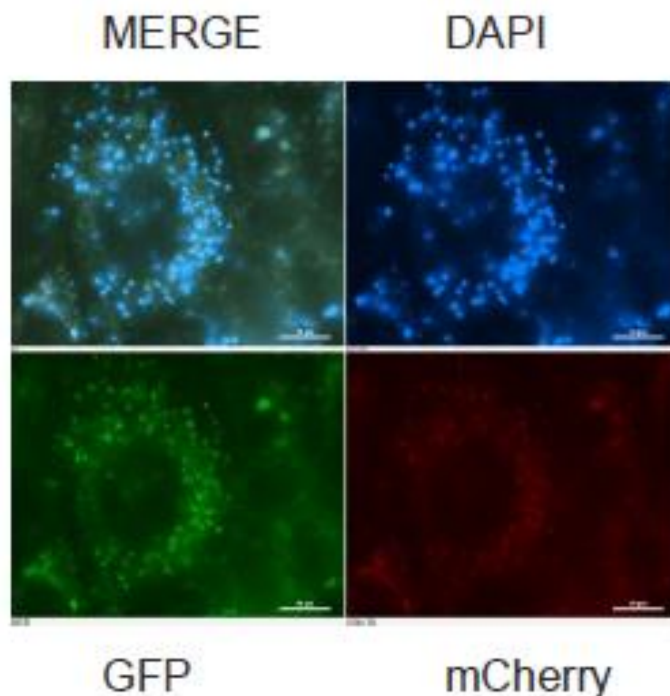


Figure 38. MK-2206 was visible in the cells as autofluorescent vesicles (scale bars are 10 µm).

The amount of autofluorescent objects was measured by automated image acquisition and image analysis from the DAPI channel at 4 hpi . As seen in Figure 39A, the amount of fluorescent vesicles rose when higher concentrations of MK-2206 are used. When compounds were added that inhibit the acidification of the endosomes (0.4 µM SaliPhe or Obatoclax, 20 µM NH_4Cl), the amount went down (Figure 39B). Probably the compound did not get trapped in the endosomes anymore, because the N-atoms of MK-2206 were not protonated anymore at the higher pH.

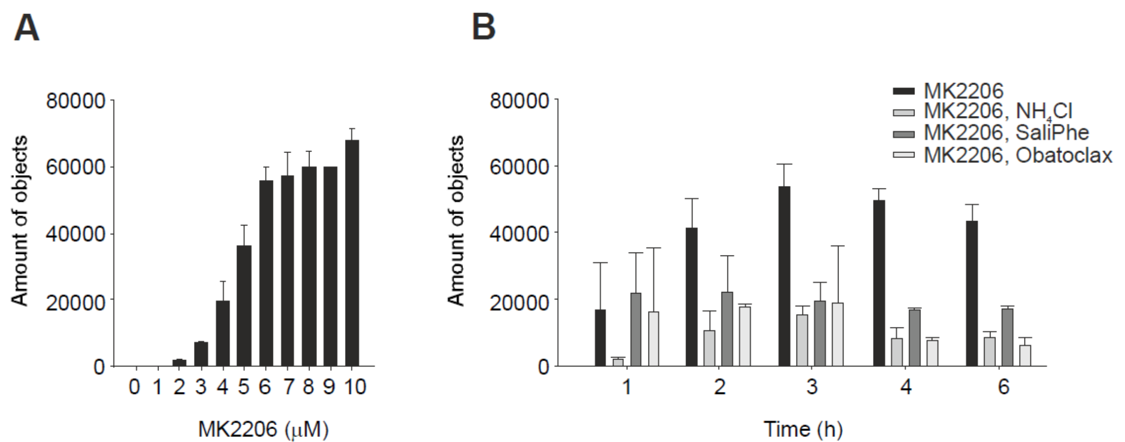


Figure 39. **A.** The number of fluorescent vesicles went up with rising concentrations of MK-2206. **B**. When compounds were added that inhibit the acidification of the endosomes, the drug was no longer held inside the endosomes.

In following tests the cells were treated with 10 µM MK-2206 and infected with moi 3. Cytokine profiling (Figure 40) revealed that MK-2206 lowered the production of cytokines in infected cells, including G−CSF, GM−CSF, GRO-α, IL1A, IL1RA, IL6, IL8, CXCL10, MIF, and CCL5. MK2206 is thus inhibiting several signalling pathways, including Akt-signaling and preventing the development of immune responses. As the levels between non infected and infected MK-2206 treated cells approximately remained the same, it can be concluded that MK-2206 is stopping the virus from releasing its vRNA into the cytoplasm.

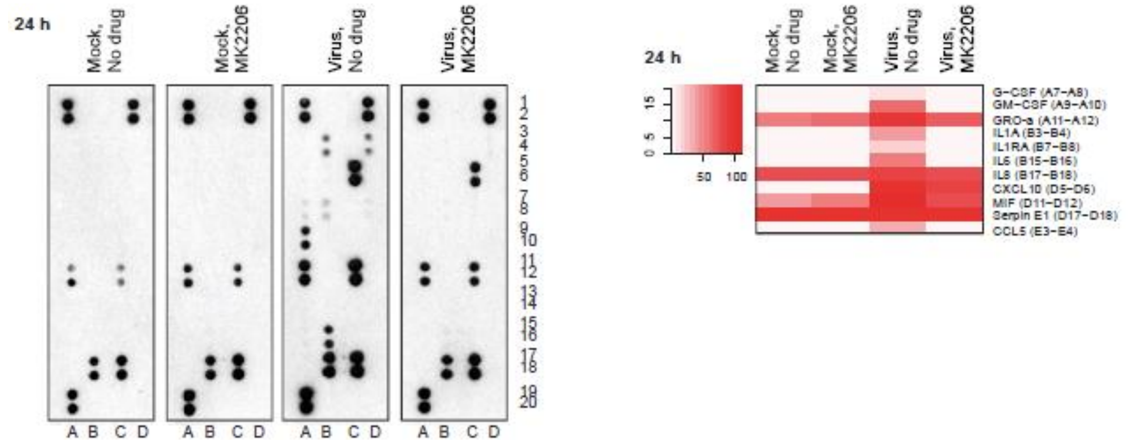


Figure 40. Membrane and heatmap of cytokine profiling at 24 hpi.

Phospho-protein profiling was done on MK-2206 treated cells at different timepoints. At 4 hpi (Figure 41), phosphorylation of EGFR (Y1086) was clearly lowered.

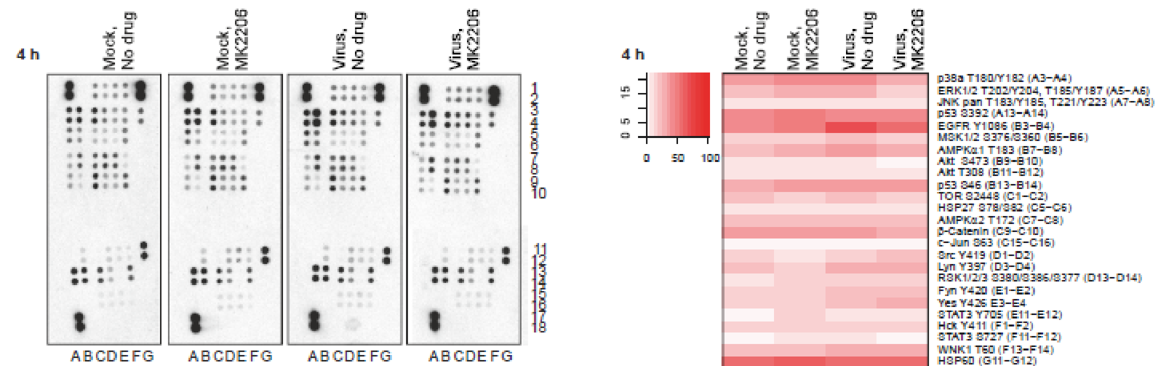


Figure 41. Membrane and heatmap of phospo-protein profiling at 4 hpi.

At 12 hpi (Figure 42), phosporylation of ERK1/1 (T202/Y204), JNK (T183/Y185 and T221/Y223), MSK1/2 (S376/S360), AKT (S473), HSP27 (S78/S82), c-Jun (S63), Lyn (Y397), RSK1/2/3 (S380/S386/S377) was prevented and de-phosphorylation of p53 (S46/S392), AMPKα1 (T183), and β-Catenin was inhibited.

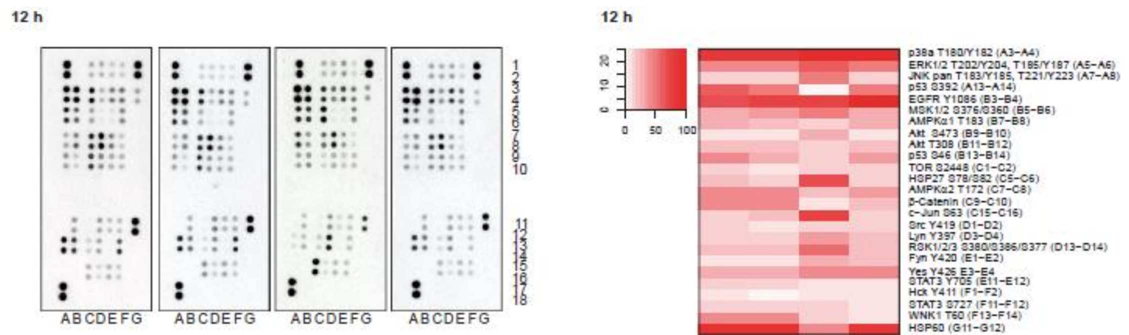
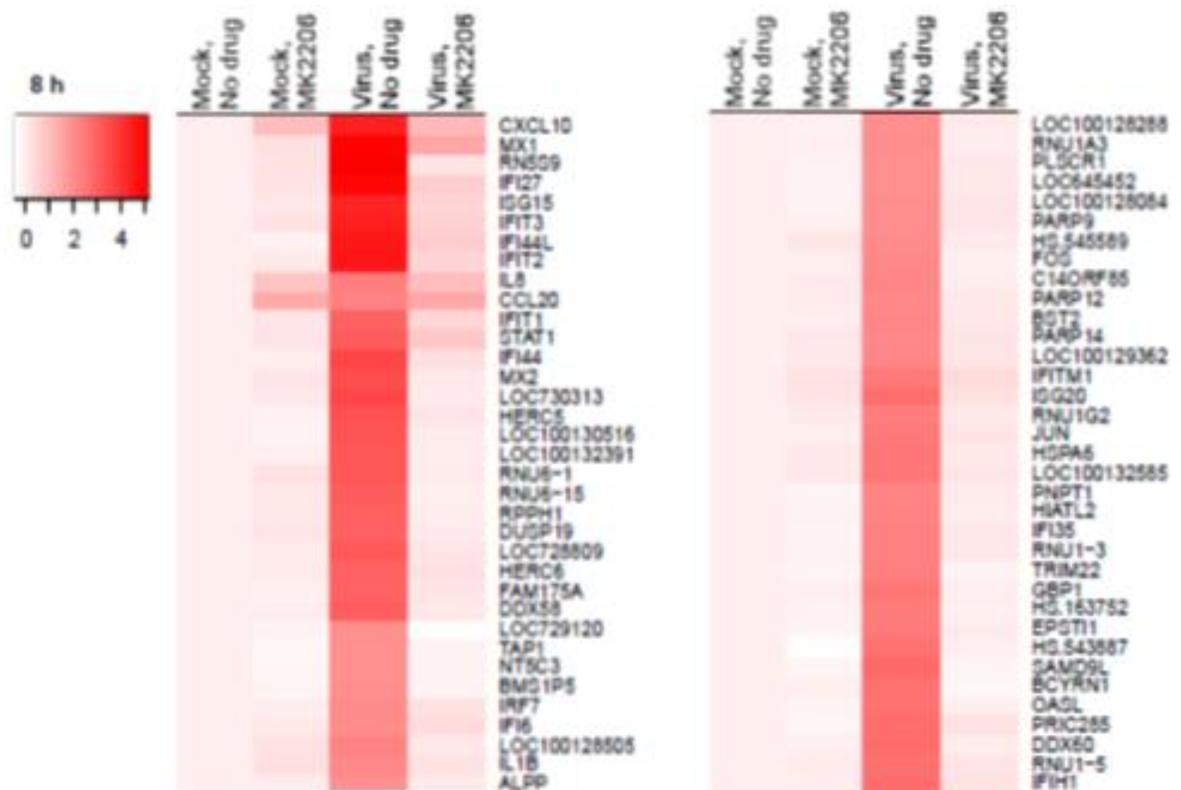


Figure 42. Membrane and heatmap of phospo-protein profiling at 12 hpi.

Whole-genome expression was done by FUGU and data analysis showed that cellular genes which were upregulated by A/Helsinki/p14/2009 infection returned to their original values. The heatmap (Figure 43) contains genes with a fold change of >2 (upregulated) and <-2 (downregulated). Out of those 70 genes, 23 belong to immune response pathways. Analysis also showed that 8 genes (LPIN1, RASD1, DHCR7, HMGCR, SC4MOL, HMGCS1, SQLE, ABCA1) involved in the lipid biosynthesis were regulated by MK-2206 in non-infected cells. This finding suggests that MK-2206 might also play a role in the lipid metabolism.

Figure 43. Whole genome gene expression profiling heatmap at 8 hpi.



To investigate if A/Helsinki/p14/2009 is able to become resistant to MK-2206, a multi-passage experiment was done on NCI-H1666 cells (Figure 44). To determine the virus titer, MDCK cells were used.

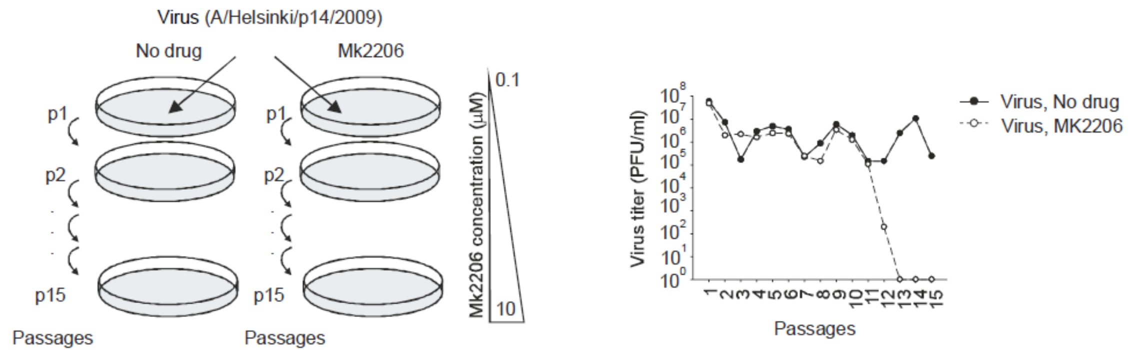


Figure 44. Multi-passage experiment clearly showed that the virus did not develop resistance to MK-2206.

The virus did not become resistant to MK-2206 as virus titers only dropped after passage 11 (48 h per passage). This corresponds to a concentration of 2 µM which is in normal circumstances just enough to inhibit the infection. This indicates that targeting host factor might be a successful strategy to fight virus infections.

5 Conclusion

MK-2206 was found to neutralize influenza A(H1N1)pdm09 infections with an exception of A/Helsinki/552/2013 at early stages of replication. The results suggest that the virus is stopped within the endosomes and thus does not enter the cytoplasm. MK-2206 was also found to be autofluorescent with a maximum fluorescence at 456 nm and is probably involved in the lipid metabolism. A/Helsinki/p14/2009 was unable to gain resistance to MK-2206.

In the future, a lot of research has to be done to find drugs that are active against a broader scale of viruses. MK-2206 is only active against influenza A(H1N1)pdm09 and therefore is not the drug that many people hope for. Ideally a drug would target a mechanism that is shared by a lot of viruses, but therefore the replication of viruses has to be clearly understood.

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