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INFLUENZA VIRUS-HOST INTERACTIONS AND THEIR MODULATION BY SMALL MOLECULES

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ACADEMIC DISSERTATION

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To my family

CONTENTS

ABBREVIATIONS

APAF1	apoptotic protease-activating factor 1
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-w	Bcl-2-like protein 2
Bcl-xL	B-cell lymphoma-extra large
Bid	Bcl-2 homology domain 3 (BH3)-interacting domain death agonist
Bim	Bcl-2-interacting mediator of cell death
BUNV	Bunyamwera virus
CCL	chemokine (C-C motif) ligand
CPE	cytopathic effect
CPSF30	cleavage and polyadenylation specificity factor
CXCL	chemokine (C-X-C motif) ligand
GM-SCF	granulocyte-macrophage colony-stimulating factor
DENV	Dengue virus
DHODH	dihydroorotate dehydrogenase
DHX9	ATP-dependent RNA helicase A
EC_{50}	half-maximal effective concentration
Echo6	human echovirus 6
FLII	protein flightless-1 homologue
H2B	histone H2B
HA	hemagglutinin
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IC_{50}	half-maximal inhibitory concentration
IFN	interferon
IL	interleukin
IP	immunoprecipitation
IP-10	IFN-γ-induced protein 10
LRRFIP2	leucine-rich repeat flightless-interacting protein 2
M1	matrix protein 1
M2	proton channel
MAPK	mitogen-activated protein kinases
Mcl-1	myeloid cell leukemia-1
MeV	Measles virus
mTOR	mammalian target of rapamycin
NA	neuraminidase
NEP	nuclear export protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NP	nucleoprotein
NS1	non-structural protein 1
PA	polymerase acid protein
PB1 N10	N-terminally truncated version of the polypeptide from PB1 codon 40
PB1	polymerase basic protein 1
PB1-F2	protein encoded the +1 reading frame of the PB1 gene
PB2	polymerase basic protein 2
PBMC	peripheral blood mononuclear cells
PI3K	phosphoinositide 3-kinase
PSi	porous silicon
SFV	Semliki forest virus
SINV	Sindbis virus
siRNA	small interfering RNA
TBEV	tick-borne encephalitis virus
THCPSi	thermally hydrocarbonized porous silicon
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor alpha
TOLLIP	Toll-interacting protein
UACA	uveal autoantigen with coiled-coil domains and ankyrin repeats
VACV	Vaccinia virus
v-ATPase	vacuolar proton-ATPase

ORIGINAL PUBLICATIONS

This thesis is based on the following publications that are referred to in the text by their Roman numerals. Additional unpublished data will be presented.

I. **Denisova O.V.**, Kakkola L., Feng L., Stenman J., Nagaraj A., Lampe J., Yadav B., Aittokallio T., Kaukinen P., Ahola T., Kuivanen S., Vapalahti O., Kantele A., Tynell J., Julkunen I., Kallio-Kokko H., Paavilainen H., Hukkanen V., Elliott R.M., De Brabander J.K., Saelens X., Kainov D.E. (2012) Obatoclax, saliphenylhalamide and gemcitabine inhibit influenza A virus infection. *J Biol Chem.* 287(42): 35324–32.

II. Kakkola L.*, **Denisova O.V.***, Tynell J., Viiliäinen J., Ysenbaert T., Matos R.C., Nagaraj A., Öhman T., Kuivanen S., Paavilainen H., Feng L., Yadav B., Julkunen I., Vapalahti O., Hukkanen V., Stenman J., Aittokallio T., Verschuren E.W., Ojala P.M., Nyman T., Saelens X., Dzeyk K., Kainov D.E. (2013) Anticancer compound ABT-263 accelerates apoptosis in virus infected cells and imbalances cytokine production and lowers survival rates of infected mice. *Cell Death Dis.* 4: e742.

III. Bimbo L.M., **Denisova O.V.**, Mäkilä E., Kaasalainen M., De Brabande J.K., Hirvonen J., Salonen J., Kakkola L., Kainov D.E., Santos H.A. (2013) Inhibition of influenza A virus infection in vitro by saliphenylhalamide loaded porous silicon nanoparticles. *ASC Nano.* 7(8): 6884-93.

IV. **Denisova O.V.**, Virtanen S., Von Schantz-Fant C., Bychkov D., Desloovere J., Soderholm S., Theisen L., Tynell J., Ikonen N., Vashchinkina E., Nyman T., Matikainen S., Kallioniemi O., Julkunen I., Muller C.P., Saelens X., Verkhusha V.V., Kainov D.E. Akt inhibitor MK2206 prevents influenza A(H1N1)pdm09 virus infection in vitro. *Submitted.*

* equal contribution

ABSTRACT

Influenza viruses cause annual epidemics and pandemics which have serious consequences for public health and global economy. The severity of infections with influenza viruses can vary from asymptomatic to life-threatening viral pneumonias frequently complicated by multi-organ failure and exacerbation of other underlying conditions. Currently, four licensed anti-influenza drugs are available for the prevention and treatment of influenza virus infections. However, resistance to the licensed antivirals develops rapidly. Therefore, there is a need for next-generation antiviral agents to combat influenza virus infections.

Recent advances in understanding influenza virus-host interactions have revealed a number of host targets for potential antiviral interventions. In particular, basic cellular functions, metabolic and biosynthesis pathways as well as the signaling cascades essential for virus replication could be modulated by small-molecule inhibitors to block virus infection. Moreover, temporal inhibition of these host functions will be less likely to induce viral drug resistance. In addition, many of the inhibitors of cellular functions are already approved or in clinical development for other diseases. Drug repurposing will facilitate their introduction for treatment of viral infections, since the pharmacokinetics and toxicity profile of these drugs are already known.

In this work, a library of small-molecule inhibitors targeting host factors and potentially interfering with influenza virus infection was built and screened. Inhibitors of vacuolar proton-ATPase (v-ATPase), Akt kinase, ribonucleotide reductase and the anti-apoptotic B-cell lymphoma-2 (Bcl-2) family proteins showed antiviral activity *in vitro*. However, ABT-263, an inhibitor of Bcl-2 family proteins, was ineffective *in vivo*. SaliPhe, an inhibitor of v-ATPase, was the most potent antiviral agent and it was effective against a broad range of influenza viruses and some other RNA viruses *in vitro*, and against a mouse adapted influenza strain *in vivo*. In order to overcome the low water solubility and high toxicity of SaliPhe, it was needed to optimize the drug's bioavailability using a porous silicon particle-based delivery system prior to embarking on future clinical trials. The results presented in this study expand the understanding of influenza virus-host interactions, and are important for rational approaches of discovering new antiviral agents.

1. REVIEW OF THE LITERATURE

1.1. Introduction

Influenza viruses cause annual epidemics and pandemics with serious consequences for public health and global economy. During the annual epidemics, influenza viruses infect 5 – 15% of the human population resulting in 3 – 5 million cases of severe illness and 250,000 – 500,000 death each year all around the world (Shirey et al, 2013). In addition, occasionally strains to which humans are naïve appear and cause pandemic outbreaks. During the past 100 years, five pandemic influenza outbreaks have occurred: "Spanish flu" (H1N1) in 1918, "Asian flu" (H2N2) in 1957, "Hong Kong flu" (H3N2) in 1968, "Russian flu" (H1N1) 1977, and "swine flu" (H1N1) in 2009. In particular, the 1918 influenza pandemic affected almost 30% of the global population and is believed to have killed over 50 million people (Johnson & Mueller, 2002).

Influenza viruses usually invade the epithelial cells of the upper respiratory tract and cause acute respiratory disease. The typical influenza symptoms are fever, headaches, chills, nasal congestion, sore throat and body aches. However, the highly-pathogenic influenza A (H5N1) virus infects the lower respiratory tract and causes viral pneumonia and acute respiratory distress syndrome. There may be multiple organ failure, caused by intense induction of proinflammatory cytokines. Recently, a new avian influenza A (H7N9) strain has been identified in China. The influenza H7N9 virus was characterized by rapidly progressive pneumonia, respiratory failure, acute respiratory distress syndrome, and fatal outcomes (39 deaths in 132 confirmed cases) (Li et al, 2013).

1.2. Influenza A virus

1.2.1. Structure and classification

Influenza A viruses belong to the *Orthomyxoviridae* family which also includes the influenza B and C types. The influenza viruses differ in several ways, i.e. genome organization, variability of the surface glycoproteins, host range and pathogenicity. Both influenza A and B viruses contain eight segments of negative-strand RNA, whereas influenza C virus contains only seven segments (Cheung & Poon, 2007).

Influenza A virus has a wide range of hosts including domestic and wild birds, pigs, horses, mink, seals, bats, as well as humans, while influenza virus types B and C infect predominantly humans (Tong et al, 2013; Webster et al, 1992). Every year, influenza virus types A and B cause epidemics in human population. However, only influenza A viruses have been responsible for pandemic outbreaks.

Influenza A virus is an enveloped virus of 80-120 nm in diameter with eight segments of negative-strand RNA encoding up to 15 proteins depending on the strain. The proteins include hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), proton channel protein (M2), nucleoprotein (NP), polymerase acid protein (PA), polymerase basic protein 1 and 2 (PB1 and PB2), non-structural protein 1 (NS1) and nuclear export protein (NEP, also known as NS2) (Medina & Garcia-Sastre, 2011). In some strains, there are additional proteins, such as PB1-F2, PB1-N40, and PA-X (Chen et al, 2001; Jagger et al, 2012; Wise et al, 2009). Recently, two novel proteins expressed from the PA segment, PA-N155 and PA-N182, have been identified (Muramoto et al, 2013).

Three viral proteins HA, NA and M1 are embedded in a lipid envelope which is derived from the plasma membrane of the infected cell (**Figure 1**). HA and NA are glycoproteins that form trimers and tetramers on the virion surface, respectively. Each RNA segment is associated with multiple copies of NP and with three polymerase subunits, PB1, PB2 and PA (Moeller et al, 2012). This large complex, called viral ribonucleoprotein (vRNP), is surrounded by a layer of M1 and stabilized by NEP. Other proteins, such as NS1, PB1-F2, PB1-N40, PA-X, PA-N155, and PA-N182, are non-structural proteins that are synthesized during virus replication in the cell (Muramoto et al, 2013).

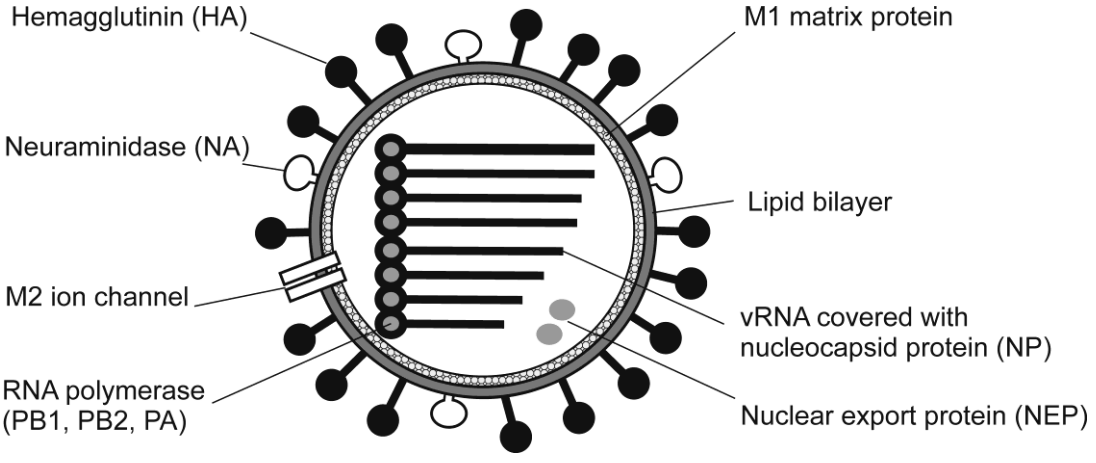


Figure 1. Schematic representation of influenza A virus.

Depending on the differences in the surface glycoproteins, influenza A viruses are classified into 162 subtypes with at least 18 HA (H1 – H18) and 11 NA (N1 – N11) subtypes having been documented to date (Tong et al, 2013). For example, influenza virus subtypes H1, H2, and H3 are found in humans, subtypes H5, H7 and H9 in birds (Medina & Garcia-Sastre, 2011).

1.2.2. The life cycle of influenza virus

The influenza virus life cycle (**Figure 2**) can be divided into the following stages: (i) attachment to the cell surface; (ii) virus entry by endocytosis; (iii) release of vRNPs into the cytoplasm; (iv) migration of vRNPs into the nucleus; (v) transcription and replication of the viral RNAs; (vi) translation of viral proteins; (vii) export of the vRNPs from the nucleus; (viii) assembly and budding at the host cell plasma membrane (Nayak et al, 2009; Samji, 2009; Sun & Whittaker, 2013).

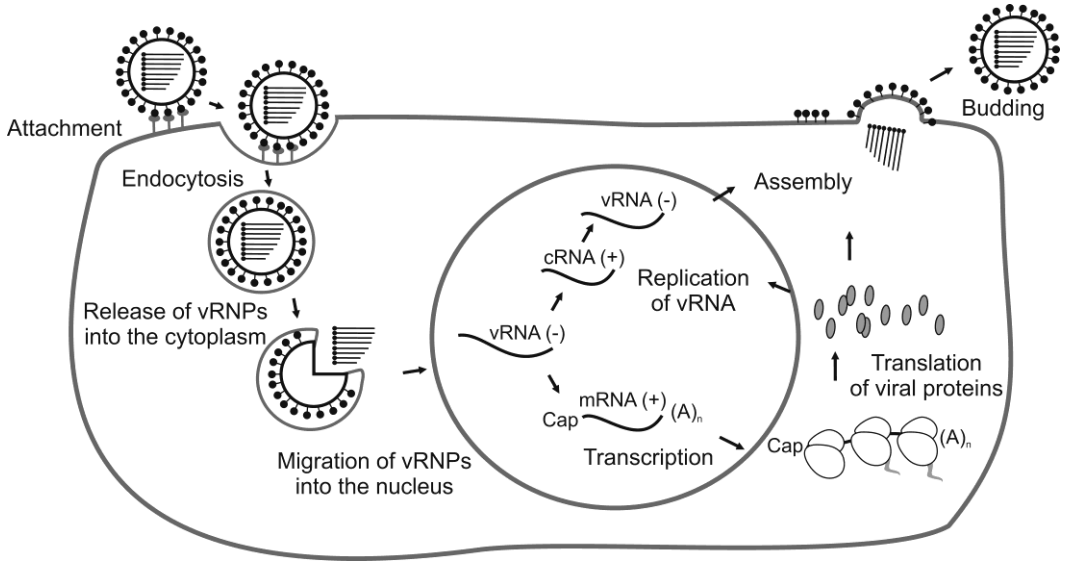


Figure 2. Influenza virus replication cycle.

HA binds to a sialic acid receptor on the plasma membrane of the infected cell. Interestingly, human influenza A strains bind to α2,6-linked sialic acid receptors, avian influenza strains bind to α2,3-linked sialic acids, and swine influenza strains can recognize both types of receptors (Skehel & Wiley, 2000; Suzuki et al, 2000). However, it has been recently shown that the avian influenza A (H7N9) virus binds to both human-like α2,6- and avian-like α2,3-linked sialic acids (van Riel et al, 2013). After binding, the virus particle enters the host cell by clathrin-dependent receptor-mediated endocytosis (Samji, 2009). The low pH (5 – 6) in the endosome induces conformational

changes in HA that trigger the fusion of the viral envelop and the endosomal membrane (Han et al, 2001). At the same time, the M2 ion channel transports protons into the virus particle interior. This evokes conformational changes in M1 resulting in the disruption of M1-vRNPs interactions (Pinto & Lamb, 2006). When fusion of the viral envelope and the endosomal membrane occurs, vRNPs are released into the cytoplasm and they migrate to the nucleus. In the nucleus, the site of influenza virus replication and transduction, vRNPs serve as templates for the production of mRNA and cRNA. The RNA polymerase formed by PB1, PB2 and PA subunits catalyzes the synthesis of mRNAs and cRNAs (Amorim & Digard, 2006). The mRNAs are exported from the nucleus to allow their translation in the cellular ribosomes. The synthesized viral proteins NP, M1, NS2 and polymerase subunits are then imported into the nucleus for vRNP assembly. The newly formed vRNPs are exported from the nucleus and directed to the apical plasma membrane where assembly of progeny virions occurs (Nayak et al, 2009). At this point, NA removes sialic acid residues from glycoproteins and glycolipids, thus allowing the new virus particles to bud off from the host cell surface and to repeat the infection cycle.

1.2.3. Evolution of influenza viruses

It is well known that influenza genes HA and NA mutate frequently. This process, referred to as antigenic drift, leads to the emergence of new virus strains with different antigenic profiles (**Figure 3A**). These new strains are not recognized by the antibodies that were produced against previous strains, and as a result an annual influenza epidemic can occur (Dixit et al, 2013; Medina & Garcia-Sastre, 2011). In addition, the segmented organization of influenza genome allows genetic reassortment between two or more viruses infecting the same cell. This process is called antigenic shift and it leads to the emergence of new subtypes (**Figure 3B**). In the case of influenza A viruses with a broad host range, reassortment events sometimes result in the emergence of new influenza subtypes to which the human population is naïve. Most of the pandemic-causing viruses over the last 100 years as well as the new avian influenza A (H7N9) virus identified in China in 2013, are thought to have emerged through genome reassortant of viruses from human, avian and/or swine hosts (Li et al, 2013; Medina & Garcia-Sastre, 2011; Shin & Seong, 2013).

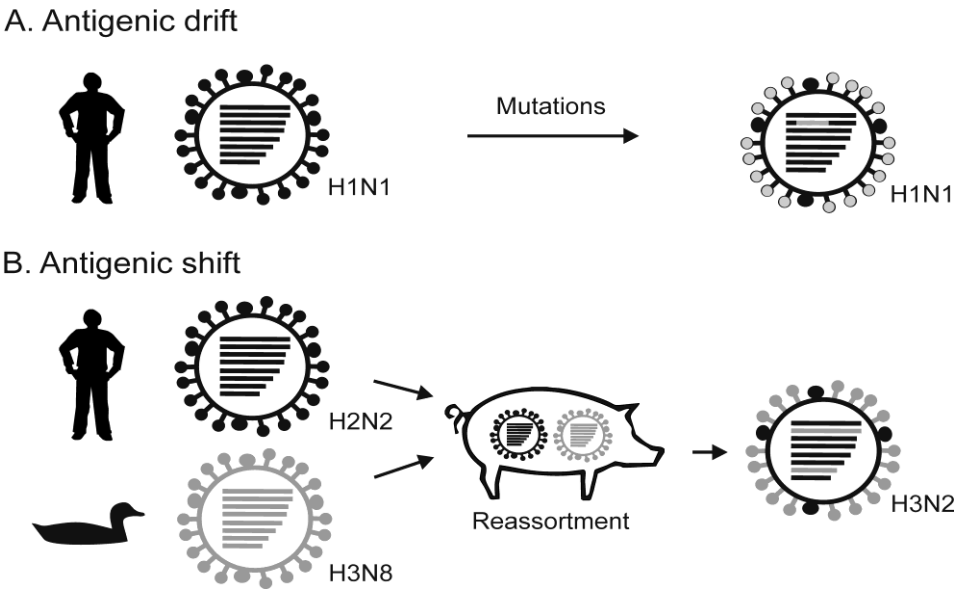


Figure 3. Evolution of influenza A viruses.

1.3. Prevention and treatment of influenza

Currently, two types of influenza virus vaccines and four directly acting antiviral drugs are approved by FDA for the prevention and treatment of influenza virus infections. Vaccination is the primary method for prophylactic protection from influenza virus infection. Routine annual influenza vaccination is recommended for persons under the age of 6 months, persons over the age of 50 years, health care workers, pregnant women and people of all ages with chronic respiratory diseases, heart or renal diseases, diabetes or immunosuppression due to disease or treatment (Baguelin et al, 2012). Due to the high mutation rate of influenza surface glycoproteins new seasonal influenza vaccines need to be produced every year, a major task since the production of influenza vaccines takes 6 to 8 months. Moreover, the vaccine should be administered about 4 weeks before the start of the next epidemic season (in the Northern Hemisphere the influenza season starts in December, in the Southern Hemisphere in May). WHO Global Influenza Surveillance and Response System issues a recommendation about the composition of influenza virus vaccines. Usually seasonal influenza vaccines contain two influenza A (H1N1 and H3N2) subtypes, and one influenza B (trivalent vaccine) or two influenza B strain (quadrivalent vaccine) (Centers for Disease & Prevention, 2013). There are two types of licensed influenza vaccines. Inactivated virus containing vaccines are available in an injectable form, whereas live

attenuated virus containing vaccines are administered as an intranasal spray (FluMist) (Lee et al, 2014; Luksic et al, 2013). In practice, the seasonal influenza vaccines will be effective in prevention and control of seasonal epidemics if they are produced and delivered in time. However, the effectiveness can differ from season to season and it will depend on the circulating influenza strains (Centers for Disease & Prevention, 2013; Gefenaite et al, 2014; Valenciano et al, 2011). Moreover, seasonal influenza vaccines are completely ineffective in the prevention of the occasional influenza pandemics caused by new emerging strains.

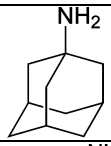
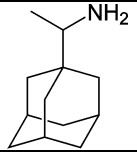
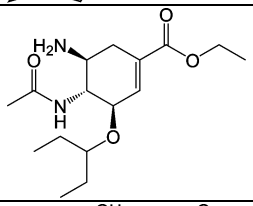
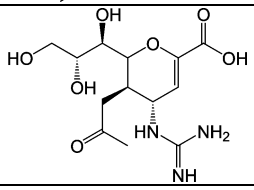
Currently, four licensed anti-influenza drugs are available to cure disease and shorten the infection period. These antivirals target two major surface glycoproteins M2 (amantadine and rimantadine) and NA (oseltamivir and zanamivir) (**Table 1**). Amantadine and rimantadine target the M2 ion channel blocking transport of protons into the virion interior and are effective against influenza virus type A, but not B and C (McKimm-Breschkin, 2013b). Both antivirals are approved for adults, amantadine in addition for children older than one year old. In 1999, FDA approved two NA inhibitors, oseltamivir (GS4104, Tamiflu®) and zanamivir (GG167, Relenza®), for treatment and prevention of acute infections caused by influenza virus types A and B. These agents act by blocking the functions of NA, release and spreading of progeny virions at the late stage of virus replication is prevented. Oseltamivir is approved for adults, whereas zanamivir is approved for adults and for children over 7 years of age.

Resistance to the licensed virus-targeted antivirals has developed rapidly. Amantadine resistance caused by a S31N mutation in the M2 protein has been described in all human (H1N1, H3N2, and H1N1pdm09) and avian (H5N1 and H7N9) influenza A strains circulating globally (Bright et al, 2005; Hayden & de Jong, 2011; Nelson et al, 2009; Zhou et al, 2013). The S31N mutation does not decrease viral replication or transmissibility (Hayden & de Jong, 2011). In addition, other amantadine resistance-conferring mutations such as A30T, L26F, and V27A have been detected (Deyde et al, 2007). Oseltamivir resistance caused by the H275Y mutation in NA has been identified among the clinical isolates of human (H1N1, H3N2, and H1N1pdm09) and avian (H5N1) influenza A viruses (Dharan et al, 2009; Hayden & de Jong, 2011; Le et al, 2005; Meijer et al, 2009). It has been demonstrated that the H275Y mutation leads to reduction of viral infectivity and virulence in seasonal influenza H1N1 viruses (Hayden & de Jong, 2011). In addition, other oseltamivir-resistance mutations in NA, such as E119V, N294S, R292K, D198N/E, I222T/V/M, and R292K have been described

(McKimm-Breschkin, 2013b; Moscona, 2009). During the 2009 pandemic, patients infected by viruses with the H275Y mutation were successfully treated with zanamivir (Uyeki, 2009).

Thus, the applicability of M2- and NA-directed licensed antiviral drugs is limited because the globally circulating influenza virus strains have acquired resistance to amantadine and/or oseltamivir (Hayden & de Jong, 2011; McKimm-Breschkin, 2013a). Nowadays, there are ongoing investigations of new potential agents targeting both virus proteins and cellular host factors. Many of these are in advanced stages of clinical development and are intended to be used in the treatment of influenza virus infection in combination therapy with licensed M2 and NA inhibitors (Haasbach et al, 2013a; Tarbet et al, 2012).

Table 1. FDA approved anti-influenza drugs.

Name	Target	Chemical structure	EC_{50}
Amantadine	M2		A: 0.15 – 1.6 μM[a]
Rimantadine	M2		A: 14 nM[b]
Oseltamivir (GS4104, Tamiflu®)	NA		A: 1.34 nM[c] B: 10.3 nM[c]
Zanamivir (GG167, Relenza®)	NA		A: 1.64 nM[c] B: 6.49 nM[c]

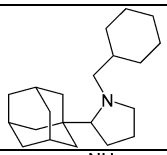
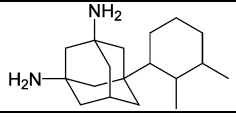
Note: EC_{50} represents effective concentration required to inhibit infectious viral yield by 50%;
[a] (Shin & Seong, 2013); [b] (Savinova et al, 2009); [c] (Yamashita et al, 2009).

1.3.1. Novel virus-directed antiviral agents

1.3.1.1. M2 ion channel blockers

Two novel compounds, I5 and I9 (**Table 2**), have been discovered by virtual screening by docking and pharmacophore modeling against influenza A (H3N2 and H1N1pdm09) viruses (Tran et al, 2011). However, detailed studies of their efficacy *in vitro* and *in vivo* are lacking.

Table 2. M2 ion channel blockers.

Name	Chemical structure	EC_{50}	Clinical development
I5		n.a.	Only virtual screen
I9		n.a.	Only virtual screen

n.a., not available.

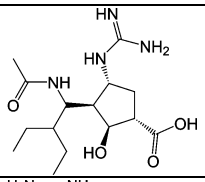
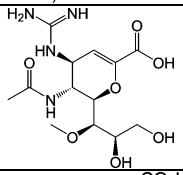
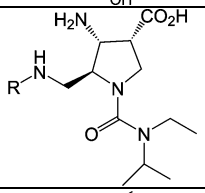
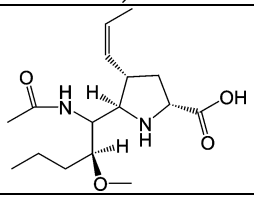
1.3.1.2. Neuraminidase inhibitors

In addition to oseltamivir and zanamivir, there are other NA inhibitors, peramivir (BCX-1812, RWJ-270201) and laninamivir (CS-8958, Inavir®) (**Table 3**) that have been used in the prophylaxis and treatment of influenza virus infection in several countries (Sidwell & Smee, 2002; Yamashita et al, 2009). Peramivir and laninamivir are already approved in Japan, and peramivir also in South Korea (Hayden, 2013). However, it has been reported that oseltamivir-resistant strains with the H275Y mutation were insensitive to peramivir treatment (McKimm-Breschkin, 2013b). At the same time, several pyrrolidine derivatives such as A-192558 and A-315675 (**Table 3**) have been shown to possess antiviral activity against influenza virus types A and B (Kati et al, 2002; Wang et al, 2001). Importantly, there is a report that A-315675 retains activity against oseltamivir- and zanamivir-resistant influenza types A and B (De Clercq, 2006).

1.3.1.3. Hemagglutinin inhibitors

HA is a critical viral protein that can potentially be targeted to treat influenza infections caused by M2 and/or NA inhibitor-resistant strains. Taking into account the fact that there are 18 HA subtypes, selection and rational drug design of broad-spectrum influenza virus inhibitors targeting HA is a challenging task. A number of investigational protein-based peptides have been described (**Table 4**). For instance, Jones and colleagues identified a 20-amino-acid peptide (EB, as an entry blocker) with a broad-spectrum antiviral activity against influenza A (H5N1) and B viruses *in vivo* and *in vitro* (Jones et al, 2006). This peptide specifically binds to HA and inhibits its attachment to the cellular receptor.

Table 3. NA inhibitors and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
Peramivir (BCX-1812, RWJ-270201)		A: 0.2 nM[a] B: 8.5 nM[a]	Licensed in Japan and South Korea; phase III for treatment of influenza A and B
Laninamivir (CS-8958, Inavir®)		A: 2.5 nM[b] B: 18.9 nM[b]	Licensed in Japan; phase III for treatment of influenza A and B
A-192558		A: 0.2 nM[c] B: 8 nM[c]	Not in clinical development
A-315675		A: 0.4 nM[b] B: 5.9 nM[b]	Not in clinical development

[a] (Kati et al, 2002); [b] (Yamashita et al, 2009); [c] (Wang et al, 2001).

Another study has described a number of *N*-stearoyl peptides that mimic sialic acid and inhibit virus attachment to the cell (Matsubara et al, 2010). Peptides C18-s2(1-8) and C18-s2 (1-5) have exhibited broad-spectrum antiviral activity against influenza A (H1N1 and H3N2) strains *in vitro.* Based on a docking simulation, the authors demonstrated that the peptides were recognized by a receptor-binding site in HA (Matsubara et al, 2010). A 16-amino-acid peptide (Flufirvitide) derived from a fusion initiation region of HA has been demonstrated to block influenza A virus infection (Badani et al., 2011). Currently, flufirvitide is in phase I clinical trials. Recently, 12-20 amino acid peptides containing highly conserved sequences of HA1 and HA2 subunits have been designed *in silico* and nine peptides have been tested against influenza A (H1N1 and H5H1) strains *in vitro* (Jesus et al, 2012). Based on the docking results, the authors proposed that the peptides bind to the HA stalk and prevent the HA conformational changes required for membrane fusion events (Lopez-Martinez et al, 2013).

Moreover, a number of small-molecule inhibitors that suppress influenza virus infection by preventing low pH-mediated conformation changes of HA and HA maturation have been described (**Table 5**). For instance, Bodian and colleagues identified

benzoquinone and hydroquinone compounds that bind to HA and stabilize its non-fusogenic conformation (Bodian et al, 1993).

Table 4. Peptides blocking HA and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
EB	NH_2-RRKKAAVALLPAVLLALLAP-COOH	4.5 μM[a]	Not in clinical development
C18-s2(1-8)	$C_{17}H_{35}CO$-ARLPRTMV-NH_2	3 – 4.2 μM[b]	Not in clinical development
C18-s2(1-5)	$C_{17}H_{35}CO$-ARLPR-NH_2	1.6 – 1.9 μM[b]	Not in clinical development
Flufirvitide	n.a.	n.a.	Phase I

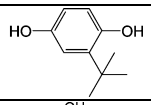
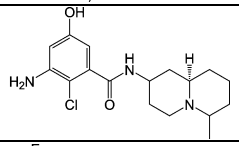
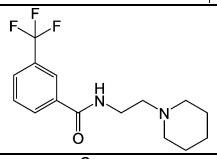
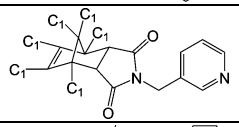
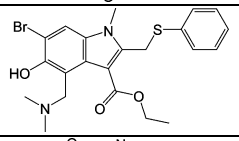
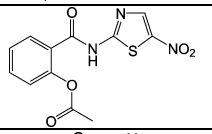
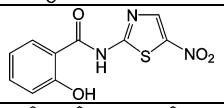
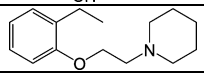
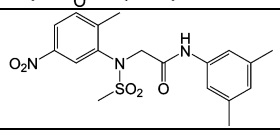
[a] (Jones et al, 2006); [b] (Matsubara et al, 2010); n.a., not available.

Recent research has revealed that a widely used food preservative *tert*-butyl hydroquinone (TBHQ) is a very promising lead compound for the development of antivirals targeting HA (Antanasijevic et al, 2013). It was demonstrated that TBHQ inhibits HA-mediated entry of influenza A (H7N7 and H3N2) viruses. Based on the limited proteolysis assay data, the authors claimed that TBHQ could bind to a specific stem-loop element and block the pH-induced conformation of HA necessary for the fusion of viral and endosomal membranes (Antanasijevic et al, 2013). Other compounds, BMY-27709, CL-61917 (N-substituted piperidine), and CL-62554, blocking the conformational changes of HA specifically inhibited replication of influenza A (H1N1 and H2N2) subtypes but not the H3N2 (Luo et al, 1996; Plotch et al, 1999). Analysis of mutant viruses resistant to these compounds revealed mutations clustered in the stem region of the HA homotrimer near to the HA2 fusion peptide part. Similarly, the antiviral drug arbidol (Umifenovir) which is widely used in Russia and China, inhibits the early membrane fusion events in influenza A and B virus infections and mutations associated with resistance to this compound have been mapped to HA2 (Boriskin et al, 2008; Leneva et al, 2009). Recently, two novel compounds, MBX2329 and MBX2546, binding in the stem region of the HA trimer and inhibiting HA mediated fusion have been identified (Basu et al, 2014).

Interestingly, salicylanilides have a wide range of biological activities including antiviral properties (Kratky & Vinsova, 2011). The nitrothiazole derivative of salicylamide, nitazoxanide (Alinia®) (**Table 5**) is an FDA-approved orally administered antiprotozoal drug used in the treatment of diarrhea in children and adults caused by *Cryptosporidium* or *Giardia*. This compound and its active circulating metabolite

tizoxanide are also known to be effective against influenza A (H1N1 and H5N9) viruses *in vitro* (Rossignol et al, 2009). The authors clearly demonstrated that during virus infection, nitazoxanide acts at the post-translational level by blocking the HA maturation therefore impairing intracellular trafficking of HA and preventing insertion into the cellular membrane. Currently, nitazoxanide is undergoing phase III clinical trials for the treatment of acute uncomplicated influenza virus infections as well as in phase II/III clinical trials for the treatment of chronic hepatitis C virus (HCV) infection.

Table 5. HA inhibitors and their antiviral activity against influenza virus infection.

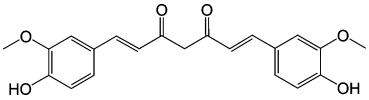
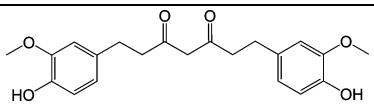
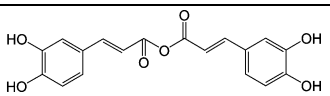
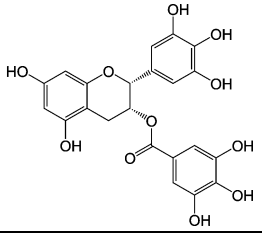
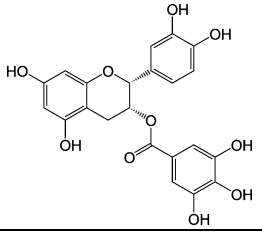
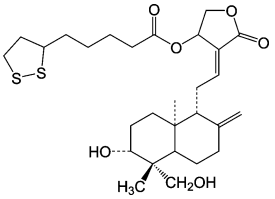
Name	**Chemical structure**	**EC_{50}**	**Clinical development**
TBHQ		6 μM[a]	Not in clinical development
BMY-27709		3 – 8 μM[b]	Not in clinical development
CL-61917		6 μM[c]	Not in clinical development
CL-62554		25 μM[c]	Not in clinical development
Arbidol (Umifenovir)		5.6 – 23 μM[d]	Approved in Russia and China; phase IV for treatment of influenza and common cold
Nitazoxanide (Alinia®)		1.5 – 3 μM[e]	Approved as antiprotozoal agent; phase III for influenza treatment
Tizoxanide		1.5 – 3 μM[e]	Not in clinical development
MBX2329		0.3 – 5.9 μM[f]	Not in clinical development
MBX2546		0.5 – 5.8 μM[f]	Not in clinical development

[a] (Antanasijevic et al, 2013); [b] (Luo et al, 1996); [c] (Plotch et al, 1999); [d] (Boriskin et al, 2008); [e] (Rossignol et al, 2009); [f] (Basu et al, 2014).

Finally, a number of natural compounds interact with the HA protein and possess a broad spectrum anti-influenza activity (**Table 6**). For example, curcumin, a natural ingredient in curry, a commonly used coloring agent and spice in food, has been found to block influenza A (H1N1 and H6N1) virus entry targeting HA *in vitro* (Chen et al,

2013). Moreover, curcumin was shown to be active against coxsackievirus B3, herpes simplex virus (HSV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV) (Kutluay et al, 2008; Rechtman et al, 2010; Si et al, 2007; Sui et al, 1993). Recently, a number of curcumin analogues with anti-influenza activities, tetrahydrocurcumin and petasiphenol, have been described (Ou et al, 2013).

Table 6. Natural compounds blocking of HA and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
Curcumin		0.17 μM[a]	Phase II/III against cancer
Tetrahydro-curcumin		15 μM[a]	Not in clinical development
Petasiphenol		14.65 μM[a]	Not in clinical development
EGCG		22 – 28 μM[b]	Phase II/III as antiviral
ECG		22 – 40 μM[b]	Phase II/III against Alzheimer's disease
AL-1		7 – 15 μM[c]	Not in clinical development

[a] (Ou et al, 2013); [b] (Song et al, 2005); [c] (Chen et al, 2009).

Moreover, it has been shown that catechins, bioactive ingredients in green tea, are able to inhibit HA. For example, epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) displayed significant activity against influenza A (H1N1 and H3N2) and B viruses *in vitro* (Song et al, 2005). Recently Kim and colleagues found that the conformational changes in HA result from EGCG-mediated viral lipid membrane damage rather than from any direct interaction between EGCG and viral HA (Kim et al, 2013). In addition, andrographolides from a herb *Andrographis paniculata* which is

widely used in Asian countries, such as 14-α–lipoyl andrographolide (AL-1), were shown to be active against avian influenza A (H9N2 and H5N1) and human influenza A (H1N1) viruses *in vitro* (Chen et al, 2009).

1.3.1.4. Polymerase inhibitors

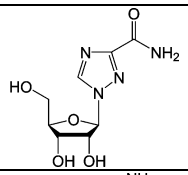
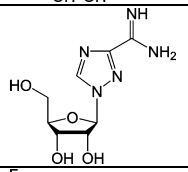
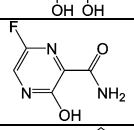
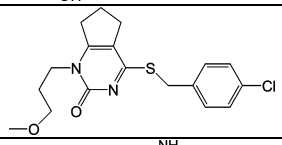
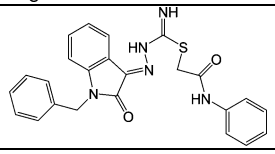
The viral RNA polymerase consisting of PB1, PB2 and PA subunits is a key enzyme responsible for the synthesis of viral mRNA and cRNA. A number of compounds blocking the functions of viral polymerase have been identified (**Table 7**), some of which are already in clinical use. Ribavirin (Virazole®) is a guanosine analog which inhibits a broad range of RNA and DNA viruses (Jonsson et al, 2008; Khan et al, 2008; Markland et al, 2000). The synthesis and antiviral activity of ribavirin were reported more than 40 years ago. Three mechanisms of action have been proposed for this compound: (i) ribavirin may target inosine 5'-monophosphate dehydrogenase, a key cellular enzyme involved in the biosynthesis of GTP, or (ii) ribavirin triphosphate may inhibit the function of a viral RNA polymerase or (iii) ribavirin may inhibit a guanine pyrophosphate "cap" on the 5'-end of viral mRNA (De Clercq, 2006; Ilyushina et al, 2008; Markland et al, 2000). An aerosol form of ribavirin has been approved for the treatment of respiratory syncytial virus infection, and in combination with pegylated interferon (IFN)-α for the treatment of chronic HCV infection (De Clercq, 2004). Viramidine (Taribavirin) is a 3-carboxamidine prodrug of ribavirin that has lower toxicity and a shorter life time *in vivo* (Wu et al, 2006). Ribavirin and viramidine were shown to exert antiviral effects against a range of influenza A (H1N1, H3N2, and H5N1) and B viruses *in vitro* and *in vivo* (Sidwell et al, 2005). Currently, viramidine is undergoing evaluation in phase II/III clinical trials for treatment of influenza virus infection and in phase III for HCV treatment (Hayden, 2013).

Favipiravir (T-705) has been shown to exert antiviral effect against influenza virus and some other RNA viruses including arenaviruses, bunyaviruses, West Nile virus, yellow fever virus, and foot-and-mouth disease virus (Baranovich et al, 2013; Furuta et al, 2009). There are experimental findings indicating that the favipiravir metabolite inhibits RNA polymerase of influenza virus without affecting the synthesis of host cellular DNA and RNA, in the way of ribavirin (Furuta et al, 2005). The authors suggested that the high-error rate viral RNA polymerase false-recognizes nucleotides more frequently compared to the cellular RNA polymerase (Furuta et al, 2009). To date, no favipiravir-resistant virus variants have been reported (Hayden, 2013). Favipiravir is

currently undergoing clinical evaluation in Japan for treatment of influenza infections with virus types A and B.

A number of other molecules targeting the activity of influenza virus RNA polymerase, have been identified by high-throughput screening and of these, compounds 367 and ASN2 are thought to act through the PB1 subunit since mutations in this protein (H456P and Y499H, respectively) have been associated with elevated resistance to these drugs (Ortigoza et al, 2012; Su et al, 2010).

Table 7. Polymerase inhibitors and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
Ribavirin (Virazole®)		2 – 22 μM[a]	Phase II/III for treatment of influenza; approved for treatment of respiratory syncytial virus and HCV
Viramidine (Taribavirin)		8 – 131 μM[a]	Phase II/III for treatment of influenza
Favipiravir (T-705)		11.4 – 17 μM[b]	Phase III for treatment of influenza
Compound 367		n.a.	Not in clinical development
ASN2		3 μM[c]	Not in clinical development

[a] (Sidwell et al, 2005); [b] (Baranovich et al, 2013); [c] (Ortigoza et al, 2012); n.a., not available.

1.3.1.5. Non-structural protein 1 inhibitors

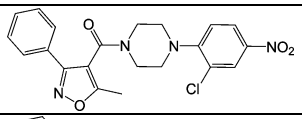
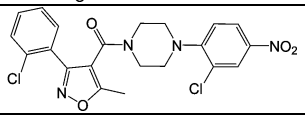
The multifunctional NS1 protein is a virulence factor of influenza virus. It has been shown that NS1 can inhibit the production of antiviral mRNAs particularly IFN-β mRNA by interacting with the cleavage and polyadenylation specificity factor (CPSF30) required for the 3'-end processing of cellular pre-mRNAs (Das et al, 2008). NS1 interacts with the second and third zinc finger domains of CPSF30 (F2F3) and *in vitro* the expression of the F2F3 fragment has been shown to inhibit viral replication by blocking the CPSF30-binding site of NS1 (Twu et al, 2006). Indeed, virus yield in F2F3-expressing cells was reduced by 35- to 60-fold in comparison to control cells. The X-ray crystal structure of the C-terminal domain of the influenza A virus NS1 protein in

complex with the CPSF30 F2F3 fragment solved at the 1.95-Å resolution suggested possible antiviral approaches aimed at disrupting this interaction (Das et al, 2008). However, as far as is known no compounds targeting the CPSF30-binding pocket of NS1 have been developed.

1.3.1.6. Nucleoprotein inhibitors

NP is the most abundant viral protein expressed during influenza virus infection. NP is a multifunctional protein, which accumulates in the nucleus at an early stage of infection and migrates to the cytoplasm during virus assembly and maturation (Kao et al, 2010). There are recent findings that NP may be a druggable target for influenza treatment and prophylaxis. For instance, Kao and colleagues identified nucleozin (FA-4; **Table 8**) as a potent inhibitor of NP (Kao et al, 2010). Nucleozin triggers the aggregation of NP and inhibits its nuclear accumulation. Nucleozin has inhibited infection of influenza A (H1N1, H3N2, and H5N1) *in vitro* and protected mice infected with avian influenza A (H5N1) virus (Kao et al, 2010). The Y289H mutation in NP was shown to be crucial in the development of nucleozin resistance. In addition, a nucleozin analog, compound 3061 (FA-2), has inhibited replication of influenza A (H1N1) virus in both cell culture and a mouse model, with resistance to this drug appearing as a result of Y52H mutation in NP (Su et al, 2010).

Table 8. NP inhibitors and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
Nucleozin (FA-4)		70 – 333 nM[a]	Not in clinical development
3061 (FA-2)		n.a.	Not in clinical development

[a] (Kao et al, 2010); n.a., not available.

1.3.1.7. Nucleic acid-based antiviral drugs

Nucleic acid-based antiviral drugs including antisense oligonucleotides and small interfering RNA (siRNA) were found to exert potent antiviral effects against influenza and other viruses (DeVincenzo, 2012; Zhang et al, 2011; Zhou et al, 2007). They specifically target viral genes but have no impact on host gene expression, avoiding potential side effects (Zhang et al, 2011). Antisense oligonucleotides targeting PA, PB1,

PB2, NP and NS1 genes were shown to be able to inhibit the replication of influenza A virus (H1N1 and H5N1) *in vitro* and *in vivo* (Wu et al, 2008; Zhang et al, 2011). Similarly, M2-specific and NP-specific siRNAs have been tested to be effective against influenza A virus (H5N1, H1N1, and H9N2) *in vitro* and *in vivo* (Zhou et al, 2007). In addition, a phosphorodiamidate morpholino oligomer AVI-7100 targeting expression of the M1 and M2 genes has been designed (Iversen et al., 2012). AVI-7100 is effective against influenza A (H1N1 and H3N2) in both mice and ferrets. Currently, AVI-7100 is in phase I clinical trials as a candidate agent for treatment of influenza infections.

1.3.2. Host-directed antiviral agents

The progress in our understanding of virus-host interactions made over the last decade has inspired alternative strategies of controlling and treating viral infection. Of these, targeting host factors essential for virus biology is an especially attractive approach since it is associated with two potential advantages. First, resistance against host-directed antivirals is expected to emerge substantially more slowly than against drugs targeting highly evolvable viral proteins. Second, compounds interacting with host factors might possess a broad-spectrum antiviral potential and, as a result, higher clinical value. Genome-wide siRNA screens have identified multiple host genes and molecular networks crucial for influenza virus (Karlas et al, 2010; Konig et al, 2010) and several other RNA virus replication processes (Li et al, 2009; Sessions et al, 2009; Zhou et al, 2008). In addition, a number of quantitative proteomic techniques have been applied for evaluating virus-host cell interactions (Dove et al, 2012; Lietzen et al, 2011; Munday et al, 2012). In view of the fact that genome-wide screens could contain false positives, Watanabe and colleagues analyzed 1449 candidate genes identified in six recently published independent genome-wide screens. They found 128 human genes affected by influenza virus that were present in at least two of the screens (Watanabe et al, 2010). In the past decade the roles of signaling pathways, including Ras-dependent Raf/MEK/ERK, PI3K/Akt (phosphatidylinositol 3-kinase), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling and protein kinase C (PCK) cascades, in efficient influenza virus replication have also been well established (Ludwig et al, 2003; Planz, 2013). Importantly, signaling pathways have proved to be ideal targets in cancer therapy, and a large number of specific and highly effective inhibitors have been generated aimed at those targets (Engelman, 2009; Fresno Vara et al, 2004; Wong et al, 2010).

This chapter summarizes our knowledge about anti-influenza virus activity of molecules targeting host proteins and host components of signaling pathways. Some of these molecules, for example DAS181 and aprotinin, are undergoing clinical trials, some are approved for treatment of influenza virus infections in some countries. The other types of host-directed antivirals have been originally developed as anticancer therapeutics and a subset of these molecules are either approved by FDA or in the clinical development stage. Given that *de novo* drug development is a notoriously lengthy and costly endeavor, repurposing cancer drugs for antiviral use opens up an exciting new avenue of clinical research and, as will be discussed in detail, it provides an innovative experimental approach to understanding influenza virus biology.

1.3.2.1. Agents cleaving sialic acid

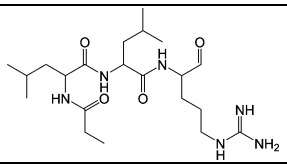
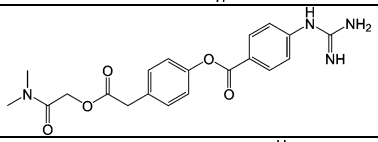
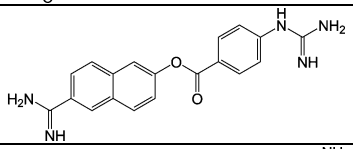
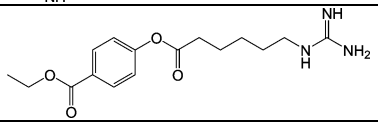
Influenza viruses require sialic acid-containing cell receptors for entry, therefore inhibitors of virus interaction with sialic acid have potential uses in therapeutic intervention. One promising candidate for influenza treatment is DAS181 (Fludase®), which is currently in phase II clinical trials (Moss et al, 2012). DAS181 is a recombinant fusion protein (46 kDa) composed of a catalytic domain from *Actinomyces vircosus* sialidase linked with a cell surface-anchoring domain of human amphiregulin (Malakhov et al, 2006). DAS181 becomes attached to a respiratory epithelium cell and cleaves off the sialic acid from the cell surface, thus preventing viral adsorption. DAS181 removes both human-like α2,6- and avian-like α2,3-linked sialic acids from host cells and it has been shown to be active against human (H3N2 and H1N1pdm09), including oseltamivir-resistant strains and avian (H5N1) influenza A viruses *in vitro* with EC_{50} values in a range of 0.04 – 0.9 nM and it is also effective *in vivo* (mice, ferrets) (Belser et al, 2007; Moss et al, 2012; Triana-Baltzer et al, 2009). These findings support the belief that DAS181 may be able to exert antiviral activity against a broad range of influenza viruses. Moreover, an inhaled administration of DAS181 was shown to protect against parainfluenza virus (Chen et al, 2011). Virus resistance to DAS181 was reported to be minimal and unstable (Triana-Baltzer et al, 2009).

1.3.2.2. Protease inhibitors

Cleavage of HA0 to HA1 and HA2 by cellular proteases is a crucial step in influenza virus infection since it is necessary for the fusion of the viral envelop and the endosomal membrane. Therefore HA-cleaving cellular trypsin-like proteases represent

potential targets for antiviral drug development. It was already demonstrated that a number of protease inhibitors widely used for the treatment of pancreatitis, including leupeptin, camostat, nafamostat (Futhan), gabexate and aprotinin (Trasylol®) (**Table 9**), are active against influenza virus types A and B *in vitro* and *in vivo* (Hosoya et al, 1992; Lee et al, 1996; Noma et al, 1998; Tashiro et al, 1987; Zhirnov et al, 2011). Interestingly, none of the protease inhibitors possessed activity against measles virus (MeV), respiratory syncytial virus or parainfluenza virus type 3 (Hosoya et al, 1992).

Table 9. Protease inhibitors and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
Leupeptin		n.a.	Not in clinical development
Camostat (Fiopan)		A: 4 μM[a] B: 11 μM[a]	Not in clinical development
Nafamostat (Futhan)		A: 1.2 μM[a] B: 4.3 μM[a]	Approved as anticoagulant
Gabexate (FOY)		A: 738 μM[a] B: 417 μM[a]	Phase IV for anticoagulation treatment
Aprotinin (Trasylol®)	58-amino acid polypeptide	A: 26 μM[a] B: 39 μM[a]	Approved in Russia for treatment of influenza; phase III in vascular surgery

[a] (Hosoya et al, 1992); n.a., not available.

Leupeptin is a small peptide produced by actinomycetes capable of inhibiting serine, cysteine and threonine proteases. Tashiro and colleagues showed that leupeptin suppresses virus replication in mice co-infected with influenza virus and *Staphylococcus aureus* (Tashiro et al, 1987). Nafamostat and gabexate are synthetic serine protease inhibitors used as anticoagulant drugs (Maruyama et al, 2011; Yuksel et al, 2003). Aprotinin is a 58-amino acid polypeptide purified from bovine lungs. It has been shown that aprotinin can suppress proinflammatory cytokines, such as interleukin (IL)-6, IL-1b and tumor necrosis factor (TNF)-α, *in vitro* and *in vivo* (Pan et al, 2011). Aerosol administration of aprotinin is now approved as an anti-influenza treatment in Russia (Zhirnov et al, 2011).

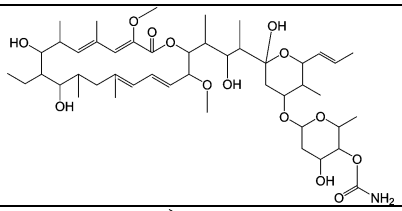
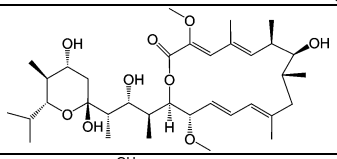
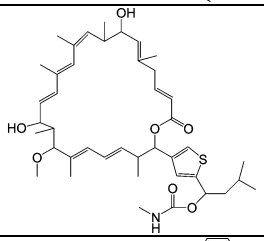
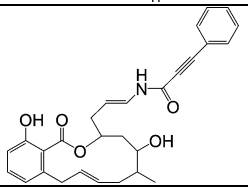
1.3.2.3. Vacuolar proton-ATPase inhibitors

The v-ATPase is a multisubunit complex with a molecular mass exceeding 850 kDa and responsible for the acidification of endosomes. v-ATPase consists of a cytosolic V1-domain, containing eight subunits (A, B, C, D, E, F, G, and H), and a trans-membrane proton translocation V_0-domain, containing five subunits (a, d, c, c′, and c″) (Kane & Smardon, 2003). Human v-ATPase plays important roles in normal physiological processes of the cell as well as in some severe diseases and cancers. Therefore, v-ATPase has proved to be a potential therapeutic target in cancer therapy (Dreisigacker et al, 2012; Lebreton et al, 2008; Wiedmann et al, 2012). Several studies have identified a role of v-ATPase in influenza virus infection (Drose & Altendorf, 1997; Guinea & Carrasco, 1995; Marjuki et al, 2011; Müller et al, 2011). Genome-wide siRNA screening revealed a number of v-ATPase genes necessary for influenza virus entry, including *ATP6V1A*, *ATP6V1B2*, *ATP6V0B*, *ATP6V0C*, *ATP60D1* and *ATP6AP1* (Karlas et al, 2010; Konig et al, 2010), and for other RNA viruses such as West Nile virus, Dengue virus (DENV), and HIV (Konig et al, 2008; Krishnan et al, 2008; Sessions et al, 2009).

There are several groups of v-ATPase inhibitors differing in their structural characteristics and mechanism of action (**Table 10**). A group of plecomacrolide antibiotics such as bafilomycin A1 and concanamycin A block the functions of v-ATPases and P-ATPases but not mitochondrial F-ATPase (Drose & Altendorf, 1997; Guinea & Carrasco, 1995). A second group of v-ATPase inhibitors is archazolides, which specifically inhibit the functions of v-ATPases (Huss et al, 2005). It has been shown that archazolid A and B can hinder the migration of invasive cancer cells *in vitro* and *in vivo* (Wiedmann et al, 2012). Benzolactone enamides represent a third group of v-ATPase inhibitors, these compounds include salicylihalamides A and B that specifically block mammalian v-ATPase (Boyd et al, 2001). It has been reported that salicylihalamide A binds irreversibly to the trans-membrane V_0-domain of v-ATPase (Xie et al, 2004). Lebreton and colleagues synthesized saliphenylhalamide (SaliPhe), a more stable phenyl derivative of salicylihalamide A (Lebreton et al, 2008). Moreover, SaliPhe selectively inhibited the growth of tumorigenic human mammary epithelial cells (Herbert et al, 2005). Müller and colleagues investigated antiviral activity of all three classes of v-ATPase inhibitors against influenza A (H1N1, H1N1pdm09, and H5N1) strains *in vitro* and compared antiviral activities of SaliPhe and bafilomycin A1 in mice. Unlike bafilomycin A1, SaliPhe provided partial protection for mice against a

lethal challenge with the mouse-adapted influenza strain (Müller et al, 2011). Additionally, v-ATPase inhibitors, bafilomycin A1 and concanamycin A, display broad-spectrum antiviral activity against influenza viruses, vesicular stomatitis virus and Semliki Forest virus (SFV) (Drose & Altendorf, 1997; Guinea & Carrasco, 1995; Perez & Carrasco, 1994).

Table 10. v-ATPase inhibitors and their antiviral activity against influenza virus infection.

Name	**Chemical structure**	**EC_{50}**	**Clinical development**
Concanamycin A		0.26 – 3.1 nM[a]	Not in clinical development
Bafilomycin A1		0.36 – 1.3 nM[a]	Not in clinical development
Archazolid B		0.43 – 1.9 nM[a]	Not in clinical development
SaliPhe		28 – 206 nM[a]	Not in clinical development

[a] (Müller et al, 2011).

1.3.2.4. Inhibitors of signaling pathways

Many cellular signaling pathways such as PI3K/Akt/mTOR, PCK, NF-κB and Raf/MEK/ERK have been demonstrated to play important roles during influenza virus replication (**Figure 4**) (Ludwig et al, 2003; Planz, 2013). Several studies have proposed a role of PI3K and its downstream effector Akt, also known as protein kinase B, in influenza virus infection (Ehrhardt & Ludwig, 2009; Ehrhardt et al, 2006; Zhirnov & Klenk, 2007). It was reported that anti-apoptotic PI3K/Akt signaling is activated by NS1 in the early/middle phases of influenza infection, therefore being able to suppress premature apoptosis during the later stages of infection (Ehrhardt & Ludwig, 2009; Zhirnov & Klenk, 2007). Moreover, Marjuki and colleagues showed that inhibition of

influenza virus-induced early activation of extracellular signal-regulated kinase (ERK) and PI3K reduce v-ATPase activity and the acidification of endosomes in infected cells (Marjuki et al, 2011). In addition, several studies have demonstrated a role of PKC in influenza virus entry (Arora & Gasse, 1998; Root et al, 2000; Sieczkarski et al, 2003). For example, PKCβII was shown to regulate the endocytic trafficking needed for influenza virus entry and infection (Sieczkarski et al, 2003).

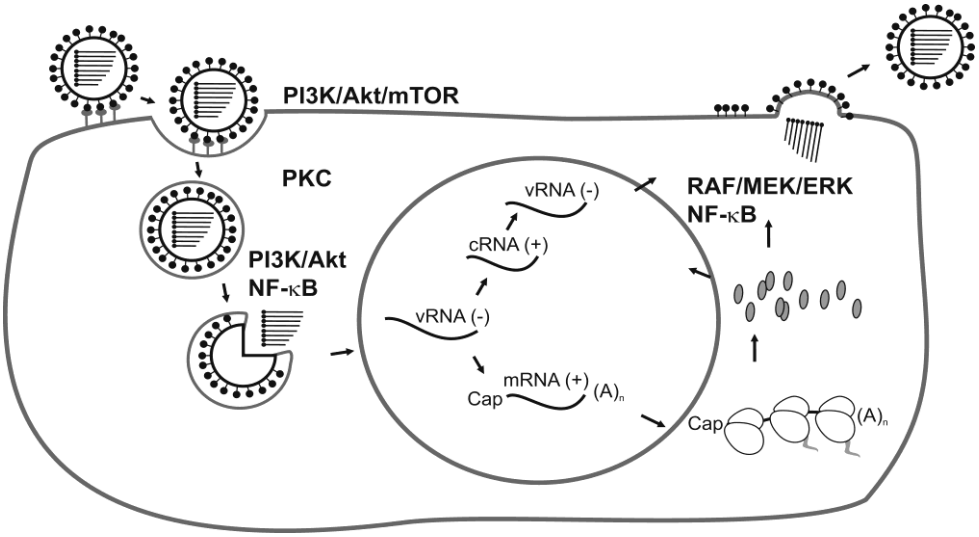


Figure 4. Signaling pathways activated during influenza virus infection.

Moreover, influenza virus has been shown to induce the NF-κB pathway to support its own replication processes via TNF-related apoptosis-induction ligand (TRAIL) and FasL-induced caspase activation promoting vRNP export (Mazur et al, 2007; Wurzer et al, 2004). In cells, NF-κB is present in a latent, inactive dimer form through association with inhibitor-of-kappa-B protein (IκK) in the cytoplasm. Many different stimuli, including TNF-α, IL-1 or some pathogens, like viruses, activate the IκB kinase (IKK) complex, which mediates phosphorylation-induced degradation of IκB in the 26S proteasome (Scheidereit, 2006). This leads to the release of the NF-κB dimer, which is transferred to the nucleus where it activates expression of its specific target genes such as IFN-β, TRAIL and Fas (Wurzer et al, 2004). Additionally, influenza virus activates the Raf/MEK/ERK signaling pathway for efficient export of vRNPs from the nucleus to the cytoplasm (Ludwig et al, 2004; Planz, 2013; Pleschka et al, 2001). It is notable that cells with activated Raf/MEK/ERK signaling pathway have been found to yield high influenza virus titers (Ludwig et al, 2004).

Two pan-PI3K inhibitors, LY294002 and wortmannin (**Table 11**), have been used in attempts to clarify the role of the PI3K/Akt pathway in influenza virus infection

(Ehrhardt et al, 2006; Zhirnov & Klenk, 2007). Treatment with LY294002 or wortmannin inhibited virus production and reduced virus titer *in vitro* (Ehrhardt et al, 2006). Moreover, LY294002 was shown to inhibit phosphorylation of Akt and to accelerate apoptosis in influenza virus-infected cells (Zhirnov & Klenk, 2007). LY294002 is a morpholino derivative of quercetin, which inhibits PI3Kα/β/γ/δ with an IC_{50} of 0.73 μM/0.31 μM/6.6 μM/1.06 μM, respectively (Chaussade et al, 2007). LY294002 inhibits cell proliferation and induces apoptosis in human colon cancer cells *in vitro* and *in vivo* (Semba et al, 2002). Wortmannin is a steroid metabolite isolated from *Penicillium wortmanni*, which inhibits PI3K and many other PI3K-related molecules (Ferby et al, 1996). Neither LY294002 nor wortmannin have progressed to clinical development due to their unfavourable pharmacological properties (Markman et al, 2010). However, a wortmannin derivative, PX-866, has better pharmacological properties and is currently being evaluated in phase II clinical trials for the treatment of solid tumors. Moreover, second-generation PI3K inhibitors, NVP-BKM120, XL765 (SAR245409), NVP-BEZ235, and NVP-BGT226, are available for oral administration and have better pharmacological properties than LY294002 and wortmannin. Currently, those compounds are in phase I/II clinical trials for the treatment of advanced solid tumors (Courtney et al, 2010; Markman et al, 2010; Mukherjee et al, 2012). Clearly, an evaluation of their antiviral potential against influenza virus infection is needed.

A genome-wide siRNA screen identified mammalian target of rapamycin complex1 (mTORC1) as being involved in influenza virus replication (Konig et al, 2010). Mata and colleagues examined naphthalimides (**Table 11**) that inhibited replication of influenza virus and vesicular stomatitis virus. Naphthalimides functioned by increasing expression of REDD1, a major negative regulator of the mTORC1 (Mata et al, 2011). Recently, Keating and colleagues demonstrated that treatment with sirolimus (also known as rapamycin) during primary infection of influenza A (H3N2) virus could achive cross-strain protection of mice against secondary infection by influenza A (H5N1, H7N9, and H1N1) viruses (Keating et al, 2013). The authors also showed that mTORC1 was required for antibody class switching and that inhibition of this pathway during vaccination promoted an antibody repertoire (Keating et al, 2013).

Hoffmann and colleagues used a cell-based high-throughput screening approach to identify distinct groups of inhibitors and enhancers of PKCs. These authors demonstrated that treatment by the commercially available PKC inhibitor rottlerin (**Table 11**) reduced replication of influenza virus *in vitro*. In contrast, activation of PKC

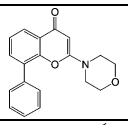
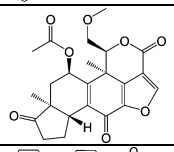
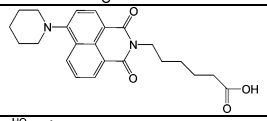
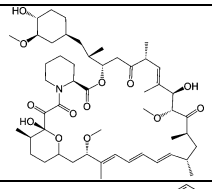
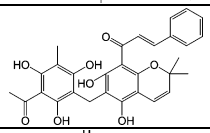
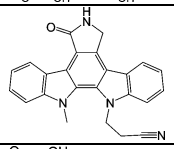
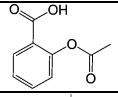
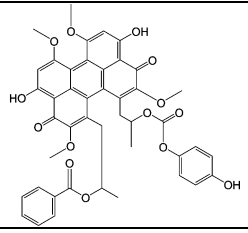
leads to enhanced virus production *in vitro* (Hoffmann et al, 2008). In their study investigating the role of PKCβII in endocytic trafficking, Sieczkarski and colleagues showed that chemical inhibition of PKC by Gö6976 and calphostin C could prevent influenza virus entry (Sieczkarski et al, 2003). Moreover, another specific PKC inhibitor bisindolylmaleimide I prevented replication of influenza virus types A and B *in vitro* (Root et al, 2000). Treatment with bisindolylmaleimide I at a micromolar concentration reduced virus yields by more than 3 orders of magnitude.

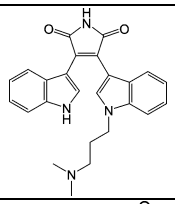
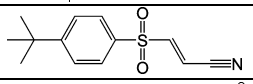
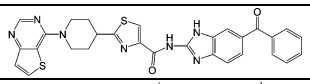
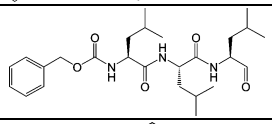
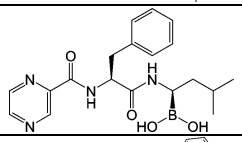
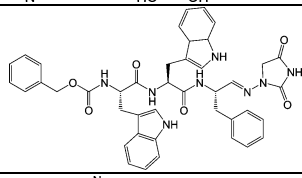
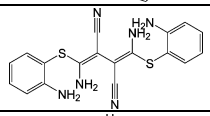
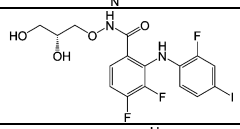
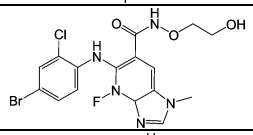
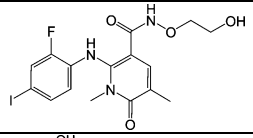
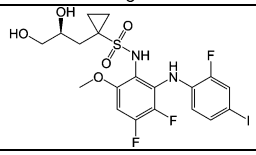
Inhibition of the NF-κB pathway has resulted in blockade of vRNP export (Mazur et al, 2007). The authors demonstrated the acetylsalicylic acid could exert an antiviral effect against several influenza viruses, including H5N1, *in vitro* and *in vivo* (H7N7 in mice). Acetylsalicylic acid (Aspirin™) (**Table 11**) is an FDA-approved analgesic and anti-inflammatory drug inhibiting cyclooxygenase (COX)-1/-2. Moreover, the acetylsalicylic acid blocks the IκB kinase (IKK) complex and therefore the phosphorylation-induced degradation of IκB. Survival rates of mice infected with influenza were significantly increased when acetylsalicylic acid and other NF-κB-inhibiting agents such as the radical scavenger pyrrolidine dithiocarbamate (PDTC) and a proteasome inhibitor MG132 were applied as an aerosol (Mazur et al, 2007). Other NF-κB inhibitors, BAY11-7085 and BAY11-7082, were also able to block influenza virus infection (Nimmerjahn et al, 2004). Recently, another NF-κB inhibitor, SC75741, was shown to prevent influenza virus propagation and to protect mice against highly pathogenic avian influenza A (H5N1 and H7N7) viruses *in vivo* (Ehrhardt et al, 2013; Haasbach et al, 2013b).

Proteasome inhibitors stabilizing IκB suppress of activation of the NF-κB signaling pathway (Haasbach et al, 2011; Russo et al, 2010; Widjaja et al, 2010). MG132 (**Table 11**) is a natural triterpene derived from a Chinese medicinal plant which binds to the active site of 20S proteasome to inhibit the proteolytic activity of the 26S proteasome complex (Guo & Peng, 2013). MG132 is widely used in basic research, but is not undergoing clinical development. Widjaja and colleagues showed that synthesis of viral RNAs depends on an ubiquitin-proteasome system, and that inhibition of proteasome activity by MG132 could modify virus replication at a post-fusion step (Widjaja et al, 2010). Another 26S proteasome inhibitor, PS-341 (Bortezomib, Velcade®), an FDA approved drug for treatment of multiple myeloma and several solid tumor types, could potently inhibit replication of influenza A virus and vesicular stomatitis virus (Dudek et al, 2010). The authors claimed that treatment of infected cells

with PS-341 resulted in induction of IκB degradation and activation of NF-κB and JNK/AP-1. Thus, PS-341 blocked influenza virus replication by inducing an antiviral state mediated by the NF-κB-dependent expression of antiviral gene products (Dudek et al, 2010). VL-01 (Virologik) is a member of a new class of proteasome inhibitors, which have been demonstrated to inhibit the replication of different influenza (H1N1 and H5N1) strains both *in vitro* and *in vivo* (Haasbach et al, 2011). Mice treated with VL-01 exhibited a reduced production of proinflammatory cytokines such as IL-1α/β, IL-6, MIP-1β, CCL5 (C-C motif chemokine 5; also RANTES) and TNF-α induced by avian influenza A (H5N1) virus (Haasbach et al, 2011).

Table 11. Selected inhibitors of signaling pathways and their antiviral activity against influenza virus infection.

Name	**Chemical structure**	**Target**	**EC_{50}**	**Clinical development**
LY294002		PI3K	n.a.	Not in clinical development
Wortmannin		PI3K	n.a.	Not in clinical development
Naphthalimide		mTORC1	31 mM[a]	Not in clinical development
Sirolimus (rapamycin)		mTORC1	n.a.	Approved as immunosuppressant drug
Rottlerin		PKC	n.a.	Not in clinical development
Gö6976		PKC	n.a.	Not in clinical development
Acetylsalicylic acid (Aspirin™)		NF-κB	n.a.	Approved as anti-inflammatory drug
Calphostin C		PKC	n.a.	Not in clinical development

Bisindolylmaleimide I		PKC	n.a.	Not in clinical development
BAY11-7085		NF-κB	n.a.	Not in clinical development
SC75741		NF-κB	53 nM[b]*	Not in clinical development
MG132		26 S proteasome	n.a.	Not in clinical development
PS-341 (Bortezomib, Velcade®)		26 S proteasome	n.a.	Approved for cancer treatment
VL-01 (Virologik)		20S and 26S proteasome	0.8 - 2.4 µM[c]	Not in clinical development
U0126		MEK	1.2 - 82 µM[d]*	Not in clinical development
PD-0325901		MEK	5 nM[e]*	Phase II against cancer. Stopped in 2008.
AZD-6244 (Selumetinib)		MEK	750 nM[e]*	Phase II against cancer
AZD-8330		MEK	40 nM[e]*	Phase I against cancer
RDEA-119 (BAY 869766)		MEK	60 nM[e]*	Phase I/II against cancer

* values obtained on A549 cells; [a] (Mata et al, 2011); [b] (Haasbach et al, 2013b); [c] (Haasbach et al, 2011); [d] (Droebner et al, 2011); [e] (Haasbach et al, 2013a); n.a., not available.

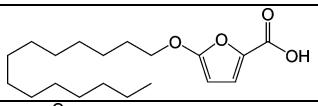
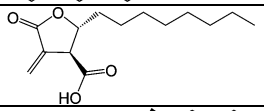
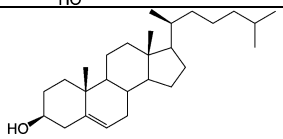
There are several studies demonstrating that chemical inhibition of mitogen-activated protein kinase (MAPK) kinases (MEK) by U0126 (**Table 11)** can exert antiviral activity against influenza A (H1N1, H5N1, and H7N7) and B viruses *in vitro* and *in vivo* (Droebner et al, 2011; Ludwig et al, 2004; Pleschka et al, 2001). In one study, inhibition of MEK resulted in nuclear retention of vRNP at a late stage of virus replication cycle (Pleschka et al, 2001). U0126 is a highly selective inhibitor of both MEK1 and MEK2 with IC_{50} values of 72 nM and 58 nM, respectively. Due to

unfavourable pharmaceutical properties, U0126 did not proceed to clinical evaluation for cancer therapy and was examined only in research (Fremin & Meloche, 2010). A recent study demonstrated that four orally available MEK inhibitors, PD-0325901, AZD-6244, AZD-8330 and RDEA-119 (**Table 11**), possessed antiviral effects against influenza A (H1N1)pdm09 *in vitro* with EC_{50} values in nanomolar range (Haasbach et al, 2013a). Moreover, a combination of these inhibitors and oseltamivir has been shown to induce a strong synergistic antiviral effect against influenza. However, further investigations of the best compound combinations are needed (Haasbach et al, 2013a). It would be interesting to test an antiviral potential of two other MEK inhibitors, trametinib and PD184352, against influenza virus as single agents or in combination with oseltamivir. Trametinib (GSK1120212) is currently approved for the treatment of patients with unresectable or metastatic melanoma with V600E or V600K mutations in serine/threonine-protein kinase B-Raf. PD184352 (CI-1040) is available for oral administration and is currently being evaluated in phase II clinical trials in cancer patients.

1.3.2.5. Inhibitors of lipid metabolism

It is known that viruses also alter cellular lipid metabolism in order that they can achieve efficient replication. Thus it has been reported that inhibition of cholesterol and fatty acid biosynthesis could disturb virus replication, maturation and budding (Blanc et al, 2013; Munger et al, 2008; Spencer et al, 2011; Taylor et al, 2011). Munger and colleagues demonstrated that chemical inhibition of acetyl-CoA carboxylase and fatty acid synthase by TOFA (5-tetradecyloxy-2-furoic acid) and C75 (trans-4-carboxy-5-octyl-3-methylene-butyrolactone) (**Table 12**), respectively, dramatically reduced influenza virus particle production (Munger et al, 2008). Recent studies revealed the role for the sterol metabolic network in the IFN-mediated antiviral response (Blanc et al, 2013; Liu et al, 2013b). It was shown that macrophage-synthesized and -secreted oxysterol, 25-hydroxycholesterol (25HC), inhibited viral growth by blocking fusion of virus and cell membranes (Liu et al, 2013b). *In vitro* 25HC blocked a broad range of enveloped viruses, including influenza A (H1N1), herpes simplex virus type 1 (HSV-1), murine gamma herpes virus 68, varicella zoster virus, HIV, Ebola, Rift Valley Fever Virus, Russian Spring-Summer Encephalitis, and Nipah viruses (Blanc et al, 2013; Liu et al, 2013b). Moreover, administration of 25HC reduces HIV infection in humanized mice (Liu et al, 2013b).

Table 12. Inhibitors of lipid metabolism and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
TOFA		n.a.	Not in clinical development
C75		n.a.	Not in clinical development
25HC		0.75 μM[a]	Not in clinical development

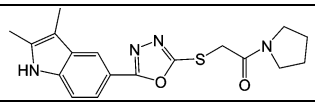
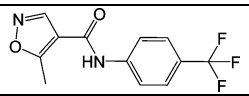
[a] (Blanc et al, 2013); n.a., not available.

1.3.2.6. Inhibitors of nucleotide metabolism

Components of the *de novo* pyrimidine biosynthesis pathway including one key enzyme, dihydroorotate dehydrogenase (DHODH), play an essential role in viral replication. During recent years, a number of compounds blocking the *de novo* pyrimidine biosynthesis have been described (**Table 13**) (Hoffmann et al, 2011; Smee et al, 2012; Zhang et al, 2012). Hoffmann and colleagues identified compound A3 which they claimed displayed broad-spectrum antiviral activity and inhibited the replication of influenza A and B viruses, Newcastle disease virus, human adenovirus, vesicular stomatitis virus, Sindbis virus (SINV), Vaccinia virus (VACV), HCV, DENV, and HIV *in vitro* (Hoffmann et al, 2011). Brequinar and NITD-982 are other inhibitors of DHODH that possess activity against a broad-spectrum of viruses (Qing et al, 2010). In the same study, the brequinar-resistant DENV was isolated. Interestingly, the brequinar-resistant virus displayed a mutation in its NS2 gene (viral RNA polymerase). Another DHODH inhibitor, D282, was active against influenza A and B viruses *in vitro* but was devoid of antiviral activity in infected mice (Smee et al, 2012). Moreover, it was shown that leflunomide, another inhibitor of DHODH, possessed antiviral activity against HIV and HSV, whereas an active metabolite of leflunomide, A77-1726 (LEF-A), was active against polyomavirus BK and delayed mortality and improved cardiopulmonary functions in influenza virus-infected mice (Aeffner et al, 2012; Bernhoff et al, 2010). Zhang and colleagues identified a nontoxic quinoline carboxylic acid, which inhibited DHODH and thus blocked influenza virus infection (Zhang et al, 2012). These authors emphasized that pyrimidines play an essential role in the inhibition of mRNA nuclear export during influenza virus infection. Recently, the same authors synthesized a new

derivative of a quinoline carboxylic acid named C44, which was reported to inhibit replication of influenza virus and vesicular stomatitis virus *in vitro* (Das et al, 2013).

Table 13. Inhibitors of the *de novo* pyrimidine biosynthesis and their antiviral activity against influenza virus infection.

Name	Chemical structure	EC_{50}	Clinical development
A3		0.178 μM[a]	Not in clinical development
D282	n.a.	6-31 μM	Not in clinical development
Leflunomide (AVARA®)		n.a.	Approved as immunomodulatory drug
C44	n.a.	41 nM[b]	Not in clinical development

[a] (Hoffmann et al, 2011); [b] (Das et al, 2013); n.a., not available.

1.4. Combination antiviral therapy

Combination therapy, i.e. simultaneous usage of antiviral drugs with different mechanisms of action, is a well established approach for the treatment of rapidly mutating viruses such as HCV and HIV (Arts & Hazuda, 2012; Casey & Lee, 2013). Combination therapy is required for maximal control of virus replication and prevention of the emergence of drug-resistance especially in immunocompromised and seriously ill patients (Hayden & de Jong, 2011; Ilyushina et al, 2006; Perelson et al, 2012). Combination therapy might minimize the adverse effects of a single-agent therapy (De Clercq, 2006; Hayden, 2013). It has been shown that combination therapy is also effective in the treatment of influenza virus infection (Hayden et al, 1984; Ilyushina et al, 2006; Ilyushina et al, 2008; Nguyen et al, 2010). As early as 1984, Hayden and colleagues showed that human IFN-α_2 in combination with rimantadine and ribavirine exerted a synergistic antiviral effect against influenza A (H3N2 and H1N1) and B viruses (Hayden et al, 1984). Exogenous IFN (alone or in combination) is approved for the treatment cancer as well as some chronic viral diseases (Finter et al, 1991; Rong & Perelson, 2010). Moreover, the feasibility and efficacy of IFN as influenza prophylaxis have been evaluated (Kugel et al, 2009). However, side effects after long term therapy and repeated administration prevented the clinical evaluation of IFNs for the treatment of respiratory diseases. The triple combination of amantadine, oseltamivir and ribavirin was shown to possess synergistic and broad-spectrum activity against drug resistant influenza A strains *in vitro* (Nguyen et al, 2010). Additionally, the triple combination therapy with amantadine, oseltamivir and ribavirin demonstrated good efficacy *in vivo*

(Seo et al, 2013). The effectiveness of a combination of oseltamivir and ribavirin has been assessed against highly pathogenic avian influenza A (H5N1) virus *in vivo* (Ilyushina et al, 2008).

Several studies examining possible pharmacokinetic interactions between currently available antivirals have been performed in healthy adults (Atiee et al, 2012; Pukrittayakamee et al, 2011). The authors reported that drug combinations (oral oseltamivir combined with intravenous zanamivir therapy; oral oseltamivir combined with intravenous peramivir therapy) were well tolerated without causing any pharmacokinetic interactions.

In addition, a number of promising antiviral agents targeting viral proteins have considerable potential alone or in combination with licensed antivirals. It was shown that favipiravir (influenza virus RNA polymerase inhibitor) combined with oseltamivir exerted a strong synergistic effect over monotherapy in mice infected with influenza A (H1N1, H3N2, and H5N1) viruses (Smee et al, 2010). In another study, the effect of combination therapy using favipiravir and peramivir was evaluated in a murine model of influenza A (H1N1)pdm09 infection (Tarbet et al, 2012).

Additionally, inhibitors targeting components of cellular signaling pathways as well as other host proteins may provide better option for combination therapy in conjunction with already approved M2 and NA inhibitors for influenza treatment and prophylaxis. For example, recent *in vitro* studies demonstrated that a combination of oseltamivir with four different orally available MEK inhibitors that are currently in phase I/II clinical trials against cancer had antiviral activity exhibiting a strong synergism (Haasbach et al, 2013a). Moreover, this supports adopting a drug repurposing strategy to achieve the development for new antivirals.

1.5. Drug repositioning and new formulations

Increased knowledge of networks in virus-host interactions has opened new horizons for alternative drug development strategies. Drug development and pharmaceutical companies are already searching for new therapeutic uses of existing drugs or lead compounds. An increasing number of small-molecule inhibitors targeting host proteins or components of cellular signaling pathways are in advanced clinical studies or already approved for treatment of cancer, diabetes, atherosclerosis and cardiovascular diseases. Several recent reports have described the problems but also the

advantages of repurposing anticancer drugs to reveal their antiviral potential (de Chassey et al, 2012; Planz, 2013). In particular, current anticancer compounds are second-generation inhibitors, meaning that they have high specificity, better pharmacological properties, less side effects and are capable of oral administration. Most of them have safety data from phase I/II clinical trials that could be helpful in antiviral drug development. However, as the authors have pointed out, anticancer and antiviral therapies differ in dosage and duration of treatment and therefore most of the side effects described for cancer patients might be irrelevant for antiviral treatment. Therefore, more clinical data is needed. One example of successful drug repurposing is the FDA-approved antiprotozoal agent nitazoxanide (Alinia®), which has broad antiviral potency against influenza virus, HCV and rotaviruses (Kratky & Vinsova, 2011).

Another way to find new therapeutic uses of existing drugs is to probe alternative administration routes. For example, it has been demonstrated that aerosol, but not oral administration of acetylsalicylic acid could reduce virus titers and promote the survival of mice infected with influenza virus (Mazur et al, 2007).

In the past decade, in addition to conventional oral or intravenous drug administration, alternative routes have been intensively studied. Micro- and nanomaterial- based drug delivery systems have a great potential for further application of drugs that do not have clinical potential due to their unfavourable bioavailability. There are several reports demonstrating that mesoporous materials and porous silicon (PSi)-based materials can be promising tools to increase dissolution and stability of drugs loaded within their mesopores (Salonen et al, 2008; Santos et al, 2011; Wang et al, 2013). These materials have a good stability and biocompatibility properties as well as a high drug loading capacity (Liu et al, 2013a; Wang et al, 2013). PSi nanoparticles loaded with anticancer agents could be additionally peptide-functionalized to achieve both targeted and effective cellular uptake and drug delivery (Wang et al, 2013). Moreover, some PSi-based materials are in widespread use in brachytherapy. For example, Brachysil^{TM} which is composed of 30 microne PSi particles encapsulating isotope [^{32}P] is currently undergoing phase II clinical trials for the treatment of solid tumors (Salonen et al, 2008).

2. AIMS OF THE STUDY

The purpose of the present study was to identify druggable host factors involved in influenza virus infection and to develop antiviral agents to be investigated in future clinical trials.

The specific aims were:

1. To develop a screening approach to search for druggable cellular factors involved in influenza virus infection;
2. To understand the molecular mechanisms of action of promising antiviral agents directed against host cell factors;
3. To begin to optimize the clinical utility of host-directed antiviral agents.

3. MATERIALS AND METHODS

This chapter briefly summarizes the materials and methods used in this study. More detailed descriptions are found in the original publications (**I - IV**).

3.1. Cells, compounds and viruses (I – IV)

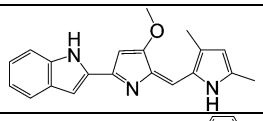
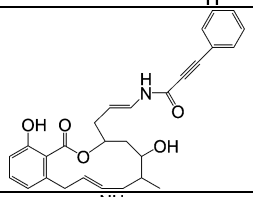
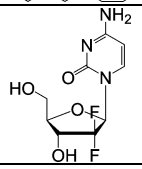
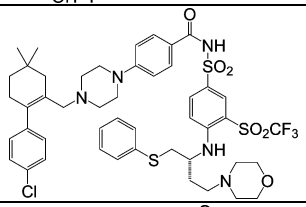
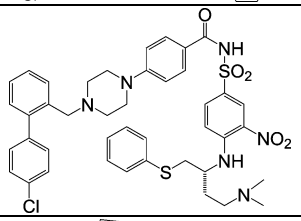
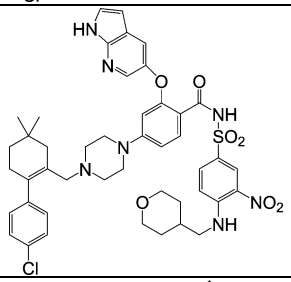
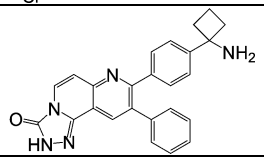
Cell types, compounds and viruses used in this study are summarized in **Tables 14**, **15** and **16**. All cells used in this study were cultured in the respectively recommended growth media and maintained at +37°C in 5% CO_2. Cells were regularly checked by PCR to ensure they were free of mycoplasma contamination. The human macrophages were obtained from peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals and buffy coats of the voluntary blood donors (Finnish Red Cross) by using lymphocyte separation medium (PAA) and cultured for seven days in Macrophage Serum-free Medium (Life Technologies) supplemented with 10 ng/ml human granulocyte macrophage-colony-stimulating factor (GM-CSF; Sigma-Aldrich).

Table 14. Cells types used in the study.

Name	Description	Study
hTERT RPE	Telomerase-expressing human retinal pigment epithelial cells	I - III
MDCK	Madin-Darby canine kidney cells	I - IV
A549	Human lung cancer cells	I, II, IV
VERO-E6	African green monkey kidney cells	I, II
BHK-21	Syrian hamster kidney cells	I, II
iSLK.219	Doxycycline-inducible human endothelial cells latently infected with Kaposi's sarcoma-associated herpes virus	II
Human macrophages	PBMC-derived macrophages from healthy donors	I, II
NCI-H1666	Human lung cancer cells	IV
NCI-H1703	Human lung cancer cells	IV
NCI-1437	Human lung cancer cells	IV
NCI-460	Human lung cancer cells	IV
NCI-H2126	Human lung cancer cells	IV
Calu-1	Human lung cancer cells	IV

All compounds were dissolved in DMSO (Sigma-Aldrich) to a concentration of 10 mM and stored at −80°C until use.

Table 15. Compounds used in the study.

Name	Chemical structure	Molecular weight	Source	Study
Obatoclax (GX15-070MS)		413.49	Selleck Chemicals	I, II, IV
SaliPhe		459.53	*	I - IV
Gemcitabine (Gemzar®)		263.198	Selleck Chemicals	I, II
ABT-263 (Navitoclax)		974.61	Selleck Chemicals	II, IV
ABT-737		813.43	Selleck Chemicals	II
ABT-199 (GDC-0199)		868.44	Active Biochem	II
MK-2206		480.39	ChemieTek	IV

* provided by Professor Jef De Brabander, Department of Biochemistry, University of Texas Southwestern Center at Dallas, Dallas, TX, USA.

The influenza A (H1N1, H3N2, and H1N1pdm09) strains were propagated in MDCK cells, whereas monkey Vero-E6 cells were used for propagation of A/PR8-NS116-GFP. Virus stocks were titrated by a plaque assay on MDCK cells. The following virus stocks were obtained from collaborators: A/Udorn/72 from Sampsa Matikainen, Finland; A/Brussels/BB/2009 from Xavier Saelens, Belgium; B/Shandong/7/97 from Ilkka Julkunen, Finland; SINV and SFV from Tero Ahola,

Finland; HSV-1 and HSV-2 from Veijo Hukkanen, Finland; Bunyamwera virus (BUNV) from Richard M. Elliott, UK.

Table 16. Viruses used in the study.

Name	Group	Family	Study
A/WSN/33 (H1N1)	(-)ssRNA	*Orthomyxoviridae*	I - IV
A/PR/8/34 (H1N1)	(-)ssRNA	*Orthomyxoviridae*	I, III
A/PR/8/34 (H1N1) (mouse-adapted)	(-)ssRNA	*Orthomyxoviridae*	II
A/PR8-NS116-GFP (H1N1)	(-)ssRNA	*Orthomyxoviridae*	I, III
A/Helsinki/P14/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/P15/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/P18/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/Vi1/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/Vi2/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/Vi3/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/543/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/629/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/551/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/628/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/552/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/568/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/604/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/668/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/598/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/526/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/678/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Helsinki/552/2013 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Brussels/BB/2009 (H1N1pdm09)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Sydney/5/1997(H3N2)	(-)ssRNA	*Orthomyxoviridae*	I, IV
A/Udorn/307/1972 (H3N2)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Chicken/Nigeria/BA211/2006 (H5N1)	(-)ssRNA	*Orthomyxoviridae*	IV
A/Anhui/01/2013 (H7N9)	(-)ssRNA	*Orthomyxoviridae*	IV
B/Shandong/7/97	(-)ssRNA	*Orthomyxoviridae*	I, II
Bunyamwera virus (BUNV)	(-)ssRNA	*Bunyaviridae*	I, II
Measles virus (MeV)	(-)ssRNA	*Paramyxoviridae*	I, II
Sindbis virus (SINV)	(+)ssRNA	*Togaviridae*	I, II
Semliki forest virus (SFV)	(+)ssRNA	*Togaviridae*	I, II
Human echovirus 6 (Echo6)	(+)ssRNA	*Picornaviridae*	I, II
Tick-borne encephalitis virus (TBEV)	(+)ssRNA	*Flaviviridae*	II
Dengue virus (DENV)	(+)ssRNA	*Flaviviridae*	II
Vaccinia virus (VACV)	dsDNA	*Poxviridae*	I, II
Herpes simplex virus type 1 (HSV-1)	dsDNA	*Herpesviridae*	I, II
Herpes simplex virus type 2 (HSV-2)	dsDNA	*Herpesviridae*	II
Kaposi's sarcoma-associated herpes virus	dsDNA	*Herpesviridae*	II

3.2. THCPSi nanoparticles loaded with SaliPhe (III)

In an attempt to analyze alternative options to DMSO to dissolve and deliver the compounds, SaliPhe was loaded into nanoparticles. The thermally hydrocarbonized PSi (THCPSi) nanoparticles were prepared from PSi films (**III**). The loading degree of SaliPhe in the mesopores of THCPSi nanoparticles was 2.88±0.11 wt %. The SaliPhe loaded nanoparticles were tested in *in vitro* experiments.

3.3. *In vitro* experiments

Drug screens (I, unpublished)

Drug screens were performed in cooperation with the High Throughput Biomedicine Unit, FIMM Technology Centre, Finland. A 384-well plate-based assay was optimized to identify the compounds that could influence influenza infection. The toxicity assay was optimized to identify inhibitors that influence cell viability. The drug screens were performed against the influenza A/PR8-NS116-GFP strain in human hTERT RPE cells (**I**), and influenza A/WSN/33 and B/Shandong/7/97 viruses in A549 cells (**unpublished**). Approximately, 2,500 cells were seeded in 25 µl of an appropriate growth using a Multidrop 384 (Thermo). After 24 h, the growth medium was changed to the virus growth medium. The drug library comprising either 201 compounds targeting host factors (**I,** Suppl. table 1) or a library with 337 compounds (including the 201 drugs and the HTB compound collection (Pemovska et al, 2013)) was added to the cells using 10-fold serial dilutions at five different concentrations starting from 10 µM with an Echo acoustic dispenser (Labsite). DMSO and benzethonium chloride were added to the control wells. The cells were infected with influenza virus at a multiplicity of infection (MOI) of 3 or left uninfected (mock). At 24 h post-infection (in hTERT RPE cells), virus-mediated GFP fluorescence was measured using a PHERAstar FS plate reader (BMG Labtech). The effective concentrations (EC_{50}) were calculated using SigmaPlot 11 software (Systat Software GmbH, Germany). At 24 h (in hTERT RPE cells) or 48h post-infection (in A549 cells), cell viability was measured using a Cell Titer Glo viability assay (CTG; Promega) according to the manufacturer's instructions. Luminescence was read using the PHERAstar FS plate reader, and inhibitory concentrations (IC_{50}) were calculated. Selectivity indices ($SI=IC_{50}/EC_{50}$) were calculated to distinguish the antiviral effect from toxic side effects of the compounds. Those compounds with SI greater than 10 were validated further.

Compound efficacy testing (I-IV)

Compound efficacy testing was performed in 96-well plates with the different cell lines listed in **Table 14**. Typically, 40,000 cells were seeded in 100 µl of the appropriate growth medium. After 24 h, the growth medium was changed and compounds listed in **Table 15** or SaliPhe-loaded THCPsi nanoparticles were added to the cells at 3-fold serial dilutions at seven different concentrations starting from 10 µM. DMSO was used as a control. Then, the cells were mock-infected or infected with different influenza strains (MOI of 3 or 10) or other viruses (**Table 16**). Cell viability was measured using the CTG assay at 24, 48 or 72 h post-infection when virus-induced cytopathic effect (CPE) was observed.

Testing of compound efficacy against Kaposi's sarcoma-associated herpes virus, MeV, VACV, TBEV, DENV, and influenza A strains H5N1 and H7N9 infections was done by collaborators based on the optimized and established protocol under biosafety level (BSL)-2 and BSL-3 conditions (**I**, **II**, **unpublished**).

Activity and toxicity scores (ATS) were calculated in order to quantitatively profile the compounds that rescued the infected cells (**I, IV**). ATS varies between -100 and +100, where negative values indicate excessive toxicity whereas the highest positive values refer to the most potent compounds. Drug sensitivity scores (DSS) were calculated to quantitatively profile those compounds that accelerated virus-induced cell death (**II**). DSS summarizes the area under the dose-response curve relative to the total area between 10% threshold and 100% inhibition (Pemovska et al, 2013).

Virus titration (I-IV)

Antiviral efficacies of compounds (**Table 15**) or SaliPhe-loaded THCPsi nanoparticles were validated by the plaque assay. Briefly, cells were non- or compound-treated and virus-infected (MOI of 0.1). Supernatants were collected at 24, 48 or 72 h post-infection. The supernatants were 10-fold diluted in virus growth media and added to MDCK cells on 6-well plates. After 1 h, the cells were overlaid with Avicel-based medium and incubated for two days. The cells were fixed with 4% formaldehyde and stained with crystal violet. Plaque forming units were calculated.

For the other viruses the titration procedure slightly differed from that described above. SINV, SFV, VACV and BUNV were titered on Vero-E6 cells, whereas A549 cells were used for titration of Echo6 virus. Other viruses were titered by collaborators based on their established protocols (**I**). The degree of inhibition mediated by a

compound was calculated as the ratio between virus titers in non- and compound-treated infected cells.

Time-of-compound-addition experiment (I, II, IV)

The cells were grown in 96-well plates and were mock- or influenza virus-infected (MOI of 3 or 10). Compounds at non-toxic concentrations were added every hour. Cell viability was measured using the CTG assay at 24 h post-infection.

Compound-competition experiment (II, IV)

hTERT RPE or NCI-H1666 cells were grown in 96-well plates. Cells were treated with obatoclax (1 μM), SaliPhe (0.4 μM), gemcitabine (10 μM) or MK-2206 (10 μM) and with increasing concentrations of ABT-263. The cells were also treated with ABT-263 (0.4 μM) and with increasing concentrations of obatoclax, SaliPhe, gemcitabine or MK-2206. Treated cells were mock- or influenza virus-infected (MOI of 3 or 10), and cell viability was measured using the CTG assay at 24 h post-infection.

Caspase assay (II)

hTERT RPE cells were grown in 96-well plates and were non- or ABT-263-treated and mock- or influenza virus-infected or RNA transfected. At 24 h post-infection or transfection, caspase 8, 9 and 3/7 activities were measured with Caspase-Glo-8, -9 and -3/7 assays (Promega) according to the manufacturer's instructions.

Metabolic labeling of proteins with [^{35}S]-Methionine

hTERT RPE cells were grown in 96-well plates and were non- or ABT-263-treated and mock- or influenza virus-infected (MOI of 3). At different time points post-infection, cells were washed twice with PBS and were incubated with methionine-free DMEM (Sigma-Aldrich) supplemented with 10% BSA and 0.11 μCi/ml [^{35}S]-labeled methionine (EasyTag EXPRESS^{35}S protein labeling mix, specific activity 1175 Ci/mmol; PerkinElmer) for 30 minutes at +37°C. Cells were washed twice with PBS and lysed in 2x SDS-sample buffer. Proteins were resolved by electrophoresis on 4-20% SDS-PAGE (BioRad) and newly synthesized [^{35}S]-labeled proteins were monitored by autoradiography using a Typhoon 9400 scanner (Amersham).

Fluorimetry (IV)

The fluorescence spectra of MK-2206 were recorded (**IV**).

RNA isolation and quantitative RT-PCR (I, II, IV)

hTERT RPE or NCI-H1666 cells were grown in 6-well plates and were non- or compound-treated and mock- or influenza virus-infected (MOI of 3). Total RNA was isolated from cells with the RNeasy Plus Mini Kit (Qiagen) and used in further quantitative RT-PCRs with the primers indicated in **Table 17** (**I**, **II**, **IV**).

Table 17. Primers used in the study

Target	5′→3′ sequence	Study
PB2	Sequence-modifying RT: (+) strand: TCAGTCCCATTGCAGCCTTGCccgccgCCACG (-) strand: GCAGAACCCAACAGAAGAGCAAtttaTttATATTTG PCR: Fwd: GCAGAACCCAACAGAAGAGC Rev: TCAGTCCCATTGCAGCCT	I, II
β-actin	Fwd: CCGACAGGATGCAGAAGG Rew: AGGAAAGACACCCACCTTGA	I, II
NS1	Fwd: GAAATGTCAMGAGACT Rew: AGAAAGCTCTTATCTCTTG	IV
huGAPDH	Fwd: GGCTGGGGCTCATTTGCAGGG Rew: TGACCTTGGCCAGGGGTGCT	IV
18S	Hs99999901_s1*	IV
IFN-β	Hs01077958_s1*	IV
IL-29	Hs00601677_g1*	IV

* TaqMan primers and probes from Applied Biosystems.

RNA transfection (I, II)

In the viral RNA transfection experiment, A/WNS/33 virus was grown in MDCK cells. Supernatants were collected at 24 h post-infection, and the virus was pelleted using ultracentrifugation at 285,000 g for 4 h. Viral RNA was purified and transfected into hTERT RPE cells using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. Six hours after transfection, the medium was replaced with growth medium with or without ABT-263. After 18 h, the cell viability and caspase activities were measured.

Knockdown of the myeloid cell leukemia-1 (Mcl-1) protein was done in hTERT RPE cells using Hs_MCL1_12 Flexi-Tube siRNA (SI04949721; Qiagen) to suppress the expression of Mcl-1 (**I**).

Transcription profiling (IV)

NCI-H1666 cells were grown in 6-well plates and were non- or MK-2206-treated and mock- or influenza virus-infected (MOI of 3). After 8 h post-infection, the cells were collected, total RNA was isolated and used in the subsequent whole genome gene expression analysis (**IV**).

Immunoprecipitation and mass spectrometry (II)

Bcl-xL-associated factors were immunoprecipitated from non- or ABT-263-treated and mock- or influenza-infected hTERT RPE cells and analyzed using liquid chromatography-tandem mass spectrometry (**II**).

Immunoblotting (I, II, IV)

Cells were grown in 96-well plates and were non- or compound-treated and mock- or influenza virus-infected (MOI of 3 or 10). At different time points post-infection, the cells were lysed in 2x SDS-sample buffer. Proteins were resolved by electrophoresis on 4-20% SDS-PAGE and transferred to polyvinylidine fluoride membranes (GE Healthcare). The membranes were blocked with 5% milk in Tris-buffered saline (TBS) and incubated with primary guinea pig anti-NS1, rabbit anti-M1 or rabbit anti-NP (1:2000, 1:2000, and 1:500 dilutions, respectively; from Ilkka Julkunen, Finland), mouse anti-LRP130 (1:100; clone G-10; Santa Cruz Biotechnology), rabbit anti-Bcl-xL (1:1000; Cell Signaling Technology), rabbit anti-Bid (1:1000; Cell Signaling Technology), rabbit anti-Bax (1:100; Santa Cruz Biotechnology), mouse anti-Bad (1:200; Santa Cruz Biotechnology), rabbit anti-UACA (1:250; Sigma-Aldrich) or mouse anti-β-actin (1:2000; Termo Fisher Scientific) antibody overnight at +4°C. After three washes, membranes were incubated for 4 h at 4°C with the representative secondary antibodies conjugated to infrared dyes 680LT or 800CW (1:20,000; Li-Cor Biosciences). The membranes were scanned with an Odyssey scanner (Li-Cor Biosciences).

Immunofluorescence assay (I-IV)

hTERT RPE or NCI-H1666 cells were grown on cover glasses in 6-well plates. Cells were non- or compound-treated and mock- or influenza virus-infected (MOI of 3 or 30) on ice for 1 h. Cells were washed twice with ice-cold virus growth media, overlaid with pre-warmed media with or without compound, and incubated at +37°C in 5% CO_2. At 1, 2, 4 or 18h post-infection cells were fixed with 4% paraformaldehyde and blocked and permeabilized with PBS with 1% BSA and 0.1% Triton X-100. The following antibodies were used in the detection of proteins, rabbit anti-Bcl-xL (1:200; clone 54H6; Cell Signaling Technology), mouse anti-Bad (1:100; clone c-7; Santa Cruz Biotechnology), rabbit anti-Tom20 (1:300; clone FL-145; Santa Cruz Biotechnology), human anti-Mcl-1 (1:100; clone 22/Mcl-1; BD Transduction Laboratories) or rabbit anti-NP and anti-M1 (1:1000; from Ilkka Julkunen, Finland). Secondary antibodies

were anti-mouse or anti-rabbit Alexa Fluor 594 or anti-rabbit Alexa Fluor 488-conjugated antibodies (1:2000 or 1:1000; Life Technologies). Nuclei were counterstained with DAPI. Images were captured with a Nikon 90i microscope and processed with NIS Elements AR software.

Phospho-protein profiling (IV)

NCI-H1666 cells were grown in 6-well plates and were non- or MK-2206-treated and mock- or influenza virus-infected (MOI of 3). After 0.5, 4 and 12 h post-infection, the cells were lysed and the phosphorylation profiles of 43 kinases and 2 related substrates were analyzed using a human phospho-kinase array (R&D Systems) according to the manufacturer's instructions. The membranes were placed on films and were exposed to X-rays and then the films were scanned. Each image was analyzed with ImageJ software (NIH, USA).

Cytokine profiling (I, II, IV)

Cytokine profiling was performed using mouse lung homogenates or supernatants of non- or compound-treated and mock- or influenza virus-infected (MOI of 3 or 10) or vRNA-transfected cells. Cytokine levels were measured using the human or mouse cytokine array panel A (R&D Systems) according to the manufacturer's instructions. The membranes were exposed onto X-ray films and the films were scanned. The scanned image was analyzed with ImageJ software. In addition, the levels of TNF-α, IFN-β and IFN-λ in the cell supernatants were assayed with ELISA (PBL Interferon Source) (**I**, **II**).

Automated image acquisition and image analysis (IV)

MDCK cells were grown in 96-well plates and treated with different concentrations of MK-2206. In a subsequent experiment, MDCK cells were treated with MK-2206 (10 μM), and in combinations with NH_4Cl (20 mM), SaliPhe (0.4 μM) or obatoclax (0.4 μM). Cells were fixed with 4% paraformaldehyde and imaged with a modular epifluorescence microscope ScanR (Olympus) to analyze the number and the mean intensity of the objects (**IV**).

Serial-passage experiment (IV, unpublished)

Human NCI-H1666 cells were grown in 6-well plates and were non- or compound-treated (MK-2206 or SaliPhe) and infected with A/Helsinki/P14/2009 (MOI

of 0.1). At 48 h post-infection, 20 µl of medium was passaged to fresh cells. During passaging, the concentration of compounds was gradually increased (MK-2206, from 0.1 to 10 µM; SaliPhe, from 0.01 to 1 µM). A total of 15 passages were performed and the virus titers were determined with plaque assay.

3.4. *In vivo* experiments

Compound efficacy testing in vivo (II)

Pathogen-free 6-7-week old female BALB/c mice were used for ABT-263 efficacy testing *in vivo*. Mice were inoculated intranasally with 50 µl PBS with or without two median lethal doses (LD50) of the mouse-adapted influenza A/PR8/8/34 strain. ABT-263 (50 mg/kg in carrier) was administered orally, and then the mice were monitored for 14 days for weight loss (**II**). Mice were sacrificed on day 6 and lung tissue dissected. Virus titers, caspase 3/7 activities and cytokine levels were measured from lungs (**II**).

Histochemistry (II)

In the experiment monitoring the degree of inflammation, the left lobes of the mouse lung slices were processed for histochemistry and the tissue slides were stained with hematoxylin and eosin (**II**).

3.5. Ethics

Virus experiments were carried out under BSL-2+ conditions and in compliance with regulations of the University of Helsinki (permit 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. Mouse influenza virus infection experiments were carried out in the Ghent University under BSL-2+ conditions and were authorized by the Institutional Ethics Committee on Experimental Animal of the VIB Department for Molecular Biomedical Research of Ghent University. Buffy coat preparations of voluntary blood donors were obtained with permission from the Finnish Red Cross Blood Transfusion Service and their use was approved by the ethics review committee of the University of Helsinki Central Hospital, Finland (165/13/03/00/2011). Collection of blood samples from volunteers was approved by the ethics review committee of the University of Helsinki Central Hospital, Finland (244/13/03/01/2012).

4. RESULTS AND DISCUSSION

4.1. Identification of host factors involved in influenza virus replication

4.1.1. Assembly of library of compounds targeting host factors (I, II, IV)

There are two main systemic approaches which can be used when searching of host factors essential for virus replication. One approach is to conduct a genome-wide siRNA screening to identify the host genes that are crucial for virus replication (Karlas et al, 2010; Konig et al, 2010; Li et al, 2009; Sessions et al, 2009). The other approach is screening of libraries of chemical compounds capable of blocking host factors for molecules that inhibit virus replication without evoking toxicity in the host cell (Zhang et al, 2012). Both approaches are widely used for understanding viral biology as well as developing alternative antiviral drugs.

To identify host factors essential for influenza virus replication it was decided to assemble a library of small-molecule inhibitors targeting cellular proteins and components of signaling pathways. The primary focus was on commercially available drugs with well described targets that were approved or under clinical development for treatment of cancer or other diseases. Small-molecule inhibitors that target the cell cycle control, apoptotic pathways, lipid metabolism, and other cellular functions that could be essential for influenza infection in the host cell were included. Moreover, the screen also contained some small-molecule inhibitors with known anti-influenza activity such as gemfibrozil and TOFA (lipid metabolism), 17-AAG and bortezomib (protein quality control), fluorouracil and cytarabine (*de novo* nucleotide biosynthesis), 3-methyladenine (autophagy), PD153035 (EGFR) and wortmannin (PI3K) (Chase et al, 2008; Dudek et al, 2010; Eierhoff et al, 2010; Meneghesso et al, 2012; Müller et al, 2012; Munger et al, 2008; Zhou et al, 2009). In addition, functional analogues of known influenza inhibitors, such as erlotinib and gefitinib (analogues of PD153035), 17-DMAG (analog of 17-AAG), carfilzomib (analog of bortezomib), and leflunomide and gemcitabine (analogues of fluorouracil) were examined. The control was a v-ATPase inhibitor, SaliPhe, which was shown to block influenza virus replication *in vitro* and *in vivo* (Müller et al, 2011). A total of 201 small-molecule inhibitors were collected into the library (**I**, Suppl. table 1). Since most of these inhibitors are approved or are under clinical development for treatment of human malignancies, it was decided to screen the library in nonmalignant cells in order to avoid cancer-specific effects of drugs. A human

telomerase-expressing retinal pigment epithelial (hTERT RPE) cell line was selected since this line has been shown to be appropriate for influenza A virus replication (Michaelis et al, 2009). Additionally, two specific drug subsets were assembled targeting the anti-apoptotic signals, especially the Akt kinase and the B-cell lymphoma 2 (Bcl-2) family members. It is well established that influenza virus activates the PI3K/Akt/mTOR signaling pathway for efficient viral replication (Ehrhardt et al, 2006; Zhirnov & Klenk, 2007). However, none of Akt inhibitors had been tested in influenza virus-infected cells. Therefore, the first set to be examined contained inhibitors targeting Akt such as MK-2206, Akt inhibitor VIII, GDC-0068, and perifosine (**IV**). The second set consisted of ABT-263, ABT-737 and ABT-199, gossypol and TW-37 that target predominantly Bcl-2, B-cell lymphoma-extra large (Bcl-xL), and Bcl-2-like protein 2 (Bcl-w) proteins (**II**). It was hypothesized that chemical inhibition of Bcl-xL, Bcl-2 and Bcl-w could have an effect on the survival of virus-infected cells.

Our library and the drug subsets comprised highly specific small-molecule inhibitors targeting several well-described host factors and components of signaling pathways. The evaluation of this library and the subsets against influenza virus infection could provide information about the importance of these host factors both for virus infection and for cell survival. Moreover, this library and subsets could be tested against other virus infections to clarify the broad-spectrum antiviral properties of those compounds. Additionally, since the library included multiple drugs that are approved or are under clinical development for treatment of cancer or other diseases, it was deemed to verify the potential of these drugs for repositioning as antiviral agents.

4.1.2. Method for searching potential antiviral agents (I, II, IV)

After assembly of the library and the drug subsets a cell-based approach was developed which allowed identification of chemical probes to study virus-host interactions. In this method, increasing concentrations of small-molecule inhibitors were tested for their ability (i) to attenuate or accelerate death of infected cells, and (ii) to suppress or induce virus replication at concentrations that would not be toxic for noninfected cells (mock control). Cell survival and virus replication were measured by cell viability and plaque assays, respectively. Importantly, these techniques are suitable for virus infections that result in virus-induced CPE. In order to obtain high CPE values, MOI of 3 or higher was used. This approach was successfully used and its validity

confirmed against different influenza strains and other viruses in different BSL-2 and BSL-3 laboratories (**I**, **II**, **unpublished**).

4.1.3. Identification of hit compounds (I, II, IV)

The library or the subsets of Akt or Bcl-2 inhibitors was screened against the influenza A/PR8-NS116-GFP strain in hTERT RPE cells (**I**, fig. 1A), A/Helsinki/P14/2009 in MDCK cells (**IV**, fig. 1B), and A/WSN/33 in human PBMC-derived macrophages (**II**, Suppl. fig. 1B). Influenza A/PR8-NS116-GFP strain expresses the $NS1_{1–116}$-GFP fusion protein which means that it is possible to monitor the antiviral efficacy of the compounds by measuring virus-mediated GFP fluorescence and i.e. it can identify those compounds that target different stages of influenza infection ranging from virus attachment to viral protein synthesis and quality control. Compound cytotoxicity tests were performed in parallel. The screen revealed that obatoclax, SaliPhe and gemcitabine markedly attenuated virus-mediated GFP expression in a dose-dependent manner and promoted survival of influenza virus-infected cells (**I**, fig. 1B). The antiviral activities of these compounds are summarized in **Table 18.** Moreover, obatoclax, gemcitabine and SaliPhe significantly reduced virus production in hTERT RPE cells by more than 3 orders of magnitude (**I**, fig. 2B).

Table 18. Identified compounds and their antiviral activity against the influenza A/PR8-NS116-GFP strain.

Name	**Cellular target**	**EC_{50}**	**Clinical development**
Obatoclax (GX15-070MS)	Mcl-1, Bcl-2, Bcl-xL	0.014 μM	Phase I/II against cancer
SaliPhe	v-ATPase	0.254 μM	Not in clinical development
Gemcitabine (Gemzar®)	Ribonucleotide reductase	0.068 μM	Approved for cancer treatment
MK-2206	Akt	n.a.	Phase I/II against cancer
ABT-263 (Navitoclax)	Bcl-xL, Bcl-2, Bcl-w	0.106 μM	Phase I/II against cancer
ABT-737	Bcl-xL, Bcl-2, Bcl-w	n.a.	Phase II against cancer
ABT-199 (GDC-0199)	Bcl-2	n.a.	Phase II against cancer

n.a., not available.

When testing antiviral effects of the subset of Akt inhibitors it was found that MK-2206 rescued MDCK cells infected with the influenza A(H1N1)pdm09 strain (**IV**, fig. 1B). None of the other Akt inhibitors demonstrated antiviral effect. It was found that ABT-

263 and its structural analogues ABT-737 and ABT-199 induced apoptosis in influenza virus-infected cells in a dose-dependent manner (**II**, Suppl. fig. 1). In contrast, gossypol and TW-37 exerted no effect on the survival of influenza virus-infected cells. A non-toxic concentration of ABT-263 was then selected and the survival of mock- or influenza virus-infected cells was monitored at different time points post-infection. The results revealed that ABT-263 promoted premature apoptosis in influenza virus-infected cells and interfered with virus replication (**II**, fig. 1). Additionally, the apoptotic cell death was dependent on MOI.

Thus, two different sets of compounds were identified: (i) obatoclax, SaliPhe, gemcitabine and MK-2206 that rescued influenza virus-infected cells and reduced virus production, and (ii) ABT-263, ABT-737 and ABT-199 that promoted premature apoptosis in influenza virus-infected cells and also reduced virus titers. Obatoclax (GX15-070) is a BH3 mimetic which inhibits Mcl-1 in addition to blocking Bcl-2 and Bcl-xL (Nguyen et al, 2007). Obatoclax overcomes Mcl-1-mediated resistance to apoptosis and currently is in phase I/II trials for the treatment of chronic lymphocytic leukemia. SaliPhe is an investigational anticancer and anti-influenza compound, which has been reported to inhibit v-ATPase activity (Lebreton et al, 2008; Müller et al, 2011). Gemcitabine (Gemzar®) is a nucleoside analog, which is FDA approved for cancer treatment. There is a report that gemcitabine blocks cellular ribonucleotide reductase required for DNA replication and repair (Cerqueira et al, 2007). MK-2206 is a potent, oral allosteric inhibitor of Akt kinases, which inhibits auto-phosphorylation of both Akt T308 and S473 (Hirai et al, 2010). Currently MK-2206 is in phase I/II trials for cancer treatment. The two BH3 mimetics, ABT-263 (Navitoclax) and ABT-737, target the anti-apoptotic proteins Bcl-xL, Bcl-2 and Bcl-w but not Mcl-1 or A1 (Tse et al, 2008). A recently discovered BH3 mimetic, ABT-199 (GDC-0199), has been claimed to selectively inhibit Bcl-2 but not Bcl-xL (Vandenberg & Cory, 2013; Vogler et al, 2013). Currently ABT-263 and its structural analogues are undergoing phase I/II trials for the treatment of chronic lymphocytic leukemia.

Taken together, these results suggest that cellular v-ATPase, Akt kinase, ribonucleotide reductase and anti-apoptotic Bcl-2 family members, such as Mcl-1, Bcl-xL, Bcl-2 and Bcl-w, represent essential host factors for influenza virus infection.

4.2. Potential mechanisms of action of promising compounds (I, II, IV)

The mechanism of action of obatoclax, SaliPhe, gemcitabine, MK-2206 and ABT-263 was investigated by testing these inhibitors in a time-of-compound-addition experiment. Appropriate cells were infected with influenza virus, and the inhibitors were added every hour. It was found that obatoclax, SaliPhe and MK-2206 added at the time of infection could inhibit early steps of influenza virus infection (**I**, fig. 3A; **IV**, fig. 2D). It has been shown previously that SaliPhe inhibited v-ATPase that is needed for acidification of endosomes and release of vRNPs into the cytoplasm (Müller et al, 2011). Thus, one could postulate that obatoclax and MK-2206 also inhibit entry stages of influenza virus infection, whereas gemcitabine which targets cellular ribonucleotide reductase blocks influenza virus at the stage of viral RNA transcription and replication. Interestingly, ABT-263 induced apoptosis in influenza virus-infected cells independently of the time of its addition (**II**, fig. 3C). To address the stage of influenza virus infection when ABT-263 triggers the cell death a compound competition experiment was conducted with obatoclax, SaliPhe, gemcitabine and MK-2206. It was found that obatoclax, SaliPhe and MK-2206, but not gemcitabine, rescued ABT-263-treated cells from influenza virus-mediated death (**II**, fig. 3A and B; **IV**, fig. 2E). These results indicate that ABT-263 sensitized cells to undergo premature apoptosis at multiple stages of influenza virus infection following viral endocytic uptake.

Viral NP or M1 proteins were then monitored in an immunofluorescence assay at different time points. Immunofluorescence experiments revealed that obatoclax, SaliPhe and MK-2206 prevented accumulation of NP in the nucleus or M1 in the cytoplasm compared to non-treated or gemcitabine-treated influenza virus-infected cells (**I**, fig. 3B; **IV**, fig. 4G and H).

Next the production of viral RNAs and the synthesis of viral proteins were compared at different time points post-infection. The results demonstrated that obatoclax, SaliPhe, gemcitabine and MK-2206 substantially affected viral RNA transcription and replication and subsequent synthesis of viral proteins (**I**, fig. 3C and D; **IV**, fig. 2F). Since ABT-263 was able to induce premature apoptosis in influenza virus-infected cells already at 8 h post-infection, production of viral RNAs and proteins declined at the same time (**II**, Suppl. fig. 3).

Based on these results it was concluded that obatoclax, SaliPhe and MK-2206 block virus entry at a stage preceding vRNA uncoating, while ABT-263 sensitizes the

release of vRNPs from the endosome to the cytoplasm, and gemcitabine inhibits transcription of viral RNAs (summarized in **Figure 5**). Thus, host factors such as Mcl-1, v-ATPase and Akt kinase are necessary for endocytic trafficking and release of vRNPs into the cytoplasm, while Bcl-xL, Bcl-2 and Bcl-w sensitize the cell to viral RNA after virus release from the endosome, and ribonucleotide reductase is essential for viral RNA transcription and replication in the nucleus of the infected cell.

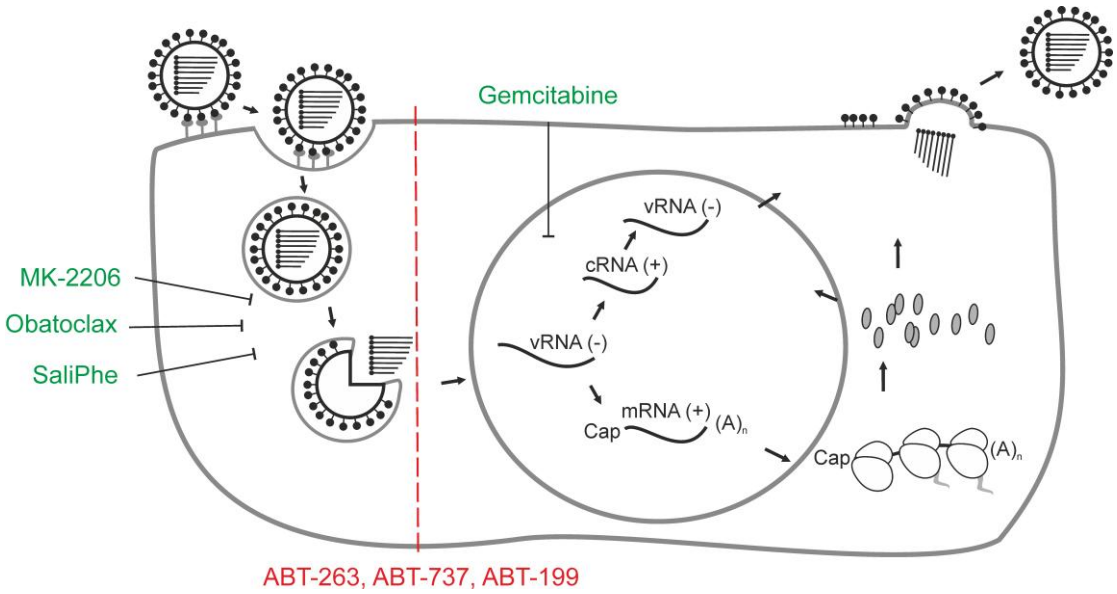


Figure 5. Schematic representation showing the stages of influenza virus replication cycle that could be blocked by obatoclax, SaliPhe, gemcitabine, MK-2206 or accelerated by ABT-263 and its structural analogues. Compounds marked with green rescue infected cells; those marked with red accelerate virus-induced apoptosis.

Obatoclax is a novel anti-influenza agent, whereas SaliPhe and gemcitabine analogues have been shown to possess antiviral activity (Meneghesso et al, 2012; Müller et al, 2011). Recently MK-2206 was demonstrated to exert an antiviral effect against HSV *in vitro* (Cheshenko et al, 2013). In order to prove that obatoclax target Mcl-1 is an essential host factor for influenza virus infection Mcl-1 was partially silenced in hTERT RPE cells by Mcl-1-specific siRNA and these cells were infected with the influenza A/PR8-NS116-GFP strain. At 24 h post-infection, the levels of influenza virus-mediated GFP expression and cell viability were analyzed. The results revealed that silencing of Mcl-1 substantially reduced influenza virus-mediated GFP expression and slightly affected the viability of the infected cells (**I**, fig. 4B). Taken together, these data suggest that cellular Mcl-1 is involved in both influenza virus infection and cellular apoptosis. In addition, it was demonstrated that Mcl-1 is

upregulated during the first hours of influenza virus infection (**I**, fig. 4C). Data from the immunofluorescence experiment revealed that Mcl-1 has a localization pattern similar to that of viral M1 (**I**, fig. 4D), indicating that Mcl-1 could be involved in virus recognition.

It has been shown that ABT-263 targets Bcl-xL, Bcl-2 and Bcl-w proteins at mitochondria in the cytoplasm and disrupts their interactions with Bcl-2 antagonist of cell death (Bad), Bcl-2-associated X protein (Bax) and Bcl-2 antagonist killer (Bak) proteins to initiate apoptosis in cancer cells (Tse et al, 2008). It was tested whether ABT-263 could exert these interactions in nonmalignant human cells infected with influenza virus. The immunofluorescence and immunoprecipitation (IP) experiments revealed that at non-toxic concentrations ABT-263 displaced Bad from Bcl-xL and mitochondria, and influenza virus facilitated this process (**II**, fig. 4 and 5).

Interactions of Bcl-xL, Bcl-2 and Bcl-w proteins are not limited to Bad, Bax and Bak. Bcl-xL has also been shown to interact with VDAC, Bim, DMN1L, Becn1, PGAM5, PUMA, p53, IKZF3, HEBP2, whereas Bcl-2 also interacts with APAF1 (apoptotic protease-activated factor 1), BBC3, BNIPL, MRPL41, TP53BP2, FKBP8, BAG1, RAF1, EGLN3 and G0S2 (Follis et al, 2013; Renault & Chipuk, 2013). In addition, the composition of Bcl-xL/Bcl-2 interactions could differ in different cell types. Based on IP and mass-spectrometry data it was confirmed that in hTERT RPE cells Bcl-xL could interact with Bad, Bax and Bak and also it was found to have novel interacting partners, such as UACA (uveal autoantigen with coiled-coil domains and ankyrin repeats), FLII (protein flightless-1 homologue), LRRFIP2 (leucine-rich repeat flightless-interacting protein 2), TOLLIP (Toll-interacting protein), TRIM21, H2B (histone H2B), DHX9 (ATP-dependent RNA helicase A), 14-3-3, PAWR, NUAK1, DAPK1, various cytoskeleton proteins and viral HA, M1, NS1, NP proteins (**II**, Suppl. table 1). The Bcl-xL interactions with pattern recognition receptors, such as LRRFIP2, H2B and DHX9, indicate that Bcl-xL is potentially involved in the sensing of viral nucleic acids, and the Bcl-xL interactions with FLII, TOLLIP and LRRFIP2 indicate that it could be also involved in TLR4 (Toll-like receptor 4) signaling cascade. Thus, novel interacting partners for Bcl-xL, and possibly for Bcl-2 and Bcl-w, were investigated.

The disruption of Bcl-xL, Bcl-2 and Bcl-w interactions with newly identified partners might contribute to ABT-263-mediated death of infected cells. It was examined whether ABT-263 had any effect on the interactions between Bcl-xL and its newly

identified protein partners in mock- and influenza virus-infected cells. SDS-PAGE analysis of immunoprecipitated Bcl-xL-interacting proteins detected differences in protein composition of IPs (**II**, fig. 5B). Mass-spectrometry and immunoblot analysis of protein candidates showed that ABT-263 in combination with influenza virus could displace UACA, Bax and Bad from Bcl-xL (**II**, fig. 5A and B). Thus, it is concluded that ABT-263 alters the composition of Bcl-xL, and perhaps Bcl-2 and Bcl-w, interactions in influenza virus-infected cells.

Since Bcl-2 proteins regulate apoptosis, the activation of caspase-8, -9, and -3/7 in response to ABT-263 treatment and influenza virus infection was monitored in hTERT RPE cells. It is well known that apoptosis can be activated by extrinsic and intrinsic stimuli that lead to activation of caspase cascades. Extrinsic stimuli activate pro-caspase-8, which in turn activates pro-caspase-3. The intrinsic pathway (also known as the mitochondrial pathway) is triggered when Bax is relocated to the mitochondria leading to cytochrome *c* release. Cytosolic cytochrome *c* interacts with APAF1 which activates pro-caspase-9 and pro-caspase-3. Caspase-3 is the terminal enzyme in both extrinsic and intrinsic pathways (Cullen & Martin, 2009).

It was observed that a combination of ABT-263 and influenza virus infection resulted in enhancement of caspase-8, -9, -3 and -7 activities, and this phenomenon coincided with the decline of cell viability (**II**, fig. 6A and B). In influenza virus-infected cells activated caspases cleaved their substrates, such as Bid, and disrupted basic cellular pathways and functions, that resulted in inhibition of transcription and translation of cellular and viral mRNAs and reduction in production of infectious virions (**II**, Suppl. fig. 3). Thus, ABT-263 is able to modulate Bcl-xL, and perhaps Bcl-2 and Bcl-w interactions, and this activates caspase-8, -9 and -3/7 to trigger apoptosis in influenza virus-infected cells. Interestingly, in hTERT RPE cells transfected with influenza virus genomic RNA, ABT-263 was also able to stimulate caspase-9 and -3/7 activity and weakly caspase-8 (**II**, fig. 6C). However, the latter effect was insufficient to trigger cell death (**II**, fig. 6D). Thus, these results indicate that ABT-263 promotes premature apoptosis only in response to replicating virus.

4.3. Effect of compounds on influenza virus-mediated cellular antiviral and pro-inflammatory responses (I, II, IV)

The innate immune response is crucial in antiviral defence and is associated with production of cytokines by the infected cell; the immune response is important in intercellular signaling and communication. The cytokines are a diverse group of small proteins which include interferons (IFN-α, IFN-β, IFN-γ, and IFN-λ), interleukins (e.g. IL-1α, IL-1β, IL-18), chemokines (about 44 members), colony-stimulating factors (CSF; GM-CSF, G-CSF) and TNF (Tisoncik et al, 2012). The cytokines are responsible for development of pro-inflammatory (IL-1, TNF, IL-6 and IL-12) and anti-inflammatory (IL-10, IL-4 and TNF-β) responses in virus-infected and in neighboring cells. Several studies have described a number of drugs that could reduce the production of pro-inflammatory cytokines, and thus these compounds could potentially be used for influenza treatment either on their own or in combination with licensed antivirals. For instance, gemfibrozil has been shown to inhibit the production of pro-inflammatory cytokines and to increase the survival of mice infected with influenza A (H2N2) virus (Budd et al, 2007). Combinations of zanamivir and inhibitors of inflammation, such as celexoxib and mesalasine, also increased the survival of mice infected with influenza A (H5N2) virus (Carey et al, 2010).

The next question asked was whether the identified inhibitors could suppress influenza virus-mediated antiviral and pro-inflammatory responses. It was found that obatoclax, SaliPhe and gemcitabine suppressed influenza virus-mediated production of pro-inflammatory cytokines including IP-10 (IFN-γ-induced protein 10; also CXCL10), CXCL1 (C-X-C motif chemokine 1; also GROα), IL-6, IL-8, CCL2 (also MCP-1), and CCL5 (also RANTES) (**I**, fig 5). Surprisingly, obatoclax inhibited, whereas SaliPhe and gemcitabine stimulated, the production of antiviral IFN-β and IFN-λ. These results indicate that obatoclax, in contrast to SaliPhe and gemcitabine, could prevent activation of cellular antiviral responses, most probably, via suppression on the Mcl-1-type I IFN signaling axis.

The effect of MK-2206 on cellular signaling pathways was also investigated. Phospho-proteome profiling revealed that MK-2206 treatment abrogated influenza virus-mediated decrease phosphorylation of Akt (T308), HSP60 and p53 (S392), and increase phosphorylation of Akt (S473), HSP27 (S78/S82), and c-Jun (S63) at 12 h post-infection (**IV**, fig. 4B). A whole-genome expression analysis and cytokine/

chemokine profiling showed that MK-2206 reduced influenza virus-mediated transcription of 23 out of 70 antiviral and pro-inflammatory genes (log fold change cut off > 2 and < -2; **IV**, fig. 5) and production of cytokines, including IP-10 (also CXCL10), IL-6, IL-8, GM-SCF (granulocyte-macrophage colony-stimulating factor; also CSF-2), IL-1α, IL-1ra (interleukin-1 receptor antagonist), and CCL5 (**IV**, fig. 6). Thus, MK-2206 prevents the development of cellular immune responses in influenza virus-infected cells. It is essential to note, that MK-2206 regulates expression of LPIN1, RASD1, DHCR7, HMGCR, SC4MOL, HMGCS1, SQLE, and ABCA1 genes (log fold change cut off > 1 and < -1) involved in lipid biosynthesis pathways. Thus, MK-2206 could modulate lipid metabolism.

The analysis of cytokine production in ABT-263 treated cells revealed that ABT-263 attenuated the influenza virus-mediated production of IP-10, IL-6, IL-8, CXCL1, CCL2, CCL5, IFN-β and IFN-λ, but stimulated the production of IL-1ra. Similar results were obtained with ABT-737 and ABT-199 (**II**, Suppl. fig. 4). Interestingly, ABT-263 treatment was ineffective in inhibition of CCL5, IL-6, IL-8, IFN-β and IFN-λ production in cells transfected with influenza virus genomic RNAs. Thus, it is concluded that ABT-263 induces premature apoptosis which limits the development of cellular antiviral and pro-inflammatory responses. Importantly, ABT-263 lowered the survival rate of influenza virus-infected mice by disrupting the host innate response (**II**, fig. 7). It is proposed that the imbalance likely resulted from an inability of the immune system to clear the virus, eventually leading to the death of the influenza virus-infected and ABT-263-treated mice. Therefore, ABT-263 was excluded from further studies.

4.4. Spectrum of antiviral activities of compounds (I, IV, unpublished)

Since different viruses use the same host factors for efficient replication, development of host-directed antivirals with broad-spectrum potential is a very promising strategy. For instance, it has been demonstrated that the protease inhibitors camostat, nafamostat, gabexate and aprotinin are active against negative-stranded RNA viruses including influenza types A and B, MeV, respiratory syncytial virus and parainfluenza virus type 3 (Hosoya et al, 1992). Moreover, the sterol metabolite 25HC was reported to inhibit a broad range of enveloped RNA and DNA viruses by blocking the membrane fusion between the virus and the cell (Liu et al, 2013b).

The possibility that Mcl-1, v-ATPase, ribonucleotide reductase and Akt could be antiviral targets for a broad range of influenza viruses was tested by examining the effects of obatoclax, SaliPhe, gemcitabine and MK-2206 against influenza A (H3N1, H1N1, H1N1pdm09, H5N1, and H7N9) and B viruses (**I, IV**). The results which are summarized in **Table 19** indicate that obatoclax and SaliPhe do exert antiviral activity against a broad range of influenza A and B viruses (**I**, table 1), while MK-2206 predominantly inhibits replication of influenza A(H1N1)pdm09 viruses as well as A/WSN/33(H1N1) at high concentrations (**IV**, fig. 3A and B). Gemcitabine activity could be limited to certain influenza strains. Moreover, it was found that obatoclax and SaliPhe exhibited antiviral activity in different cell types, whereas gemcitabine activity was cell-type specific (**I**, Suppl. fig. 1).

Table 19. Viruses tested *in vitro* for their susceptibility to selected inhibitors.

Virus name	Obatoclax	SaliPhe	Gemcitabine	MK-2206
A/WSN/33(H1N1)	+	+	+	+
A/PR/8/34 (H1N1)	+	+	+	–
A/Helsinki/P14/2009 (H1N1pdm09)	n.a.	+	n.a.	+*
A/Udorn/307/1972 (H3N2)	n.a.	+	n.a.	–
A/Sydney/5/1997(H3N2)	+	+	+	–
A/Chicken/Nigeria/BA211/2006 (H5N1)	+	+	–	–
A/Anhui/01/2013 (H7N9)	+	–	–	–
B/Shandong/7/97	+	+	+	–
BUNV	+	+	–	+
MeV	–	–	–	n.a.
SINV	+	+	+	n.a.
SFV	+**	+**	+	–
Echo6	+	–	+	-
VACV	–	–	–	n.a.
HSV-1	–	–	+	–

"+" possess antiviral activity, "–" no antiviral activity; * antiviral activity against other influenza A(H1N1)pdm09 strains listed in **Table 16**, except A/Helsinki/552/2013 (H1N1pdm09) strain that is insensitive to MK-2206 treatment; ** no reduction of virus titers; n.a., not available.

To examine whether these host factors are essential for other virus infections, the same inhibitors were tested against four enveloped RNA viruses (BUNV, MeV, SINV, SFV), two enveloped DNA viruses (HSV-1 and VACV) and one non-enveloped RNA virus (Echo6) (**Table 19**). Of these, SFV is similar to influenza virus in that it is known to utilize endocytosis to enter the cell. It was found that obatoclax efficiently attenuated infections and reduced virus production of BUNV, SINV and Echo6, whereas SaliPhe

suppressed infection of BUNV and SINV. Obatoclax and SaliPhe were able to rescue SFV-infected cells, however no reduction in the numbers of infectious virus particles was observed. Moreover, gemcitabine suppressed HSV-1, SINV, SFV and Echo6 virus infections.

In summary, SaliPhe and obatoclax exerted antiviral activity against a broad range of influenza and some other RNA viruses. Obatoclax was toxic to cells during prolonged incubation periods and, for this reason, was excluded from further studies. Thus, subsequent studies focused on SaliPhe as the most potential antiviral agent. However, it will be interesting to test the antiviral potential of another selective inhibitor of Mcl-1, maronopyrrole A (maritoclax), which has higher specificity and better pharmacological properties in comparison to obatoclax (Doi et al, 2012).

4.5. Development of resistance (unpublished)

The increasing clinical use of licensed antivirals has been associated with the global emergence of amantadine- and/or oseltamivir-resistant influenza strains (Hayden & de Jong, 2011; Zhou et al, 2013). Resistance results from both antigenic drift and antigenic shift events specific for influenza viruses. Therefore, current prevention options for influenza infections are limited and there is a need for developing next-generation antiviral agents. Although, it was emphasized above that targeting host factors essential for virus biology would be associated with a decreased likelihood of resistance emergence, examples of virus resistance to potential host-directed antivirals have been described in the literature (Qing et al, 2010; Triana-Baltzer et al, 2009).

The next experiment assessed whether continued inhibition of cellular v-ATPase by SaliPhe could lead to the emergence of resistant influenza virus variants by conducting a serial-passage experiment. During passaging the concentration of SaliPhe was gradually increased. It is argued that this strategy does provide enough time for virus resistance to emerge. A total of 15 passages were performed and the virus titer determined after each passage. The results showed that the virus titer in the presence of SaliPhe (0.35 μM, passage #8) decreased, and was not restored in further passages (**Figure 6**). These results confirm the hypothesis that the influenza A/Helsinki/P14/2009 strain cannot efficiently adapt to selective pressure associated with the reduction of v-ATPase activity. Thus, it is concluded that SaliPhe is a promising antiviral agent exhibiting a high-barrier to case resistant virus variants.

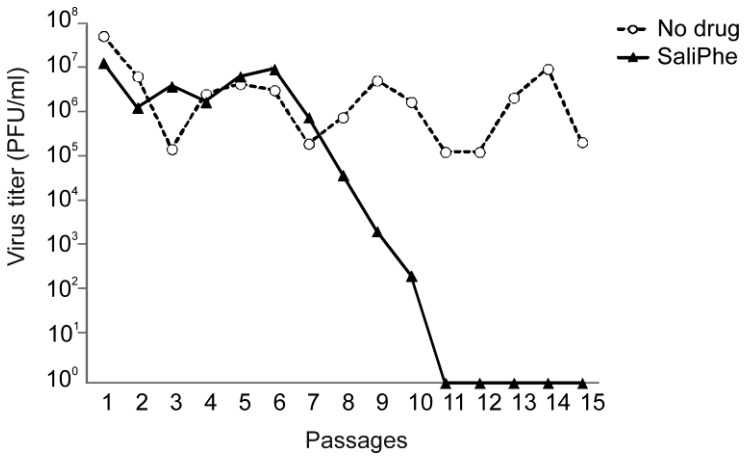


Figure 6. Influenza A/Helsinki/P14/2009 virus titers from the serial-passage experiment on NCI-H1666 cells.

4.6. Optimization of SaliPhe clinical utility (III)

SaliPhe is a small-molecule inhibitor of v-ATPase, a potential target for antitumor chemotherapy (Lebreton et al, 2008). Moreover, on particle-induced osteolysis *in vivo* SaliPhe was shown to inhibit bone destruction and suppress inflammatory response (Qin et al, 2012). In addition, SaliPhe is a potential antiviral drug. It has been shown previously that SaliPhe increases the survival rates of mice infected with lethal doses of a mouse-adapted influenza strain (Müller et al, 2011). However, SaliPhe has a low water solubility and high toxicity that are major bottlenecks for the evaluation of this compound in the clinic (Müller et al, 2011).

In an attempt to increase the solubility and bioavailability of SaliPhe and to reduce side-effects, it was decided to evaluate the PSi nanoparticle-based delivery system that encompasses several advantageous properties for drug delivery. It has been shown that PSi-based materials increase dissolution of otherwise poorly water-soluble drugs loaded within its mesopores (Bimbo et al, 2011; Salonen et al, 2008). SaliPhe was loaded into thermally hydrocarbonized porous silicon (THCPSi) nanoparticles with a loading degree 2.88±0.11 wt% and then the drug releasing properties of SaliPhe-loaded THCPSi nanoparticles were investigated. It was observed that at pH 7.4 THCPSi nanoparticles released approximately 30% of the loaded SaliPhe during the first 15 minutes (**III**, fig. 3). In contrast, the pure form of SaliPhe could achieve only a 5% dissolution within the same time period. Importantly, the THCPSi nanoparticles allowed the delivery of SaliPhe in the absence of DMSO, and they increased the drug dissolution rates in comparison to the pure drug.

The SaliPhe-loaded THCPSi nanoparticles were further investigated for their ability to inhibit the influenza A/PR8-NS116-GFP virus infection *in vitro*. It was observed that SaliPhe-loaded THCPSi nanoparticles like SaliPhe dissolved in DMSO efficiently inhibited influenza virus-mediated GFP expression and reduced virus titers by more than two orders of magnitude (**III**, fig. 5 and fig. 7). In addition, SaliPhe-loaded THCPSi nanoparticles exerted less cytotoxic effects than SaliPhe dissolved in DMSO (**III**, fig. 4 and fig. 5). Thus, the therapeutic window of SaliPhe-loaded THCPSi nanoparticles was wider in comparison with SaliPhe dissolved in DMSO.

This data provides the first proof-of-principle of the potential of THCPSi nanoparticles to delivery antiviral agent to virus-infected cells and to inhibit the infection. Optimization of administration routes, such as inhalation or intravenous injection, is currently envisioned, and further preclinical evaluation is warranted for the development of PSi nanoparticles-based delivery systems as a potential platform for targeted delivery of antiviral compounds *in vivo*.

5. FUTURE DIRECTIONS

Currently, the usage of licensed anti-influenza drugs is limited due to the globally circulating drug-resistant influenza strains. It is essential to develop of new potential antivirals targeting cellular host factors or components of signaling pathways in order to control and treat future influenza virus epidemics and pandemics. Host-directed antiviral agents represent a very attractive approach because the likelihood of resistance emergence is very low and host-directed antivirals might possess broad-spectrum antiviral properties.

The drug screening described in Study **I** focused on the cellular requirements for virus entry, uncoating, nuclear import, and viral RNA transcription/translation. Another screen was performed against wild type influenza A and B viruses in order to identify host factors involved in other stages of influenza virus in A549 cells. The screen revealed compounds identified in the previous screen (**I**) and a number of potential antiviral-agents (unpublished). The hit list and EC_{50} values are presented in **Table 20**. Additionally, their antiviral activity was tested against avian influenza A (H5N1 and H7N9) strains. It is clear that further investigation of their mechanism of action and their antiviral efficacy *in vivo* is needed.

Table 20. Identified compounds and their antiviral activity against influenza infection.

Name	**Target**	**EC_{50} (μM)***			
		A(H1N1)[a]	A (H5N1)[b]	A(H7N9)[c]	B[d]
Flavopiridol (Alvocidib)	CDK	0.60	0.34	0.47	0.50
SNS-032 (BMS-387032)	CDK	0.32	0.38	1.00	0.50
Dinaciclib (SCH727965)	CDK	0.12	0.04	0.04	0.04
DCC-2036 (Rebastinib)	Abl	0.04	–	0.04	2.50
PIK-75	PI3K	0.08	0.08	–	0.13
Homoharring-tonine	60S ribosome	–	–	–	0.12

* values obtained on A549 cells; "–" no antiviral activity;
[a] A/WSN/33; [b] A/Chicken/Nigeria/BA211/2006; [c] A/Anhui/01/2013; [d] B/Shandong/7/97.

6. CONCLUSIONS

A screening approach was developed to identify druggable host factors involved in influenza virus replication and it was efficiently used to reveal cellular Mcl-1, v-ATPase, ribonucleotide reductase, Akt kinase and Bcl-xL. The detailed molecular mechanisms of action of potential antiviral agents, such as obatoclax, SaliPhe, gemcitabine, MK-2206 and ABT-263, targeting these host proteins, have been elucidated. These potential antiviral agents demonstrated a broad spectrum of antiviral activities *in vitro*. However, ABT-263, an inhibitor of Bcl-2 family proteins, was ineffective *in vivo*. SaliPhe showed the greatest as an antiviral agent with activity against a broad range of influenza A and B viruses and some other RNA viruses *in vitro* and against a mouse adapted influenza strain *in vivo*. In order to overcome a low water solubility and high toxicity of SaliPhe, its bioavailability was improved using a porous silicon particle-based delivery system for use in future clinical trials. The results presented in this thesis expand the understanding of virus-host interactions, and provide a novel perspective for ways to adopt a rational approach in the discovery of new antiviral agents.

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