Master's thesis

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Commonly prescribed medicines interfere with cellular response to influenza A virus infection

Master's thesis in pharmacy
Supervisor: Denis E. Kainov
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Abstract

Background
Every year, millions of people are infected with influenza A viruses (FLUAV). Many of these people have underlying chronic diseases, such as such as arthritis, diabetes and hypertension, thus are reliant on medical treatment. Medicines can suppress immune responses, enhance pain sensitivity and disturb behavior during infections. However, such immune- and neuro-modulating effects remain elusive for many commonly prescribed medicines.

Aim
The aim of this thesis is to examine if commonly prescribed medicines could interfere with cellular responses to influenza virus infection.

Method
We identified 45 medicines commonly prescribed in Central Norway and tested their effect on viability, transcription and metabolism of mock- and A/WSN/33(H1N1)-infected retinal pigment epithelial (RPE) cells and viral replication. A drug-target interaction network of the compounds was constructed to identify potential implication for FLUAV-host cell interaction.

Results
Cell viability assay revealed that non-toxic concentration (10 μM) of 45 compounds were ineffective against FLUAV.

We found that the drugs differently affected cellular gene transcription and metabolism of mock- and FLUAV-infected cells. Furosemide, for instance, induced viral gene expression most, whilst metformin was the only compound exhibiting excessive down-regulation of one viral gene, PB1-F2.

Most compounds displayed down-regulation of hypoxanthine, guanosine and d-ribose 5-phosphate.

The drug-target interaction network showed that many compounds, such as losartan, ramipril, valsartan and cetirizine could target and modulate FLUAV-host cell interaction through various cellular pathways.

Conclusion
Most cases of hospitalizations and death due to influenza occur among the elderly. Medicines used for treatment of underlying conditions could modulate virus-host interactions and either attenuate or accelerate the disease. Here, we shed new light on the mechanisms of action of the selected compounds, which after further experiments in vitro and in vivo, can be used to reduce the mortality, maximize the number of healthy life years, and improve the quality and cost-effectiveness of medical care.

Sammendrag

Bakgrunn
Hvert år blir millioner av mennesker smittet med influensa A-virus. Mange av disse menneskene har underliggende kroniske sykdommer, slik som leddgikt, diabetes og høyt blodtrykk, og er derfor avhengige av medisinsk behandling. Legemidler kan undertrykke immunresponsen, øke smertefølsomheten og påvirke ulike effekter ved infeksjon. Slike immun- og nevromodulerende effekter er derimot ukjent blant mange av de mest foreskrevne legemidlene.

Mål
Målet med denne oppgaven var å undersøke om kjente forskrevne legemidler kunne forstyrre den cellulære responsen ved en influensa infeksjon.

Metode
Vi identifiserte de 45 mest forskrevne legemidlene i Midt-Norge og testet deres effekt på celledødelighet, transkripsjon og metabolisme av mock- og A/WSN/33 (H1N1)-infiserte ritnale pigment-epitel (RPE) celler og viral replikasjon. Et interaksjonsnettverk mellom forbindelsene og mulige målmolekyller ble konstruert for å identifisere mulig interaksjon mellom influensa A-virus og vertscellene.

Resultater
Cytotoksisitets- og effektanalysene viste at ikke-toksiske konsentrasjoner (10 μM) av de 45 forbindelsene var virkningsløse mot FLUAV.

Forbindelsene påvirket cellulær transkripsjon av gener og metabolisme av mock- og FLUAV-infiserte celler forskjellig. Blant annet påvirket furosemid genuttrykket mest, mens metformin var den eneste forbindelsen som viste kraftig nedregulering av ett viral gen, PB1-F2.

De fleste forbindelsene viste nedregulering av hypoksantin, guanosin og d-ribose 5-fosfat.

Interaksjonsnettverket viste at mange av forbindelsene, slik som losartan, ramipril, valsartan og cetirizin, kunne påvirke og modulere virus-vertscelle-interaksjonen gjennom ulike cellulære veier.

Konklusjon
De fleste tilfeller av sykehusinnleggelser og død grunnet influensa, forekommer blant eldre. Legemidler som brukes til behandling av underliggende sykdommer kan modulere virus-vert-interaksjoner og enten dempe eller akselerere sykdommen. Her kaster vi nytt lys over virkningsmekanismene til de valgte forbindelsene, som etter ytterligere eksperimenter in vitro og in vivo, kan brukes til å redusere dødelighet, maksimere antall sunne leveår, og forbedre kvaliteten og kostnadseffektiviteten til medisinsk behandling.

Acknowledgements

This thesis marks the end of my Master of Science studies in Pharmacy - and was carried out at the Department of Clinical and Molecular medicine (IKOM), Faculty of Medicine and Health Science (MH), at the Norwegian University of Science and Technology (NTNU) in Trondheim, from February 2019 till June 2021.

During this time, I became mother to my lovely daughter Leah. It has been a wonderful experience to finish off my studies, and at the same time, becoming a parent. This would not be possible without my boyfriend, Jørn. Thank you for all the help.

Starting off this thesis, the ongoing pandemic had not yet shed light over us, however, I feel this thesis has made me more aware of possible, but not wanted, pandemics in the future, as well as the importance of vaccines to protect high-risk groups.

I wish to thank everyone involved in this, and especially Dr. Denis Kainov and Aleksandr Ianevski. Without you, and your patience with me, I would never have landed with my studies. Thank you so much.

I would also like to thank my best friend, Carolina, for motivating me throughout the project.

Hege Ramstad,

June 2021.

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List of Abbreviations

ADR	Adverse drug reaction
ATC	Anatomic, therapeutic, chemical-classification system
CC50	Half-maximal cytotoxic concentration
CDC	Centers for Disease Control and Prevention
CTG	Cell Titer Glow assay
CXCLs and CCLs	Chemocines
DDD	Daily defined dose
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide solution
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
EC50	Half-maximal effective concentration
ECE	Embryonated hen eggs
FBS	Fetal bovine serum
FHI	Norwegian Institute of Public Health (no: Folkehelseinstituttet)
FLUAV	Influenza A virus
FLUBV	Influenza B virus
HA	Hemagglutinin
H1N1	Specific subtype of FLUAV, expressing hemagglutinin type 1 and neuraminidase type 1
IFN	Interferon
ISG	Interferon-stimulated genes
M1	Matrix 1 protein
M2	Matrix 2 protein
MDCK	Madin Darby Canine Kidney cells
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NCD	Non-communicable disease
NEP/NS2	Nuclear export protein

NS1	Non-structural protein 1
PA, PB1, PB2	Subunits of influenza virus polymerase complex
PRR	Pattern recognition receptor
PB1-F2	Alternate reading frame of PB1-gene
RNase 1	Ribonuclease L
RNA	Ribonucleic acid
RPE	Retinal pigment epithelial cell
SA	Sialic acid
SaliPhe	Saliphenylhalamide
vRNA	Viral ribonucleic acid
vRNP	Viral ribonucleoprotein
OAS	2’-5’-oligoadenylate synthase family
WHO	World Health Organization

1. Introduction

Circulating in all parts of the world and being the most common cause of seasonal epidemics, both influenza virus A (FLUAV) and influenza virus B (FLUBV) annually causes millions of acute respiratory infections in humans worldwide, accompanied by three to five million cases of hospitalizations and 250.000–500.000 deaths, consequently affecting both the public health and global economy (1-4). Opposed to FLUBV, only FLUAV is known to have caused pandemic outbreaks due to its ability to cross the interspecies barrier, where a novel subtype emerges from an animal origin further transmitting to humans (4).

Anyone is susceptible to become infected with influenza, and the most commonly described symptoms are fever, sore throat, runny nose, cough, headache, muscle- and joint pain, fatigue and inflammation of upper and lower respiratory tracts (1-4). A successful recovery largely depends on an efficient functioning of both the immune- and nervous-system. Although symptoms range from mild to severe, most cases of hospitalizations and deaths occur among high-risk groups, including pregnant women, young children, the elderly, patients with immunosuppressive conditions and patients with non-communicable diseases (NCDs), where the risk of severe diseases or complications during infection is elevated (1, 3, 4). The latter, being the interest of this thesis, is also known as chronic diseases, such as asthma, diabetes, cardiovascular- and chronic kidney-disease (5). For instance, in patients with asthma, a possible influenza infection may cause further inflammation and contraction of the already inflamed and swollen airways, leading to severe asthma attacks and worsening of the patients' asthma symptom control. An infection could also elevate the risk of developing secondary infections such as pneumonia and other acute respiratory diseases, which may lead to hospitalization (6).

Pursuant to the World Health Organization (WHO), NCDs tend to last for a long period of time, perhaps even lifelong, due to a combination of genetic, physiological, environmental and behavioral factors, possibly leading to premature death and reduced life quality (5). Fortunately, medicines to treat NCDs are available, mostly even in low-cost generic forms (7). The medicines safety, quality and efficacy are strictly regulated to ensure safe medical treatment through the pharmaceutical industry (8). However, any substance with a therapeutic effect may give rise to unwanted adverse effects (ADRs). Both healthcare professionals and consumers can spontaneously report ADRs at any time, thus strengthening a drug's safety profile. However, some ADRs may be unidentified during which time they are reported, even long after hitting the public market (9).

Medicines prescribed for treatment of NCDs may modulate viral replication as well as the body's immune- and neurological-responses to infection. Such immuno- and neuro-modulating effects still remain elusive for many marketed medicines (10). To identify possible immuno- and neuro-modulating effects of the most prescribed medicines in Central Norway we used an in vitro approach, which has been developed by Söderholm, S. et. al., 2016 (11-12). We identified eight drugs which interfere with FLUAV-host cell interactions, including transcription of cellular and viral genes and cellular metabolism.

1.1 FLUAV structure

There are four types of seasonal influenza viruses (A, B, C and D) (1), however, only FLUAV and FLUBV are of clinical relevance to humans (1, 2, 4). We will focus on FLUAV. The genome of FLUAV and its protein functions is already described in previous publications (4, 13, 14). However, the most predominant key-facts will be reiterated here. Figure 1.1 illustrates the structure of FLUAV and where the proteins are located, whilst table 1.1 lists the protein functions. Notably, not all proteins are expressed in virions, but only in already infected host cells (4).

FLUAV belong to the family Orthomyxoviridae, characterized by being segmented, negative-sense single-strand RNA segments (vRNA) (4). The genome consists of eight vRNAs, each encoding for at least one protein (4, 14). Two of the gene segments encode pre-RNAs that produce nonstructural protein 1 (NS1) and alternatively, by mRNA-splicing, nuclear export protein (NEP/NS2) and matrix M1/proton channel M2 protein. These proteins are only expressed in host cells (4). The NS1 protein is highly expressed in infected cells and has multiple functions during viral replication, including functioning as an interferon (IFN) antagonist, thereby countering the IFN-induced proteins released by the host cell, whose aims are to inhibit viral replication, triggering the immune system and alarming neighboring cells (4, 15), see section 1.2. NEP/NS2 protein enables nuclear export of viral ribonucleoprotein (vRNP) complexes (4).

FLUAV are enveloped viruses, meaning they are coated with a lipid bilayer. Located underneath the lipid bilayer, the M1 protein forms a matrix layer, binding the vRNPs (4, 14). The transmembrane M2 protein traverse through the lipid bilayer, allowing ions to cross the membrane (14). Four gene segments encode mRNAs translating into nucleoproteins (NP) – forming a major component of the vRNP complex – and the polymerase subunits PA, PB1 and PB2 (4). An alternate reading frame of the PB1 gene gives PB1-F2. PB1-F2 is only expressed in infected host cells, and studies have shown that PB1-F2 has pro-apoptotic activity, targeting the host's mitochondrial inner membrane, thus enhancing virus-induced cell death (4, 14).

The remaining two gene segments encode mRNAs which translates into the transmembrane proteins hemagglutinin (HA), a surface glycoprotein, and neuraminidase (NA), an enzyme that cleaves sialic acid (SA) groups from glycoproteins (4, 13, 14). FLUAV express different subtypes of HA and NA based on their structure, all of which are potential antigens to the host (2, 4). Currently, there are 18 known subtypes of HA (H1-18) and 11 subtypes of NA (N1-11). Yet, only a limited number of the subtypes (H1, H2, H3 and N1, N2) are capable of infecting humans, such as H1N1 (4, 14). Vaccines are developed based on different subtypes of HA and NA, see section 1.3.2.

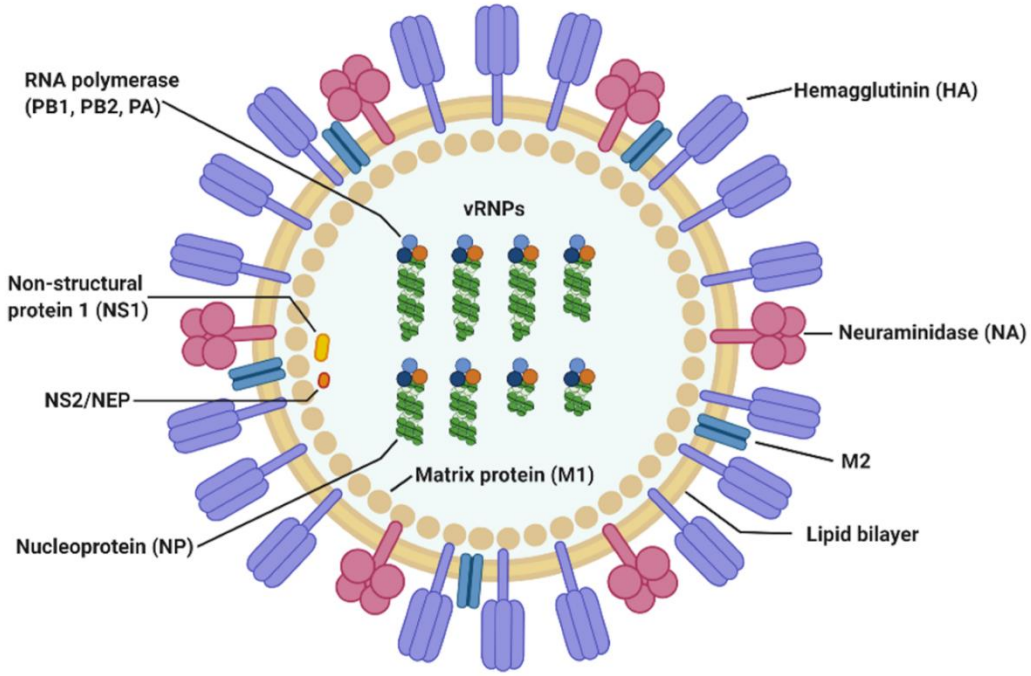

Figure 1.1 Structure of influenza A virus

The genome of FLUAV consist of eight single-stranded viral RNA segments, each encoding for at least one protein (HA, NA, M1, M2, NP, NS1, NS2, PA, PB1, PB2, and PB1-F2). HA, NA and M2 are transmembrane proteins, located in the viral envelope (lipid bilayer). M2 and NS2/NEP are products of spliced mRNAs of M1 and NS1. An alternate reading frame of PB1 gives PB1-F2 (not shown). NS1, NEP and PB1-F2 are only expressed in infected cells (4). Figure retrieved from (16).
***FLUAV**: influenza A virus*

Table 1.1 The encoded proteins of FLUAV and their functions

Encoded proteins	Protein function(s)
HA	Surface glycoprotein: recognizes SA-receptors of the host cell and fuses the viral envelope with the target cell.
M1	Matrix protein: binds vRNPs underneath the lipid envelope
M2	Ion channel: allows proteins to pass through the lipid envelope and facilitates the process of uncoating vRNPs
NA	Surface glycoprotein: involved in the process of budding
NEP/NS2	Nuclear export of RNA
NP	RNA binding protein: nuclear import regulation
NS1	Interferon antagonist protein: regulates host gene expression
PA	Polymerase subunit
PB1	Polymerase subunit
PB1-F2	Pro-apoptotic activity
PB2	Polymerase subunit

The encoded proteins of FLUAV and their functions. PA, PB1 and PB2 have different functions in the polymerase complex. Their functions are not discussed here. HA and NA are recognized by the immune system as foreign particles, resulting in antibody protein production (4, 14).
Table retrieved and modified from (13).
***SA**: sialic acid, **vRNPs**: viral ribonucleoproteins*

1.2 Replication cycle of FLUAV and host dependent factors

To undergo viral replication and reproduction, FLUAV must enter a host cell and release its genome (4, 14). FLUAV is highly infectious and spreads mainly through droplets made when an infected person sneezes, speaks or coughs, and less often through contact-surfaces (1, 4, 17). As FLUAV replicates, mutations can occur every now and then in the genes encoding the main human antigenic proteins HA and NA (18). Minor changes are termed genetic drift and occur continuously, whereas accumulation of genetic drift can allow emerging viruses to infect already immune protected hosts, although they have been previously infected or vaccinated (4, 14, 18). The occurrence of genetic drift is the main target for anti-FLUAV vaccines (18), see section 1.3.2. Greater mutations which result in a new subtype, possibly having a mixture of HA and NA of the two or more original strains, is called genetic shift, conceivably giving rise to new pandemic outbreaks (2, 4). Figure 1.2.2 illustrates examples of host factors involved in FLUAV replication, whilst figure 1.2.3 summarizes the replication cycle as well as cellular antiviral responses.

1.2.1 Viral replication

When transmitted, the first step of viral replication happens as HA recognizes receptors with SA expressed in human epithelial cells of the retinal or respiratory tract, or other immune cells, such as dendritic cells, type II pneumocytes or alveolar macrophages (4). When HA binds to SA, the viral envelope fuses with the host cell's membrane, triggering endocytosis (4, 14). At this stage, the virus is contained within an endosome, still cut off from the cytoplasm of the host cell and cannot undergo viral replication (4, 19). However, as the endosome is being transported closer to the perinuclear region of the cell - the area just around the nucleus - the M2 ion channels allows protons to move across the membrane, gradually acidifying the endosome, thus facilitating the uncoating and exposure of the viral genome (4, 14). This process triggers HA-mediated fusion of viral and endosomal membranes, as well as degradation of M1 protein, eventually freeing vRNPs into cytoplasm (4). Containing the viral genetic information, the vRNPs enters the nucleus of the host cell, where viral polymerase transcribes negative-sense vRNA into positive-sense mRNA (4, 14). Cap-dependent endonuclease of vRNA polymerase produces capped RNA primers, initiating viral mRNA synthesis (20). The mRNAs are then translated into various proteins by the ribosomes of the host cell (4, 14).

As the viral proteins are being made by the cell's own organelles, the replication of new virus particles begins (4, 19). Inside the nucleus, NS1 protein inhibits transcription and pre-mRNA processing and mRNA nuclear export, while the viral polymerase complex (consisting of PB1, PB2 and PA) and NEP replicate vRNPs via complementary RNA intermediates (4, 14, 15). As the newly synthesized vRNPs leave the nucleus, they gather in the plasma membrane of the host cell, where new virus particles are ready for budding (4, 14, 21). The process of budding requires NA-activity and involves cleaving terminal SA residues off glycoconjugates on both the virus particles and the host cell, resulting in new virus particles ready to infect other cells (4, 21).

1.2.2 Host factors involved in replication of FLUAV

Due to its humble genome, FLUAV have evolved multiple strategies to interact with a potential host cell to replicate and reproduce. Several studies have identified hundreds of host factors and numerous cellular pathways that FLUAV avail itself on to accomplish its life cycle, many of which are presented in the articles by Shim, J. S. et. al., (2017) and Tripathi, S. et. al., (2015) (4, 22). The following presents a step-by-step overview of some of the most prominent virus-host interactions involved in the replication cycle of FLUAV, all of which are redeemed in figure 1.2.2.

When found on the outside of living cells, FLUAV itself is metabolically inert. Once interactions between viral HA and cellular SA have been established, cellular clathrin, epsin-1, Rab 5/7/10 and COPI is required to help facilitate endocytic uptake of the virus (4). The next step is to release the viral genome into cytoplasm (4, 14). The process of uncoating and release of vRNPs is derived from the host cell's extracellular serine proteases, which are activated when cellular vATPases acidifies the interior of the late endosomes when near the perinuclear region (4, 21). This process causes cleaving off HA and degradation of M1 protein that binds the vRNPs underneath the lipid bilayer, thereby liberating the vRNPs into cytoplasm (4). The uncoated vRNPs can then enter the nucleus through the nuclear pore complex (NPC) mediated by importins located in the cytoplasm

(4). As presented in the article by Shim, J. S. et. al., (2017) and further recapped in figure 1.2.2, many host proteins are necessary to accomplish transcription of vRNAs and the process of splicing of NS1/NEP and M1/M2 pre-mRNAs, as well as transportation of viral mRNAs into cytoplasm and translation into functional proteins (22). The virus also uses the cells' own control mechanisms to control the condition of the newly synthesized proteins through cellular chaperones and chaperonins. The newly synthesized proteins are then ready for budding and release of new virions, all of which requires several host factors and cellular pathways (4).

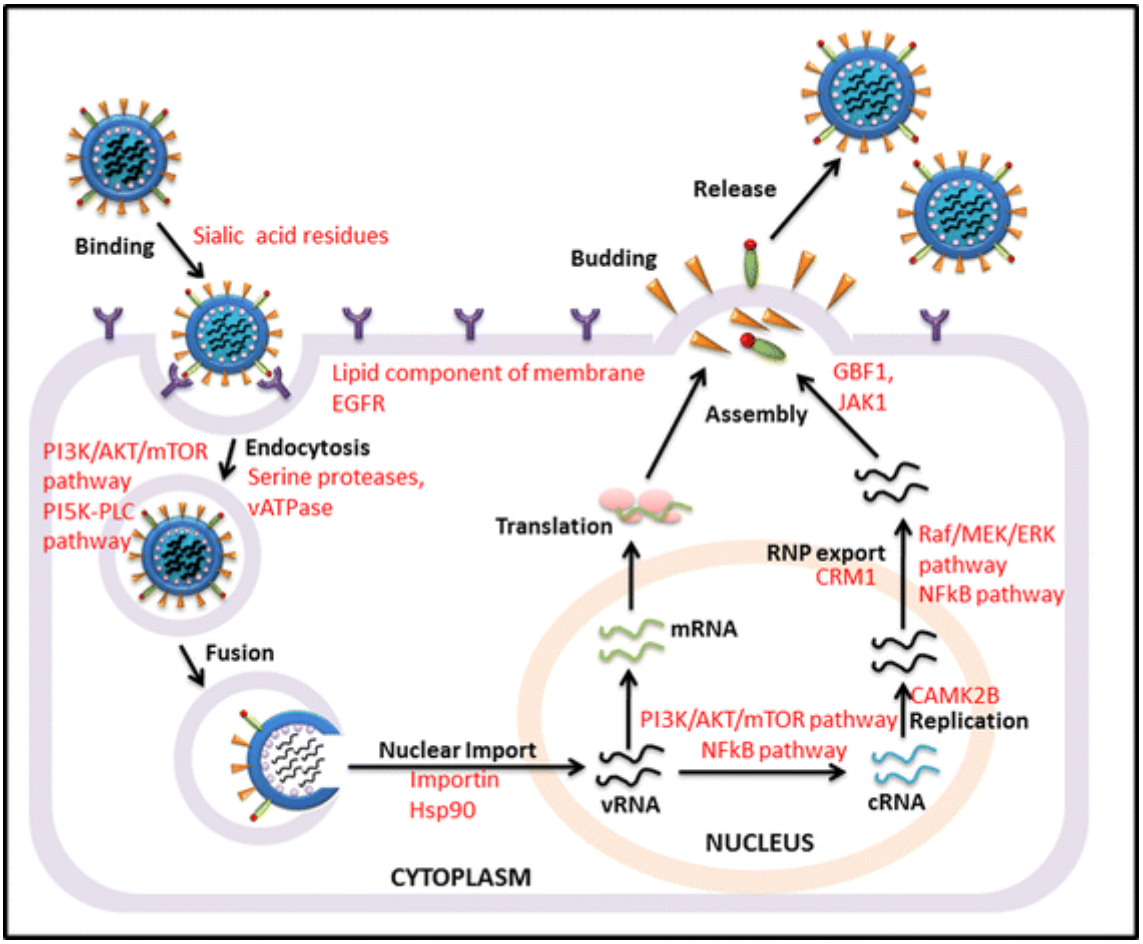

Figure 1.2.2 Examples of host factors involved in FLUAV replication

Due to its simple genome, FLUAV have evolved multiple strategies to complete its life cycle through interactions and utilizations of the host cell's own cellular factors, pathways and organelles. It has been identified several hundreds of host factors and numerous cellular pathways that FLUAV uses to replicate itself (4, 22). Some of the most prominent virus-host interactions are presented. Figure retrieved from (22).
***FUAV**: Influenza A virus*

1.2.3 Cellular antiviral responses

Multiple host sensors detect infections of FLUAV, resulting in a colossal interplay between the infected host cell, non-infected neighboring cells as well as innate and adaptive immune cells. Here, some antiviral responses in the infected cell, drawn from the article by Shim, J. S. et. al., (2017), will be presented (4). Figure 1.2.3 summarizes the cellular antiviral responses.

Within the cell, various pattern recognition receptors (PRRs) recognize the vRNAs as foreign particles, initiating an inflammatory downstream signaling response where transcription and secretion of type 1 IFNs are initiated (4, 14). Additionally, high amounts of vRNAs activates the cellular antiviral proteins IFITM1 and SAMD9 which prevent further fusion of the viral envelope as well as the endosomal membrane in self and non-infected neighboring cells (21). This results in a type 1 IFN-mediated autocrine loop that facilitates the expression of IFN-stimulated gene (ISG) expression in the infected cell, and when secreted, in non-infected neighboring cells, thus inducing an antiviral state (4, 14). To counter this effect – once transcribed – FLUAV NS1 can antagonize the IFN-induced proteins and continue its replication cycle (4).

During the antiviral state, the ISGs encode various proteins with antiviral properties, including the enzyme ribonuclease L (RNase 1) which, together with the 2’-5’-oligoadenylate synthase (OAS) family, degrades vRNAs in cytosol, and interleukins (ILs), chemokines (CXCls and CCLs) and other cytokines which aims to recruit immune cells to the site of infection, destroy the infected cell and prevent further infection (4, 14).

Alternatively, the infected cell can undergo apoptosis to prevent further infection when large amounts of vRNA or its replications intermediates is sensed by PRRs in cytosol of the infected cell, signaling anti-apoptotic Bcl-2 proteins (4). The Bcl-2 protein family then release pro-apoptotic proteins that regulate the permeabilization of the mitochondrial outer membrane (MoMp), degradation of the energy providing compound ATP, and activation of caspase 3, an enzyme that cleaves peptide bonds – all which induce cellular stress, resulting in cell death (4).

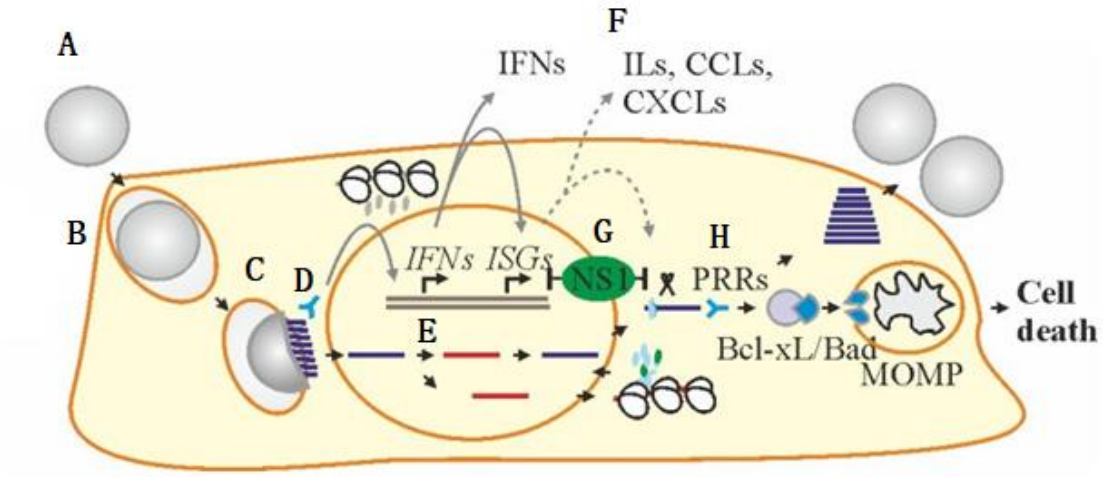

Figure 1.2.3 Replication cycle of FLUAV and cellular antiviral responses

(A) *Viral HA recognizes and binds to cells expressing surface receptors carrying sialic acid residues (4, 14).* ***(B)*** *Once bound, the FLUAV particles are taken up by endocytosis (4, 14).* ***(C)*** *Gradual acidification of the endosome occurs as the endosome is being transported to the perinuclear region of the host cell, whilst M2 protein allows ions to pass through the membrane (4, 14).* ***(D)*** *HA-mediated fusion of viral and endosomal membranes is triggered, and degradation of M1 protein leads to release of vRNPs into cytoplasm, which then is transported into the nucleus (4, 21).* ***(E)*** *Viral polymerase transcribes negative sense vRNA into positive-sense mRNA, which is translated into various proteins by the ribosomes of the infected cell (4, 14).* ***(F)*** *PRRs sense vRNAs as foreign particles, initiating transcription of IFN genes. Transcribed IFNs facilitate expression of ISGs, which results in encoding of RNAses that degrade vRNAs, as well as ILs, CXCls, CCLs and other cytokines that recruit immune cells to the site of infection (4).* ***(G)*** *FLUAV NS1 overcomes antiviral state by binding with vRNA, cellular DNA or others cellular factors, thereby resuming viral replication (3,8).* ***(H)*** *PRRs recognizes large amounts of vRNA, signaling anti-apoptotic Bcl-2 proteins that release pro-apoptotic proteins to initiate MoMP, ATP degradation and caspase 3 activation, resulting in cell death (4, 14). Figure retrieved and edited from (4)*

1.3 Anti-FLUAV drugs and vaccines

Anti-FLUAV drugs and vaccines are used to control influenza, conceivably by preventing infection, shortening the course of the disease or by alleviating symptoms (1, 3, 23, 24, 25, p. 2). The American agency, Centers for Disease Control and Prevention (CDC) and the Norwegian Institute of Public Health (FHI), help provide guidelines on the use of antiviral drugs and vaccines against influenza. Both agencies recommend anti-influenza treatment to high-risk groups due to elevated risk of developing severe complications such as hemorrhagic bronchitis or either primary viral or secondary bacterial pneumonia during a potential infection (23, 24). Noteworthy, patients with NCDs are specifically mentioned, and particularly those with pulmonary-, cardiovascular-, renal- or hepatic disease, metabolic disorders, neurologic or neurodevelopment conditions as well as patients with severe obesity and patients with reduced immune system (23, 24, 25, p. 3). The complications can develop within as little as a few hours, whilst shortness of breath, low oxygen levels (cyanosis), coughing of blood, pulmonary edema or fatality, can develop within 48 hours after the first onset of symptoms (26). This reveals the importance of preventing infections through vaccines and anti-FLUAV drugs.

1.3.1 Anti-FLUAV drugs

Currently, there are six approved anti-influenza drugs. In the United States, Food and Drug Administration (FDA) have approved four drugs (oseltamivir, zanamivir, peramivir, baloxavir marboxil) for use during the 2020-2021 influenza season, all of which have activity against FLUAV and FLUBV (24). The two remaining drugs, amantadine and rimantadine, only target FLUAV (25, p. 3). Figure 1.3 illustrates at which stage of the viral replication the anti-FLUAV drugs exert their mechanism of action.

Oseltamivir phosphate (Tamiflu®), zanamivir (Relenza®) and peramivir (Rapivab®) are NA-inhibitors. These drugs hinder virions from budding off the host cell, thus preventing reproduction in potential new target cells (4, 21, 24). Oseltamivir is the only available drug in Norway (27). Baloxavir marboxil (Xofluza ®) targets viral polymerase by inhibiting cap-dependent endonuclease, thus interfering with transcription of vRNA and thereby blocks viral replication (14). Amantadine and rimantadine inhibit proton transport in M2 ion-channels in FLUAV, and thereby prevents acidification of late endosomes containing virus, thus obstructing viral replication (4). Due to current surveillance and resistance data of the virus, amantadine and rimantadine are not recommended for use, as FLUAV carries high levels of resistance against these drugs (25, p. 3).

In the article by Söderholm, S. et. al. (2016), they found that the anticancer drug gemcitabine allowed for activation of immune responses, thus concluding that gemcitabine could be further developed as an anti-FLUAV drug (11). Interestingly, this could indicate that cancer patients receiving gemcitabine as treatment could be protected from FLUAV infection, however, this cannot be concluded without further research. Also, studies have found that antifungal itraconazole used in adults to treat infections caused by fungus, could inhibit FLUAV, while boosting IFN-response and unbalancing cholesterol metabolism, thus indicating that the drug could possess adverse events on FLUAV infected patients with certain underlying inflammatory or metabolic disorders (28).

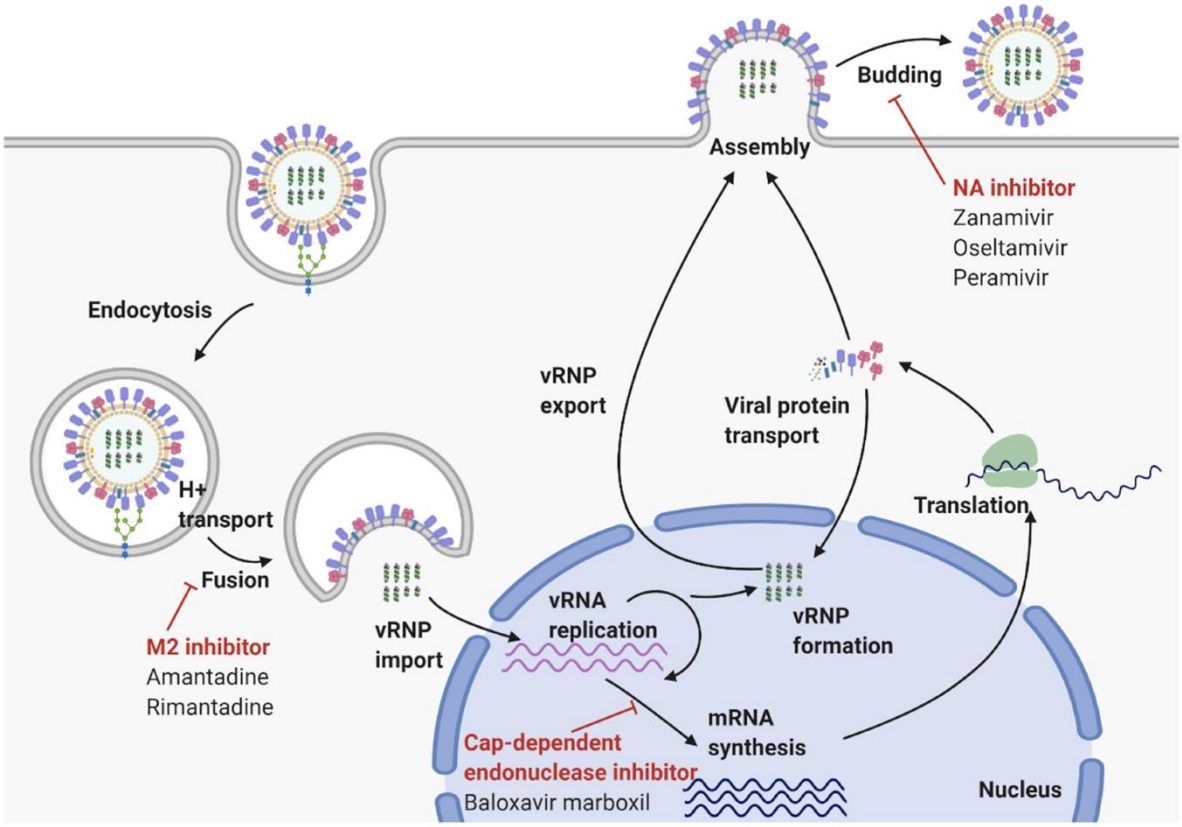

Figure 1.3 Mechanism of action of anti-influenza drugs

Oseltamivir, zanamivir and peramivir inhibit NA. This hinders the process of budding. Baloxavir marboxil inhibit cap-dependent endonuclease, thus blocking viral replication. Amantadine and rimantadine prevents acidification of endosomes containing virus by inhibiting proton transport in M2 ion-channels in FLUAV, obstructing viral replication (4, 14). However, due to levels of resistance, the latter are not recommended for use. Figure retrieved from (16).

1.3.2 Influenza vaccines

Immunization of influenza virus can be derived either from a natural source, such as human-to-human encounters and less commonly via contact surfaces, or seldom through animal-to-human transmissions. It can also be provided through artificial sources such as vaccines (1, 3, 17, 30). In Norway, the marketed vaccines are available either as inactivated or subunit injections, or as an attenuated vaccine administrated as nasal spray (30).

When a person has been infected with influenza, the immune system often provide protection for several years against later infections with the same virus, as well as cross-immunity of virus of close resemblance (29, 30). However, due to accumulation of genetic drift over time, the virus becomes less recognizable and antigenically different to the host, making it possible to retrieve influenza infections more than once (14, 18). As a result, the World Health Organization (WHO) selects strains to be included into vaccines twice a year for upcoming influenza epidemics based on previous epidemics and continuous global surveillance of influenza (23). From a view of current literature, the details regarding the fundamentals of vaccine immunology will not be presented here, due the colossal interplay between the innate and adaptive immune system. However, a short summary will be presented.

When the vaccine is administrated, the patient is subjected to a weakened or dead form of the virus, where antigen presenting dendritic cells recognize viral components and adjuvant, thus displaying it on the cell surface (23, 30). Adjuvant, such as aluminum salts, are added to certain vaccines to increase the capacity of the vaccine, as well as providing a strong and long-lasting immune response (30). The displayed antigen is then recognized by native T-cells, which then signals B-cells to make antibodies using the displayed antigen as a template (31). The antigens used in vaccines consist of two FLUAV strains (H1N1, H3N2) and one (trivalent vaccine) or two FLUBV strains (quadrivalent vaccine) (Victoria, Yamagata) (32).

Most currently approved influenza vaccines are manufactured using embryonated hen eggs (ECEs), a method first used in the 1940s. This method has been highly used due to vast experience regarding large-scale production and extensive safety data concerning administration to humans, however, it also carries some disadvantages, as some viruses do not grow well in ECEs. It also requires large amounts of pathogen-free eggs, and occasional breakdown in sterility could lead to large scale quantities of bulk rejections. Consequently, alternative influenza virus cultivation systems have been developed, such as Madin Darby Canine Kidney cells (MDCK) and Vero cells. Vaccines made from cell lines are immunogenic, and allergies to egg proteins can be avoided. Compared with Vero cells, MDCK cells have shown to be most suitable to obtain primary isolates of influenza virus, due to faster replication and higher yield, thus reducing the chances of accumulation of mutations of the HA-protein consequently altering the matching and vaccine effectiveness (33).

The efficacy of the vaccines are about 60 percent, meaning six out of ten vaccinated individuals are protected against influenza during the season. Though, patients who falls ill even when vaccinated have a milder disease course, less complications and comorbidities than non-vaccinated patients (1, 23). The efficacy relies mainly on how well-matched the selected strains used in the vaccines for the expected season is, compared with the ongoing circulating viruses (23).

2. Hypothesis

There are many medicines approved for treatment of different diseases or conditions. Most of us use some of these medicines at some point in our lives. For instance, we can use the over-the-counter medicine such as paracetamol to relieve headache or as an antipyretic, or perhaps we use some medications to treat NCDs. **We hypothesized that some of the drugs could target host factors involved in FLUAV replication and, thereby, interfere with virus-host interactions and possess ADRs.**

3. Aim and objectives

Identification and prediction of ADRs is challenging (14, 16). Söderholm, S. et. al. (2016) described an in vitro approach and tested anticancer saliphenylhalamide (SaliPhe), SNS-032, obatoclax and gemcitabine on FLUAV-host cell interactions (16-18). Our aim is to exploit the method for discovering ADRs of commonly prescribed therapeutics.

To discover ADRs of commonly prescribed drugs, we developed following objectives:

1) To identify 45 most dispensed drugs in our region;
2) To test the effect of these drugs on cell toxicity and FLUAV replication;
3) To evaluate the effect of these drugs on transcription of viral and cellular genes;
4) To evaluate the effect of the drugs on metabolism of non- and infected cells;
5) To identify ADRs and evaluate clinical relevance.

Thus, we will expand the spectrum of ADRs of existing therapeutics and identify drugs.

4. Materials and Methods

4.1 Compounds

To identify the most dispensed medicines in Central Norway in 2019, we searched the Norwegian Prescription Database (www.norpd.no), ATC/DDD-version 2021. The database contains data about dispensed drugs in different regions of Norway (54). We obtained the 45 most dispensed medicines in Central Norway by collecting all drugs registered in the database based on their ATC-codes, selecting all age groups, both sexes and daily defined dosage (DDD) in 2019. The ATC-classification system classifies the compounds according to which organ or organ system they act on and their therapeutic, pharmacological and chemical properties (34). Table 4.1 lists the compounds, their suppliers and catalogue numbers.

To obtain 10 mM stock solutions, compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Steinheim, Germany) or milli-Q water. The solutions were stored at −80 °C until use.

Table 4.1: The compounds used, their suppliers and catalogue numbers

Drug	CAS	MW	Formula	Cat N	Purity, %	Supplier
17α-Ethynylestradiol	57-63-6	296	C20H24O2	E4876-100MG	≥98	Sigma Aldrich
4-Acetamidophenol	103-90-2	151	C8H9NO2	102330050	98	Acros Organics
Acetylsalicylic acid	50-78-2	180	C9H8O4	AC158180500	99	Acros Organics
Amlodipine	88150-42-9	409	C26H31ClN2O8S	CAYM14838	≥98	Cayman Chemicals
Atorvastatin	134523-03-8	559	C33H35FN2O5	CAYM10493	≥98	Cayman Chemicals
Bumetanide	28395-03-1	364	C17H20N2O5S	CAYM14630	≥98	Cayman Chemicals
Candesartan	139481-59-7	440	C24H20N6O3	sc-217825	≥98	Santa Cruz Biotechnology
Cetirizin	83881-52-1	389	C21H27Cl3N2O3	89126-50MG	≥98	Sigma Aldrich
Cyanocobalamin	68-19-9	1355	C63H88CoN14O14P	DRE-C11798500		LGC Standards
Desloratadine	100643-71-8	311	C19H19ClN2	CAYM16931	≥98	Cayman Chemicals
Desogestrel	54024-22-5	310	C22H30O	CAYM23651	≥95	Cayman Chemicals
D-Pantothenic acid	79-83-4	219	C9H17NO5	HY-B0430	≥98	MedChemExpress
Drospirenone	67392-87-104	367	C24H30O3	CAYM23347	≥98	Cayman Chemicals
Enalapril	75847-73-3	376	C20H28N2O5	J60750.03	≥97	Alfa Aesar
Escitalopram	128196-01-0	324	C20H21FN2O	CAYM22405	≥98	Cayman Chemicals
Esomeprazole	161973-10-0	767	C34H42MgN6O9S2	CAYM17326	≥95	Cayman Chemicals
Etonogestrel	54048-10-1	324	C22H28O2	CAYM21062	≥98	Cayman Chemicals
Fluticasone propionate	80474-14-2	445	C25H31F3O5S	462101000	≥96	Acros Organics
Folic acid	59-30-3	441	C19H19N7O6	J62937.06	≥97	Alfa Aesar
Furosemide	54-31-9	331	C12H10ClN2O5S	448970010	≥97	Acros Organics
Hydroxocobalamin	13422-5 51-0	1346	C62H89CoN13O15P	CAYM24099	≥95	Cayman Chemicals

Drug	CAS	MW	Formula	Cat N	Purity, %	Supplier
Insulin aspart	116094-23-6	5826	C256H387N65O79S6	EPY0000349		LGC Standards
Lercanidipine	132866-11-6	612	C36H41N3O6	HY-B0612A	98.5	MedChemExpress
Levonorgestrel	797-63-7	312	C21H28O2	CAYM10006	≥95	Cayman Chemicals
Levothyroxine	25416-653	817	C15H12I4NNaO5	FT48192	≥97	Carbosynth
Losartan	114798-26-4	423	C22H23ClN6O	FL39656	≥97	Carbosynth
Metformin	1115-70-4	166	C4H12ClN5	sc-202000	≥99	Santa Cruz Biotechnology
Metoprolol	51384-51-1	267	C15H25NO3	sc-264643	97	Santa Cruz Biotechnology
Mometasone furoate	83919-23-7	521	C27H30Cl2O6	CAYM21365	≥98	Cayman Chemicals
Naproxen	22204-53-1	230	C14H14O3	CAYM70290	≥99	Cayman Chemicals
Nicotinic acid	59-67-6	123	C6H5NO2/HOOC5H4N	128290050	99.5	Acros Organics
Nifedipine	21829-25-4	346	C17H18N2O6	CAYM11106	≥98	Cayman Chemicals
Pantoprazole	102625-70-7	383	C16H15F2N3O4S	CAYM21345	≥98	Cayman Chemicals
Ramipril	87333-19-5	417	C23H32N2O5	FC27676	≥98	Cymit Quimica
Riboflavin	83-88-5	376	C17H20N4NaO9P	A11764.14	98	Alfa Aesar
Salbutamol	18559-94-9	239	C13H21NO3	CAYM21003	≥98	Cayman Chemicals
Salmeterol	89365-50-4	416	C25H37NO4	HY-14302	99.7	MedChemExpress
Sertraline	79559-97-0	306	C17H18Cl3N	462190010	≥98	Acros Organics
Simvastatin	79902-63-9	419	C25H38O5	458840010	98	Acros Organics
Tamsulosin	106463-17-6	445	C20H29ClN2O5S	CAYM24020	≥98	Cayman Chemicals
Thiamine	67-03-8	337	HC12H17ON4SCl2	148990100	99	Acros Organics
Valsartan	137862-53-4	436	C24H29N5O3	sc-220362	≥98	Santa Cruz Biotechnology
Venlafaxine	99300-78-4	277	C17H27NO2	HY-B0196A	98	MedChemExpress
Vitamin D2	50-14-6	397	C28H44O	CAYM11791	≥98	Cayman Chemicals
Vitamin D3	67-97-0	385	C27H44O	CAYM11792	≥98	Cayman Chemicals

The 45 most dispensed medicines in Central Norway in 2019, sorted alphabetically. The table lists the compounds, their suppliers and catalogue numbers.

4.2 Cells

Madin–Darby canine kidney cells (MDCK, American Type Culture Collection (ATCC)) were grown in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley, Scotland) supplemented with 100 U/mL penicillin and 100 ug/ml streptomycin mixture (Pen/Strep; Lonza, Cologne, Germany), 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum (FBS; Lonza, Cologne, Germany). Human telomerase reverse transcriptase-immortalized retinal pigment epithelial (RPE, ATCC) cells were grown in DMEM-F12 medium supplemented with Pen/Strep, 2 mM L-glutamine, 10% FBS, and 0.25% sodium bicarbonate (Sigma-Aldrich, St. Louis, USA).

4.3 Viruses

Human influenza A/WSN/33(H1N1) virus (FLUAV) was generated using eight-plasmid reverse genetics system in HEK293 and Vero-E6 cells, as described previously by Hoffmann et. al., 2000 (35). All the experiments with viruses were performed in BSL2 laboratory in compliance with the guidelines of the national authorities using appropriate biosafety laboratories under appropriate ethical and safety approvals.

4.4 Microscopy

Approximately 4×10^4 RPE cells were seeded per well in 96-well plates. The cells were grown for 24 h in DMEM-F12 medium supplemented with 10% FBS, and Pen/Strep. The medium was replaced with DMEM-F12 medium containing 0.2% bovine serum albumin, 2 mM L-glutamine, and 1 µg/mL TPSK-trypsin. The compounds were added to the cells in 3-fold dilutions at seven different concentrations starting from 100 µM. SaliPhe, ABT-263 and DMSO were added to the control wells. SaliPhe inhibits endocytic uptake of FLUAV by targeting cellular vATPase, which protects cells from virus-mediated death (10). ABT-263 inhibits anti-apoptotic Bcl-2 proteins, which facilitates death of cells with vRNAs (10-12). RPE cells were infected with FLUAV or mock at multiplicity of infections (moi) of 1.

4.5 Cell viability assays

RPE cells were treated with compounds or control compounds as described above and infected with FLUAV virus at moi 1. After 48 hours of infection, the medium was removed from the cells. The viability of mock- and virus-infected cells were measured using Cell Titer Glow assay (CTG; Promega, Madison, USA). The luminescence was read with a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany).

The half-maximal cytotoxic concentration (CC50) for each compound was calculated based on viability/death curves obtained on mock-infected cells after non-linear regression analysis with a variable slope using GraphPad Prism software version 7.0a. The half-maximal effective concentrations (EC50) were calculated based on the analysis of reporter protein expression or the viability/death of infected cells by fitting drug dose–response curves using four-parameter (4PL) logistic function f(x):

$$f(x) = A_{min} + \frac{A_{max} - A_{min}}{1 + (\frac{x}{m})^{\lambda}}$$

, where f(x) is a response value at dose x, Amin and Amax are the upper and lower asymptotes (minimal and maximal drug effects), m is the dose that produces the half-maximal effect (EC50 or CC50), and λ is the steepness (slope) of the curve. A relative effectiveness of the drug was defined as selectivity index (SI = CC50/EC50).

4.6 Transcriptomics analysis

We infected RPE cells with FLUAV at moi 1. After 8 h we isolated total RNA using a RNeasy Plus minikit (Qiagen). 384 TruSeq Stranded mRNA libraries were prepared in 96 sample batches. Sequencing was done on HiSeq (HSQ-700358) instrument (set up: SR 1 x 70 bp + dual index 8 bp) using HiSeq Rapid SR Cluster Kit v2 sequencing kit, RapidRunV2 flow cell (up to 300M reads per flowcell), RTA version: 1.18.64. Reads were aligned using the Bowtie 2 software package version 2.3.4.1 to the reference influenza A/WSN/1933 or human GRCh38 genome. For viral genome, sequence alignments were converted to Binary alignments using SAMtools version 1.5. Number of mapped and unmapped reads that aligned to each gene were retrieved with SAMtools idxstats. For human genome, number of mapped and unmapped reads that aligned to each gene were obtained with featureCounts function from Rsubread R-package version 2.10.

4.7 Metabolomics analysis

We infected RPE cells with FLUAV at moi 1. After 24 hours we collected cell culture medium. Metabolomics analysis was performed as described previously (60). Briefly, 10 µL of labelled internal standard mixture was added to 100 µL of the sample (cell culture media). About 0.4 mL of solvent (99% ACN and 1% FA) was added to each sample. Insoluble fraction was removed by centrifugation (14 000 rpm, 15 min, 4 °C). The extracts were dispensed in Ostro™ 96-well plate (Waters Corporation, Milford, MA, USA) and filtered by applying vacuum at a delta pressure of 300–400 mbar for 2.5 min on Hamilton StarLine robot's vacuum station. The clean extract was collected in a 96-well collection plate and placed under the Ostro™ plate. The collection plate was sealed and centrifuged for 15 min, 4000 rpm, 4 °C and placed in auto-sampler of the liquid chromatography system for the injection. Sample analysis was performed on an Acquity UPLC-MS/MS system (Waters Corporation). The auto-sampler was used to perform partial loop with needle overfill injections for the samples and standards. The detection system, a Xevo® TQ-S tandem triple quadrupole mass spectrometer (Waters), was operated in both positive and negative polarities with a polarity switching time of 20 msec. Electro spray ionization (ESI) was chosen as the ionization mode with a capillary voltage at 0.6 KV in both polarities. The source temperature and desolvation temperature of 120 and 650 °C, respectively, were maintained constantly throughout the experiment. Declustering potential (DP) and collision energy (CE) were optimized for each compound. Multiple reaction monitoring (MRM) acquisition mode was selected for quantification of metabolites with individual span time of 0.1 s given in their individual MRM channels. The dwell time was calculated automatically by the software based on the region of the retention time window, number of MRM functions and also depending on the number of data points required to form the peak. MassLynx 4.1 software was used for data acquisition, data handling and instrument control. Data processing was done using TargetLynx software and metabolites were quantified by calculating curve area ratio using labelled internal standards (IS) (area of metabolites/area of IS) and external calibration curves.

4.8 Bioinformatics analysis

Transcriptomics and metabolomics data were log2 transformed for linear modelling and empirical Bayes-moderated t-tests using the LIMMA package (61). To analyze the differences in transcripts or metabolites levels, a linear model was fit to each transcript or metabolite. The Benjamini–Hochberg method was used to correct for multiple testing. The significant transcript and metabolites were determined at a Benjamini–Hochberg false discovery rate (FDR) controlled at 10%. The heatmaps were generated using the pheatmap package (https://cran.r-project.org/web/packages/pheatmap/index.html) based on log2-transformed profiling data. Gene (GSEA) and metabolite (MSEA) set enrichment analysis tools were used to retrieve pathways (http://software.broadinstitute.org/gsea/index.jsp; https://www.metaboanalyst.ca/).

4.9 Cellular targets of FLUAV-host cell interaction

Cellular targets of FLUAV-host cell interaction were visualized using the STITCH web-tool (ref 10.1093/nar/gkv1277).

5. Results

5.1 The 45 most dispensed medicines in Central Norway

The 45 most dispensed medicines were selected using the Norwegian Prescription Database (www.norpd.no) as described above. Table 5.1 lists the active compounds, ATC-codes and DDDs for each compound. Table 5.1 also lists the indications of the main diseases or conditions the active compounds are used to treat, all of which are obtained from Felleskatalogen (www.felleskatalogen.no).

Several of the selected compounds have structural similarities, thus closely sharing the same mechanisms of actions, reflecting the assigned ATC-codes. I.e., both salmeterol (ATC-code: R03AK06) and salbutamol (ATC-code: R03AC02) are used against asthma, due to their agonism of β2-adrenoceptors, leading to smooth muscle relaxation and bronchodilation. However, due to a long side chain in salbutamol, salbutamol has longer half time than salmeterol, thus giving the compounds slightly different ATC-codes. Figure 5.1 depicts the structural similarities between all the selected compounds, which co-reflects the ATC-system. Some compounds, however, such as metformin and insulin aspart do not share same chemical properties but are clustered due to their similar indications.

Table 5.1 The most dispensed medicines in Central Norway in 2019

ATC	Active compounds	Indications	DDD
C10AA05	Atorvastatin	Hypercholesterolemia	24 480 740
B01AC06	Acetylsalicylic acid	Pain, fever or inflammation	16 113 802
C08CA01	Amlodipine	Hypertension and coronary artery disease	10 990 638
C09CA06	Candesartan	Hypertension	9 404 137
A02BC02	Pantoprazole	Erosive esophagitis and Zollinger-Ellison syndrome	8 626 254
R06AE07	Cetirizine	Hay fever, allergies, angioedema, and urticaria	8 377 836
C09AA05	Ramipril	Hypertension and congestive heart failure	8 034 213
N02BE01	Paracetamol	Pain and fever	7 548 361
C10AA01	Simvastatin	Hypercholesterolemia	7 117 959
H03AA01	Levothyroxine sodium	Thyroid hormone deficiency	6 701 185
G03AA07	Levonorgestrel	Birth control (in combination with the estrogen ethinylestradiol), emergency birth control	6 677 552
G03AA07	Ethinylestradiol	Birth control and treatment of menopausal symptoms in combination with progestins	6 677 552
B03BB01	Folic acid	Folate deficiency	6 598 841
A12AX	Vitamin D2	Vitamin D deficiency	5 675 283
A11CC05	Vitamin D3 (colecalciferol)	Vitamin D deficiency	5 332 710
R06AX27	Desloratadine	Allergic rhinitis, nasal congestion	5 309 712

C07AB02	Metoprolol	Hypertension and coronary artery disease	5 264 848
A02BC05	Esomeprazole	Gastroesophageal reflux disease, erosive esophagitis, duodenal ulcers	5 152 910
N06AB10	Escitalopram	Depression, generalized anxiety disorder	4 728 187
A10BA02	Metformin	Type 2 diabetes, polycystic ovary syndrome	4 351 201
G03AC08	Etonogestrel	Birth control	4 276 000
M01AE52	Naproxen	Pain and fever caused by inflammation	4 031 853
C09CA03	Valsartan	Hypertension and congestive heart failure	3 514 084
C09CA01	Losartan	Hypertension	3 491 802
G03AC09	Desogestrel	Birth control and menopausal symptoms	3 301 519
B03BA03	Hydroxocobalamin	Vitamin B12 deficiency	3 182 950
R03AC02	Salbutamol	Asthma	3 018 486
A11E	D-Pantothenic acid*	Vitamin B deficiency	2 872 185
A11E	Thiamine*	Vitamin B deficiency	2 872 185
A11E	Riboflavin*	Vitamin B deficiency	2 872 185
A11E	Nicotinic acid*	Vitamin B deficiency	2 872 185
C03CA01	Furosemide	Hypertension and edema	2 864 836
R03AK06	Fluticasone propionate	Asthma, allergic rhinitis, atopic dermatitis	2 668 274
C08CA13	Lercanidipine	Hypertension	2 667 274
B03BA01	Cyanocobalamin	Vitamin B12 deficiency	2 406 614
C03CA02	Bumetanide	Heart failure	2 354 487
N06AB06	Sertraline	Depression	2 129 315
A10AB05 and A10AD05	Insulin aspart	Diabetes mellitus type 1 and 2	2 101 161
G04CA02	Tamsulosin	Benign prostatic hyperplasia, kidney stones, acute urinary retention	2 065 873
R01AD09	Mometasone furoate	Symptoms in nose caused by allergy or polyps	1 968 160
N06AX16	Venlafaxine	Depression, general anxiety disorder	1 840 761
R03AK06	Salmeterol	Asthma	1 833 358
C08CA05	Nifedipine	Hypertension and angina pectoris	1 773 040
C09AA02	Enalapril	Hypertension, diabetic kidney disease and heart failure	1 770 279
G03AA12	Drospirenone	Birth control and menopausal symptoms	1 725 864

*The 45 most dispensed medicines in Central Norway in 2019 sorted by daily defined dosage (DDD). *In Norway, there are no marketed drugs with d-pantothenic acid (vitamin B5), thiamine (vitamin B1), riboflavin (vitamin B2) and nicotinic acid (vitamin B3) separately. However, these are included due to high DDD. Examples of marketed drugs with different compositions of these vitamins are B-Tonin, Nycoplus B-kompleks and TroBe.*

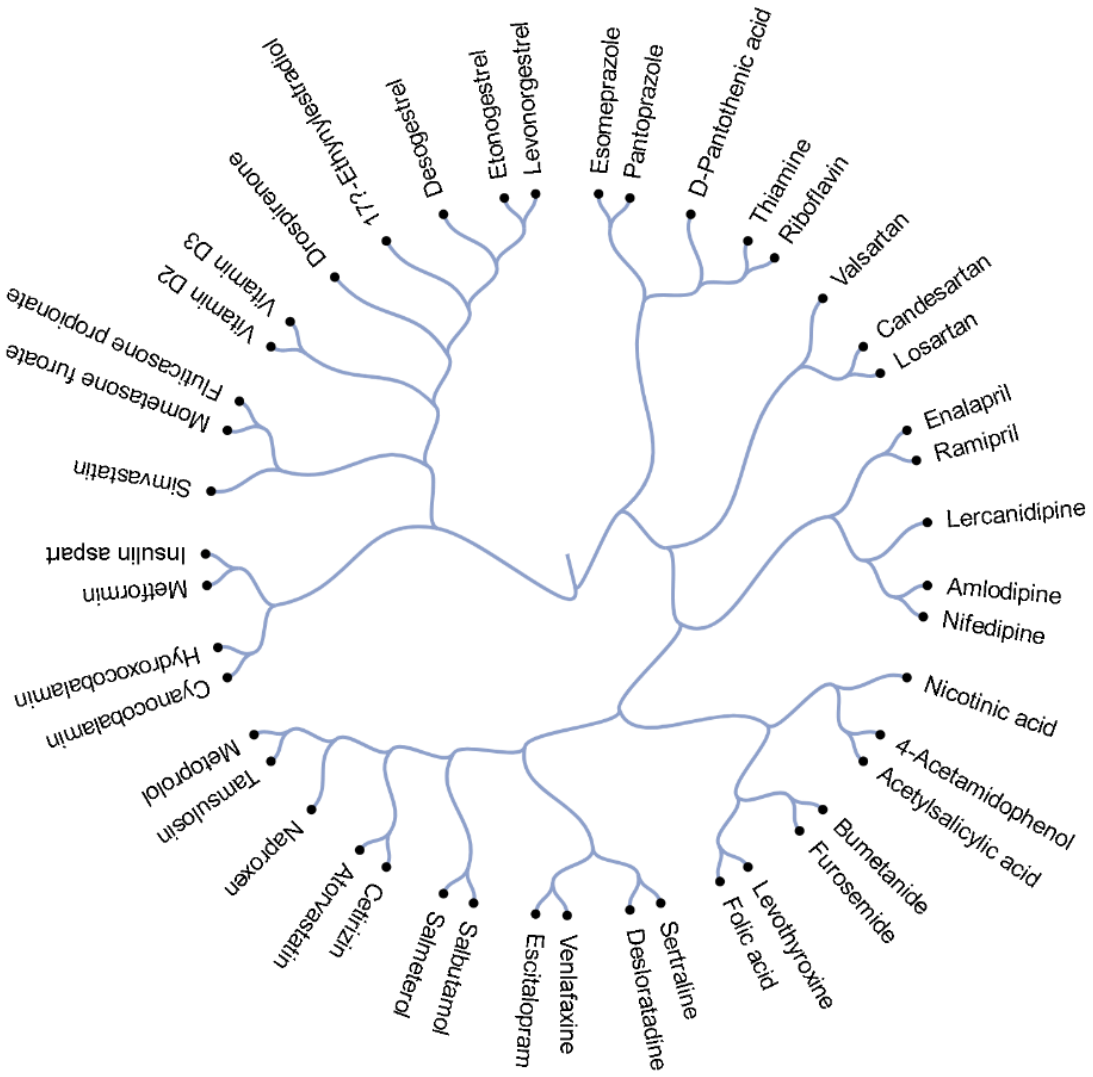

Figure 5.1 The selected compounds clustered, based on their structural similarity

The 45 most prescribed drugs in Central Norway in 2019 depicting their structural similarities, calculated by ECPF4 fingerprints and the Tanimoto coefficient. Compounds are grouped by similar molecules together using clustering. I.e., candesartan and losartan are angiotensin II receptor antagonists used to treat hypertension, mometasone furoate and fluticasone propionate are corticosteroids commonly prescribed against allergy, and esomeprazole and pantoprazole are proton pump inhibitors (PPI) used to treat reflux and ulcers.

5.2 Cell viability effect in mock- and FLUAV-infected RPE cells

In order to examine whether compounds could affect viability of mock- and FLUAV-infected RPE cells, half-maximal cytotoxic and half-maximal effective concentrations of each compound were determined and plotted using a non-linear regression model and a 4PL model, respectively. The effect of compounds on viability of mock- and FLUAV-infected cells are presented in figure 5.2.

We found that most compounds in mock-infected cells did not exhibit cytotoxicity in concentrations up to 100 µM, 48 hours post treatment. Eight compounds, however, were found to attain cytotoxicity at lower concentrations. The compounds exhibiting cytotoxicity below 100 µM are presented in table 5.2 together with their calculated CC50-values. Using the 4PL model as previously described, we found that RPE cells treated with compounds and infected with FLUAV did not affect cell viability 48 hours post addition and infection.

To avoid possible loss of data due to cell death, we chose to proceed the transcriptomics and metabolomics analysis with 10 µM of each compound.

Table 5.2 Compounds indicating higher cytotoxicity (CC50 < 100 µM)

Compound	CC50-value
Amlodipine	28,5
Desloratadine	46,7
Desogestrel	34,5
Salmeterol	29,3
Sertraline	17,5
Simvastatin	48,3
Vitamin D2	42,1
Vitamin D3	48,8

Half-maximal cytotoxic concentrations (CC50) on mock-infected RPE cells treated with amlodipine, desloratadine, desogestrel, salmeterol, sertraline, simvastatin, vitamin D2 and vitamin D3 indicated higher cytotoxicity at lower concentrations, compared with the compounds excluded from the table (CC50>100 µM). CC50-values were calculated and plotted using a non-linear regression model.

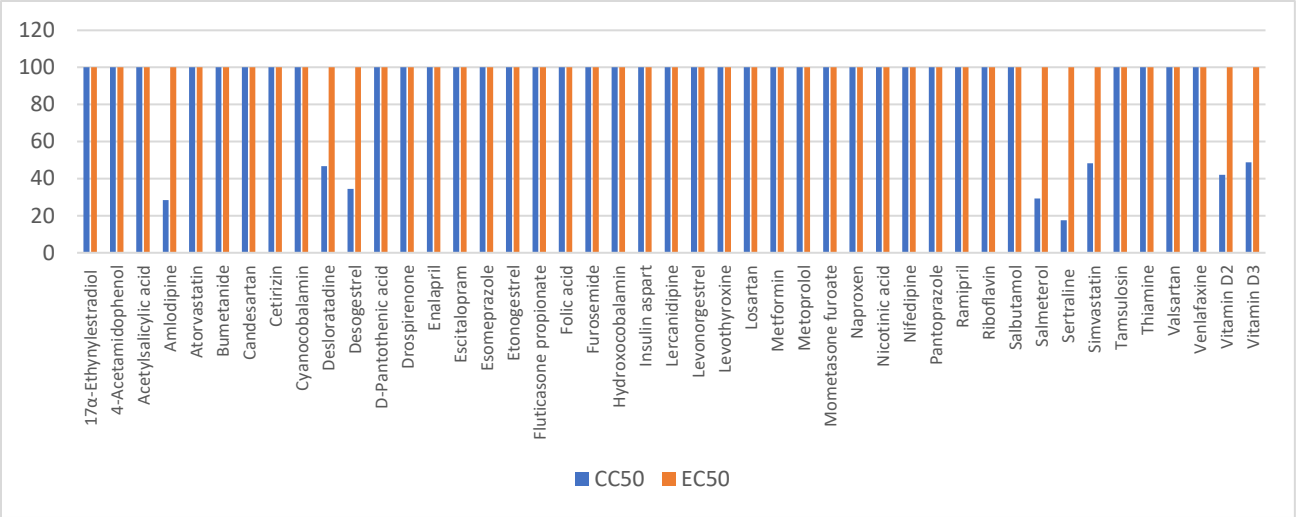

Figure 5.2 Effect of 45 compounds on the viability of mock- and FLUAV-infected human RPE cells.

*(**Blue**): RPE cells were treated with increasing concentrations of each compound, respectively. The viability of the cells was determined after 48 hours with the CTG assay. CC50 values were calculated and plotted. The CC50 >100 µM are shown as 100 µM. Mean ± standard deviation (SD); n = 3 (experimental replicates).*

*(**Orange**): RPE cells were treated with increasing concentrations of each compound and infected with FLUAV (moi, 1). The viability of the cells was determined after 48 hours with the CTG assay. EC50 values were calculated and plotted (Mean ± SD; n = 3).*

5.3 Transcriptional effects in mock- and FLUAV-infected RPE cells

To evaluate the effects of the compounds on transcription of host and viral genes, we infected RPE cells with FLUAV at moi 1 or mock. After 8 hours, we isolated total RNA and sequenced mRNA. Heatmaps of the most variable host genes were constructed, using human GRCh38 genome as reference. Figure 5.3.1 and 5.3.2 presents the most variable host genes found on mock- and FLUAV-infected cells, respectively, clustered, according to the number of mapped and unmapped reads. To evaluate the most variable viral genes affected by treatment, influenza A/WSN/1933 genome was used as reference. The transcriptional effects of viral mRNAs of each compound are shown in figure 5.3.3. Due to substantial amount of data, nine compounds were selected, reflecting the differences in host and viral transcriptional effects. Table 5.3.1 summarizes the transcriptional differences found in these compounds.

We found that all compounds affected transcription of host genes in mock-infected cells to certain extent, see figure 5.3.1. For instance, amlodipine increased the expression of IGFBP1, CTH, ATF3, PTGS2, KCP and ATP6VOD2 five to six folds compared to control cells, whilst FAM111B, CCNE2 and CDCA7 were significantly downregulated (4-5 folds). FLUAV induces expression of IFNs and ISGs. In FLUAV-infected cells, the same trend became apparent, only with other genes, see figure 5.3.2. Most of the compounds affected most genes in a relative manner compared with control cells, however, as for amlodipine, C3 and CADM1 become slightly upregulated when added compared with control. Amlodipine also showed predominant upregulation of OASL, IFIT2 and OAS1 and downregulation in ARHGAPA42, in line with control cells.

Lastly, we found that most compounds altered the transcriptional effects of viral proteins, see figure 5.3.3. Here, furosemide showed the most abundant effect, upregulating the genes encoding M2, M1, NA, NP, PB1, HA, PA, PB2, NS1 and NS2.

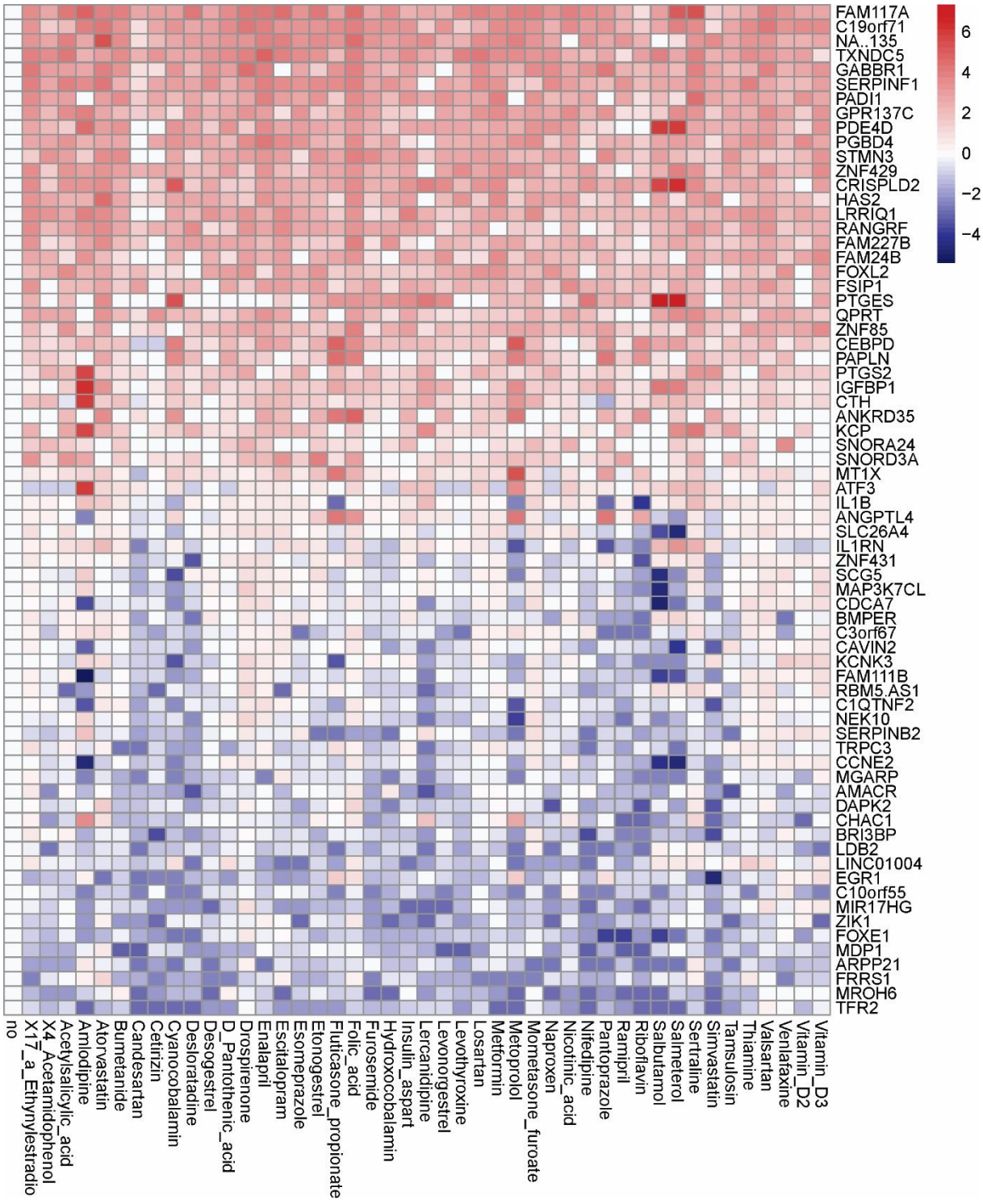

Figure 5.3.1 Effect of 45 compounds on mRNA levels in RPE cells.

RPE cells were treated with 10 µM compounds. After 8 hours, total RNA was isolated, and mRNA was sequenced. A heatmap of 70 most variable mRNAs affected by treatment is shown. Rows represent gene symbols, columns represent samples. Each cell is coloured according to the log2-transformed and quantile-normalized expression values of the samples, expressed as FC relative to the average of DMSO-treated controls.

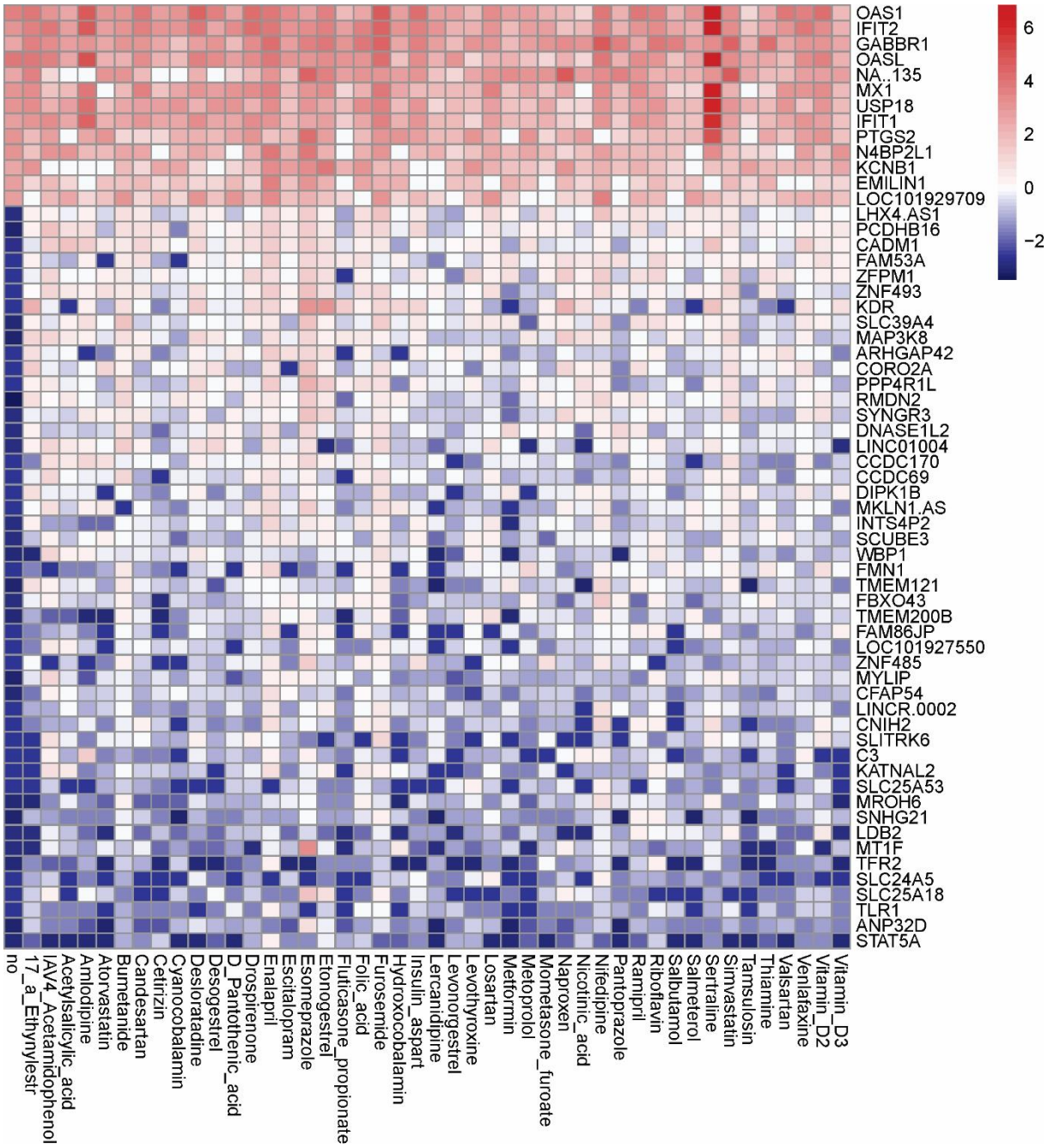

Figure 5.3.2 Effect of 45 compounds on mRNA levels in FLUAV-infected RPE cells.

RPE cells were treated with 10 µM compounds and infected with FLUAV (moi, 1). After 8h total RNA was extracted, and mRNA was sequenced. A heatmap of the most variable genes affected by FLUAV infection is shown (2.5 < log2FC < -2.5). Rows represent gene symbols, columns represent samples. Each cell is coloured according to the log2-transformed and quantile-normalized expression values of the samples, expressed as FC relative to the average of DMSO-treated mock-infected controls.

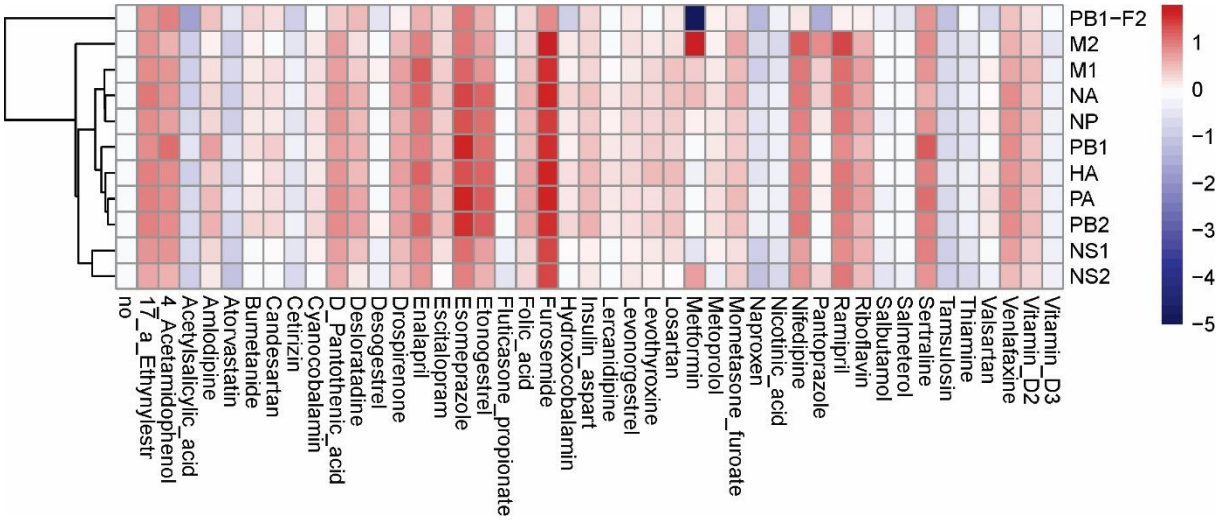

Figure 5.3.3 Effect of 45 compounds on viral mRNA levels in FLUAV-infected RPE cells.

RPE cells were treated with 10 µM compounds and infected with FLUAV (moi, 1). After 8h total RNA was extracted, and mRNA was sequenced. A heatmap of viral genes affected by treatment is shown (2.5 < log2FC < -2.5). Rows represent gene symbols, columns represent samples. Each cell is coloured according to the log2-transformed and quantile-normalized expression values of the samples, expressed as FC relative to the average of non-treated FLUAV-infected controls.

Table 5.3.1 Summarized effect of nine selected compounds on mRNA levels in mock-infected and FLUAV-infected RPE cells, and viral mRNA levels in FLUAV-infected RPE cells.

Compound	Mock-infected cells	FLUAV-infected cells	Viral genes
Amlodipine	Up/Down	FLUAV lowers drug-induced genes	Activates viral genes
Cyanocobalamin	Up	FLUAV lowers drug-induced genes	No effect on viral genes
Fluticasone	Up	FLUAV lowers drug-induced genes	No effect on viral genes
Metoprolol	Up	FLUAV lowers drug-induced genes	Suppresses viral genes
Pantoprazole	Up	FLUAV lowers drug-induced genes	Suppresses viral genes
Salbutamol	Up/Down	FLUAV lowers drug-induced genes	Suppresses viral genes
Salmeterol	Up	FLUAV lowers drug-induced genes	Suppresses viral genes
Sertaline	Up	Slight reduction in drug-induced genes	Activates viral genes
Furosemide	No	No effect in drug-induced genes	Activates viral genes

The table summarizes the effect on mRNA levels of nine selected compounds, based on the heatmaps presented in figure 5.3.1-5.3.3. Most compounds exhibited up-regulation on mRNA levels in mock-infected cells. Amlodipine and salbutamol exhibited both up- and downregulation, whilst furosemide showed no change. In FLUAV-infected cells, all compounds lowered the drug-induced genes found in figure 5.3.2, except sertraline and furosemide that had slight to no effect in these genes. The selected drugs affected viral transcription differently. Furosemide, sertraline and amlodipine activated the viral genes and metoprolol, pantoprazole, salbutamol, salmeterol and sertraline suppressed the viral genes, whilst cyanocobalamin and fluticasone had no effect on viral gene transcription.

5.4 Metabolic effects in mock- and FLUAV-infected RPE cells

To evaluate the effects of the drug treatment on cellular metabolism we infected RPE cells with FLUAV at moi 1 or mock. After 24 hours we collected cell culture media and analyzed the polar metabolites. We constructed heatmaps with the most variable metabolites affected by treatment and FLUAV infection, as previously described. The compounds' effect on metabolism of RPE cells are shown in figure 5.4.1.

We found that most compounds had slight to no effect on most metabolites, except hypoxanthine, guanosine and D-Ribose 5-Phosphate, which in most compounds were significantly down-regulated (4-6 folds). Octanoylcarnitine was slightly up regulated in all compounds, ranging from 0,5 to 2,4 folds.

We found that infecting treated RPE cells with FLUAV altered composition of the polar metabolites. Here, FLUAV was found to increase the concentration of L-Kynurenine and inosine and lower the concentration of spermidine and NAD in most compounds. Figure 5.4.2 presents the effect of the compounds on metabolism of FLUAV-infected RPE cells.

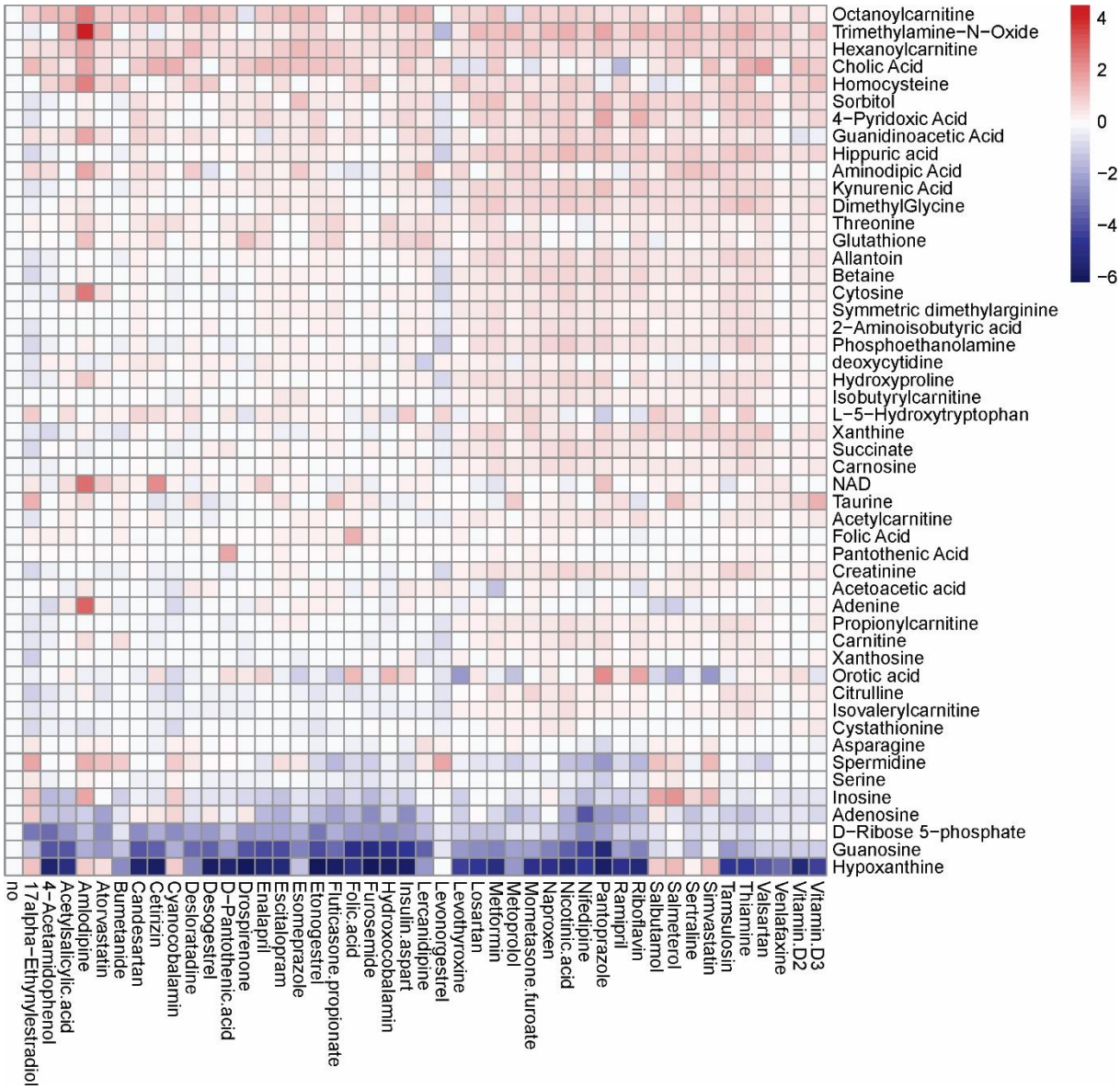

Figure 5.4.1 Effect of 45 compounds on the metabolism of RPE cells.

RPE cells were treated with 10 µM compounds. After 24 h the media were collected, and polar metabolites were analyzed using LC-MS/MS. A heatmap of 50 most variable metabolites affected by treatment is shown. Rows represent metabolites, columns represent samples. Each cell is coloured according to the log2-transformed and quantile-normalized values of the samples, expressed as FC relative to the average of DMSO-treated controls.

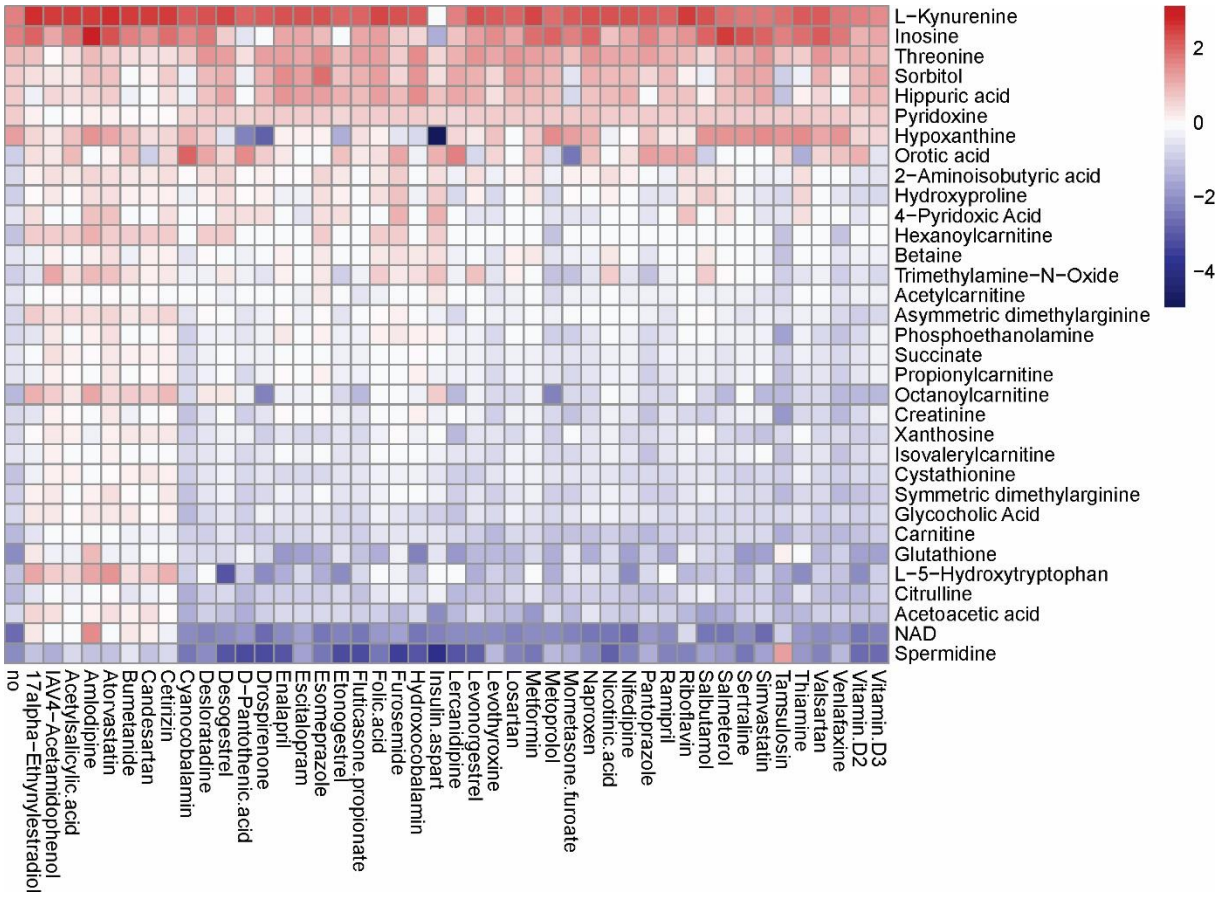

Figure 5.4.2 Effect of 45 compounds on the metabolism of FLUAV-infected RPE cells.

RPE cells were treated with 10 µM compounds and infected with FLUAV (moi, 1). After 24h the media were collected from the cells, and polar metabolites were analysed using LC-MS/MS. A heatmap of most variable metabolites affected by FLUAV infection is shown (0.5 < logFC < -0.5). Rows represent metabolites, columns represent samples. Each cell is coloured according to the log2-transformed and quantile-normalized values of the samples, expressed as FC relative to the average of DMSO-treated mock-infected controls.

Table 5.4.1: Summarized effect of nine selected compounds on metabolism of mock-infected RPE cells and FLAUV-infected RPE cells

Compound	Mock-infected cells	FLUAV-infected cells
Amlodipine	Up	FLUAV up-regulates drug-induced metabolites
Cyanocobalamin	Up/down	FLUAV up- and down-regulates drug-induced metabolites
Fluticasone	Down	FLUAV up- and down-regulates drug-induced metabolites
Metoprolol	Up/Down	FLUAV up- and down-regulates drug-induced metabolites
Pantoprazole	Up/Down	FLUAV up- and down-regulates drug-induced metabolites
Salbutamol	Up	FLUAV up- and down-regulates drug-induced metabolites slightly
Salmeterol	Up/Down	FLUAV up- and down-regulates drug-induced metabolites
Sertaline	Up/Down	FLUAV up- and down-regulates drug-induced metabolites
Furosemide	Down	FLUAV down-regulates drug-induced metabolites

The table summarizes the effect on metabolism of nine selected compounds, based on the heatmaps presented in figure 5.4.1 and 5.4.2. Most compounds displayed down-regulation of hypoxanthine, guanosine and d-ribose 5-phosphate. RPE cells treated with compounds and infected with FLUAV showed altered composition of the polar metabolites. Here, FLUAV was found to increase the concentration of L-Kynurenine and inosine and lower the concentration of spermidine and NAD in most compounds.

5.5 Cellular targets of the drugs and their potential implication for FLUAV-host cell interaction

We constructed a drug-target interaction network of the 45 most prescribed drugs in Central Norway in 2019. The visualization was performed using the STITCH web-tool (ref 10.1093/nar/gkv1277) and is presented in figure 5.5. The edge width and color darkness indicate the degree of data support for the connection. The chemical-protein interactions include both direct targets of the compounds as well as their downstream targets.

CXCR4, ALB, HRH1, RHOA, ADRA2B, PNP, and MMAB proteins associated with FLUAV replication are marked with red-dashed circles (4, 22). Thus, Niacin (CXCR4 and PNP); Losartan, Ramipril, Aspirin, Thyroxine, Valsartan (ALB); Cetirizine, Citalopram (HRH1); Atorvastatin, Simvastatin (RHOA); Metoprolol, Niacine, Setraline, Candesartan (ADRA2B) and hydroxocobalam (MMAB) could target and modulate FLUAV-host cell interaction.

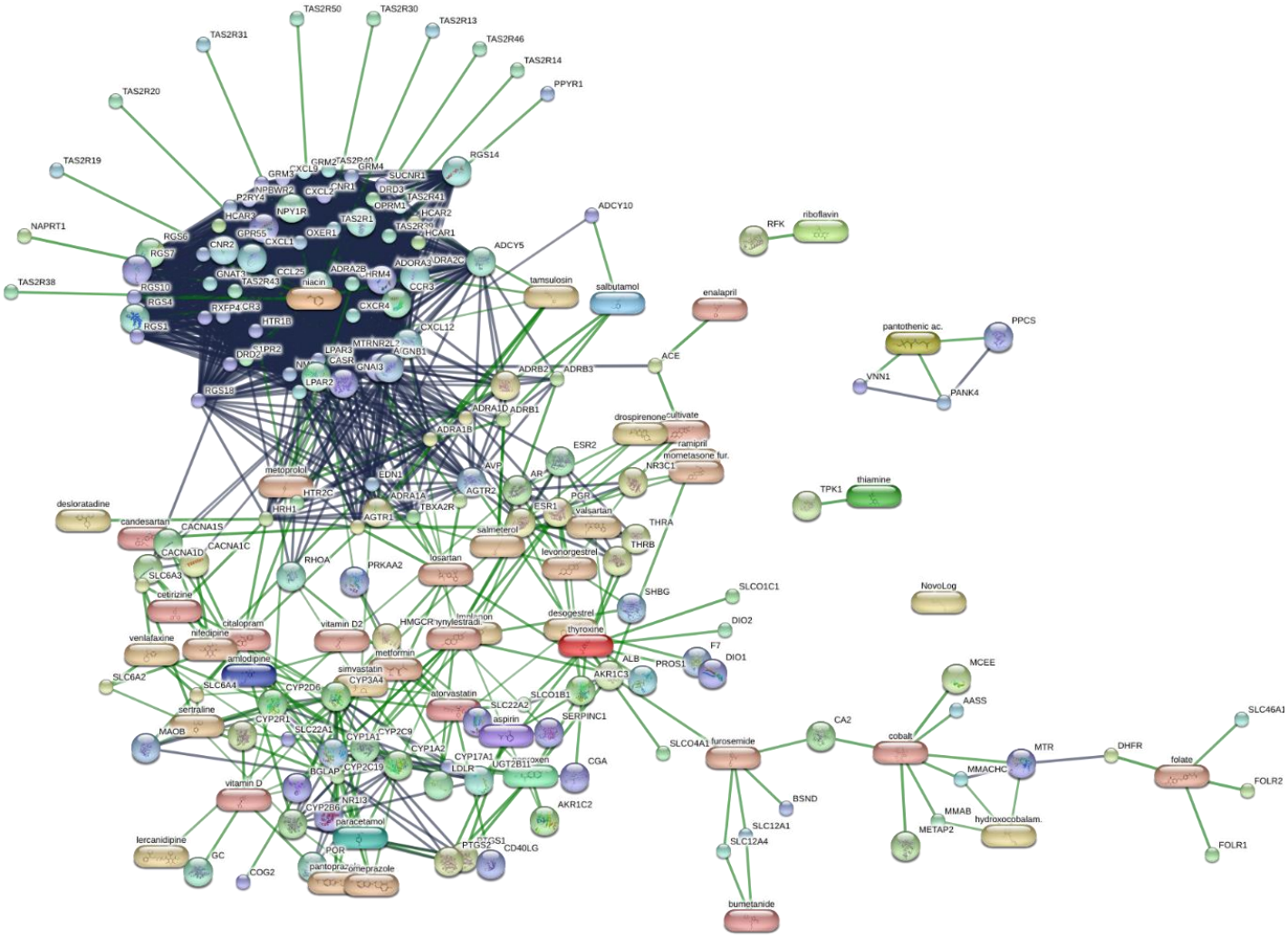

Figure 5.5 Cellular targets of 45 drugs and their potential implication for FLUAV-host cell interaction.

Chemical-protein interactions between direct targets of the compounds and their downstream targets. Proteins associated with FLUAV replication (marked with red-dashed circles) indicate that losartan, ramipril, valsartan, cetirizine, citalopram, atorvastatin, simvastatin, metoprolol, niacin, sertraline and candesartan could target and modulate FLUAV-host cell interaction (4, 22).

6. Discussion

The pharmaceutical industry is one of the most strictly regulated industries in the world, ensuring that medicines approved with marketing authorization are safe and effective, and that the benefits of the drugs outweigh potential risks and ADRs to the patients (8-11). We decided to investigate whether the most commonly prescribed medicines in Central Norway could affect FLUAV-host cell interaction and thus be beneficial or harmful in the course of FLUAV infection.

6.1 Selection of compounds

To find the most commonly prescribed medicines, we used the Norwegian Prescription Database, which contains information about medicines dispensed from pharmacies based on prescription from doctors. Over-the-counter medicines, however, such as ibuprofen and paracetamol as well as medicines to treat allergies, heartburn, constipation, diarrhea and more, are not included into this database. Thus, the actual use of medicines may differ from what we collected from the database. Moreover, the database is based on the number of tablets sold per inhabitant, not accounting for actual consume per person (36). However, this database provides a pointer of which medicines are most commonly prescribed. We decided to study the immuno-modulatory effects of the 45 most prescribed medicines.

6.2 Cytotoxicity and efficacy assays

Cell viability assay revealed that all compounds reached half-maximal cytotoxic concentrations at higher concentrations than 10 μM. We also found that the compounds were ineffective against FLUAV at concentration below 10 μM. Thus, we chose to proceed the study using 10 μM of each compound to prevent feasible cell death and data loss. The concentrations we used do not necessarily relate to actual concentrations found in vivo at recommended doses to treat the different diseases or conditions.

6.3 Transcriptomics analysis

To evaluate the effect of the compounds on transcription of viral and cellular genes, we used transcriptomics analysis. We found that the compounds affected transcription, both in mock-infected cells and FLUAV-infected cells. Interestingly, we found that compounds having the same mechanisms-of-actions, such as simvastatin and atorvastatin, salbutamol and salmeterol, candesartan and valsartan differently affected the same genes tested. Studies have shown that many medicines possibly hold multiple physiologic targets, conceivably explaining why both ADRs occur as well as why pharmacological and clinical effects can differ (4, 9, 14). This may also explain why different genes were affected in different degrees, however, from this we cannot conclude without further studying.

When analyzing the heatmaps we found that many of the genes up-regulated by the compounds were down-regulated during FLUAV-infection. This could indicate that FLUAV down-regulates the drug-induced genes, but it could most probably be because an ongoing viral infection changes the cells need of other proteins when found in a cellular antiviral state. For instance, high amounts of vRNAs in cytosol initiates transcription and secretion of type 1 IFNs, resulting in type 1 IFN-mediated autocrine loop that facilitates the expression of ISG expression in the infected cell, and when secreted, in non-infected neighboring cells (4, 14).

The transcriptomics analysis of the viral genes, suggest that many of the compounds induce viral gene expression. For instance, furosemide, highly up-regulated all viral genes, which could indicate that this compound may lead to more serious infection of FLUAV. Further studies are needed to see if these drugs facilitate FLUAV replication. Interestingly, metformin, was the only compound exhibiting excessive down-regulation of one viral gene, PB1-F2, which could enhance virus-induced cell death (4, 14). This could indicate that patients using metformin perhaps have shorter course of the disease, or experience fewer or milder symptoms, however, this must be further studied.

6.4 Metabolomics analysis

We found that all compounds differentially affected the metabolism of mock- and FLUAV-infected cells. However, some key-findings could be drawn. Most compounds down-regulated the metabolism of hypoxanthine, guanosine and d-ribose 5-phosphate. This could indicate that the synthesis of DNA and RNA, as well as NADPH is downregulated. When infecting the RPE cells with FLUAV, metabolism changed. L-kynurenine was down regulated in cells treated with all compounds, except hydroxocobalamin.

6.5 FLUAV-host cell interaction

We found that losartan, ramipril, valsartan, cetirizine, atorvastatin, simvastatin, metoprolol, sertraline, candesartan and hydroxycarbamide could target and modulate FLUAV-host cell interaction through various cellular pathways, thus possibly affecting the course of the disease and symptoms. However, further studies need to be conducted to draw a conclusion.

6.6 Future studies

This study included one cell-line and one FLUAV strain. To understand how genetic variations contribute to virus-host interaction, it would be interesting to see if primary human cells would give different results. It would be interesting to see whether other cell lines would affect the transcriptional and metabolic effects differently, compared with the one we used. It would also be interesting to see if our findings could be reproduced in other pathogen-host systems. Our system biology approach could also be used by pharma companies during pre-clinical development of new medicines to detect side effects or perhaps even new indications.

7. Conclusion

Although symptoms of FLUAV-infection range from mild to severe, most cases of hospitalizations and death occur among high-risk groups, where the risk of severe diseases or complications are elevated (1-4). CDC and FHI recommend anti-influenza treatment to high-risk groups, stating the importance of preventing infection or alleviating symptoms and shortening the course of the disease in high-risk patients (23, 24, 30). We found that many compounds differently affected cell transcription and metabolism, thus indicating they have several physiological targets. We also found that gene expression and metabolism changed in cells treated with each compound and infected with FLUAV. This is most probably because the cell is activated into an antiviral state, thus different proteins are needed compared with non-infected cells. Interestingly, however, we found that compounds closely sharing the same mechanisms-of-actions differently affects the gene-expression and metabolism, both in non-infected cells and infected-cells. This could indicate why the compounds may have differently reported side effects, as well as whether some of the compounds could lead to more serious FLUAV infections in patients treated, compared with others. However, this needs to be further studied. The identified properties of the compounds are most probably associated with on- and off-target effects, i.e., the compounds may target several essential host factors involved in synthesis and metabolism of important immuno- and neuro-modulators. However, it is important to perform follow-up omics analyses to get consistent results.

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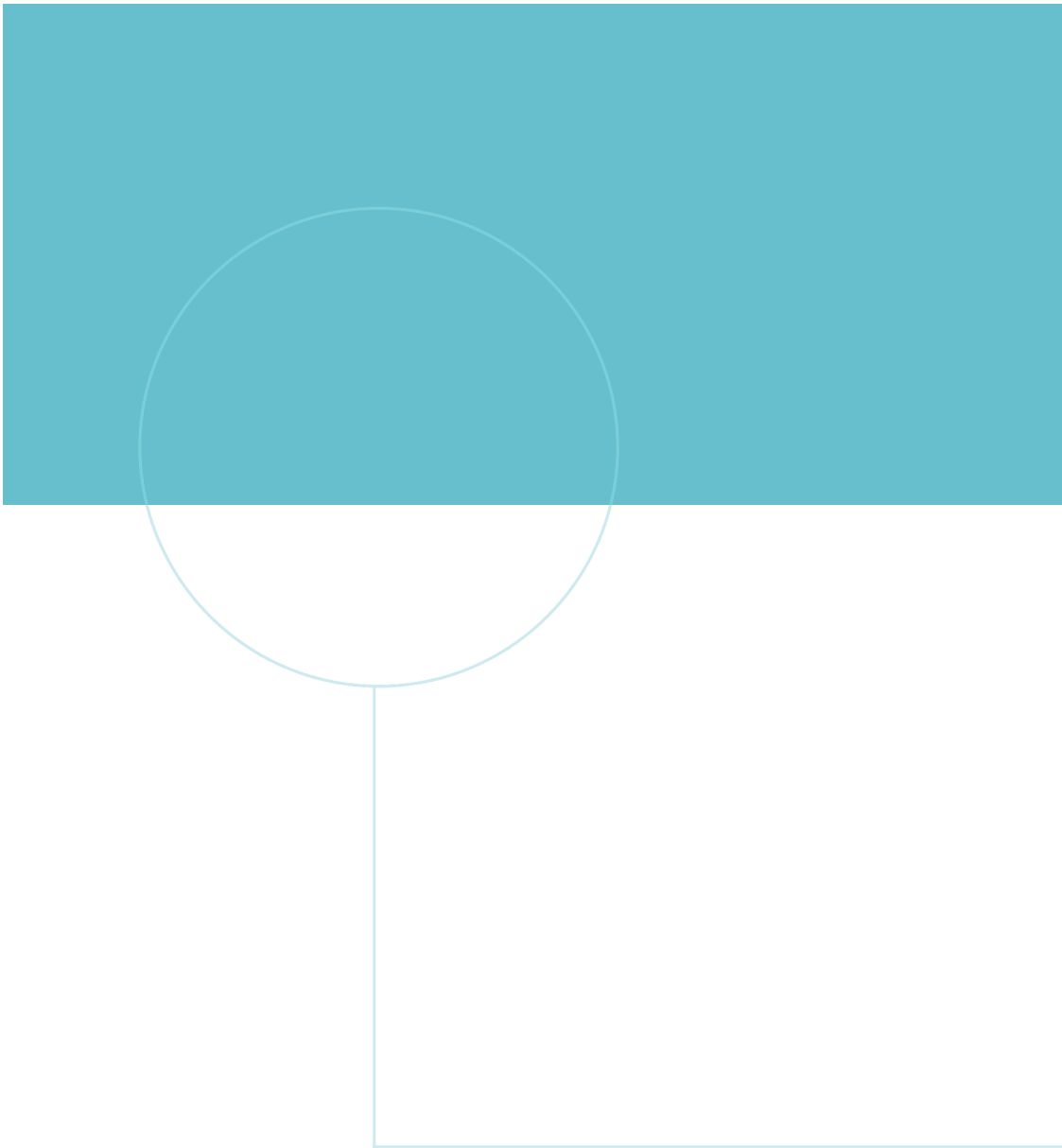

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