Antiviral Molecules of Enteroviruses

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Abstract

Enteroviruses have a major impact in today's world. They are responsible for a large portion of harmless infection but also cause life-threatening diseases, poliomyelitis being perhaps the best known example. Although vaccines have proven effective in the abolishment of polio, they cannot be considered as a final solution since they hold no effect on infections that are already taking place. For this, compounds inhibiting the proliferation of the viruses, antivirals, are needed. The research in search for such compounds against enteroviruses has been ongoing for decades, yet no drugs have been put to clinical use so far. Many potent antivirals have been made, a few of them going through clinical trials, but have always proven inefficacious against the diverse range of pathogens and their high mutation rate that provides quick resistance. This work aims to offer an introduction to the enteroviruses and an in depth coverage of many of the antivirals devised against them. This includes the prototype capsid binders, the antivirals created against the non-structural proteins of the viruses as well as the compounds inhibiting the cellular factors involved in the infection found quite recently. The concluding remarks aim to provide some insight to the future of the field and the approaching of antienteroviral treatments.

Tiivistelmä

Enteroviruksilla on suuri merkitys nykymaailmassa. Ne ovat vastuussa sekä suuresta osasta harmittomia tartuntoja että hengenvaarallisista taudeista. Jälkimmäisistä parhaiten tunnettu esimerkki lienee polioviruksen aiheuttama polio. Vaikka rokotteet tarjoavat suojaa poliolta ja joiltain muilta taudeilta, niistä ei ole lopulliseksi ratkaisuksi, sillä ne eivät auta jo tartunnan saaneita ihmisiä. Tätä varten tarvitaan antiviraaleja, yhdisteitä, jotka estävät virusten lisääntymistä. Tutkimus yhdisteiden kehittämiseksi enteroviruksia vastaan on jatkunut jo vuosikymmeniä, mutta ainuttakaan lääkettä ei ole vielä saatu yleiseen hoitokäyttöön. Monia potentiaalisia yhdisteitä on tänä aikana kehitetty ja joitakin testattu kliinisissä kokeissa, mutta niiden suorituskyky ei ole riittänyt koko laajaa taudinaiheuttajaryhmää ja suuresta mutaatioherkkyydestä aiheutuvaa nopeaa resistenttien viruksien kehittymistä vastaan. Tämä pro gradu-tutkielma pyrkii tarjoamaan lyhyen esittelyn enteroviruksista ja syvemmän tarkastelun niitä vastaan kehitetyistä antiviraaleista. Tämä sisältää ensimmäisenä kehitetyt kapsidiin sitoutuvat molekyylit, ei-rakenteellisia viruksen proteiineja vastaan suunnitellut antiviraalit sekä virusinfektiossa hyödynnettyjä solun proteiineja estävät yhdisteet. Yhteenvetokappale pyrkii tarjoamaan ajatuksia kehitystyön tulevaisuudesta ja alati lähenevistä antienteroviraalisista hoidoista.

Preface

This work was done during the time span from September to December 2016 at the Nanoscience Center of University of Jyväskylä. The supervisors of the thesis were Dr. Tanja Lahtinen and Dr. Varpu Marjomäki. The literature search for the work was mainly conducted with the use of Web of Science™ literature service with the complementary use of the Google search engine. The searches included the combinations of the terms enterovirus, rhinovirus, picornavirus, antiviral, inhibitor and the names of the different drugs and their targets (e.g. capsid, 3A, OSBP). Additionally the references and authors in the found articles were used for more literature.

I would like to thank both of my supervisors; Dr. Tanja Lahtinen for her motherly supervising and support since my B.Sc. work and Dr. Varpu Marjomäki for offering the possibility to work with such interesting topics and for withstanding most of the interdisciplinary confusion of moving to an area between chemistry and biology. I thank Karolina Sokołowska and my other colleagues for providing encouragement and useful advice during the writing process. Thanks to all the people who have taught and helped me with both my studies and research. I look forward to working with all of you. Last I would like to thank my family and especially my parents Jola and Seppo for supporting me in my choices, regardless of what they may be.

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Abbreviations

ATA	aurinitricarboxylic acid
CC_{50}	50% cytotoxic concentration
CV	coxsackievirus
DNA	deoxyribonucleic acid
EC_{50}	50% effective concentration
EV	enterovirus
fmk	fluoromethyl ketone
GuaHCl	guanidine hydrochloride
HFMD	hand, foot, and mouth disease
HRV	human rhinovirus
Hsp90	heat shock protein 90
IC_{50}	50% inhibitory concentration
IRES	internal ribosomal entry site
MIC	minimum inhibitory concentration
mRNA	message ribonucleic acid
OBSP	oxysterol binding protein
PI4KIIIβ	phosphatidylinositol-4-kinase III beta
PV	polio virus
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
SI	selectivity index
siRNA	small interfering RNA

UTR	5′ untranslated region
VPg	viral protein genome-linked

1 Introduction

Viruses are nanometer scale particles that are inactive in the open, but once inside their target cells, they quickly become more lively.[1] In other words, they are cellular parasites, carrying DNA or RNA genome inside a protein structure called viral capsid, some additionally covered by a lipid layer stolen from their previous host cells. A viral infection on a cell begins with a collision of virus and a binding site, most often a receptor on a cell that a virus is able to infect. The viral genome is then released into the cell and either works by itself in cytoplasm (lytic cycle) or is incorporated to the host cell DNA (lysogenic cycle).[2] The genome is translated by the host ribosomes and the proteins produced from this then start repurposing the cell for the production of new viral particles, virions. This includes hijacking some of the cell's enzymes for this as well as the work from newly formed viral enzymes. Eventually the cell becomes a virus factory at the expense of its own functions. Finally the different virion building blocks come together to form a virion that is then released from the cell to look for a new recipient. In a lytic cycle the cell is killed due to destructive exit strategy of viral particles, while at the end of lysogenic cycle the host cell remains alive as the virus most often exits enveloped in the host lipid membrane. The bipartite nature of the virus; a lifeless particle having such complex biological functions and liveliness in its host cells has always been a topic of debates, whether they are living organisms or not.[3,4]

The diversity of 3705 species[5] of viruses in 2015 has been divided into different orders, families, subfamilies and genera by the classical Linnaean hierarchical system. The classification is based on the nature of their genome, whether it's RNA or DNA, the symmetry of their capsid, whether a lipid membrane exists and the dimensions of the capsid.[1] Additionally, the Baltimore classification system divides the viruses in seven different classes depending on the type of their genome and the pathway of nucleic acid replication they use to produce mRNA.[6] The nucleic acid strand can be coded either in negative sense or positive sense. Positive sense strand can be translated straight into proteins by ribosomes, while negative strand ones need a complementary intermediate before protein synthesis. In addition the nucleic acid strand can be either a single or a double strand in viruses. To make things more complicated, the different species of viruses can be divided into different subspecies, serotypes, by their response to antibody

neutralization tests.[7] This means each viral serotype of the same species have different antigens on their surface that bind to antibodies in these tests. This work focuses on the enterovirus (EV) genera, containing over two hundred serotypes that are responsible pathogens for very severe conditions as well as major culprits for the common cold.

While already the diversity between viral species poses a large challenge for the development of treatments against disease caused by them, they are also rapidly evolving agents. This is due to the error-prone nature of translation of nucleic acids, which can happen in a few different ways.[8] For example just a singular base pair in a nucleic acid strand can change causing a point mutation, or the strand can be broken and joined to another strand in a genetic recombination. As a result, even after development of treatment to specific viral diseases, the viruses might become resistant to this drug in a short interval. The primary weapon against viruses thus far has been putting the human immune system to work. This is achieved through the use of vaccines. Vaccines are a neutralized variant of the virus, such as an empty capsid, that causes an immune system reaction in the host organism and after which the immune system gains antibodies against this specific infection.[2] Thanks to vaccines, the world has already been eradicated of serious illnesses like small pox[9] and some, such as polio[10], have been contained to only a few countries. However, vaccines cannot cure infections already caused by viruses, which means that the demand for antiviral drugs remains. Antiviral compounds, both synthesized and natural ones, target the different viral proteins and other factors involved in the progress of an infection, inhibiting the capability of the virus to produce new viral particles. For enteroviruses, no antiviral drugs have been approved for use as treatment, although the research has been ongoing for decades. Yet many potential drugs have been reported over the years. These drugs, their development, efficacy, current status and future will be the main focus of this work.

2 Enteroviruses

The picornaviridae family of the picornavirales order uses humans and other vertebrates as host animals. More commonly known as picornaviruses, they are defined as small plus-strand RNA agents covered in an icosahedral capsid, without a lipid membrane.[11] These Baltimore class IV viruses size to around 30 nanometers and compared to other viruses, differ so that the first molecule produced after infection is a protein, instead of another nucleic acid molecule. Belonging to this family is the genus of enteroviruses (EV). As the name suggests, the main route of entry to the host is through the fecal-oral route, using feces or respiratory secretions as an intermediate. To this date, 71 different serotypes of enteroviruses among 12 species have been identified. Although EVs cause many diseases that will be discussed in detail in section 2.4, only polio and EV71[12] have available vaccines and no antiviral therapies are available. This is due to the high mutation rate of the viral genome.

2.1 Genome and proteins

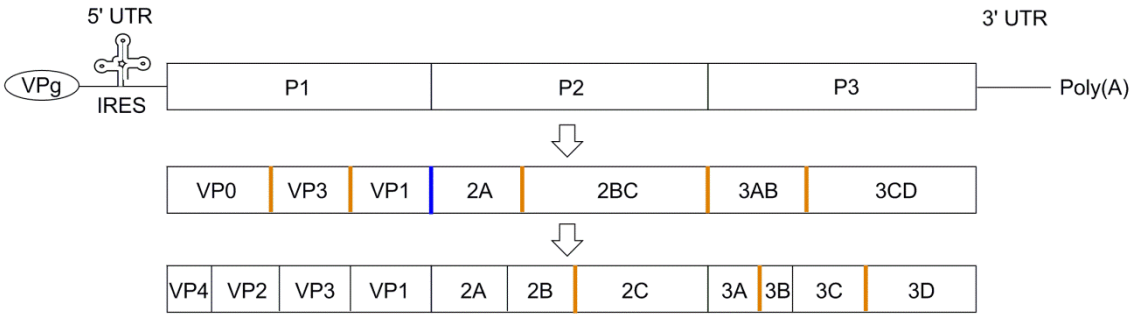


Figure 1. Structure of the genome of enteroviruses and the processing pathway of its complementary polyprotein. The protein cleavages conducted by $2A^{pro}$ and $3C^{pro}$ are colored as blue and orange respectively.[13]

The genome of an enterovirus is usually approximately 7500 bases long, singular RNA-strand.[11] In the 5′-end the genome is connected to the viral protein genome-linked (VPg).[13] To begin the translation of viral mRNA, enteroviruses have an internal

ribosomal entry site (IRES), as a replacement for 5′ cap found in eukaryotic mRNA, which binds to host cell ribosomes and starts the process. The IRES is on both sides of 1000 nucleotides in length and assumes secondary structures that have a high similarity among enteroviruses.[14] After IRES the viral protein coding part of the genome starts. This contains all the information for genetic coding of capsid, enzymes and other proteins for a new virus (fig. 1). The viral genome ends with a long chain of adenosine in the 3′-end. The protein encoding area is divided in three parts P1-P3 depending on the proteins that these areas code. However the viral protein is translated as a single polyprotein that is then cleaved by the viral enzymes to yield the functional molecules for the virus. Some combinations of two proteins have also their own functions before being separated. P1 of the genome is responsible for the structural proteins VP1-4 that build up the virus capsid. P2 and P3 encode the nonstructural proteins of the viruses. From P2 polyprotein, enzymes 2A-C are cleaved by 2A and 3C protease enzymes. P3 polyprotein produces proteins 3A-D in the same way. Thus these areas contain the information for all the proteins of the virus that are needed to complete a viral cycle.

2.2 Viral cycle

With enteroviruses, the viral cycle is also started with the viral capsid attaching to its target host cell's receptor. The binding to receptor is located to a depression or a canyon on the interface between five capsid proteins, the hydrophobic pocket.[13] The receptors on the cell that the viruses bind to, vary quite a lot, but can be categorized to some extent. However, it should be emphasized that a single viral genus does not necessarily use only one pathway for infection but multiple pathways can be employed. Popular domains for EV attachment are some immunoglobulin receptors.[15] For example, human rhinoviruses (HRV) use intercellular adhesion molecule-1[16], polioviruses abuse nectin-like molecule-5 receptor, also known as polio virus (PV) receptor[17] and coxsackie viruses take advantage of a epithelium component named after it, the coxsackie and adenovirus receptor.[18] Another receptor class used by EVs are the different integrins and their ability to recognize a three amino acid sequence.[15] This behavior has been observed with echo- and coxsackievirus (CV) A9. Decay accelerating factor -receptor is a commonly used pathway by enteroviruses, however it doesn't bind to the hydrophobic

pocket and often works in tandem with some other receptor for a successful entry to the cell.

After a successful attachment to the host cell surface, endocytosis ensues and the viral particle is taken inside the cell in a vesicle.[15] This event can also happen through multiple different pathways, connected to the nature of the receptor the virus is bound to, but the most common ones are caveolin, clathrin and neither -mediated endocytosis. The clathrin pathway involves a protein by the same name forming pits on the cell's inner surface that then get internalized and a subsequent uncoating of the protein produces an endosome inside the cell. Unique for this pathway, the conditions in the endosomes become acidic, which the viruses actually require for a successful entry to the cell. Caveolin-mediated pathway is governed by the protein with a same name, and requires lipid rafts and cholesterol on the cell surface to function properly. Caveolin pathway has been shown at least for echo- and coxsackieviruses. As an example of viral endocytosis, poliovirus is a good example that requires neither of these and employs other measures after binding to the poliovirus receptor.[19]

The binding to the host cell receptor, or the acidic conditions in the endosomes, subject the EV particles to conformational changes, forming so called A particles or 135S particles.[20] What happens is the ejection of VP4 protein from inside of the virus and externalization of an N terminal region in the VP1. This makes the virus surface hydrophobic, making direct binding to vesicle membranes possible. After this the A particles are then converted into empty capsids which has led to the conclusion that the procedure is part of RNA injection into the cytoplasm. The exact mechanism for membrane permeability isn't known but evidence has been shown for the involvement of the externalized VP1 region[20] and the released VP4[21].

The viral RNA released into the cell cytoplasm is read from 5′ untranslated region (UTR) by 40S ribosomal subunit until the polyprotein synthesis site is encountered.[14] More accurately, the initiation is directed by the IRES unit in UTR. Now the other 60S ribosomal subunit attaches and the first polyprotein synthesis is carried out in the cell. The newly formed viral enzymes restrict many cell functions to make way for viral

replication. One of the most important is the hindrance of host cell protein synthesis by the cleavage of eukaryotic initiation factor 4G, connected to the protein complex responsible for mRNA binding to ribosomes.[22] This is done by the viral 2A protease. As only eukaryotic mRNA uses a protein cap structure to bind to ribosomes and start protein synthesis, the lack of functional 4G factor ultimately shuts down the host cell protein synthesis. For this to happen, also the polyadenosine-binding protein gets shut down by 2A or 3C protease.[23] Furthermore, the viral proteins, mostly 2A and 3C, inhibit the nucleus-cytoplasm trafficking in multiple ways[24], disrupt the cytoskeleton with cleavage of dystrophin[25] and suppress the cellular responses to the infection by cleavage of RNA helicase MDA-5[26], regulatory factor 7[27], mitochondrial anti-viral signaling protein and an interferon beta inducing protein[28].

After making the cell into a virus protein factory, for a complete virus particle, a new plus-strand RNA molecule is needed. To achieve this, a negative-strand complementary RNA template is needed. The process is started with the uridylylation (reaction with uridine monophosphate) of VPg at the beginning of the genome by viral RNA polymerase 3D (fig. 2).[29] This reaction is further assisted by a secondary structured RNA chain, located in the 2A protein genome, called the cis-acting replication element[30] and a similar structure that protects the susceptible RNA molecule, 5′ cloverleaf cis-acting replication element.[31] The uridylated VPg then gives a site for the 3D enzyme to produce the mirrored negative strand RNA strand from the original. This strand is then used as a template for new plus strand RNA molecules that can be used in either protein synthesis or new virions.

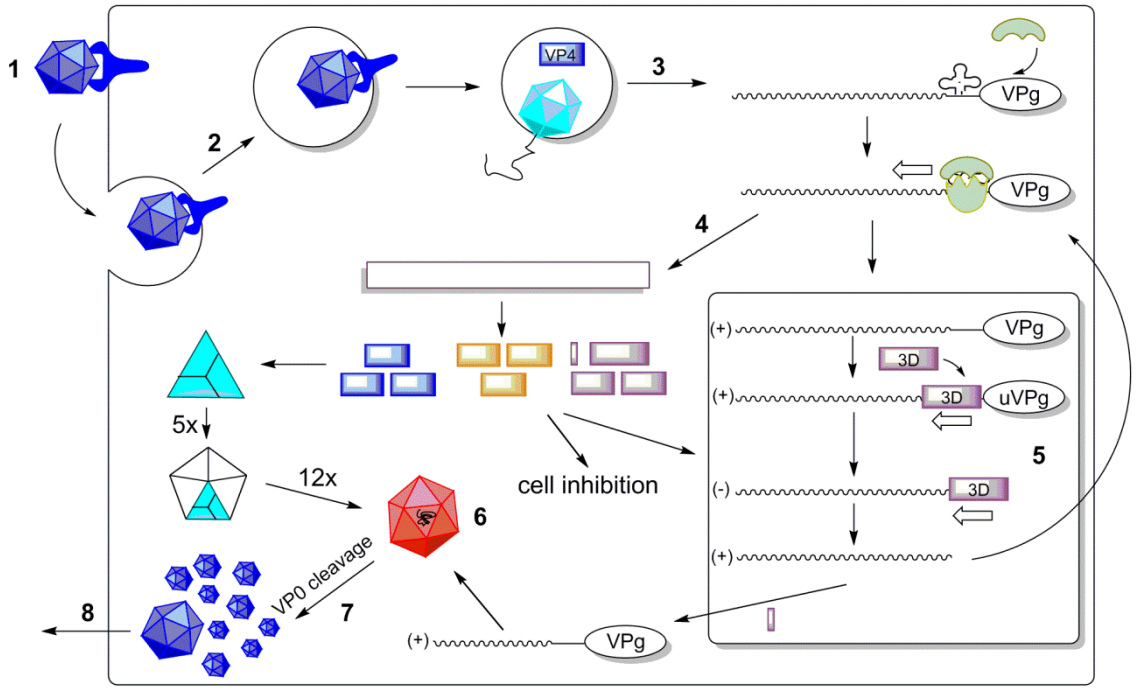


Figure 2. Depiction of the viral cycle of enteroviruses. 1. Attachment 2. Endocytosis 3. RNA release 4. Polyprotein synthesis 5. Synthesis of new RNA strands in virus induced membrane structures 6. Encapsidation 7. Maturation by the cleavage of VP0 8. Virion release through either lytic or nonlytic pathway.[13]

The structural proteins containing polyprotein P1, liberated from the rest by 2A protease, is processed further to start the capsid forming process. This includes protein folding issued by host cell heat shock protein 90 (Hsp90) and cleavage of VP0, VP1 and VP3 by 3CD protease from each other.[13,32] VP0 contains both VP2 and VP4 still joined together. The three capsid proteins then form spontaneously a building block for the capsid, a protomer. Five of these units then come together to form a pentamer and in turn, twelve pentamers form a viral capsid. Some viral species require glutathione to stabilize the pentamers.[33] The encapsidation of a new RNA strand and transformation of a virion begins from an interaction between 2C enzyme and capsid protein VP3.[34] It is hypothesized that the RNA complex synthesizing new plus strand RNA attracts pentamers to encapsidate the RNA.[35] The RNA itself is thought to facilitate addition of more pentamers. The thus formed provirion then goes through maturation where the VP0 is cut in an RNA-induced cleavage to yield VP2 and VP4.

For the new virions to escape the host cells they were created in, both lytic and non-lytic release is employed by enteroviruses.[13] In the lytic pathway the cell membrane is broken and thus the new viral particles are released from the cell. The non-lytic pathway takes advantage of alteration of host cell membrane structures. Already in the replication phase, the infection causes single membrane tubular vesicles to be formed that are used as sites for RNA replication.[36] As the encapsidation is connected to the newly forming RNA, also that happens in the vicinity of these membranes. In the later part of infection the single walled membranes change to double-membrane structures, enclosing cytoplasm around them. On a molecular level the formation of these membranes is as follows; protein 3A from the virus recruits Golgi-specific BFA-resistance factor 1, which in turn causes an ADP-ribosylation factor 1 to form a complex with the previous protein and become bound to the cell membrane.[13] This complex activates the coat complexing proteins, inducing vesicle formation. Furthermore, the 3A protein recruits another protein, phosphatidylinositol-4-kinase III beta (PI4KIIIβ), to the membrane, causing increased phosphate content on the cell membrane. Then a oxysterol-binding protein (OSBP) is attracted to the membrane concentrated with the phosphates and starts exchanging them for cholesterol.[37] The increased cholesterol content is thought to be the inducing factor for the vesicle formation. The importance of understanding the aforementioned process in detail has been proven by obstructing this process chemically, which ended the replication of virus in the host cells.[38]

2.3 Classification

While segregating the constantly and rapidly evolving enteroviruses in different classes might seem like an arbitrary task, it gives important information on characteristics and similarities between different species and greatly contributes to the understanding of them. Previously the division between polio-, coxsackie A and B -, echo-, and rhino viruses was done based on their pathogenesis.[39] Coxsackie viruses were distinguished from polio viruses by their ability to cause paralysis in mice, A genus causing flaccid paralysis versus spastic one caused by B genus. Echo viruses were determined by the fact that no diseases were associated with them. Additional viral species were named after the animals they were found on; simian EVs found on monkeys, porcine EVs

found in pigs and bovine EVs found in cows. While the nomenclature from this time still lives strong, the more recently found enterovirus species have been named with consecutive numbers (EV68 onwards), in order of discovery. For classification purposes pathology proved insufficient determinant.

The next step in categorizing was distinction by antigens.[39] Each enterovirus serotype is antigenically distinct and thus can be neutralized by different antibodies. The serologic studies separating the different EVs by their sensitivity to antibodies have produced the presently known 71 different human EV serotypes. However serology offers little help for the classification of different serotypes in their own groups. The methods for more modern subgrouping have developed with the study of viral genomes as the polymerase chain reaction (PCR) machinery became more common. An attempt to sequence enteroviral VP2 protein led to the distinction of four major phylogenetic groups.[39,40] However, insufficient correlation with serotype led to a choice of other sequence. As VP1 was declared as the most dominant for viral immunoresponse, Oberste *et al.*[40] compared the sequence of this domain between different serotypes and divided EVs into four major groups as well based on their sequences. These groups consist of CVA16-like, CVB-like, PV-like and EV68/EV70 like viruses, with a few others falling out of these groups. The non-human EVs and rhinoviruses form their own phylogenetic clusters. All in all, today's official nomenclature puts enteroviruses into nine different species EV A-J, A-D consisting of aforementioned groups respectively.[5] EV-B is the biggest group of these, containing all the echoviruses, CVB1-B6 and CVA9. Species E and F are species found on bovine, G in porcine and H in simian sources. Additionally three different rhinovirus species A-C are found in the EV family. As a result of the classification of enteroviruses, not only has the understanding of researchers about the common factors in these agents increased, but also rapid identification of EV species has been made possible with the design of versatile primers together with PCR.[41,42] In the future these methods may prove invaluable in quick determination of species of EV infection and thus fast way of determining best course for treatment of many diseases caused by these viruses.

2.4 Diseases and society

Enteroviruses affect the lives of people through the many different diseases that their infections cause. These diseases manifest themselves from the common cold to meningitis, paralysis and even death.[11] Probably the best known agent in the EV family is the polio virus, which is also considered the prototype of the genus, causing poliomyelitis. While 72% and 24% of infections are a- or mildly symptomatic respectively, the less than 1% of infections that caused flaccid paralysis in subjects made the disease well known.[10] This is due to spreading from initial infection site in the pharynx to the central nervous system. In 1952 this led to over 21,000 cases of paralysis and in severe cases the inability to breath was treated with machines called iron lungs that took advantage of negative pressure to keep breathing going for patients until the infection passed. However after the introduction of the first vaccine in 1955 and the polio eradication program, only 34 cases of wild PV in Pakistan, Afghanistan and Nigeria have been reported in 2016 so far.[43]

A more recent agent has been the hand, foot, and mouth disease (HFMD) caused by EV-A71 in Asia-Pacific region.[44] In this area, EV71 has been causing major outbreaks of HFMD in small children, the biggest one locating to Taiwan, where 1-5 million people were infected by the virus. Although the disease manifests in most cases as blisters in the hand, foot and mouth area together with mild flu symptoms, like PV it is also possible that patients develop neurological symptoms, such as encephalitis and meningitis that have also led to paralysis or even death, due to encephalomyelitis of the brainstem.[45,46] While the Asia area is not the only place where EV71 outbreaks have been experienced, the massive epidemics there are a determining factor for the effort put to finding treatments for the disease caused by the virus. Together with CVA16, EV71 is the most common cause for HFMD.

In general, among the previously mentioned diseases, enteroviruses are also known to cause upper and lower respiratory diseases, pleurodynia (Bornholm disease), herpangina, conjunctivitis, gastroenteritis, myopericarditis, pancreatitis, hepatitis and even type 1 diabetes.[47] EVs are the most common reason, in over 90% of cases the

inflammation is caused by enteroviral infection. While coxsackie resembling viruses are the most common to cause more acute symptoms in patients, all other enterovirus types are also inclined to have stronger effect than mild fever. Even the Echo viruses, first isolated from asymptomatic patients, are now known to cause various diseases.[48] Interestingly, coxsackieviruses have been associated with onset of delayed neurological conditions, after the infection, such as schizophrenia, amyotrophic lateral sclerosis and even coma. Additionally CVB1 has been identified as the reason for type 1 diabetes, due to the infection leading to an autoimmune attack on insulin producing cells.[49]

Unlike EVs, rhinoviruses are infectious only through the respiratory pathway, instead of the fecal-oral route.[47] HRVs are the most common cause of the flu.[50] While the symptoms are mostly harmless, they pose a great threat to people with asthma or other respiratory conditions and can in severe cases develop into pneumonia or bronchiolitis.[47] What's more, with the common cold caused by HRVs, the economic losses suffered annually are estimated to be 40 billion dollars in the USA only.[13] The severity and commonness of the diseases drive forward the antiviral and vaccine research against EVs. This determines the impact the viruses have in the society. While severe compilations or mortality are usually considered the driving factor, also the billions lost due to the common cold and work days lost call for a cure to avoid these.

3 Antiviral molecules

Antiviral molecules are compounds that disrupt some function in the viral cycle of its target virus and this way, inhibits the infection of the virus in the organism.[51] Unlike antibiotics meant for the destruction of bacteria, antivirals only distract viruses in a way that their reproduction is hindered significantly. This can be achieved by interactions with the proteins of the virus or inhibition of the cellular factors abused by the virus. While the first antivirals were discovered without any knowledge about the specific working mechanisms of their targets, the increasing knowledge in this discipline has given way for massive boost to antiviral drug development in recent years. For

enteroviruses, all the functionalities described in chapter 2.2 and the further elucidations to the EV viral cycle in the future are potential targets for inhibition. The structural elucidation of different viral proteins, identification of interaction sites for antiviral molecules and the subsequent design and synthesis consume enormous amount of work. In addition to all this, the mutation prone nature of viruses, especially EVs, may quickly produce antiviral resistant strains of the same virus. Sometimes all that is needed is a change of one amino acid in the viral genome to render an antiviral drug useless.

Humans themselves aren't defenseless against viral infections. In mammals, the first line of defense against them is the interferon system.[52] Interferons are proteins called cytokines that are meant to inhibit infections. The contamination of the biological system with viral particles and the viral products, most notably double stranded RNA, leads to synthesis of hundreds of interferons. This in turn sets off the immunologic response with stimulation of certain genes in cells and controlled apoptosis, cell death, of infected ones. With a complex set of interactions with interferons, viral nucleic acids and cells, different cells serve their own functions in the purification of the system from the infection. The roles of the infected cells and healthy ones differ as well. However, viruses have evolved to circumvent and manipulate the functions of the immune system. This happens through interfering with interferon induced gene expression or protein synthesis, minimizing the interferon response, inhibiting the signaling of interferons, blocking the antiviral activity of induced enzymes or just being insensitive to interferons themselves.[53] When the immune system successfully repels an infection, memory cells are created to respond to the same infection in the future. Vaccines assist this pathway by mimicking an infection in the system and thus inducing immunity to a pathogen. Interferons offer also alternative route for antiviral design. For example antiviral drugs can be designed to activate the immune system by blocking the pathways for viruses to avoid interferon system in the ways described earlier. Vaccines offer no help to an infection already taking place in the system and for this reason antivirals are needed, even for diseases with working vaccines available.

3.1 Antiviral strategies

Approaches in devising new antivirals against enteroviruses most often, but not only, involves the interaction or binding between the antiviral molecule and a target protein. The two main targets for binding are either the various proteins produced in the viral cycle or the proteins in cells that the viruses abuse for their replication cycle.[51] Targeting viral proteins offers unique and specific sites for antiviral activity, but is more vulnerable to mutation in viruses. Cellular proteins are more attractive due to the fact that these components are not likely to develop resistance. Naturally, inhibiting the proteins that are a part of cellular machinery, also stop them from accomplishing their actual cellular function. Most often the binding happens in the active sites of the proteins or enzymes, but also allosteric attachment has been reported.[54] The binding itself can be based on weak interactions or it can be covalent. In competition for active sites, the antivirals need to have a higher affinity for the binding site of target receptor/enzyme compared to the original substrate. If the 3D structure for the target molecule and binding site for the antiviral is known after successful protein crystallization or other means a complementary molecular compound can be devised with computer modelling and its binding affinity can be predicted. Additionally, from synthetic measures a structure-activity relationship can be derived. As is usually the case, antiviral molecules synthesized are identified from a plethora of closely related molecule derivatives and from their antiviral potency a relationship can be established to help with future designing processes.

3.2 Target molecule features

While the main quality of antiviral molecules is their ability to hinder viral infection, there are still many other attributes that are required before it is considered ready to use. Naturally, they are desired to be as universal as possible, broad-spectrum antivirals that cover multiple different types of viruses. The designed antiviral needs to have a high inhibition against their targets, but low cytotoxicity towards cells. Usually, an antiviral is considered potent when its 50% effective concentration (EC_{50}), the concentration

needed to inhibit virus growth by 50%, is in the micro molar scale. Applying the drug for use in patients applies new requirements for the potential antiviral. For this, the drug needs to be structurally stable and able to withstand the metabolic system without being converted to other compounds. Oral administration is always the preferred pathway, which means the drug additionally has to survive the acidic and enzymatic conditions of the gastrointestinal tract and be absorbable to blood circulation as well. Finally, as with all drugs, the side effects from antivirals should be mild or even nonexistent. This is especially true when designing drugs meant to treat entero- and rhinoviral diseases, which are most often quite mild themselves.

3.3 Evaluation methods

To determine the capability of new potential antiviral compounds, the testing is usually started from the studies conducted *in vitro*; outside of biological context, oftentimes in petri dishes in cell cultures. These tests show the initial antiviral activity of a compound when comparing two cell cultures infected with specific serotype of virus with and without the presence of the compound. Trying the effect against many different serotypes gives information about the wide spectrum activity of the new compound. *In vitro* studies can also include the measurement of activity of specific, purified proteins, such as nonstructural proteins of the virus or cell factor proteins, without including cells at all. Measuring the amount of inhibition compared to the concentration of the tested compound gives important point of reference when comparing the efficacy of many compounds. The terms to describe this are MIC (in older research), IC and EC, minimum inhibitory concentration, inhibitory concentration and effective concentration, respectively. Minimum inhibitory concentration means the lowest concentration when visible inhibition of the target happens. Often times the 50% reduction in growth is used in most cases (IC_{50}, EC_{50}) and sometimes also the 90% inhibition is reported. The toxicity of the compounds is also routinely determined using CC_{50} (50% cytotoxic concentration) value, the concentration required to reduce cell viability to half. Dividing this value with the EC_{50} or similar gives the selectivity index (SI) for the drug, giving a value for its efficacy.

After examining the activity of an antiviral *in vitro*, the next step in evaluation is testing the compound in animals. The *in vivo* tests, tests taking place in a living organism are most often conducted in mice but also monkeys and dogs have been used as well. In drug use in humans, the antivirals should withstand the enzymes and other factors in the organism attempting to break it down. This is important since the concentration in humans needs to be high enough to have an antiviral effect. With *in vivo* studies, valuable information is gained about the antiviral's possible metabolic products and the amount of drug that reaches blood circulation or is there at a point of time after an injection. For EV antivirals the desired pathway is oral and few antivirals have required additional structural variation to increase the bioavailability of the compounds. Finally the potential antivirals enter clinical trials with humans, testing against both experimentally induced and natural infections of the target viruses.

3.4 Capsid interacting antiviral molecules

For enteroviruses, the structural elucidation of the viral capsid of rhinovirus 14 was the first step in the clarification of the structure of EVs.[55] Hence it is only logical that the first antivirals designed against them were also concentrated to binding to the viral capsid. There are multiple aspects to the functions of the capsid structure that can be interfered with. These include hindrance of attachment, entry or uncoating, as described in chapter 2.2. One way of achieving this has been by misleading viruses with antivirals mimicking the binding receptors of the particles.[56] However, the focus on the capsid binders against EVs was quickly focused on the hydrophobic pocket, a pore in the capsid canyon between VP1 and VP3.[57] The pocket is originally occupied by a fatty acid, which can be replaced easily with another substrate. It is proposed that the binding of a substrate affects the rigidity and hinders the ability of the virus to interact with its host.[58] Currently, the three most potent compounds among the plethora developed along the way, contain pleconaril, vapendavir and pocapavir. Pleconaril specifically has been the prototype molecule in the development of capsid binders. The problem in using capsid binding antivirals has been the fast pace that EVs develop a resistance for them[59] but nevertheless, some are still being tested for medicinal use.

3.4.1 WIN compounds and Pleconaril

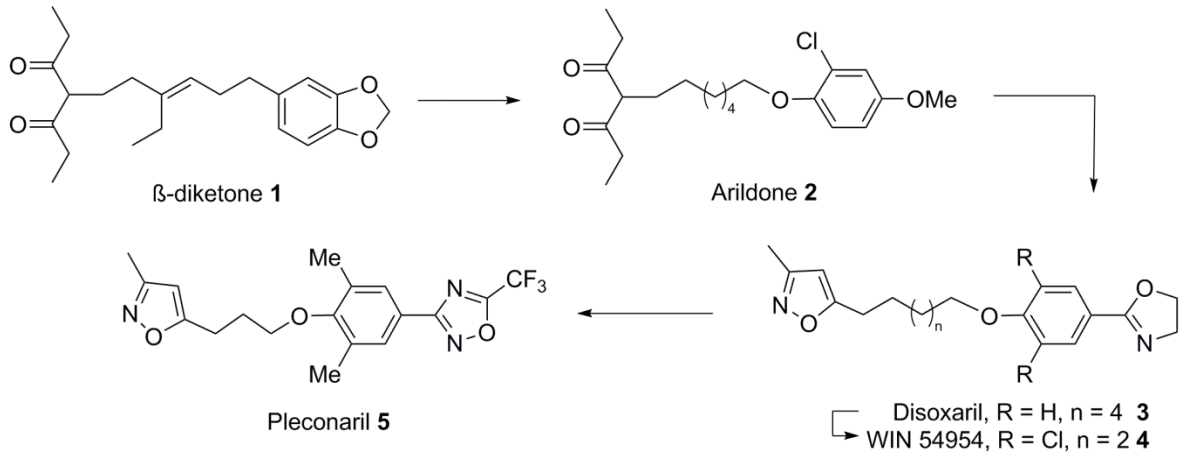


Figure 3. Synthetic development of Pleconaril.[59]

The beginning of a group of capsid binding antivirals called WIN compounds, was created with the accidental creation of a beta diketone compound (**1**) in an attempted synthesis in hormone mimetics.[59] The further development at Sterling-Winthrop led to arildone (**2**) being synthesized after several modifications to the first intermediate. While already prohibiting paralysis caused by polio, the activity was attempted to develop further while also addressing the instability of the beta-diketone moiety. Replacing the diketone group with an isoxazole, along with substituent modifications to the benzene ring of the molecule created disoxaril (**3**) that could be administered even orally for successful polio inhibition. Disoxaril (**3**) fell short in the first clinical studies, showing poor bioavailability (~15%) and inducing the formation of crystal urea in patients. *o*-Dichlorination of the benzene ring and optimizing the length of the aryl chain fixed the bioavailability with increased antiviral activity in WIN 54954 (**4**). However also the neurological toxicity of the compound was increased, it went through a rapid metabolizing and even caused reversible hepatitis. The final changes included shortening the aryl chain to only three carbons long for increased spectrum of activity, replacing the chlorine atoms with methyl groups and adjusting the heterocycle substituent to oxadiazole to assess the metabolic and toxicity issues. Finally the oxadiazole methyl group was replaced with trifluoromethyl moiety to prohibit metabolic cleavage at this site and thus pleconaril (fig. 3, **5**) was produced.

The antiviral capability of WIN compounds has developed steadily towards pleconaril (**5**). Already arildone (**2**) showed minimum inhibitory concentration values at only 0.2 µM concentrations against poliovirus in HeLa cells.[60] With disoxaril (**3**) the MIC values against polioviruses were already a fraction of the previous with 0.004-0.03 µg/ml.[61] More extensive studies on the drug also revealed a broad spectrum of antiviral action versus 9 different serotypes of EV as well as against 33 HRVs with MIC values ranging between 0.004-0.17 µg/ml and 0.004-6.2 µg/ml respectively. This antiviral was already successfully used together with enviroxime against a resistant poliovirus.[56] The chlorinated WIN 54954 (**4**) showed slightly increased efficacy against 50 different HRVs it was tested against. The MIC was in the range of 0.007-2.2 µg/ml and EC_{80} (80% inhibition of virus) with only 0.28 µg/ml while activity on EVs stayed similar.[62] Thus the increase in spectrum of the antiviral was 20-fold. While coming up with derivatives to counter toxicity and metabolic issues the potency of the drug was first increased to EC_{80} of 0.3 µM with a oxadiazole derivative against 54 serotypes of HRV[63] and one more step later the resulting pleconaril has been subject to vigorous studies since its conception.

Pleconaril (**5**), having been tested on 215 clinical isolates of EV serotypes, showed EC_{50} activity at concentration of ≤0.03 µM and EC_{90} inhibition at ≤0.18 µM with all of the tested serotypes.[64] However, it should be noted that the drug shows no effect on EV71. While showing great promise in tests in cell cultures, the various clinical trials the drug has went through have been ambiguous. On mice, infection of CVA9, CVA21, CVB3 were all successfully treated with pleconaril. On humans the drug was tested first on life-threatening infections. Enteroviral hepatitis was cured on neonates in 2 out of 3 cases and use on chronic meningoencephalitis lead to improvement of condition in 78% of patients.[59] Additionally a phase III study with almost 2100 patients infected with picornavirus was shown to reduce symptoms of the common cold. Trials showing significant reduction in symptoms caused by EVs include purposeful infection with CVA21, treatment of EV meningitis both in adults and children and a few others.[57] However clinical studies exist also showing completely opposite results. A double blind placebo-controlled trial on infants with EV meningitis reported no significant effect by pleconaril, but rather drug accumulation and some adverse effects. In another phase II trial attempting to use pleconaril nasal spray to treat HRV infections, no significant effect was reported.[13] Even though a favorable safety profile has been reported to the

drug[57], it was rejected for use against common cold, due to safety and resistance concerns.[65] Such concerns are generally justified due to observation of resistant mutant strains against the antiviral that show the general susceptibility of capsid binders to quick surfacing of resistant strains.[66,67]

3.4.2 Vapendavir, pirodavir and oxime ethers

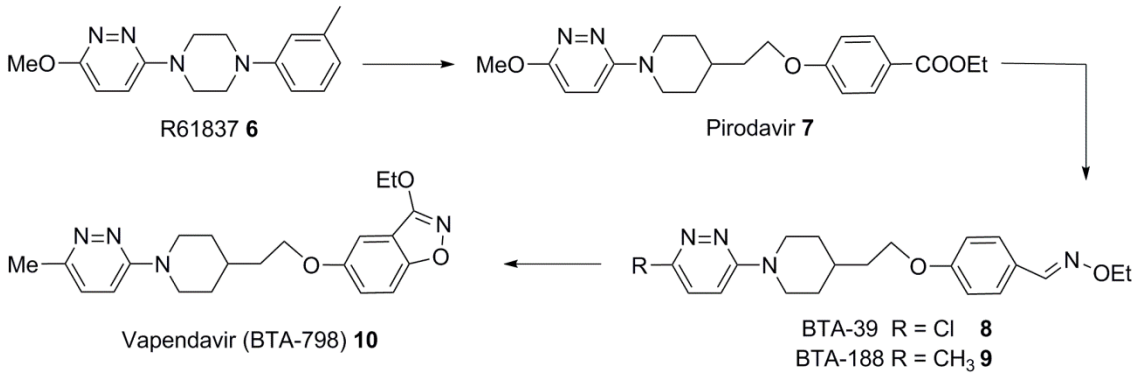


Figure 4. Synthetic development of vapendavir.[59]

The development of the oxime ethers was started in Janssen Research Foundation with the creation of R61837 (**6**), a pyridazine derivative with piperazine and phenyl functional groups attached in a chain, a phenyl-pyridazinamine (fig. 4).[68] Already the next generation of the drug design led to the inception of pirodavir (**7**) with a 500-fold increase in antiviral ability. The effect was credited to capsid binding as increased stability to acid and heat were observed. To address the bioavailability issues, the ester group in pirodavir was converted to an oxime ether, creating BTA-188 (**9**). In tandem, by replacing the 6-methyl group with chlorine, BTA-39 (**8**) was synthesized. The most recent analogues of the oxime ether antiviral groups were devised with allocation of a bicyclic benzoxazole group to the phenyl ring's place.[69] The logic behind this was the estimation of increased half-life and oral bioavailability due to better hydrolytic stability. Twenty such benzoxazole or benzothiazole derivatives were designed, synthesized and tested. While another derivative showed the greatest antiviral activity

(10 times that of pleconaril (**5**) and same compared to pirodavir (**7**)), it was BTA-798 (**10**), later named vapendavir, which was chosen for clinical testing.

Unlike pleconaril (**5**), the oxime ethers show a high inhibition against rhinoviruses. Already the first predecessor to the compound group, the R61837 (**6**), showed inhibition with 100 different serotypes of HRV, although mostly against serotype group B.[68] Additionally the concentrations required were extremely high with an EC_{80} value of 32 µg/ml. With pirodavir (**7**) the required concentration for EC_{80} was already as low as 0.064 µg/ml and showing same effect on HRV-A -group as well. Additionally, it had effect on 16 enteroviruses, albeit with higher concentration of 1.3 µg/ml for EC_{80}. Both drugs underwent clinical trials. R61837 (**6**) showed substantial reductions in infection of HRV-A9 when administered prophylactically, but use after inducing the infection had no clinical effects.[59] Also pirodavir (**7**) fell short in its clinical trials, as testing with naturally occurring HRV infections showed no efficacy.[56] However this was deduced to be the fault of low solubility of the drug in water and resulting hydrolysis of the ester bond that resulted in an inactive form. The two derivatives had great EC_{50} values against the 59 HRV strains they were tested on, ranging from 0.0005 to 0.0067 µM. They expressed the desired improved bioavailability values of 62-63% in rats and 21-28% in dogs. Increasing the bioavailability further with the development of BTA-798 (**10**) the bioavailability of the drug was enhanced while the potency of the drug remained the same. Vapendavir (**10**) has been gone through a few clinical trials in treatment of HRV infections in patients with asthma or chronic obstructive pulmonary disease. The drug has gone through a phase I and two phase II (a and b) studies.[13,59,70] The phase II studies on people with naturally acquired HRV infection showed significant reduction in severity and incidence. In 2015 a third phase II clinical trial was announced. Contrary to pleconaril (**5**), vapendavir (**10**) has been shown to have an effect also against the EV71.[71]

3.4.3 Pocapavir and SCH imidazoles

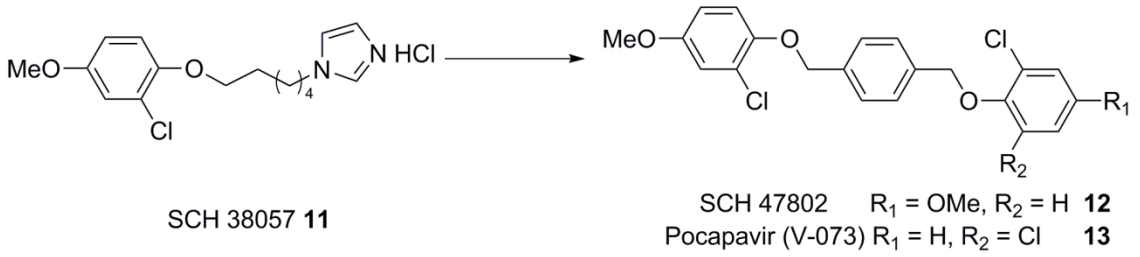


Figure 5. The synthetic development of Pocapavir.[59]

In an attempt to find an antiviral against polio viruses, a first generation drug, 1-[6-(2-chloro-4-methoxyphenoxy)-hexyl]imidazole (**11**) was developed.[59] SCH 38057 (**11**), as the molecule is called, contains an imidazole unit and tri-substituted phenyl ring connected with a heptyl chain (fig. 5). In the following generation of five analogues, the imidazole group was abandoned and five different linkers between two *o*-chloro-*p*-methoxy-phenols were synthesized. From the five derivatives, the SCH 47802 (**12**) proved the most potent, containing a 1,4-oxymethylbenzene group between. By adjusting substituents on the second phenol to two *ortho*-chlorines, a close derivative called SCH 48973, also known as V-073 and later coined as pocapavir (**13**) was conceived.

The antiviral effect of the first generation drug SCH 38057 (**11**) was limited, requiring high concentrations *in vitro* to achieve the EC_{50} values; between 10.2 and 29.1 µM depending on EV/HRV serotype.[72] Nevertheless, the compound was also tested on mice against CVB3 and echovirus 9 infections. An oral administration of 60 mg/kg and subcutaneous injection of 20 mg/kg saved the animals from death. Without much surprise, the next generation SCH 47802 (**12**) was shown to have the same effect the same oral dose of the antiviral.[73] However, the EC_{50} values were tremendously lower, between 0.03 and 10 µg/ml. A pharmacokinetic analysis of murine plasma showed correspondence between the oral dosage and EC_{50} concentrations. Among the five derivatives tested against PV-C2, SCH 47802 was superior with EC_{50} at 0.01 µg/ml, while for the rest the value varied between 0.08-0.62 µg/ml. However the drug showed poor response against the coxsackie viruses it was tested on. Finally with such a small

structural change, pocapavir (SCH 48973 (**13**)) displayed similar high efficacy, but on more serotypes comparing to its precursor.[74] The EC_{50} of the drug was 0.9 µg/ml on 80% of the 154 clinical EV isolates from humans. Additionally the oral dosage was diminished to 3-20 mg/kg for an effective antiviral effect. Pocapavir (**13**) has yet to go through extensive clinical trials on patients and has only been tried on few cases of neonatal sepsis so far.[75] However the results from the initial testing showed promise with reduction of symptoms and diminishing of EV found on the samples. More studies on patients would be required but the attractiveness of the antiviral is reduced by its non-existent effect on rhino viruses. Due to the lack of broad spectrum activity, additional development on the drug has been halted.[57]

The binding of the compounds differs from the previously described capsid binders greatly. The successful crystallization and characterization of SCH 38057 (**11**) complexed with rhinovirus HRV-C14 showed that the binding site of the compound is at the end of the hydrophobic pocket. Crystallographic studies of pocapavir (**13**) coupled with poliovirus 2 (PV-C2) elucidated the site further, showing the antiviral to bind within the beta-barrel of VP1, close to the area where natural pocket factors do.[59] The binding site also affects the mode of action for the drug. With the SCH compounds, the virus particles are able to attach to their host cells, however the antiviral hinders the activity after the first steps of uncoating process.[72]

3.4.4 Pleconaril derived isoxazoles and pyridyl imidazolidines

Since its conception, pleconaril (**5**) has served as a foundation for a few new EV capsid binders that have been developed in its footsteps. The resistance of a CVB3 strain named “Nancy” drove Makarov *et al.*[76] to synthesize and test multiple derivatives to pleconaril. The derivatives were alternated from pleconaril as such, that the trifluoromethyl oxadiazole-moiety of the drug was replaced with a phenyl group with various substitution. The synthesized [(biphenyloxy)propyl]isoxazoles were mono-, di- and tri-substituted from various positions with different substituents and their antiviral activity was measured. A 4-fluoro substituted (fig. 6 (**14**)) derivative produced the best

results against the tested viruses that were the Nancy strain, HRV-A2 and HRV-C14 with EC_{50} being 1.34 µg/ml and 0.009 µg/ml respectively. However the drug had no effect on HRV-C14. Continuing their studies on the Nancy strain, Makarov *et al.*[77] explored the effect of changing substituents on the middle phenoxy ring of pleconaril and the effect they had in the antiviral ability. Both pleconaril and the previously synthesized 4-fluoro isoxazole derivative were functionalized with one or two methyl, halide or methoxy groups and the compounds were tried on different EV serotypes. Best results against six different CVB3 Nancy mutants were shown by a 3-Br substituted derivative (**15**). However testing other serotypes of coxsackie virus genus and HRV showed mixed results with different derivatives, others working on some serotypes and then having no effect on the other, showing the difficulty of achieving a broad spectrum capsid binding antivirals. In present times Makarov's and the groups research has led them to pyrazolo-pyrimidine derivatives, most promising of them a three ring system containing *p*-trifluoromethyl aniline, pyrazolo[3,4-*d*]pyrimidine and a phenyl ring.[78]

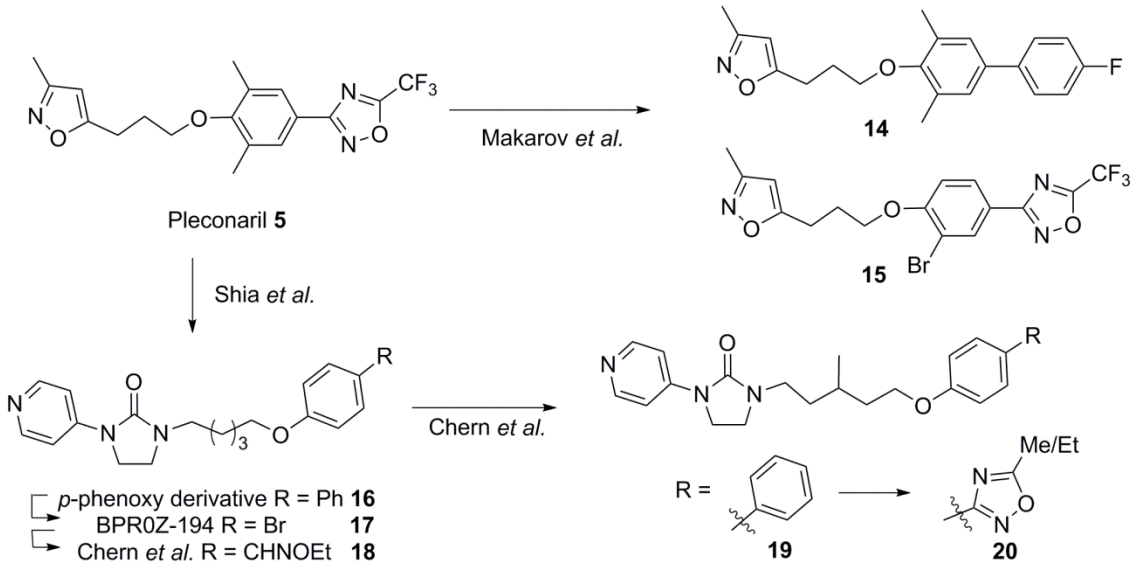


Figure 6. The synthetic development of further pleconaril derivatives.[76,77,79–82]

Another group working on new compound based on pleconaril (**5**) is the group led by Shia. The group's work was also driven by the lacking effect of pleconaril, although versus EV71. With computer assisted drug design, using pleconaril and other WIN compounds as base skeletons, a series of pyridyl imidazolidinones was designed.[79] The computational analysis displayed that an imidazolidinone derivation and changing the

oxadiazole substituent to an aryl substituent would lead to antiviral activity against the HFMD virus and thus a plethora of corresponding compounds were synthesized and tried against the EV-A71. Following the computational deductions, the group evaluated different substitution, both in the *p*-phenoxy and N-imidazolidinone positions and the greatest antiviral effect was displayed by a phenyl and pyridine substitution respectively (fig. 6 (**16**)). This derivative had the EC_{50} value of 0.05 µM against EV71. Although not the most effective molecule in the series (EC_{50} = 0.50 µM), BPR0Z-194 (**17**) (1-[5-(4-bromophenoxy)pentyl]-3-(4-pyridyl)-2-imidazolidi-none), was tested additionally against EV-D68, coxsackievirus A9 and A24 and echovirus 9, showing activity against all of them.[80] While the EC_{50} values against these were 7.78, 0.38, 0.1 and 0.81 µM respectively, the drug was also ineffective against over half of the other tested serotypes and was shown to be extremely susceptible to mutations in a single VP1 amino acid at 192 position.

Taking place in the same department as the previous research, Chern *et al.*[81] revisited substitution of phenoxy moiety, but with oxime ether functional groups this time. With the evaluation of differently substituted oxime ethers, the group concluded the simple oxime ethyl ether (**18**), familiar from a vapendavir precursor BTA-39 (**8**), to be the most effective antiviral against EV71 with EC_{50} of 0.001 µM. Additionally the length of the allylic chain was evaluated and found to be most optimal at five carbons. As their final effort, the group explored the possible alterations to the said allyl chain.[82] Both oxygen and nitrogen as well as different phenyl substitutions to the middle of the chain were explored as well as other allylic chains. However the only increase to the antiviral capability was from the introduction of a simple methyl group to the middle of the chain (**19**). Finally a third re-evaluation of the phenoxy substituents led back to an oxadiazole with a methyl or ethyl arm giving the group (**20**) best results in their work against EV71 (EC_{50} 0.0009 and 0.0005 µM respectively). However the wide spectrum capabilities were not increased. The (S)-(+) enantiomer was identified to be ten times more powerful than (R)-(-). The imidazolidinone compounds have shown great potential as a specific drug against EV71 *in vitro* and a recent study showed similar effect also in experiments done *in vivo*.[83]

3.4.5 MDL pyridines and phenoxybenzenes

A company called Merrell Dow has focused their antiviral development against HRV on different derivatives of pyridine and phenoxybenzene derivatives. Studies on the compounds began with the discovery of 2-(3,4-dichlorophenoxy)-5-nitrobenzonitrile (MDL-860, (**21**)) in a screening of 800 nitrobenzene derivatives for antiviral activity.[84,85] MDL-860 (**21**) showed promise for broad spectrum of activity, exhibiting inhibition against 80% of the 90 HRV and 10 EV serotypes it was tested on with 1 µg/ml concentration. Additional *in vivo* experiment displayed efficacy on mice, protecting them from coxsackievirus B3 and A21. Although MDL-860 (**21**) had shown greatest activity in testing, another pyridine derivative and potent HRV antiviral from the screening, 2-(3,4-dichlorophenoxy)-5-(methylsulfonyl)pyridine (**22**), was used as a scaffold for further development (fig. 7). Changes to the ether bridge linker were explored using carbon and nitrogen, but the antiviral activity of the compounds stayed similar. Interestingly, the capsid interacting mechanism of MDL-860 (**21**) and the sulfonyl derivatives differs, despite the similar structure. MDL-860 (**21**) hinders the virus after initial uncoating, while the sulfonyl compounds are active already during it.[59] Desire to avoid the toxic nitro substituent and develop the drug further, the functional groups on pyridine were altered and evaluated.[86] A cyano group in 2-position of the pyridine was identified as beneficial for the antiviral capability of the drug and further syntheses of alternative C-3 substituents showed that an ethyl thiol arm (**23**) increased the antiviral capability the most. However the required concentrations for EC_{50} effect were still quite high around 3 µg/ml at best against 11 different HRV serotypes.

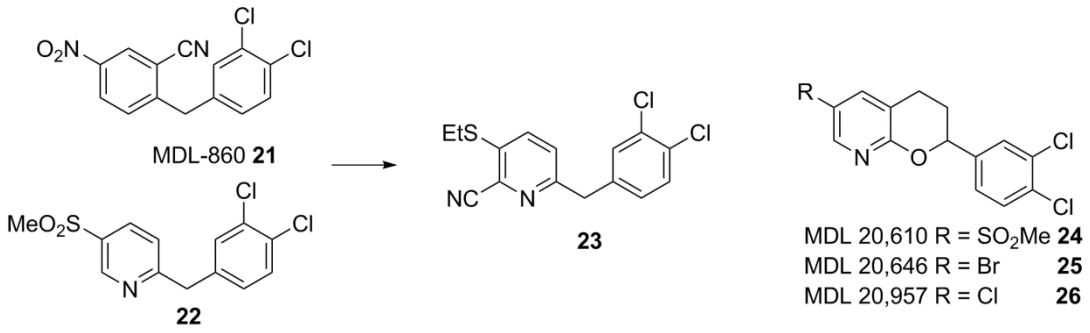


Figure 7. MDL-860 and its derivatives.[84–86]

The research on the compounds continued from MDL-860 (**21**) with the identification of three phenoxy-2H-pyrano[2,3-b]pyridine derivatives that showed very low inhibitory concentrations against 32 serotypes of HRV.[87] The phenoxy group of the compounds was substituted with two chlorine atoms at carbons 3 and 4 and three different substituents and their effect on the *m*-position of the pyridine was evaluated. The variables were methylsulfonyl (MDL 20,610 (**24**)), bromine (MDL 20,646 (**25**)) and chlorine (MDL 20,957 (**26**)) groups and showed 50% effective concentrations (EC_{50}) of 0.03, 0.006 and 0.006 µg/ml against tested serotypes respectively. MDL 20,957 (**26**) showed the greatest antiviral activity, showing 99% reduction in viral shredding with only 0.004 µg/ml concentration however also disturbing cellular metabolism lower concentration (5 µg/ml) compared to MDL 20,610 (**24**) (20 µg/ml). Interestingly the MDL 20,610 (**24**) was also identified to have significantly wide spectrum activity, spreading over rotaviruses of different animals, paramyxovirus and a variety of EVs with EC_{50} values ranging 0.8-1.5 µg/ml. However, no *in vivo* trials on the compounds have been reported.

3.4.6 Flavans and chalcones

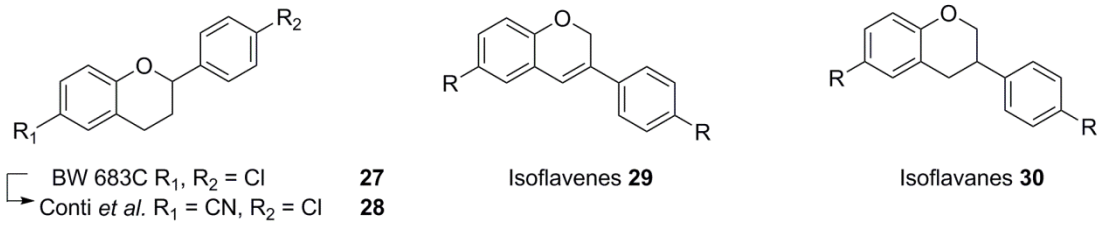


Figure 8. Flavans, isoflavenes and isoflavanes.[88,89]

Both flavans and chalcones, were identified as a potential HRV capsid binding inhibitors with promising sub-micro molar concentrations *in vitro* for EC_{50} effect.[59] Additionally the cytotoxic effects on cells of these compounds tends to be a thousand fold higher to the EC_{50}. Flavans are a species of benzopyran (benzene ring and pyran fused together) with a phenyl ring bound to the 2-position of the molecule. After synthesis of a group of flavan derivatives by Bauer *et al.*[88], 4',6-Dichloroflavan

(BW 683C (**27**)) was found to be the most potent molecule among them with EC_{50} between 0.007-0.17 µM.[90] The clinical trials on the antiviral showed no improvement against an experimental infection with HRV, although the bioavailability of the compound was good. In an attempt to improve the compounds, Conti *et al.*[89] designed another group of 3-isoflavenes (**29**), isoflavanes (**30**) and flavans with varying substituents (fig. 8). While the changes to the molecule framework proved to be ineffective, a simple change of one or both chlorine substituents to cyanide not only increased the drug activity against HRV-1B but additionally showed antiviral activity under 1 µM concentration against some other EV genus such as CVB4, echovirus 6, EV-A71 and PV-C2. The best effects were shown with 4'-chloro,6-cyano substitution (**28**). Since then, further attempts to create improved antivirals has been attempted with all three frameworks, adding a ketone to the pyran ring[91], separating the phenyl group with a diene chain or a methine bridge[91], heteroatomics of the phenyl ring[92] as well as also heteroatomic substituents[93] to no avail. Yet positive results were achieved in inhibiting the 3D polymerase, described in chapter 3.5.7.

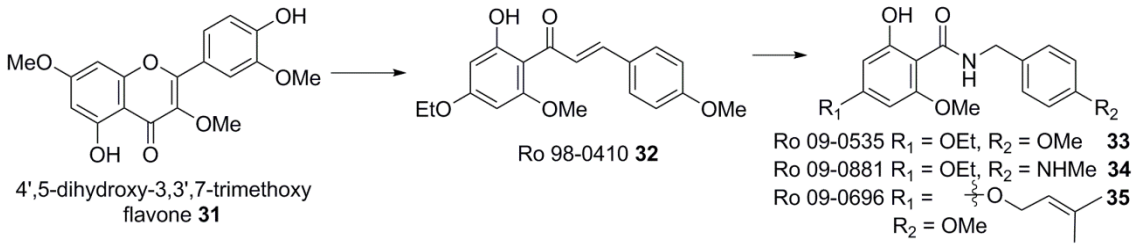


Figure 9. The different chalcone derivatives synthesized at Roche.[94,95]

Chalcones are a group of α,β-ketones with phenyl rings on both positions (α and β). They serve a large variety of biological activity and hold also potential for antiviral activity against rhinoviruses. The prototype molecule was developed by Roche research center by Ishitsuka *et al.*[94] with the exploration of analogs to 4',5-dihydroxy-3,3',7-trimethoxy flavone (**31**). The newly identified 4'-ethoxy-2'-hydroxy-4,6'-dimethoxychalcone (Ro 98-0410, fig. 9 (**32**)) was found effective against 46 of the tested 53 HRV serotypes with EC_{50} of 0.03 µg/ml. Against enteroviruses the antiviral had no effect. Following the development of this antiviral, further enchantments were done to increase the drugs' efficacy tenfold at best.[95] A significant improvement was achieved with the exchange of the diene chain into an allylic α-amine. From these

derivatives, exchanging the substituent at 4' position to (3-methylbut-2-en-1-yl)oxy group (Ro 09-0696 (**35**)) or at 4 position to methyl amine (Ro 09-0881 (**34**)), together with the original substituents (Ro 09-0535 (**33**)) provided the best alternatives. The inhibiting concentrations were <0.002-0.003 µg/ml while the cytotoxicity remained the same. The drugs have gone through a few trials; prophylactically treated controlled HRV-9 infection with phosphorylated pro-drug of Ro 09-0410 (**32**), intranasal administration of Ro 09-0410 (**32**) and a challenge of HRV resistant to or dependent on Ro 09-0410 (**32**).[59] Only the latter test provided some inhibition of infection, with the susceptible virus losing its infectivity and even the drug-resistant type losing some of its power.

3.4.7 Other capsid binders

Perhaps inspired by the vapendavir precursors described in chapter 3.4.2, SDZ compounds with a piperazinyl moiety have been studied. Already the first compound presented, SDZ 35-682 (**36**) containing a pyridine ring on the other and a 2-hydroxy-3-(4-cyclohexylphenoxy)propyl group as the other piperazine substituent was promising (fig. 10).[96] The compound showed antiviral effect against several HRV and echovirus 9 with 0.1 µg/ml concentrations, however also showing up to tens of µg/ml required for the EC_{50} to be reached on many other serotypes. The drug's efficacy was proven in an animal model against echovirus 9, displaying protection from paralysis and death. However, the best results on the compounds were achieved with a completely different molecule. By substituting the piperazine ring with benzothiazine and a thiazole carboxyethylester, SDZ 880-061 (**37**) was synthesized, among other derivatives.[97] The antiviral was able to produce EC_{50} on 85% of 89 HRV serotypes tested with 3 µg/ml, but more importantly 31 of these with concentrations of 0.003 µg/ml or less. The compound was shown to bind similarly as WIN compounds and blocking virus attachment, although not causing such drastic changes in conformation.

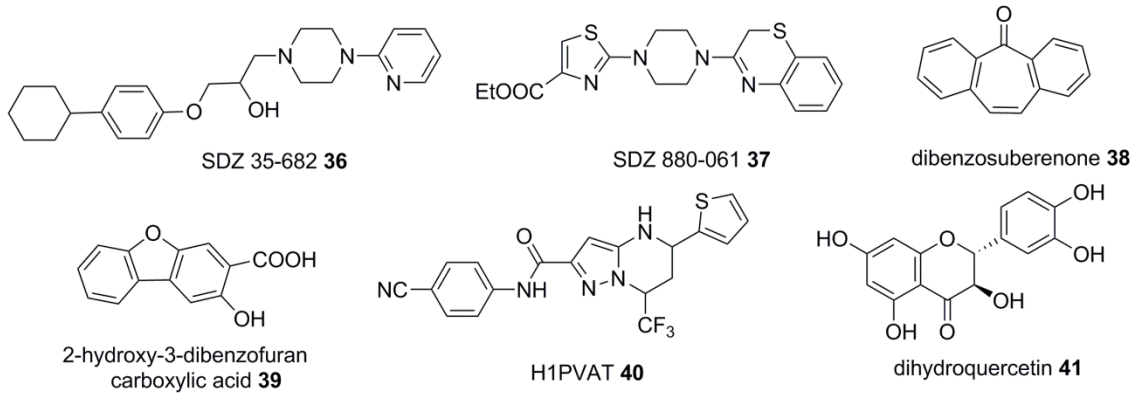


Figure 10. Some of the other developed capsid binders.[96–100]

From a group of dibenzofurans and dibenzosuberols (cyclo heptanol), one derivative from each was identified as an HRV inhibiting compound.[98] 2-hydroxy-3-dibenzofuran carboxylic acid (**39**) and dibenzosuberenone (fig. 10, (**38**)) were deemed the most promising, however showed 50% inhibitory concentration (IC_{50}) inhibition with quite high concentrations in the tens of µM. What makes these compounds interesting however, is that they are at the same time anti-inflammatory and could provide a bifunctional drug for respiratory inflammation caused by HRV infection.

The research on capsid binding compounds hasn't been so active in the third millennium but still a few compounds have been reported. One such compound with greater potential was the H1PVAT (**40**) that was shown to have great antiviral efficacy against the three different PV serotypes.[99] Almost all of the tested 45 poliovirus strains were all inhibited with EC_{50} values less than 1 µM. However the drug doesn't have an effect in any other EVs. The pyrazolo-pyrimidine molecule is substituted with a *p*-cyano aniline from the five-ring and trifluoromethyl and thiophene groups from the six member ring side. The drug holds great potential for the end game treatments needed against polio. An interesting wood extract that closely resembles the Ro 09-0410 (**41**) precursor in structure, dihydroquercetin (**41**), was shown to inhibit coxsackie virus B4 induced pancreatitis in mice.[100] Finally the group of Johan Neyts has published two more capsid binding antivirals, but their effects were mostly limited to HRV-C14 and were inferior to compared pleconaril (**5**) in most cases.[101,102] First one was a 2-ethanol connected two substituted benzene rings containing molecule and the other a combination of parts from pleconaril and pirodavir (**7**) compounds.

3.5 Antivirals for non-structural viral proteins

Compared to capsid binders, antivirals for non-structural viral proteins hold a much larger potential.[113] While the viral capsid proteins and the hydrophobic pocket do not serve a very specific task and thus singular mutations can happen easily without much disruption to viral functionality, the seven other proteins are quite different. One non-structural protein of the virus serves one or more very specific functions (motifs). Their structure needs to be very precise for them to maintain their functionality and mutations anywhere in the protein may cause conformational changes that render it useless. The capsid proteins were in the focus of antiviral research for decades, due to their known crystal structure. With the emerging of structural characterization of the other proteins, they are starting to become more attractive research targets.

Often the description of these antivirals is categorized in proteolytic enzyme antivirals and replication machinery affecting antivirals.[113] However in this work, no difference will be made, but antivirals against specific proteins will be described one after another. This is due to the fact that individual proteins may have multiple motifs, and all their specific functions have not been elucidated as of yet. A few enzymes identified as crucial contain the viral proteases $2A^{pro}$ and $3C^{pro}$ in proteolytic activity and $3D^{pol}$ for replication cycle as RNA polymerase. The information available for designing antivirals for non-structural proteins is limited, as it is connected to the successful structural characterization of their targets. Thus the development among these compounds is tightly connected to first establishing the crystal structure of the enzymes. Other option is to mimic the substrate the enzyme is responsible of reacting with. As with any proteins, the binding of antivirals on them can happen straight to the active site, or anywhere else on the protein allosterically, causing conformational changes rendering the protein nonfunctional.

3.5.1 2A protease targeted antivirals

The 2A protease of EVs and rhinoviruses has been identified as the most active proteolytic powerhouse in the viruses, falling second only to the 3C protease.[59] The structure of the protease is known from the successful crystallization from HRV-A2[103], CVB4[104] and EV-A71[105] sources. While the RNA sequence of the HRV-A2 doesn't match very well to the two others and the surface of the protein is quite different, the three dimensional structures of all three have high similarity. EV71 shares 71% of its sequence with CVB4. The HRV-A2 crystal structure showed a four-stranded and a six-stranded anti-parallel β-barrels as the building blocks for the protein. The catalytic center in the proteins was cysteine, histidine and aspartic acid, same amino acids for all three viruses. Despite the high conservation of the active site and the known structures of the proteins, only few antiviral candidates have surfaced so far.

The simplest compounds tested *in vitro* were the thiol alkylating reagents discovered in the attempt of characterizing the enzyme.[59] It was found that iodoacetamide and N-ethyl maleimide inhibit the 2A enzyme activity with 79% and 84% respectively, in 10 mM concentration by alkylating the thiol group in cysteine. Another inorganic molecule inhibiting the EV proteases is nitric oxide. This was shown in a study, where CVB3 induced myocarditis was treated with nitric oxide donating molecules.[106] Treatment of mice with glyceryl trinitrate and isosorbide dinitrate the former showed 86% survival rate for the disease. The only other group of synthetic compounds developed so far were the homophthalimides created by Wang *et al.*[107] that was discovered by blind screening, restricting both 2A and 3C proteases. The molecules are N- and 4-substituted 1,3-dione derivatives of isoquinoline framework, inhibiting both 2A and 3C proteases. Synthesizing eleven derivatives of these compounds with N-substituents varying as different alkyl thiol, mesyl and ethyl esters and three 4-substituents, a candidate inhibiting HRV-A2 was devised. The N-ethyl propionate, 4-acetophenone substituted homopthalimide LY353352 (fig. 11 (**42**)) inhibited the 2A protein of the virus with EC_{50} of 3.9 μM in a cell based testing. Despite the initial success, no further work on the compounds was done.

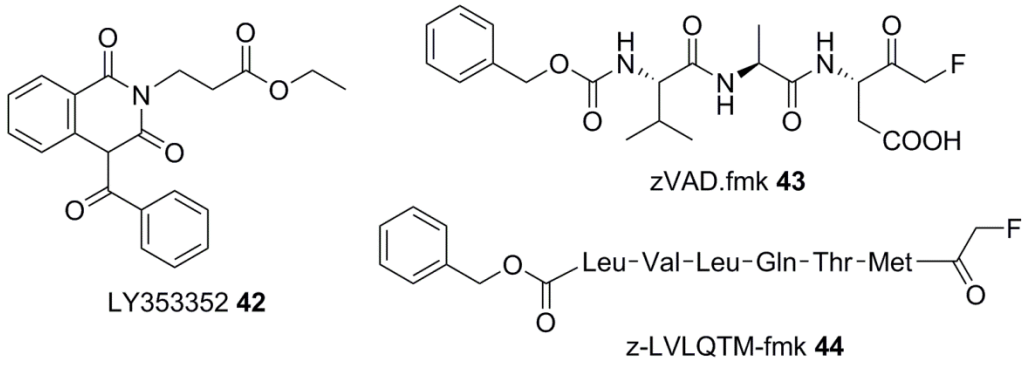


Figure 11. Three $2A^{pro}$ inhibitors.[107–109]

A few peptide based inhibitors for $2A^{pro}$ have been reported. It has been shown that the commercially available elastase (a class of proteases) inhibitors have a clear effect on the viral protease as well. The inhibitors tested were methoxysuccniyl-Ala-Ala-Pro-Val-chloromethylketone (EC_{50} = 65 µM), elastatinal (EC_{50} = 7 µM) and the former has been shown to also reduce viral yields on PV-C1, CV-A21 as well as HRV-A2 in HeLa cell cultures.[110] Another protease class, caspase, has its own group of commercial inhibitors, peptides functionalized with a fluoromethyl ketone (fmk) moiety. The fmk group in the inhibitors binds covalently to the thiol group in cysteine, thus causing irreversible inhibition. Deszcz *et al.*[108] showed the effect of the commercial compounds benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD.fmk, fig. 11 (**43**)) and benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (zIETD.fmk) with EC_{50} values of 5.6 µM and 7.7 µM respectively *in vivo*. Developing the molecule further to increased selectivity, the same research group achieved an improved version by replacing the aspartic acid in the oligo amino acid chain to methionine.[111] With this change, it was shown that the cleavage of a common target for 2A, eukaryotic translation initiation factor 4 gamma 1, was completely halted with only 0.01 µM concentration. Following the same reaction in cell cultures against HRV-A2 showed the same result, but against CVB4 the required concentration was 20 times higher. The fmk moiety was put to work also after the discovery of a pseudo substrate for the 2A enzyme in another study. The discovery of a strongly binding six amino acid chain LVLQTM for the 2A active site led Falah *et al.*[109] to try the similar substitutions to this oligo peptide and test the antiviral activity of the z-LVLQTM-fmk (**44**) compared to the previously reported compounds. Monitoring HRV-A2 activity in A549 cell infection, the 50% inhibitory concentration was found to be 0.3 µM. Additionally the drug was tested in mice against the same HRV-A2 infection and the results revealed

diminished amount of viral titers with the use of compound. The same molecule was then tried on the viral 2A protease of EV71 and was shown to reduce viral titers over a hundredfold at best in cell cultures.[112]

3.5.2 3C protease powerhouse

A lion's share of the proteolytic cleavages is done by the $3C^{pro}$, both on the viral protein as well as on a variety of cellular targets, making it the most active viral protease of EVs and HRVs.[113] Among these actions, it also releases itself from the P3 polyprotein as a 3CD complex that serves its own purpose in maturation of new virions and binds to the 5′-non coding end of RNA during replication. The $3C^{pro}$ has been successfully crystallized and determined from few different serotypes starting with HRV-A2, HRV-B14, PV-C3 in the 1990s and leading to CVB3, EV-A71 and EV-D68 more recently. A good visualization of the protease is provided by Wang *et al.*[114] for the EV-A71 extract. The ample amount of information has shown a chymotrypsin-like folding for the protein that is highly conserved among the different viruses. For EV-B93 the enzyme folds into two β barrels, consisting from six antiparallel protein strands each, packed into ~90° orientation from each other. Interestingly, the catalytic site on the enzyme is highly conserved among the different serotypes and even holds a great similarity to the one found on $2A^{pro}$; cysteine and histidine. The third amino acid of the catalytic triad is glutamic acid, instead of aspartic acid.

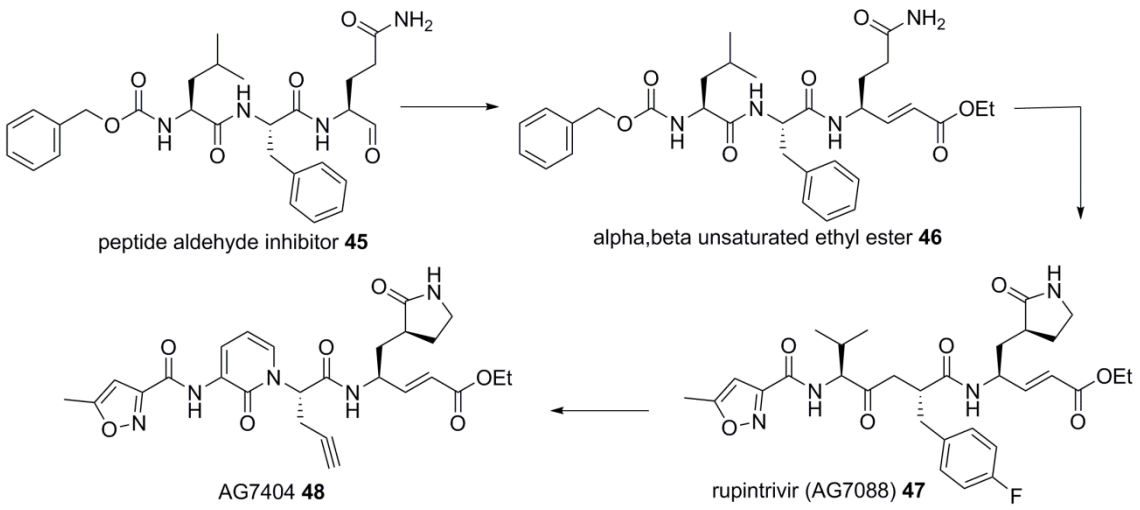


Figure 12. The development of rupintrivir.[59]

The research on the first 3C inhibitors started with the exploration of peptide mimetics corresponding to the preference of the HRV 3C protein to cut a P_1-glutamine-$P_{1'}$-glycine site in polyproteins.[59] Additional bias was observed with non-polar peptides in the side chain. In the beginning, an approach of competitive peptide binding was chosen. The first generation attempted electrophilic anchoring with the functionalization of a chain of four amino acids with an aldehyde form of glutamine. This however led to a spontaneous cyclization reaction and as a result a methionine sulfone as a glutamine dipeptide mimic was tried and discarded due to low antiviral activity. First inhibitors, in the sub micro molar scale were devised with the introduction of a third peptide group with a bigger alkyl and aryl amide group (fig. 12 (**45**)).[115] The research continued to tetra peptides which however led to no improvement against the HRV-B14. With the increase in knowledge and computational technology, a new approach was chosen with the development of peptidic inhibitors containing a Michael acceptor.[59] In these compounds, the mode of action comes from the Michael acceptor moiety present between the P_1 and $P_{1'}$ positions, forming a covalent bond with the cysteine nucleophile, thus rendering the enzyme useless. The first of these compounds were tetra peptides reported by Hanzlik *et al.*[116] after the development of more general cysteine protease inhibitors, but a large portion of the antiviral development was carried by Agouron Pharmaceuticals. The scientists at the company studied carboxybenzyl protected tripeptide (Cbz-leucine-phenylalanine-glutamine (LFQ)) with tens of different Michael acceptors attached to the glutamine.[117] From these studies, an α,β unsaturated ethyl ester was identified as a compound with most potential (**46**), showing antiviral activity

against three HRV serotypes. Further structural alterations based on the structure-activity relationship of the previous findings revealed that the α,β unsaturated carboxylic acids, esters with α substituents, different ketones, amide containing Michael acceptors etc. showed reduced or no antiviral activity compared to the ester. Moving on further in the drug optimization, improving the amino acid chain was attempted, by a meticulous and systematic evaluation of different alterations by Matthews *et al.*[118,119]. For example already the exchange of glutamine to a five carbon ring lactam functional group increased the antiviral activity 5-fold. Additionally 4-substitution with fluorine in phenyl alanine or replacement of the amino acid to an ethylene group showed potential, as did replacing the peptide bond and leucine with a pyridinone moiety and turning the N-terminal into isoxazole carboxamide, although a thiocarbamate group displayed better antiviral activity.

All the above mentioned research finally led to the development of AG7088 (**47**), later coined as rupintrivir.[120] The compound combined the best of all the previous studies; the α,β-unsaturated ethyl ester as the Michael acceptor, P_1 5-member ring lactam replacing the glutamine, replacement of the P_2 nitrogen with a carbon and the isoxazole carboxamide in the N-terminal position instead of thiocarbamate for lower metabolic susceptibility. The crystal structure reported by Wang *et al.*[114] shows rupintrivir bound to the active site of $3C^{pro}$ of EV71. The following *in vitro* studies on the drug showed immense and wide spectrum antiviral effect. The EC_{50} values against 48 serotypes of HRV had a mean of 0.023 µM ranging between 0.003-0.081 µM and mean EC_{90} of 0.082 µM. The tests on CVA21, CVB3, EV-D70 and echovirus 11 showed slightly lower EC_{50} of 0.147, 0.183, 0.007 and 0.014 µM respectively. The practically non-cytotoxic (CC_{50} = >1 mM) effect on cells gave the drug a great therapeutic index. Further tests on clinical isolates of HRV all displayed nanomolar EC_{50} activity[59]. Thus the antiviral was taken to clinical testing, where it was administrated intranasally after the observation of high liver enzymatic hydrolysis on it.[121] Rupintrivir was shown to be well tolerated by humans and limited the symptoms in an experimental infection with HRV, by the use of an antiviral nasal spray in phase I and II studies.[122,123] However it was reported that the antiviral was not able to reduce symptoms in natural infection studies.[124] In a subsequent study the research aim was focused on developing an orally bioavailable derivative in hopes of a more successful drug.[119] In this process the AG7404 (**48**) was devised and shown to withstand metabolic activity in dogs and

monkeys. The changes required for the effect compared to rupintrivir were switching the fluorophenyl P_2 moiety into an ethylene group and introduction of pyridinone ring in the P_3 position. This derivative was still shown to maintain its antiviral capability with EC_{50} less than 0.25 μM against 36 HRV serotypes, 5 clinical HRV isolates and 8 EV serotypes with mean values being EC_{50} = 0.075 μM and EC_{90} = 0.197 μM. AG7404 was shown to be safe through oral administration on humans but regardless, no further clinical testing was pursued with the antiviral.[124]

Other compounds against the 3C protease have been developed as well, containing both peptidic and non peptidic molecules. Sharing a high similarity with rupintrivir, only different from the P_3 and P_4 positions, SG85 was discovered from a series of $3C^{pro}$ inhibitors.[125] Evaluation of alternative P_3 substitution and capping molecules proved the replacement of leucine's methyl group with t-butoxy group and capping with simple benzyl group to provide the best results. SG85 was tested against EV71, 5 HRVs, echovirus 11 and PV-C1 giving EC_{50} values 0.18, 0.05-1.7, 26 and 47 μM respectively. Using the tripeptide chain familiar from the first rupintrivir precursors, diazomethyl ketones were developed in correspondence to the cleavage site HRV-B14.[126] The Michael acceptor part was replaced with a diazomethyl group and the antiviral was shown to cause accumulation of the 3CD protein. Although inhibiting the enzyme with EC_{50} of 1 μM, the antiviral activity against HRV-B14 and A16 was quite modest (EC_{50} = 30 μM). More potent activity was shown by a series of α-ketoamides synthesized by Chen *et al.*[127]. Adding an extra carbonyl on the P_1 side the group evaluated different protein substituents, achieving best results with replacing the glutamine with a simple propyl group. The resulting molecule had an inhibiting potential of EC_{50} of 0.17 against pure enzyme and 0.85 μM for HRV-B14. Similarly as with the 3A protein, also the $3C^{pro}$ can be inhibited with nitric oxide. For the 3C, this was attempted with the use of S-nitrosothiols as NO donors.[128] Introducing a methylnitrosothiol group as a $P_{1'}$ substituent to a six peptide chain, Xian *et al.*[128] were able to inhibit the function of the $3C^{pro}$. While the research on the 3C inhibition has focused on the peptidomimetic compounds, some non-peptidic variants have been reported as well.[59] However, with the diminishing alikeness to the natural substrate with these compounds, also the selectivity often suffers, even in the event of a successful antiviral activity. Reich *et al.* achieved moderate results with combining a Michael acceptor to a benzamide molecule, mimicking the P_1 part of the natural substrates. With

these compounds as well, the antiviral potency was increased with the use of α,β-unsaturated molecules in the 5-position of the benzamide, for a moderate efficacy against HRV-A16 (EC_{50} = ~1 µM) with different piperazinyl substituents at 3-position. Similarly Webber *et al.* reported antivirals based on an α-ketoamide group connected to a 2,3-dioxindole framework and while great enzymatic suppression was shown, the antiviral activity went hand-in-hand with the cytotoxicity of different derivatives.

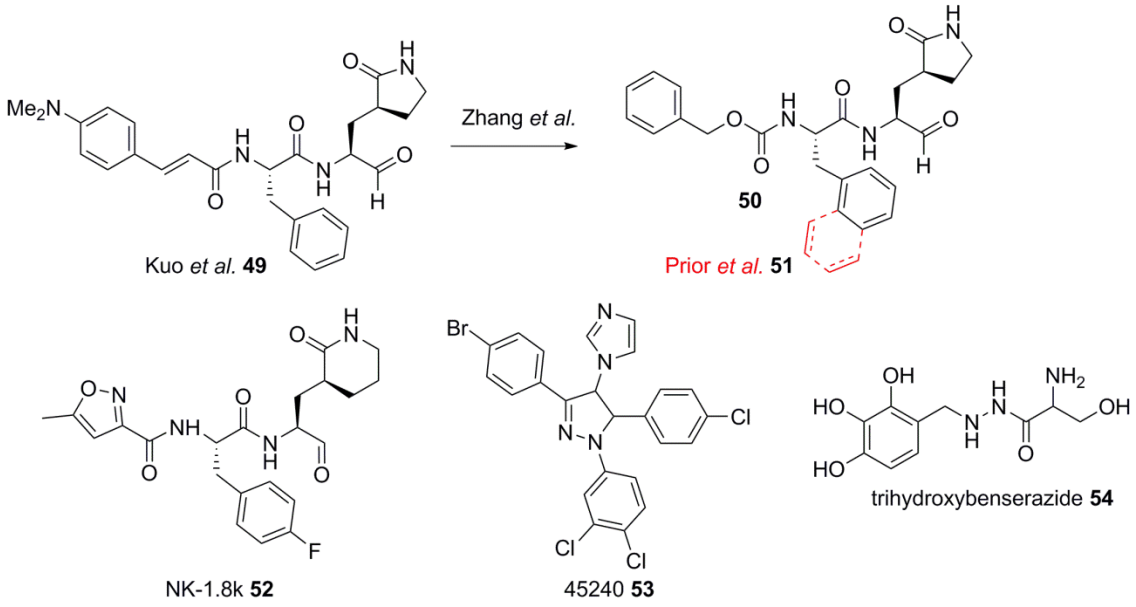


Figure 13. Antivirals for $3C^{pro}$ of EV71.[129–134]

The research on inhibiting the $3C^{pro}$ has re-sparked recently with the search for antivirals against the epidemics caused by the EV-A71. Still, many of these compounds are based on altering the Rupintrivir structure. In an effort to simplify the synthesis of the prototype antiviral, Kuo *et al.*[129] removed the P_3 moiety completely, linking a series of cinnamoyl derivatives with a peptide bond to the phenyl alanine (**49**). Although the antiviral capability of the compounds was diminished by this, the replacement of the whole $P_{1'}$ group with an aldehyde provided an antiviral with a great inhibition on the EV-A71. The best result was achieved with the *p*-dimethylamine substituted cinnamoyl group, yielding an EC_{50} value of 0.018 µM against the target virus. Further optimization to the aldehyde moiety published by Kuo has been already studied with Zhang *et al.*[130] displaying increased substrate binding with replacement of cinnamoyl moiety with methoxybenzene (fig. 13 (**50**)). Prior *et al.*[131] synthesized a series of molecules varying

the phenylalanine substitution with naphtyl derivatives and trying new Michael acceptors with the aldehyde. The aldehyde replacement showed now improvement to the antiviral ability but switching to 1-naphtylalanine (**51**) gave an increase in the antiviral activity against EV71. Molecular screenings against 3C have revealed two potential antivirals. The NK-1.8k (**52**) was tested against EV-D68 and EV-A71 strains inhibited them with 50% effective concentration of 0.09 μM.[132] The molecule bears a high resemblance to aldehyde functionalized rupintrivir, only missing its P_3 completely and sporting one extra carbon in the lactam ring. In another screening, a completely different compound containing dihydropyrazole ring with two phenyl groups and a benzimidazolylaminotoluene group was found.[133] This compound and two others with varying N-allyl chain were all found to have an inhibitory effect on 3C proteases of CVB3, EV71 and HRV-B14 in the EC_{50} range of 3.3-8.5 μM. Another triphenyl and imidazole substitutions containing dihydropyrazole (45240 (**53**)) showed even greater inhibition ranging 0.5 to 1.7 μM and could be a valuable start for a novel species of 3C inhibitors. Another set of a novel 3C protease inhibitor of CVB3 has been explored by Kim *et al.*[134], the benserazides. The antiviral effect is issued through a unique allosteric inhibitory pathway, making the compounds an interesting candidate. A trihydroxybenserazide (**54**) connected with an allylic chain to a phenol, showed high inhibitory activity on the enzyme with 0.07 μM concentration, superior among the tested derivatives. A few more potential antivirals against the EV-A71 have been identified from natural flavonoids that inhibit the $3C^{pro}$ such as rutin[135], fisetin[135] and luteoloside[136]. While the flavonoids only restrict the enzyme with EC_{50} of 110, 85 and 360 μM respectively, they also exhibit very low cytotoxicity. All in all, the research on inhibiting EVs and HRVs through the disruption of $3C^{pro}$ has been done to great extent but the focus has been mostly on peptidomimetics of the active site substrates. A few more recent developments however display the great potential that is still hidden in the field, for both non-peptidic as well as allosteric inhibition of the enzyme.

3.5.3 The viroporin 2B

Not much is known about the enteroviral protein 2B. Research on it has showed a small 50-120 amino acid protein with at least one hydrophobic surface for lipid layer

interaction.[137] It is hypothesized that the molecule forms oligomers that function as porous cellular membrane ion/protein channels. Research conducted by van Kuppeveld's group showed them to localize to the endoplasmic reticulum and the Golgi complex, reducing their Ca^{2+} levels.[138] They are also deduced to affect protein trafficking in some way. An accurate structure for the protein is unknown and together with the fact that it serves quite small function in the EV viral cycle has made it less attractive target for antivirals hence no specific antivirals against it has been reported.

In an earlier study, Gazina *et al.*[139] showed the importance of ion transportation, especially Ca^{2+} in rhinovirus infection. The study was conducted by observation the effect of various Ca^{2+} and Na^{+} inhibitors had on HRV-A2 and the Ca^{2+} inhibitors were shown to slow the production of new viruses strongly enough for an inhibitory concentration to be determined. Ca^{2+} inhibitors verapamil, diltiazem and nifedipine showed EC_{50} values between 7-12 µM with 99% inhibition at 50, 100 and 75 µM respectively. Also the Na^{+} inhibiting compounds displayed antiviral effect with 5-(N,N-ethyl-isopropyl)amiloride as the best with EC_{50} of 7 µM and 25 µM for 99% inhibition. Many anti-viroporin compounds for other viruses have been developed[137] but none of them have been tried on EVs or HRVs, maybe since it has been proven that their 2B protein differs so much even in the *picornaviridae* family, that their function is different.[138] In the future however the 2B viroporin might receive some limelight as a potential antiviral target.

3.5.4 The multipotent protein 2C

The non-structural protein 2C of enteroviruses is a real jack-of-all-trades. While all the functions of the enzyme are not clear as of yet, it has been associated with RNA replication and binding, membrane rearrangement and both encapsidation as well as uncoating of viruses.[13] An ATPase domain has been identified in the protein, with three conserved motifs A, B and C, which usually indicates a helicase (RNA unwinding) enzyme activity. The ATPase function has been shown to be true, but the proof for the helicase behavior has been inconclusive. Due to its multipotency, the 2C protein makes

for an attractive target for antivirals. However the lack of a known structure has hindered the progress in this regard. Still, multiple 2C active antivirals have already been developed with varied clarity on the working mechanism of the molecules.

Guanidine hydrochloride (GuaHCl (**55**)) is a small molecule of two amide and one imine group attached to the same carbon.[59] The large amount of studies on the compound has shown activity against PVs, some coxsackieviruses and echoviruses and in the process, the mode of action has led to the viral protein 2C. More accurately the antiviral effect was associated with the inability of protein to bind with host membranes.[140] While a clear antiviral effect has been shown, the concentrations required for significant effects are very high. A study on echovirus 9 displayed some inhibition only at millimolar concentration range[141] and while *in vivo* testing on mice with lethal CVA16 infection showed diminished mortality[142], the doses needed were very high, causing tremors as side effects. However GuaHCl still holds some promise on to be used in combination with some other drugs, such as 2-(α-hydroxybenzyl)-benzimidazole (HBB (**58**)) as the two 2C inhibitors were shown to have a synergistic effect for increased potency for both compounds.[141] Similar results were shown by Eggers by treating echovirus 9 and CVA9 infections on new born mice with 0.02 ml of 10 mM HBB (**58**) and 100 mM guanidine solutions, while same amounts of only one drug led to death of all the specimens.[143]

HBB (fig. 14 (**58**)) is the first of the few benzimidazole derivatives that have been developed and tested against the 2C protein.[144] The first tests on PV among 19 new potential compounds, HBB was not the most effective, but outshone the others as less cytotoxic, yet still inhibiting the viral replication in concentration of 0.25 μg/ml. While another derivative in this study, 6-methoxy-4-nitrobenzimidazole, had a more favorable *in vitro* values (effective concentration half of that of HBB's (**58**)) experiments on mice showed no improvement in mortality, while using HBB (**58**) led to death of only one test subject. Observing the spectrum of antiviral activity, Eggers and Tamm observed the antiviral to have a wide spectrum activity against all polio serotypes as well as a wide selection of CVB and echoviruses but almost none against CVA viruses.[145] Few further explorations by Tamm *et al.*[146,147] into further benzimidazole derivatives have

shown the type of compound to be a very prolific source for antiviral research, leading also to the development of enviroxime that will be discussed in chapter 3.5.5.[148]

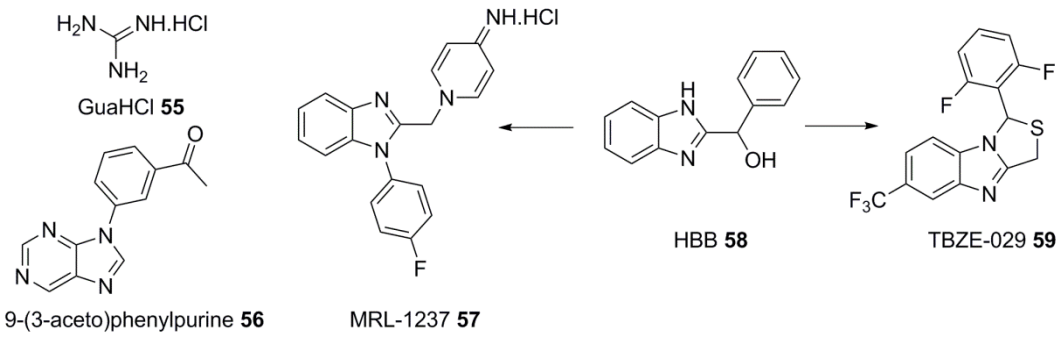


Figure 14. A collection of 2C inhibiting antivirals.[59,144,149–151]

However it was only during this millennium, that two new potential 2C benzimidazole inhibitors have been reported. The first one is MRL-1237 (**57**) that was created with the replacement of the phenyl ring with a 1-methylpyridin-4-imine, removing the hydroxyl group and adding *p*-fluorophenyl as an N-substituent compared to HBB (**58**).[149] Testing the antiviral activity against four poliovirus strains and two CVB strains showed improvement compared to its predecessor, however no studies on a wider spectrum have been conducted. A murine model testing *in vivo* against CVB3 that MRL-1237 (**57**) was most effective against brought mortality to 0% compared to control group's 57%.[152] The second quite recent group of compounds synthesized in the group of Neyts exhibits more alterations. Adding a thiol ring to create 1H,3H-thiazolo[3,4-a]benzimidazole moieties, the antiviral potency of different *o*-dihalophenyl substituents at C-1 and trifluoromethyl, methoxy and fluoro groups at C-6 were explored.[153] The trifluoromethyl moiety on the C-6 position showed a significant increase in potency in general as for the phenyl substituents, a combination of chloride and fluoride showed the greatest effect in this case. The described molecule and another derivative, the *o*-difluoro moiety (TBZE-029, (**59**)) of the molecule showed EC_{50} of 0.41 µg/ml and 1.2 µg/ml against CVB3. However, no similar magnitude on other type of viruses (CVA, echo virus, HRV and PV) tested was observed, although the two echoviruses and one CVA showed some restraint. Researching the target of TBZE-029 (**59**) compound's antiviral effect with genotyping of resistant strains of CVB3, the location in the genome was identified close to the motif C in the 2C protein.[151] The adenosinetriphosphatase

function remained intact in the presence of the antiviral. As GuaHCl (**55**), HBB (**58**), MRL-1237 (**57**) also test resistant to the same strains as TBZE-029 (**59**), there is indications that the antiviral effect of these drugs would be on the nucleoside triphosphate hydrolase or helicase motif on the 2C. Closely related in structure to the benzimidazoles, a bunch of 9-arylpurine have been reported with low µM antiviral activity against CVB3.[150] It was identified, that a 6-chloro substituted purine with a 9-(3-aceto)phenyl group (**56**) was the most prominent compound in this group against CVB3 (EC_{50} = 7.3 µM) and showed also activity against many CVA serotypes, as well as echovirus 9, but none on PV, HRV and EV71. What is interesting is that the purine derivative does not exhibit cross-resistance to TBZE-029 (**59**) which suggests a different mode action for this antiviral. Some additional testing on amine substituents to the aryl ring and new ones on the purine ring were attempted, increasing the antiviral capability to some extent, however the virus serotypes the drug was ineffective against remained the same.[154]

Hydantoin (5-(3,4-dichlorophenyl) methylhydantoin (**60**)) has also been shown to reduce the replication polioviruses and CVA21 in high concentrations.[155] The lowest tested concentration, 25 µg/ml, showed a reduction of 94-99% on all aforementioned viruses, while showing some restriction on CVB3 and rhinoviruses. While the antiviral concentrations seem quite high, what is interesting about the compound is its mode of action. Unlike the previously mentioned 2C inhibiting molecules, hydantoin (fig. 15 (**60**)) has been shown to affect the viral assembly and the cleavage of the 2BC protein.[155,156] While the antiviral potency of the antiviral is not phenomenal, the different mode of action could open another approach to inhibiting the 2C enzyme.

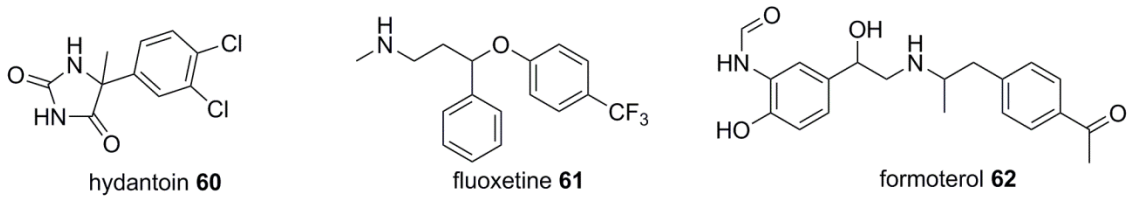


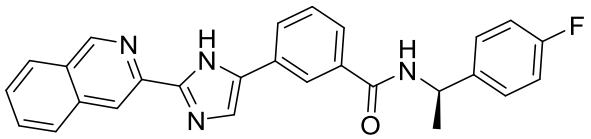
Figure 15. Molecular structures of hydantoin, fluoxetine and formoterol.[155,157,158]

Multiple screening studies of libraries of small molecules or drugs have reported a few more antivirals with the capability of inhibiting some EVs through the 2C disruption. One of them is the serotonin reuptake inhibitor, fluoxetine (**61**) that is better known for its commercial name, the antidepressant Prozac. Fluoxetine (fig. 15 (**61**)) was found in a screening by Zuo *et al.*[157] together with few other compounds to restrict the growth of CVB3 significantly. The concentration required for 50% inhibition of the virus was 2.3 μM, which corresponds to the levels in a person's bloodstream during treatment of depression. However further evaluation of the drug by Ulferts *et al.*[159] revealed that while the drug had moderate activity against EV-B and D (EV68 and EV71) species, the tested serotypes of species A and C were unaffected. Two other screening studies have also shown fluoxetine's (**61**) effect but also displayed tens of other candidates with EC_{50} concentrations in the micromolar range in CVB3 assays.[158,160] Other compounds found multiple times included zuclopenthixol, dibucaine and mefloquine, a common antimalarial drug. However a drug displaying unusual wide spectrum capabilities unlike 2C inhibitors was formoterol (**62**) and to no surprise the activity was not identified to take place there. It had EC_{50} between 0.30-2.36 μM against a tested serotype from each EV species as well as HRV-A and B and promises great potential for antiviral use, although the action mechanism is still unknown.

3.5.5 Membrane protein 3A

The 3A protein is another membrane-active, small, hydrophobic protein in the enterovirus genus.[70] Similar to the viroporin 2B it associates itself with the cellular membranes of the cell and controls them as described in section 2.2. The crystal structure of the protein is unknown, which has prohibited the development of site specific antivirals. Many antivirals were previously presented as 3A inhibiting compounds due to mutation selection, such as enviroxime (**78**), AN-12-H5 (**88**), GW5074 (**81**), itraconazole (**89**), T-00127-HEV1 (**82**) and -HEV2 (**87**). Later they were all shown to be inhibitors of one of the many cell membrane proteins recruited by the 3A protein during replication endosome formation.[161] Thus these compounds will be discussed in length in their respective sections in chapter 3.6. One antiviral with ambiguous effect on the 3A and connected cellular proteins is the TTP-8307 (**63**)

detected from a screening assay (fig. 16).[162] The molecule contains a fluorophenyl group joined in a chain with benzamide, imidazole and isoquinoline moieties respectively (fig. 16). The antiviral exhibits good inhibition of polioviruses and some HRV serotypes (EC_{50} = 0.091-0.99 µM), while having no effect on others nor on EV-A71, with CVs showing some effect (EC_{50} = 1.2 and 5.34 µM). While the efficacy, both in concentration and wide spectrum capabilities, compared to enviroxime is lower and the drug tests cross-resistant to the comparison, it still brings some new insight to the area. Although the researchers reported the resistant strain mutations to some extent locate to the same hotspots in the genome as with enviroxime, they also note the importance of other mutations and their differences, calling for additional research to determine the accurate working mechanism of TTP-8307 (**63**).[162]



TTP-8307 **63**

Figure 16. Chemical structure of TTP-8307.[162]

3.5.6 Protein 3B: the genome linking viral protein

Although the smallest protein in the viral genome, 3B (also known as VPg) still serves a crucial part in the RNA replication process.[13] The uridylylation of VPg catalyzed by the 3D enzyme serves as the first step to RNA replication after which the newly formed uridylylated product serves as a primer for the synthesis of new RNA. Although structural information of the VPg is available, for example through the crystallization of 3D/VPg complex from EV-A71[163], the focus of research has been on the 3D polymerase. However it should be noted that the three known $3D^{pol}$ structures show quite different binding sites for the VPg. Still no solely VPg inhibiting antivirals have been reported thus far, so the RNA replication inhibiting processes will be covered in the next chapter with 3D protein.

3.5.7 The RNA-dependent polymerase 3D

The 3D protein of enteroviruses, also known as the RNA-dependent RNA polymerase (RdRp), is a polymerase enzyme, serving as a catalytic center for both positive and negative strand RNA synthesis.[70] The $3D^{pol}$ works together with multiple other viral proteins (e.g. the VPg), cellular factors and requires Mg^{2+} and Mn^{2+} ions as cofactors. As such, the RdRp is an irreplaceable part of the viral cycle and together with the fact that human cells lack any enzymes with similar function, they make for one of the most attractive targets for antivirals. The targets offered by the $3D^{pol}$ include inhibition of binding of the cofactors to the enzyme or attacking the active site/RNA synthesis itself. The disruption of the polymerase itself can be done with either nucleoside or non-nucleoside compounds, that either incorporate themselves to the newly synthesized genome or inhibit the enzyme itself. The effect of the antiviral leads to an increased amount of mutation during RNA synthesis which in turn makes the new genome lose its viability, leading to mutagenesis. Usually the resistance against these drugs causes an increase in the fidelity of the RdRp, and thus a decrease in the amount of mutations in RNA synthesis. This in turn hinders the capability of virus to produce resistant strains against other antivirals, making the compounds highly potential for cocktail treatments. The structure for different $3D^{pol}$ proteins with or without substrate has been solved at least for poliovirus, CVB3, EV-A71, HRV-1, -14 and -16.[113] On the CVB3 enzyme reminds a cupped hand with three fingers. The fingers serve as subdomains for the secondary substrates while the active site resides at the "palm" of the hand and also is the most conserved subdomain, where two aspartic acid residues, coordinated by metal ions catalyze the RNA polymerization reaction. As such, the $3D^{pol}$ offers many targets for designing new antivirals but also their mechanism of action is not so easy to determine.

Starting with the nucleoside analogues of antivirals, ribavirin (**64**) is one of the oldest and most studied among them.[164] 1-β-D-ribofuranosyl-1-H-1,2,4-triazole-3-carboxamide (fig. 17 (**64**)) was developed as a guanosine analog containing a triazole-carboxamide moiety in place of the purine. Already the first tests showed extraordinarily widespread activity ranging both DNA and RNA viruses, including poliovirus, CVB1 and rhinoviruses. The exact mechanism of the antiviral has remained

unknown until this day. Different propositions made in studies of different viruses include inhibition of inosine 5′-monophosphate dehydrogenase, viral capping, viral polymerases or maintaining immunomodulatory responses.[70] Another theory is the incorporation of the drug into the newly synthesized genomes, causing loss in viral infectivity. The efficacy of ribavirin (**64**) with *in vitro* tests against EV-D68, HRV-87 and EV-A71 was limited, required high concentrations (EC_{50} = 135, 106 and >1100 µM respectively) and was ineffective against EV71.[165] A long time after the inception of ribavirin (**64**), Graci *et al.*[166] discovered a much more potent purine analogue with the variation of N-6-substitution. The best out of the eight derivatives tested, the compound containing a 6-hydrazinyl group inhibited 90% of proliferation of PV and CVB3 with 0.5 mM concentration. Another group of nucleoside analogues against EVs are cytidine derivatives, developed by the inspiration of 5-hydroxy-2'-deoxycytidine, a human immunodeficiency virus antiviral.[167] Cytidine, a RNA molecule (ribose and cytosine combined) was used as a framework and by changing the 5-substituents with hydroxide, bromide, nitro- and amide groups, the nitro derivative (**65**) showed the most promise. The compound inhibited the PV RNA polymerase and although no accurate concentrations for inhibition were reported, the drug at 2 mM concentration used against PV and CVB3 had 33-fold and 12-fold increased potency compared to ribavirin. Similarly NITD-008 (**66**), an adenosine derivative, first discovered as an antiviral against *flaviviridae*, also exhibits activity against EV-A71.[168] Surprisingly, functionalizing the deoxyribose ring with an ethylene led to antiviral activity (EC_{50} = 0.67 µM) against wildtype virus with the effect lasting even in murine model studies, 5 mg/kg dosage leading to abolishment of symptoms and death.

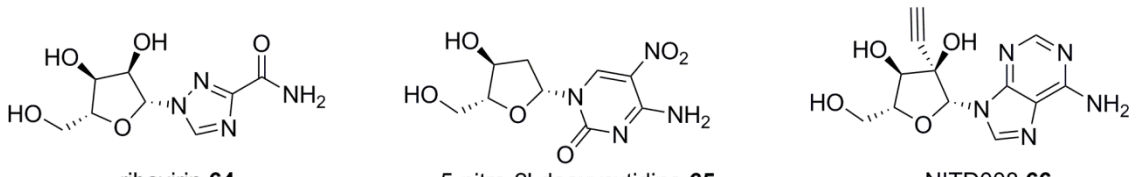


Figure 17. Nucleoside analogues for $3D^{pol}$ inhibition.[164,167,168]

The non-nucleoside inhibitors of the $3D^{pol}$ are many and work through various different mechanisms, some better known than others. Amiloride (**71**), an ion transport blocker in cells since 1967, was more recently shown to inhibit also CVB3 through RNA

replication.[169] Studying the mechanism in more depth, Gazina *et al.*[169] showed that the antiviral effect is induced through competitive binding to $3D^{pol}$ with Mg^{2+} and nucleoside triphosphate sites. While amiloride by itself shows antiviral activity against the CVB3, substituting it with a 5-(N,N-ethyl isopropyl)-group leads to a compound requiring only 6% in concentration for the same effect compared to amiloride. Antivirals hindering the proper binding of other cofactors include the GPC-N114 (**68**), DTrip-22 (**70**), aurintricarboxylic acid (ATA, **69**) and BPR-3P0128 (**67**) (fig. 18). The latest addition of these, the GPC-N114 (2,2'-[(4-chloro-1,2-phenylene)bis(oxy)] bis(5-nitro-benzonitrile)) was shown to hold a widespread activity against an array of 12 EV and HRV serotypes covering all different species of these viruses with EC_{50} value ranging from 0.13 µM to 1.73 µM.[170] The binding mode of the bis-nitrocyanophenoxy 3,4-substituted chlorobenzene was displayed with the crystallization together with CVB3 enzyme as well as location of the resistant strain mutations to locate to the position where the acceptor nucleotide for a new base-pairing nucleotide resides. BPR-3P0128 on the other hand has been shown to be a highly potent compound against the EV-A71 with EC_{50} = 0.0029 µM.[171] The potential drug combines a 4-carboxylic acid, 6-bromo derivative of quinolone substituted from 2-position with a pyrazol group that in turn is substituted with a dimethylphenyl group. While the actual mechanism of antiviral effect remains ambiguous, the compound inhibits also the uridylation of VPg, contrary to other $3D^{pol}$ inhibitors. Similarly, ATA (**69**) was distinguished as an inhibitor of EV71 with an effective concentration at 2.9 µM.[172] While the compound is known for the binding of nucleic acids to enzymes, the accurate mechanism in the case of EV71 was only incorporated with the 3D. The compound itself contains three 2-hydroxybenzoic acid moieties, connected by a single sp^2 carbon from the 5-position, sharing a ketone isomerization between the hydroxyl groups.

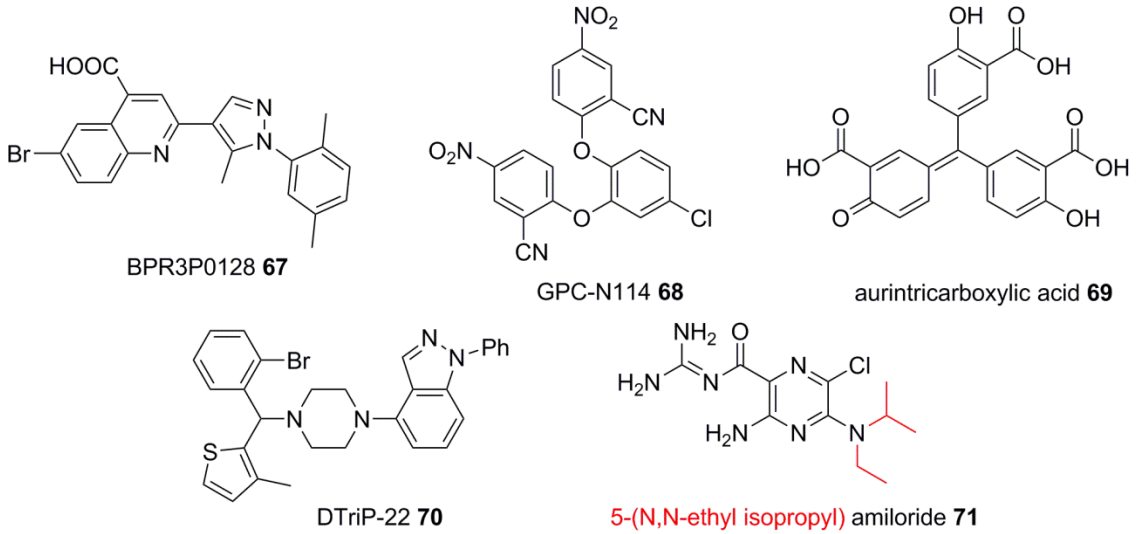


Figure 18. Non-nucleoside 3D^{pol} inhibitors.[169–173]

An inhibitor of triphosphate nucleotide entry on the 3D complex, DTriP-22 (**70**) is an important derivative in the group of pyrazolo pyrimidine compounds, designed as antivirals.[173] The compound was the result of extensive structure-activity relationship studies conducted by Chern *et al.*[174] after the discovery of a novel pyrazolo [3,4-*d*]pyrimidine in a screening program for anti enteroviral compounds. The initial hit containing a phenyl N-substituent and a 4-benzhydryl-piperazyl as a 4-substituent, 24 other derivatives were synthesized to evaluate best antiviral among the group of compounds. These included different phenyl, pyridine and sulfur heterocycle substituents as replacement for one or both of the benzhydryl rings. Changing the phenyl substituent to anything else killed the antiviral effect. Thus DTriP-22 (**70**) was conceived, containing an *o*-bromophenyl and 3-methylthiophene instead of pure phenyl rings in the structure (fig. 18). While the original derivative already displayed EC_{50} values ranging between 0.05-0.94 µM against 15 EV serotypes[174], the DTriP-22 (**70**) exceeding 625 in SI against EV71 and EC_{50} ranging 0.08-1.69 µM against 4 EV71 genotypes, 11 other EVs and HRV-2, the compound holds promise as a broad spectrum 3D inhibitor.[173]

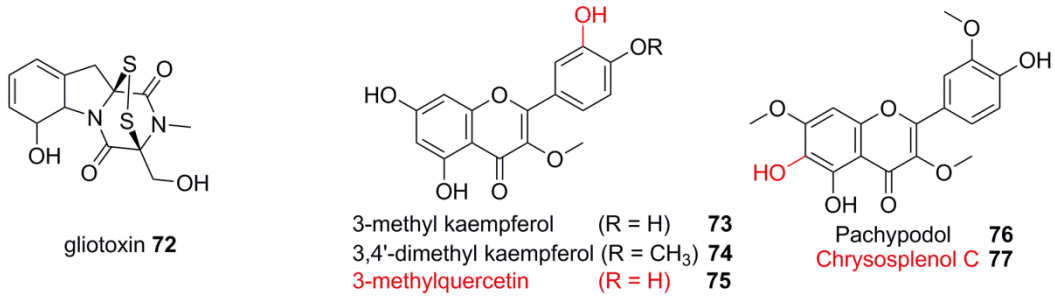


Figure 19. 3D^{pol} inhibitors extracted from natural sources. Pachypodol inhibits the PI4KIIIβ.[59,175–178]

Nature has also contributed its share of 3D^{pol} inhibitors. A metabolite extract from a fungal species called gliotoxin (**72**) was first identified as an antiviral against polio virus *in vitro* as well as with a daily dose of 0.2 mg/kg with testing in monkeys.[59] While studying the mode of action of the toxin, Rodriguez and Carrasco pinpointed the antiviral activity on the 3D polymerase and showed that the compound stops the synthesis of both plus and minus strand RNA.[179] Extraction from a few species of plants has yielded multiple flavonoid species that hold great antiviral potential, sharing codenames and close structural similarity with the flavans described in chapter 3.4.6, main difference being the change of pyran ring into aromatic 4-pyranone moiety. These compounds include 3-methylquercetin (**75**) from *Euphorbia grantii*[175], 3-methyl and 3,4'-dimethyl kaempferol (**73, 74**) from *Psiadia dentata*[176] and chrysosplenol C (**77**) and 6,7,8-trimethoxycoumarin from *Pterocaulon sphacelatum*[177] (fig. 19). All the compounds exhibit antiviral effect through inhibition of RNA synthesis. 3-methylquercetin exhibited even greater potency, displaying high antiviral effect with 90% inhibition in testing against PV-C1 and CVB4 at concentration of 0.01 µg/ml. Similar effect was also shown on mice with CVB4 infection. The antiviral effect of chrysosplenol C (**77**) against PV was EC_{50} = 0.27 µg/ml (0.75 µM). From the two kaempferols (**73, 74**), the single methylated form showed superior inhibition with EC_{50} of 1 µM, with higher potency compared to 3-methylquercetin (**75**). Some synthetic efforts have also been made to achieve more potent flavonoids through the synthesis a series 2-styrylchromones as flavone vinylogues with variation of substitution with methoxy-, hydroxyl-, fluoro- and chloro-groups but no significant improvements to the antiviral capabilities were shown.[59] Other synthetic compounds shown to inhibit EVs include 2-furylmercury chloride (EC_{50} = 0.02 µM vs. HRV-2), 5,5'-diphenyl-3,3'-diisothiazole disulfide (vs. PV-C1), anti-oxidant pyrrolidine dithiocarbamate (activity

shown on PV, CVB3 and HRVs) and YZ-LY-0[180] (EC_{50} = 0.29 µM vs. EV-A71) displaying the high diversity and amount of compounds that the $3D^{pol}$ can be inhibited with.

3.6 Cell function inhibiting antivirals

Similarly as the non-structural protein triumph over capsid proteins as antiviral targets, cell's own function beat the proteins 2A-3D in the same category; the chances for the drug becoming nonfunctional due to mutation are zero.[70] In other words, the chances for drug resistance developing are really low. However this does not mean that the compounds inhibiting cellular functions will always remain effective, as viruses have also been shown to be able to change their pathway of operation and not use the cell protein that is being inhibited.[13] Naturally, additional problems may arise with taking these antivirals to higher *in vivo* models or clinical use as these drugs also inhibit the function of the protein in their original task in the cells as well. The latest identified targets for inhibition are the PI4KIIIβ, OSBP, Hsp90 chaperone and depleting the cell of glutathione.[13] As described previously, PI4KIIIβ and OSBP are abused in the manipulation of cell membranes for EV replication purposes. Hsp90 and glutathione are both employed during the assembly of a new virion.

3.6.1 Phosphatidylinositol-4-kinase III beta (PI4KIIIβ)

As described earlier the PI4KIIIβ is a host factor recruited by the virus for the production of membrane structures for viral replication processes' needs.[13] While previously the compounds inhibiting this protein were described as 3A antivirals, nowadays they have been identified to target this kinase enzyme. One of the most recent antivirals shown to affect the PI4KIIIβ in 2012, enviroxime (fig. 20 (**78**)) was long thought to be a potent 3A inhibiting antiviral.[181] Regardless of the mode of action enviroxime (2-amino-1-(isopropyl sulfonyl)-6-benzimidazole phenyl ketone oxime

(**76**)), first reported in 1980, has since been shown to be a potent inhibitor of both rhino- and enteroviruses. On rhinoviruses enviroxime was reported to completely inhibit the tested 12 serotypes with 0.2 µg/ml concentration.[182] Testing enviroxime and another derivative, enviradene (**79**) (oxime moiety replaced with propene) against 11 EV-D70 and 15 CVA24 isolates, both compounds inhibited 50% of cytopathogenic effect with mean concentrations of 0.17 µg/ml for enviradene and 0.13 µg/ml for enviroxime against all tested viruses.[183] Additional testing on EV-D68, HRV-87 and EV-A71 gave EC_{50} values of 0.29, 0.19 and 1.0 µM respectively.[165] Enviroxime was tested in multiple clinical trials displaying mixed results.[59] Prophylactic treatment against rhinoviruses showed some improvement in reduction in symptoms, however the tests never reached clinical significance. Additionally oral administration of the drug led to side-effects, such as vomiting and abdominal pain. The poor water-solubility and bioavailability was incorporated as the main short comings of the drug and compounds improving these factors were devised. As a compound with superior bioavailability, enviradene was chosen as the precursor for these improvements.[184] Evaluating six different substituents in place of the methyl group in the propene chain, Victor *et al.*[184] discovered the acetylene replacement (**80**) to not only triple the antiviral potency against PV-C1 but also increased the compound plasma levels in a monkey model. Further exploration of another six substituents to the phenyl ring's *p*-position, a fluoride group increased the bioavailability in same tests by 14%, however at the cost of halving of the antiviral potency *in vitro*. Further research into other nitrogen substitution and addition of one more nitrogen to the imidazole ring, different sulfonyl groups and further propene group functionalities was done by Hamdouchi *et al.*[185,186] but no significant improvement compared to enviroxime was achieved.

Through a screening with small interfering RNA (siRNA), Arita *et al.*[161] were able to identify PI4KIIIβ as a target for enviroxime-like compounds GW5074 (**81**) and T-00127-HEV1 (fig. 20 (**82**)). Additionally the plant derived oxoglaucine (**84**) and pachypodol (Ro 09-0179, fig. 19 (**76**)) were shown to inhibit the same kinase.[178] Originally designed as a Raf-1 inhibitor, GW5074 was found in a drug screening of pharmacologically active compounds.[187] Testing against EV-A71 and all three poliovirus genus inhibited both species with EC_{50} of 2.0 and 2.7 µM respectively. The compound itself is an indolin-2-one, connected from 3-position with a methine bridge to 3,5-dibromo-4-hydroxybenzene. Due to higher specificity towards PI kinases, along

with other PI kinase inhibitors PIK93 (**85**), exhibits a higher antiviral potential compared to the previously mentioned antivirals. The PIK93 has been only tested against PV but proved itself the most potent among PI4KIIIβ inhibitors (EC_{50} = 0.14 μM) but also exhibited cytotoxicity to some extent (CC_{50} = 12 μM). Pachypodol (4',5-dihydroxy-3,3',7-trimethoxyflavone (**76**)) extracted from a Chinese medicinal herb[188], was shown to inhibit 20 HRV serotypes, 4 echoviruses, CVA21 and B1 and PV with the EC_{50} range of 0.03-0.54 μg/ml. Additionally an orally bioavailable diacetylated Ro 09-0298 reduced mortality of mice with lethal CVB1 infections. Oxoglaucine, a planar molecule of two dimethoxy benzenes, pyridine and cyclohexanone, has a similar efficacy against PV with EC_{50} of 0.51 μM.[178]

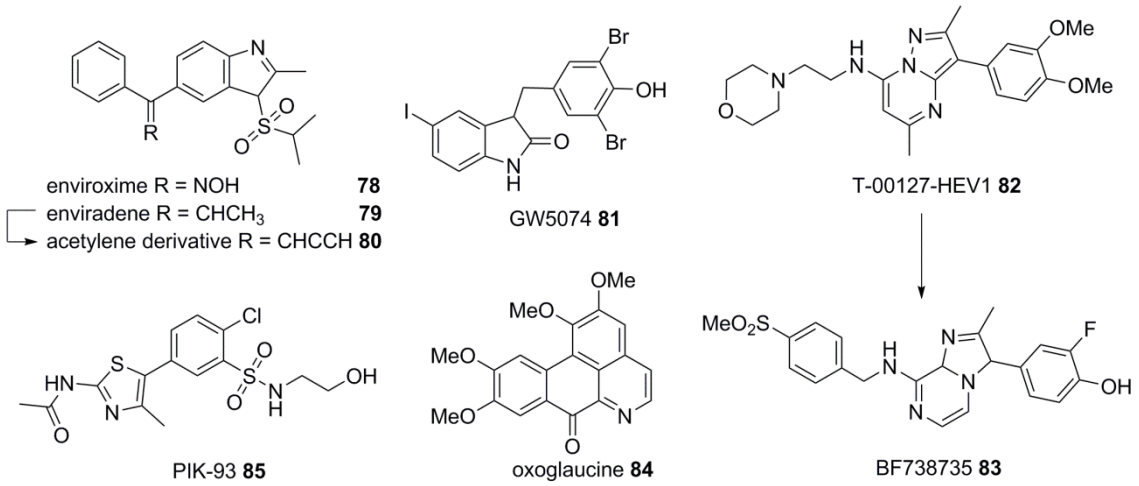


Figure 20. Molecular formulae of enviroxime and other PI4KIIIβ inhibiting compounds.[161,178,182,184,189]

The compounds exhibiting better therapeutic index with high efficacy are the T-00127-HEV1 (**82**) and a later developed close analogue, BF738735 (**83**). T-00127-HEV1 (**82**) was identified from a screening of 72,000 compounds as a potential inhibitor against PV and EV-A71 with low cytotoxicity and affecting replication step.[161] The found antiviral had a 50% effective concentration of 0.77 μM against PV and 0.73 μM against EV-A71. A low cytotoxicity gave it a SI of ≥162. Unfortunately the use of the antiviral *in vivo* was lethal to mice.[13] The structure of the antiviral is 2,5-dimethylpyrazolo[1,5-a]pyrimidine base, substituted with dimethoxy benzene from C-3 and morpholino group from C-7 connected with an ethylamine chain.

Closely resembling the T-00127-HEV1, on BF738735 (fig. 20) the benzene ring is substituted with hydroxyl and fluoride groups, while the ethylamine is joined with a mesylbenzene with slight alterations on the base framework as well.[189] The compound was thoroughly tested *in vitro* with 15 HRV and 10 EV serotypes covering all enterovirus groups and had a high efficacy ranging between 0.004-0.071 µM and with the lowest SI being 371. A close structural derivative (mesylbenzene exchanged to pyridine and hydroxyl group to methoxy), due to insufficient pharmacokinetic properties of BF738735 (**83**), was used to study the bioavailability and antiviral effect *in vivo*. The drug was administrated in mice, 1 mg/kg intravenously or 5 mg/kg orally to treat CVB4 induced pancreatitis. The drug was well tolerated by specimens with good plasma levels of the antiviral in circulation and a complete inhibition with 25 mg/kg and some inhibition with 5 mg/kg dose was observed, making it a good candidate for clinical testing of PI4KIIIβ inhibition.

3.6.2 Inhibitors of oxysterol binding protein

In their studies on PI4KIIIβ inhibition, Arita *et al.*[190] identified that the antiviral AN-12-H5 did not affect the activity of the same cell factor compared to others. Setting out to find the target of this compound, the researchers revealed through siRNA sensitization assays the oxysterol-binding protein family I as the target for the compound. In addition the natural ligand of OSBP, 25-hydroxycholesterol (**86**) as well as a new compound, T-00127-HEV2 (**87**), were identified as inhibitors of this cellular protein. AN-12-H5 (fig. 21 (**88**)) was discovered in a screening of a chemical library to find antiviral compounds against PV and EV71.[191] From four different derivatives of the same group of molecules, AN-12-H5 (**88**) inhibited both PV and EV71 with EC_{50} of 1.1 and 0.55 µM showing highest promise. However, another compound, AN-23-F6 was a more potent inhibitor of EV71 (EC_{50} = 0.15 µM, SI = 550). Similarly, T-00127-HEV2 was also a result of a screening with a large chemical library.[190] Highly resembling the 25-hydroxycholesterol, T-00127-HEV2 (**87**) has the allylic side chain replaced with a hydroxyl group, dimethyl group in 16-position and the hydroxyl group is ether bound to 2-tetrahydropyran. Both compounds showed inhibitory action against a PV pseudovirus infection, the natural ligand cholesterol more weakly, EC_{50} being 2.8 µM and

T-00127-HEV2 (**87**) at higher efficacy with EC_{50} of 0.66 µM. However no measurement on wide spectrum activity has been conducted with any actual viruses.

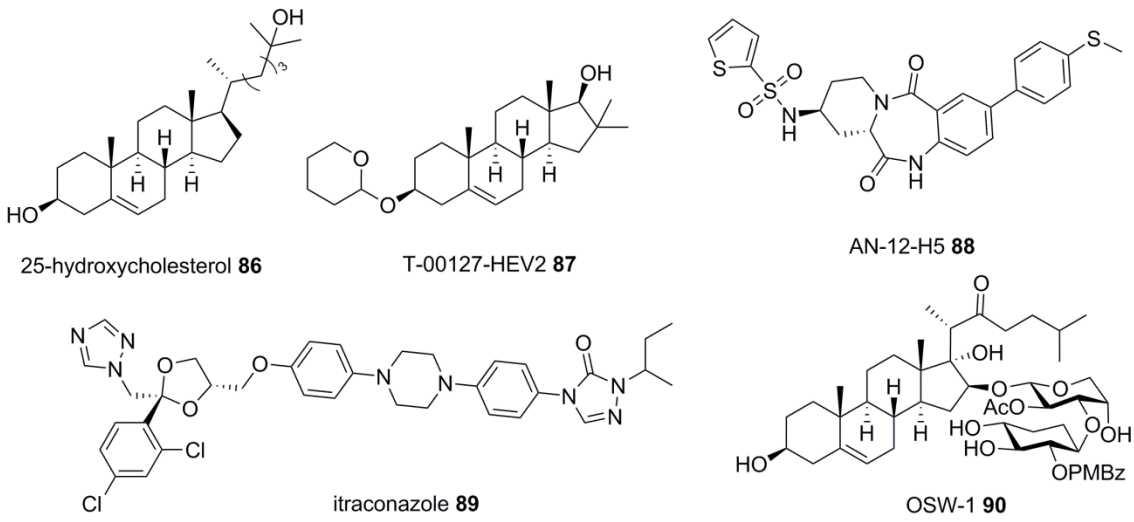


Figure 21. OSBP inhibiting molecules.[190–193]

OSW-1 (**90**) and itraconazole (**89**) are both compounds that have attracted attention as potential anti-cancer drugs quite recently and were also tested as potential antiviral molecules.[192,193] OSW-1 (**90**) is a specific inhibitor of the OSBP, extracted from natural sources. It also shares a high resemblance with the 25-hydroxylcholesterol, containing additionally a hydroxyl group at the 17- and a disaccharide resembling group at the 16-position in the molecule. The antiviral showed a high efficacy against tested EVs; EV-A71, CVA21, HRV-A2 and HRV-B14 with EC_{50} values ranging between 0.0024-0.0094 µM on all tested serotypes. Itraconazole (**89**) was first discovered in 1984 as an antifungal drug and has been approved in USA since 1992. Thus a lot of information is available on the *in vivo* activity of the drug and is readily available for straight clinical testing against viral infections. While this makes the compound an attractive antiviral option, the *in vitro* capabilities were inferior compared to OSW-1, with 10 EV serotypes and HRV-14 EC_{50} values on the range 0.3-1.6 µM (mean 0.73 µM).

3.6.3 Assembly involved cell factors

The two cell factors involved in the maturation and assembly of the viral capsid proteins with the current knowledge are the chaperon heat shock protein 90 (Hsp90) and glutathione.[13] Hsp90 participates in the folding of the P1 capsid precursor and its proper processing after. The inhibition of Hsp90 thus leads to improper folding and decrease in production of new virions. Geldanamycin (**91**) and its more bioavailable derivative 17-AAG (**92**) (17-allyamino-17-demethoxygeldanamycin) are both well-known inhibitors of Hsp90 (fig. 22). Although the role of the Hsp90 has been shown for the enteroviruses[194], no comprehensive studies on the effect for its inhibition against EV infections have been reported. However, the validity of Hsp90 as a target for antivirals has been shown on multiple other virus types, including the inhibition of herpes simplex 1 proliferation *in vitro* with geldanamycin (EC_{50} = 0.093 µM)[195]. Hsp90 still holds unknown potential as EV antiviral target, which needs to be clarified in future studies.

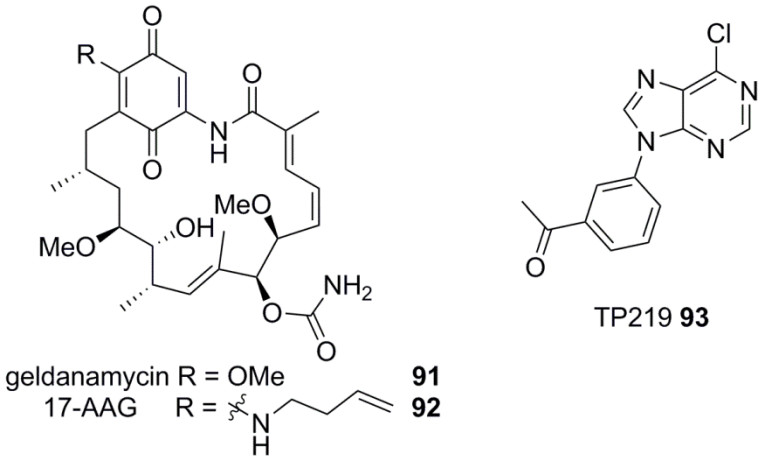


Figure 22. Assembly affecting antiviral molecules.[195,196]

The role of glutathione in the assembly of a new virion capsid structure is the transition of a VP0, VP1 and VP3 protein complex into a pentamer of five units.[13] This cell cofactor was first discovered by Mikami *et al.*[197] on echovirus 9 and confirmed to include all types of EV, testing with PV-C3, CV-B5, CVA10, and A16 with the use of buthionine sulfoximine, a glutathione synthesis inhibitor. The concentrations to block cell death however were quite high, 100 µM. After this discovery, a glutathione

scavenging molecule, TP219 (**93**) was reported.[196] The compound, 6-chloropurine with *m*-acetobenzene as N-substituent, did not show wide spectrum viability. Although the antiviral exhibited EC_{50} values at a millimolar range, the effect was also shown to be completely abolished by a surface-exposed methionine in VP1, which already exists in wildtype EV71 for example. Although glutathione-depleting compounds have been tolerated well as part of a cancer treatment *in vivo*, the previous observation makes glutathione a less attractive target for antivirals.

3.7 Viral RNA as a target

With the current knowledge on the enteroviruses, not many antiviral targets remain after covering both the structural and nonstructural proteins of the virus as well as the cellular factors involved. However the viral RNA itself, so far left untouched, also offers a potential target for EV inhibition. To access inhibition of viruses through this pathway has been discovered in the form of RNA silencing.[56] The method uses short (21-25 nucleotides) double stranded RNA molecules mimicking the sequence on the gene to be silenced. A successful example of this was achieved by Phipps *et al.*[198] who targeted the sequences encoding VP4, 2C and 3D proteins of HRV-A16 *in vitro*. Inhibition of these sequences gave a strong antiviral response, EC_{50} varying between 0.73 and 0.93 nM. A similar effect while targeting 5′ uncoding region of poliovirus was observed as well.[199] However, the investigators observed a creation of resistant strains to this antiviral approach as well. This could be circumvented with the inhibition of multiple different gene sequences at the same time. Another downfall of RNA use as a drug is its susceptibility to enzymatic disintegration. Some alternate nucleotide structures have been used as RNA inhibitors. Yuan *et al.*[200] targeted the untranslated region of CVB3 genome both from the 5′ and 3′ ends with phosphorothioate DNA. The difference to regular DNA is the replacement of an oxygen double bonded to the phosphorus in DNA with a sulfur atom. The UTR (both 3′ and 5′) areas serve vital roles in the viral translation and the inhibition with the phosphorothioate DNA showed antiviral activity, with the most potent location in the genome being in the 3′ terminus. The researchers observed a decrease of 87.6% in viral transcription and 40.1% in VP1 synthesis. Additional test *in vivo* on mice showed the oligonucleotide to be able to decrease the

replication of CVB3 by 68% in specimens. Another group attempting the use of peptide-conjugated phosphorodiamidate morpholino oligomers in mimicking single stranded DNA for EV inhibition was also successful.[201] Testing various targets in the genome against HRV-14, CVB2, PV-C1 the IRES was identified the most valuable target. The highest antiviral activity was achieved from targeting 22 nucleotides in the IRES, a sequence that is conserved in 99% of all EVs and HRVs. This oligonucleotide, coined EnteroX, was tested on mice against PV-C1 infection and shown to improve the survivability by 80%. This displays the viability of targeting the genome of enteroviruses among all the other explored antiviral targets explored in this work.

4 Conclusions

Enteroviruses and rhinoviruses are a genus of positive single stranded RNA picornaviruses protected by a viral capsid of ca. 30 nm in diameter. The genome contained inside the capsid comprises the four structural proteins and seven proteins with enzymatic and other activity, containing the capability to overtake its target cell making it a factory for new viral particles, virions. After virus binding and entry, a viral cycle takes place in cells, leading to synthesis of new viral protein molecules and RNA that are assembled into whole virions which in the end of the viral cycle escape the host cell to start the process again by attaching to a new cell. The enterovirus genus, named after their gastroenteric entry pathway to their host, contains 71 different serotypes that have humans as their targets. Similarly, human rhinoviruses target the respiratory system in humans, spanning over a hundred different viruses. The EVs were previously categorized into four groups; polioviruses, coxsackieviruses A and B and echoviruses by their pathology and while the nomenclature still lives strong, the official division is to four different species from A to D. HRVs on the other hand are divided to species A-C. HRVs are the largest group of viruses causing the common cold that causes yearly large economical losses all around the world due to missing work and visiting doctors. In addition they are also involved in chronic pulmonary disease that is expected to become the leading cause of death in the near future. Enteroviral infections on the other hand, range from mild symptoms to complications causing death. Many cause some

form of meningitis, most notorious being the poliomyelitis caused by the polioviruses. More recent sicknesses are the epidemics of hand, foot and mouth disease caused by EV71 in the far East.

The work to create antivirals against EV and HRV infections has been ongoing for decades but despite the efforts, not a single drug has made it past the clinical testing. While a plethora of antivirals have been developed, the requirements for these compounds have been set high since the beginning. The most important requirements are antiviral activity with low concentrations against wide spectrum of both EVs and HRVs. Additionally the developed drugs are desired to be bioavailable through oral administration and have no/very low side effects, not stronger than the infections cause themselves. The most potent antivirals against EVs are represented in table 1. The development of new antivirals has gone closely hand in hand with both the general understanding and the structural knowledge of their targets. Thus the first target for the antiviral research was the capsid of EVs and the hydrophobic pocket identified after structural elucidation by Rossmann *et al.*[55]. The unyielding research on capsid binders gave inception to three major antivirals (among many others); the prototype antienteroviral pleconaril (**5)**, vapendavir and pocapavir. However the capsid binders are highly susceptible to resistant mutations so focus shifted towards nonstructural proteins of EVs, as their structures were uncovered. They make for ideal targets as their similarity compared to host cell and thus possible side effects from drugs are very low. The research on these proteins has also been highly prolific and essentially all the proteins apart from 2B and 3B have their own antivirals available. From these the 2A, 3C and 3D are above others due to their known structures, but also the 2C has already many antivirals available, despite its structure still being unknown. The 3D especially has offered multiple sites as inhibition targets. The most potent compounds in this class include for example rupintrivir and its bioavailable derivative AG7404, fluoxetine and ribavirin. More recently, the role of some of the cellular factors have been identified, namely PI4KIIIβ, OSBP, Hsp90 and glutathione. The inhibition of these cellular factors has been already shown to have a wide spectrum effect on small doses, especially for the OSBP.[192] Although the risk for resistant mutation is lowered, targeting the cellular factors always involves the risk of higher amount of side effects in use *in vivo*. Additionally RNA interference with small strands of oligonucleotides has been explored

to some success. However no antivirals against human enteroviruses nor rhinoviruses have reached clinical use and so the research continues on.

Table 1: Table of some antiviral molecules covered in this work

Product name	Target viral function	State of development	Comments
Pleconaril (**5**)	Hydrophobic pocket	Many clinical studies concluded	
Vapendavir (BTA-798) (**10**)	Hydrophobic pocket	Clinical studies conducted	
Pocapavir (V-073) (**13**)	Hydrophobic pocket	Initial clinical sample done	
TTP-8307 (**63**)	2A	*in vitro* effect shown	
Guanidine HCl (**55**)	2C	*in vivo* required concentrations too high	Tested in tandem with HBB
HBB (**58**)	2C	*in vivo* effect shown	
MRL-1237 (**57**)	2C	*in vivo* effect very high	Only CVBs tested
Fluoxetine (**61**)	2C	Used clinically as antidepressant	
z-LVLQTM-fmk (**44**)	3A	*in vivo* effect shown	mechanism ambiguous
Rupintrivir (AG7088) (**47**)	3C	Poor bioavailability, discontinued	
AG7404 (**48**)	3C	Clinical testing halted	Tested safe for oral administration
NK-1.8k (**52**)	3C	*in vitro* effect shown	EV71 and EV68
Ribavirin (**64**)	3D	Development halted	
NITD0008 (**66**)	3D	*in vivo* effect shown	
Amiloride (**71**)	3D	*in vitro* effect shown	Mg^{2+} Mn^{2+} transport
GPC-N114 (**68**)	3D	*in vitro* effect shown	
Gliotoxin (**72**)	3D	*in vivo* effect shown	
DTrip-22 (**70**)	3D	*in vitro* effect shown	Broad spectrum
BPR-3P0128 (**67**)	3D	*in vitro* effect shown	Tested on EV71 only
3-methyl quercetin (**75**)	3D	*in vitro* effect shown	Tested on PV-C1 and CVB4
Enviroxime (**78**)	PI4KIIIβ	Mixed results in clinical trials. Discontinued	
T-00127-HEV1 (**82**)	PI4KIIIβ	*in vitro* effect shown	Lethal to mice
BF738735 (**83**)	PI4KIIIβ	High potency *in vitro*, *in vivo* effect shown	
PIK93 (**85**)	PI4KIIIβ	High potency *in vitro*	Some cytotoxicity
Pachypodol (**76**)	PI4KIIIβ	*in vivo* effect shown	
Itraconazole (**89**)	OSBP	*in vitro* effect shown	Used as antifungal clinically since 1992.
OSW-1 (**90**)	OSBP	High potency *in vitro*	
AN-12-H5 (**88**)	OSBP	*in vitro* effect shown	EV71 tested only
T-00127-HEV2 (**87**)	OSBP	*in vitro* effect shown	PV pseudovirus tested
25-hydroxycholesterol (**86**)	OSBP	Small *in vitro* effect	Natural ligand of OSBP
Geldanamycin (**91**)	Hsp90	Effect on herpes simplex 1 shown	Not tested on EVs
TP219 (**93**)	Glutathione	*in vitro* effect shown	Resistance gained easily
EnteroX	Viral RNA	*in vivo* effect shown against PV	22 nucleotide siRNA

Although many antivirals with good activity against a wide spectrum of enteroviruses have been developed, many targets for future antivirals still remain unexplored. Although the structure of the $3C^{pro}$ is quite well known, only recently Wang *et al.*[54] reported the first inhibitor on an allosteric site on the protein, revealing that still a lot of work remains undone concerning the protease. Another bottle neck for the field is the lack of known structures for the 2C and 3A although many antivirals against 2C already exist.[113] The structural elucidation of both can be expected to push the development of new antivirals. The study of inhibiting cellular targets abused by the virus is still in its infancy and although the effect has been already shown *in vivo*, clinical trials with humans are yet to be conducted. No antivirals have yet been put to clinical use, due to concerns regarding the selection for resistant mutants against individual antivirals and their efficacy against in wide enough spectrum on natural EV and HRV infections. As a solution for this, employing many antivirals at the same time, the so called ‘cocktail therapy’ has been suggested and initially tested.[113,202] The initial study showed that although, no significant synergistic boost could be observed between the examined antivirals, they neither inhibited each other, showing some promise for the method. Further studying the possibilities of such combinations might finally bring the first clinically applicable treatments against enterovirus infections that the world desperately needs.

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