

## ABSTRACT

Title of Dissertation: IDENTIFICATION AND CHARACTERIZATION OF  
NEW SMALL MOLECULE INHIBITORS OF  
PICORNAVIRUS REPLICATION

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The *Picornaviridae* family consists of positive-strand RNA viruses that are the causative agents of a variety of diseases in humans and animals. Few drugs targeting picornaviruses are available, making the discovery of new antivirals a high priority. Here, we identified and characterized three compounds from a library of kinase inhibitors that block replication of poliovirus, coxsackievirus B3, and encephalomyocarditis virus. The antiviral effect of these compounds is not likely related to their known cellular targets because other inhibitors targeting the same pathways did not inhibit viral replication. Using an *in vitro* translation-replication system, we showed that these drugs inhibit different stages of the poliovirus life cycle. A4(1) inhibited the formation of a functional replication complex, while E5(1) and E7(2) affected replication after the replication complex had formed. A4(1) demonstrated partial protection from paralysis in a murine model of poliomyelitis. Poliovirus resistant to E7(2) had a single mutation in the 3A protein. This mutation was previously found to confer resistance to enviroxime-like

compounds, which target either PI4KIII $\beta$  (major enviroxime-like compounds) or OSBP (minor enviroxime-like compounds), cellular factors involved in lipid metabolism and shown to be important for replication of diverse positive-strand RNA viruses. We classified E7(2) as a minor enviroxime-like compound, because the localization of OSBP changed in the presence of this inhibitor. Interestingly, both E7(2) and major enviroxime-like compound GW5074 interfered with the viral polyprotein processing. Multiple attempts to isolate resistant mutants in the presence of A4(1) or E5(1) were unsuccessful, showing that effective broad-spectrum antivirals could be developed on the basis of these compounds. Studies with these compounds shed light on pathways shared by diverse picornaviruses that could be potential targets for the development of broad-spectrum antiviral drugs.

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INHIBITORS OF PICORNAVIRUS REPLICATION

By

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University of Maryland, College Park in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2016

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## Dedication

This thesis is dedicated to my family and friends for always being there for me, and to Michael for his unconditional love and unwavering support.

## Acknowledgements

First and foremost, I would like to acknowledge my adviser, Dr. George Belov for his advice and mentorship through my graduate career. He always inspired me to think outside the box and encouraged me to develop into an independent scientist. I would also like to thank my committee members Drs. Liqing Yu, Jeffrey DeStefano, Yanjin Zhang, and Meiqing Shi for their time and their suggestions for my project.

I would like to thank the members of the lab, Dr. Ekaterina (Katya) Viktorova and Jules Nchoutmboube. Katya was always willing to lend a hand in preparing reagents. She also taught me many laboratory techniques and protocols. Jules was always there to listen to my complaints about negative results and to share in my excitement when experiments worked. I will always be grateful to Katya and Jules for their friendship and support.

I would like to thank all of the members of my family for encouraging me to never give up on my dreams: to my mom for her love and encouragement; to my dad, who was always excited to hear about my research; to my Grandma and Grandpa for their support and for our conversations on poliovirus and HeLa cells; to my Grammy for her constant confidence in me; and to my Pop-pops, one of the greatest, brightest men I have ever known. I wish you were here to see me achieve my dream.

I would like to thank my friends Caitlin, Meghan, and Kelsey for always being there for me. I could not ask for better friends. I would also like to thank my dogs Cody, Sophie, and Eliza for being the best anti-stress medicine ever.

Most importantly, I would like to thank my husband, Michael, for listening to me prattle on about poliovirus and virology every day for five years. Without your love and encouragement I would not be where I am today.

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## List of Abbreviations

BFA: Brefeldin A

CVB3: Coxsackievirus B3

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethylsulfoxide

EMCV: Encephalomyocarditis virus

ER: Endoplasmic reticulum

FBS: Fetal bovine serum

GuHCl: Guanidine hydrochloride

HCV: Hepatitis C virus

HRV: Human rhinovirus

IPV: Inactivated polio vaccine

IRES: Internal ribosome entry site

kDa: Kilodalton

OPV: Oral polio vaccine

OSBP: Oxysterol binding protein

PCR: Polymerase chain reaction

PH: Pleckstrin homology

PI4KIII $\beta$ : Phosphatidylinositol-4-kinase III  $\beta$

PI4P: Phosphatidylinositol-4-phosphate

PV: poliovirus

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

UTR: Untranslated region

## Chapter 1: Introduction to Picornaviruses and Poliovirus

### 1.1 Classification of Picornaviruses and Picornavirus-associated Diseases

The *Picornaviridae* family within the order *Picornavirales* comprises a diverse group of nonenveloped, positive-sense RNA viruses that infect both humans and vertebrate animals (Table 1-1). This family of viruses is divided into twenty-nine genera; some genera contain only one representative, while others encompass multiple species of viruses. The *Enterovirus* genus, for example, is subdivided into twelve clusters comprising 316 individual viral species. The viral particles have an average diameter of 30 nm and they encapsidate a small RNA genome.

Many picornaviruses can infect humans; some infections are asymptomatic, but others can cause severe disease. The *Enterovirus* genus includes many important viruses, such as enterovirus 71, coxsackievirus A16, human enterovirus D68, human rhinoviruses, coxsackievirus B3, and poliovirus. Enterovirus 71 and coxsackievirus A16 are common causative agents of hand, foot, and mouth disease in children; however, children infected with enterovirus 71 may experience neurological manifestations, such as meningitis, encephalitis, and poliomyelitis-like paralysis (98). Human enterovirus D68 is an emerging respiratory virus identified in California in 1962. This virus can cause a mild cold-like illness or can lead to severe respiratory infections requiring hospitalization (125). Human rhinoviruses are the cause of the common cold and are responsible for the loss of billions of dollars each year in medical costs and work absence (52, 130). Human rhinoviruses may also be associated with the development of severe respiratory illness in

susceptible populations (29). Coxsackievirus B3 is one of the most common causes of viral myocarditis; 10-20% of patients progress to chronic cardiac disease (98). Group B coxsackieviruses are also associated with the development of type I diabetes (87). Hepatitis A virus, the lone member of the *Hepatovirus* genus, is the causative agent of acute viral hepatitis. Although most patients recover from this disease, in severe cases, necrosis of the liver leads to fulminant hepatitis and liver failure (99). Foot-and-mouth disease virus of the *Aphovirus* genus is the causative agent of a highly infectious febrile and vesicular disease of cloven-hoofed animals, including cattle and pigs. The high morbidity of this disease bestows a large economic burden on the livestock industry (16).

**Table 1-1. The *Picornaviridae* family with example viruses and natural hosts.**

<b>Genus</b>	<b>Species</b>	<b>Natural Hosts</b>
<i>Apthovirus</i>	Foot-and-mouth disease virus Equine rhinitis A virus Bovine rhinitis A virus Bovine rhinitis B virus	Cows, Sheep, Pigs, Goats, Horses
<i>Aquamavirus</i>	Aquamavirus A	Seals
<i>Avihepatovirus</i>	Avihepatovirus A	Waterfowl
<i>Avisivirus</i>	Avisivirus A	Turkeys
<i>Cardiovirus</i>	Encephalomyocarditis virus Theilovirus (eg: Saffold virus)	Rodents
<i>Cosavirus</i>	Cosavirus A	Humans
<i>Dicipivirus</i>	Cadicivirus A	Dogs
<i>Enterovirus</i>	Enterovirus A (eg: Coxsackievirus A16, EV 71) Enterovirus B (eg: Coxsackievirus B3) Enterovirus C (eg: Poliovirus, Coxsackievirus A1) Enterovirus D (eg: EV D68) Enterovirus E (eg: Bovine enterovirus group A) Enterovirus F (eg: Bovine enterovirus group B) Enterovirus G (eg: Porcine enterovirus) Enterovirus H (eg: Simian enterovirus) Enterovirus J (eg: Simian enterovirus) Human rhinovirus A Human rhinovirus B Human rhinovirus C	Humans, Non-human primates, Pigs, Monkeys, Cows, Wild Boar
<i>Erbovirus</i>	Erbovirus A	Horses
<i>Gallivirus</i>	Gallivirus A	Poultry
<i>Hepatovirus</i>	Hepatovirus A (eg: Hepatitis A virus)	Humans, Small Mammals
<i>Hunnivirus</i>	Hunnivirus A	Cows
<i>Kobuvirus</i>	Aichivirus A Aichivirus B Aichivirus C	Humans, Cows, Dogs, Cats, Rodents, Pigs
<i>Kunsagivirus</i>	Kunsagivirus A	Birds
<i>Megrivirus</i>	Melegrivirus A	Poultry
<i>Mischivirus</i>	Mischivirus A	Bats
<i>Mosavirus</i>	Mosavirus A	Rodents, Birds
<i>Oscivirus</i>	Oscivirus A	Birds

**Table 1-1 continued. The *Picornaviridae* family with example viruses.**

<b>Genus</b>	<b>Species</b>	<b>Natural Hosts</b>
<i>Parechovirus</i>	Parechovirus A (eg: Human parechovirus) Parechovirus B (eg: Ljungan virus)	Humans, Ferrets, Rodents
<i>Pasivirus</i>	Pasivirus A	Pigs
<i>Passerivirus</i>	Passerivirus A	Birds
<i>Rosavirus</i>	Rosavirus A	Humans, Rodents
<i>Sakobuvirus</i>	Sakobuvirus A	Cats
<i>Salivirus</i>	Salivirus A	Humans, Non-human primates
<i>Sapelovirus</i>	Sapelovirus A Sapelovirus B Avian sapelovirus	Pigs, Birds, Monkeys, Sea lions, Mice
<i>Senecavirus</i>	Senecavirus A (Seneca Valley virus)	Pigs
<i>Sicinivirus</i>	Sicinivirus A	Poultry
<i>Teschovirus</i>	Teschovirus A	Pigs
<i>Tremovirus</i>	Tremovirus A	Birds



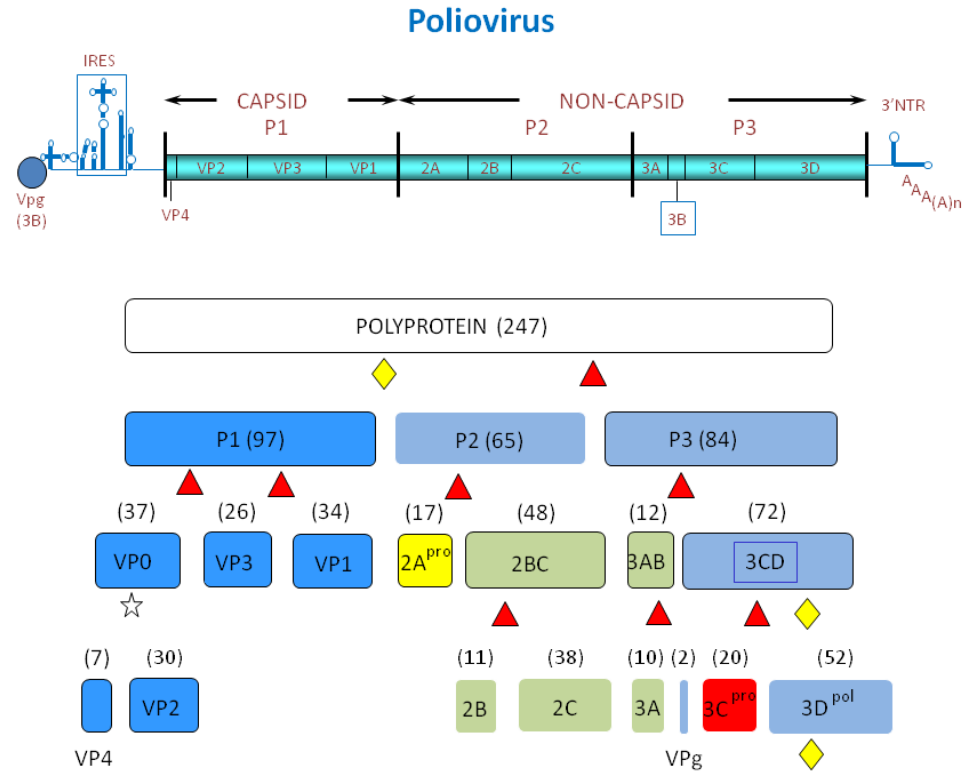
Poliovirus (PV), the causative agent of poliomyelitis, is the prototype member of the *Picornaviridae* family and one of the best-studied animal viruses. There are three serotypes of poliovirus (PV1, PV2, and PV3) which share between 70%-80% sequence homology (63). Poliovirus is restricted to humans due to the specific requirement of the poliovirus receptor (PVR or CD155) in virus entry, however a transgenic mouse that expresses the poliovirus receptor has been developed to model human poliomyelitis (83).

Poliovirus is transmitted through the fecal-oral route and is thought to replicate in the oropharyngeal and gastrointestinal mucosa (2). The exact course through the host has not been well-established. The virus is hypothesized to reach the draining lymph nodes and then spread throughout the bloodstream, establishing viremia (75). In most cases, patients develop neutralizing antibodies against the virus and the disease progresses no further. This is known as abortive poliomyelitis, in which patients may experience mild gastrointestinal symptoms (75). In around 1% of cases, poliovirus penetrates the blood-brain barrier through an unknown mechanism to invade the central nervous system where the virus infects motor neurons (75). Poliomyelitis has several different manifestations: 1) spinal poliomyelitis, which is associated with flaccid paralysis of the extremities and trunk; 2) bulbar poliomyelitis, or paralysis of the respiratory muscles leading to difficulties in breathing; and 3) bulbospinal poliomyelitis, which is a combination of the two (75, 98).

## **1.2 Poliovirus Genome Organization**

Poliovirus is the prototype member of the *Picornaviridae* family. The poliovirus genome RNA is approximately 7500 nucleotides in length. The 5' untranslated region

(UTR) is covalently linked to viral protein VPg (3B) and contains an internal ribosome entry site (IRES) and other secondary structures important for replication and translation. The coding region is divided into the P1 region, which encodes the structural proteins that make up the capsid (VP1, VP2, VP3, and VP4), and the P2 and P3 regions, which encode nonstructural proteins necessary for viral replication (2A-2C and 3A-3D, respectively). The 3' UTR, which contains structures important for replication, is followed by a polyA tail of around 60 residues (Figure 1-1) (75).



**Figure 1-1. Diagram of the poliovirus genome and polyprotein processing.** Poliovirus has a genome of positive-strand RNA of around 7500 nucleotides. The 5' end is covalently-linked to a viral protein VPg and contains an internal ribosome entry site (IRES). The open reading frame is divided into three regions: the P1 region encodes the structural proteins that make up the capsid, while the P2 and P3 regions encode the nonstructural proteins important for replication. The 3' end contains a polyA tail of around 60 residues. Upon entry into the cell, the genome is translated into a large polyprotein that is cleaved by the viral proteases 2A, 3C, and 3CD into protein precursors and individual proteins. The red triangles represent sites cleaved by the 3C/3CD protease. The 3CD protease preferentially cleaves the structural proteins, while 3C preferentially cleaves the non-structural proteins. The yellow diamonds represent sites cleaved by the 2A protease, and the white star represents a site of autocatalytic processing.

### 1.3 Poliovirus Life Cycle

Upon binding to the poliovirus receptor CD155, the virus enters the cell through receptor-mediated endocytosis. The viral RNA is released into the cytoplasm where VPg is cleaved from the 5' end of the RNA (143). The RNA is translated into a single polyprotein which is subsequently cleaved *in cis* and *in trans* by viral proteases 2A, 3C, and 3CD into the structural and nonstructural proteins. Specifically, the 3CD protease preferentially cleaves the structural proteins, the 3C protease processes the nonstructural proteins, and the 2A protease cleaves the P1 region from the P2P3 region (76). Viral protease 2A also cleaves translation initiation factor eIF4G to shut down the cap-dependent translation of host mRNAs (73). Translation occurs in a cap-independent manner, with ribosomes binding to the IRES. Following translation, replication takes place in the cytoplasm on membranous structures (23). The initiation of both minus-strand and plus-strand synthesis by the viral RNA-dependent RNA polymerase 3D<sup>pol</sup> is primed by uridylylated viral protein VPg (42). Newly synthesized positive-strand RNA can be transcribed into negative-strand RNA, used as a template for continued translation of viral proteins, or be targeted for encapsidation (4). The formation of the viral capsid in the cytoplasm begins with the cleavage of the P1 precursor into VP0, VP1, and VP3 by the 3CD protease. These structures form the procapsid, which associates with viral RNA. The cleavage of VP0 into VP2 and VP4, which is thought to be autocatalytic, results in the production of the mature virus particle containing 60 copies of each capsid protein (67). Viruses are released into the extracellular milieu by cell lysis, although recent evidence has suggested that poliovirus particles are also released non-lytically within phosphatidylserine lipid-enriched vesicles (31, 75, 79).

All positive-strand RNA viruses replicate their genomes on membranes in the cytoplasm of infected cells. These membranous structures are thought to (i) provide a structural scaffold for the formation of the replication complexes, (ii) increase the local concentration of factors involved in poliovirus replication, and (iii) protect the replication intermediates like dsRNA from recognition by cellular cytosolic sensors of the innate immune system, such as RIG-I or MDA-5 (85). Infection with picornaviruses leads to the proliferation and rearrangement of intracellular membranes to form the replication complexes. Poliovirus proteins containing 2C, 2B, and 3A have membrane-binding domains and expression of 2BC and 3A rearranges the intracellular membranes into structures similar to those seen upon natural infection (32, 41). These proteins recruit viral and host proteins to the replication complexes to facilitate replication of the viral genome (49).

These membranous structures are dynamic throughout the infectious cycle— electron tomography images of cells infected with poliovirus or coxsackievirus B3 have revealed the presence of mainly single-membrane tubular compartments at early time points post infection when the viral genome is replicating at high levels. These structures transform into predominately double-membrane vesicles at around six hours post infection. The double-membrane vesicles are similar in structure to autophagosomes, suggesting that the autophagy pathway plays a role in their formation (23).

## 1.4 Vaccine Development

Within the *Picornaviridae* family, successful human vaccines have been developed only for hepatitis A virus and poliovirus, and experimental vaccines directed against enterovirus 71 are at the stage of clinical testing. Currently, neither hepatitis A virus nor poliovirus has been eradicated with the existing vaccines.

### 1.4.1 Hepatitis A Vaccines

Hepatitis A virus, the lone member of the *Hepatovirus* genus, causes acute viral hepatitis associated with jaundice, fever, fatigue, nausea, and diarrhea. Less than 1% of patients develop fulminant liver failure, with increased age correlating with higher risk (99). Two types of hepatitis A vaccines were developed in the early 1990s: an inactivated vaccine (Havrix, Vaqta, etc.) used throughout the world, and a live-attenuated vaccine most commonly used in China and India (18, 99). To create the inactivated vaccine, a cell culture-adapted HAV strain is grown in MRC-5 cells. After virus extraction by cell lysis, the virus is purified by various techniques, including filtration, and inactivated with formaldehyde (18). Studies have shown that inactivated vaccine recipients remain seropositive for 25 years (109). The live-attenuated vaccines, which are 95% effective, consist of HAV strains adapted to growth in human diploid cells at 32 °C (99, 147). Since the introduction of the Hepatitis A vaccine in the United States, disease incidence has declined rapidly, demonstrating the high efficacy of the vaccination campaign.

### **1.4.2 Poliovirus Vaccines**

Although poliovirus was first described as the causative agent of poliomyelitis by Karl Landsteiner and Erwin Popper in 1909, evidence of poliomyelitis is apparent in an ancient Egyptian stele, which portrays a priest with a withered leg (34, 75). Sporadic cases of poliomyelitis were reported throughout history, however it wasn't until the mid-twentieth century that poliovirus became a wide-spread health concern. Before the twentieth century, most children were likely exposed to poliovirus while they were still protected by maternal antibodies. Improved sanitary practices and hygiene resulted in exposure of children at a later age at which they were no longer protected by maternal antibodies (75). Better hygiene and the increase in population density both likely contributed to the massive polio epidemics of the mid-twentieth century. These outbreaks were controlled with the introduction of the inactivated polio vaccine (IPV) and the live-attenuated polio vaccine (OPV).

In 1953, over 20,000 poliomyelitis cases were reported in the United States alone. With the introduction of the inactivated Salk vaccine in 1955, the incidence of disease dropped 25-fold. This downward trend continued with the introduction of the live-attenuated Sabin vaccine, which became the preferred vaccine, in 1961. By 1979, wild type poliovirus was eradicated in the United States. In 2000, the United States began to exclusively use the Salk vaccine for immunizations, because the live-attenuated Sabin vaccine, although effective in inducing immunity, had a low risk of reverting to a pathogenic phenotype. Although delayed, with extensive vaccination campaigns that involved coordinated international efforts, poliovirus has almost been eradicated.

Currently, two countries remain where poliovirus is still endemic: Afghanistan and Pakistan.

The first poliovirus vaccine, made from inactivated virus, was developed by Jonas Salk in 1955. As poliovirus has three antigenically-distinct serotypes, immunization against one serotype does not confer protection against the others. Therefore, all three serotypes were grown in Vero cells and inactivated with formaldehyde. This vaccine is administered intramuscularly and effectively protects against poliovirus challenge. After its introduction, poliomyelitis cases dropped by ~95% in the United States over five years (128). The Cutter Incident, in which the vaccine was improperly inactivated due to the accumulation of viral aggregates during production, resulted in poliomyelitis outbreaks in the western United States in 1955 (106). To prevent this tragedy from reoccurring, vaccine production changed; inactivated virus is now passed through filters to remove the viral aggregates (128).

The live-attenuated Sabin vaccine was developed in the late 1950s by Albert Sabin and introduced to the population in 1961. The three vaccine strains were derived through passage in cell culture at suboptimal temperatures or passage through an animal host. The goal was to isolate viruses that could replicate in the gastrointestinal tract but were unable to invade the central nervous system, and that could replicate in a human host without reverting to a pathogenic phenotype (75). Sequencing of the vaccine strains found 57 nucleotide substitutions within the Sabin 1 strain, two substitutions within the Sabin 2 strain, and ten substitutions within the Sabin 3 strain (113). The trivalent vaccine is administered orally and effectively confers a strong mucosal immune response against all three serotypes.



Both the Salk vaccine and Sabin vaccine have their advantages and disadvantages. Because the Salk vaccine consists of inactivated virus, there is no chance for the virus to mutate back to a pathogenic phenotype, causing this vaccine to be preferred in the United States and other developed countries. However, although effective and safe, production of this vaccine requires the growth of a large amount of wild type virus, and the possibility of accidental release of wild type virus would remain a threat after eradication. In addition, the Salk vaccine does not induce a strong mucosal immune response, and it is more expensive than the live-attenuated vaccine (98).

In contrast to the Salk vaccine, the Sabin vaccine is administered orally, is relatively inexpensive, and confers strong intestinal immunity. However, because this vaccine consists of live virus, in rare cases (~1 in 2.7 million cases), vaccination can lead to vaccine-associated paralytic poliomyelitis in immunocompromised individuals (1, 113). In addition, the vaccine strain can mutate back to the neurovirulent form, leading to vaccine-derived poliovirus that can circulate in human populations, causing sporadic outbreaks of poliomyelitis in unvaccinated individuals (98).

## **1.5 Antiviral Compounds**

Although effective vaccines have been developed for humans for polio and hepatitis A, other picornaviruses have no vaccines available; nor is it feasible to develop a vaccine for viruses such as the rhinoviruses, which have so many serotypes that vaccine development would be all but impossible. In addition, the reversion of vaccine-derived poliovirus shed by people immunized with OPV to the pathogenic phenotype make the development of antiviral compounds for poliovirus an important safety measure for

controlling disease outbreaks (3, 113). The discovery and development of broad-spectrum antiviral compounds is a promising alternative to vaccine development.

The conventional targets for antiviral compounds are viral proteins. In general, antiviral compounds that specifically target viral proteins are less toxic because they do not interfere with normal cellular processes. One downside of inhibitors that target viral proteins is the high likelihood of emergence of resistant viruses, especially for small RNA viruses due to their high mutation rate. Poliovirus, for example, readily generates resistance to compounds targeting viral proteins (118), imposing significant restrictions on the repertoire of targets for drug development. Resistance is well documented to the prospective anti-picornavirus compounds pleconaril and V-073, restricting their use to cases of life-threatening infections and public health emergencies (136). Another disadvantage of antiviral compounds that target viral proteins is that these inhibitors are typically specific to a single group of closely-related viruses.

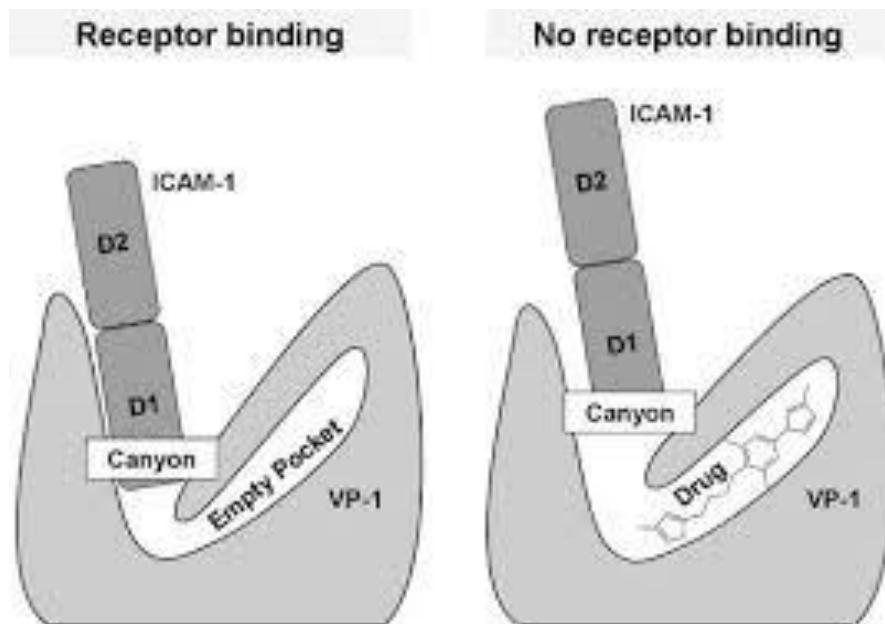
An alternative option is the development of antiviral compounds that target host proteins required for the virus life cycle. These inhibitors would affect a broad range of viruses that share the use of the host protein in their life cycle. In addition, it is hypothesized that resistance would be less likely to occur as host proteins remain stable during the course of infection. Currently, the emergence of resistant mutants is accepted as an unavoidable pitfall; however, it is possible to find drugs that will not easily induce resistant virus. Inhibition of at least one cellular protein, chaperon Hsp-90 involved in the folding of the poliovirus capsid proteins, was shown to be refractory to the emergence of resistant mutants *in vitro* and *in vivo* (57). A disadvantage of antiviral compounds that target host proteins is the increased likelihood of inducing host toxicity.

### 1.5.1 Antiviral Compounds Targeting Attachment and Entry

Antiviral compounds that target different steps of the virus life cycle have been identified. The first step of the viral life cycle is attachment and entry. Many picornaviruses utilize receptors belonging to the immunoglobulin superfamily. These include the poliovirus receptor (PVR or CD155) for poliovirus, the intercellular adhesion molecule-1 (ICAM-1) for major-group rhinoviruses, and the coxsackie-adenovirus receptor (CAR) for group B coxsackieviruses. For members of the *Enterovirus* genus, the four capsid proteins (VP1, VP2, VP3, and VP4) arrange to form grooves or “canyons” surrounding the vertices at each five-fold axis of symmetry (127). VP1-3 are exposed to the environment, while VP4, a small protein covalently linked to a myristic acid at its N-terminus, is internally located (9). Within each canyon lies a pocket of conserved hydrophobic residues in which a small lipid molecule (the canyon factor) resides. The cellular receptors bind to these grooves, releasing the lipid molecule from the pocket (8). The resulting conformational change is thought to initiate the uncoating process.

The WIN compounds, developed by Sterling-Winthrop, occupy the hydrophobic pocket preventing virus-receptor interaction and virion uncoating (Figure 1-2). Studies on the structure of the major-group rhinovirus HRV-14 capsid bound to WIN compounds show that the compounds bind deep in the hydrophobic pocket, inducing a large conformational change that both prevents virus-receptor interaction and stabilizes the virion to block uncoating of the capsid (40). In contrast, binding of WIN compounds to minor-group rhinovirus HRV-1A does not produce as large of a conformational change due to the differing topology of the virus (86). Accordingly, the receptor is able to attach

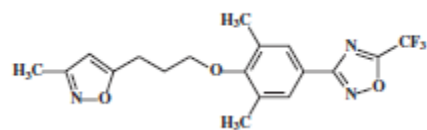
to the virus; however, the uncoating process is still blocked due to the loss of virion flexibility (62, 108). The same phenomenon is observed with WIN compound-bound poliovirus (62).



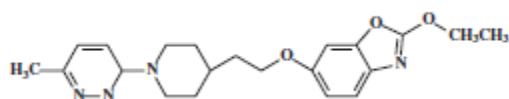
**Figure 1-2. Mechanism of action of WIN compounds blocking virus-receptor binding.** **Left:** Diagram of the ICAM-1 receptor successfully binding to the virus capsid. **Right:** Binding of WIN compounds deep in the hydrophobic pocket produces a conformational change that prevents virus-receptor interactions. Figure taken from De Palma, AM (8).

The most recognized WIN compound is WIN63843, more commonly known as pleconaril (Figure 1-3). Preliminary studies of pleconaril demonstrated its rapid plasma clearance and broad-spectrum activity within the *Enterovirus* genus. In a phase III clinical study, patients who received pleconaril reported reduced duration and severity of symptoms of the common cold compared to patients who received a placebo. Up until 2002, pleconaril was distributed to patients on a compassionate-basis. In 2003, the FDA rejected pleconaril as a treatment of rhinovirus infection, as they were unsure about the drug's safety profile (8). Currently, pleconaril is being developed as a nasal spray by Merck Sharp & Dohme Corp. for the treatment of the common cold and asthma exacerbation upon rhinovirus infection (8). Results from a phase II clinical study are not yet available.

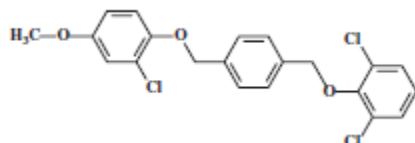
Other capsid-binding inhibitors undergoing clinical trials include BTA-798 and V-073 (Figure 1-3). BTA-798 is a broad-spectrum anti-rhinovirus compound that is 10-fold more potent than pleconaril *in vitro*, and is currently being developed by Biota as a treatment for high-risk rhinovirus-infected patients (136). V-073 is a broad-spectrum anti-poliovirus compound being developed by ViroDefense.



**Pleconaril (WIN63843)**



**BTA-798**



**V-073**

**Figure 1-3. Structures of capsid-binding inhibitors pleconaril, BTA-798, and V-073.**

### 1.5.2 Antiviral Compounds Targeting Viral Replication Proteins

A variety of compounds targeting steps of enterovirus replication have been identified, including inhibitors of proteases 2A and 3C/3CD, the 2C protein, and the RNA-dependent RNA polymerase 3D.

The enterovirus proteases are responsible for cleaving the polyprotein upon translation into precursor and individual proteins that are responsible for viral replication. The 2A protease cleaves at the Tyr-Gly pair between the P1 region that encodes the structural proteins and the P2P3 region that encodes the nonstructural proteins. An alternative 2A cleavage site in the P3 region produces the proteins 3C' and 3CD', which serve an unknown function, but are thought to be nonessential for replication (91). The 3CD protease is primarily responsible for cleavage of the individual capsid proteins, while the 3C protease preferentially targets Gln-Gly bonds within the P2P3 polyprotein precursor (76). In addition to viral proteins, the enterovirus proteases also cleave several host proteins. For example, poliovirus, CVB3, and HRV hijack the host translation machinery via 2A-mediated cleavage of the translation initiation factor eIF-4G (92).

Peptides containing a Gln-Gly bond to serve as competitive inhibitors of the 3C protease have been synthesized and assessed for antiviral activity. One of these compounds, rupintrivir (also known as AG7088), was designed to irreversibly bind to the HRV 3C protease (Figure 1-4). This compound shows broad-spectrum antiviral activity against a broad range of HRV serotypes and EV-71 (25, 149). HRV variants repeatedly passaged *in vitro* accumulated multiple mutations in 3C that only minimally reduced susceptibility of the drug (26). Intranasally-administered rupintrivir was well-tolerated in



healthy individuals and a substantial amount was recovered by nasal wash nine hours post-administration (69). In a phase II double-blind study, rupintrivir nasal spray treatment reduced rhinovirus titers and the proportion of volunteers that were positive for rhinovirus, but did not reduce the frequency of the clinical cold (64). Development was ceased, because rupintrivir did not effectively inhibit rhinovirus in phase II clinical trials studying the effect of the drug upon natural infections (115).

The enterovirus 2A protease is another potential drug target, although no anti-2A protease drugs are currently undergoing clinical trials. Several 2A-targeting drugs have been identified, including the thiol alkylating agents iodoacetamide and N-ethylmaleimide, which bind irreversibly to the cysteine within the 2A protease active site and effectively inhibit enzymatic activity by 79% and 84%, respectively (Figure 1-4) (84). In addition, the elastase-specific inhibitors methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MCPK) and elastatinal (Figure 1-4) mimic the protease cleavage site and have been shown to inhibit the 2A protease activity of both poliovirus and HRV-14 (104). A six-amino acid peptide (LVLQTM) that bound to the HRV 2A protease and inhibited replication of HRV in both A549 cells and mice was also shown to inhibit EV-71 2A protease activity and virus replication, however the efficacy of the drug in humans has not been demonstrated (50).

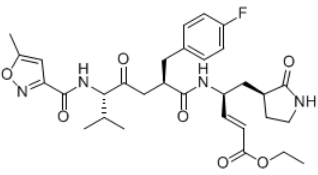
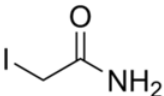
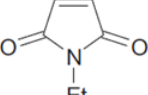
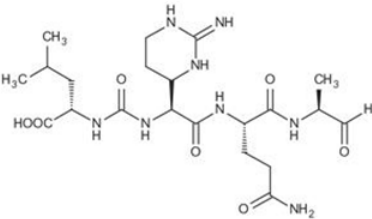
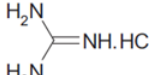
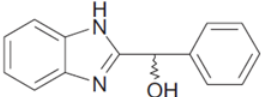
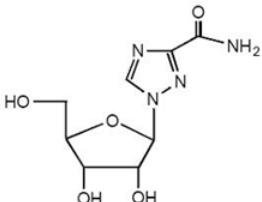
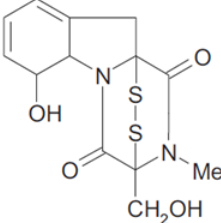
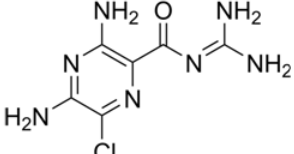
The compound guanidine HCl (GuHCl), which has been shown to inhibit the replication of poliovirus, coxsackievirus A16, echovirus, and FMDV, results in a defect of negative-strand synthesis, but not positive-strand synthesis (Figure 1-4) (30, 35, 66, 80). Poliovirus and FMDV resistant to GuHCl contain mutations that map to the 2C protein, implicating that GuHCl targets a 2C-dependent function (122). Although its

function is not well understood, the 2C protein is important for virus replication. This protein contains membrane-binding and RNA-binding domains, has nucleotide triphosphatase activity, and has been demonstrated to function as an RNA helicase (146). GuHCl is thought to inhibit the ATPase activity of poliovirus 2C (118). However, this drug would not be a good candidate for antiviral therapy, due to its high toxicity (118). Another compound that has been determined to act upon 2C due to mutation profiles of resistant viruses is 2-( $\alpha$ -hydroxybenzyl)-benzimidazole (HBB) (Figure 1-4). This drug inhibits replication of poliovirus, group B coxsackieviruses, some group A coxsackieviruses, and some echoviruses (80).

Inhibitors of the 3D polymerase can be divided into two types: nucleoside analogs and non-nucleoside analogs. Nucleoside analogs imitate the normal substrate of the polymerase, often, but not always, resulting in chain termination after their incorporation. One such example is the compound ribavirin (Figure 1-4), which contains a purine-like moiety attached to a ribose sugar and is currently approved to treat HCV infection in combination with interferon treatment (103). Ribavirin treatment has been shown to increase the mutation frequency of both poliovirus RNA and HCV replicon replication, breaking the error threshold tolerated by RNA viruses. This phenomenon is termed “error catastrophe” (36). RNA viruses normally maintain a delicate balance between viral fitness and the high mutation rate due to the error-prone RNA-dependent RNA polymerase. Breaking this threshold by increasing the viral mutation rate leads to a decline in viral fitness. A single amino acid change in poliovirus 3D confers resistance to this compound by increasing the fidelity of the polymerase (117).

Examples of non-nucleoside inhibitors include the compounds gliotoxin and amiloride (Figure 1-4). Gliotoxin is a fungal metabolite that has been shown to inhibit both plus- and minus-strand synthesis of poliovirus RNA. The *in vitro* activity of purified 3D polymerase was inhibited by addition of gliotoxin, suggesting that 3D is the target of this compound (126). The reduced form of gliotoxin was not inhibitory to viral RNA synthesis, in contrast to the active, oxidized form that contains a disulfide bridge. The proposed mechanism of action hypothesizes that the disulfide bridge of the compound interacts with a sulfhydryl group on the 3D polymerase, inhibiting viral RNA synthesis (139).

Amiloride is an ion-channel inhibitor that also has anti-enteroviral activity. The drug was shown to inhibit initiation of minus-strand synthesis, but not plus-strand synthesis of CVB3 in a cell-free system. Further studies showed that amiloride inhibited uridylylation of VPg, a process that is required for both plus- and minus-strand synthesis. Crystal structure analysis of the CVB3 3D polymerase in complex with VPg proposed a VPg-binding site on the back of the polymerase (61). Using this model and computational molecular docking analysis, amiloride was hypothesized to compete with VPg to bind to the VPg-binding site on the back of the 3D polymerase (111).

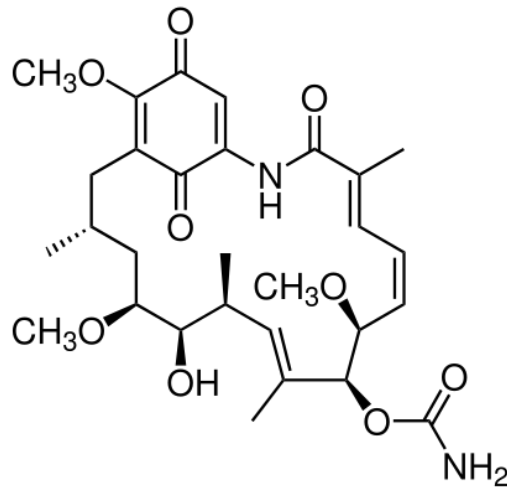
Drug	Target
	<p><b>Rupintrivir (AG7088)</b></p> <p>3C protease- many HRV serotypes, EV71</p>
	<p><b>Iodoacetamide</b></p> <p>2A protease- poliovirus</p>
	<p><b>N-ethylmaleimide</b></p> <p>2A protease- poliovirus</p>
	<p><b>Elastatinal</b></p> <p>2A protease- poliovirus, HRV-14</p>
	<p><b>Guanidine HCl</b></p> <p>2C- poliovirus, several coxsackieviruses, echoviruses, FMDV</p>
	<p><b>HBB</b></p> <p>2C- poliovirus, several coxsackievirus B serotypes, some coxsackievirus A serotypes, some echovirus serotypes</p>
	<p><b>Ribavirin</b></p> <p>3D- many RNA viruses</p>
	<p><b>Gliotoxin</b></p> <p>3D- poliovirus</p>
	<p><b>Amiloride</b></p> <p>3D- coxsackievirus B3</p>

**Figure 1-4. Structures of antiviral compounds that target viral nonstructural proteins.** Antiviral drugs can inhibit the function of the viral nonstructural proteins, including the 3C protease, the 2A protease, 2C, and the 3D polymerase.

### **1.5.3 Antiviral Compounds Targeting Cellular Proteins: Capsid Assembly**

An alternative option is the development of antiviral compounds targeting host factors important for viral replication. Compounds targeting several cellular proteins involved in the picornavirus replication cycle have been identified, although none have been approved for clinical use. Geldanamycin is a specific inhibitor targeting chaperone Hsp-90 (Figure 1-5). The enterovirus precursor P1 interacts with Hsp-90, along with Hsp-70 and other cofactors, to fold in the correct conformation for processing into individual capsid proteins (57). Thus, inhibition of Hsp-90 blocks correct folding and capsid maturation. Geldanamycin exhibits antiviral activity against many different viruses *in vitro*, including hepadnaviruses, vaccinia virus, herpes simplex I virus, and hepatitis C virus (28, 70, 71, 112), although it is unclear for some of these viruses whether antiviral activity is due to inhibition of proper folding of viral proteins or other cellular proteins important for replication (57). Geldanamycin is a potent inhibitor of enteroviruses with low toxicity *in vivo*; this is likely due to the redundancy of chaperone activity in the host (136). Interestingly, poliovirus resistant to geldanamycin has not been reported, suggesting that poliovirus has evolved to strictly and specifically utilize Hsp-90 during the capsid maturation process (57).

Another inhibitor of capsid assembly is L-buthionine-sulfoximine (BSO), an inhibitor of glutathione synthesis. Glutathione is an intracellular reducing agent that is involved in regulating oxidative stress. Pre-treatment of cells with BSO reduced the viral titer of CVB3, CVB4, and HRV14, although the level of viral RNA replication and protein translation was not affected. Fractionation of the cell lysate revealed the presence of empty viral capsids, suggesting that glutathione or a glutathione-dependent process is involved in the formation of infectious viral particles (132). Studies with the inhibitor TP219, a free glutathione scavenger, demonstrated a direct interaction between glutathione and capsid protein VP1 (137). Virus resistant to these two inhibitors contained mutations that mapped to the VP1 and VP3 proteins at the capsid protomer interfaces (95, 137). The mutant viruses were more heat-resistant, suggesting that the interaction between glutathione and the capsid acts to stabilize the virus during the formation of mature viral particles (95, 137).



**Geldanamycin**

**Figure 1-5. Structure of geldanamycin.** Geldanamycin targets the host chaperone Hsp-90, which plays a role in folding the P1 polyprotein into the proper conformation for processing into the individual capsid proteins.

#### 1.5.4 Antiviral Compounds Targeting Cellular Proteins: Replication

The compound enviroxime, which targets phosphatidylinositol 4-kinase III  $\beta$  (PI4KIII $\beta$ ), is highly effective against enteroviruses and rhinoviruses. PI4KIII $\beta$  phosphorylates the phosphatidylinositol lipid family at the D4-position of the inositol ring to produce phosphatidylinositol-4-phosphate (PI4P) (43). Under physiological conditions, PI4KIII $\beta$  is recruited to the Golgi where it catalyzes the production of PI4P. PI4P lipids play important roles in cellular secretion and the recruitment of effector proteins, such as lipid transfer proteins (39). Upon enterovirus infection, PI4KIII $\beta$  is recruited to the sites of viral replication by the 3A protein, where it catalyzes the production of a PI4P-lipid enriched environment (33). The PI4P-enriched environment is important to enterovirus replication, because inhibition of PI4KIII $\beta$  results in inhibition of viral replication (68, 142). The PI4P lipid was proposed to bind the viral 3D polymerase to form the replication complex for viral RNA synthesis, although no data support this hypothesis *in vivo* (68).

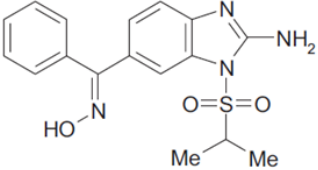
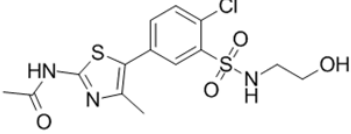
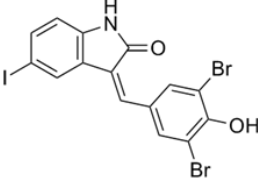
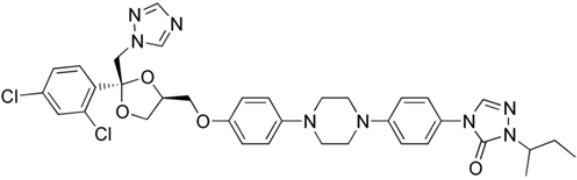
In several clinical trials, the effect of enviroxime on rhinovirus-infected volunteers has been variable. For some cases, enviroxime significantly reduced the duration and shedding of rhinovirus, while in others the reduction in overall clinical score and virus titer in nasal washes was not statistically significant (120, 121). Additionally, volunteers reported gastrointestinal side effects such as vomiting and abdominal pain with oral administration of the drug, likely due to the compound's poor solubility in water (120); however, these side effects were eliminated upon treatment with aerosolized enviroxime-containing liposomes (58).



Viruses that are resistant to enviroxime contain a G5318A mutation in the 3A protein (Ala70Thr). This mutation also confers resistance to a diverse group of inhibitors of picornavirus replication lacking any obvious structural similarity, thus designated “enviroxime-like compounds”. Enviroxime-like compounds have been divided into two groups of inhibitors (Figure 1-6). Major enviroxime-like compounds have been shown to inhibit PI4KIII $\beta$ , while minor enviroxime-like compounds show little to no inhibitory effect on PI4KIII $\beta$  and instead induce relocation of oxysterol binding protein (OSBP) to the Golgi apparatus (5). Minor enviroxime-like compounds, but not major enviroxime-like compounds, have also been shown to inhibit EMCV and HCV replication, suggesting the importance of OSBP in the life cycle of distantly-related positive-strand RNA viruses (5, 6, 45, 74).

OSBP binds oxysterol and is involved in maintaining cholesterol homeostasis (5, 97, 107). OSBP contains a pleckstrin homology (PH) domain that is responsible for its targeting to the Golgi; this domain has also been shown to be necessary for the binding of OSBP to PI4P (138). OSBP also has a sterol-binding domain, and several members of the OSBP family in yeast have been shown to be involved in extracting and delivering cholesterol to and from membranes (124). OSBP has been shown to act as a sterol/PI4P exchanger in which sterol transfer to the Golgi is coupled with PI4P transfer to the ER where PI4P is hydrolyzed by an integral ER membrane protein (27). Enterovirus replication organelles are enriched in PI4P and free cholesterol, both of which are important for enterovirus replication (114). Depletion of free cholesterol with the inhibitor methyl- $\beta$ -cyclodextrin (M $\beta$ CD) has been shown to inhibit poliovirus and CVB3 replication, while restoration of free cholesterol rescues replication (114). The cholesterol

content of membranes influences membrane fluidity and curvature, properties which may be important for a functioning viral replication complex.

Compound	Target
	<p><b>Enviroxime</b></p> <p><b>PI4KIII<math>\beta</math></b></p>
	<p><b>PIK93</b> (Major enviroxime-like compound)</p> <p><b>PI4KIII<math>\beta</math></b></p>
	<p><b>GW5074</b> (Major enviroxime-like compound)</p> <p><b>PI4KIII<math>\beta</math></b></p>
	<p><b>Itraconazole</b> (Minor enviroxime-like compound)</p> <p><b>OSBP</b></p>

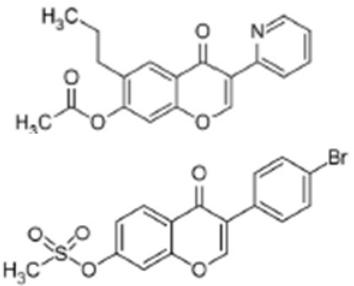
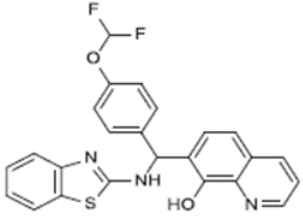
**Figure 1-6. Structures of enviroxime and some major- and minor-group enviroxime-like compounds.**

### 1.5.5 Antiviral Compounds: Induction of the Innate Immune Response

The innate antiviral response is triggered when pathogen-associated molecular patterns (PAMPs), like dsRNA, are recognized by pattern recognition receptors (PRRs), such as RIG-I and MDA5. Upon PAMP recognition, RIG-I or MDA-5 undergo a conformational change and recruit proteins such as the adapter molecule MAVS, which is associated with the outer mitochondrial membrane. This signaling cascade results in the phosphorylation, dimerization, and translocation of transcription factor IRF-3 to the nucleus where IRF-3 binds to its promoter to initiate transcription of interferon and other antiviral genes. Many viruses attempt to shut down host innate immune signaling to promote virus replication and dissemination through the host (78). The hepatitis C virus (HCV) protease NS3-4A has been shown to cleave the adaptor molecule MAVS to suppress RIG-I-inducing signaling, and poliovirus induces cleavage of RIG-I (10, 12, 93). Hepatitis A virus prevents IRF-3 phosphorylation by inhibiting activation of kinases, IKK $\epsilon$  and TBK1 (53), and MDA-5 was shown to degrade in poliovirus-infected cells (11). An appropriate innate immune response is important for controlling RNA virus infection; therefore agonists of innate immune signaling pathways represent potential broad-spectrum targets for antiviral therapeutics.

A series of isoflavone compounds (KIN100 and KIN101) have been identified that act as agonists of the innate immune response by activating IRF-3 (Figure 1-7). These compounds inhibited HCV (KIN100 only) and influenza virus replication (KIN100 and KIN101) (17). The compound KIN101 was able to induce IRF-3 translocation in the presence of HCV NS3-4A, signifying that KIN101 is able to induce the innate immune response despite viral countermeasures (17). An additional family of hydroxyquinoline

compounds (KIN1400 and analogs) (Figure 1-7) has been identified that have broad-spectrum antiviral activity against members of the *Flaviviridae* family (West Nile virus, Dengue virus, HCV), *Orthomyxoviridae* family (influenza A virus), *Paramyxoviridae* family (respiratory syncytial virus), *Filoviridae* family (Ebola virus), and *Arenaviridae* family (Lassa virus) (116). The broad-spectrum activity suggests that these compounds may also be effective against the *Picornaviridae* family. The compound KIN1400 induces the expression of innate immune genes in an IRF-3- and MAVS-dependent manner (116). Importantly, treatment with this family of compounds resulted in low or undetectable amounts of IFN and induced gene expression patterns differing from IFN treatment, which is associated with many negative side-effects (55, 116). These families of compounds represent promising candidates for the development of broad-spectrum therapeutics.

Drug	Target
 <p>The top structure is KIN100, a benzoxazinone derivative with a methyl group, an acetate group, and a pyridine ring. The bottom structure is KIN101, a benzoxazinone derivative with a methylsulfonate group and a 4-bromophenyl group.</p>	<p>HCV</p> <p>HCV, influenza A virus</p>
 <p>The structure of KIN1400 is a complex molecule featuring a benzothiazole ring system, a hydroxyl group, and a 4-(difluoromethoxy)phenyl group.</p>	<p>HCV, West Nile virus, Dengue virus, influenza A virus, respiratory syncytial virus, Ebola virus, Lassa virus</p>

**Figure 1-7. Structures and targets of innate immune response agonists.**

## Chapter 2: Methods

**Cells and Viruses.** HeLa cells were grown in high glucose Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS). Poliovirus type 1 Mahoney, Coxsackievirus B3, and encephalomyocarditis virus were propagated in HeLa cells. Titers were determined by standard plaque assays.

**Kinase inhibitors.** Two libraries of cell-permeable kinase inhibitors were purchased from Calbiochem. Wortmannin was from Sigma-Aldrich; LY294002 was from Cell Signaling Technology. BF738735 and TTP-8307 were gifts from Prof. Frank van Kuppeveld (University of Utrecht, The Netherlands). Compounds were dissolved in DMSO to a stock concentration of 10 mM.

**Plasmids.** The pXpA-RenR plasmid coding for the polio replicon in which the P1 structural region is replaced with a gene encoding for the *Renilla* luciferase was described previously (19). The plasmid coding for a similar Coxsackie B3 replicon was a kind gift from Prof. Frank van Kuppeveld (University of Utrecht, The Netherlands). The pXpA-P2P3 plasmid contains poliovirus cDNA coding for nonstructural proteins under the control of a T7 RNA polymerase promoter. pXpA plasmids contain the ribozyme sequence that generates the authentic 5'UU sequence of poliovirus RNA upon T7 RNA polymerase transcription (65). The pPV $\Delta$ P1 plasmid was a gift from Dr. Natalya Teterina (NIH). It contains poliovirus cDNA coding for the nonstructural proteins (P2P3) under the control of a T7 RNA polymerase promoter but does not contain the ribozyme sequence resulting in 5'GG sequence on the RNA generated by T7 RNA polymerase. Polio RNAs were generated using MEGAscript T7 transcription kit (Ambion) and

purified as described elsewhere (21). The pTM-2Apol poly plasmid, which contains a T7 promoter, was a gift from Dr. Natalya Teterina (NIH).

**Polio replicon assays.** Polio RNA replication assays were performed essentially as described previously (19). HeLa cells were grown on a 96-well plate overnight. Transfection mix with purified *Renilla* luciferase poliovirus replicon RNA (10 ng RNA/well) was prepared with mRNA TransIt transfection reagent (Mirus) according to the manufacturer's protocol. After 5 min of incubation at room temperature, the transfection mix was combined with normal cell growth medium supplemented with cell-permeable Endu-Ren *Renilla* luciferase substrate (Promega). For the initial library screening experiments, medium on the 96 -well plate was replaced with this master mix (75  $\mu$ l/well) and every inhibitor was added in a single well of a 96-well plate (1  $\mu$ l of 75x solution in DMSO). DMSO (positive control) and 10  $\mu$ M of BFA (negative control) were added into 8 wells (one column on the 96 plate each), thus also providing a measurement of the reproducibility of the signals from individual wells. After addition of compounds, the plate was incubated on a shaker for 2 min. For characterization of the selected inhibitors, the transfection master mix was divided into aliquots sufficient for transfection of the required number of wells (at least 16 wells per sample), and the inhibitors (corresponding amount of DMSO in controls) were added directly to the master mix before transfection. After the transfection, medium was added to the cells, and the plates were sealed with an optical clear film and incubated directly in the M5 (Molecular Devices) or Infinite M1000 (TECAN) plate readers equipped with heated cameras at 37  $^{\circ}$ C. The luciferase signal was measured every hour for 16 hours. Results were plotted using GraphPad Prism statistical software with error bars showing standard deviation.



Total replication was calculated based on the integrated luciferase signal (area under curve).

**In vitro translation and replication assays.** HeLa S10 cell extracts were prepared and translation-replication reactions were performed essentially as previously described (54). Translation mixtures contained 2 mM guanidine-HCl to prevent replication. An aliquot of translation reaction was mixed with 1  $\mu$ l of EasyTag Express Protein Labeling Mix [ $^{35}$ S] (Perkin Elmer) to detect newly-synthesized proteins. Translation reactions were incubated at 34 °C for 3.5 hours and then centrifuged at 15,000 rpm to collect the membrane-associated poliovirus replication complexes. The pellet was resuspended in a replication buffer containing [ $^{32}$ P]- labeled  $\alpha$ CTP (Perkin Elmer), without guanidine-HCl. Replication reactions were carried out for 1 h at 37 °C. Total RNA was isolated and purified with the RNAeasy Mini Kit (Qiagen). Proteins from  $^{35}$ S-labeled translation reaction were resolved by SDS-PAGE gel. RNA was denatured for 45 min in a glyoxal buffer at 65 °C and resolved on a glyoxal-containing denaturing agarose gel. The gel was stained with ethidium bromide to assess the total RNA content, dried, and exposed to a radiographic film to reveal the newly-synthesized polio RNA. Image density quantitation was performed with ImageJ software (NIH).

**VPg uridylylation assay.** VPg uridylylation assay was performed essentially as described (135) with minor modifications. *In vitro* translation was performed as described above. Following translation, replication complexes were collected via centrifugation at 15000 rpm and resuspended in a replication buffer without guanidine-HCl containing [ $^{32}$ P]- labeled  $\alpha$ UTP (Perkin Elmer). In the negative control, 2 mM of guanidine HCl was added to the replication buffer. Replication reactions were carried out for 1 h at 37 °C.

Following replication, the replication complexes were spun down at 15000 rpm and resuspended in 1X protein sample buffer. Samples were fractionated on a polyacrylamide-Tris-Tricine gel. Image density quantitation was performed with ImageJ software (NIH).

**Cell viability assay.** Cell viability was measured with the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega).

**Antibodies.** Rabbit polyclonal anti-GBF1 antibodies were a gift from Dr. Nihal Altan-Bonnet (NIH). Rabbit polyclonal antibodies against PI4KIII $\beta$  were from Millipore. Mouse monoclonal antibodies against calnexin were from Sigma-Aldrich. Rabbit polyclonal anti-poliovirus 3D antibodies were produced by Chemicon using full length recombinant 3D as an antigen. Anti-polio 2C and anti-polio 3A mouse monoclonal antibodies were a gift from Prof. Kurt Bienz (Basel University). OSBP antibodies were a gift from Prof. Frank van Kuppeveld (University of Utrecht, The Netherlands).

**Analysis of proteins associated with poliovirus replication complexes.** *In vitro* translation was performed as described above. The membranous pellets containing replication complexes were collected by centrifugation and resuspended in 50  $\mu$ l of 1X protein sample buffer. Aliquots of 20  $\mu$ l were run on 10% or 15% SDS-PAGE gels and proteins were analyzed by Western blots.

**Solubilization of inhibitors in water using molecular containers M1 and M2.**

The ability of molecular containers M1 and M2 to increase the solubility of water-insoluble compounds A4(1), E5(1), and E7(2) was performed using the methodology described previously (94). The concentration of the A4(1), E5(1), and E7(2) compounds in the resulting solutions was determined by proton nuclear magnetic resonance

spectroscopy by comparing the integrals for protons of the inhibitors with the integral of the singlet at 8.3 ppm for 1,3,5-benzene tricarboxylic acid used as an internal standard.

**Murine model of poliomyelitis.** TgPVR21 mice expressing poliovirus receptor (81, 82) were randomized into three groups (each containing 5 males and 5 females) to be infected with poliovirus and treated with: [1] compound A4(1) solubilized using molecular container M2 dissolved in PBS, [2] PBS, and [3] empty molecular container M2 dissolved in PBS. Groups of two mice (one male, one female) were treated with the same substances, but mock-infected with the virus. Mice received the first intraperitoneal injection of A4(1) solution (10 mg/kg) (or corresponding amount of control solutions) 3 hours before intramuscular challenge with 10 PD<sub>50</sub> of type I poliovirus Mahoney. The animals received the second injection 24 hours after viral challenge (Day 2) and were not treated after that since mice treated with A4(1) showed signs of toxicity. The mice were observed for the signs of paresis/paralysis for 4 days, until all animals were paralyzed. Animal care protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of the FDA Center for Biologics Evaluation and Research.

**Serial passages.** For the first passage, a HeLa cell monolayer grown on a 6-cm plate was infected with poliovirus at 10 pfu/cell. After virus attachment, media containing 10% FBS and indicated concentrations of inhibitors were added. The samples were frozen at 6 hours post infection. After three cycles of freeze-thawing to release the intracellular virus, one-tenth of the viral material was used for subsequent passages and the rest was stored for further analysis. Every five passages, standard plaque assays were performed to determine virus titer.

**Identification of resistant mutations.** Resistant viruses were picked from individual plaques and viral RNA was isolated with the QIAamp Viral RNA Mini Kit (Qiagen) following the Spin Protocol. RNA was reverse transcribed into cDNA with the MonsterScript 1<sup>st</sup>-Strand cDNA Synthesis Kit (Illumina). Three overlapping cDNA fragments covering the entire polyprotein coding region were amplified by PCR using Phusion PCR kit (New England Biolabs) and were commercially sequenced (Genewiz Inc.). Primers for PCR and sequencing are available upon request.

**Immunofluorescence Assay.** HeLa cells were transfected with pEGFP-hOSBP, a plasmid that expresses the human OSBP gene. The following day, cells were treated with 10  $\mu$ M of itraconazole (ITZ) or E7(2). DMSO was added as the positive control. Cells were imaged at 1 hour post-treatment and 8 hours post-treatment. Immunofluorescence was performed by J. Strating and L. Albulescu (Utrecht, the Netherlands).

**Vaccinia-Induced Expression of Viral Proteins.** HeLa or HEK 293T cells plated on 12-well plates were transfected with pUC, pTM-2A-3', or pTM-2A-3' G5318A using Mirus TransIt 2020 Reagent. The following day, cells were infected with 10 pfu/cell of T7-expressing vaccinia virus. After one hour, media containing DMSO or 10  $\mu$ M enviroxime-like compounds was distributed to the wells. Cells were incubated at 37 °C for four hours, and then cells were lysed with mild lysis buffer and prepared for Western blotting to analyze the translation of poliovirus proteins.

## Chapter 3: Identification and Characterization of Broad-spectrum Inhibitors of Picornavirus Replication

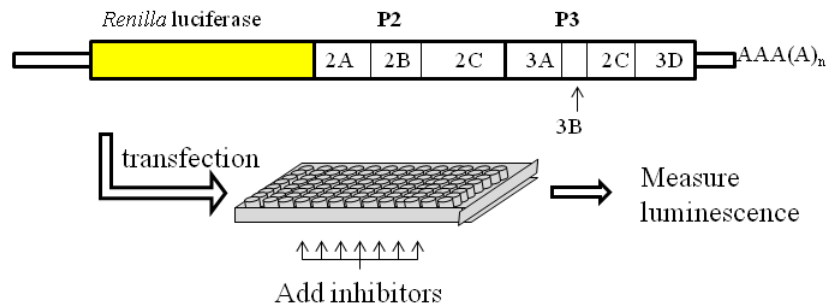
### 3.1 Introduction

The *Picornaviridae* family includes many viruses that cause disease in both humans and animals. Unfortunately, at this time, vaccines have been developed for only Hepatitis A virus and poliovirus, making the discovery of anti-picornaviral drugs an important undertaking. To this end, we screened a library of cell-permeable kinase inhibitors intending to identify novel metabolic pathways important for poliovirus replication; however, we determined that the antiviral effect of the inhibitors A4(1), E5(1), and E7(2) was not mediated by their annotated targets. All three compounds were effective against poliovirus and related enterovirus coxsackievirus B3 (CVB3), as well as against much more distantly-related encephalomyocarditis virus (EMCV). Separate assessment of individual steps in the viral replication showed that A4(1) blocked both formation and functioning of the poliovirus replication complexes. E5(1) and E7(2) prevented assembly of the functional replication complexes but did not interfere with the replication reaction. Replication complexes assembled in the presence of the compounds were fully competent in the synthesis of uridylylated VPg primer, but could not support effective RNA replication. Evaluation of A4(1) *in vivo* in a murine model of poliomyelitis showed that while this compound had significant toxicity, it delayed the development of the disease. This section describes the identification and characterization of three novel anti-picornaviral compounds.

## 3.2 Results

### **Identification of small molecule inhibitors that block enterovirus replication.**

In order to identify possible small molecule inhibitors of enterovirus replication, we screened a library of 192 cell-permeable kinase inhibitors for their effect on polio replication. For this screen, we used polio replicon RNA which expresses the polio nonstructural proteins and a gene encoding *Renilla* luciferase instead of the P1 structural proteins (Figure 3-1). This RNA is translation- and replication-competent, but without the capsid proteins, is unable to produce progeny viruses. Cells transfected with the replicon are incubated in media containing Endu-Ren, the cell-permeable *Renilla* luciferase substrate. Luciferase expression can be monitored in live cells automatically. The level of luminescence corresponds to the level of replicon replication. This system bypasses virus entry and egress, limiting the selection to inhibitors of translation and replication.



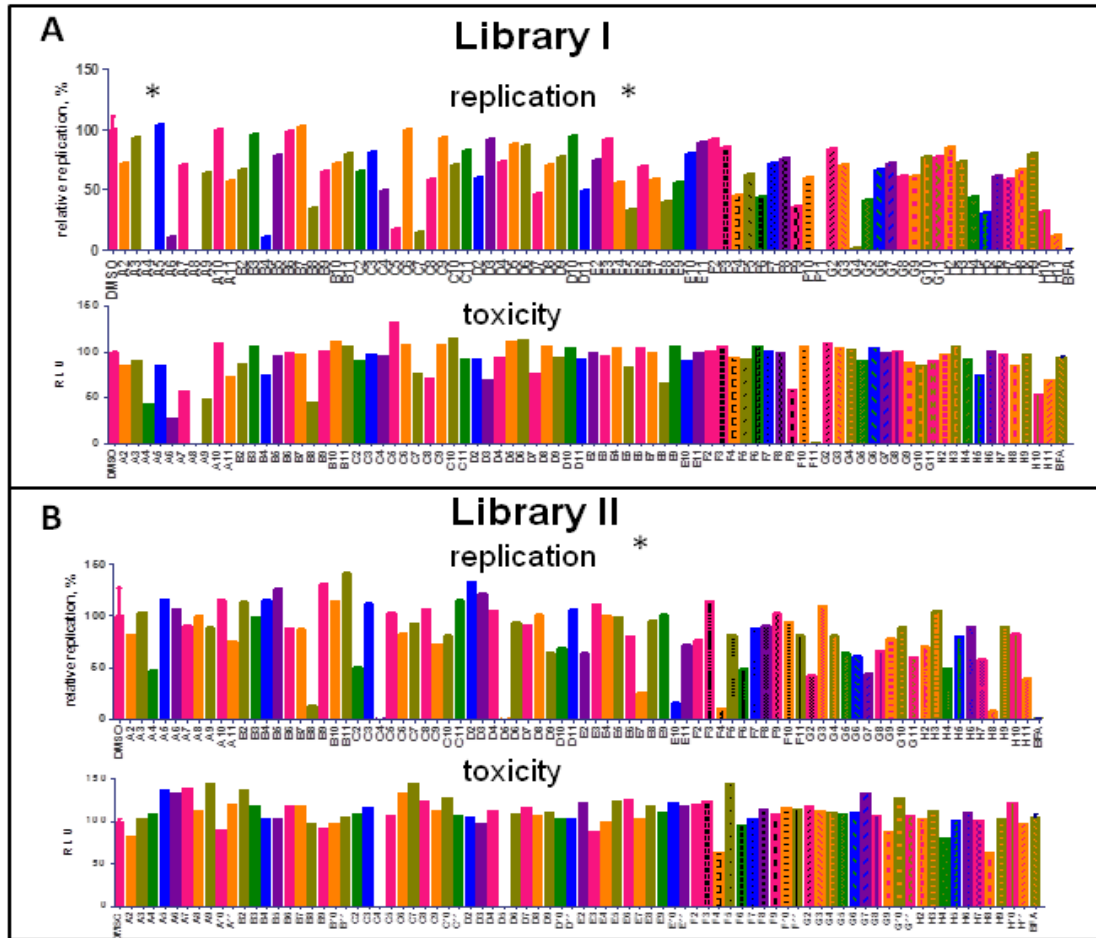
**Figure 3-1. Diagram of poliovirus *Renilla* replicon and experimental scheme.**

We transfected HeLa cells with the polio replicon in media containing Endu-Ren and the inhibitors. DMSO was added to the positive control and brefeldin A, a known inhibitor of polio replication, was added to the negative control. Luminescence was measured with an Infinite M1000 (TECAN) plate reader every hour for 16 hours to construct a replication curve. The effect of the inhibitors on cell survival was measured after replication.

In our screen, we identified several compounds that effectively inhibited poliovirus replication without significant cell toxicity. Among others, we identified Flt3 inhibitor II (Figure 3-2A, C5), which was previously reported to inhibit poliovirus replication (7), thus validating our screening approach. We repeated the screen using a similar replicon construct based on CVB3 (another enterovirus) RNA. The compounds that suppressed polio replication were equally effective against CVB3 (data not shown), highlighting the similarity of the replication process of the two viruses and the possibility of the development of broad-spectrum therapeutics.

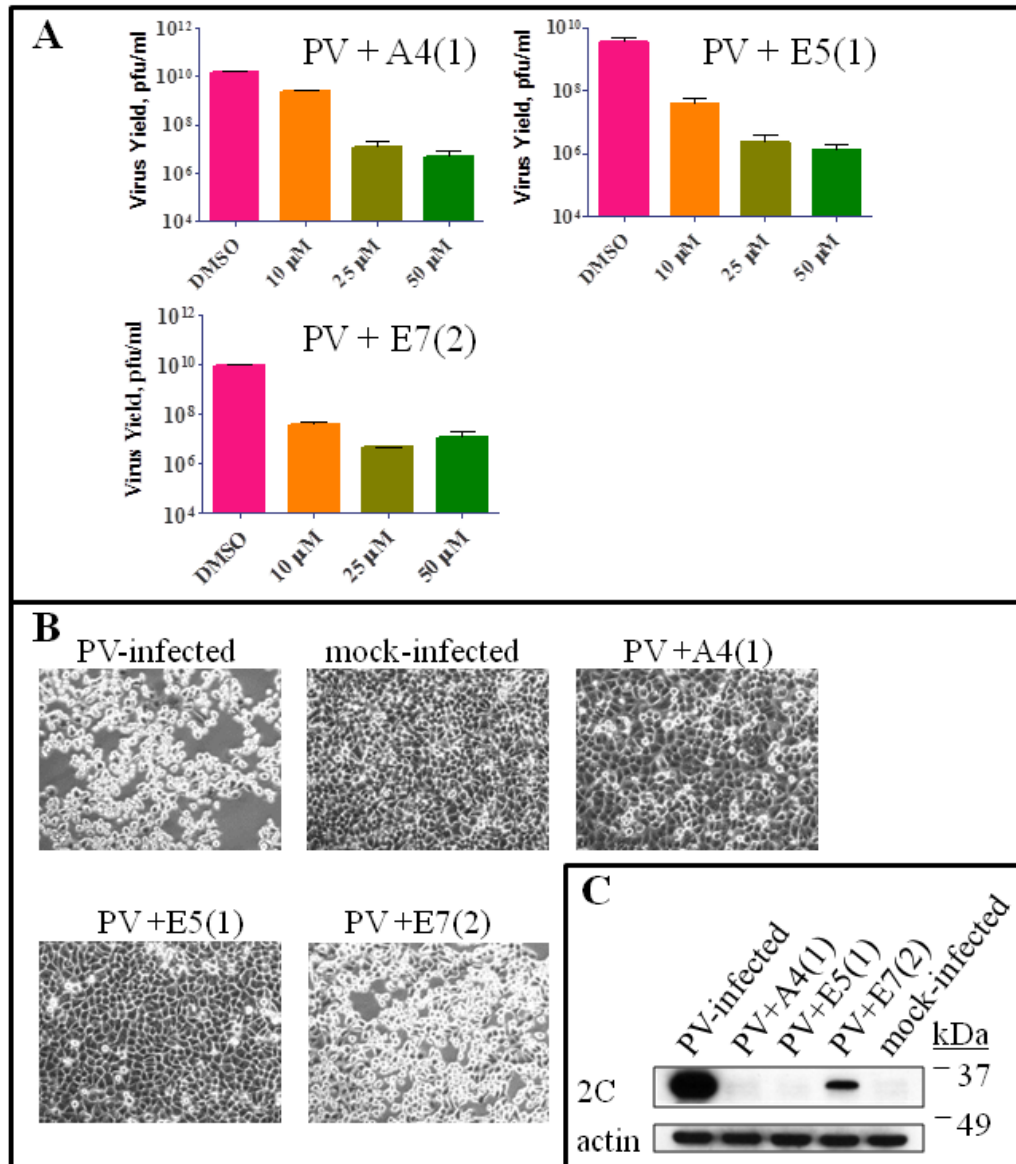
Next, we determined the effect of the compounds showing significant inhibition of replicon replication on virus yield in the conditions of natural infection. We infected HeLa cells with 10 pfu/cell of poliovirus and incubated the cells with each inhibitor after virus attachment. The cells were frozen at 6 hpi and subjected to three cycles of freeze-thawing to release the intracellular virus. The virus titer of each sample was determined with a plaque assay. Interestingly, many of the inhibitors effective in the replicon assay did not significantly affect virus propagation, suggesting that their effect was mediated by inhibition of the RNA transfection process rather than actual replication. Three compounds, Akt inhibitor IV, henceforth called A4(1), PDGF Receptor Tyrosine Kinase Inhibitor III (E5(1)), and Indirubin Derivative E804 (E7(2)), all which considerably reduced polio replicon replication in the initial screen, effectively suppressed virus infection in cell culture and did not show significant cellular toxicity (Figure 3-2). These compounds were chosen for further characterization.





**Figure 3-2. Effect of kinase inhibitors on polio replication.** Representative experiments showing poliovirus replicon replication in the presence of 10  $\mu$ M of kinase inhibitors from library I (A) and library II (B). HeLa cells grown on a 96-well plate were transfected with polio replicon RNA with a *Renilla* luciferase gene. After transfection, each inhibitor was added in one well in the 96-well plate (X-axis labels). The cells were incubated for 16 h in the presence of a cell-permeable *Renilla* luciferase substrate. Total integrated luciferase signal measured each hour over 16 hours of observation is plotted. Data is normalized to control replication in the presence of DMSO (solvent); BFA served as a negative control. Toxicity is measured in the same wells of a 96-well plate after the replication experiment is finished.

To further characterize the antiviral activity of A4(1), E5(1), and E7(2) in cell culture, we infected HeLa cells with 10 pfu/cell of poliovirus and incubated the cells with 10  $\mu$ M, 25  $\mu$ M, or 50  $\mu$ M of each inhibitor after virus attachment. The cells were frozen at 6 hours post infection and subjected to three cycles of freeze-thawing to release the intracellular virus. The virus yield in each sample was determined in a plaque assay. All three inhibitors reduced the titer of poliovirus in a dose-dependent manner (Figure 3-3A). Accordingly, poliovirus-infected cells incubated in the presence of 25  $\mu$ M of A4(1) and E5(1) did not show signs of viral CPE at least up to 8 hours post infection (Figure 3-3B). The protective effect of the compounds directly correlated with the inhibition of accumulation of the viral proteins (Figure 3-3C). In spite of the strong suppression of viral propagation, E7(2) was not as protective to infected cells compared to the other two inhibitors, although the development of CPE was still delayed compared to the control sample (Figure 3-3B). Mock-infected cells incubated with any of the inhibitors for 8 hours were essentially indistinguishable from DMSO (solvent) control (data not shown).



**Figure 3-3. A4(1), E5(1), and E7(2) inhibit poliovirus.** **A.** HeLa cells were infected with 10 pfu/cell of poliovirus and incubated in the presence of the inhibitors for 6 hpi. Virus yield was determined by plaque assay. **B.** HeLa cells infected with 10 pfu/cell of poliovirus were incubated for 8 hpi in the presence of 25 μM of the inhibitors (DMSO in control). **C.** Accumulation of the viral protein 2C in HeLa cells infected with 10 pfu/cell of poliovirus in the presence of 25 μM of the inhibitors (DMSO in control) at 4 hpi.

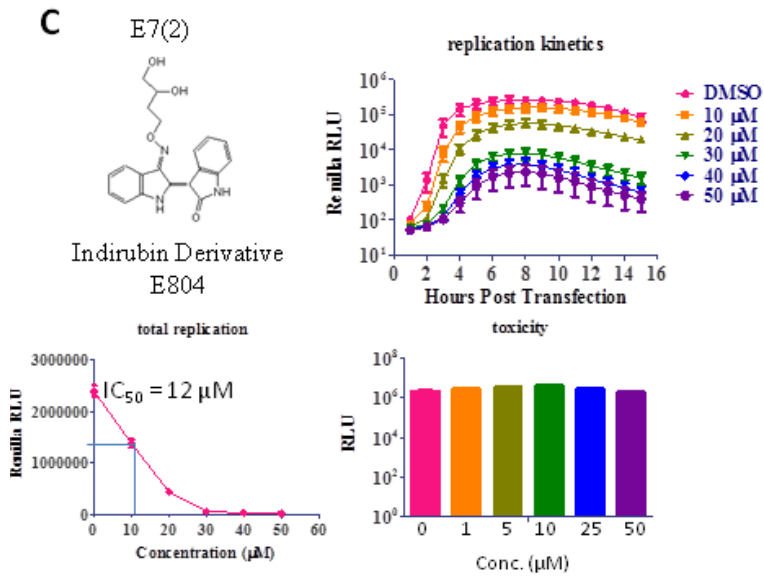
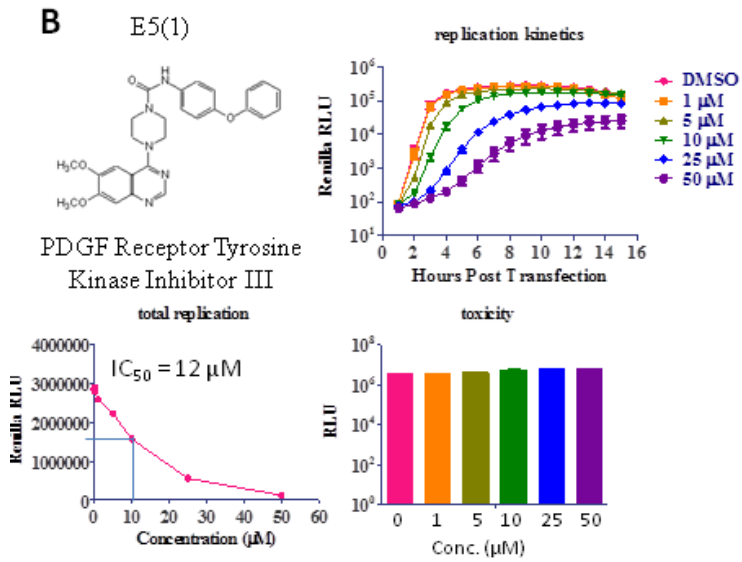
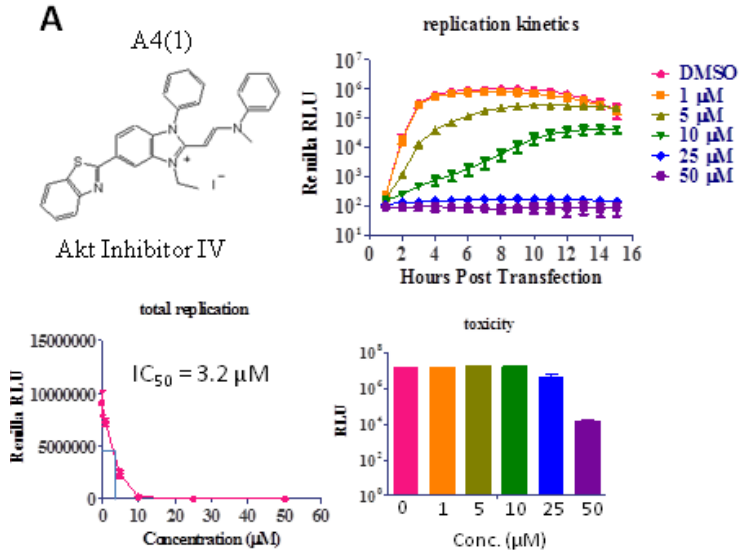
**The selected inhibitors suppress polio replication in a dose-dependent manner but their antiviral effect is likely not related to their annotated cellular targets.** To determine the IC<sub>50</sub> for the selected inhibitors, we performed the polio replicon replication assay in the presence of different concentrations of the compounds. Cellular toxicity of the treatment was assessed after the replication (~18 hours of inhibitor treatment). The most effective was A4(1) with the IC<sub>50</sub> of 3.2 μM, followed by E5(1) and E7(2), with an IC<sub>50</sub> of 12 and 16 μM respectively. We observed noticeable cytotoxicity with 50 μM treatment with A4(1), however lower concentrations of this inhibitor as well as all treatment conditions with the other two inhibitors were well-tolerated by the cells (Figure 3-4). In the presence of E7(2), especially at higher concentrations, the luciferase signal was noticeably decreasing towards the end of the experiment (Figure 3-4C) likely reflecting death of cells harboring the poliovirus replicon in the presence of this compound. Activation of the cellular apoptotic program in conditions of suppressed poliovirus infection was described previously for inhibitors of translation and replication (24).

A4(1) has been shown to inhibit PI3 kinase-dependent activation of Akt signaling by inhibiting a kinase upstream of Akt, but downstream of PI3K (77). However, Akt Inhibitor V (tricitiribine), which prevents activation of all Akt isoforms (148), and Akt inhibitor X had no significant effect on poliovirus replicon replication (Figure 3-2A (A5, A7), and data not shown). This controversy prompted us to further test the importance of the Akt pathway for polio replication. We pre-treated cells for 2 h before replicon RNA transfection with 25 μM of wortmannin and LY294002, the well-known strong inhibitors of PI3-kinase dependent Akt activation (51). The replication assay was also performed in

the presence of 25  $\mu$ M of these inhibitors. These treatment conditions had minimal effect on the efficiency of replication compared to that of A4(1) (not shown), arguing that inhibition of PI3K-Akt pathway is not detrimental to polio RNA replication, at least in cell culture, and that the antiviral activity of A4(1) is unrelated to this pathway.

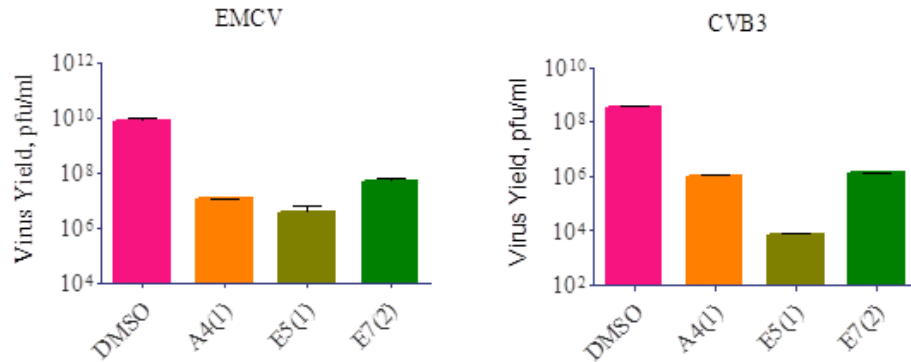
Similarly, the antiviral effects of the other two selected inhibitors seem to be not related to their annotated cellular targets. E5(1) targets PDGF receptor family of tyrosine kinases (100), however other PDGF receptor tyrosine kinase inhibitors, PDGFR inhibitors II and IV, had no significant effect on replication (Figure 3-2A, (E4 and E6), and data not shown).

E7(2) inhibits cyclin-dependent kinases as well as Src kinase activity resulting in inhibition of the Stat3 signaling pathway (105). Other inhibitors targeting Src kinase (SU6656) and cyclin-dependent kinases (kenpaullone, aminopurvalanol A, SB 218078 and SU9516) had minimal or no effect on replicon replication (Figure 3-2B, (E2, F5, A9, H4, H10), and data not shown). We conclude that the observed antiviral action of the small molecule inhibitors A4(1), E5(1), and E7(2) is due to their effect on yet unknown cellular or viral targets.



**Figure 3-4. The kinase inhibitors interfere with polio replication in a dose-dependent manner.** Molecular structure of the inhibitors tested is shown. HeLa cells grown on 96-well plates were transfected with a polio replicon RNA with a *Renilla* luciferase gene. Inhibitors were added after transfection and the cells were incubated for 16 h in the presence of a cell-permeable *Renilla* luciferase substrate. The signal was measured every hour for 16 hours post transfection. Replication kinetics and total replication (integrated *Renilla* luciferase signal) are shown. Integrated signal plot was used to determine IC<sub>50</sub> of the inhibitors. Cell toxicity was measured after the replication experiments in the same plate. **A.** Akt inhibitor IV (A4(1)). **B.** PDGF receptor tyrosine kinase inhibitor III (E5(1)). **C.** Indirubin derivative E804 (E7(2)).

**A4(1), E5(1), and E7(2) block propagation of diverse picornaviruses.** In order to determine the effect of the inhibitors on other members of the *Picornaviridae* family, we infected HeLa cells with 10 pfu/cell of CVB3 or EMCV in the presence of 25 μM of each inhibitor. EMCV belongs to the *Cardiovirus* genus in the *Picornaviridae* family and is significantly different from poliovirus and CVB3, both members of the *Enterovirus* genus. All inhibitors reduced propagation of these viruses similar to their effect on poliovirus. E7(2) reduced EMCV yield by about two logs while A4(1) and E5(1) reduced the virus yield by three logs (Figure 3-5). A4(1) and E7(2) reduced CVB3 yield by two logs and E5(1) by more than 4 logs (Figure 3-5). This shows that the three inhibitors have a broad-spectrum effect on picornavirus replication, suggesting that they target replication components conserved even among distantly related viruses.



	EMCV		CVB3	
	Average Titer (pfu/ml)	Fold Change (inhibitor/control)	Average Titer (pfu/ml)	Fold Change (inhibitor/control)
<b>DMSO</b>	7.30E+09	1	3.20E+08	1
<b>A4(1)</b>	1.14E+07	1.56E-03	9.75E+05	3.05E-03
<b>E5(1)</b>	3.70E+06	5.07E-04	6.70E+03	2.09E-05
<b>E7(2)</b>	5.00E+07	6.85E-03	1.29E+06	4.03E-03

**Figure 3-5. A4(1), E5(1), and E7(2) inhibit diverse picornaviruses.** HeLa cells were infected with 10 pfu/cell of EMCV or CVB3 and incubated for 8 hpi in the presence of 25  $\mu$ M of the inhibitors. Virus yield was determined by plaque assay. The table denotes the average titer (pfu/ml) calculated from two dilutions, and the fold change of the average titers compared to the DMSO control.

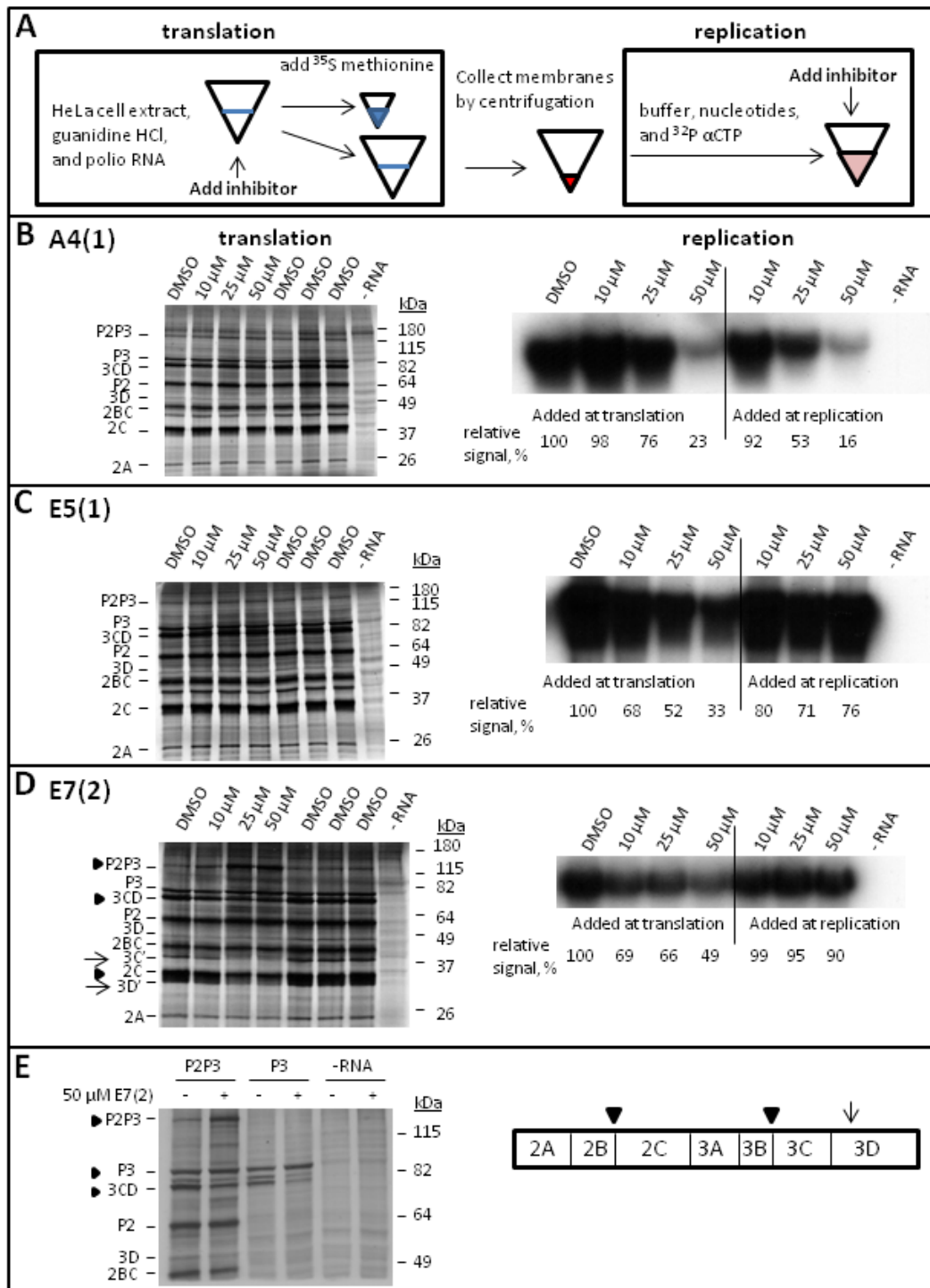


### **The inhibitors block different steps of the poliovirus replication cycle. In**

order to determine the possible mechanisms of action of the inhibitors, we analyzed their effects on replication-competent polio RNA expressing the P2-P3 region but lacking the P1 structural proteins (P2P3) using an *in vitro* system that separates translation from replication (Figure 3-6A). During the first step, the RNA is mixed with crude HeLa cell extract in the presence of a low concentration of guanidine HCl to inhibit replication. A portion of each sample is mixed with [<sup>35</sup>S]-methionine to visualize the level of viral translation. After the translation reaction, the replication complexes are spun down and the replication assay is performed by resuspending the complexes in guanidine-HCl-free replication buffer supplemented with [<sup>32</sup>P]-CTP, which becomes incorporated in newly-synthesized RNA. We tested the effects of each inhibitor on P2P3 translation and replication, with DMSO serving as the positive control.

A4(1) inhibited RNA replication in a dose-dependent manner when added either to translation or to replication reactions, but had more pronounced effect if present during the replication step, suggesting that it can directly block proper functioning of the replication complexes. This compound had no effect on the efficiency of the RNA translation or polyprotein processing (Figure 3-6B). E5(1) also had no apparent effect on polyprotein expression and processing, but if the inhibitor was present during the translation step, subsequent replication was inhibited in a dose-dependent manner. If the inhibitor was added during the replication step it had no effect on the RNA replication. Thus, E5(1) prevents formation of the functional replication complexes but does not interfere with the RNA replication once the complexes are assembled (Figure 3-6C). E7(2) was effective if added during the translation step, showing that it interferes with the

formation of the replication complexes, but not their functioning. In contrast to the other two inhibitors, E7(2) had a strong effect on polyprotein processing. Alteration of viral polyprotein processing in the presence of E7(2) may contribute to its inhibition of RNA replication. Specifically, it resulted in significant accumulation of the unprocessed P2P3 polyprotein and a decrease of 3CD, 2C, 3D', and 3C' accumulation (Figure 3-6D). Most of the viral nonstructural proteins are processed by 3C protease, however the cleavage products 3C' and 3D' are the result of the alternative cleavage of 3CD by the 2A protease. The strong effect of E7(2) on the 3C-dependent processing was confirmed in the experiment where only the P3 (3ABCD) fragment was expressed in the presence or in the absence of E7(2). Like in the case of the full length P2P3, processing of the P3 into 3BCD and 3CD was significantly suppressed in the presence of the compound (Figure 3-6E). Thus, E7(2) causes a defect in both 2A- and 3C-dependent cleavage.

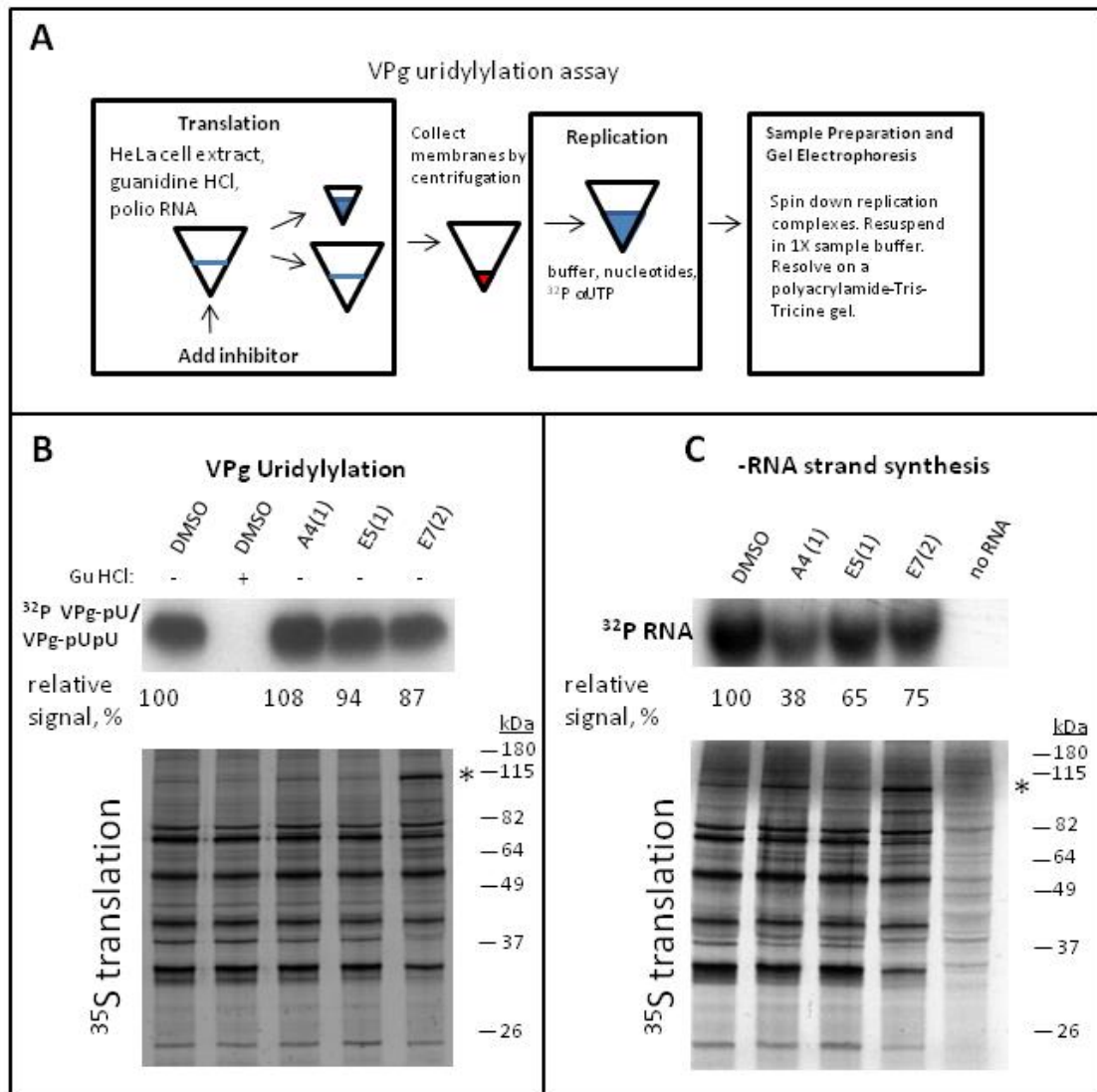


**Figure 3-6. A4(1), E5(1), and E7(2) inhibit formation and/or functioning of poliovirus replication complexes. A.** Scheme of the two step *in vitro* translation-

replication system. The inhibitors were added either during the translation step (formation of the replication complexes) or replication step (functioning of the replication complexes). DMSO (solvent) was added as a control where indicated. An aliquot of the *in vitro* translation mixture was labeled with  $^{35}\text{[S]}$ -methionine to measure viral polyprotein synthesis and processing. Replication was performed in the presence of  $^{32}\text{[P]}$ - $\alpha\text{CTP}$  to label the newly-synthesized RNA. **B.** A4(1) **C.** E5(1) **D.** E7(2). **E.** Effect of E7(2) on P3 processing. Scheme summarizes the result of these and other translation experiments performed in the presence of E7 and shows the most affected proteolytic sites. Arrowheads indicate 3C-dependent cleavage, and thin arrows indicate 2A-dependent cleavages.

**The inhibitors have a modest effect on minus-strand synthesis and do not block VPg uridylylation.** To further discern the effects of the inhibitors on replication, the ability of the P2P3 transcript to support VPg uridylylation was examined (Figure 3-7A). Uridylylated VPg acts as a primer for both positive- and negative-strand synthesis. After *in vitro* translation, the replication complexes were collected and resuspended in the presence of  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ . Guanidine HCl was added to the negative control, because it has been shown to inhibit VPg uridylylation (13). After the replication reaction, samples were run on a SDS-Tris-Tricine-polyacrylamide gel to quantify the levels of VPg uridylylation. The level of VPg uridylylation of the inhibitor-containing samples was comparable to the positive control, implying that the inhibitors do not block replication at this step in the viral life cycle (Figure 3-7B).

After translation, positive-strand RNA is transcribed into negative-strand RNA, which is used as a template for synthesis of more positive-strand RNA. We next wanted to determine if these inhibitors inhibited negative-strand synthesis. To this end, we used  $\Delta$ P1 RNA, which expresses the poliovirus nonstructural proteins and has a 5'-GG sequence instead of the typical 5'-UU sequence, so although translation and negative-strand synthesis are unaffected, it is unable to produce plus-strand RNA (15). An *in vitro* assay using  $\Delta$ P1 RNA showed that all three compounds inhibited minus-strand synthesis to varying degrees, with A4(1) exhibiting the strongest effect (Figure 3-7C).



**Figure 3-7. A4(1), E5(1), and E7(2) inhibit polio RNA replication but not VPg uridylylation.** **A.** Scheme of the VPg uridylylation experiment. **B.** The effect of 50  $\mu$ M of the inhibitors on VPg uridylylation. The inhibitors were present during RNA translation step (formation of the replication complexes). <sup>32</sup>[P]-uridylylated VPg is shown. Guanidine-HCl was added as a negative control. An aliquot of the *in vitro* translation mixture was labeled with <sup>35</sup>[S]-methionine to measure viral polyprotein synthesis and processing. **C.** The effect of 50  $\mu$ M of the inhibitors on the negative RNA

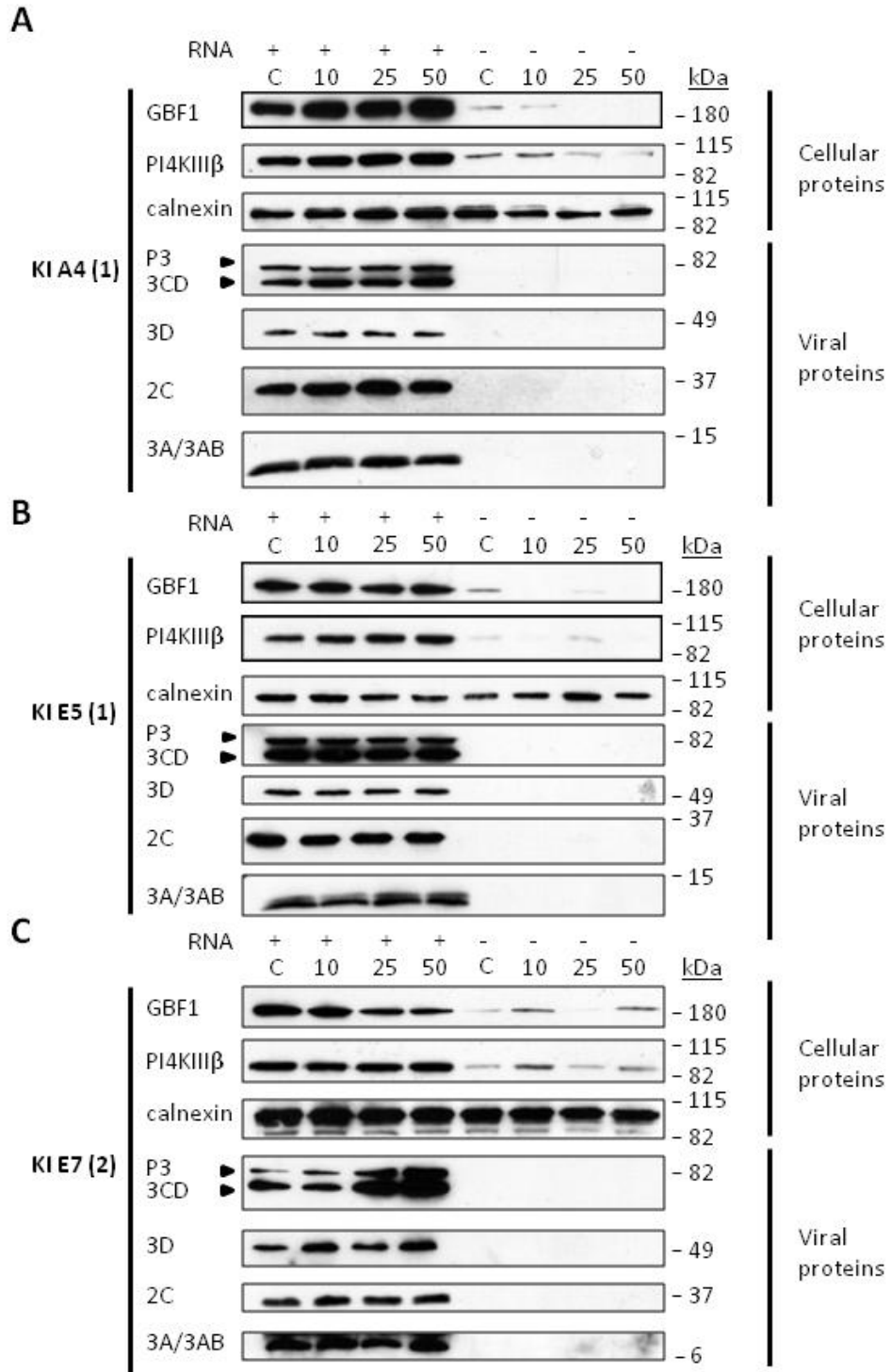
strand synthesis. The inhibitors were present during the translation step (formation of the replication complexes). Replication of the polio template with an extra 5'-GG sequence was performed in the presence of  $^{32}\text{P}$ - $\alpha\text{CTP}$  to label the newly-synthesized RNA. An aliquot of the *in vitro* translation mixture was labeled with  $^{35}\text{S}$ -methionine to measure viral polyprotein synthesis and processing. Asterisks indicate accumulation of unprocessed P2P3 polyprotein in the presence of E7(2).

**E7(2) affects binding of cellular and viral factors to membranes.** Proteins from the cellular secretory pathway are known to be recruited to the poliovirus replication complexes and to facilitate RNA replication. GBF1, a guanine nucleotide exchange factor for a small cellular GTPase Arf1, is recruited to the replication complexes of poliovirus and CVB3 through interaction with the viral protein 3A; inhibition of GBF1 with brefeldin A strongly suppresses viral replication (20, 90, 144, 145). Another cellular factor, phosphatidylinositol kinase III beta (PI4KIII $\beta$ ), was shown to be important for replication of different positive strand RNA viruses and was proposed to be recruited to the replication complexes in a GBF1-dependent manner (68). To determine if the new small molecule inhibitors of viral replication interfere with the recruitment of these important host factors to the replication complexes, we translated polio RNA in HeLa cell extract in the presence of the inhibitors, collected the membranes, and analyzed the associated proteins by Western blot. The presence of A4(1) in the translation reaction resulted in a somewhat dose-dependent increase of both GBF1 and PI4KIII $\beta$  recruitment to membranes (Figure 3-8A). E5(1) did not have any significant effect on polio-specific association of these proteins with membranes (Figure

3-8B). Interestingly, samples incubated with E7(2) showed a dose-dependent decrease of recruitment of GBF1 to the membranes, but had no effect on PI4KIII $\beta$ , supporting the recent report that recruitment of these proteins may be independent of each other (Figure 3-8C) (47). Similarly, we did not see any significant effect on the association with membranes of another cellular protein, acyl-CoA binding protein 3 (ACBD3) that was shown previously to be able to modulate replication of diverse picornaviruses including poliovirus (60, 131, 134) (data not shown). Since GBF1 recruitment to membranes was diminished in the presence of E7(2), we tested if its inhibitory effect on viral replication can be rescued by over-expression of GBF1. We monitored polio replicon replication in cells transfected with a GBF1-expressing vector in the presence of 10 and 25  $\mu$ M of E7(2). No differences were observed compared to the control samples transfected with an empty vector (data not shown), arguing that the reduced amount of GBF1 recruited to the replication complexes in the presence of this inhibitor is not a limiting factor for the viral replication.

We similarly investigated the effect of these compounds on the recruitment of the viral proteins to membranes. A4(1) and E5(1) did not significantly affect binding to membranes of the viral proteins 2C, 3A, 3D and some uncleaved precursors containing these sequences (Figure 3-8A, B). On the other hand, E7(2) induced significant increase of accumulation on the membranes of the polyprotein fragment P3 and 3CD but not of any other assessed viral proteins (Figure 3-8C). Thus, the replication complexes formed in the presence of E7(2) display aberrant composition of the cellular and viral factors, suggesting that this compound interferes with normal recruitment of some proteins to membranes.





**Figure 3-8. Recruitment of the host and viral proteins to the replication complexes in the presence of the inhibitors. Polio RNA was translated in HeLa cell**

extract in the presence of the inhibitors. The membranes were collected by centrifugation and the membrane-associated proteins were analyzed by Western blot. Calnexin is shown as a loading control. Recruitment of the cellular proteins GBF1 and PI4KIII $\beta$  and poliovirus proteins P3, 3CD, 3D, 2C, and 3A/3AB to membranes in the presence of **A.** A4(1), **B.** E5(1), and **C.** E7(2).

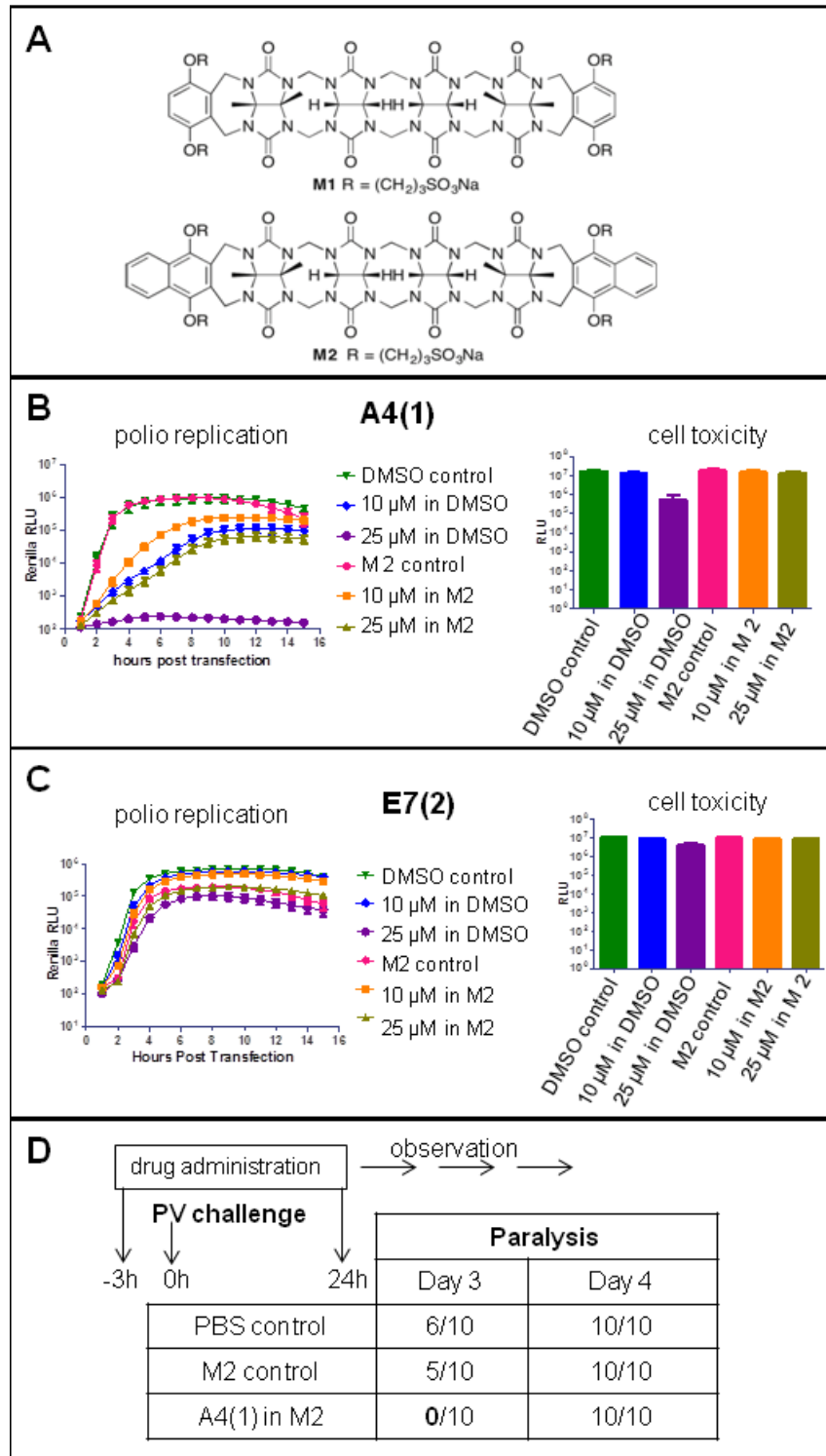
**A4(1) delays the onset of paralysis in a murine model of poliomyelitis.** The novel anti-picornaviral compounds A4(1), E5(1), and E7(2) are practically insoluble in water which significantly limits their usefulness as antiviral drugs for *in vivo* studies. To enhance their solubility, we utilized two novel acyclic cucurbit[n]urils molecular containers known as M1 and M2 (94) (Figure 3-9A). These containers have been previously shown to increase the solubility of at least ten pharmacologically-relevant drugs and have been shown to be nontoxic to several cell lines and tolerated by mice at doses as high as 1230 mg/kg (94). Based on the large aromatic surfaces of inhibitors A4(1), E5(1), and E7(2), we decided to first test solubilization using M2, which features the larger aromatic naphthalene rings as sidewalls. We separately mixed each insoluble inhibitor (1 mg) with M2 (14 mM, 0.7 mL) in 20 mM sodium phosphate buffer (pH 7.4, RT). The mixture was then filtered and the concentration of each inhibitor was determined by <sup>1</sup>H NMR by comparing the integrals of the inhibitor with those of 1,3,5-benzene tricarboxylic acid added as an internal standard of known concentration. Container M2 (14 mM) increased the solubility of A4(1) up to 2.01 mM (1.24 mg/ml) and E7(2) up to 0.90 mM (0.33 mg/ml), but did not solubilize E5(1). Accordingly, we

tested inhibitor E5(1) with container M1, but once again we were unable to enhance its solubility.

We evaluated if the M2 container-solubilized A4(1) and E7(2) retained their antiviral activity in the polio replicon replication assay. Empty container M2 did not have any inhibitory effect on polio RNA replication (Figure 3-9B and C, replication). The M2 container-dissolved A4(1) reduced polio replicon replication although not as effectively as the DMSO-dissolved compound (Figure 3-9B). The M2 container-dissolved E7(2) did not inhibit replicon replication at both 10  $\mu$ M and 25  $\mu$ M (Figure 3-9C), suggesting a strong molecular interaction between the inhibitor and the M2 container. M2 container-dissolved compounds also did not show increased cellular toxicity compared to the DMSO-dissolved ones (Figure 3-9B and C, toxicity). Since M2 container-dissolved A4(1) retained its antiviral properties in cell culture, we further evaluated it as an antiviral drug in a murine model of poliomyelitis (82).

A group of 10 transgenic mice expressing human poliovirus receptor (5 males and 5 females) were inoculated intraperitoneally with 10 mg/kg of A4(1) in complex with container M2 in PBS on day 1, three hours before intramuscular challenge with 10 PD<sub>50</sub> of poliovirus type I Mahoney. Control groups were treated with either empty container M2 in PBS or PBS alone. Mice received the second dose of the compound or control solution at 24h post infection. The treatment was stopped after the second dose due to signs of toxicity in animals treated with A4(1). Hind limb paralysis developed in both control groups treated with container M2 only (5 mice out of 10) and PBS (6 mice out of 10) on Day 3, while mice treated with A4(1) did not show signs of the disease. On Day 4,

mice in all groups were paralyzed (Figure 3-9D). Thus, in spite of its toxicity, A4(1) demonstrated protective anti-poliovirus potential *in vivo*.



**Figure 3-9. A4(1) retains antiviral activity in cell culture and delays poliovirus-induced paralysis *in vivo* after solubilization enhancement with molecular**

**container M2. A.** Chemical structures of acyclic CB[n]-type molecular containers M1 and M2. **B, C.** HeLa cells grown on 96-well plates were transfected with a polio replicon RNA with a *Renilla* luciferase gene. DMSO-dissolved or container M2-dissolved A4(1) (**B**) or E7(2) (**C**) were added after transfection and the cells were incubated for 16 h in the presence of a cell-permeable *Renilla* luciferase substrate. The signal was measured every hour for 16 hours post transfection. Cell toxicity was measured after the replication experiments in the same plate. **D.** A4(1) delays the onset of paralysis in a murine model of poliomyelitis. Container M2-dissolved A4(1) in PBS (14 mM M2 / 2.01 mM A4(1)) was administered to TgPVR21 mice at 10 mg/kg 3 hours before challenge of mice with 10 PD<sub>50</sub> of Type 1 wild type poliovirus (Mahoney strain), and the second dose 24 h after the challenge. Control groups were treated with PBS or container M2 in PBS only.

### 3.3 Discussion

In this study, we identified and characterized three novel molecules with anti-picornavirus activity. These compounds similarly blocked infection by poliovirus and another related enterovirus, CVB3, as well as a much more distantly related EMCV from the *Cardiovirus* genus. Enteroviruses and cardioviruses are known to be markedly different in their response to at least some other inhibitors targeting either viral or cellular proteins. Low concentrations of guanidine-HCl strongly interfere with enterovirus but not cardiovirus replication. The effect of this compound is believed to be mediated by inhibition of the ATPase activity of the enterovirus protein 2C (14, 102, 119, 123). Similarly, brefeldin A, an inhibitor of cellular protein GBF1, strongly inhibits replication of poliovirus and some other picornaviruses but is completely ineffective against

cardioviruses (20, 56, 72, 89, 101). The identification of compounds that can equally block replication of cardioviruses and enteroviruses suggests that at least some components in the replication complexes of these viruses are conserved. The inhibitors were selected for their activity in the replicon assay. As the introduction of replicon RNA into the cells by transfection skips virus attachment and entry, the inhibitors likely target viral protein translation or RNA replication.

Since the compounds effectively blocked infection by such diverse picornaviruses as enteroviruses and cardioviruses, it is likely that they are targeting cellular rather than viral proteins. Originally, we anticipated that screening libraries of characterized kinase inhibitors may reveal the cellular metabolic pathways that may be important for viral infection. Our results show, however, that the antiviral action of the inhibitors described is unlikely to be mediated through their annotated targets, since other inhibitors of the same kinases often showed markedly different effects on viral replication. Interestingly, A4(1) was also previously reported to block replication of negative-strand RNA viruses, including respiratory syncytial virus and vesicular stomatitis virus, by an unknown mechanism unrelated to the Akt pathway targeted by this molecule (48). This inhibitor also showed negative effect on replication of Vaccinia virus (48), suggesting that these diverse viruses may share a requirement for the same cellular factor(s).

The experiments with the *in vitro* translation-replication system revealed that all inhibitors interfered with RNA replication, while having minimal effect on uridylylation of the viral protein VPg, which serves as a primer for positive- and negative-sense RNA synthesis. A4(1) interfered with both formation and functioning of the replication complexes. E5(1) and E7(2) only blocked formation of the replication complexes, but

could not stop the replication if added to the already-formed complexes. Neither A4(1) nor E5(1) showed any effect on translation of the viral proteins, while E7(2) interfered with the processing of the polyprotein. Alteration of viral polyprotein processing in the presence of E7(2) may have contributed to its inhibition of RNA replication. Since the inhibitors were effective in the *in vitro* system which is based on post-nuclear cell lysate, they likely directly inhibit formation and/or functioning of the viral replication complexes rather than induce alteration of cellular metabolism making cells generally non-permissive for viral infection. The inhibitory effect in the *in vitro* system required higher concentration of the compounds than in the *in vivo* assays such as replicon replication or virus propagation. A similar phenomenon was also previously observed for another inhibitor of poliovirus replication, brefeldin A, which acts by blocking activity of cellular protein GBF1 (21, 38). This may reflect the fact that in live cells, translation and replication of the viral RNA are not separated and inhibition of any process is amplified in a feedback loop, while in the two step *in vitro* system, the amount of available RNA templates is the same regardless of the previous replication, so higher concentration of the compounds may be required to achieve similar inhibitory effect on the replication readout signal.

We were able to partially evaluate protective properties of A4(1) *in vivo* in a murine model of poliomyelitis. To render the compound water soluble, we used the novel molecular container M2 (94). Unfortunately, A4(1) was noticeably toxic to mice and we had to stop its administration after only two doses, however even this limited treatment delayed the onset of paralysis. It is likely that slight modification of the chemical



structure of the inhibitors identified in this study may render them more water-soluble and increase their antiviral potency *in vivo*.

## Chapter 4: Selection of Resistant Mutants

### 4.1 Introduction

Poliovirus is a positive-strand RNA virus that mutates readily due to its low fidelity RNA polymerase. The emergence of poliovirus resistant to drug treatment has been well-documented in literature. Studies of resistant mutants can lead to the discovery of novel host proteins and metabolic pathways essential for the virus life cycle. For example, poliovirus resistant to brefeldin A (BFA), a fungal metabolite that blocks the secretory pathway by inhibiting the regeneration of Arf-GTP from Arf-GDP by the guanine nucleotide exchange factor GBF1 (110), acquires mutations in the 2C and 3A proteins. Further studies of poliovirus resistant to BFA revealed the role of 3A in the recruitment of GBF1 to the replication complex (22).

In order to gain insight on the mechanism of action of the inhibitors, we passaged poliovirus in increasing concentrations of the inhibitors to promote the development of mutations. Multiple attempts to select mutants resistant to compounds A4(1) and E5(1) were unsuccessful, showing that these drugs may be developed into superior antiviral therapeutics. A resistant poliovirus mutant selected upon propagation in the presence of E7(2) contained a mutation in the viral protein 3A that was previously reported to confer resistance to a group of so-called enviroxime-like compounds, strongly suggesting that E7(2) has a similar mechanism of action (5, 6).

## 4.2 Results

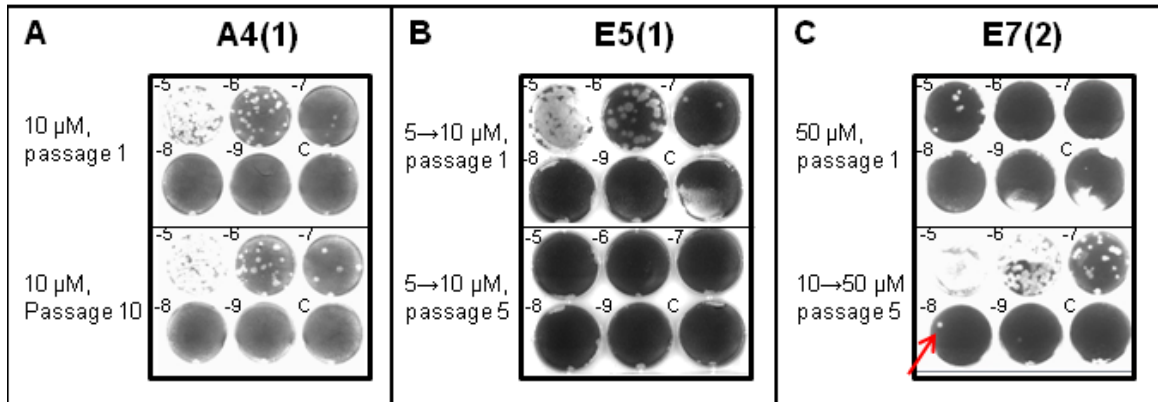
**A4(1) and E5(1) are refractory to emergence of resistant mutants, while resistance to E7(2) is conferred by a mutation previously shown to rescue replication in the presence of enviroxime-like compounds.** To get a deeper insight into the mechanism of inhibition of the viral life cycle and to evaluate the potential of the inhibitors as antiviral therapeutics, we attempted to isolate resistant mutants by serially passaging poliovirus in the presence of the compounds. For the first passage, HeLa cells were infected with poliovirus at an MOI of 10 pfu/cell; cells and extracellular media were harvested at 6 hpi and subjected to three cycles of freeze-thawing to release the intracellular virus. An aliquot of this material was used for the next round of infection and the rest was stored for later analysis. Initially we passaged the virus in the presence of 50  $\mu$ M of the inhibitors. In these conditions, no resistant mutants emerged to any of the inhibitors. When we passaged virus in the presence of 10  $\mu$ M A4(1) up to passage 20, the virus titer was about 2 logs lower than control replication (not shown), but after sequencing, we found no mutations leading to amino acid changes (Figure 4-1A).

Propagation of poliovirus in the presence of 10  $\mu$ M of E5(1) resulted in complete disappearance of the virus at passage 5. We then lowered the inhibitor to 5  $\mu$ M and propagated the virus for several passages to enrich the population for possible mutations that may facilitate selection of resistant mutants at a higher concentration. At 5  $\mu$ M E5(1), the virus replicated to about 0.5-1 log lower level than control replication (not shown). We took the material collected at passage 10 at 5  $\mu$ M and continued passaging at 10  $\mu$ M of the inhibitor. Again, after passage 5 we saw complete disappearance of the virus showing that no resistant mutants could be selected in these conditions (Figure 4-

1B). Thus, we were unable to select for viruses resistant to A4(1) or E5(1) under the conditions used. This suggests that these inhibitors would potentially be good candidates for the development of antivirals, as the possibility of the virus becoming resistant to the compound by acquiring mutations is low.

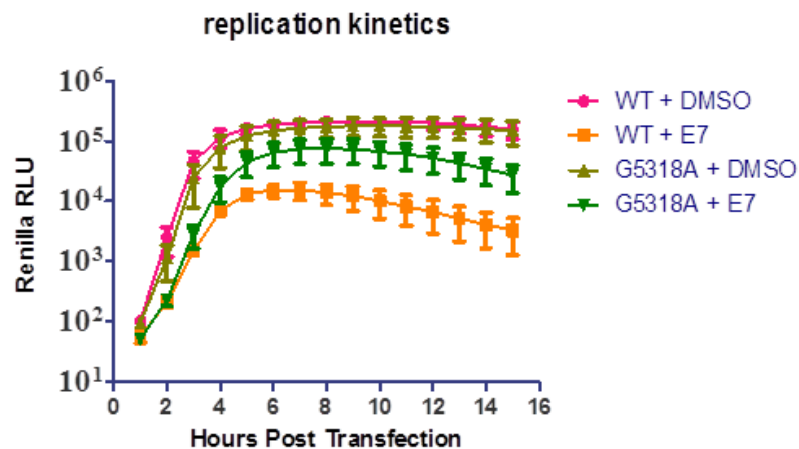
After initial failure to isolate resistant mutants at 50  $\mu$ M of E7(2), we propagated the virus for 10 passages at 10  $\mu$ M of the inhibitor and then used that material for 5 subsequent passages at 50  $\mu$ M. This scheme resulted in selection of the resistant population (Figure 4-1C). We isolated two individual plaques from passage 5 at 50  $\mu$ M of E7(2) and sequenced the entire coding region of the viral RNA. We found that both of them contained one nucleotide substitution G5318A, resulting in an amino acid change from alanine to threonine in the hydrophobic region of the 3A protein. This mutation has been previously isolated in viruses resistant to enviroxime and enviroxime-like compounds which are known to inhibit PI4KIII $\beta$  or oxysterol binding protein (OSBP) family I (5, 6). To confirm that this mutation was responsible for the resistance to E7(2), we genetically engineered the corresponding change into the poliovirus replicon RenR-P2P3 that has a gene encoding for *Renilla* luciferase instead of the structural proteins. We transfected HeLa cells with the WT RenR-P2P3, and RenR-P2P3 G5318A. The transfection media contained Endu-Ren, the cell-permeable *Renilla* substrate, and either 25  $\mu$ M or 50  $\mu$ M of E7(2). DMSO was added as a positive control. Luminescence was measured with an Infinite M1000 (TECAN) plate reader every hour for 16 hours. Both RNAs replicated to similar levels in the absence of E7(2), showing that both RNAs are viable and the G5318A mutation does not have an inhibitory effect on replication in this system (Figure 4-2). Thus, A4(1) and E5(1) are novel strong inhibitors of poliovirus

replication which cannot be overcome by resistant mutations, at least in HeLa cells, while E7(2) is another enviroxime-like compound targeting a 3A-dependent process in the viral life cycle.



**Figure 4-1. Selection of poliovirus mutants resistant to the inhibitors.**

Poliovirus yield is determined in a plaque assay on HeLa cell monolayers. Log of dilution factors are indicated, C denotes the control uninfected wells. **A.** Poliovirus yield after passage 1 (top panel) and 10 (bottom panel) in the presence of 10  $\mu\text{M}$  of A4(1). **B.** Poliovirus yield after passage 1 at 10  $\mu\text{M}$  of E5(1) of the population previously passaged 10 times at 5  $\mu\text{M}$  of the inhibitor (top panel), and disappearance of the virus after 4 subsequent passages at 10  $\mu\text{M}$  of E5(1) (bottom panel). **C.** Poliovirus yield after passage 1 at 50  $\mu\text{M}$  of E7(2) (top panel), and propagation of the resistant population selected after 5 passages at 50  $\mu\text{M}$  after 10 preliminary passages at 10  $\mu\text{M}$  of the inhibitor (bottom panel). The red arrow points to a plaque formed by a resistant virus.



**Figure 4-2. The G5318A mutation rescues replication of the *Renilla* replicon in the presence of E7(2).** HeLa cells grown on 96-well plates were transfected with wild type RenR-P2P3 (WT) or mutant RenR-P2P3 (G5318A). DMSO or 50  $\mu$ M E7(2) were added after transfection. Cells were incubated for 16 h in the presence of a cell-permeable *Renilla* luciferase substrate, and the signal was measured every hour for 16 hours post transfection.

#### 4.4 Discussion

In this study, we passaged poliovirus in the presence of A4(1), E5(1), and E7(2) to promote the emergence of resistant viruses. Drug resistance is a common problem that researchers face in the development of antiviral drugs against positive-strand RNA viruses. These viruses rapidly gain resistance to drugs that target viral proteins due to the nature of their low fidelity RNA polymerase. Antiviral drugs that target host factors are an intriguing alternative, as host proteins are more genetically stable than viral proteins. In addition, these drugs have the potential to inhibit all viruses that utilize the host protein in their life cycle. We were unable to isolate virus resistant to A4(1) and E5(1), which suggests that these inhibitors target host proteins. Although poliovirus resistance to inhibitors of host proteins has been documented in some cases (110, 142), poliovirus resistant to the drug geldanamycin, which inhibits host chaperone protein Hsp90, has not been isolated (57). A4(1) and E5(1) could potentially be effective broad-spectrum antiviral drugs with chemical modification to increase their solubility and decrease their effective concentration and toxicity. Further experiments should be performed to identify the targets of A4(1) and E5(1), as they may offer valuable insight into novel host pathways involved in the poliovirus life cycle.

Poliovirus resistant to E7(2) was found to contain a single point mutation in the hydrophobic region of the 3A protein (G5318A) that resulted in an amino acid change (Ala70Thr). This mutation has been isolated in poliovirus resistant to enviroxime and enviroxime-like compounds. Enviroxime-like compounds are divided into two classes: major enviroxime-like compounds that target PI4KIII $\beta$  and minor enviroxime-like



compounds that target OSBP. The current model proposes that PI4KIII $\beta$  is recruited to the replication complex by the viral 3A protein where it produces a PI4P-lipid enriched environment. The PI4P lipids recruit OSBP via the protein's PH domain, resulting in the accumulation of unesterified cholesterol at the replication complexes (129). Interestingly, in yeast, mutated OSBP that can no longer bind to PI4P is still recruited to the Golgi in an Arf1-dependent manner (129); interaction between OSBP and Arf1 was later described in mammalian cells (59). Arf1 is recruited to the enterovirus replication complex by the 3A protein, suggesting an alternative pathway for OSBP recruitment via the hijacked secretory pathway.

In the literature, other mutations that convey resistance to enviroxime and related compounds have been described. In enviroxime-resistant poliovirus, most isolates contained the Ala70Thr amino acid substitution; other point mutations resulting in amino acid changes were not as common, but were acquired consistently in or around the hydrophobic region of 3A (142). HRV14 resistant to enviroxime also acquired mutations in the 3A protein at the N-terminal region (142). Resistant poliovirus and HRV14 replicated to wild type levels in the absence of inhibitor, indicating that the 3A protein is tolerant of mutations in the hydrophobic region and the N-terminal region (142). Mutations 3A-Val45Ala and 3A-His57Tyr (as well as 3A-Ala70Thr) restored replication of CVB3 in the presence of major enviroxime-like compounds GW5074 and PIK93. These mutations did not restore PI4KIII $\beta$  activity; interestingly, resistant CVB3 gained the ability to replicate independently of this host factor and its isoforms, indicating that viruses indeed gain resistance to compounds targeting host factors involved in the viral life cycle (140). The ease of selection of resistant mutations among enteroviruses treated

with enviroxime-like compounds, as well as their demonstrated ability to replicate independently of the targeted host metabolic pathway, suggests that this class of compounds and others targeting this particular pathway would not be ideal antiviral drug candidates.

## Chapter 5: Characterization of the Mechanism of Inhibition of Poliovirus Replication by Enviroxime-like Compounds

### 5.1 Introduction

In the previous chapter, we isolated poliovirus resistant to E7(2) that contained a single point mutation G5318A (3A-Ala70Thr) in the hydrophobic domain of the 3A protein. This suggests that E7(2) is a so-called enviroxime-like compound, as enteroviruses resistant to this family of compounds contain this mutation or equivalent mutations in the 3A protein (142). The 3A and 3AB proteins play important roles in the viral life cycle. The 3A and 3AB proteins are associated with membranes via 3A's hydrophobic membrane-binding domain. The 3A protein is involved in the recruitment of GBF1 and PI4KIII $\beta$  to the replication complexes (46); expression of 3A alone is sufficient to block anterograde transport, inhibiting the secretory pathway (44). The 3AB protein is thought to anchor the 3B (VPg) protein to the membranes at the replication complexes prior to priming synthesis of plus- and minus-strand replication (88). The appearances of mutations that confer resistance to enviroxime-like compounds in 3A suggest that 3A or 3AB are involved in the PI4KIII $\beta$ /OSBP metabolic pathway.

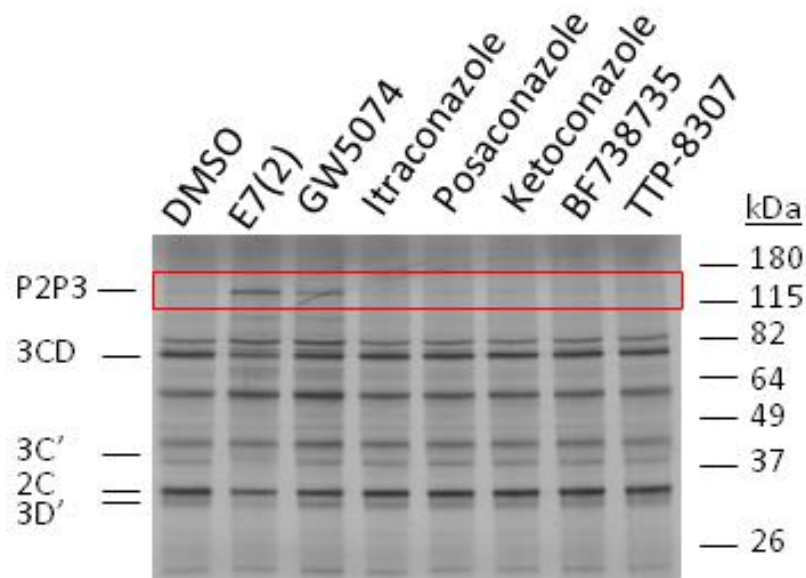
Several major and minor enviroxime-like compounds have been described. The major enviroxime-like compounds include PIK93, GW5074, BF738735, AN-12-H5, and T-00127-HEV1. These compounds all affect the activity of PI4KIII $\beta$  (6, 141). Minor enviroxime-like compounds that target OSBP include TTP-8307, OSW-1, itraconazole, and posaconazole (5, 133). Major and minor enviroxime-like compounds show no structural similarities, with the exception of their hydrophobic properties. The only feature that remains consistent is the G5318A mutation that confers virus resistance to

enviroxime-like compounds. This mutation has been shown to generate weak resistance to minor-enviroxime-like compounds and strong resistance to major enviroxime-like compounds (5). The goal of this chapter is to characterize E7(2) as a major or minor enviroxime-like compound and to compare the effects of these compounds on poliovirus translation and processing.

## 5.2 Results

### **Some, but not all, enviroxime-like compounds inhibit polyprotein processing.**

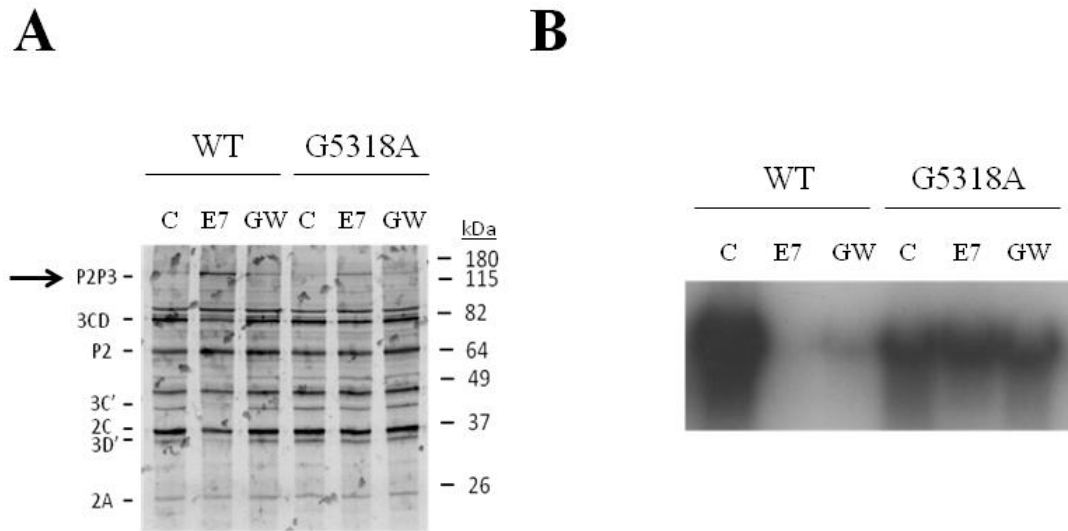
Previously, we showed that E7(2) treatment caused a defect in polyprotein processing in the cell-free translation system. In order to determine if this phenomenon was specific to E7(2), we performed an *in vitro* assay in which P2P3 RNA was translated in HeLa cell extract. We added 50  $\mu$ M of E7(2) or various enviroxime-like compounds to each sample, and an equivalent amount of DMSO was added to the control reaction. A portion of each sample was mixed with [<sup>35</sup>S]-methionine to visualize the level of viral translation. The compounds GW5074, BF738735, and PIK93 are classified as inhibitors of PI4KIII $\beta$  (6, 96), while the inhibitors itraconazole (ITZ), posaconazole (POS), and TTP-8307 target OSBP (5, 133). We found that, of all the enviroxime-like compounds tested, E7(2) and GW5074 caused a similar defect in polyprotein processing (Figure 5-1, PIK93 data not shown). However, other major enviroxime-like compounds did not affect processing, suggesting that E7(2) and GW5074 have an alternative or an additional effect on the poliovirus life cycle that is not shared with the other enviroxime-like compounds.



**Figure 5-1. E7(2) and GW5074 affect the processing of the P2P3 polyprotein.**

*In vitro* translation was performed in HeLa cell extract in the presence of 50  $\mu$ M of the listed major and minor enviroxime-like compounds. DMSO (solvent) was added as the solvent control. Ketoconazole is an inactive control compound. A portion of each reaction was mixed with  $^{35}$ [S]-methionine to assess viral polyprotein synthesis and processing. Several poliovirus proteins are indicated on the left. The red box highlights the defect in processing of the P2P3 polyprotein in the presence of E7(2) and GW5074.

**The G5318A mutation partially rescues the processing defect in the cell-free translation system.** We have previously shown that the G5318A mutation rescues replicon replication in the presence of E7(2) (Figure 4-2). In order to determine if the G5318A mutation rescues the polyprotein processing defect seen with E7(2) and GW5074, *in vitro* translation and replication assays were performed. During the first step, WT P2P3 RNA or G5318A P2P3 RNA was incubated in crude HeLa cell extract in the presence of 2 mM of guanidine HCl to inhibit replication. DMSO, E7(2), or GW5074 were added at the translation step. A portion of each sample was mixed with [<sup>35</sup>S]-methionine to visualize viral translation. After the translation reaction, the replication complexes were spun down and resuspended in buffer containing [<sup>32</sup>P]-CTP, which becomes incorporated in the newly-synthesized RNA. We saw that the mutation partially rescued processing of the polyprotein in samples containing E7(2) and GW5074 (Figure 5-2A), however, rescue came at the cost of diminished replication (Figure 5-2B). The partial rescue of the processing defect shows that the defect is not a side effect of the inhibitor treatment and is at least partially involved in the mechanism of inhibition.

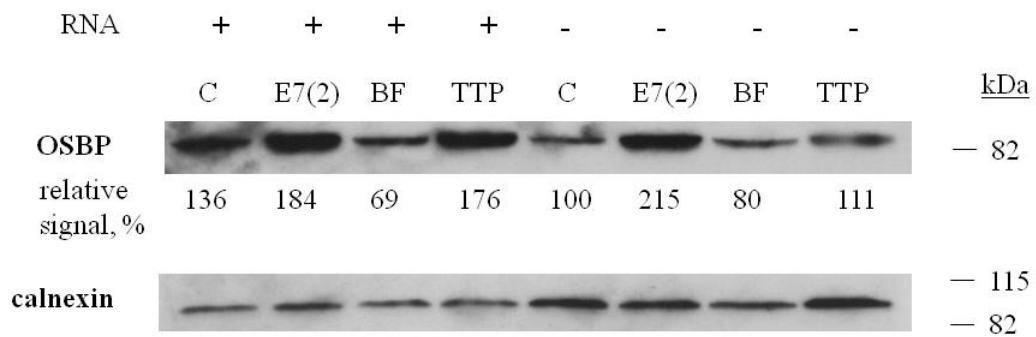


**Figure 5-2. Rescue of polyprotein processing (A) and replication (B) in the presence of E7(2) with G5318A P2P3 RNA.** The G5318A mutation partially rescues the polyprotein processing defect and replication in the presence of 50  $\mu$ M of E7(2) and 10  $\mu$ M of GW5074, but with the cost of lower replication in the absence of inhibitors. The inhibitors were added during the translation step. DMSO was used as the positive control. An aliquot of the *in vitro* translation mixture was labeled with  $^{35}$ [S]-methionine to assess viral translation and processing of WT and G5318A P2P3 RNA (A). The arrow points to the processing defect of the P2P3 polyprotein seen in the presence of E7(2) and GW5074. Poliovirus proteins are indicated on the left. (B) Replication of WT or G5318A P2P3 RNA was performed in the presence of  $^{32}$ [P]- $\alpha$ CTP to label the newly-synthesized RNA. C = DMSO, E7 = 50  $\mu$ M of E7(2), GW = 10  $\mu$ M of GW5074.

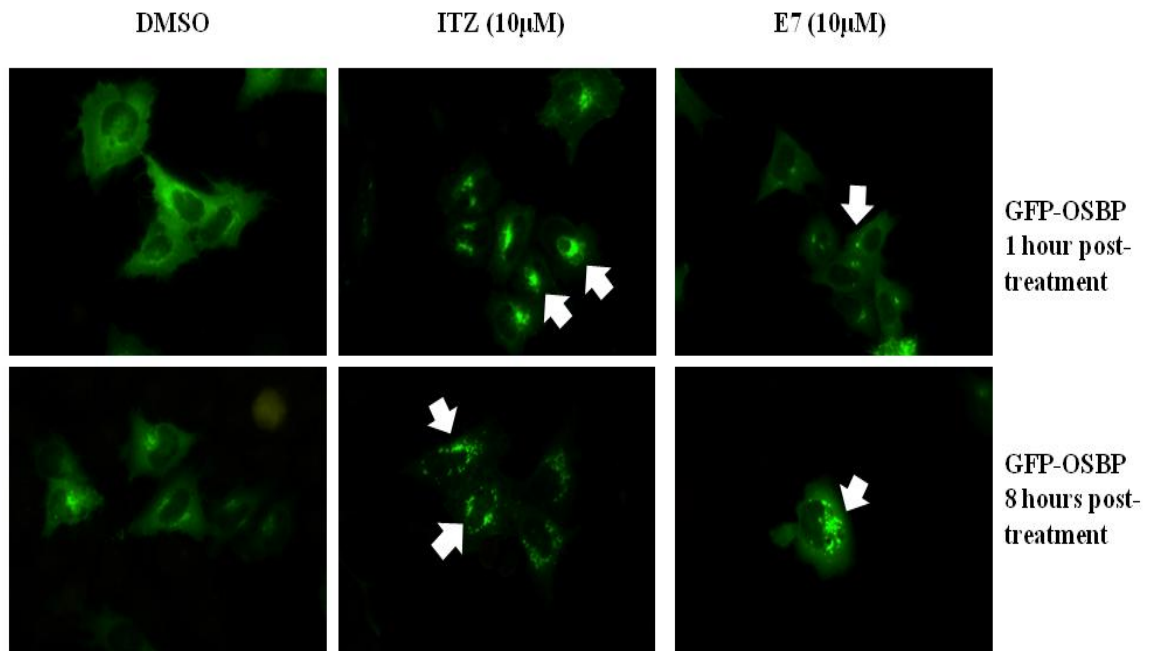
**E7(2) is a minor enviroxime-like compound.** Previously, we showed that E7(2) did not affect recruitment of PI4KIII $\beta$ , the target of major enviroxime-like compounds, to the replication complex (Figure 3-8C). This and the drug's inhibitory effect on EMCV led us to hypothesize that E7(2) is a minor enviroxime-like compound that targets OSBP. In order to determine if E7(2) changes the recruitment of OSBP at the replication complex membranes, we translated P2P3 RNA in HeLa cell extract in the presence of the inhibitors, collected the membranes, and analyzed the recruitment of OSBP by Western blot. We observed that the addition of E7(2) in both the presence and absence of poliovirus resulted in the accumulation of OSBP at the membranes. Thus, this inhibitor modifies the recruitment of OSBP to likely result in an imbalance of PI4P and cholesterol at membranes. We also observed this effect in the presence of minor enviroxime-like compound TTP-8307 (Figure 5-3).

In addition, in cells transfected with GFP-OSBP, treatment with 10  $\mu$ M E7(2) resulted in the relocalization of OSBP from the Golgi to small, punctate clusters in the perinuclear region at 8 hours post treatment, possibly the result of Golgi fragmentation (Figure 5-4). This effect was also observed in cells treated with 10  $\mu$ M of ITZ, a minor enviroxime-like compound that has been shown to bind to and inhibit the PI4P/cholesterol exchange function of OSBP (133). These data suggest that E7(2) is a minor enviroxime-like compound that targets OSBP.





**Figure 5-3. Accumulation of OSBP at the membranes in the presence of E7(2) and TTP-8307.** P2P3 RNA or -RNA (negative control) was translated in cytoplasmic HeLa cell extract in the presence of 50  $\mu$ M of the inhibitors or DMSO. After translation, the membranes were collected by centrifugation and the pellets were resuspended in sample buffer for Western blot analysis of membrane-associated proteins. Accumulation of OSBP at the membranes is shown and calnexin serves as the loading control. The relative signal of OSBP accumulation was calculated with ImageJ software. C = DMSO, BF = BF738735, TTP = TTP-8307.

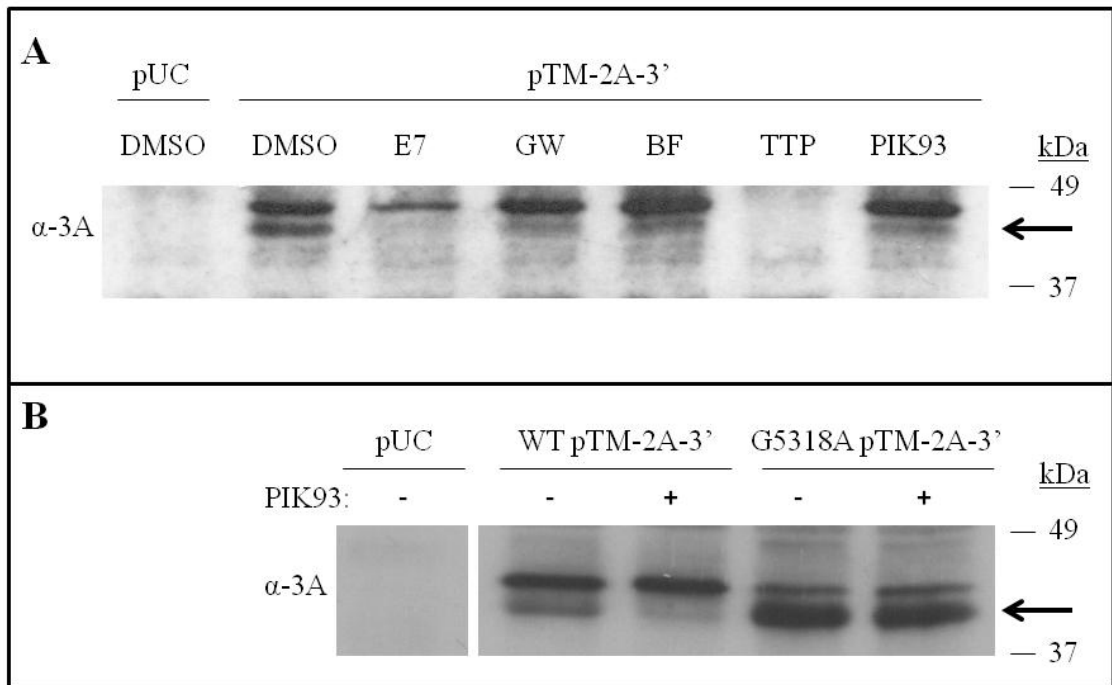


**Figure 5-4. Treatment with E7(2) results in the relocation of OSBP.** HeLa cells were transfected with a plasmid expressing GFP-OSBP. The following day, the cells were treated with 10  $\mu$ M of itraconazole (ITZ) or E7(2). DMSO was added as the positive control. Cells were imaged at 1 hour post-treatment and 8 hours post-treatment to observe OSBP localization. Figure courtesy of Dorobuntu, et al.

### **Major enviroxime-like compounds induce the same defect in poliovirus**

**polyprotein processing.** The ability of the same mutation in the poliovirus protein 3A to confer resistance to diverse enviroxime-like compounds strongly suggests that all of them interfere with the same step of the replication cycle. In order to identify a possible common mechanism underlying the inhibitory action of enviroxime-like compounds, we performed an in-depth examination of their effect on viral polyprotein processing. To investigate poliovirus polyprotein expression and processing independent of replication of the genome RNA, we used a Vaccinia-T7-based expression system. We transfected HeLa cells with pTM-2A-3', a plasmid encoding the poliovirus nonstructural proteins under the control of a T7 promoter. Cells transfected with pUC served as the negative control. The following day, we infected the cells with Vaccinia virus expressing T7 RNA polymerase. After infection, cells were incubated in media containing 10  $\mu$ M of enviroxime-like compounds. Cells were lysed at 4 hpi and the samples were run on a Western blot to look at the expression of viral proteins. The preparation of the samples involved the fractionation of the cellular lysate. Therefore, we observed either a defect in the processing or a defect in membrane association of a 3A-containing precursor in the presence of 10  $\mu$ M of all major enviroxime-like compounds tested in cells expressing viral proteins (Figure 5-5A). This defect was consistently seen in both HeLa and HEK293T cells. Minor enviroxime-like compounds TTP-8307 and to a lesser extent E7(2) seem to inhibit Vaccinia-induced expression of poliovirus proteins (Figure 5-5A and data not shown). Major enviroxime-like compounds did not inhibit the expression of other poliovirus proteins (2C, 3B, 3D, data not shown). The effect of other minor enviroxime-like compounds on Vaccinia-induced expression is currently being examined.

In order to determine if this defect in 3A processing is important, we transfected HeLa cells with WT pTM-2A-3' and G5318A pTM-2A-3', followed by Vaccinia infection to express the T7 RNA polymerase. Cells were incubated in media containing DMSO or 10  $\mu$ M PIK93 after infection. Cells were lysed at 4 hpi and samples were run on a Western blot. In cells expressing WT poliovirus proteins, we saw the defect in the 3A-containing protein (Figure 5-5B). Interestingly, in cells expressing G5318A 3A, accumulation of the 3A-containing protein is strongly increased, even in the absence of inhibitors (Figure 5-5B). Further experiments are being performed to identify this protein and its role during infection.



**Figure 5-5. Major enviroxime-like compounds share a defect in a 3A-containing protein that is rescued by G5318A 3A.** HeLa cells were transfected with **A**) pUC or WT pTM-2A-3' or **B**) pUC, WT pTM-2A-3', or G5318A pTM-2A-3'. The following day, cells were infected with 10 pfu/cell of Vaccinia expressing the T7 RNA polymerase. After infection, the cells were incubated in media containing DMSO or 10  $\mu$ M of the listed inhibitors. At 4 hpi, cells were lysed and samples were collected for Western blot analysis with poliovirus 3A antibodies. The arrows point to the 3A-containing protein of interest. GW = GW5074, BF = BF738735, TTP = TTP-8307.

### 5.3 Discussion

Enviroxime-like compounds have been described to target either PI4KIII $\beta$  (major enviroxime-like compounds) (6, 141) or oxysterol-binding protein (OSBP) (minor enviroxime-like compounds) (5), host proteins involved in creating a PI4P and cholesterol enriched environment of the replication membranes. Why such environment could be conducive for virus replication remains unknown. It was demonstrated that viral RNA-dependent RNA polymerase 3D could directly bind PI4P in a biochemical assay, but whether this is important *in vivo* remains to be established. The appearance of resistant mutations only in the 3A protein strongly suggests that the function of this and/or other 3A-containing fragments of the viral polyproteins are affected by membrane perturbations induced by enviroxime-like compounds. Inhibition of PI4P production and cholesterol exchange at the replication complex likely influences the physical properties of the replication complex to effect viral replication and/or translation. This seems to suggest that in the presence of E7(2) (a minor enviroxime-like compound) or GW5074 (a major enviroxime-like compound), the conformation of the polyprotein on the imperfect replication membranes leads to a defect in polyprotein processing by both 2A and 3C proteases. However, we did not observe a defect in P2P3 polyprotein processing with other enviroxime-like compounds (major and minor) tested with the *in vitro* translation assay in HeLa cell cytoplasmic lysate. This suggests that these two inhibitors have an additional effect on viral translation that is not seen with other enviroxime-like compounds tested. This is not completely unexpected; small molecules such as these often have off-target effects, especially at higher concentrations. Importantly, we observed that all enviroxime-like compounds tested prevented accumulation of a

particular 3A-containing polyprotein fragment and that the resistant mutation strongly increases such accumulation both in the presence and in the absence of the inhibitors. It is not yet clear if this fragment is important for functioning of the replication machinery, however this result indicates that the inhibitory action of enviroxime-like compounds is likely mediated by their interference with proper processing of the viral proteins and/or their binding to replication membranes in the modified lipid environment.

## Chapter 6: Conclusion

Picornaviruses are the causative agents of a wide variety of diseases in humans and animals. Although this family of viruses causes significant morbidity and mortality, only two vaccines are available for humans— vaccines against poliovirus and hepatitis A virus. For other picornaviruses, the development of antiviral therapeutics is more practical, as the large number of serotypes presents a challenge for vaccine development. Even for poliovirus, antiviral drugs could limit outbreaks caused by vaccine-derived poliovirus and are considered an important safety component in the post-eradication world. To date, no antiviral compounds have been approved to treat any picornavirus infections in clinical practice.

In this report, we identified and characterized three compounds with anti-picornavirus activity by screening commercially available libraries of cell-permeable kinase inhibitors. The compounds were effective against poliovirus, CVB3, another related enterovirus, as well as a much more distantly-related EMCV from the *Cardiovirus* genus (Figure 3-3 and 3-5). The broad-spectrum activity of A4(1), E5(1), and E7(2) suggests that these inhibitors target conserved metabolic pathways that are important for the replication of diverse viruses.

A major problem in antiviral drug development is the emergence of resistant mutants. Picornaviruses are known to easily overcome compounds targeting cellular or viral proteins (6, 37, 117, 136). After multiple passages, we were able to select resistant viruses to E7(2) (Figure 4-1). The resistant mutation was localized in the hydrophobic domain of the 3A protein and was also described in virus resistant to enviroxime and enviroxime-like compounds; we classified E7(2) as a minor enviroxime-like compound



that targets OSBP. Interestingly, we have preliminary data that suggest another property—a defect in the processing or the membrane association of a 3A-containing protein— shared by major enviroxime-like compounds (Figure 5-5). Further experiments are being performed to identify this protein and how it is involved in the poliovirus life cycle.

Importantly, multiple attempts to obtain poliovirus mutants resistant to A4(1) and E5(1) were unsuccessful, making these broad-spectrum inhibitors of picornavirus replication promising candidates for drug development.

Future experiments will include the chemical modification of A4(1) and E5(1) to increase their antiviral efficiency, reduce their toxicity, and increase their solubility. Currently, the  $IC_{50}$ 's of A4(1) and E5(1) are around 3.2  $\mu$ M and 12  $\mu$ M respectively, while the ideal  $IC_{50}$  of antiviral drugs is in the nanomolar range. We can optimize the chemical structure of the compounds by analyzing the antiviral activity of A4(1) and E5(1) analogs in the polio replicon replication assay. This system would allow us to readily compare multiple derivatives of these inhibitors to develop structure-activity relationships (SAR) that would guide further design of effective drugs based on A4(1) and E5(1). We could then enhance the solubility of the most effective compounds in the acyclic cucurbit[n]urils molecular containers M1 and M2, if necessary, and analyze their anti-poliovirus activity and toxicity in a murine model.

We will also aim to identify the mechanism of action of A4(1) and E5(1). We would implement a selection enrichment system to identify potential cellular targets of these inhibitors. Briefly, we would transfect HeLa cells with a cDNA expression library, followed by infection with poliovirus and incubation with A4(1) or E5(1). In the majority

of cells, productive infection would be blocked by the inhibitors; only cells overexpressing potential cellular targets of the drugs would progress through infection leading to cell lysis and the release of the plasmids responsible for the rescue of productive infection. We can then isolate and propagate these plasmids, and then repeat the transfection process to select for plasmids that express proteins that rescue infection in the presence of the compounds.

The other approach would be to use biotinylated derivatives of A4(1) and E5(1) to identify proteins bound to these inhibitors. Briefly, the cytoplasmic lysate of mock- and poliovirus-infected cells will be incubated with biotinylated A4(1) or E5(1). The protein complexes will then be collected with streptavidin beads and identified by mass spectrometry analysis. Alternatively, we could run cellular proteins on a native gel, transfer the proteins to a PVDF membrane, and incubate the membrane in biotinylated A4(1) or E5(1). Proteins bound to these compounds would be visualized with a fluorescent streptavidin conjugate. These proteins could then be excised from the native gel and analyzed by mass spectrometry analysis. Identified protein targets can then be confirmed via siRNA knockdown for their effect on poliovirus replication.

Therefore, these molecules we identified in this study provide important tools for understanding the fundamental aspects of the formation and functioning of the picornavirus replication complexes as well as for the development of broad-spectrum antiviral drugs.

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