Gemcitabine Inhibits Poliovirus Replication

and Interferes with Viral RNA Elongation

BY

Zhuoran Zhang
B.S., Fudan University, Shanghai, China, 2011

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology and Immunology
in the Graduate College of the
University of Illinois at Chicago, 2016

Chicago, Illinois

Defense Committee:

Zheng W. Chen, M.D., Ph.D., Chair and Advisor
Alan McLachlan, Ph.D.
Lijun Rong, Ph.D.
Howard Lipton, M.D.
Richard Novak, M.D., Department of Medicine

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List of Abbreviation

PV	poliovirus
cVDPV	circulating vaccine derived poliovirus
OPV	oral poliovirus vaccine
IPV	inactivated poliovirus vaccine
RdRp	RNA-dependent-RNA-polymerase
GnHCl	Guanidine Hydrochloride
FMDV	foot-and-mouth disease virus
EV	enterovirus
RV	rhinovirus
CV	coxsakievirus
OSBP	oxysterol-binding protein
ORP4	OSBP-related protein 4
PI4KIIIβ	phosphatidylinositol 4-kinase III beta
Hsp	heat shock protein
dFdC	2', 2'-difluoro-2'-deoxycytidine
dFdU	difluorodeoxyuridine
dFdC-TP	dFdC triphosphate
dFdU-TP	dFdU triphosphate
dFdC-DP	dFdC diphosphate
IRES	internal ribosome entry site

Summary

Poliovirus (PV) is the causing agent of polio disease. Despite of the control of polio pandemic by vaccination, we are facing emerging challenges such as circulating vaccine derived poliovirus (cVDPV) and chronic PV excreters. There is no antiviral available to treat PV infection. In consistent to the Global Polio Eradication Initiation, one or two PV antiviral are required to treat chronic PV excreters and reach the end game of PV eradication. They are also essential to maintain a polio-free world post eradication.

In an effort to identify novel anti-poliovirus, a cell-based high-throughput screening (HTS) assay was developed. Taking advantage of a luciferase-expressing PV chimera (PV-GLuc), compounds that inhibit viral replication can be rapidly identified by the reduction of the luminescence signals. The PV-GLuc construct confers growth kinetic similar to the wild type PV in terms of plaque forming unit (PFU) as well as viral RNA copy numbers. It also continuously generates luciferase protein throughout the incubation up to 60-hour post infection. After validating the characteristics of the chimera construct and optimizing the HTS, this assay turns out to be a highly sensitive and reliable tool for polio antiviral discovery.

We applied the HTS assay to screen a FDA approved compound library. 11 hits were selected from the 1,200 compounds screened. These hits inhibited at least 80% of viral replication indicated by reduction of luciferase signals while kept more than 70% of cell viability as measured by intra-cellular ATP level. These hits were further verified and characterized using

wild type PV and one of the hits, Gemcitabine, was confirmed to be the best PV antiviral candidate with IC_{50} of 0.3µM.

Gemcitabine (2', 2'-difluoro-2'-deoxycytidine, dFdC) is a nucleoside analog which has been widely used in cancer chemotherapy. Its effective metabolite, dFdC triphosphate (dFdC-TP) is incorporated into DNA and RNA chain and obtains the anti-tumor effect by inhibiting DNA and RNA chain elongation. To elucidate its mechanism of action as PV replication inhibitor, a cell-free assay was conducted using purified PV RNA-dependent-RNA-polymerase (RdRp) and single-stranded viral RNA template. Data from the *in-vitro* elongation assay demonstrates dFdC-TP inhibits viral RNA elongation in a dose-dependent manner. This is consistent to our observations in cell cultures. Therefore, we propose that the metabolite of Gemcitabine, dFdC-TP, inhibits PV replication by blocking viral RNA replication and probably interacts with the viral RdRp.

An *in vivo* study taking advantage of our recently developed non-human primate PV oral infection model is being conducted to evaluate the therapeutic effect of Gemcitabine against PV infection. Since RdRp is conserved among picornavirus species, we propose that Gemcitabine could be the leading compound for broad-spectrum antiviral of picornavirus species.

Abstract

(This chapter represents my own poster abstract presented in the UIC Research Forum on April 2016)

While we are approaching the end game of global eradication of circulating wild-type polioviruses(PV), vaccination with oral poliovirus vaccine (OPV) has led to emergence of circulating vaccine-derived poliovirus (cVDPV) and vaccine-associated paralytic poliomyelitis (VAPP). Complete cessation of all poliovirus infections may require stopping use of OPV and formulating improved vaccines and new antiviral drugs. Currently, no licensed drugs are available to treat chronically infected poliovirus excreters. Here, we created a modified PV expressing Gaussia Luciferase (PV-GLuc) and developed a cell-based high-throughput screening (HTS) antiviral assay. Using the validated HTS assay, we screened the FDA-approved drug library of compounds and identified candidate agents capable of inhibiting PV replication. We then characterized anti-poliovirus activity for the best hit, Gemcitabine, a nucleoside analogue used in tumor chemotherapy. We found that Gemcitabine inhibited PV Mahoney replication with IC_{50} of 0.3μM. It completely protected HeLa cells from PV-induced cytopathic effects at 5μM, without detectable toxicity for cell viability. Furthermore, Gemcitabine metabolite directly inhibited the ability of PV RNA polymerase to synthesize or elongate PV RNA. Since PV RNA polymerase is somehow conserved among species in *Picornaviridae* family, Gemcitabine may be further developed as an attractive broad-spectrum antiviral for PV and others.

Chapter I. Introduction

1.1. Current Polio Disease Status and Application of Anti-poliovirus

Poliovirus (PV) is the causing agent of polio (poliomyelitis) disease which used to induce tremendous public health problems. PV spreads through fecal-oral route by contaminated food or drinking water. Once infected, the virus replicates in the patient's intestine and is released to the environment through contaminated stool. One in 200 PV infected patients will develop irreversible paralysis, usually in one side or both sides of their legs, due to the damages of neurons in the central nerve system that control muscle movement. In more severe situations, patients may suffer from difficulties in breathing and swallowing when neurons in the brain stem controlling chest muscles are affected. These patients had to be supported by special equipment such as tank respirator, also known as "Iron Lung", until they recovered and could breathe independently.

1.1.1. International Polio Transmission

PV caused 350,000 poliomyelitis cases a year when World Health Organization (WHO) launched Global Polio Eradication Initiation in 1988. Thanks to the successful immunization and global polio surveillance, that number rapidly dropped down to 359 cases in 2014[1]. Although worldwide pandemic of polio disease has been controlled, regional and international PV transmissions occurred from time to time[2]. In May, 2014, WHO declared global public health emergency of international PV transmission in Afghanistan, Cameroon, Equatorial Guinea, Ethiopia, Israel, Nigeria, Pakistan, Somalia and the Syrian Arab Republic[3, 4]. International polio

transmission not only threats population in the affected countries, but also lays heavy burden of importing polio diseases on neighboring countries. In 2013, Israel suffered from a PV outbreak despite the fact that it has been certified as polio-free by WHO in 2002 and it has high immunization coverage of IPV since 2005[5, 6]. The international PV transmission and PV outbreak in certified polio-free countries indicate that the existing vaccination and surveillance system are not sufficient to achieve the goal of PV eradication[7].

1.1.2. Circulating Vaccine Derived Poliovirus (cVDPV)

In addition to the international PV transmission, we are facing other challenges such as circulating vaccine derived poliovirus (cVDPV)[8-11], which caused 52 out of the 359 polio cases in 2014[1]. There are two polio vaccines available, oral PV vaccine (OPV) and inactivated PV vaccine (IPV). Unlike OPV which provides systemic immunity to immunized individuals, IPV generates humoral immunity in the blood but lacks mucosal immunity, which could lead to prolonged viral shedding after PV infection in certain immune compromised patients[12]. On the other hand, OPV replicates for limited period of time after immunization in the GI tract [13], where it could convert into cVDPV by mutation or recombination with other enterovirus (EV) [14-18] and be excreted into the environment. For example, a virulent Sabin type 2 derived cVDPV strain was identified in the seawater from Brazil in 2014 while the original application of OPV was traced back to more than 8.5 years ago based on molecular genetic analysis, indicating unidentified viral circulation after cessation of OPV vaccination[19]. As a result, even though OPV will be gradually replaced by IPV according to the strategy of Global PV Eradication Initiation, cVDPV will remain a risk of PV reintroduction years after PV eradication. Since no

safer and more effective PV vaccine is available to replace OPV, PV antiviral could be the last line of defense against cVDPV.

1.1.3. Chronic PV Excreters

Besides that, certain immune compromised patient may continuously shed PV after infection and become chronic PV excreters. For example, an immune-compromised patient in Taiwan was found to excrete cVDPV over 10 months after the development of poliomyelitis. The patients received OPV vaccination about one and half year before the occurrence of symptoms. Sequencing results reveals at least five cVDPV lineages derived from OPV, suggesting chronic asymptomatic PV replication and infection[20]. It is estimated that there are 30 asymptomatic chronic PV excreters in the world, while only 4 cases have been reported[21]. The unidentified PV excreters could be a critical step in the generation of cVDPV and serves as additional source of PV infection. Therefore, antiviral is necessary to treat chronic excreters and stop the "undercover" PV circulation.

In summary, polio disease remains a threat to the global public health. Stopping the use of OPV, as well as formulating improved vaccines and new antiviral drugs are essential to achieve the goal of complete cessation of all PV infections.

1.2 Virology of Poliovirus

1.2.1. Structure of PV Viral Particle

PV is the prototype of Picornaviridae family. It is a non-enveloped virus with a diameter of about 30nm[22]. PV virion capsid consist 60 copies of each of viral proteins VP1, VP2, VP3 and VP4. VP1, VP2 and VP3 are on the exterior surface of virion, while VP4 is located at the inner side of viral particle attaching to the viral RNA genome[23]. The receptor binding domain sits at the bottom of the hydrophobic pocket on the viral particle. PV contains a small single-stranded positive RNA genome of 7.5kb in size. Viral protein VPg, which is critical for the initiation of the viral RNA replication, attached to the 5'-end of the positive RNA genome[24].

1.2.2. Viral Entry

CD-155, also known as PV receptor (PVR), is the cellular receptor for PV viral entry. CD-155 is only found in Old World primates and humans[25]. It is widely expressed on epithelial and endothelial cells. In addition to the small intestine epithelial cells, CD-155 is also expressed in tissues such as heart, brain, placenta, lung, liver, skeletal muscle, and kidney[26]. After binding to CD-155 on the cell surface, PV virion is internalized by receptor-induced endocytosis. Binding to CD-155 induces conformational change of the virion capsid, including the exposure of VP4 from the inner-side of viral particle to the exterior-side of the viral particle[27]. VP4 and the N-terminal extension of VP1 protein insert into the membrane of the endocytic vesicle and form a pore to release the viral RNA genome into the cytoplasm. High-resolution imaging of poliovirus and coxsakievirus A16 confirms the formation of the hole on the endosome membrane[28, 29]. Cryo-EM imaging also detects viral RNA genome exposed from the expanded viral particle[30]. However, detailed procedures in the viral RNA release are still poorly understood.

1.2.3. Viral Polypeptide Translation and Procession

Once get access to cytoplasm, the positive-strand viral RNA initiates protein translation through the internal ribosome entry site (IRES) in the 5' non-coding region to generate one single polypeptide[22]. The polypeptide consist three regions, P1, P2 and P3. P1 contains structural proteins while P2 and P3 contain non-structural proteins. After translation, the polypeptide is processed by viral proteases into functional structural and non-structural viral proteins. The polypeptide processing begins with cleavage between P1 and P2 region by $2A^{pro}$. The released non-structural proteins then mediate viral RNA replication. Almost all of the non-structural proteins and their immature precursors are involved in the viral RNA replication, such as $2A^{pro}$, 2BC, 2B, $2C^{NTPase}$, 3AB, $3CD^{pro}$ and RNA-dependent-RNA-polymerase (RdRp)$3D^{pol}$[22].

1.2.4. Cell Membrane Rearrangement

Rearranged cell membranes that form single or double-layered vesicles are observed in PV and other picornaviruses infected cells[31, 32]. Further studies revealed that viral $3D^{pol}$ co-localizes with these vesicles, indicating these structures serve as viral RNA replication complex where viral RNA synthesis takes place [33]. Co-expression of recombined 3A and 2BC (precursor of 2B and 2C proteins) mimics the formation of these structures in naïve cells[34]. Recombined 3A and 2C proteins expressed in the cells directly bind to the ER membrane and recruit other host factors involved in cell membrane rearrangement, such as phosphatidylinositol-phosphate-4-kinase III beta (PI4KIIIβ)[35], phosphatidylinositol-4 phosphate (PI4P)[36] and valosin-containing protein (VCP/p97)[37]. PI4KIIIβ and VCP/p97 were found to directly interact with 2BC protein. Pre-treatment by PI4KIIIβ or VCP/p97 inhibitors block viral RNA synthesis in the

infected cells, while the presence of these inhibitors after the initiation of RNA replication complex had no effect[35]. $3D^{pol}$ was recruited after the formation of the viral-induced membrane vesicles by direct interaction between $3D^{pol}$ and 3AB protein (the precursor of 3A protein). Specific $3D^{pol}$ mutant that lacks binding activity to 3AB but maintains RNA polymerase activity leads to reduction in viral RNA synthesis[38]. These observations indicate that 2C, 3A proteins as well as their precursors play important roles in the initiation of viral RNA replication.

1.2.5. Viral RNA Replication

PV uses VPg-pUpU, which is cleaved from uridylylated 3AB protein, as primer for both plus and minus-strand RNA replication[24]. *In vitro* experiment using a cell-free system shows that VPg could be robustly elongated into VPg-pUpU in the presence of purified $3D^{pol}$ using a poly-A template[39]. Interestingly, when using PV RNA in the cell-free system, a *cis*-replicating RNA element (cre) located in the 2C protein coding region (*cre*(2C)) instead of the poly-A tail serves as the template for uridylylation of VPg protein by $3D^{pol}$[40]. Deletion of the *cre*(2C) sequence or adding an additional U suppressed the initiation of viral RNA synthesis, however, this can be rescued by introducing a similar sequence in the VP1 coding region in the human rhinovirus 14. After uridylylation, VPg-pUpU slides from *cre*(2C) to the 3'-end of plus or minus viral RNA to serve as the primer for RNA chain elongation, but the mechanism of VPg-pUpU translocation is still not clear[24]. After initiation, the RNA replication begins with the synthesis of minus-strand RNA using the plus-strand genomic RNA as template. $3D^{pol}$ first produces double-stranded intermediate RNA (RF). After the production of RF, $3CD^{pro}$ binds to the 5'-end of the plus-strand to unwind the double-stranded RNA[41, 42]. At the same time, $2C^{ATPase}$ binds to the 3'-end of

the minus-strand[43, 44], which may facilitate the binding of VPg-pUpU and $3D^{pol}$ to initiate the synthesis of plus strand RNA.

1.2.6. Encapsidation

Several viral and host factors are involved in the assembly of infectious PV virion. The viral structural domain P1 was first cleaved into VP1, VP3 and VP0 by viral $3C^{pro}$[22, 45]. Host factor heat shock protein 90 (Hsp90) is required as a chaperon for the correct folding of P1 as well as its cleavage[46]. One piece of each VP1, VP3 and VP0 proteins first form a 5S protomer and five protomers form a 14S pentamer. The assembly of 75S empty viral capsid requires 60 copies of VP1, VP3 and VP0 proteins. The newly assembled empty viral capsid is recruited to the viral RNA replication complex to load positive-strand viral RNA genome. Recent studies reveal that $2C^{ATPase}$ directly binds to VP3 during morphogenesis and mutations that inhibit the bindings suppresses viral growth[47, 48]. Since $2C^{ATPase}$ binds to the minus-strand RNA during viral RNA replication, it might play important role in recruiting the newly assembled empty capsid to the viral genomic RNA[45]. After loading of viral RNA genome, VP0 is cut into VP2 and VP4 to form mature viral virion.

1.3. PV Antiviral Development

Currently no antiviral is available to treat PV infection. As recommended by the National Research Council, two antivirals with different mechanisms of action are necessary for the end game and preventing possible polio outbreaks post eradication[13]. The main purpose of the application of PV antivirals is to treat prolonged PV excreters (e.g. B cell related immune compromised patients who continuously excrete PV after infection for a long period of time)

and block the transmission of cVDPV. It will also be given to the community where PV outbreak occurs so as to protect the population from acute PV infections and prevent PV pandemic. Simulation models suggest that PV antivirals can significantly benefit the eradication and maintain a polio-free world[21, 49]. The models indicate that a robust PV antiviral with 90% effectiveness can significantly reduce the risk of prolonged viral shedding in immune-compromised patients[21] and contribute to the health and economic benefits[49]. As a result, there is an urgent call for PV antiviral development.

1.3.1. Capsid Binders That Inhibit Viral Entry

Capsid binder is one of the most classic categories in antiviral. Capsid binders usually target the hydrophobic pocket of PV receptor binding domain and inhibit conformational change that is required for the viral entry and uncoating of viral genome[50, 51]. Several compounds under this category, such as Pleconaril (WIN63843), Vapendavir (BTA-798), and Pocapavir (V-073) have been developed and applied in clinical trials.

Pleconaril is one of the WIN compound series that confers broad-spectrum antiviral effect[52]. It confers IC_{50} of 0.03μM to all of the clinical isolates of EV serotypes and IC_{90} less than 0.18μM. It also protected animals from lethal coxackievirus infection. However, it obtained controversial results in the clinical trials, with potent inhibitory effects against viral infection but indication of toxicity among the involved patients[53-55]. Currently Pleconaril is under phase II clinical study for enteroviral infection in neonates, but the results has not been released since 2013[56]. Another WIN compound, WIN51711, has also been reported to inhibit

poliovirus replication *in vitro*[57], however, no clinical study information is available regarding this compound.

Vapendavir is another capsid binder which is originally developed for rhinovirus[58, 59]. Currently a phase IIb clinical study has been completed using Vapendavir to treat rhinovirus infected asthma patients, and another phase IIb clinical study has been launched in 2015 to test it on moderate-to-severe asthma patients with rhinovirus infection[60].

Pocapavir was intentionally developed by ViroDefense Inc. for the Global Polio Eradication Initiation and showed antiviral effects *in vitro*[61, 62]. It has been tested in a phase II clinical trial in Sweden in 2012, however, no further information has been released[63]. Recently it was used to treat a coxsackievirus infection case in neonate[64].

Although capsid binder is the most classic and extensively studied category in PV antiviral development, due to the low fidelity of PV RdRp, resistant strains rapidly arise along with the treatment. For example, in a study to characterize PV variants resistant to Pocapavir, all variants exhibited single amino acid mutation in either VP1 or VP3 region[65]. These mutants were stable when cultured without the presence of drug treatment and conferred identical growth pattern comparing with the WT strain. One possible strategy to overcome this problem is combined therapy using another drug with different mechanism of action. For example, Vapendavir and Pocapavir were tested in combination with a 3C protease inhibitor Compound 1

(AG7404)[66]. In that study, although shared resistance was observed against both of the two capsid binders, the application of Compound 1 in addition to Vapendavir or Pocapavir conferred inhibitory effects against resistant strains. Therefore, antiviral with different targets are useful to increase efficacy and reduce the risk of resistance.

1.3.2. Viral Protease Inhibitors

During viral replication, the RNA genome of poliovirus is first translated into one single polypeptide and processed into mature viral proteins by viral proteases, such as $2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$[22, 67]. Each protease recognizes a unique peptide site and plays a critical role in the viral life cycle. In addition to that, the viral protease also cuts cellular factors that are important for viral sensing, so as to block antiviral signaling and immune responses[22]. As a result, PV proteases are attractive targets for antiviral development.

Two $3C^{pro}$ inhibitors, Ruprintrivir (AG7088) and Compound 1 (AG7404) have been tested in clinical trials. Ruprintrivir was originally designed based on the structure of rhinovirus to form stable covalent binding with $3C^{pro}$, so as to inhibit viral replication by irreversibly blocking viral protease activities[68]. Ruprintrivir also overcame viral sensing inhibition induced by $3C^{pro}$ and boosted robust antiviral signaling and proinflammatory cytokine production in the host cell[69]. Although Ruprintrivir exhibited promising inhibitory effect against rhinovirus and EV replication in preclinical studies [70-72], it failed to achieve significant reduction in the viral load in clinical trials. As a result, further development was suspended for Ruprintrivir[73]. Compound 1 was developed based on Rupintrivir with improved bioavailability [73, 74]. It conferred inhibitory

effect against EV $3C^{pro}$ but not foot-and-mouth disease virus (FMDV)[75]. Although Compound 1 displayed potent antiviral activity in vitro and completed phase I clinical study, its clinical development was discontinued[76].

1.3.3. Viral RNA Replication Inhibitor

1.3.3.1. $2C^{ATPase}$ Inhibitor

PV 2C protein has been reported to be involved in various steps in the viral life cycle. 2C protein plays important role in RNA replication initiation[77, 78]. It also induces host cell membrane rearrangement and modifies membrane trafficking machinery that favors viral replication[35, 37, 79]. Its interaction with viral capsid proteins VP3 is also required for morphogenesis and encapsidation of infectious virion[47, 48, 80]. In addition, 2C protein also interacts with host factors to inhibit antiviral immune responses. For example, it binds to Protein Phosphatase 1(PP1) and blocks IKKβ phosphorylation in the NF-κB signaling pathway[81, 82]. Although several 2C protein inhibitors have been identified, their mechanisms of action are not clearly elucidated, and none of the 2C protein inhibitors have been tested in clinical trial.

Guanidine hydrochloride (GnHCl) is a well-known PV replication inhibitor that targets 2C protein[23]. It inhibits the initiation of negative-strand RNA synthesis through 2C protein[77], and the inhibitory effects can be diminished by either of two single amino acid mutation on 2C protein[83]. Further studies reveals that GnHCl also interferes with other functions of 2C protein such as its binding to the cellular membranes[84] and its ATPase activities[85]. Interestingly, GnHCl dependent mutants were found when poliovirus was continuously passaged

in the presence of the drug[86, 87], indicating that GnHCl might interfere with the conformational change of 2C protein to perform its functions. The development of GnHCl dependent mutants also suggests that although 2C protein is relatively conserved and involved in multiple steps of the viral life cycle, it might not be an ideal target for drug development.

Two compounds with similar structures, HBB and MRL-1237 were identified to inhibit viral RNA replication of poliovirus and coxsakievirus, but is not effective against rhinovirus or FMDV[88-90]. Analysis of resistant strains found mutations accumulated in the 2C protein, suggesting that it might be the target of the compounds, but the precise mechanism is not clear[91-93]. Dependent strains are also identified for these compounds[93]. Another 2C protein inhibitor, TBZE-029, inhibits coxsackievirus and EV 68 replication and mapping of resistant mutations also leads to the 2C protein[94, 95]. It confers robust inhibitory effect against the EV species with an IC_{50} of 1.2μg/mL. Interestingly, HBB, MRL-1237 and TBZE-029 shared some resistant mutations, indicating that they might have similar mechanisms of action[94].

1.3.3.2. 3A Protein Inhibitor

3A protein is another multifunctional viral protein that plays a critical role in EV replication. It binds to the cellular membrane and recruits host factors that are essential to form RNA replication complex with $3D^{pol}$ [96]. Although many 3A protein inhibitors have been reported, none of these have been tested in clinical trials.

Enviroxime was originally developed for rhinovirus infection with IC_{50} of 0.02 μg/mL[97]. It was also test on 11 EV70 and 15 cocksackie A24 isolates with IC_{50} ranging from 0.01-0.65 μg/mL[98]. Further studies suggested that viral RNA replication was inhibited by Enviroxime[99]. Analysis of the resistant mutants revealed that all of them shared one single amino acid mutation in the 3A protein and site-directed mutagenesis confirmed that the mutation is sufficient to generate resistance to Enviroxime, suggesting that the 3A protein is likely the target for Enviroxime[100, 101]. Functional assays confirmed that Enviroxime specifically inhibits positive-strand RNA replication in poliovirus infected cells, which is consistent with the mutagenesis analysis[100]. However, site-directed mutagenesis approaches failed to demonstrate direct binding between Enviroxime and 3A or several other viral proteins, indicating that Enviroxime could interact with the RNA replication complex containing multiple viral and host proteins[102]. Enviroxime and several analogues have been tested on animals or patients against rhinovirus infection, but the results were not ideal. Although some of the symptoms were reduced in the patients after Enviroxime treatment, no significant improvement was achieved in the tested groups comparing with the control groups, along with high level of side effects[103-106]. The poor effectiveness of Enviroxime *in vivo* is partly due to the low bioavailability and rapid removal of the drug after administration. Enviroxime also failed to prevent rhinovirus infection in healthy volunteers when the drug was applied before viral challenge[107].

Itraconazole, which was originally approved by FDA as a triazole antifungal drug, was found to inhibit EV68, EV71, poliovirus and coxsackievirus replication *in vitro*[108]. It conferred IC_{50} of 1.2 μM to EV 71. Mutations V51L and V75A in EV71 3A protein conferred resistance to Itraconazole treatment. Interestingly, the mutant strains do not exhibit cross-resistance between Itraconazole and posaconazole or enviroxime-like compound GW5074, which are also known to inhibit 3A protein. This observation indicates different binding sites for these compounds on 3A protein. Recent studies reveal host factors oxysterol-binding protein (OSBP) and OSBP-related protein 4 (ORP4) as targets of Itraconazole[109]. Itranconazole was also identified in our PV antiviral screening assay, however, it requires high concentrations to protect HeLa cells from the cytopathic effect of poliovirus and exhibits potential toxicity to the host cells at that concentration. Therefore, Itraconazole could be a leading construct for drug development, but further modification is required to improve its efficacy and reduce toxicity.

Several other EV and rhinovirus 3A inhibitors have been recently reported, such as TTP-8307, which induced cross-resistant mutations with Enviroxime in 3A protein hydrophobic domain[110]. Another Enviroxime-like reagent, T-00127-HEV1 was found to inhibit phosphatidylinositol 4-kinase III beta (PI4KIIIβ), indicating that this host factor might be essential for the formation of the viral RNA replication complex[111]. Other PI4KIIIβ inhibitors have been reported to exhibit antiviral effects, such as GW5074 and PIK93[111]. Considering the fact that these inhibitors suffered from cross-resistance, they are likely to perform similar

mechanism of action in blocking viral replication. This could also be one of the reasons why these drugs are halted in preclinical investigations.

1.3.3.3. RNA-dependent-RNA-polymerase (RdRp) $3D^{pol}$ Inhibitor

PV RdRp $3D^{pol}$ is an attractive target for PV antiviral development. As a conserved part, RDRP is considered as a potential target for broad spectrum antivirals. $3D^{pol}$ inhibitors can be divided into two classes, the nucleoside analogs and non-nucleoside analogs[112]. For example, the nucleoside analog Ribavirin is reported to induce lethal mutagenesis in PV and thus inhibit viral replication[113, 114]. In consistent to this observation, resistant strains against Ribavirin exhibited one single amino acid mutation in RdRp and conferred increased fidelity when cultured in the absence of the drug [115]. Another nucleoside analogue, 2'-C-methylcytidine (2'-C-MetCyt), exhibited an EC_{50} of 6.4±3.8μM and EC_{90} of 10.8±5.4μM against FMDV-induced cytopathic effect. In addition to that, a 5-nitrocytidine cytidine analogue was reported to inhibit PV and coxsackievirus B3 (CVB3) infection, and the 5-nitrocytidine triphosphate inhibited RdRp activity with a K(d) of 1.1±0.1 μM. This drug achieved 30-fold better inhibitory effect than Ribavirin at the concentration of 2mM.

Non-nucleoside analogs, on the other hand, bind to $3D^{pol}$ with different mechanisms. For example, Amiloride competes with nucleoside triphosphates and Mg^{2+} for the binding site on the $3D^{pol}$ so as to inhibit viral RNA replication[116]. Further investigations revealed that Amiloride also blocks the binding site of VPg on $3D^{pol}$, which is critical for the uridylyation of VPg. As a result, the initiation of the viral RNA replication is inhibited due to the lack of VPg-

pUpU primers[117]. Interestingly, $3D^{pol}$ mutants with increased fidelity showed resistance to the treatment of Amiloride, indicating that Amiloride may also serve as a mutagenic reagent[118]. Another compound, GPC-N114 was recently identified to inhibit the elongation activity of $3D^{pol}$, and crystal structural analysis revealed that GPC-N114 competes for the RNA-binding channel with the template-primer complex so as to block the initiation of replication[119].

In addition to the compounds listed above, there are several other RdRp inhibitors whose mechanisms of action are not clear. Aurintricarboxylic acid (ATA) was found to specifically inhibit the RdRp activity but does not affect viral polyprotein translation or $3C^{pro}$ activity[120], however, its exact mechanism of action has not been elucidated. Gliotoxin, a fungal metabolite, was identified as a specific viral RNA elongation inhibitor of both plus and minus strand synthesis[121-123]. Gliotoxin has been tested on a monkey model for poliovirus infection, however, it showed high level of toxicity in the animals[124]. DTrip-22, which was identified as a RdRp inhibitor by its resistant strains, inhibits viral RNA elongation by preventing rNTPs from entering the cavity of RdRp[125]. Similarly, BPR-3P0128 reduced RNA accumulation and inhibits EV71 replication with EC_{50} of 2.9 nM, but its mechanism of action is not clear[126].

Despite of numerous candidates identified and the commitment of enormous effort, none of the RdRp inhibitors have been tested in clinical trials.

1.3.4. Morphogenesis / Encapsidation Inhibitors

After the viral polypeptide is translated and processed into functional structural and non-structural proteins by various viral proteases, the assembly of the viral particle is the critical step in the viral life cycle that involves several viral and host factors[22, 45]. The proper assembly of viral particles requires the help of host factors such as heat shock protein 90 (Hsp90) and Hsp70 as chaperones. Host factor Glutathione (GSH) is also important to stabilize the precursor and the mature viral particle during assembly[45]. The 2C protein, as we described before, is also involved in the assembly of the viral particles by direct interaction with viral capsid protein VP3[47, 48, 80].

There are few drugs available targeting this step. For example, Hsp90 inhibitor Geldanamycin suppressed PV, RV and CV replication in cell cultures[46]. Geldanamycin binds to the ATP/ADP binding pocket on Hsp90, indicating that it might inhibit viral replication by interfering with the folding and processing of the viral structural proteins[127]. However, no resistant strain was found against Geldanamycin treatment, indicating it is difficult for PV to bypass the requirement of Hsp90 in viral particle assembly[127]. L-Buthionine Sulfoximine (BSO), which specifically inhibits the biosynthesis of GSH, has also been shown to suppress RV, EV and CV strains in cell cultures[128-131]. Empty capsids, instead of mature virions, were recovered from the BSO treated cells, despite the fact that viral RNA replication was not affected[129]. Consistent with these observations, TP219, another reagent that directly binds to GSH also conferred robust inhibitory effect on the assembly of PV and CV[130].

5-(3,4-dichlorophenyl) methylhydantoin (Hydantoin) inhibited the assembly of PV viral particle by inhibiting post-translational cleavage of the viral structural proteins in the cell-free system as well in the infected cells[132]. However, the inhibitory effect of Hydantoin is reversible, indicating that it might bind to the precursor of the structural proteins to block or delay the cleavage[132]. On the other hand, resistant mutants to Hydantoin were recovered with single amino acid mutation in 2C protein coding region. Since 2C is also involved in the assembly of viral particles, this could be an additional target of the drug[133].

In summary, although compounds targeting various steps in the PV life cycle have been discovered, few of them have been tested in the clinical trials. Most of the compounds fail to achieve robust inhibitory effect *in vivo* while some of the compounds are aborted due to high levels of adverse reactions. The only compounds remaining in the pipeline are the three capsid binders as we described before. Although they have been tested in phase II or phase IIb clinical trials, no results or future development plans have been released. As a result, there is an urgent call for novel formulation of PV antiviral drugs.

Chapter II. Material and Methods

2.1. Generation of Poliovirus Construct Expressing Gaussia Luciferase (PV-GLuc)

Poliovirus attenuated vaccine Sabine strain complete cDNA was obtained from Vignuzzi Lab at Institut Pasteur, France. *Gaussia Luciferase* (*GLuc*) gene was cloned from pSV40-GLuc Control Plasmid (New England BioLabs, MA). *GLuc* was inserted into the end of the VP1 region by overlap extension PCR method [134] using following primers: 5'-GGATGGTACGCTTACACCCCTCTCCACCAAGGACTTAACGA-CTTACGGTTTTGGTCATGGCGGAGGTGGGGGAGGTGAATTCATGGGAGTCAAAGTTCTGTTTGCCC-3', and 5'-CCGCTTTGTTTTGGTGTCCGAATCCATATGTGGTCAGATCCTCGAGGTCACCACCGGCCCCCTT-3'. The primers contain overlap with both *GLuc* gene and poliovirus cDNA VP1 region. Briefly, the *GLuc* gene was first cloned by PCR amplification using the described primer pier. The PCR products were purified using gel extraction kit (Qiagen, CA) and used as mega-primer in the second round of PCR amplification to insert *GLuc* gene into poliovirus cDNA. The insertion of *GLuc* was confirmed by PCR extension using primers: 5'-CCCGTGGTGACATCTGA-GTA-3' and 5'-CGAGAAATGGGACGACTACAC-3'.

The PV-GLuc plasmid was passed in *E. coli* SURE 2 strain (Agilent Technologies, CA) to avoid mutation and deletion in the plasmid. The PV-GLuc cDNA was *in vitro* transcribed into RNA using T7 RiboMAX™ Express Large Scale RNA Production System (Promega, WI). The RNA product was used to transfect HeLa cells through electroporation. The transfected HeLa cells were incubated in DMEM culture medium with 10% FBS (Gibco, MA) at 37°C with 5% CO^2 for three days until complete cytotoxicity was observed. The culture medium was centrifuged to remove the cell fragments and the

supernatant containing PV-GLuc replicons were collected. The supernatants were concentrated using 100 filter KD (Millipore, MA) by centrifuging at 4500rpm for 30min. The filtered supernatant were diluted by DMEM culture medium containing 10% FBS into the final volume of 3mL and kept at -20°C as stock vial.

2.2. Cell-based High-throughput Screening Assay

The screening assay was performed on 96-well or 384-well plates using automated workstation (PerkinElmer, MA). The compound library screened is Prestwick Chemical Library (Prestwick Chemical, CA) For 96-well plate, 5×10^4 cells were inoculated per well with 100µL complete culture medium (DMEM culture medium containing 10% FBS). Cells were incubated overnight at 37°C with 5% CO^2 to form cell monolayer. After incubation, culture medium was removed and replaced by 60µL fresh complete culture medium containing designated compound and 40µL viral solutions. For 384-well plate, 10^4 cells were inoculated per well with 30µL complete culture medium and incubated overnight at 37°C with 5% CO^2. After incubation, culture medium was removed and replaced 30µL fresh complete culture medium containing 10µM designated compound using gridding tool (V&P Scientific, CA). After that, 10µL viral solution containing 2×10^5 PFU PV-GLuc was added per well and incubated for another two days. The plates were kept in 37°C with 5% CO^2 for another two days. Before testing the viral replication, virus were inactivated by heating at 56°C for 30min.

Viral replication was examined by reading the luminescent signals using BioLux *Gaussia* Luciferase Assay Kit (New England BioLabs, MA). A duplicate plate not containing virus was conducted to test the cytotoxicity of the compound to the HeLa cells. Cell viability was measured by intra-cellular ATP level

using CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI)[135]. For the set of plates with PV-GLuc infection, each compound's inhibitory effect on poliovirus replication (E_c) was calculated as following: $E_c = 1 - L_c / \mu_n \times 100\%$, where μ_n is the mean of negative controls with PV-GLuc only on the specific plate, while L_c is the read of luminescence signal for the specific compound. For the set of plates without PV-GLuc infection, the cell viability rate for each compound on HeLa cells (V_c) was calculated as following: $V_c = L_c / \mu_p \times 100\%$, where μ_p is the mean of positive controls with HeLa only without any compound treatment, while L_c is the read of luminescence signal for specific compound.

Z-factor of the high-throughput screening assay is calculated as following:

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

σ_p and σ_n are the variances of positive and negative controls, while μ_p and μ_n are the means of positive and negative controls.

2.3. Plaque Assays

10^6 HeLa cells were inoculated each well on 12-well plate with 2mL complete culture medium. The plate was kept in 37°C with 5% CO^2 overnight to form cell monolayers. 2×DMEM culture medium and 0.6% agarose were prepared before the experiment. To prepare 2×DMEM culture medium, 27g DMEM powder (Sigma, MO) and 2.4g sodium bicarbonate (Thermo Fisher, MA) were dissolved and mixed in 1L sterile water. After that, 40mL FBS (Gibco, MA) and 20mL Penicillin/Streptomycin (Gibco, MA) were added and filtered by 0.22μM filter (Millipore, MA). To prepare 0.6% agarose, 0.6g ultra-pure agarose

powder (Invitrogen, MA) was dissolved in 100mL sterilized water by heating. 2×DMEM culture medium was kept in 37°C water bath prior to the experiment, while 0.6% agarose was kept in 40°C water bath. To infect the HeLa cells, the culture medium was removed and the cell monolayers were washed once with PBS (Gibco, MA). 200µL viral solution with designated PFU were added to the center of the cell monolayers and incubated at 37°C with 5% CO^2 for 10min to allow virus to infect the HeLa cells. After incubation, extra-cellular virus was removed by washing once with PBS. 1mL 2×DMEM culture medium (37°C) and 1mL 0.6% agarose (40°C) were mixed and immediately added to cover the cell monolayers. The plate was incubated at 37°C with 5% CO^2 up to three days until cytotoxicity effects were observed. The covers were discarded. The cell monolayers were washed once using PBS and fixed by 2% formaldehyde at room temperature for 10min. After fixation, the cell monolayers were washed once using PBS and stained by crystal violet solution (Sigma, MO) for 10min at room temperature. After staining, the cell monolayers were carefully washed by water to remove extra staining solution and air-dried to read the plaques.

2.4. qRT-PCR

Viral RNA was extracted using RNeasy kit (Qiagen, CA) and converted into cDNA using MEGAscript T7 Kit (Thermo Fisher, MA). To perform qRT-PCR, 1µL cDNA was loaded with 10µL TaqMan Universal PCR Master kit (Applied Biosystem, MA), 1µL primers piers 5'- AGGTCAGATGCTTGAAAGC-3' and 5'-TCCACTGGCTTCAGTGTT-3', 1µL TaqMan MGB probe 5'-CACAGTCCGTGAAACGGTGG-3' (Life Technologies, MA) and 7µL ddH_2O. The mixture was then loaded onto Applied Biosystem Standard 7300 Real Time PCR system (Thermo Fisher, MA). Thermal cycle started with 50°C for 2min following

by 95°C for 10min, then repeated 40 rounds at 95°C for 15s, and finished by 60°C for 1min. Samples containing 10^6, 10^5, 10^4, 10^3, 10^2 copies of viral RNA were used as standard controls.

2.5. *In vitro* Elongation Assay

The *In vitro* Elongation Assay was performed as described before [136]. Briefly, recombined poliovirus RNA dependent RNA polymerase $3D^{pol}$ and single-stranded RNA template was incubated with normal A/U/G-TP with or without CTP or the triphosphate form of Gemcitabine, 2',2′-difluoro-2'-deoxycytidine triphosphate (dFdC-TP, BOC Science, CA). $3D^{pol}$ was kindly provided by Dr. Peersen (Colorado State University, Ft. Collins, CO) [137, 138] and viral cDNA lacking VP1 region was kindly provided by Dr. Flanegan (University of Florida, Gainesville, FL). The viral cDNA was digested by restriction enzymes SpeI or AvrII (New England BioLabs, MA), and transcribed into ssRNA in vitro using T7 RiboMAX™ Express Large Scale RNA Production System (Promega, WI). Recombined $3D^{pol}$ and ssRNA template were first mixed in 4μL initiation buffer containing 10μM $3D^{pol}$ RNA, 0.4μM RNA templates, 500μM of normal r-A/U/G-TP (Applied Biosystem, MA), 50mM NaCl (Thermo Fisher, MA), 1.5mM $MgCl_2$ (Fisher, MA), 4mM TCEP (Pierce™ TCEP-HCl, Life Technologies, MA), 50mM HEPES(pH6.5, Invitrogen, MA) and 1 unit/μL RiboLock RNase inhibitor (Life Technologies, MA). 500μM of normal r-CTP was added in the positive control, while no r-CTP or dFdC-TP was added in the negative control. Designated amount of r-CTP and/or dFdC-TP were added in the experimental groups. The mixture was incubated at room temperature for 15min to form elongation complex. Following that, the elongation complex was locked by adding 16μL elongation buffer containing 356mM NaCl, 200μM r-A/U/G-TP, 1.5mM $MgCl_2$, 4mM TCEP and 50mM HEPES (pH 6.5). The mixtures were then incubated at room temperature for another 2 hours. After incubation, the reaction was quenched by mixing with 16mM

EDTA and the RNA products were extracted using phenol-chloroform-isoamyl alcohol solution (Sigma, MO). To extract the RNA, 1 volume sample was first mixed with 1volume of phenol-chloroform-isoamyl alcohol (125:24:1, pH 4.5), vortex for 1 min and spin at 13,000rpm for 2min. The upper, aqueous phase was transferred to a fresh tube and mixed with 1 volume of chloroform-isoamyl alcohol (24:1), vortexed for 1 min and centrifuged at 13,000rpm for 2 min. The upper, aqueous phase was then transferred to a new tube and mixed with 0.1 volume 3M sodium acetate (pH5.2, Life Technologies, MA) and 1 volume of isopropanol. The mixture was placed on ice for 15min and centrifuged for 10min. The supernatant was carefully removed and the pellet was washed once with 1mL 70% ethanol. The pellet was air-dried and dissolved in ddH_2O. The RNA products were then examined by gel electrophoresis and stained by ethidium bromide.

Chapter III. Setup and Optimize The Cell-Based High-Throughput Screening (HTS) Anti-Polioviral Assay

3.1 Introduction

3.1.1. HTS Technologies in Drug Discovery

Integrating advanced technologies including automated operation platform, highly sensitive detection system, large-scale compound libraries, and data processing methods, HTS is one of the most favorable strategies in the drug discovery field[139]. The concept of HTS can be traced back to the first invention of microtiter plates by Dr. Gyula Takatsky during 1950s, but the maturation of HTS as a discipline of biological studies happened after the appearance of automated-operation system and detection system after 1990s[140]. Taking advantage of the advanced technologies, HTS greatly miniaturized the assay scale by increasing well density from 96-well to 1536-well plates and reducing the volume of compounds from 200µL to less than 10µL per test[141]. These achievements enable us to rapidly test over 10,000 compounds per day, or even over 100,000 compounds per day by ultra-high-throughput screening (uHTS). In addition to that, the small volume needed for the test assay also enables us to study samples that are difficult to obtain, such as high-quality natural products[142]. Besides the application in the pharmaceutical industry, HTS is also widely used to target complicated biological and biophysical studies in academia[141].

In this study, we proposed to develop a novel cell-based PV HTS assay, which can be used for antiviral development as well for basic research of PV virology e.g. viral entry, viral replication and assembly. This assay can also be applied to study the host factors that are crucial for the life cycle of the virus using siRNA methods. Together, this cell-based PV HTS assay should be a useful platform for various studies.

3.1.2. Detection Technologies

One of the key factors for the HTS system is the rapid signal detection strategy. Since the HTS system is continuously dealing with a large scale of testing libraries, it is essential to have a detection technology with short readout time and high reliability between each single test. Many methods have been developed for rapid detection in the HTS assays, e.g. fluorescence or luminescence based technologies, nuclear-magnetic resonance, affinity based technologies, surface plasmon resonance and microarray technologies[139]. Among these, fluorescent or luminescent based methods, either by direct detecting light signal or by measuring the interaction between two fluorophores, are the most widely accepted ones.

Fluorescent protein is one of the most widely used reporters in the biological studies. Fluorescent protein has been used to study gene expression since the 1990s[143]. Fluorescent protein has many outstanding features that make it a favorable reporter for biological studies. First, it does not require the addition of substrate or cofactor to generate light signals. It also confers low toxicity to the host cells and can be continuously produced throughout the incubation. Besides that, each fluorescent protein has a unique excitation and emission

wavelength, making it perfect to distinguish specific signals of interested. In combination with confocal microscopy, it can also be used to detect the location of the fusion protein in the live organs[144].

Fluorescence protein has been used in various drug screening studies. For example, green fluorescence protein (GFP) expressing vectors was transfected to *Mycobacterium tuberculosis* (Mtb) and the decrease in the fluorescence signals was used to evaluate the effect of drugs against Mtb replication[144-146]. Similarly, HIV constructs carrying GFP were developed and the antiviral effects can be measured by the reduction of the fluorescent signals in the infected cells[147]. A recombinant human cytomegalovirus construct expressing GFP was also developed for antiviral screening assays[148]. The fluorescent protein can also be transfected into cell lines under the control of viral promoters and used to screen for compounds that inhibit this specific target[149].

Small fluorescent molecules other than fluorescent proteins are also widely used in the drug discovery. Owing to the fluorescence polarization characteristic, the binding of the small fluorescent molecule to other molecules can be detected by the changes in rotation rate. As a result, these small fluorescent molecules are perfect tools for the detection of direct interaction between compounds and their targets[150]. For example, in a study to screen for Dengue viral-entry inhibitors, Fluorescein isothiocyanate (FITC) was conjugated to a peptide recognizing a hydrophobic pocket on the viral envelope, and the inhibitory compounds were identified by

detecting reduction in the fluorescent polarization signals[151]. Similarly, a fluorescent-labeled peptide was used in a search for inhibitors that block the nucleotide binding site of HIV-1 Rev[152].

Beside fluorescence polarization, fluorescence resonance energy transfer (FRET) method can more precisely detect the direct interaction between two molecules[153]. Two fluorophores are included in the FRET system serve as donor and acceptor. The acceptor fluorophore has an excitation wavelength which overlaps with the emission wavelength of the donor. The energy released from the donor fluorophore will be directly transferred to the acceptor when these two fluorophores are close to each other (e.g. 10nm or less). As a result, the emission signal from the acceptor instead of the donor fluorophore will be observed. This method is usually used to screen for kinetic inhibitors of enzymes. In these cases, enzyme and substrate are labeled with donor or acceptor fluorophores, and the changes in the emission wavelength reflects dissociation of the duplex[154, 155].

Luminescence-based reporter is another tool widely used in HTS assays for biochemical and biomedical studies. Like fluorescence, luminescence-based reporter is measured by the light signal generated through the catalysis of luciferin substrate by luciferase protein in the presence of Mg^{2+} and ATP. Unlike fluorescence, which acquires excitation energy through absorption of photons with a specific wavelength, luminescence uses the chemical energy from catalysis of the substrate to generate a light signal. As a result, a luminescence signal usually

confers higher signal/noise rate then fluorescence and is less likely to be interfered by non-specific signals in the environment[156]. Many luciferases from different species have been identified and are commercially available, such as firefly luciferase[157], *Renilla* luciferase[158], *Gaussia* luciferase[159], etc. For example, firefly luciferase has been used to screen for viral entry inhibitors for HIV, influenza virus and Ebola virus[160-162]. These luciferases are used alone or in combination to each other in HTS assays screening for inhibitors of certain targets[157, 163, 164]. In addition to that, since the activity of the luciferase relies on the chemical energy from ATP, it has also been used to measure the ATP concentration, which can be used as a parameter for cell proliferation and viability[135].

3.1.3. Development of PV Replicon Expressing Luciferase Reporter

Many efforts have been devoted in developing poliovirus constructs that express various exogenous proteins. Due to the high immunogenicity and simplicity in manipulation, poliovirus has been proposed as the vaccine vector expressing various antigens. Taking advantage of the viral proteases that process the polyprotein into functional viral proteins, the inserted exogenous proteins can be released by introducing an artificial viral protease recognition site. There are two strategies to introduce foreign genes into the poliovirus genome. The first one is to replace the P1 region, which encodes viral structural proteins by the foreign protein. For example, VP2 and VP3 encoding sequences in the P1 region were replaced by human immunodeficiency virus-1 (HIV-1) Gag and Pol to serve as HIV vaccine constructs[165, 166]. In this case, the modified viral construct was considered as minireplicon, which could replicate only when helper virus or plasmid expressing the missing parts were co-transfected. The RNA

genome of the minireplicon is relatively stable, however, the efficiency of the chimera virus replication is restricted due to the requirement of the helper virus or plasmid.

Another strategy is to insert the exogenous gene directly while keeping the original viral elements intact. Usually the foreign gene is inserted either at the 5'-end of the P1 region or in the middle of the P1 and P2 regions. For example, antigens from hepatitis A virus (HAV), hepatitis B virus (HBV), simian immunodeficiency virus (SIV), HIV-1, rotavirus, etc. have been inserted into the PV replicon using this strategy to generate vaccine constructs[167-171]. This strategy has been used to express exogenous proteins other than antigens, such as bacterial chloramphenicol acetyltransferase (CAT), firefly luciferase and green florescent protein (GFP)[172, 173]. It showed that the recombined viral construct continuously express large amount of exogenous proteins up to five passages, and the activities of the foreign proteins can be detected in the cell culture after viral replication. However, for the large insert, such as the 67kDa firefly luciferase, it was rapidly depleted from the viral RNA genome after 20-hour of incubation[172]. These observations indicate that although the second strategy does not require the presence of helper virus or plasmid, the RNA genome of the recombined replicon is unstable and the virus has the tendency to deplete the insertion. Therefore, this strategy is not suitable for long-term protein expression, but it could be useful for transient studies, such as a PV reporter replicon for HTS screening assay.

A PV replicon containing a proper reporter gene is essential for the HTS anti-polioviral screening system and is very useful to study the pathology of PV infection *in vivo*. We attempted to develop two recombined PV replicons expressing GLuc or GFP reporters. The development of the GFP expressing construct was not successful, probably due to the large size of the reporter gene inserted. The GLuc expressing construct (PV-GLuc), on the other hand, was successfully developed.

GLuc is the smallest luciferase protein known in the world with the size of only 19kDa[174]. The 558b coding sequence makes it suitable to be inserted into the tiny PV genome. It was reported by another group that the GLuc can be successfully inserted into the PV genome and the replicon expressing GLuc can replicate in the HeLa cells[175]. Despite the small size of the insertion, the GLuc reporter gene was still rapidly depleted from the viral construct after four passages, leading to the decrease in the luminescent signal generation. Interestingly, the luminescent signal came back after eight passages and deep sequencing of the recovered viral genomes revealed a mixture of mutants containing various partial deletions of the *GLuc* reporter gene or viral elements[175].

We hypothesize that although the *GLuc* reporter gene will be depleted after continuous passages and eventually leads to a mixture of partial deletion mutants, however, it could still be useful for a transient replication in a limited period of time. Therefore, we validate the PV-GLuc construct and its application in the HTS assay.

3.2. Generate and Define Recombinant Poliovirus Producing Gaussia Luciferase (PV-GLuc).

GLuc reporter gene was introduced into the genome of the attenuated Poliovirus vaccine Sabin strain to generate PV-GLuc construct (Fig. 1). The *GLuc* was inserted at the 3'-terminal of the poliovirus VP1 by overlap extension PCR cloning strategy[134]. Given that there is a natural poliovirus 2A protease recognition site at the end of VP1, an additional 2A protease recognition site was added in front of the GLuc reporter gene. As a result, the GLuc protein was cut by the viral 2A protease and released from the viral polyprotein after translation.

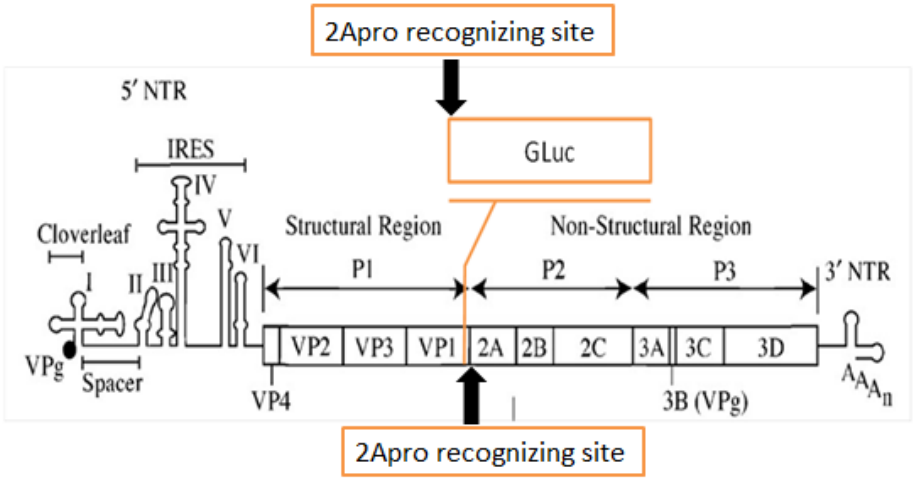


Figure 1. Illustration of the PV-GLuc construct.
GLuc reporter gene was introduced into the poliovirus genome at the end of VP1 coding sequence. An original $2A^{pro}$ recognition site is located at the end of VP1 coding sequence. An artificial $2A^{pro}$ recognition site was introduced in front of the reporter gene to facilitate the release of GLuc from the polypeptide after translation.

To examine the characteristics of the PV-GLuc replicon, HeLa cells were infected by either PV-GLuc or wild type PV Mahoney (WT-PV). The PV-GLuc was able to generate plaques on HeLa cells two days after incubation, although the plaques were smaller than those generated by WT-PV (Fig. 2a). While PV-GLuc replicated with similar kinetics as WT-PV up to 72 hours post infection, the titers were significantly lower: the Mahoney strain generated more than 10^7 PFU/ml after 72-hour incubation, while PV-GLuc achieved over 10^6 PFU/ml (Fig. 2b). Indeed, it is well known that Sabin 1 strain replicates with titers one order of magnitude below the wild type Mahoney strain[176]. qRT-PCR assay also revealed a continuous increase of both Sb-GLu and WT-PV viral RNA copy numbers up to 72 hours post infection (Fig. 2c). Together, these results indicate that the PV-GLuc replicon is able to replicate robustly and induce cytopathic effects on HeLa cells in a pattern quite similar to WT-PV up to 72 hours post infection.

GLuc was produced along with the replication of PV-GLuc and accumulated in the cytoplasm. After incubation, cells were lysed to release GLuc into culture medium. Heating at 65°C for 20 min completely inactivated PV-GLuc virus but did not affect the activity of GLuc (data not shown). Consistent with the plaque assay and qRT-PCR results, luminescent signal was observed in the PV-GLuc samples and increased throughout the period of incubation (Fig. 2d). 2mM Guanidine Hydrochloride (GnHCl) served as control as it could inhibit poliovirus replication by interfering with viral 2C protein. The luminescence signal did not increase when 2mM GnHCl was present in the culture medium (Fig. 2d), indicating that the GLuc production was inhibited when viral replication was blocked. In summary, while the PV-GLuc virus was developed and

validated for its ability to replicate and generate the luminescent signal as a consequence of viral replication, levels of luminescent signals could be used to reflect PV-GLuc replication in the HeLa cells.

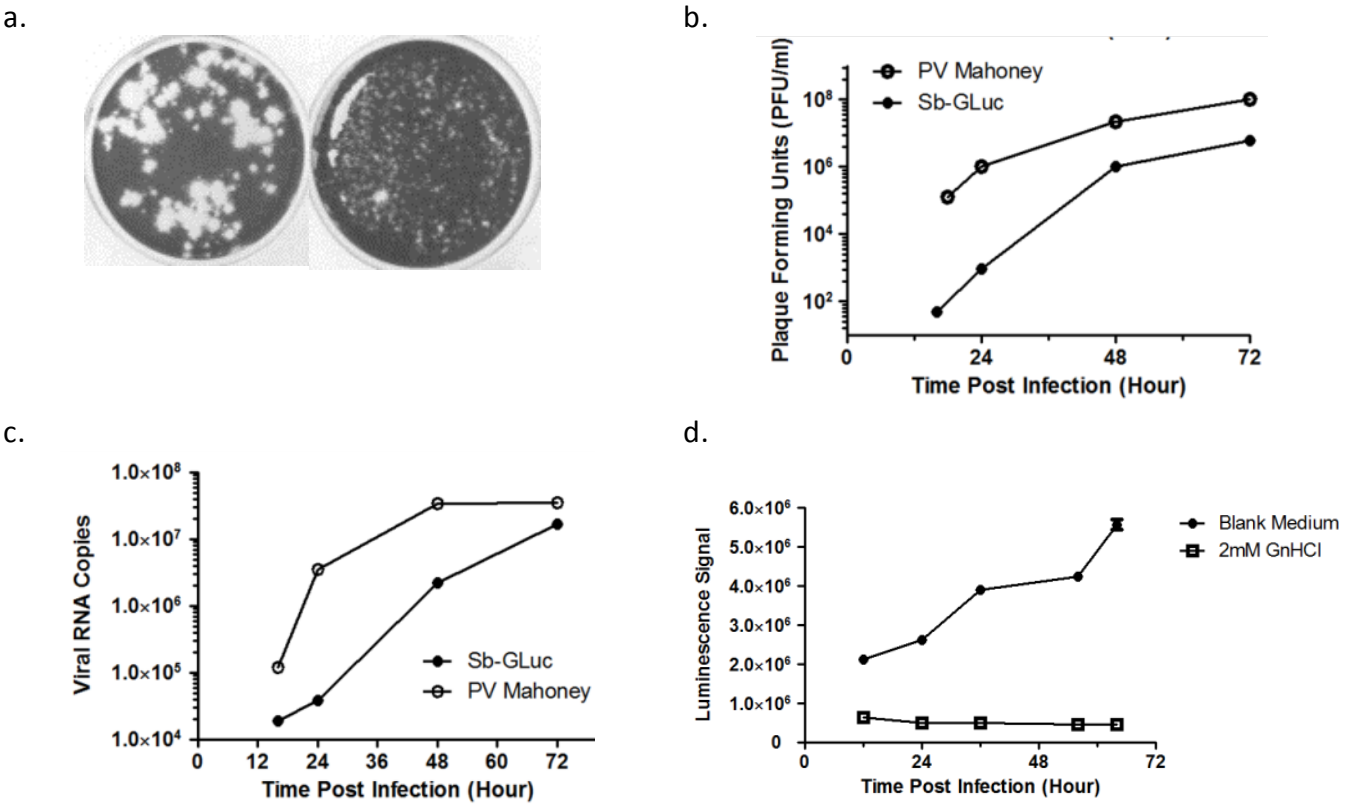


Figure 2. Generate and define recombinant poliovirus expressing Luciferase (PV-GLuc).
a) Representative pictures of the plaque assay results using PV-GLuc particles (right) and WT-PV (left) after 72-hour incubation. b) Plaque forming units (PFUs) of PV-GLuc and WT-PV. HeLa cells were infected by PV-GLuc or WT-PV and the viral particles were harvested after 16, 24, 48 and 72-hour incubation by centrifuge, and the viral PFUs were examined by plaque assays. c) Viral RNA copy numbers of PV-GLuc and WT-PV. HeLa cells were infected by PV-GLuc or WT-PV and the viral particles were harvested after 16, 24, 48 and 72-hour incubation by centrifuge, and the viral RNA copy numbers were examined by qRT-PCR. d) Luminescent signals curves of PV-GLuc incubated w/wo the presence of 2mM GnHCl. Luminescent signals were assessed after 12, 24, 36, 56, and 64-hour incubation.

3.2. Validate and Optimize The Cell-Based High-Throughput Screening Anti-Polioviral Assay

We then utilized the PV-GLuc virus to develop a cell-based high-throughput screening (HTS) assay. We hypothesize that compounds capable of inhibiting PV-GLuc replication can be identified by reduced luminescent signals compared to the blank controls.

To validate the application of PV-GLuc on HTS assay, a pilot experiment was conducted using 96-well plates. The un-diluted viral solution, as well as the 5, 25, or 125 times diluted viral stock (10^6 PFU/ml) were used to infect HeLa cells. As a control for background noise, we measured residual luminescence in the viral stocks that may have been carried over during production of PV-GLuc virus.

After 24-hour incubation, an increase in the luminescent signals was observed in all of the groups except 2mM GnHCl treated group and the background controls (Fig. 3a). The samples infected by 5-fold diluted viral solution gave the highest luminescent signal of over 2×10^6 units after 24-hour incubation (Fig. 3a, left panel). At 48-hour after infection, samples infected by 25-fold diluted viral solution gave the highest luminescence signal of about 5×10^6 units (Fig. 3a, right). A six-fold increase of luminescent signal was also observed in samples infected with 125-fold diluted viral solution during the second 24-hour incubation. However, the luminescent signal remained unchanged in the un-diluted group, and only slightly increased in the 5-fold diluted samples during the second 24-hour incubation (Fig. 3a). One possible explanation is that although a higher titer of PV-GLuc led to rapid start in viral replication and GLuc generation, most of the cells were immediately killed, which limited the amount of GLuc generated. In case

of infection using a lower titer of PV-GLuc, the virus continuously replicated and spread to the rest of the cells so that more GLuc were generated throughout the incubation.

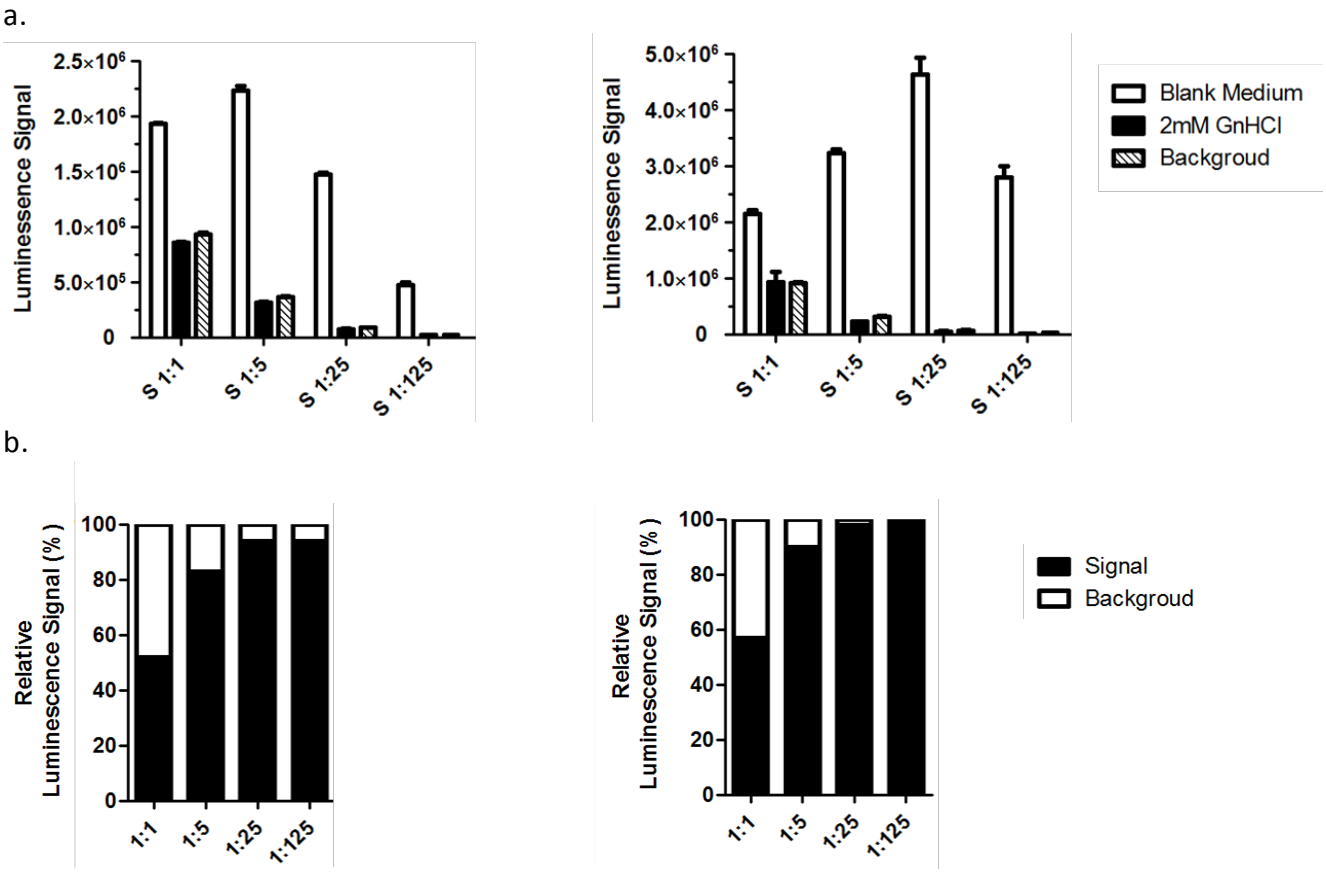


Figure 3. Validate and optimize the High-throughput intra-cellular screening assay.
a) Luminescent signals at 24 hours post infection (left) and 48 hours post infection (right). 5×10^4 HeLa cells were infected by 4×10^4 PFU (1:1), 8×10^3 PFU (1:5), 1.6×10^3 PFU (1:25) or 3.2×10^2 PFU (1:125) PV-GLuc. The white bar represents PV-GLuc incubated without treatment, the black bar represents PV-GLuc incubated in the presence of 2mM GnHCl, and the grey bar represent PV-GLuc incubated without HeLa cells to measure the luminescent signals in the background. b) Percentage of background in total luminescent signals at 24 hours post infection (left) and 48 hours post infection. White bar shows the net luminescent signals, while black bar represents the background.

For the samples infected by un-diluted viral solution, pre-existing GLuc contributed to almost half of the total signal (Fig. 3b). The background was significantly reduced when viral solution was extensively diluted. The background was less than 10% in the samples infected by 25- or 125-fold diluted viral solutions after 24-hour incubation, and less than 1% after 48-hour incubation (Fig. 3b). Therefore, 100-fold diluted viral solution (10^4 PFU/ml) was used in the HTS assay.

The Z-factor of this assay was calculated as 0.877 by three positive and negative controls. Collectively, the results suggest that the cell-based HTS assay is robust and reliable.

Chapter IV. Identification and Characterization of Gemcitabine and Other Anti-Poliovirus Agents

4.1. Gemcitabine

4.1.1. Application of Gemcitabine in Cancer Chemotherapy

Gemcitabine is originally developed by Eli Lily and Company as an antiviral reagent. Later studies revealed that it inhibited the growth of leukemia in cell culture, thus it was repurposed to be anti-cancer drug in subsequent development. It was approved by the FDA for cancer chemotherapy in 1996 and was registered by the trade-mark name of Gemzar. Gemcitabine has been used alone or in combination with other drugs to treat various types of cancer, such as ovarian cancer, breast cancer, non-small cell lung cancer and pancreatic cancer[177]. Generally Gemcitabine was given on Day 1 and Day 8 of each 21-day cycle at the dosage of 1000 – 1250 mg/m^2, which is equal to 27-34 mg/kg. Since Gemcitabine will be rapidly digested in the small intestine, usually it is administrated intravenously (i.v.). Common adverse reactions usually include minor or acute gastrointestinal tract, liver and hematological responses. Serious adverse reactions are rare but might include liver, kidney, hematological and neural damages[177]. Infusion time of 60min or longer might increase the possibility of adverse reactions, thus 30min infusion time is recommended. Over 90% of the drug is rapidly excreted through urine after administration[178].

Recently, a phase I study of daily or every-other-day Gemcitabine treatment with reduced dosage was conducted on advanced cancer patients[178]. Instead of i.v. infusion, oral administration of Gemcitabine was performed in the form of capsules. The patients received up to 8mg daily for 14 days in a 21-day cycle or up to 20mg every-other-day for 21 days in a 28-day cycle. Among these patients, Gemcitabine was well-tolerated except one patient who was given the drug 8mg daily. That patient passed away at day 9 after the start of the second 21-day cycle due to toxicity of the drug. The most common adverse reactions observed in both groups were nausea (37% of patients) and vomiting (30% of patients). Hematologic toxicity was mild as determined by Common Toxicity Criteria. Gastrointestinal-related toxicities contributed to 53% and 50% of the grade 1 and grade 2 adverse reactions. Only 4 patients who received daily dosage of 4mg Gemcitabine experienced serious side effects including vomiting and nausea. Some of the patients in both groups showed improved outcome of the diseases[178].

4.1.2. Mechanism of Action of Gemcitabine in Cancer Chemotherapy

Gemcitabine replaces the hydrogens at 2’ positions by fluorine (2', 2'-difluoro-2'-deoxycytidine, dFdC) to serve as a cytidine analog. dFdC is also converted into difluorodeoxyuridine (dFdU) by cytidine deaminase in the body[179]. Once up-taken by cells, dFdC and dFdU will be phosphorylated into their effective metabolite, dFdC triphosphate (dFdC-TP) and dFdU triphosphate (dFdU-TP)[180], which compete with normal deoxycytidine or uridine triphosphate (dCTP or UTP) to be incorporated into DNA or RNA chains. Only one more nucleotide can be added following dFdC-TP or dFdU-TP, thus block chain elongation and prevent dFdC-TP or dFdU-TP from being removed by proofreading[179]. *In vivo* test found that 80% of

dFdC-TP and dFdU-TP was recovered from the cellular DNA while the rest 20% was recovered from cellular RNA[178].

In addition to that, the diphosphate metabolite of dFdC (dFdC-DP) also inhibits ribonucleotide reductase so as to block the conversion of deoxycytidine diphosphate (dCDP) from cytidine diphosphate (CDP)[181]. This further exhausts the source of normal dCTP and eventually boosts incorporation of the drug metabolites into the DNA or RNA of cancer cells.

4.1.3. Antiviral Effect of Gemcitabine

Gemcitabine was originally developed as an antiviral reagent, and many recent researches revealed that Gemcitabine inhibit various pathogens *in vivo* and *in vitro*. For example, Gemcitabine has been reported to inhibit influenza A virus replication in vitro, however, its mechanism of action was not elucidated[182]. It also inhibited 90% of *Mycoplasma pneumoniae* (Mpn) growth at the concentration of 2μg/mL[183]. Gemcitabine also inhibited *Staphylococcus aureus* growth *in vitro* and protected mice from fatal *S. pyogenes* infection[184]. Gemcitabine has been used in combination with other drugs to treat HIV infection[185, 186]. It has also been tested alone in a murine AIDS model and conferred inhibitory effect against LP-BM5 murine leukemia virus (LP-BM5 MuLV) infection[187]. Recently, Gemcitabine was also reported to inhibit CVB3 and EV71 in combination with Ribavirin[188].

In this study, we reported that Gemcitabine inhibits PV replication with IC_{50} of 0.3μM. After careful study of its mechanism of action, we demonstrated that Gemcitabine acts as a $3D^{pol}$

inhibitor that directly interferes with viral RNA elongation. Therefore, we proposed that Gemcitabine could be a prospective PV antiviral as well as a potential broad spectrum picornavirus antiviral.

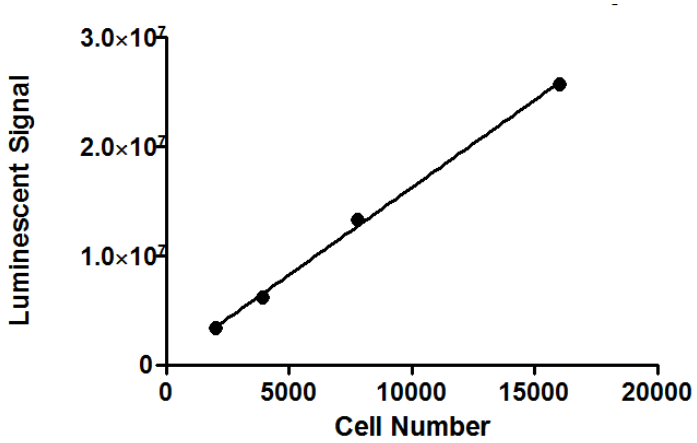


Figure 4. Linear correlation of intra-cellular ATP level and cell viability.
2,000, 3,900, 7,800 or 16,000 HeLa cells were inoculated on 96-well plate and incubated overnight. Cells were lysed and intracellular APT level was tested by CellTiter Glo Kit (Promega). Three repeats of each cell number were conducted and measured separately. The results were analyzed using linear regression.

4.2. Identify Prospective Anti-Poliovirus Agents Including Gemcitabine Using the Cell-Based HTS Assay

The Prestwick Compound Library containing 1,200 compounds of FDA-approved drugs was used for the initial screening, and 2mM GnHCl was included as positive control. Cell viability was tested by measuring the intracellular ATP levels. Different number of HeLa cells was inoculated in 96-well plates and their intra-cellular ATP level was measured. Clear linear correlation was

observed between the number of HeLa cells inoculated and their ATP levels (Fig. 4), indicating that the reading of the intracellular ATP level can accurately reflect the viability of the HeLa cells in the range from 2,000 cells to 16,000 cells.

Table 1. Compounds identified from the high-throughput screening assay.

Index	Ec	Vc	Compound Name
2B16	96.00%	79.40%	Itraconazole
2J05	92.80%	87.90%	Thiostrepton
2K14	82.10%	81.50%	Meclocycline sulfosalicylate
2M19	83.00%	103.10%	Ketoconazole
3A05	80.40%	80.10%	Gefitinib
3F21	82.80%	88.40%	Ethaverine hydrochloride
3G18	81.90%	88.80%	Ribostamycin sulfate salt
4G10	85.10%	75.40%	Cyproterone acetate
4G17	81.80%	92.00%	Zuclopenthixol hydrochloride
4I07	94.10%	91.20%	Azapropazone
4N07	96.60%	87.90%	Gemcitabine

Table 2. Other forms of nucleoside analogs do not remarkably inhibit PV replication.

Index	Ec	Vc	Compound Name	Mechanism of Action
01A03	62.71%	47.54%	Azaguanine-8	Purine antimetabolite
01C17	0.00%	93.78%	Idoxuridine	deoxyuridine analog
01O08	0.00%	102.77%	Didanosine	Adenosine analog
02C16	0.00%	99.37%	Lamivudine	Cytidine analog
02N12	43.97%	92.40%	Cytarabine	Cytidine analog
03F06	0.00%	97.53%	Abacavir Sulfate	Guanosine analog
03N22	36.25%	91.76%	Stavudine	Thymidine analog
04C14	0.00%	86.80%	Trifluridine	deoxyuridine analog
04F15	24.46%	85.79%	Fludarabine	Purine analog
04G07	60.28%	94.58%	Ribavirin	Guanosine analog

The inhibitory effect of each compound against poliovirus replication was reflected by Ec. For example, Ec of GnHCl is 98%, which means GnHCl inhibits 98% of viral replication compared to the untreated control. Vc was used to reflect the viability of cells after incubation in the presence of each compound. Vc of 2mM GnHCl is 95%, which means 95% of the cells remain alive after 48-hour incubation (data not shown).

We set the criteria that qualified compound should achieve over 80% Ec and over 70% Vc. 11 compounds were identified following this criterion (Table 1). One inhibitory test and one cell viability test was performed for each compound. Among these, Itraconazole and Gemcitabine were the best hits conferring Ec of 96.0% and 96.6%. Itraconazole's Vc is 79.4% while Gemcitabine's Vc is 87.9%. Another compound Ketoconazole similar to Itraconazole was also identified with Ec of 83.0% and Vc of 103.1%.

Since Gemcitabine has been reported as a nucleoside analog of cytosine and extensively used in cancer chemotherapy[76, 189], we comparatively evaluated Gemcitabine and other forms of published nucleoside analogues including anti-tumor drugs and HIV antivirals. The results demonstrated that only Gemcitabine, but not other forms of nucleoside analogs, could achieve remarkable inhibition of poliovirus replication (Table 2).

Collectively, our validated cell-based HTS antiviral assay allowed us to identify prospective anti-poliovirus agents including Gemcitabine.

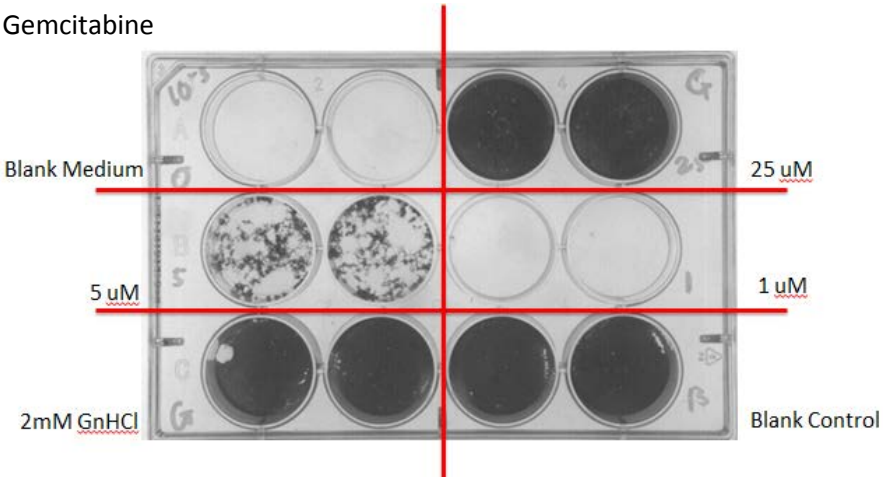
4.3. Characterize Gemcitabine and Other Anti-Poliovirus Agents for the Ability to Inhibit PV Replication

We then sought to verify and characterize Gemcitabine, Itraconazole and Ketoconazole for their antiviral effects on WT-PV using plaque assays. All of the HeLa cells were killed within 48 hours of incubation without treatment (Fig. 5). 25μM Itraconazole and Ketoconazole confirmed partial protective effect on HeLa cells (Fig. 5b, c). In contrast, 25μM Gemcitabine completely protected HeLa cells from WT-PV-mediated damages, without a single plaque being observed (Fig. 5a). Since Gemcitabine conferred the best inhibitory effect against WT-PV, we focused on the following verification experiments.

First, PV-GLuc was used to explore the inhibitory characteristics of Gemcitabine against poliovirus. IC_{50} was calculated as 2.6μM based on luminescent signal (Fig. 6a). qRT-PCR detecting PV-GLuc RNA copies gave rise to similar results and revealed IC_{50} as 1μM (Fig. 6b). Concurrently, Gemcitabine at concentrations of up to 100μM did not affect cell viability as indicated by intra-cellular ATP level (Fig. 6c).

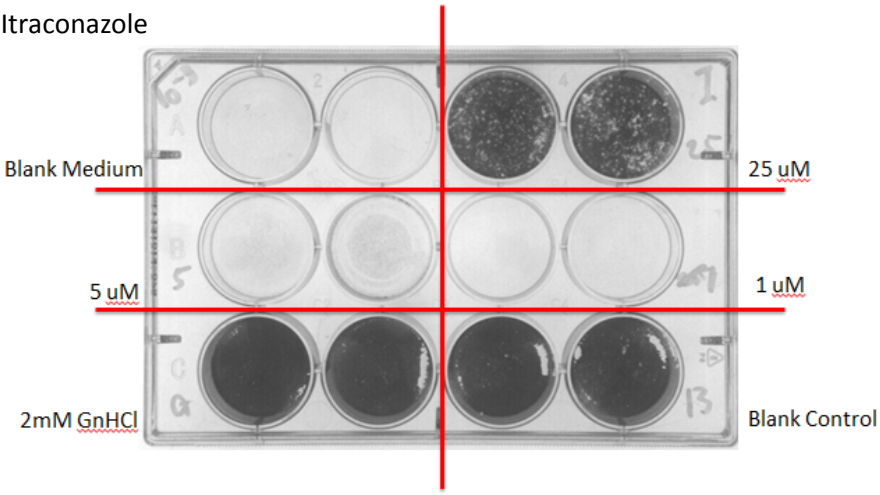
a.

Gemcitabine



b.

Itraconazole



c.

Ketoconazole

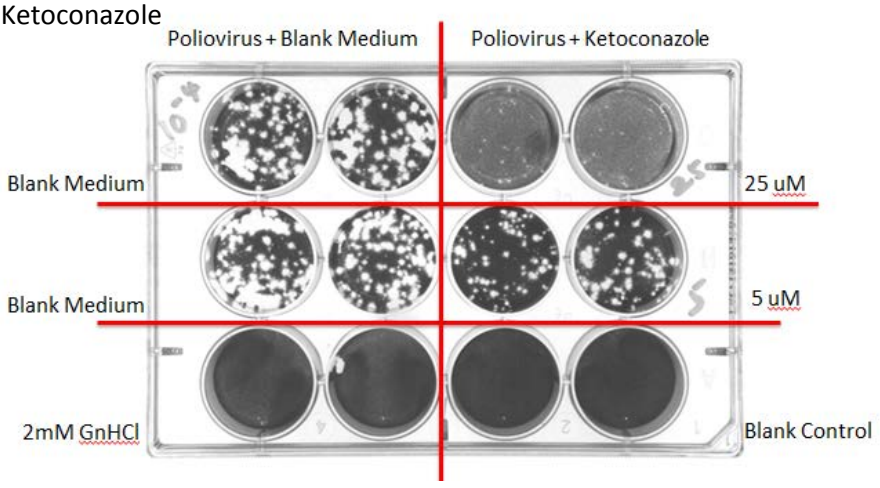


Figure 5. Gemcitabine, Itraconazole and Ketoconazole confer protective effects on HeLa cells against WT-PV infection.
a) 10^6 HeLa cells were infected by 2×10^5 PFU WT-PV (M.O.I. = 0.2) and treated with 0, 5 or 25μM of Gemcitabine. b) 10^6 HeLa cells were infected by 2×10^5 PFU WT-PV (M.O.I. = 0.2) and treated with 0, 5 or 25μM of Itraconazole. c) 10^6 HeLa cells were infected by 2×10^4 PFU WT-PV (M.O.I. = 0.02) and treated with 0, 5 or 25μM of Ketoconazole.

Secondly, WT-PV was used to further evaluate Gemcitabine's antiviral effect. A single dose of 25μM Gemcitabine introduced at the beginning of the incubation was able to reduce up to 99% of PFU as well as viral RNA copies at the first 24-hour incubation, although only 60% reduction was observed at 48-hour post infection (Fig. 7). It was possible that Gemcitabine was exhausted after 24-hour incubation, which led to a loss of antiviral effect. To test this, Gemcitabine was added every 24 hours during the incubation. No noticeable increase in the viral RNA copies was observed in the samples that were treated by 25 μM Gemcitabine every 24 hours (Fig. 8a). IC50 was determined as 0.3μM by measuring the RNA copy numbers and dosages over 2μM conferred > 90% inhibition on WT-PV replication (Fig. 8b). Similar results were obtained from plaque assays (Fig. 8c).

Thus, while Gemcitabine exerts antiviral effect on both PV-GLuc and wild type poliovirus Mahoney, the compound doses of up to 100 uM do not affect cell viability as reflected by the intra-cellular ATP levels. These results therefore suggest that Gemcitabine could be a potential anti-poliovirus agent with high efficiency and limited toxicity.

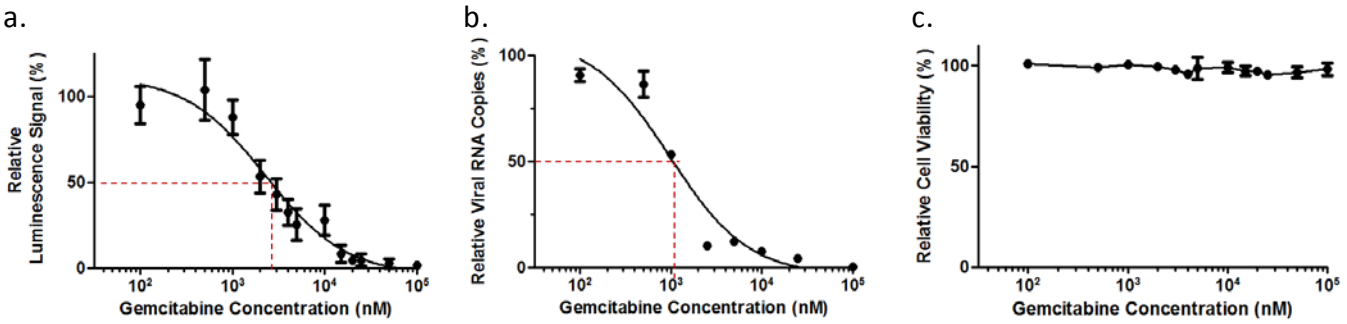


Figure 6. Verify and characterize Gemcitabine using PV-GLuc.
a) Luminescent signals of PV-GLuc incubated in the presence of various concentrations of Gemcitabine for 48 hours. 4×10^5 HeLa cells were incubated with 10^5 WT-PV (M.O.I = 0.25) The IC_{50} of Gemcitabine against PV-GLuc was calculated as 2.5μM. b) Viral RNA copy numbers of PV-GLuc (M.O.I = 0.25) incubated in the presence of various concentrations of Gemcitabine for 48 hours, measured by qRT-PCR. The IC_{50} of Gemcitabine against PV-GLuc was calculated as 1μM. c) Cell viability of HeLa cells incubated in the presence of various concentrations of Gemcitabine without viral infection for 48 hours, measured by intra-cellular ATP levels.

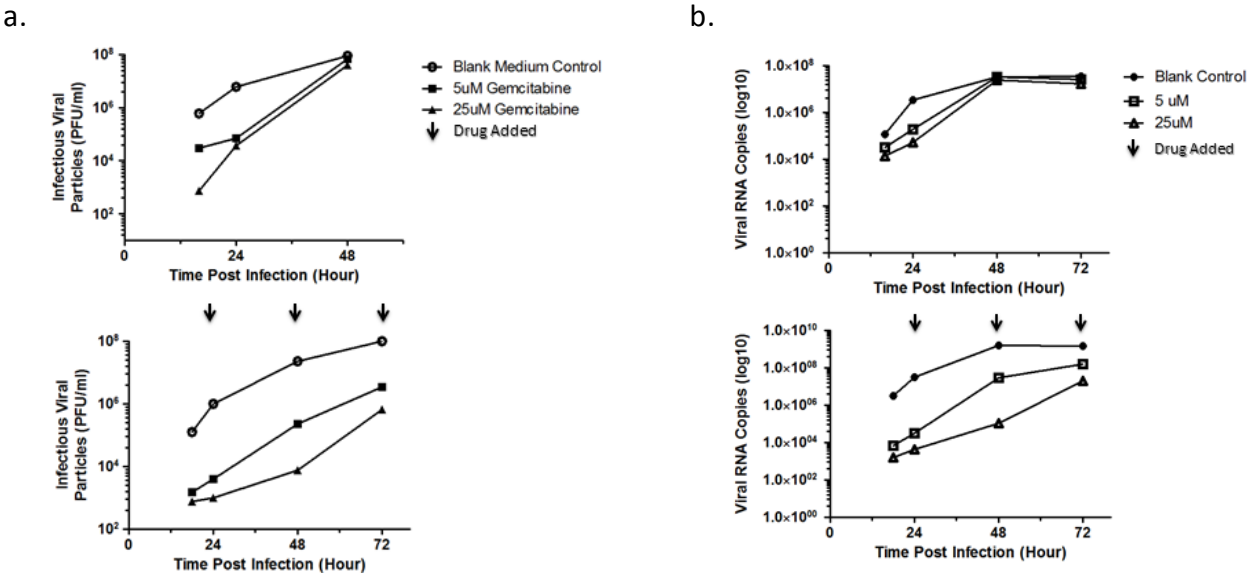


Figure 7. Daily dosage of Gemcitabine confers inhibitory effect against PV.
a) PFUs of WT-PV treated by Gemcitabine. WT-PV 10^6 HeLa cells were infected by 2×10^4 PFU WT-PV (M.O.I. = 0.02) and treated with 0, 5 or 25μM of Gemcitabine. The drug was delivered either at the

beginning of the incubation (upper) or every 24-hour (down). The arrows indicate the administration of additional dosages. Virus were harvested after 16, 24, 48 and 72-hour incubation, and the PFU were determined by plaque assays. b) Viral RNA copy numbers of WT-PV treated by Gemcitabin. WT-PV 10^6 HeLa cells were infected by 2×10^4 PFU WT-PV (M.O.I. = 0.02) and treated with 0, 5 or 25μM of Gemcitabine. The drug was delivered either at the beginning of the incubation (upper) or every 24-hour (down). The arrows indicate the administration of additional dosages. Virus were harvested after 16, 24, 48 and 72-hour incubation, and the viral RNA copy numbers were determined by aRT-PCR.

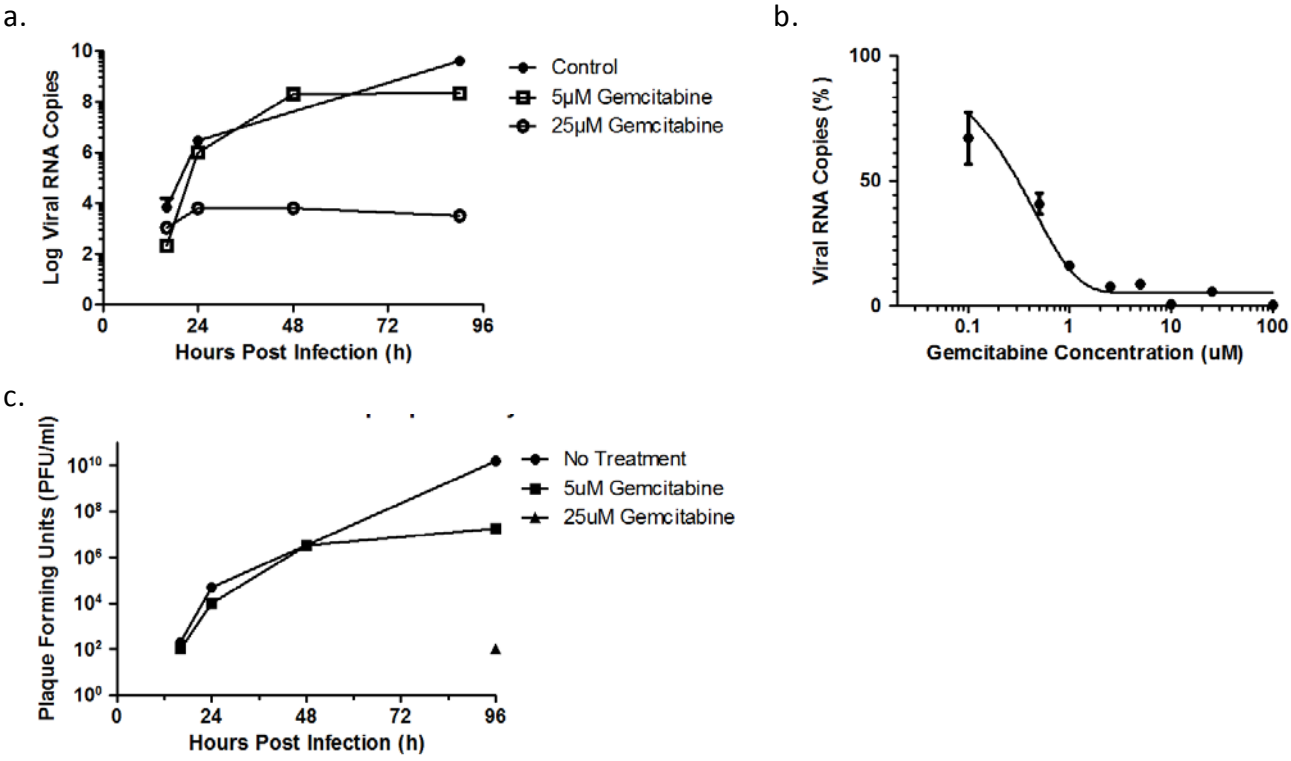


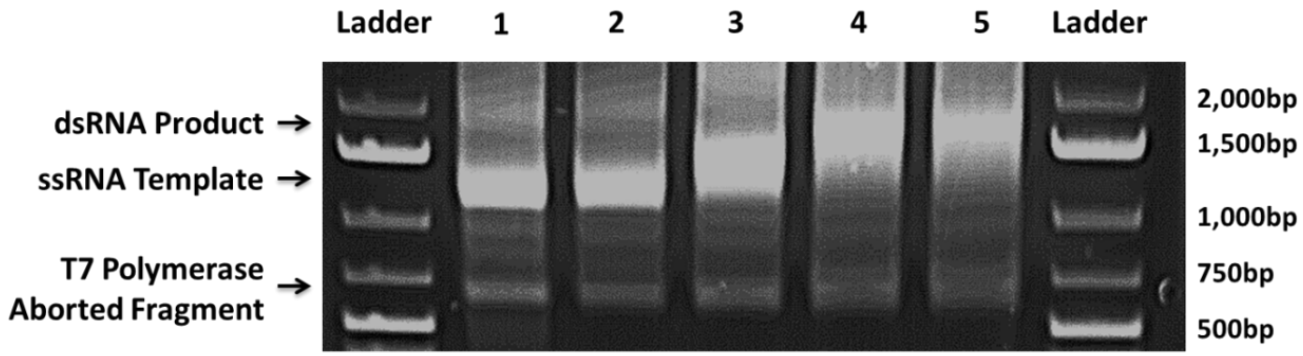
Figure 8. Verify and characterize Gemcitabine for the ability to inhibit poliovirus replication.
a) Viral RNA copy numbers of WT-PV incubated in the presence of 0, 5 and 25μM of Gemcitabine for 48 hours. 4×10^5 HeLa cells were infected by 100 PFU WT-PV, and Gemcitabine was added every 24 hours during the incubation. b) Viral RNA copy numbers of WT-PV incubated in the presence of various concentrations of Gemcitabine for 48 hours. 4×10^5 HeLa cells were infected by 100 PFU WT-PV, and Gemcitabine was added every 24 hours during the incubation. Viral RNA copy numbers were determined by qRT-PCR. c) 4×10^5 HeLa cells were infected by 100 PFU WT-PV, and 0, 5 or 25μM of Gemcitabine was added every 24 hours during the incubation. Virus was harvested after 16, 24, 48 and 72-hour incubation, and the PFU were determined by plaque assays.

4.4. Gemcitabine Suppression of PV Replication Was Consistent with the Ability of Its Metabolite dFdC-TP to Inhibit Elongation or Synthesis of PV RNA

Gemcitabine is extensively used in cancer chemotherapy since Gemcitabine is metabolized as a nucleoside analog of cytosine. It replaces the hydrogens at 2' positions by fluorine (2', 2'-difluoro-2'-deoxycytidine, dFdC). In the case of cancer chemotherapy, dFdC is up-taken by tumor cells and phosphorylated into its effective metabolite, dFdC-TP, which can be incorporated into DNA or RNA chains and block chain elongation[76, 189]. We presumed that Gemcitabine metabolite interferes with poliovirus RNA elongation and thereby inhibits viral replication. To test this, we took advantage of the cell-free polio RNA synthesis system, in which recombinant PV RNA-dependent RNA polymerase synthesizes double stranded RNA (dsRNA) product based on single stranded RNA (ssRNA) template[136]. This cell-free system would allow us to directly test whether dFdC-TP metabolite derived from Gemcitabine could affect poliovirus RNA elongation.

Gemcitabine metabolite dFdC-TP significantly reduced synthesis or elongation of PV dsRNA in a dose-dependent manner (Fig. 9). Specifically, dFdC-TP alone without r-CTP (lane 2, Fig. 9) led to complete inhibition of dsRNA synthesis or elongation, which was identical to that of the negative control (lane 1, Fig. 9). Based on the up-shifted patterns of dsRNA, apparent inhibition of dsRNA elongation was observed when dFdC-TP and r-CTP was added in a 1:1 ratio(lane 3, Fig. 9), and weak inhibition was found when dFdC-TP and r-CTP was added in a 1:10 ratio (10-fold reduction of dFdC-TP, lane 4, Fig. 9). The dose-dependent inhibition of dsRNA elongation

supports our hypothesis that Gemcitabine suppression of PV replication consists with the ability of its metabolite to inhibit poliovirus RNA elongation.



ssRNA template	+	+	+	+	+
5uM 3Dpol	+	+	+	+	+
r-A/T/U-TP	500uM	500uM	500uM	500uM	500uM
r-CTP	-	-	500uM	500uM	500uM
dFdC-TP	-	500uM	500uM	50uM	-

Figure 9. Gemcitabine metabolite dFdC-TP inhibits the ability of PV RNA polymerase (3Dpol) to synthesize PV dsRNA.

10μM Recombined $3D^{pol}$ was incubated with 0.4μM linear ssRNA fragment of PV (lower band) w/wo dFdC-TP for 2 hours, and the production of dsRNA (upper band) was analyzed using electrophoresis and stained by EB. Lane 1 is the negative control in which 500μM of r-A,U,G-TP was added in absent of r-CTP or dFdC; Lane 5 is the positive control in which 500μM of all four r-NTPs were added; 500μM dFdC-TP was added in addition to 500μM r-A,U,G-TP in Lange 2; 500μM dFdC-TP and 500μM r-CTP was added in addition to 500μM r-A,U,G-TP in Lange 3; 50μM dFdC-TP and 500μM r-CTP was added in addition to 500μM r-A,U,G-TP in Lane 4.

Chapter V. Discussion

In the current study, we created a modified PV expressing Gaussia Luciferase (PV-GLuc) and developed a cell-based high-throughput screening (HTS) antiviral assay. The validated HTS assay allowed us to identify the best hit, Gemcitabine, as potent anti-PV agent candidate. While Gemcitabine inhibited PV Mahoney replication with IC50 of 0.3μM, it completely protected HeLa cells from PV-induced cytopathic effects at 5μM, without detectable toxicity for cell viability. Furthermore, Gemcitabine metabolite directly inhibited the ability of PV RNA polymerase to synthesize or elongate PV RNA. Our findings are consistent with the current efforts to develop therapeutic drugs for treatment of chronically infected poliovirus excreters.

Our cell-based HTS assay appears to be robust and reliable, with Z-factor of 0.877. The modified viral replicon containing 558b GLuc reporter gene provides a useful tool for both HTS assay and evaluation of viral replication, as it is able to replicate and induce plaques in the host cells in the pattern similar to the wild type PV. GLuc generates strong luminescent signals up to 72 hours along with the viral replication (Fig. 3.2). Our cell-based HTS system is also of merit in that it can identify compounds targeting various steps of the viral life cycle, including viral entry, replication, release, etc. In addition, cell-based HTS assay can also reveal host factors involved in the viral life cycle. For example, Itraconazole, one of the compounds we identified, was also shown by another group to inhibit the enterovirus replication by blocking host factor Oxysterol-binding Protein (OSBP) mediated cholesterol transportation between endoplasmic reticulum

(ER) and Golgi[109, 190]. Therefore, this cell-based HTS assay should be a useful tool for translational and basic research.

The identification of Gemcitabine as the best hit in our cell-based HTS leads to the subsequent definition of anti-poliovirus function. 25μM Gemcitabine completely protects the HeLa cells from the PV infection and inhibits viral RNA replication up to 90h post infection. With IC_{50} of 0.3μM, Gemcitabine exhibited limited cytotoxicity to HeLa cells at the concentration up to 100μM. Although Gemcitabine was also reported to inhibit influenza A virus replication in vitro[182], its mechanism of action was not elucidated. In contrast, Gemcitabine suppression of PV replication is consistent with the ability of its metabolite dFdC-TP to inhibit PV dsRNA elongation or synthesis. Our cell-free system using recombinant RdRp and ssRNA template clearly shows a dose-dependent inhibition of RNA elongation when dFdC-TP is present. This result suggests that Gemcitabine's antiviral effect is at least partly achieved by inhibition of viral RNA elongation.

The inhibition of PV RNA synthesis by Gemcitabine metabolite is also in line of the report in the cancer research indicating that Gemcitabine acts as a cytidine analog, which is extensively used in the chemotherapies for various cancers[189, 191]. Notably, we have evaluated other forms of nucleoside analogues including anti-tumor drugs and HIV antivirals together with Gemcitabine. None of those known nucleoside analogs achieve such remarkable inhibition of poliovirus replication as Gemcitabine does (Table 4.2). Even the well-known nucleoside analog

Ribavin capable of inhibiting PV replication by increasing the mutation rate of RdRp[113, 114], can only reduce 60.3% of viral replication, whereas Gemcitabine readily achieves 96.6% reduction of PV replication. Moreover, although most of the compounds showed limited cytotoxicity, some of those nucleoside analogs are found to be toxic to the HeLa cells. For example, Azaguanine-8 reduced the intra-cellular ATP level to 47.54%, whereas Gemcitabine had almost no cytotoxicity effect on HeLa cells at concentrations of up to 100μM.

PV RNA-dependent RNA polymerase (RdRp) is conserved among members in the *Picornaviridae* family. Crystal structure seven conserved motifs which plays critical role for viral RNA replication and are shared by picornavirus species[192, 193]. Mutagen experiment using Ribavirin revealed that poliovirus as well as other picornavirus are living at the edge of mutation, while any increase in the mutation rate in the RNA replication will introduce unfavorably high amount of mutations in the viral genome and eventually lead to fatal defects in the viral genome replication [113, 114]. Gemcitabine could potentially serve as a leading compound of broad-spectrum antiviral against PV and other picornavirus species.

The appreciable anti-PV potency and low cytotoxicity of Gemcitabine support further studies to evaluate Gemcitabine for therapeutic effects on PV infection in recently-developed nonhuman primate model of oral poliovirus infection leading to paralytic poliomyelitis.

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VITA

NAME: Zhuoran Zhang

EDUCATION: B.S. Biology, Fudan University, Shanghai, China, 2011

Ph.D., Microbiology and Immunology, University of Illinois at Chicago, Illinois, 2016

HONORS: CCTS Pre-doctoral Education in Clinical and Translational Science Fellowship, 2014

Hui-Chun Chin & Tsung-Dao Lee Undergraduate Research Scholar, 2010