CHARACTERIZATION OF VIRAL ENTRY INHIBITORS

A Dissertation

by

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ABSTRACT

Hepatitis C virus (HCV), Human Immunodeficiency virus (HIV) and Herpes Simplex virus (HSV) are pathogenic viruses known to cause liver disorder, acquired immunodeficiency and skin lesions, respectively. Although current therapies have played substantial roles in the fight against these pathogens, their use is limited and for the most part does not result in viral eradication. Moreover, most antivirals target viral encoded structures which overtime foster the development of resistant strains. Hence, antivirals aimed at preventing initial infection represent a promising strategy for viral combat.

This dissertation focuses on the characterization of viral entry inhibitors and their potential use. The first compounds evaluated come from the phenothiazines family, widely used as antipsychotic drugs. Phenothiazines were shown to suppress HCV entry by intercalating into cholesterol-rich membrane domains of target cells thus reducing viral-host fusion.

The second candidates studied are two members of the H_1-anthistamines currently used for allergy treatment. Both compounds strongly reduce HCV entry, likely at the fusion step, and its inhibition was associated with cholesterol content in the virion and host cells, pointing to the use of an NPC1L1-receptor dependent mechanism.

Lastly, we evaluated the antiviral activity of PD 404,182 (PD) as an alternate treatment for HCV-HIV coinfected patients as well as its potential use as an anti-HIV microbicide. PD is able to reduce viral entry of the three pathogens through physical

disruption of virions releasing the nucleic acids into the surrounding medium. Moreover, PD possesses several qualities pointing to its use as a potential microbicide.

DEDICATION

To my family

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Viral entry

Viruses, originally referred to as filterable agents, are obligate parasites that lack replication machinery and are therefore highly dependent on their host cells. Viruses consist of a core of RNA or DNA generally surrounded by a protein, lipid or glycoprotein coat, or some combination of the three. Once inside the cell, the goal of a virus is to use the host intracellular replication machinery for virus replication and production. Depending on the virus, different strategies are utilized to mediate efficient infection. Many enveloped viruses take advantage of the cells' natural endocytosis mechanism, while others fuse directly with the host's plasma membrane. Many locations have been described as potential penetration sites for viruses, including plasma membrane, early/mature/late endosomes, lysosomes, macropinosome and endoplasmic reticulum (ER). After internalization viruses exploit the cytoplasmic transport system of the cells to reach the site of replication.

The entry process can be broken down into five discrete steps: attachment, viral uptake, endosomal escape and penetration/fusion, intracellular transport and uncoating.

1.1.1 Attachment

Virus infection commences with the interaction between the virion and the host surface. Cell surface molecules that serve as binding partners comprise a large spectrum of proteins, lipids and glycans, and are distinguished between attachment factors or true

receptors (1, 2). Attachment factors enable viruses to bind and concentrate on the cell surface. Such interactions are relatively nonspecific and do not induce changes in the virion structure. Conversely, true receptors bind viruses, induce conformational changes in viruses to promote their entry and activate signaling pathways that aid in the infection process. Interactions between the envelope viral proteins and cellular receptors are often highly specific, dictating virus' host and tissue tropism. Due to their multivalent nature, viruses usually bind to multiple surface cell surface receptors forming receptor-rich microdomains that can promote transbilayer signaling, support membrane curvature generation and activate endocytosis (3).

1.1.2 Viral uptake

Endocytosis is a process by which macromolecules are taken into cells through endosomes. While some viruses enter cells through direct fusion with the plasma membrane, most viruses use at least one of the several endocytic routes such as clathrin-mediated endocytosis, caveolar endocytosis, macropinocytosis and some poorly characterize variations of these (4).

1.1.2.1 Clathrin mediated endocytosis

Clathrin mediated endocytosis is a common uptake mechanism for viruses of small and intermediate size (50-200nm). During clathrin-mediated endocytosis, interaction of the ligand and a specific receptor induces clustering of the receptor-ligand complex in coated pits on the plasma membrane. Such pits then invaginate and pinch off the plasma membrane, forming intracellular clathrin coated vesicles. Coat removal of the vesicles results in early endosomes. Multiple early endosomes can fuse with each other

to form late endosomes which eventually fuse with the lysosomes. Molecules internalized through this pathway experience a decline in pH, which is necessary to trigger the fusion process for some viruses (5).

1.1.2.2 Caveolar endocytosis

Although not as common, some viruses, especially small non-enveloped viruses, use caveolar endocytosis to enter the host. Internalization takes place in small fitting vesicles devoided of a clathrin coat (6). Following internalization, viruses are transported to early and late endosomes, eventually reaching the ER where the initial uncoating and penetration occurs (7).

1.1.2.3 Macropinocytosis

Macropinocytosis is an actin dependent pathway for the nonspecific uptake of fluids, solutes and particles in response to the activation of cell surface molecules. It is an alternate entry route predominantly used by larger viruses (≥250nm). The process results from the activation of a complex signaling pathway that modifies the actin cytoskeleton dynamics. Virus-containing macropinosomes move deeper into the cytosol where acidification, maturation and fusion with the endosome or lysosome takes place (8).

1.1.2.4 Fusion at the plasma membrane

Direct entry through the plasma membrane requires the fusion process to happen at neutral pH. Such route is only used by enveloped viruses and allows delivery of the genomic material into the cytosol (9). The internalization process involves the fusion of

the enveloped virus with the plasma membrane after fusion-promoting viral proteins are recognized by specific cell receptors.

1.1.3 Endosomal escape and penetration

Upon internalization via endocytic pathways, viruses must escape their intracellular compartments for efficient genomic delivery. Escape mechanisms depend on the nature of the virus: enveloped vs non-enveloped. Enveloped virions utilize membrane fusion to cross the membrane barrier and reach the cytoplasm, while non-enveloped viruses use pore formation or membrane disruption (10).

Internalized virions are directed to early endosomes or remain as cargo in newly formed macropinosomes. Since both pathways are targeted to hydrolase-filled lysosomes, cargos in both compartments experience pH drops with time. Transport encompasses a maturation process that involves endosomal acidification, followed by the formation of intraluminal vesicles and finally microtubule movement to the perinuclear region for some viruses (11). Depending on the virus, penetration can occur in the early endosomes, late endosomes or macropinosomes, with late penetration requiring more acidic pH and additional factors involved in the endosomal maturation process (12).

1.1.3.1 Fusion dependent mechanism

Delivery of genomic material and accessory proteins into the cytosol is an active process initiated by the virus. In the case of enveloped animal viruses, penetration is mediated by the viral fusion proteins. Fusion proteins are classified as class I, class II and class III based on key structural features. Class I proteins are trimeric in both their

prefusion and postfusion state, and their fusogenic domain is predominantly composed of alpha helices at the N-terminal. Class II proteins are mostly made of beta sheets. Their fusion peptides are located in internal loops. Unlike class I, class II proteins undergo an oligomeric rearrangement from the prefusion dimer to a stable homotrimer. Class III proteins resemble a combination of the original two classes as they acquire a similar post fusion trimeric structure as class I, but are predominantly made of beta sheets (13). Upon exposure to the right cue - pH, lipid composition, proteolytic activity or redox reactions - fusion proteins undergo conformational changes so that exposed hydrophobic residues can insert into target membranes to form a bridge between the virus and the endosomal membrane. Once in close contact, viruses fuse with the limiting membrane of the endocytic vacuoles from the luminal side, enabling cytosolic translocation of the viral genome.

1.1.3.2 Fusion independent mechanism

Non-enveloped viruses use a fusion independent mechanism to deliver their genomes. To date two alternatives have been proposed: membrane disruption and pore formation (14). Membrane disruption requires electrostatic interactions between the hydrophilic peptide group on the virus and the lipid head group in the membrane. The process is usually described by four steps: (i) positively charged viral peptide monomers bind to negatively charged phospholipids, (ii) peptide monomers aligned so that their hydrophilic groups face the phospholipid head groups or water molecules, (iii) the hydrophobic group of the peptides reorients towards the hydrophobic core of the membrane leading to (iv) disruption of lipid bilayer curvature and membrane

disintegration. In pore formation, also known as the barrel-stave mechanism, hydrophobic surfaces of viral amphipathic α-helices interact with the lipid core of the membrane, and their hydrophilic surfaces point inward producing an aqueous pore (15).

1.1.4 Intracellular transport

Once in the cytosol, viruses and viral capsids reach their replication site via the cytosolic transport machinery, such as microtubule-based motors. Transport of virus from the ER to the nucleus can follow one of the two routes. The first route requires the interaction of virions with nuclear import receptors allowing them to target their capsid or genome to the nuclear pore complexes. This approach is not feasible for large virions that cannot pass though the nuclear pores. Alternatively, genomic DNA is directly transported from the ER into the nucleus across the inner nuclear membrane or by releasing their genome directly into the nuclear pore (16).

1.1.5 Uncoating

Release of the genomic material form a protective confined capsid structure is usually the last step of the entry program as genomic material cannot be easily moved once release from the capsid. In general, virus uncoating involves a stepwise process that occurs during different stages of entry. It is highly dependent on the nature of the virus and in some cases relies on host cell factors.

1.2 Targeting viral entry of human pathogens

Human Immunodeficiency virus (HIV), Herpes simplex virus (HSV) and Hepatitis C virus (HCV) are pathogenic viruses known to cause acquired immunodeficiency, skin lesions and liver disorders, respectively. Even though life

extending treatment exits for all three pathogens, infection by these viruses is considered incurable. For HSV, viral latency plays a key role in the therapy outcome. The clearance of HIV is hampered by latency and rapid evolution of escape mutations that abrogate recognition by neutralizing antibodies and cytolytic T lymphocytes. For HCV, therapy mainly relies in unspecific antivirals and is riddled with serious side effects. Thus, prevention strategies as well as antivirals with novel modes of action are among the favored contenders for future therapeutics. This section will provide a brief introduction of these three human pathogens, a detailed description of their entry processes, current treatments and direction of future therapeutics relating to the entry step.

1.2.1 Hepatitis C virus (HCV)

Hepatitis C virus is a member of the Hepacivirus genus belonging to the family of *Flaviviridae*. First identified as the causative agent of non-A, non-B hepatitis in 1989, HCV is a human pathogen and the leading cause of cirrhosis, hepatocellular carcinoma and liver failure (17-19). There are seven major genotypes of HCV around the world, with sequence variations of about 30-35% between genotypes and 20% between subtypes (20-22). Different HCV genotypes are predominantly found in different geographic regions, with genotype 1 being the most prevalent in the United States and Western Europe (23). An estimated 130 million people worldwide are currently afflicted by HCV and there is no vaccine available. Current treatment relies on unspecific antiviral agents like pegylated interferon in combination with ribavirin. In 2011 the first direct antiviral agents were added to the standard-of-care interferon/ribavirin therapy for genotype 1 infected individuals, which improved the cure rates, but also increased the

frequency of adverse effects (24). Moreover, HCV replicates with a high mutation rate due to lack of proofreading activity of the viral RNA-dependent RNA polymerase. This combined with a high viral turnover rate, results in a population of newly synthesized viral genomes with different degrees of fitness and 'quasispecies' (24). The HCV quasispecies is able to evolve under a variety of selective pressures, facilitating the virus to escape the body's immune defense and develop drug resistant variants. Due to a combination of non-diagnosis, treatment failure, treatment avoidance, and treatment discontinuation, only approximately 2% of the HCV-infected individuals in the United States are successfully treated (25).

Hepatitis C virus is a hepatotropic enveloped positive-sense RNA virus. Its 9.6 kb genome encodes a single large polyprotein that is processed by viral and cellular proteinases to produce the virion structural proteins (core and glycoproteins E1 and E2), P7, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (26). Once inside the host, replication takes place in lipid rich ER derived compartments close to the nucleus known as membranous webs. Progeny virions are assembled in close proximity to lipid droplets and are released through to the cell's secretory pathway. Aside from the cell free infection process, an alternative route of transmission has been suggested in which progeny viruses are directly transmitted from an infected cell to an uninfected neighboring cell (27).

1.2.1.1 HCV entry mechanism

HCV entry into hepatocytes is a highly coordinated and multi-step process mediated by the viral envelope glycoproteins (E1 and E2) and host cell factors. E1 and

E2 are type I transmembrane proteins with a short C-terminal transmembrane domain and an N-terminal extracellular domain. Both proteins are synthesized at the rough ER as part of the polyprotein precursor, and are cotranslationally cleaved off by the cellular signal peptidase (28). Glycans present in the N-terminal ectodomains of E1 and E2 play a major role in E1E2 folding, HCV entry and are crucial for evading host immune responses by masking the immunogenic envelope epitopes (29, 30). C-terminal transmembrane domains play a major role in the biogenesis of the E1E2 complex and the membrane fusion process (31). Heparan sulfate glycosaminoglycans and low density lipoprotein receptors represent the first attachment sites for HCV prior to interaction with scavenger receptor class B type 1 (SR-B1), the tetraspanin cluster of differentiation 81 (CD81), claudin-1 (CLDN1), Occludin (OCLD) and Niemann-Pick C1-Like 1 (NPC1L1) (32). SR-B1 is an 82 kDa glycoprotein highly expressed in the liver and steroidogenic tissues (33). It harbors two transmembrane domains and is involved in bidirectional cholesterol transport at the cell membrane. Evidence suggests that the hypervariable region 1 of E2, comprising the first 27 amino acids, interacts with SR-B1's extracellular loop (34). CD81 is a ubiquitously expressed 25 kDa tetraspanin protein with four transmembrane domains, two extracellular loops and one intracellular loop. CD81 was the first candidate receptor shown to interact with a soluble truncated form of E2 and is a critical factor of HCV entry (35-37). CLDN-1 is a 23 kDa transmembrane protein expressed in all epithelial tissue but predominantly in the liver (38). It resides in the tight junction were it regulates paracellular permeability and polarity. Evidence suggests that CLDN-1 is likely involved at a post binding HCV entry

step (39, 40). CLDN-1 associates with CD81 in a variety of cells and mutations at residues 32 and 48 in CLDN-1's extracellular loop ablate its association with CD81 and the viral receptor activity, suggesting that complex formation is essential for HCV entry (41, 42). OCLD is a 65 kDa protein with four transmembrane regions and two extracellular loops. It is expressed in tight junctions of polarized cells where it partakes on cell-cell adhesion and anchoring of the junctional complex to the cytoskeleton. Confocal microscopy analyses demonstrated that OCLD accumulates in the ER and colocalizes with the E2 viral glycoprotein (43). Association between E2 and OCLD was further confirmed by co-immuno precipitation pull down assays (44). NPC1L1 is a cell surface cholesterol-sensing 13 transmembrane-domain receptor. It is highly expressed on the apical surface of intestinal enterocytes and the bile canicular membrane of human hepatocytes (45), and is responsible for intestinal cholesterol absorption (46) and regulation of biliary cholesterol concentration (47). Despite the lack of evidence for direct interaction between NPC1L1 and HCV, antibody mediated receptor blockage and silencing of NPC1L1 impaired HCVcc infection. Moreover, treatment of cells with ezetimibe, an NPC1L1 specific antagonist, inhibited the uptake of all major HCV genotypes *in vitro* (48).

Although the sequence of receptor engagement is not exactly known, it is speculated that the process starts with the interaction of E1E2 with SR-BI. The complex then encounters CD81 and is translocated to the tight junction. Clathrin-mediated endocytosis transports the incoming virus-receptor complexes to the early and late endosomes (2). The acidic environment in the late endosome triggers virus fusion with

the host membrane (1). Subsequently, the viral genome is released into the cytosol where protein translation, replication and assembly of new viruses occur.

1.2.2 Human immunodeficiency virus (HIV)

HIV is an enveloped virus belonging to the Lentivirus genus, of the *Retroviridae* family. Since its discovery in 1981, HIV has infected over 60 million people worldwide and caused more than 25 million deaths (49). Primary targets of HIV are activated CD4+ T helper lymphocytes although the virus can infect several other cell types. Two types of HIV, HIV-1 and HIV-2, exist and they differ by about 40% in genomic sequence. Based on genetic variations four major groups of HIV-1 -M, N, O and P- have been identified, with group M responsible for the majority of the infections. Group M has been further divided into 9 clades that differ by 15-20% in their DNA sequences. Clade B is the most common clade in the Americas, Europe and Australia, whereas clade C predominates in southern Africa (50). HIV can stay dormant in the proviral form within a cell, usually resting CD4+ T lymphocytes, and can be reactivated to resume active virus production (51). HIV can be transmitted by contaminated blood and blood products, from infected mother to baby and most frequently through vaginal or anal intercourse (52).

HIV is a diploid positive sense RNA virus. Its genome contains nine open reading frames that produce fifteen proteins. Once inside the cell, the viral RNA is reverse transcribed via reverse transcriptase into double stranded DNA which is subsequently transported to the nucleus and integrates into the host chromosome. The integrated provirus is then transcribed and translated by the host's machinery to generate

polyproteins that are autocatalytically cleaved and processed, leading eventually to the formation of nascent viruses that bud from the host cell.

Current HIV therapy, known as HAART, comprises five major groups of drugs: nucleoside/nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, integrase inhibitors, protease inhibitors and entry inhibitors (53). Although effective, HAART therapy does not eliminate virus reservoirs or cure infections. Furthermore long term HAART usage is associated with the emergence of HIV resistant variants and side effects (54-56).

1.2.2.1 HIV entry mechanism

Entry of HIV into target cells is a complex, multi-stage process that involves coordinated sequential interactions between the viral proteins and the host cell receptors. HIV virions are surrounded by a lipid membrane containing two embedded envelope glycoproteins: a surface glycoprotein gp120 and the transmembrane glycoprotein gp41. Both proteins originate from a single polyprotein, gp160, which is cleaved by furin or furin like proteases in the Golgi compartment prior to being incorporated in the viral membrane (57). Initial contact between HIV and the target cell is driven by non-specific electrostatic interactions, between the positively charged domains on gp120 and negatively charge proteoglycans on the cell surface, and/or by specific interactions between virion incorporated host proteins and their ligands (58-60). Once in close proximity, gp120 can bind to the primary receptor CD4, a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. CD4, expressed on immune cell populations, contains four extracellular immunoglobulin domains, a single pass

transmembrane domain and a short cytoplasmic tail suggested to participate in intracellular signaling. CD4 binding induces a conformational transition from an unbound state to a bound state which exposes a coreceptor binding site (61, 62). CCR5 and CXCR4, coreceptors most often used by HIV, are seven-pass G protein coupled receptors with an extracellular N-terminal tail, three intracellular and extracellular loops and a C-terminal cytoplasmic tail (63). Binding of gp120 to a coreceptor triggers a second conformational change in the transmembrane subunit gp41 that results in the exposure of the hydrophobic fusion peptides and its insertion into the host membrane. This brings the viral and cellular membranes to close proximity and initiates the formation of a fusion pore that allows the release of the viral genetic material into the cytosol.

1.2.3 Herpes simplex virus (HSV)

Herpes Simplex virus is a double stranded DNA virus belonging to the *Herpesviridae* family, and one of the most common viruses acquired by humans (64). There are two serotypes of HSV: HSV-1 and HSV-2. They are distinguished by antigenic differences in their envelope proteins. The former is primarily associated with oral and facial lesions although some strains have been reported to cause genital infection (65). It is mainly transmitted by oral secretions and non-genital contact. It is estimated that 57% and 50-90% of the individuals in the USA and worldwide, respectively, are seropositive for HSV-1. HSV-2 predominantly causes genital herpes and is mainly transmitted sexually by genital secretions. It has an estimated seroprevalecence of 20% in North America (66). HSV has the ability to avoid the host

immune system through a non-replication state known as latency. As a result, lifelong infection is established with periods of reactivation stimulated by different cues (67). The success of HSV is aided by its broad cell tropism, as it has the ability to infect multiple cell types by exploiting various receptors. Thus the pathways of HSV entry are dependent on the host cell being targeted (68).

HSV is an enveloped virus composed of an electron dense core encapsulating the double stranded DNA, an isocohedral capsid, a tegument and 16 envelope proteins. The genome of HSV consists of two covalently linked components with unique sequences—UL (unique long) or US (unique short)—flanked by large inverted repeats. The genomes encodes at least 80 proteins, with approximately half of them involved in virus structure and replication, and the other half in interaction with the host cell and the immune system. Following viral entry, expression of alpha, beta and gamma proteins is initiated. Alpha proteins, products of the intermediate early genes, are involved in transcriptional regulation and the control of beta protein synthesis. Beta proteins or early proteins include the DNA polymerase and transcription factors, and are involved in DNA replication. The late proteins, gamma proteins, are initiated after DNA genome replication and comprise the structural proteins of the virion. Upon completion of transcription and DNA replication, nucleocapsids assembled in the nucleus are loaded with viral genome and bud through the double nuclear membrane. Viruses can either leave the cells via the exocytosis pathway, through plasma membrane budding or disseminate by spreading from cell to cell using methods such as cell-to-cell fusion, virion fusion across tight junctions, transfer across synapses and the recruitment of actin-

containing structures to spread the virus directly from an infected to an uninfected cell (69).

Current therapies for HSV infection rely on replication and fusion inhibitors. Acyclovir (ACV) introduced in 1980, is one of the oldest antiviral drug used for HSV treatment (70). Once phosphorylated by the viral thymidine kinase and cellular kinases, ACV functions as a substrate for the viral DNA polymerase inducing chain termination and arresting HSV replication. Due to limited ACV bioavalability, efforts were placed on the development of drugs that retain similar safety and efficacy while improving oral bioavailability. Such efforts allowed the development of several ACV prodrugs and derivatives currently classified as nucleoside, nucleotide and pyrophosphate analogs that either function as substrates for or inhibit the viral DNA polymerase (71). In addition to viral replication inhibition, N-docosanol is the only marketed HSV-1 drug that inhibits viral entry. It is a 22-carbon aliphatic alcohol that inhibits HSV-1 infection by targeting the cell membrane and modifying the regions necessary for fusion of enveloped viruses (72). While effective, it is only approved in topical form for treatment of herpes labialis but not recurrent genital herpes or ocular infections, the predominant diseases caused by HSV worldwide (73). Although replication inhibitors have been proven successful in the prevention of primary infection in neonates (74) and in reducing the duration of infection outbreaks, none are able to abolish viral latency and drug resistance usually arise after long term usage (75).

1.2.3.1 HSV entry mechanism

HSV entry is initiated by the attachment of glycoprotein B (gB) and glycoprotein C (gC) to cell surface heparan sulfate proteoglycans. Although both glycoproteins are present on both serotypes, gC plays a critical role in HSV-1 attachment (76), while gB is more important for HSV-2 (77). gB and gC can also bind to DC-SIGN, facilitating attachment during infection of dendritic cells. After attachment, virions travel to the cell body via a lateral movement along the length of filopodia. Once it approaches the desired receptors, it can enter the host via endocytosis, phagocytosis like mechanism, or direct fusion. In either case, the membrane fusion requires essential participation from viral glycoproteins gB, gD, gH and gL. Membrane fusion is initiated by the interaction between gD and one of the following receptors: herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor family; nectin-1 and nectin-2, two members of the immunoglobulin superfamily; and specific sites in heparan sulfate 3-O-sulfated heparin sulfate (78). Interaction of gD with the appropriate receptor allows tight anchoring of the virus to the plasma membrane of the host, bringing them close to juxtaposition (79). gD receptor binding leads to the insertion of gB fusion loops into the host membrane and promotes the formation of gB-gH/GL complex (80). Formation of the complex helps convert gB from a prefusion to a postfusion conformation capable of forming a fusion pore (81). Pore formation completes the fusion process allowing the release of the tegument protein and viral capsid into the cytoplasm. Upon release in the cytoplasm, the tegument surrounded nucleocapsid is carried to the nuclear membrane

where the nucleocapsid docks onto a nuclear pore complex and the DNA genome enters the nucleus.

1.2.4 Different methods to inhibit viral entry

While current therapies have played substantial roles in the fight against HCV, HIV and HSV, their use is limited and for the most part does not result in eradication of the virus. One way to overcome such constraint is the use of entry inhibitors that prevent initial viral infection.

1.2.4.1 HCV

As the entry process is essential for initiation of infection, intrahepatic spread and maintenance of infection, HCV research efforts have focused on the development of antivirals targeting the virus particles or host factors indispensable for HCV uptake.

1.2.4.1.1 Polyclonal & monoclonal neutralizing immunoglobulins

The first *in vitro* proof of concept was demonstrated by Farce et al. when preincubation of infectious HCV with autologous polyclonal serum or hyperimmune anti HVR1 (first 27 aa of E2) serum conferred protection in chimpanzees (82). Encouraged by this outcome, numerous human and non-human antibodies targeted against E1 and E2 have been successfully tested pre-clinically in small animals or HCVpp and/or HCVcc cell culture system (83). Only Civacir®, HCV1 and HCV-AB68 were evaluated in clinical trials. While Civacir® proved to be safe and well tolerated no effect on HCV RNA levels and HCV reinfection after liver transplantation was observed (84, 85). HCV-AB68 usage resulted in a modest and transient decrease of HCV RNA levels in some patients during Phase I, but failed to have significant effects during a Phase II

study in HCV infected liver transplant patients (86, 87). HCV1 significantly suppressed viral load and delayed the time of viral rebound in Phase II trials, but resistant mutants emerged in all treated subjects (88). Such disappointing results were attributed by high variability of HCV envelope glycoproteins and the difficulty in reaching the appropriate dose after administration.

1.2.4.1.2 Carbohydrate binding agents

Since both E1 and E2 are highly glycosylated, several molecules that can interact and thereby inhibit envelope protein-cellular receptor interactions have been developed. Among them are lectins including cyanovirin N and griffithsin, and other carbohydrate binding agents such as GNA, HHA and pradimicin A. All molecules showed nanomolar to micromolar antiviral activity when tested *in vitro* (89-91). Furthermore griffithsin's activity was corroborated *in vivo* using humanized mice. Because glycosylation sites are highly conserved, viral escape mutants will be retarded providing an effective way of suppressing entry.

1.2.4.1.3 Viral targeting inhibitors

In addition to the above mentioned agents, small molecules and peptides known to target viral particles directly have been described. The polyphenol epigallocatechin-3-gallate (EGCG) was shown to interfere with virion attachment through direct viral targeting (92, 93). Moreover, it prevents cell-free infection and cell-to-cell transmission of HCV in a genotype independent manner. Serum amyloid A and CLDN1 derived peptide, CL58, were also shown to inhibit HCV uptake by directly interacting with the viral envelope glycoproteins (94, 95).

1.2.4.1.4 Targeting host factors

Host factors represent ideal targets for antiviral therapy as they are less likely to mutate overtime. Studies focused on the interaction between virions and receptor/attachment factors have allowed the development of small molecules and antibodies against all identified HCV binding partners. The NPC1L1 antagonist ezetimibe (48), protein kinase inhibitors, erlotinib and dasatinib (96), and SR-B1 antibody mAb16-71 (97) show the highest potential as they have either already received FDA approval or have been shown to successful suppress HCV infection when used as a prophylactic *in vivo*. Additional small molecules targeting downstream attachment steps have also been developed/discovered including well know fusion inhibitors concanamycin A, bafilomicyn and chloroquine. The most promising to date is REP9AC, an amphipathic oligonucleotide, propose to inhibit the fusion step and shown to be effective and safe in clinical trials involving chronically infected HBV patients (98).

Despite the above mentioned efforts the SR-B1 antagonist ITX-5061, is the only HCV entry inhibitor that has entered clinical testing.

1.2.4.2 HIV and HSV

Although extensive research has been devoted to the development of HIV and HSV vaccines (99, 100), the scope of this document will focus on the use of microbicides as prevention strategies. Moreover, given the well documented interplay between HSV and HIV, microbicides with dual action are preferred. Microbicides are compounds that can be applied to the vaginal or rectal mucosa aiming at preventing or

reducing sexually transmitted infections. In general viral microbicides can be divided into (i) nonspecific compounds with broad antiviral activity and (ii) specific molecules targeting essential viral or host cell proteins.

Four major types of vaginal HIV microbicides have been developed with varying degrees of clinical success: surfactants, buffers, entry inhibitors, and antiretroviral agents. Surfactants are the first microbicides to enter phase III clinical trials and they inactivate pathogens through the disruption of membranes or changes in the cell membrane structure. Studies conducted with surfactants nonoxynol-9 (N-9) and SAVVY® failed to provide protection against HIV acquisition, with N-9 found to increase the risk of HIV infection (101-103). Buffers comprise chemicals/agents used to supplement or enhance natural immune defenses. BufferGel®, ACIDFORM and recombinant/engineered *Lactobacillus* are among the most well-known examples. Unfortunately, BufferGel®, designed to maintain a healthy vaginal milieu, did not alter the risk of HIV infection when tested alongside 0.5% PRO 2000® in Phase III clinical trials (104). Entry inhibitors include co-receptor blockers, polyanionic sulfate and sulphonated polymers that prevent virions from attaching to and fusing with the host cell membrane. PRO 2000®, Carraguard®, cellulose sulfate and dextrin 2-sulfate have been evaluated as potential microbicides. Despite encouraging results from animal studies and *in vitro* none of them prevented HIV acquisition in large scale human trials (105-112). Other entry inhibitors being considered are VivaGel^{TM}, sodium rutin sulfate, PPCM, Spm8CHAS, and cyanovirin-N (113-116). Recent development of microbicides has shifted to the use of antiretroviral drugs that form part of the HAART drug cocktail as

they have an already established efficacy. Tenofovir, an adenosine nucleotide analog, is one of the active ingredients in several studies including CAPRISA 004, VOICE and FACTS 001 (117, 118). Although reduction in HIV incidence was observed in CAPRISA 004 and is being corroborated in FACTS 001, the VOICE study was halted because interim results showed no difference between the experimental and placebo group (119). To date Truvada ®, comprising two nucleoside analogs, tenofovir and emtricitabine, is the only FDA-approved anti-HIV prophylactic therapeutic and offered a 44% reduction in HIV transmission during clinical trial (120). Although encouraging, many concerns have been raised regarding the transmission of drug resistant mutants and its failure to reduce HIV acquisition in the VOICE trial.

As with HIV, surfactants, buffering agents and entry inhibitors have been considered as potential anti-HSV microbicide candidates. Although surfactant N-9 demonstrated *in vitro* activity against HSV, given the results obtained with HIV and subsequent work demonstrating the cytotoxic and inflammatory responses of N-9 any potential benefit was outweighed (101, 121-123). The next series of candidates considered were sulfated and sulfonated polymers; PRO 2000®, cellulose sulfate and Carraguard®. Potential concerns were raised when several candidates lost anti-HSV activity *in vitro* and in murine models if HSV was introduced in seminal plasma (124). Lack of efficacy against HSV was observed for PRO 2000® in Phase IIIb clinical trials (125). Carraguard® showed no protection against HIV and other sexually transmitted infections, although HSV acquisition and recurrences were not reported (111). Moreover, a combination of Carragurad® and zinc, known as PC 710, showed higher

efficacy of HSV-2 protection compared to HIV-1 when tested in animal models (113). Lastly, cellulose sulfate efficacy trials against HIV were prematurely halted due to a significant increase in HIV acquisition, thus its effect against HSV was not evaluated (112). Other compounds with similar mechanism of action were in developmental/initial clinical stages as of 2009 are: VivaGel^{TM}, PPCM, Spm8CHAS, amphipathic DNA polymers, sodium rutin sulfate and SAMMAN (113-116, 126, 127). Two acid buffering agents, BufferGel® and ACIDFORM, were also advanced to clinical trials. BufferGel® demonstrated short duration of action and failed to provide protection against HSV in clinical trials (125). Additional strategies being explored include defensins and siRNA. Defensins were shown to inhibit HSV infection *in vitro* through multiple mechanisms (128), while small interfering RNA sequences targeting viral proteins provided mice protection against lethal HSV-2 challenge (129, 130). The most recent success story has come from the CAPRISA 004 trial which not only reduced HIV-1 transmission by 39% but also reduced the risk of HSV-2 acquisition by 51% (117).

1.3 Research objectives

The focus of this research is to characterize entry inhibitors that target molecules indispensable for viral entry not encoded by the viral genome, thus retarding the emergence of viral resistance. The following chapters contained a detailed description of the experimental techniques used to characterize each molecule, and the conclusions drawn from the obtained results.

Chapter II focuses on the discovery and characterization of the phenothiazines family as strong entry inhibitors of HCV. Specifically phenothiazines were shown to

suppress HCV infection by intercalating into cholesterol-rich domains of target cells, modulating its fluidity and consequently reducing fusion between the host and viral membranes.

Chapter III describes the characterization of two first generation H1-antihistamines. Like phenothiazines, both compounds suppress HCV entry likely at the fusion step. Their antiviral action was proven to be highly associated with cholesterol content in the host and virion, suggesting the use of an NPC1L1 dependent pathway.

The last two chapters explore the potential of PD as an alternate treatment for HCV and HIV coinfected patients as well as its use as a prevention strategy for the acquisition of sexually transmitted viruses like HIV and HSV. Chapter IV demonstrates the ability of PD to inactivate virions directly through the interaction with a structural molecule not encoded by the viral genome. For HIV this interaction is later accompanied by irreversible disruption of the virion rendering the particle non-infectious. Chapter V focuses on the use of PD as an anti-HIV microbicide. PD was proven to be highly stable in environments encountered by microbicides. Moreover, PD was able to efficiently inhibit HSV infection, a virus known to enhance viral acquisition of HIV.

CHAPTER II

PHENOTHIAZINES INHIBIT HCV ENTRY LIKELY BY INCREASING THE FLUIDITY OF CHOLESTEROL-RICH MEMBRANES[*]

2.1 Overview

Despite recent progress in the development of direct-acting antiviral agents against hepatitis C virus (HCV), more effective therapies are still urgently needed. We and others have previously identified three phenothiazine compounds as potent HCV entry inhibitors. In this study, we show that phenothiazines inhibit HCV entry at the step of virus-host cell fusion, by intercalating into cholesterol-rich domains of the target membrane and increasing membrane fluidity. Perturbation of the alignment/packing of cholesterol in lipid membranes likely increases the energy barrier needed for virus-host fusion. A screening assay based on the ability of molecules to selectively increase the fluidity of cholesterol-rich membranes was subsequently developed. One compound that emerged from the library screen, topotecan, is able to very potently inhibit the fusion of liposomes with cell culture-derived HCV (HCVcc). These results yield new insights into HCV infection and provide a platform for the identification of new HCV inhibitors.

Data from Figures 2.2C, 2.6 and 2.8B were generated at University of Lyon by Dr. Eve-Isabelle Pécheur

2.2 Introduction

Hepatitis C virus (HCV) infects at least 130 million people worldwide and is the major cause of chronic liver disease. Infected patients are at risk of developing fibrosis, cirrhosis and liver cancer (17-19). Although identified in 1989, advances in treatment have been augmented by the development cell culture-grown HCV (HCVcc) in 2005 (35, 131, 132). No vaccine is available, and the current treatment for HCV infection involves a weekly injection of pegylated alpha interferon and a twice-daily weight-based dose of ribavirin for 24- to 48-weeks. This standard of care is plagued by a long duration, limited efficacy and serious side effects (133). Although the recent addition of new direct acting antivirals (DAAs) targeting HCV NS3-4A protease – telaprevir and boceprevir – to the anti-HCV therapeutic arsenal have improved the cure rates, they must be used in combination with interferon, as HCV has a remarkable ability to overcome a single DAA. Telaprevir and boceprevir only work in patients infected with genotype 1 HCV, and are both not very effective in patients who did not respond to the pegylated interferon-ribavirin therapy (134). In addition, both telaprevir and boceprevir appear to worsen the already problematic side effects of the standard therapy such as rashes and anemia (24). Currently approved DAAs and most molecules in the pipeline are protease inhibitors, nucleoside inhibitors, non-nucleoside inhibitors and NS5A inhibitors (135). A major obstacle in combating HCV is the low fidelity of the viral replication machinery, enabling the virus to quickly develop resistance (136). To date, ITX-5061 is the only inhibitor of HCV entry that has entered clinical testing. ITX-5061 blocks a post-binding step in the viral entry process by directly interacting with the entry

factor scavenger receptor B1 (SR-B1) (96). New DAAs targeting entry steps critical to viral infection with additive potency when combined with existing DAAs and exhibiting low cytotoxicity are highly desirable.

HCV is an enveloped, positive-sense RNA virus belonging to the *Flaviviridae* family. The 9.6-kb viral genome encodes a single large polyprotein that is processed by viral and cellular proteases to produce the virion structural proteins (core and glycoproteins E1 and E2), P7, and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). HCV infection involves multiple steps. Viruses first attach to target cells via glycosaminoglycans and low-density lipoprotein (LDL) receptors. After recruitment to the membrane, HCV binds sequentially to entry factors involving SR-B1, tetraspanin CD81, Niemann-Pick C1-like 1 (NPC1L1) cholesterol (Cho) uptake receptor and proteins of tight junctions, i.e., CLDN1 and OCLD (32). HCV then enters cells at the tight junction via clathrin-mediated endocytosis and fuses with the host membrane in the late endosome. Progress in defining the molecular mechanism of HCV entry raises the opportunity to exploit new viral and host targets for therapeutic intervention. Entry inhibitors have the potential to limit the expansion of the infected cell reservoir, prevent re-infection after liver transplantation and complement the many protease and polymerase inhibitors currently under development. Although the discovery of drugs targeting the entry stage is still in its infancy, antibodies against SR-B1 (137), CD81 (138), and CLDN1 (40), as well as a number of small molecule inhibitors, have recently been developed and are able to effectively block HCV entry (92, 93, 139-144).

Phenothiazines are a group of nitrogen- and sulfur-containing tricyclic compounds that were first synthesized by Bernthsen in 1883. Phenothiazines with dialkylaminoalkyl groups and small groups substituted at positions 10 and 2, respectively, were found to interact with the dopamine receptors and have exhibited valuable activities such as neuroleptic, antiemetic, antihistaminic, antipruritic, analgesic and antihelmintic activities (145). To date, more than 100 phenothiazines have been used in clinic to treat psychotic disorders, and over 5,000 phenothiazine derivatives have been synthesized. Other receptors that can be modulated by phenothiazines include histamine H1, adrenergic α1 and α2, muscarinic (cholinergic), and serotonergic receptors (145). In addition to neurotransmitter receptors, phenothiazines have also been reported to bind to calmodulin and block its calcium signal-transduction activity, inhibit clathrin-coated pit formation, and activate rynodine receptors (146). Antiviral and anti-microbial activities have also been described for phenothiazines and related compounds (147).

Our lab and others recently identified three phenothiazines – fluphenazine, trifluoperazine and prochlorperazine – as potent HCV entry inhibitors (148, 149). In this work, we wanted to understand the antiviral mode of action of this family of compounds, which presumably inhibit HCV entry through a common mechanism of action. This information will assist in future endeavors to identify new and more potent inhibitors of HCV entry. We found that phenothiazines inhibit the virus-cell fusion step of the HCV life cycle by intercalating into the host cholesterol-rich membrane. In the presence of phenothiazines, cholesterol-rich membranes become more permeable to water molecules, leading to increased membrane fluidity. We subsequently developed a high-

throughput screening assay. We screened a library of 2,752 compounds and identified a molecule, topotecan, that dose-dependently inhibits HCVcc-liposome fusion. This study suggests that alteration of target cholesterol-rich membrane fluidity may be a novel mode for suppressing HCV entry and should facilitate the identification of new HCV inhibitors with unique mode of action.

2.3 Materials and methods

2.3.1 Cells, plasmids, compounds and reagents

Huh-7.5 cells and plasmids encoding HIV gag-pol (39) and the envelope proteins of HCV H77/J6 (39) and vesicular stomatitis virus (VSV) were kindly provided by Prof. Charles Rice (Rockefeller University, NY). HEK 293T cells were purchased from Invitrogen (Carlsbad, CA). Trifluoperazine, prochlorperazine, mesoridazine, promazine, trifluprom azine and *cis*-flupentixol were purchased from Sigma-Aldrich (St. Louis, MO). Chlorpromazine and thioridazine were from MP Biomedicals (Solon, OH). Fluphenazine and bafilomycin were from Alfa Aesar (Ward Hill, MA) and Axxora (San Diego, CA), respectively. All phenothiazine compounds were dissolved in dimethyl sulfoxide (DMSO) to a 10 mM stock concentration. Bafilomycin was dissolved in DMSO to a 250 μM stock concentration. Laurdan and Prodan fluorescent probes were purchased from Anaspec (Fremont, CA). Laurdan and Prodan were dissolved in methanol and DMSO to a final concentration of 0.5 mM and 10 mM, respectively. The human anti-CD81 JS-81 monoclonal antibody (MAb) was obtained from BD Biosciences (San Jose, CA). CellTiter-Glo Luminescent Cell Viability assay kit and BioLux Gaussia Luciferase assay kit were purchased from Promega (Madison, WI) and

New England Biolabs (Ipswich, MA), respectively. The growth medium for all cell culture work was Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/l glucose, 4.0 mM L-Glutamine, and 110 mg/l sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X non-essential amino acids (Thermo Scientific HyClone, Logan, UT). Dulbecco's Phosphate-Buffered Saline (DPBS) was purchased from Thermo Scientific HyClone (Logan, UT). Octadecyl rhodamine B chloride (R18) was purchased from Invitrogen (St Aubin, France), and all other lipids (99% pure) were from Avanti Polar Lipids (Alabaster, AL).

2.3.2 Production of HCVcc and pseudotyped lentiviruses

The production and titer determination of Jc1 Gluc HCVcc (150) in Huh-7.5 cells was performed as previously described (148). Jc1 Gluc HCV contains the *Gaussia luciferase* (Gluc) reporter gene between HCV genes encoding the p7 and NS2 proteins. Pseudotyped lentivirus were produced by co-transfecting 293T cells with plasmids encoding HIV gag-pol (39), a provirus pTRIP-Gluc (148) and the appropriate envelope protein, using TransIT reagent (Mirus, Madison, WI) following the manufacturer's protocol. The supernatants containing the pseudoparticles were collected, pooled and filtered (0.45 μm pore size) at 48 h post transfection and then stored at 4°C for up to 1 week or at -80 °C for long-term storage. For production of lentivirus pseudotyped with envelope protein from HCV genotype 1a H77 (H77 HCVpp), HCV genotype 2b J6 (J6 HCVpp) and vesicular stomatitis virus (VSV-Gpp), plasmids H77 E1E2 pcDNA3, J6 E1E2 pcDNA3 and pVSVG, respectively, were used (39). A control pseudotyped

lentivirus lacking any envelope protein (Env$^-$pp) was generated using the same protocol, except that the envelope protein-encoding plasmid was replaced with the empty vector (pcDNA3).

2.3.3 HCVcc infection assays

To determine whether phenothiazines are virucidal, Jc1 Gluc HCVcc (6.4×10^5 50% tissue culture infective doses [$TCID_{50}$]/ml) was incubated with phenothiazines (50 or 5μM), PD 404,182 (150 μM) or DMSO (0.5%) for 1 h at 37°C, and the virus-compound mixtures were diluted 100-fold in growth medium and used to infect Huh-7.5 cells seeded 4-6 h earlier in 96-well plates at 3.2×10^4 cells/well. For controls, virus and drugs were diluted 100-fold separately and mixed before infecting Huh-7.5 cells. Cells were thoroughly washed 14-16 h post-virus inoculation to remove residual drug and virus. Supernatant Gluc activity was measured 48 h post infection. The percentages of virus entry and spread were determined relative to those of the DMSO control.

To determine whether phenothiazines act on the host cells, Huh-7.5 cells were infected with Jc1 Gluc HCVcc at various times after drug removal. Briefly, Huh-7.5 cells were seeded in 48-well plates at 4×10^4 cells/well. After attachment, these cells were treated with phenothiazines (5 μM), PD 404,182 (150 μM), bafilomycin (10 nM) or DMSO (0.5%) for 2 h at 37°C. For Set 1-3, these cells were washed thoroughly to remove residual drugs and then inoculated with Jc1 Gluc HCVcc (multiplicity of infection [MOI] = 1) or VSV-Gpp (100 fold-dilution) at 0, 4 or 24 h post-drug removal at 37°C. At 15 minutes post-virus inoculation, these cells were thoroughly washed to remove any remaining viruses and returned to the 37°C/5% CO_2 incubator. For Set 4,

cells were infected with the same viruses, but in the presence of the drug, and were continuously incubated in drug-containing medium after the infection period. Supernatant Gluc activity was measured 72 h post infection, normalized to viable cell levels, and used as an indication of viral infection.

To determine the anti-HCV activity of phenothiazines and topotecan , Huh-7.5 cells (1.6×10^4 cells/well) seeded 4-6 h earlier were infected with Jc1 Gluc HCVcc (MOI = 0.01) in the presence of increasing concentrations of the compounds. Supernatant Gluc activity was measured 48 to 72 h post infection and normalized to the DMSO (0.02-0.5%) treatment control. The cell viability was measured using CellTiter-Glo assay (Promega, Madison, WI) to gauge the compound toxicity. The 50% inhibitor concentration (IC_{50}), 50% effective concentration (EC_{50}) and 50% cytotoxic concentration (CC_{50}) were calculated using the sigmoidal fit function in Origin Lab (OriginLab, Northampton,MA).

2.3.4 Synchronized HCVcc/pp infection assay

To determine the step of entry inhibited by phenothiazines, we carried out a synchronized infection assay. Huh-7.5 cells were seeded in 48-well plates at 4×10^4 cells/well. The next day, virus-cell attachment was initiated by incubating the cells with Jc1 Gluc HCVcc (MOI = 1) at 4°C for 1.5 h. Unbound viruses were removed by thorough washing with complete growth medium, and then infection of bound viruses was initiated by moving the cell culture plates to a 37°C/5% CO_2 incubator. Fluphenazine (5 μM), bafilomycin (10 nM), JS-81 (2 μg/ml) or DMSO (0.05%) were added to the cells at different time points after the temperature shift. Cells were washed

thoroughly with complete growth medium 5 hours post-drug addition. Supernatant Gluc activity was measured 48 hours post infection using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA) and used as an indication of viral infection.

For HCVpp, Huh-7.5 cells were seeded in poly-L-lysine treated 96-well flat bottom tissue culture plates at $1.4x10^4$ cells/well and incubated at 37°C and 5% CO_2 for 5-6 hours to allow cell attachment. Compounds were added to the appropriate wells at the indicated time-points. For pseudoparticle infection, HCVpp, and Env$^-$pp were added to cells at 1:5 dilution. The cells were then transferred to a 4°C centrifuge and spinoculated for 3 hours at 300 x*g* to facilitate pseudoparticle attachment. Before resuming incubation at 37°C/5% CO_2, cells were washed 4 times with 100 µl of cold growth media to remove unbound pseudoparticles and pre-existing Gluc, and 100 µl of fresh media, either containing or lacking the relevant drugs, were added to the cells. Supernatant Gluc activities were quantified 72 h later (using BioLux Gaussia Luciferase assay kit), normalized to viable cell levels (determined by CellTiter Glo Luminescent Cell viability assay kit) in the relevant wells and used to report the levels of lentiviral pseudoparticle entry.

2.3.5 HCVpp/cc-liposome fusion assay

To evaluate the ability compounds to inhibit HCV fusion *in vitro,* we carried out HCV-liposome fusion assay. Fusion between HCVpp and liposomes was assayed as described elsewhere (151). H77 HCVpp collected from the cell supernatant was purified and concentrated 100-fold by use of ultracentrifugation devices to a titer of ~10^7 IU/ml. One microliter of liposomes (25 µM lipid final) composed of phosphatidylcholine (PC),

cholesterol and R18 (65:30:5 mol%) were added to a 37°C thermostated cuvette containing 20 µl concentrated H77 HCVpp (~$2x10^5$ viral particles) in phosphate-buffered saline (PBS) at pH 7.2. Fluphenazine, trifluoperazine or promazine dissolved in DMSO was added to the mixture at a final concentration of 2.5, 5, 10 or 20 µM. After thermic equilibration, fusion was initiated by adding 2 µl diluted HCl (final pH 5.0). Lipid mixing was measured as the dequenching of R_{18} (excitation 560 nm, emission 590 nm), resulting in an increase of the fluorescence signal. Recordings were performed in a Tecan Infinite-1000 spectrofluorometer. Maximal R_{18} dequenching was measured after addition of Triton X-100 to the cuvette at 0.1% final concentration. Fusion between JFH-1 HCVcc and liposomes was determined similarly. Freshly prepared JFH1 HCVcc, concentrated 100-fold through a 20% sucrose cushion was used in the fusion experiment (152).

For the modified HCVpp-liposome fusion experiment, 20 µl concentrated H77 HCVpp or liposomes (working suspension at a 1:20 dilution from stock) was preincubated with fluphenazine, trifluoperazine or promazine at a 5 µM final concentration for 3 min at 37°C. This mixture was then diluted 10-fold with PBS to reach 0.5 µM final drug concentration, and 1 µl untreated liposome or 20 µl HCVpp was added, respectively. Fusion was determined in the same way as described above.

The study of the effect of topotecan on HCVcc membrane fusion was performed as described previously (152). Briefly, JFH-1 HCVcc (~$1.6x10^5$ particles), suspended in 150 mM NaCl, 10mM Tricine-NaOH, pH 7.4, was added to a cuvette containing R18-labeled PC-cholesterol liposomes (15 µM final lipid concentration) in the absence or

presence of increasing concentrations of topotecan. After temperature equilibration, fusion was initiated by HCl addition to the cuvette, and kinetics were recorded using a dual-channel PicoFluor hand-held fluorometer (Turner Biosystems, Sunnyvale, CA) operated under the "rhodamine" channel (excitation and emission wavelengths 540 ± 20 and > 570 nm, respectively). Maximal R18 dequenching was measured after the addition of 0.1% Triton X-100 (final concentration) to the cuvette.

2.3.6 Fluorescence spectroscopy

To determine whether phenothiazines affect membrane fluidity, we calculated the generalized polarization of fluorescent dye Laurdan and Prodan incorporated into the liposomes in the absence or presence of phenothiazines. POPC (11-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), sphingomyelin (SM) and cholesterol (Cho) (Avanti Polar Lipids, Inc, Alabaster, AL) dissolved in chloroform at 10 mg/ml were mixed at molar ratios of 1:0:0 (100% POPC), 2.3:0:1 (70% POPC plus 30% Cho) or 3.9:1:2.3 (54% POPC plus 14% SM plus 32% Cho). To remove solvent, the lipids were first dried under a stream of nitrogen and then lyophilized. The lyophilized lipid mixtures were resuspended in DPBS at 400 µM final lipid concentration, sonicated at room temperature in a water bath sonicator for 10 min, and extruded repeatedly through a 100-nm polycarbonate membrane filter (Avanti Polar Lipids, Inc.) to obtain uniformly sized liposomes. Extruded liposomes were stored at 4°C for up to a week. For GP determination, liposomes (200 µM final concentration) were first incubated with Laurdan (5 µM final concentration) or Prodan (15 µM final concentration) for 15 minutes at room temperature in the dark. Increasing concentrations of the compounds

were added to the mixture and 100 µl/well of liposome-drug mixture was transferred to a white 384-well plate. The plate was incubated at 23°C for 30 min in dark and the fluorescence spectra were collected in a Gemini EM Spectrofluorometer (Molecular Devices, San Francisco CA) with an excitation wavelength of 310-350 nm for both dyes and the emission spectra recorded at 440 and 480 nm for Prodan and 440 and 490 nm for Laurdan. GP was calculated according to the equation (153):

$$GP = \frac{I_B - I_R}{I_B + I_R}$$

where I_B and I_R are the fluorescent intensities at the blue and red edges of the emission spectrum, respectively. Data were corrected for background signal measured with liposomes deprived of a probe. After the fluorescence spectra were measured, the plate was returned to a 37 °C incubator for another 30 min, after which the spectra were measured again.

2.3.7 Screen for inhibitors

A high-throughput screening assay was developed based on the ability of a compound to selectively increase the GP of cholesterol-rich liposomes in comparison to the DMSO control. The library screening was conducted at The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB). Liposomes composed of POPC-alone or POPC, Cho and SM at a 3.9:1:2.3 molar ratio (200 µM final concentration) were incubated with Prodan (15 µM final concentration) for 15 minutes at room temperature in the dark. Eighty microliters of the mixture was dispensed into each well of white 384-well plates using a Matrix *Well*Mate liquid dispenser (Matrix,Hudson, NH). One hundred nanoliters of the

drug library (ranging from 2-5 mg/ml) was then added to each well by use of a Seiko D-TRAN XM3106-31 PN 4-axis Cartesian robot.pin transferor (Caliper Life Science, Waltham, MA). The plates were incubated in the dark for 30 minutes at room temperature, and the fluorescence spectra were collected in a SGM 610 FlexStation III Spectrofluorometer (Molecular Devices, San Francisco CA) with an excitation wavelength of 310 nm and the emission spectra recorded at 440 and 480 nm. We screened the libraries Biomol 4 (Enzo Life Sciences, Plymouth Meeting, PA) and Chembridge 3 for molecules whose GP differ from that of the 0.25% DMSO control by a value of >0.05 (positive hit). Although we screened only 2,752 compounds in this study, this assay is amenable to high-throughput screening.

2.3.8 Replication inhibition and quantitative RT–PCR

To determine the effect of topotecan on viral replication, we quantified the amounts of HCV RNA in the appropriate cells. Huh-7.5 cells were electroporated with Jc1 Gluc HCV genomic RNA according to a previously described protocol (148) and seeded in 24-well plates (1.4×10^5 cells/well). The desired compounds were added to the medium 6 hours post electroporation. The medium was replaced with fresh, compound-containing medium at 48 h post electroporation, and the supernatant Gluc activity was determined using a BioLux Gaussia Luciferase assay kit (New England biolabs, Ipswich, MA) at 72 h post electroporation. After removing all the supernatant, these cells were washed once with Dulbecco's phosphate-buffered saline (Thermo Scientific HyClone, Logan, UT) and underwent one freeze-thaw cycle at -80 °C before RNA extraction using an EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA). The amount of HCV RNA

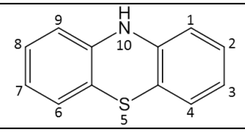
was quantified via TaqMan quantitative reverse transcription-PCR (qRT-PCR) (qScript One-Step FAST Kit, Quanta Biosciences, Gaithersburg, MD), using previously described primers (154).

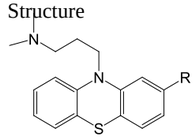
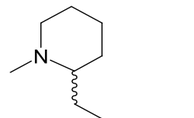
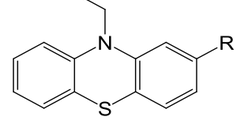
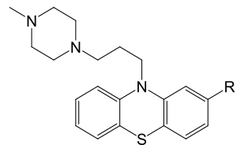
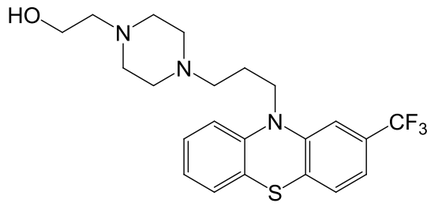
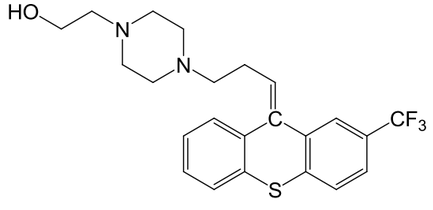
2.3.9 Virus entry inhibition assay

To determine the effect of topotecan on HCV entry, uptake of HCVpp in the presence of the compound was evaluated. Huh-7.5 cells were seeded in poly-L-lysine treated 96-well flat bottom tissue culture plates at 1.8 $x10^4$ cells/well and incubated at 37°C and 5% CO_2 for 5-6 hours to allow cell attachment. Compounds were added to the appropriate wells 1 h before transduction. HCVpp and Env$^-$pp were added to the cells at 1:5 dilution. The next day, these cells were washed 4 times with 100 µl/well of complete growth medium to remove unbound pseudoparticles and pre-existing Gluc reporter, and 100 µl of fresh medium were added to each well. Supernatant Gluc activity was quantified at 48 h post washing using BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA). The presented HCVpp entry data represent differences in supernatant Gluc activities between HCVpp- and Env$^-$pp-transduced cells at the specified compound concentrations.

To determine the effect of phenothiazines on HCV entry, Huh-7.5 cells were seeded in poly-L-lysine treated 96-well flat bottom tissue culture plates at 1.4$x10^4$ cells/well and incubated at 37°C and 5% CO_2 for 5-6 hours to allow cell attachment. Compounds were added to the appropriate wells 1 h before transduction. HCVpp and Env$^-$pp were added to the cells at 1:5 dilution. VSV-Gpp was added to the wells at 500-fold dilution.

Table 2. 1 Phenothiazines/thioxanthene used in this study and their anti-HCV properties.



Compound class	Compound subclass	Compound	R group	Structure	IC_{50} (μM)[a]	CC_{50} (μM)[a]	CC_{50}/IC_{50}
Phenothiazine	Aliphatics	Chlorpromazine	Cl		1.47±0.32	8.76±1.68	6
	Aliphatics	Promazine			2.28±0.43	>12	>5.3
	Aliphatics	Triflupromazine	CF_3		1.20±0.43	8.15±0.52	6.8
	Piperidines	Mesoridazine			1.77±1.01	>12	>6.8
	Piperidines	Thioridazine			0.78±0.31	5.28±0.84	6.8
	Piperazines	Prochlorperazine	Cl		0.92±0.11	7.60±1.49	8.3
	Piperazines	Trifluoperazine	CF_3		0.69±0.13	6.29±0.61	9.1
	Piperazines	Fluphenazine			0.37±0.01	5.65±0.09	15.3
Thioxanthene		*cis*-Flupentixol			0.25±0.07	5.22±0.23	20.9

[a] IC_{50} and CC_{50} values were calculated using a Gluc reporter HCVcc infection assay and represent the average of two independent experiments (error, SD).

The next day, cells were washed 4 times with 100 µl growth media to remove drugs and unbound pseudoparticles, and 100 µl of fresh growth media were added to the cells. Supernatant Gluc activities were quantified 48 h post infection using BioLux Gaussia Luciferase assay kit (New England biolabs). Cell viability in the absence of infection was quantified using CellTiter Glo Luminescent Cell Viability assay kit (Promega).

2.3.10 Statistical analysis

Statistical significance between different samples was evaluated using Student's *t*-test. A p-value of <0.01 was considered statistically significant. All analysis was done using Microsoft Excel.

2.4 Results

2.4.1 Identification of additional phenothiazine-like HCV inhibitors

Since phenothiazines and like compounds have been used extensively in humans, we first explored the anti-HCV activity of 6 additional FDA-approved phenothiazine and similar compounds using a Gluc HCVcc assay (Table 2.1). All tested compounds exhibited anti-HCV activity at submicromolar to micromolar concentrations and were able to specifically inhibit HCV entry (Figure 2.1). The most potent compound, *cis*-flupentixol, exhibited an IC_{50} of 0.25 µM and a therapeutic index (CC_{50}/IC_{50}) of 20. Comparing the anti-HCV potencies of different phenothiazines, we noticed that molecules with a piperazine ring at position 10 appeared to be slightly more potent than those with a tertiary amine (prochlorperazine > chlorpromazine; trifluoperazine, fluphenazine > triflupromazine), and the presence of a propanol group on the piperazine ring (fluphenazine) further increased the anti-HCV potency.

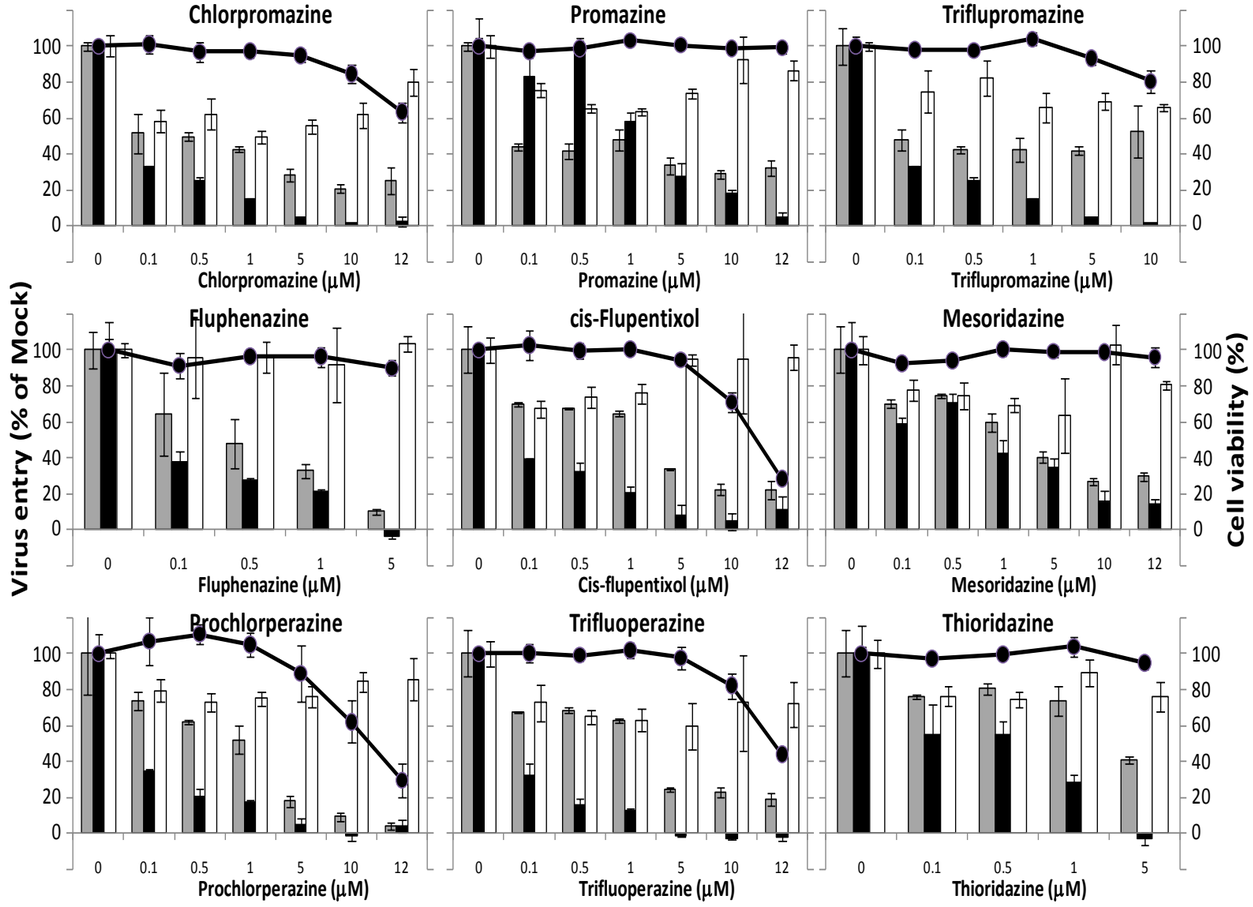


Figure 2. 1 Dose-response of different phenothiazine compounds on the entry of H77 HCVpp (grey bars), J6 HCVpp (black bars) and VSV-Gpp (white bars) into Huh-7.5 cells. Huh-7.5 cells were infected with H77/J6 HCVpp (5-fold dilution) or VSV-Gpp (500-fold dilution) in the presence of drugs for overnight. The supernatant gluc activities were quantified 48 h post infection. Cell viability (black circles) in the absence of pseudoparticle transduction was used to gauge the compound cytotoxicity. All values are expressed as percentages of 0.6% DMSO-treated cells (mock) after subtraction of signals obtained from Env-pp control pseudoparticles lacking envelope proteins and represent the mean of 2 independent experiments (error, SD). Note that negative values were occasionally computed for J6 HCVpp due to weak absolute signals, resulting in the measured Gluc activity for J6 HCVpp being lower than the signal for the corresponding Env-pp control. These results are within the experimental error range.

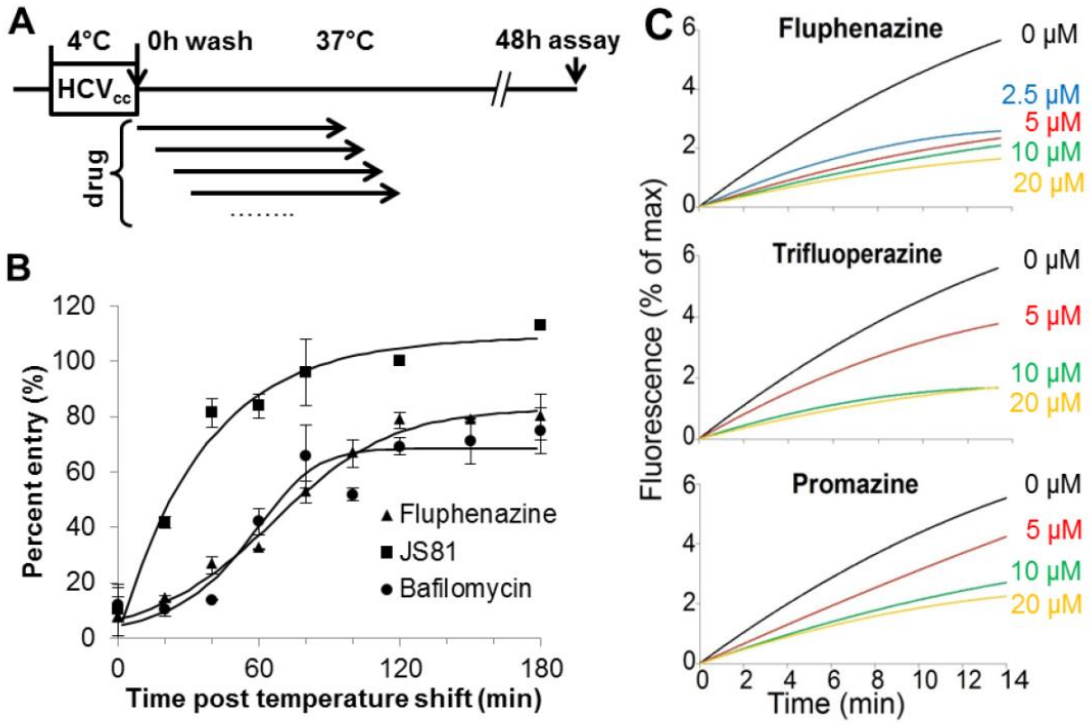


Figure 2. 2 Phenothiazines inhibit HCV fusion. (A) Schematic of synchronized HCVcc infection assay. Huh7.5 cells were incubated with Jc1 Gluc HCVcc (MOI 1) for 1.5h at 4°C, washed extensively and shifted to 37°C. Fluphenazine (5 µM), bafilomycin (10 nM), JS-81 (2 µg/ml) or DMSO (0.05 %) was added at different times after the temperature shift and removed 5 hours post compound addition. Supernatant Gluc activity was measured 48 hours post infection, normalized to the DMSO control and used as the indication of infection efficiency. **(B)** Fluphenazine inhibits HCVcc entry at a step similar to bafilomycin and later than JS-81. Fluphenazine (5 µM), JS-81 (2 µg/ml) or bafilomycin (10 nM) was added to the appropriate wells at different times post temperature shift. Representative data from at least 5 independent experiments are represented. The error-bars represent the standard deviation of triplicate samples in that experiment. **(C)** *In vitro* liposome fusion assay confirms that fluphenazine, trifluoperazine and promazine dose-dependently inhibit HCV fusion. Concentrated H77 HCVpp (~$2x10^5$ particles) was mixed with R-18 dye labeled liposomes and increasing concentrations of the phenothiazines. Fusion between HCVpp and liposome was triggered by the addition of HCl to lower the solution pH. Dequenching of R-18 dye, corresponding to fusion between HCVpp and liposome, was monitored by fluorescence increase. X-axis corresponds to the duration of the fusion process. Representative curves from at least 4 independent experiments are presented. Data generated at University of Lyon by Dr. Eve-Isabelle Pécheur.

A trifluoromethyl group at position 2 of the phenothiazine nucleus also enhanced the overall anti-HCV activity (trifluoperazine > prochlorperazine; triflupromazine > chlorpromazine).

2.4.2 Phenothiazines inhibit HCV fusion

HCV entry involves three main steps: (i) attachment of virions to the cell surface, (ii) movement of virus particles from the cell surface to the tight junction through interaction with different receptors, and (iii) entry into host cell through clathrin-mediated endocytosis and fusion of the viral membrane with the endosome upon acidification (155). To elucidate the anti-HCV mechanism of action of phenothiazines, we first determined the entry step inhibited by phenothiazines in a synchronized infection experiment (Figure 2.2A). Fluphenazine retained maximum entry inhibition when added after the temperature shift to 37°C, indicating that this compound inhibits a post-attachment step of HCV entry. Similar profiles were obtained for all the other phenothiazine inhibitors in a similar assay using HCVpp (Figure 2.3). The inhibitory activity of fluphenazine was lost at a later time than that of CD81 antibody (JS-81) and at a step similar to that of inhibition by bafilomycin, an H+-ATPase inhibitor that blocks the fusion of HCV by suppressing endosome acidification, indicating that fluphenazine, and possibly other phenothiazines, inhibit HCV entry at a step later than CD81 binding, likely during fusion (Figure 2.2B).

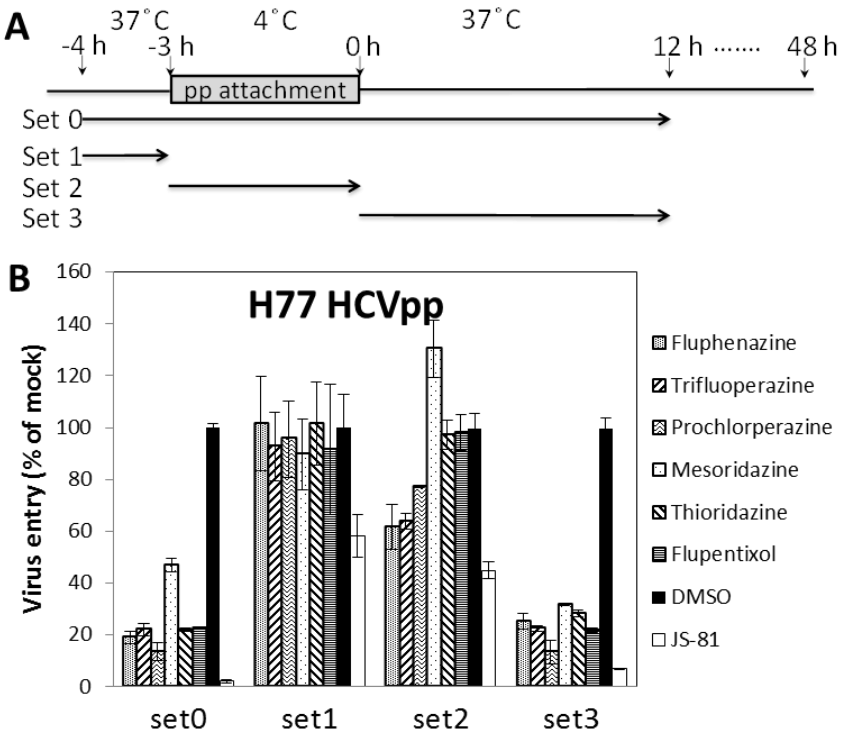


Figure 2. 3 Time-dependent effects of compounds on entry of H77 HCVpp into Huh-7.5 cells. (A) Experimental scheme. Huh-7.5 cells were spinoculated with H77 HCVpp (1:5 dilution) at 4 °C for 3 h. The cells were thoroughly washed to remove any unbound viruses and transferred to 37 °C incubator to initiate viral entry. The black arrows indicate the presence of the relevant inhibitors. JS-81 (2 µg/ml) and DMSO (0.5%) were used as the positive and negative control, respectively. These cells were washed 12 h post temperature shift to remove all the drugs. The supernatant Gluc activities were quantified 72 h later and used as indication of viral infection. **(B)** All compounds inhibit HCVpp entry at a step following virus attachment to cells.

To determine the effect of phenothiazines on HCV fusion directly, we carried out an *in vitro* HCVpp-liposome fusion experiment. For this experiment, we chose fluphenazine, trifluoperazine, both phenothiazines with piperazine substitution at position 10, and promazine, which has an aliphatic substitution at this position. All three phenothiazines dose-dependently inhibited HCVpp-liposome fusion *in vitro* (Figure 2.2C), with fluphenazine exhibiting the strongest fusion inhibition, followed by trifluoperazine and promazine, consistent with their anti-HCV potencies determined via Gluc HCVcc assay (Table 2.1). This activity was not due to unspecific molecular quenching of R18 fluorescence (data not shown) and was therefore fully attributable to fusion inhibition. These results confirm the fusion-inhibitory activity of these three phenothiazines. The ability of phenothiazines with both piperazine and aliphatic substitutions at position 10 to inhibit HCV fusion suggests that the fusion inhibition is independent of the substitution at position 10 and is likely a feature shared by other phenothiazines. In addition, since proteins are absent from the liposome, these data also indicate that a cellular protein(s)/receptor(s) is not required for phenothiazine-mediated HCV fusion inhibition.

2.4.3 Phenothiazines inhibit HCV fusion by acting on the host cell

We sought to elucidate whether phenothiazines act on cells or the virus. To determine whether phenothiazines are virucidal, Jc1 Gluc HCVcc was mixed with fluphenazine, prochlorperazine or trifluoperazine and then diluted 100-fold (pre-treatment) or each component was first individually diluted and then mixed (control) (Figure 2.4A).

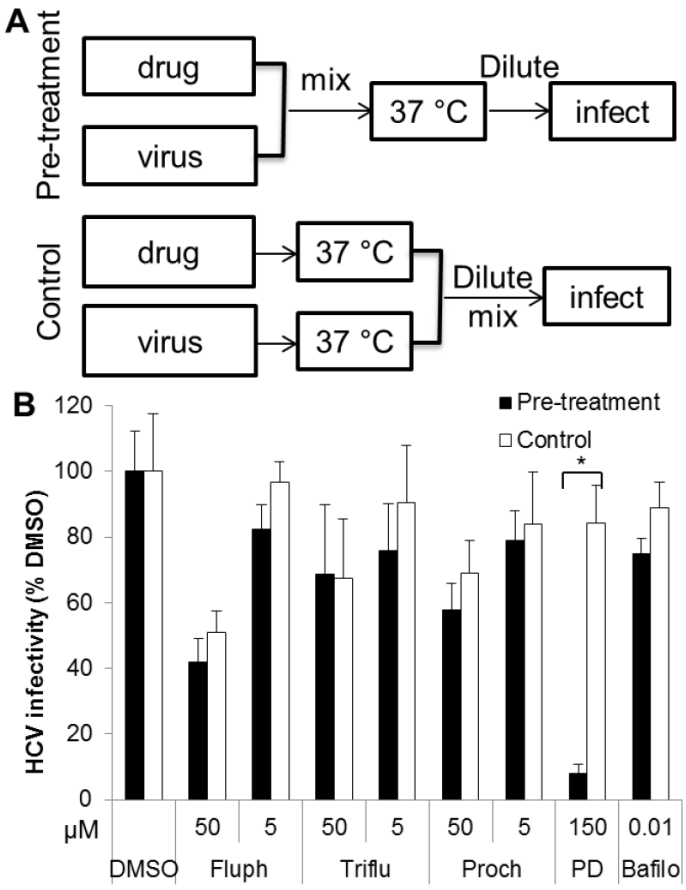


Figure 2. 4 Phenothiazines do not act directly on HCV. (A) Schematics of experiments to determine the compound's effect on virions. In the 'pre-treatment' group, Jc1 Gluc HCVcc (6.4 x 10^5 $TCID_{50}$/ml) was incubated with the appropriate compound at 37°C for 1h, then diluted 100-fold and used to infect Huh-7.5 cells. In the 'control' group, the same amount of HCVcc and drug were incubated separately at 37°C for 1h, diluted 100-fold, then mixed and used to infect Huh-7.5 cells. The final titer and the concentration of HCVcc and the drugs, respectively, are identical between the 'pre-treatment' and the 'control' groups. **(B)** Viruses pretreated with phenothiazines retain similar infectivity as the control. Supernatant Gluc activity was measured 48h post infection and normalized to the DMSO control (0.5 %). Fluph, fluphenazine; Triflu, trifluoperazine; Proch, prochloperazine; PD, PD 404,182; Bafilo, bafilomycin. The values and error bars represent the mean and standard deviation, respectively, of at least 2 independent experiments. Statistical significance was determined by the Student's *t* test (*, $p < 0.01$).

The infectivity of HCVcc samples pretreated with phenothiazines was similar to that observed with the control (non-pretreated) (Figure 2.4B). The positive control, the virucidal compound PD 404,182, reduced HCV infectivity by >90% during the same period (156). These results indicate that phenothiazines do not inhibit HCV entry by inactivating the virus directly.

To evaluate whether phenothiazines interact with the host cell, Huh-7.5 cells were treated with phenothiazine at 37°C for 2 h and extensively washed to remove residual drug prior to infection with Jc1 Gluc HCVcc or VSV-Gpp for 15 min at 0, 4 or 24 h post-drug removal (Figure 2.5A). Cells pretreated with phenothiazines became significantly more resistant to infection by HCVcc, but not VSV-Gpp, suggesting that phenothiazines inhibit the entry of HCV by acting on the host cell (Figure 2.5B). Cells treated with the control compound bafilomycin were similarly resistant to both HCVcc and VSV-Gpp infections. It is worth noting that although some phenothiazines are able to inhibit clathrin-coated pit formation and endosome acidification, this effect is likely not at play in the observed phenothiazine-mediated HCV inhibition phenomenon, because inhibition of VSV-Gpp, which also enters cells through clathrin-mediated endocytosis (157), was not observed at the same concentration of phenothiazines (Figure 2.5B). Collectively, these results suggest that phenothiazines inhibit HCV entry by acting on a non-protein host cell component, likely the lipid membrane.

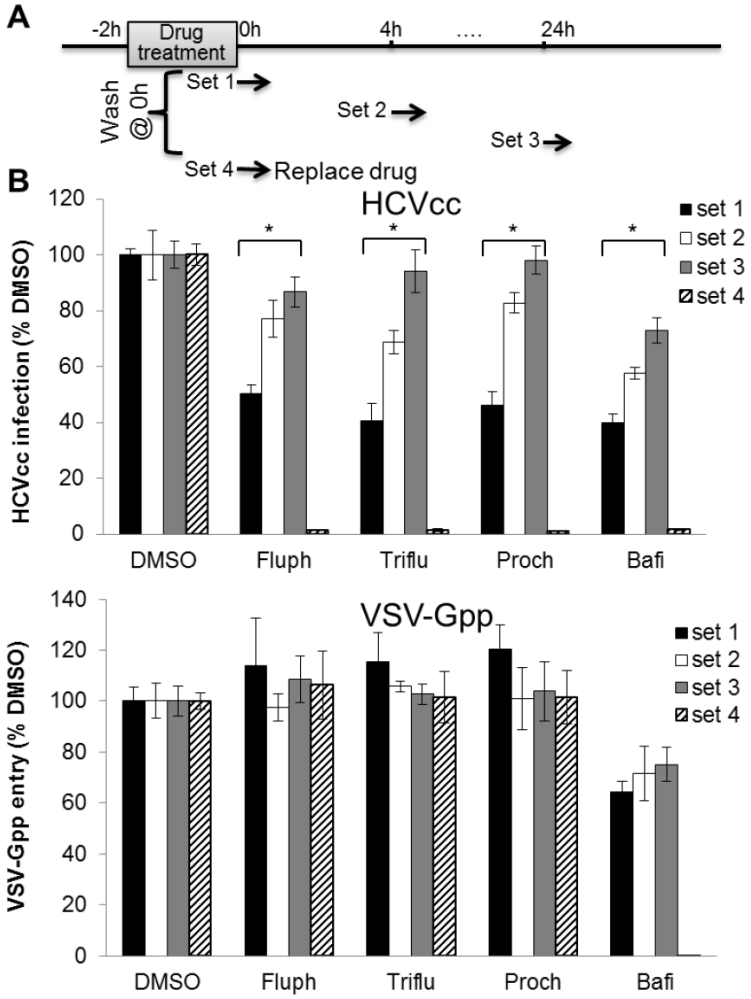


Figure 2. 5 Phenothiazines inhibit HCV by acting on the host cells. (A) Schematic of experiment to determine whether phenothiazines inhibit HCVcc entry by acting on the host cells. Huh-7.5 cells were treated with the specified compounds at 37°C for 2 h, extensively washed at t = 0h and then infected with Jc1 Gluc HCVcc (MOI ~1) or VSV-Gpp (diluted 500-fold) for 15 min at 37 °C at 0, 4 or 24 h post compound removal (Set 1-3). For Set 4, the cells were infected with the same viruses in the presence of the drugs. **(B)** Cells pretreated with phenothiazines became resistant to HCVcc but not VSV-Gpp infection, indicating that phenothiazines selectively inhibit HCV entry. The supernatant Gluc activity was measured at 48 h post drug removal, normalized first to the cell viability and then to the cell-viability-normalized DMSO control (0.5%). The values and the error bars represent the mean and the standard deviation, respectively, of at least 2 independent experiments. Fluph, fluphenazine, 5 µM; Triflu, trifluoperazine, 5 µM; Proch, prochloperazine, 5 µM; Bafi, bafilomycin, 10 nM. Statistical significance was determined by Student's *t test* (*, $p < 0.01$).

To confirm that phenothiazines inhibit HCV by interacting with the target lipid membrane, we designed a modified liposome-HCV fusion experiment (Figure 2.6A) in which liposomes or virus are pre-treated with the appropriate compound prior to dilution of the mixture and addition of the other component. In set 1, cholesterol-rich liposomes were pre-incubated with phenothiazines (5 μM). This mixture was diluted 10-fold to lower the drug concentration and then mixed with HCVpp. In set 2, HCVpp was pre-incubated with phenothiazines (5 μM), and the mixture was diluted 10-fold prior to addition of liposomes. In both sets, the final concentration of phenothiazine in the mixture was 0.5 μM, a concentration that is expected to have little to no effect on liposome-HCVpp fusion. In set 3, liposomes, phenothiazines (final concentration 5 μM) and HCVpp were all mixed together at the same time. Finally in set 4, liposomes and HCVpp were mixed in the absence of phenothiazines. We reasoned that if phenothiazines inhibit HCV-membrane fusion by interacting with the liposomal membrane, set 1 (liposomes pretreated with phenothiazine) would exhibit a similar extent of fusion inhibition to set 3 (all components mixed together), despite the much lower phenothiazine concentration at the time of fusion in set 1, while set 2 (HCVpp pretreated with compound) would exhibit minimal fusion inhibition.On the other hand, if phenothiazines interact with the virus directly, set 2 would exhibit a similar extent of fusion inhibition to that of set 3. Stronger fusion inhibition was observed in sets 1 and 3 than in set 2 (Figure 2.6B), providing further evidence that phenothiazines inhibit HCV fusion by acting on the target liposome/cell membrane.

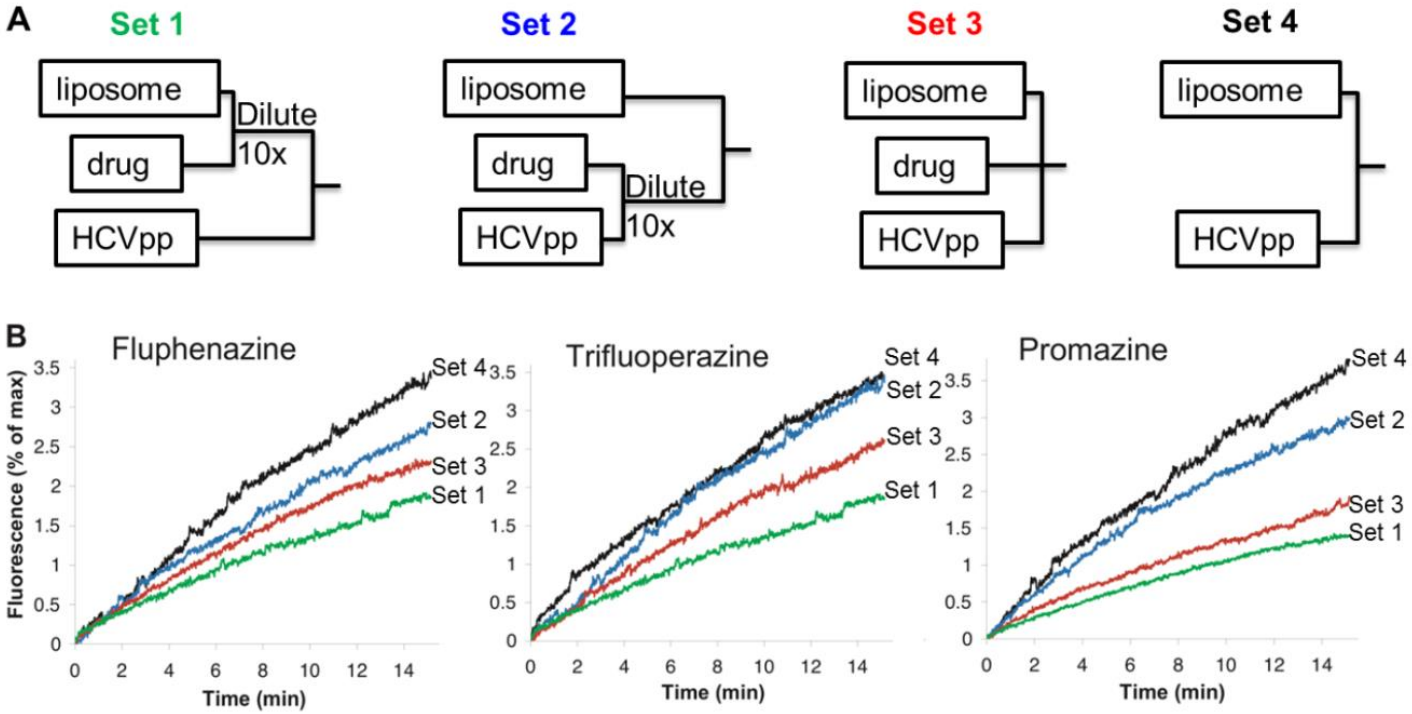


Figure 2. 6 Phenothiazines inhibit HCVpp-liposome fusion by interacting with the target membrane. (A) Modified drug addition protocol. Set1: Phenothiazine and liposomes were premixed and diluted 10-fold prior to the addition of HCVpp; Set 2: HCVpp and phenothiazine were premixed and diluted 10-fold prior to the addition of liposome; Set 3: Liposomes, phenothiazine and HCVpp were mixed together; Set 4: Liposomes and HCVpp were mixed in the absence of phenothiazine. The final concentration of phenothiazine in Set 1&2 is 10% of that in Set 3. **(B)** Fusion between HCVpp and liposomes was initiated by decreasing the pH to 5.0 (time 0) and recorded as R18 fluorescence dequenching over time. Concentrated H77 HCVpp (~$2x10^5$ particles) was used in each assay. Representative curves from at least 4 independent experiments were presented. Data generated at University of Lyon by Dr. Eve-Isabelle Pécheur.

Set 1 exhibited slightly stronger fusion inhibition than set 3, despite the 10-fold lower concentration in set 1 at the time of fusion. This may have been due to the presence of additional vesicles (e.g. exosome) in the concentrated HCVpp samples used in set 3, which could have competed with R18-labeled liposomes for the phenothiazines during membrane fusion.

2.4.4 Phenothiazines inhibit HCV fusion likely by increasing the target membrane fluidity

To gain insight into how phenothiazines inhibit HCV-membrane fusion, we studied their effect on lipid membrane fluidity. The lipophilic nature of phenothiazines enables this class of molecules to intercalate into lipid membranes and alter their fluidity (158). However, this effect was never evaluated at these low concentrations or in membranes with high cholesterol concentrations. Membrane fluidity was gauged by the GP generated by the fluorescent dyes Laurdan or Prodan. Both Laurdan and Prodan probes are lipophilic dyes able to insert into lipid bilayers and become fluorescent. The GP value is higher in rigid/ordered lipid membranes, as fewer water molecules have access to the probes embedded inside the membrane (159). The Laurdan probe inserts deep in the hydrophobic core of the lipid membrane, while Prodan preferentially partitions to the lipid head groups (160).

We determined the effect of phenothiazines on the GP of liposomes composed of 100% POPC or POPC with 30 mol% cholesterol. Membranes composed of 100% POPC resemble the basal cellular membrane, while those containing the additional cholesterol mimic lipid raft-containing membranes (161). Phenothiazines significantly reduced the

GP of cholesterol-rich, but not cholesterol-free membranes, in a dose-dependent manner (Figure 2.7). These results suggest that phenothiazines specifically reduce the rigidity of cholesterol-rich membranes. Since similar GP reductions were observed for both the Laurdan and Prodan dyes, phenothiazines likely insert deep in the hydrophobic core of the lipid membrane. It is possible that phenothiazines also intercalate into POPC membranes, but since these membranes are naturally very fluidic, the presence of phenothiazines does not appear to further increase the membrane fluidity at the low concentrations used. The ability of phenothiazines to significantly reduce the GP of cholesterol-rich but not cholesterol-free membranes may account for its minimal toxicity at the tested concentrations. We also determined the effect of phenothiazines on liposomes containing both cholesterol and sphingomyelin at a composition that more closely resembles lipid rafts (161). Incorporation of sphingomyelin did not have much effect on GP reduction (Figure 2.7), suggesting that cholesterol may be the major effector of phenothiazines. Lipid rafts are believed to be the location of HCV-cell fusion (151). Collectively, our data indicate that an increase of lipid raft membrane fluidity could be a means through which phenothiazine-induced HCV entry inhibition occurs.

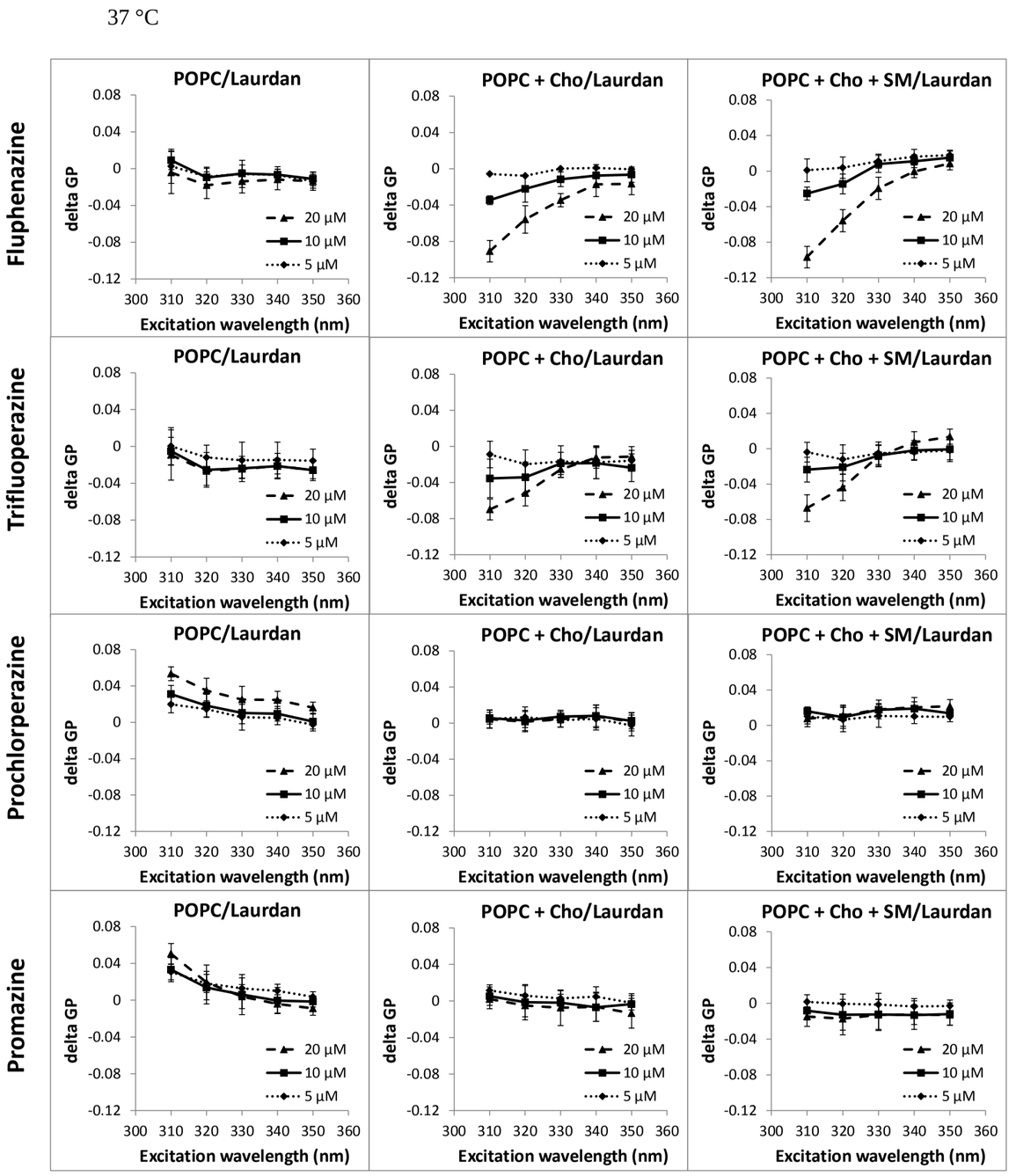


Figure 2. 7 Phenothiazines alter membrane fluidity. Liposomes composed of molar percentage: 100% POPC (left), 70% POPC 30%Cho (center) or 54%POPC 32%Cho 14%SM (right) were incubated with 5 µM Laurdan or 15 µM Prodan for 15 minutes at room temperature. Increasing concentrations of phenothiazine or DMSO (1.25%) were added to the mixture followed by a second incubation at the specified temperature for 30 minutes. Shifts in red and blue emission were measured at 440 and 480/490 nm after excitation at 310-350 nm. Delta GP = $GP_{phenothiazine}$-GP_{DMSO}. Values represent the mean of 2 independent experiments (error, SD). POPC; 11-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Cho; Cholesterol. SM; Sphingomyelin.

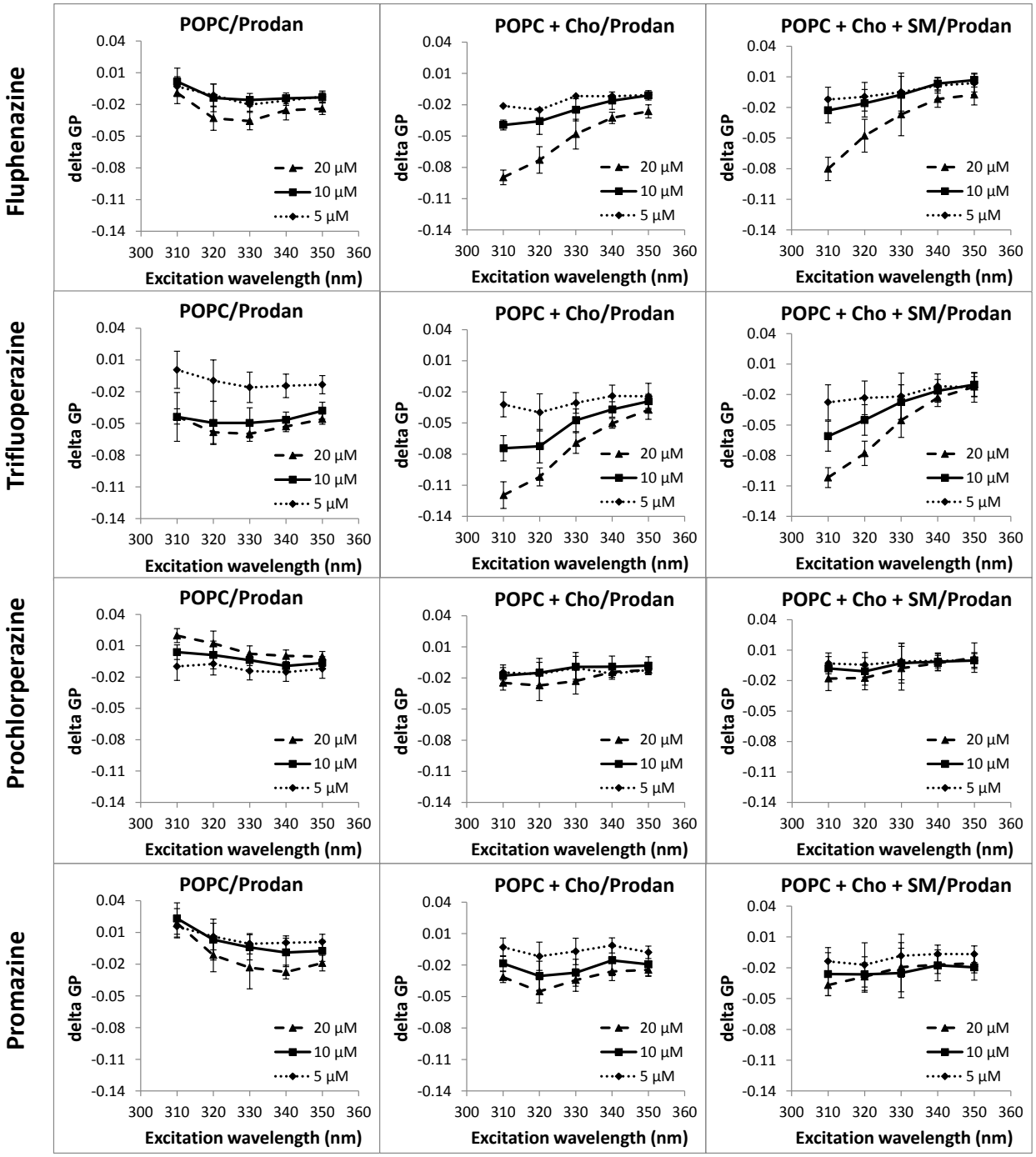


Figure 2.7 Continued.

23 °C

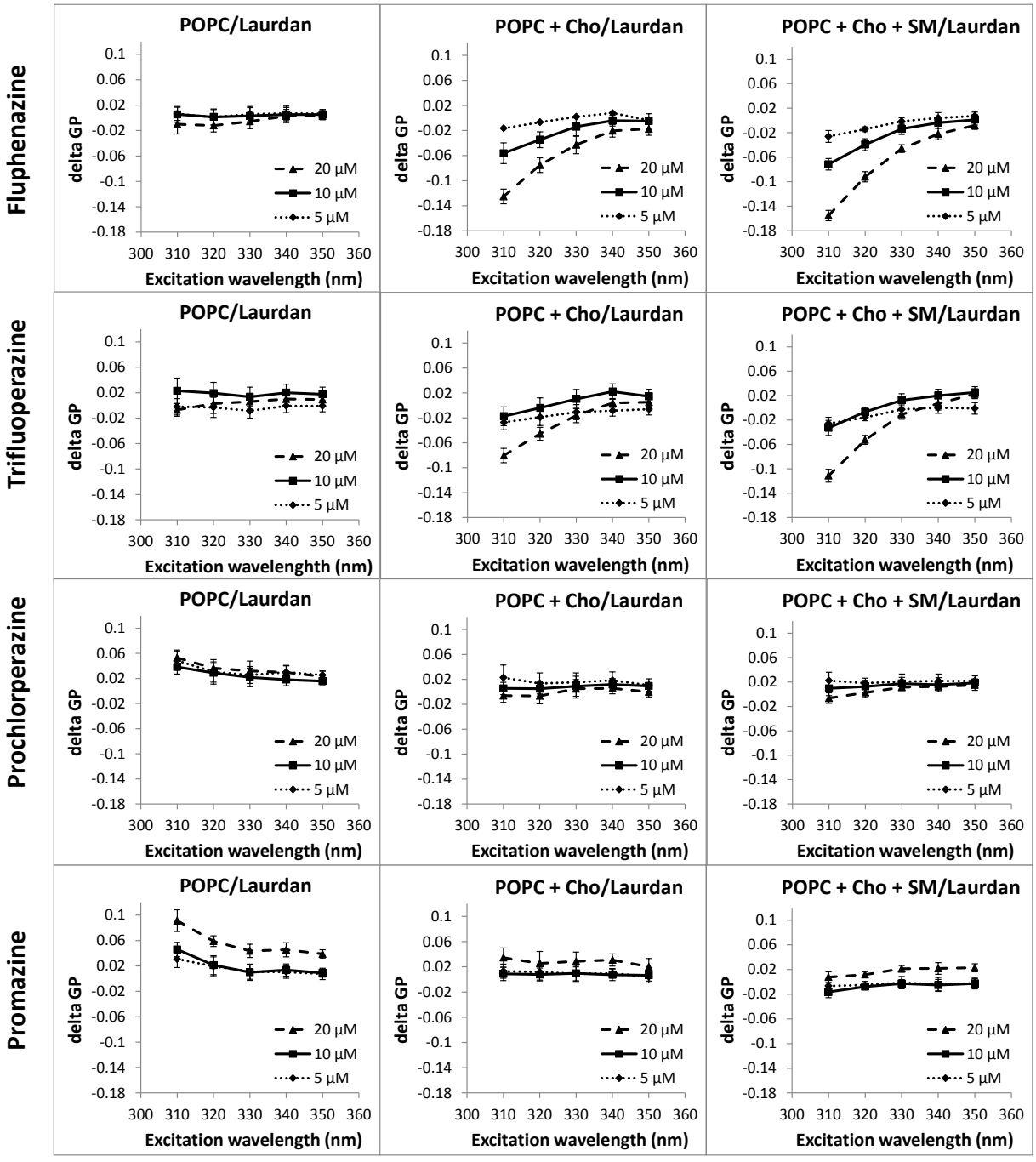


Figure 2.7 Continued.

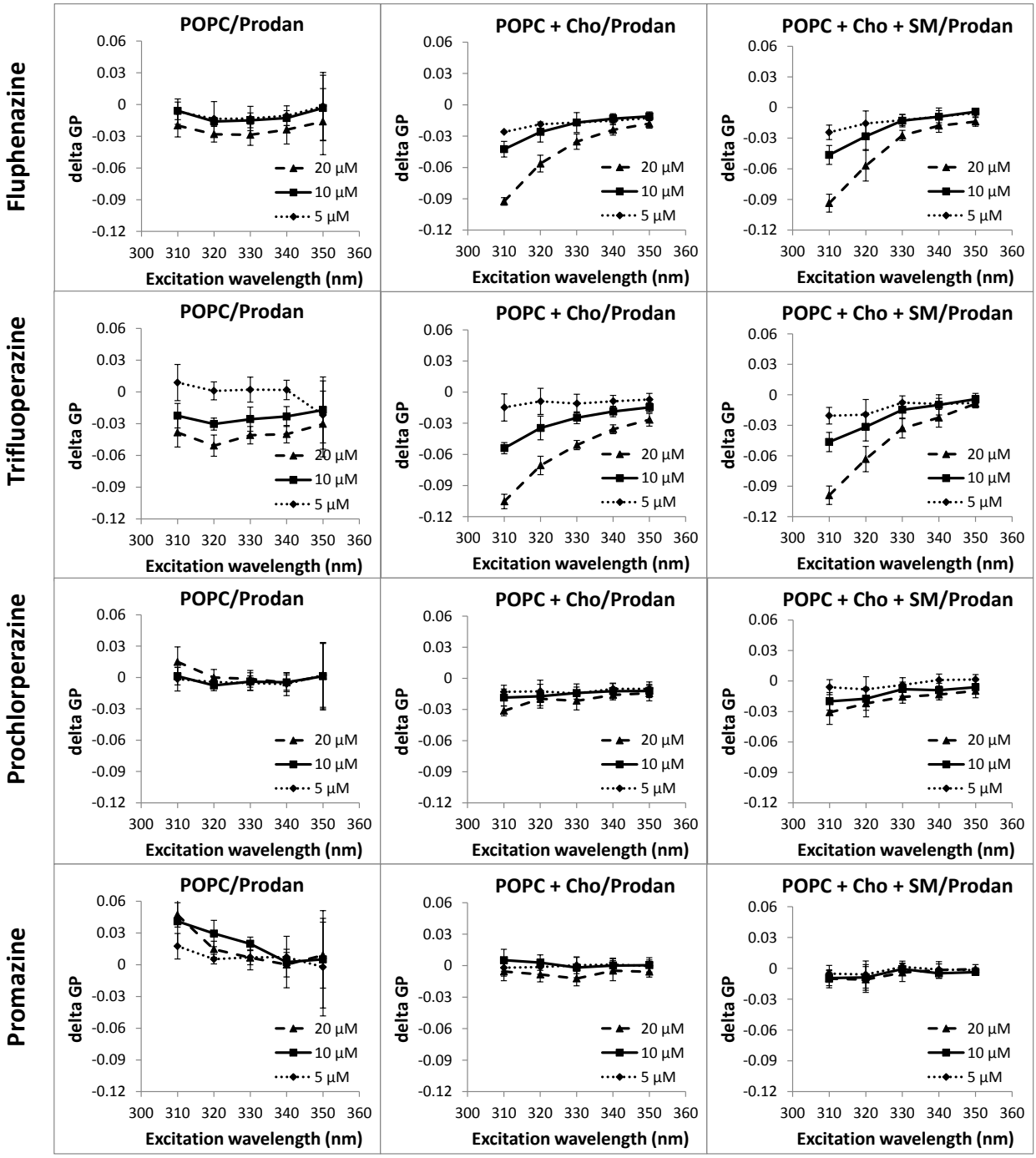


Figure 2.7 Continued.

2.4.5 Screening for additional HCV fusion inhibitors

Based on the above results, we hypothesized that compounds capable of increasing the fluidity of cholesterol-rich membranes will be able to inhibit HCV entry. We developed a screening assay using cholesterol-containing liposomes incorporating the Prodan dye and then screened 2,752 compounds. One compound, topotecan, was found to preferentially increase the fluidity of cholesterol-rich membranes at concentrations comparable to those approved for therapy in humans (162) (Figure 2.8A). An *in vitro* membrane fusion assay confirmed that topotecan inhibits HCVcc-liposome fusion (Figure 2.8B). This result underscores the importance of membrane fluidity on HCV entry and validates our membrane fluidity-based screening approach for HCV entry inhibitor discovery. Topotecan dose-dependently inhibits HCVcc infection in cell culture, with an estimated EC_{50} of 0.2 µM (Figure 2.8C) and CC_{50} of 88.1 µM (Figure 2.9). However, the inhibitory effect of topotecan was mainly due to its inhibition of HCVcc replication rather than entry (Figure 2.10) (see Discussion).

2.5 Discussion

Insight gained into HCV entry over the last few years has allowed for the discovery and development of inhibitors acting at different stages of viral uptake. Addition of new entry inhibitors to the current therapies will increase the resistance barrier, inhibit expansion of the infected pool and reduce the rate and extent of re-infection after liver transplantation (163). We and others recently identified 3 phenothiazine compounds – trifluoperazine, fluphenazine and prochlorperazine – as inhibitors of HCV entry (148, 149).

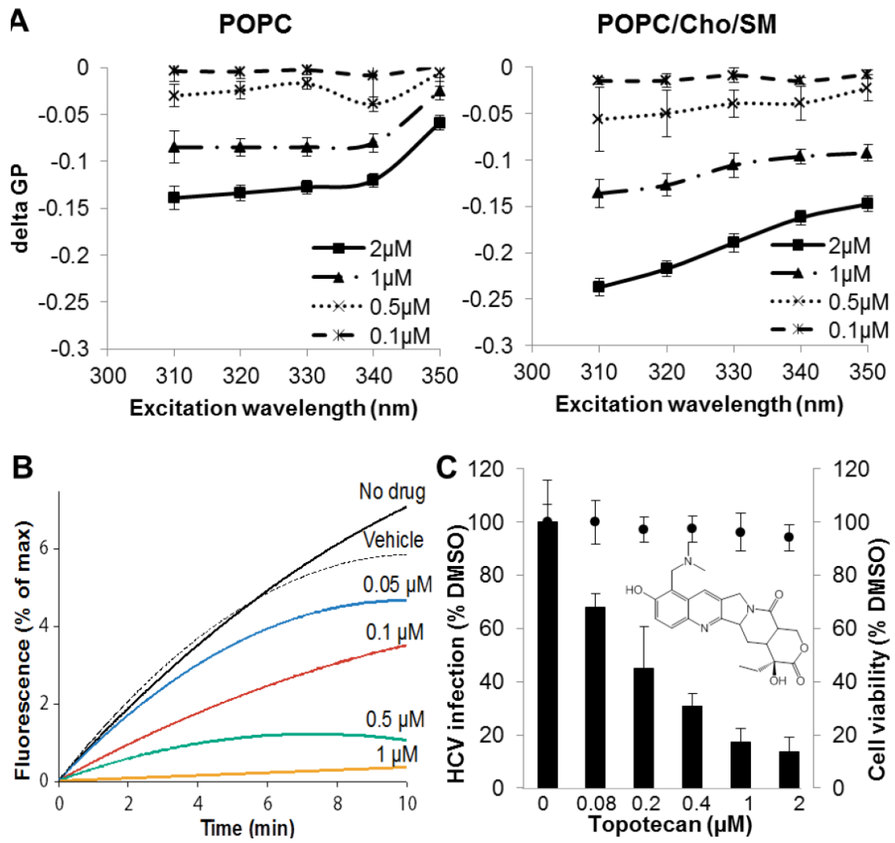


Figure 2. 8 Characterization of topotecan. (A) Topotecan preferentially increases the fluidity of cholesterol-rich membrane (POPC+Cho+SM) compared to cholesterol-free membrane (POPC). Liposomes composed of pure 11-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) or POPC with 32 mol-% cholesterol (Cho) and 14 mol-% sphingomyelin (SM) were incubated with Laurdan (5μM) or Prodan(15μM) for 15 minutes at room temperature prior to the addition of topotecan (0.1, 0.5, 1 and 2 μM) or DMSO (1%). The mixture was incubated at 37°C for another 30 minutes and the fluorescence shifts were determined. Delta GP = $GP_{topotecan}$-GP_{DMSO}. The values and the errors represent the mean and the standard deviation, respectively, of 2 independent experiments. **(B)** Topotecan dose-dependently inhibits HCVcc-liposome fusion *in vitro*. HCVcc (~$1.6x10^5$ particles) was mixed with R-18 dye labeled liposomes in the presence of increasing concentrations of topotecan. Fusion between HCVcc and liposome was triggered by the addition of HCl to lower the solution pH. Dequenching of R-18 dye was monitored by fluorescence increase. X-axis corresponds to the duration process. Data generated at University of Lyon by Dr. Eve-Isabelle Pécheur **(C)** Topotecan dose-dependently inhibits HCVcc infection in cell culture. The chemical structure of topotecan is shown in the inset. Huh-7.5 cells were infected with Jc1 Gluc HCVcc (MOI 0.01) in the absence or the presence of increasing concentrations of topotecan. Infectivity was quantified by measuring the supernatant Gluc activity 72 h post infection and normalized to the DMSO (0.02%) control (black bars). Drug cytotoxicity in the absence of HCV infection was determined by CellTiter-Glo assay (solid circles). The values and the errors represent the mean and the standard deviation, respectively, of 2 independent experiments.

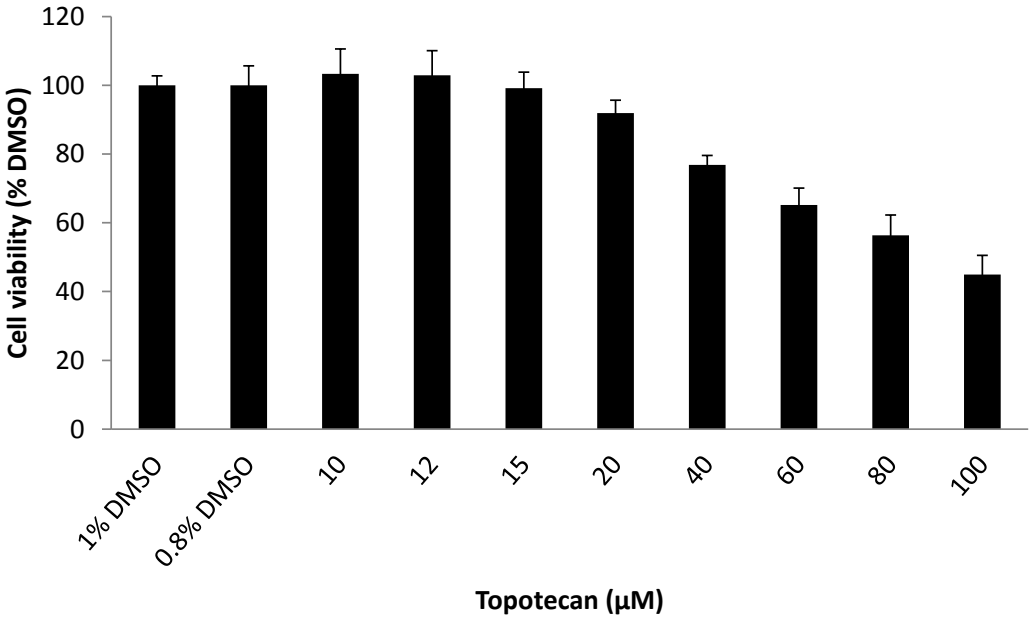


Figure 2. 9 Cytotoxic profile of topotecan. Huh-7.5 cells were treated with increasing concentrations of Topotecan for 72 h. Cell viability was determined by CellTiter-Glo assay to gauge compound cytotoxicity profile. Values represent the mean of 2 independent experiments carried out in triplicates (error, SD).

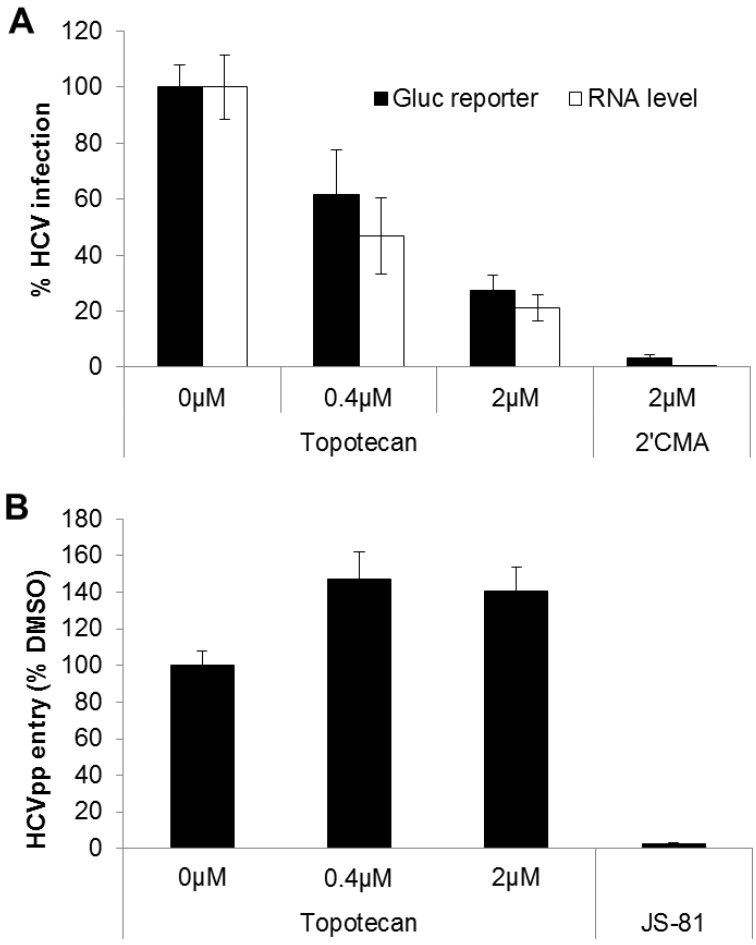


Figure 2. 10 Topotecan primarily inhibits HCV replication. (A) Effect of topotecan on HCVcc replication. Huh-7.5 cells were electroporated with Jc1 Gluc HCV genomic RNA and treated with topotecan, DMSO (0.125%) or 2'CMA at 6 h post electroporation. The amount of Gluc reporter in the supernatant and the intracellular HCV RNA level were quantified 72 h post electroporation. 2'CMA: 2'-C-methyladenosine. **(**B**)** Topotecan does not inhibit H77 HCVpp entry. Huh-7.5 cells were transduced with 5-fold diluted H77 HCVpp or Env¯pp in the presence of topotecan, JS-81 (2μg/ml) or DMSO (0.02%). The supernatant Gluc reporter activity was measured 48 h post compound removal, normalized to signals from Env¯pp, and used as an indication of the level of infection. Values represent the mean of 2 independent experiments (error, SD).

Phenothiazines are a large class of chemicals, many of which are currently used in clinic to treat psychotic disorders (145). To determine whether other phenothiazines can also inhibit HCV infection, we tested 6 additional FDA-approved phenothiazines and similar molecules and discovered that all these molecules exhibit anti-HCV activity (Table 2.1). The most potent inhibitor, *cis*-flupentixol, exhibited an IC_{50} of 0.25 μM and a therapeutic index of 20. The presence of a piperazine ring at position 10 enhances but is not required for HCV entry inhibition. The presence of a trifluoromethyl group at position 2 also appears to enhance anti-HCV activity. This information should assist future structure-activity relationship studies to identify more potent phenothiazine-based anti-HCV inhibitors.

We showed that phenothiazines inhibit HCV-cell-fusion by specifically interacting with the host/target membrane. Incorporation of phenothiazines into the leaflets of the target membrane increases the water permeability/fluidity of cholesterol-rich membranes (Figure 2.7) and reduces the rate of virus-liposome lipid mixing and hemifusion (Figure 2.6). The effect of lipid composition on viral infection, particularly the influence of cholesterol and spingolipids, has been studied widely. Many viruses enter host cells via cholesterol-rich microdomains (lipid raft), such as the West Nile, Ebola and Marburg, herpes simplex and vaccinia viruses, retroviruses and alphaviruses (164). In some cases, the cholesterol-dependence is due to clustering of viral receptors in the lipid raft, while in other cases it is due to specific interactions between the viral envelope glycoproteins and (a lipid of) the target membrane, as is the case for the fusion protein of alphaviruses and cholesterol (165). Concerning HCV, both phenomena are believed to occur. *In vitro*

cell culture studies have shown that HCV entry is adversely affected by cholesterol depletion (166, 167). The cholesterol absorption receptor NPC1L1 was recently identified as an HCV entry factor (48), which forms cholesterol-enriched microdomains together with flotillins (168). Concerning the tetraspanin HCV receptor CD81, two cholesterol binding sites have been mapped in the three-dimensional model of this molecule (169). It therefore appears that cholesterol might play a role in HCV entry through the local mobilization of receptors at specific membrane regions.

Our *in vitro* fusion studies showed that the presence of cholesterol significantly enhances the fusion of both HCV envelope protein-pseudotyped lentiviruses and cell culture-produced virions with liposomes, further confirming the important role of cholesterol in HCV-mediated fusion (151, 152, 170). In these receptor-free assays, cholesterol is likely to play a direct role in the fusion process.

Cholesterol is one of the most important lipid species in eukaryotic cells and has several different functions. Two of the primary and essential roles of cholesterol are to decrease permeability and increase the stability of the membrane bilayer (171). Membranes rich in cholesterol have a rough surface due to clustering of cholesterol molecules into small patches (microdomains) (172) and the different membrane thickness of cholesterol-rich regions (173, 174). The local inhomogeneity and curvature in the target membrane can influence the early interaction of a fusion protein/peptide with the target membrane, and ultimately, virus fusion (175, 176). The ability of phenothiazines to significantly increase the fluidity of cholesterol-rich membranes indicates that these molecules could interfere with cholesterol clustering and decrease

the packing of cholesterol-rich microdomains, leading to reduced local inhomogeneity, which is important for HCV fusion.

A second possibility, which is not completely independent of the first one, is that the incorporation of phenothiazines can affect interaction of the 3β-OH of cholesterol with the HCV envelope protein/fusion peptide. The 3β-OH participates in H-bond interactions with the head groups of various lipids, water in the solvent, and membrane proteins. In addition, 3β-OH can influence the folding of peptides at the water-membrane interface (177). The 3β-OH in cholesterol is required for the fusion of Semliki Forest virus (178, 179) and the optimal fusion of HCV with liposomes (151). Insertion of phenothiazines in the cholesterol-rich membrane may adversely affect the interaction of 3β-OH with HCV envelope proteins, thus inhibiting HCV-cell fusion.

Building upon our observation that HCV entry can be inhibited by increasing target membrane fluidity, we developed a screen using liposomes and the lipophilic dye Prodan. We screened a library of 2,752 compounds and identified a molecule, topotecan, that preferentially increases the fluidity of cholesterol-rich membranes (Figure 2.8A). Using a well-established HCVcc-liposome fusion assay (151, 152), we showed that topotecan dose-dependently inhibits HCVcc-membrane fusion *in vitro*, validating the screen as a tool to discover inhibitors of virus-cell fusion (Figure 2.8B). However, in cell culture assays, the anti-HCV activity of topotecan appears to derive primarily from its inhibition of HCV replication rather than entry, with an estimated EC_{50} ~0.2 μM (Figure 2.10). Topotecan (Hycamtin®) is a topoisomerase I inhibitor that acts by stabilizing the ssDNA-topoisomerase I complex and causing DNA cleavage (180). It is currently used

in chemotherapy for various cancers. In addition to topoisomerase, topotecan is known to affect many other cellular pathways, including down-regulation of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway (181) and disruption of hypoxia inducible factor-1 (HIF-1) signaling pathway (182). Topotecan is also a substrate of the ABC transporters P-glycoprotein (P-gp/MDR1) and breast cancer resistance protein (BCRP), and is actively cleared by the cell (183). The lack of HCV entry inhibition by topotecan in cell culture assays may be due in part to active cellular extrusion and/or intracellular trafficking of the compound. Nevertheless, the ability of topotecan to inhibit HCV replication may warrant additional clinical studies of this compound.

In conclusion, our studies shed light on the mechanism of action of phenothiazines as inhibitors of HCV entry and showed, for the first time, that alteration of target host cell membrane fluidity can inhibit HCV entry. It is possible that the same mechanism is responsible for the antiviral activities of phenothiazines toward other viruses, such as inhibiting the budding of measles and herpes simplex viruses (147). Based on these insights, we developed a high-throughput screen for modulators of cholesterol-rich membrane fluidity and screened a library of 2,752 compounds. One hit from this screen – topotecan – was found to both increase the fluidity of cholesterol-rich membranes and inhibit the fusion of these membranes with HCV. Targeting an entry step independent of viral proteins may also be an effective way to retard the development of drug resistance and inhibit HCV deletion mutants, which were found to reduce the antiviral effects of

interferon therapy for chronic hepatitis C patients (184). This study represents an exciting new paradigm to explore additional membrane-targeting antivirals.

CHAPTER III

BENZHYDRYLPIPERAZINE COMPOUNDS INHIBIT CHOLESTEROL-DEPENDENT HCV ENTRY*

3.1 Overview

Hepatitis C virus (HCV) infection remains a serious global health problem that lacks an effective cure. Although the addition of protease inhibitors in combination with the current standard-of-care interferon/ribavirin therapy has improved sustained virological response of genotype 1 infected patients, it also exacerbates already problematic side effects. Thus, new HCV antivirals are still urgently needed. Using a whole cell-based assay previously developed in our laboratory, we screened 30,426 compounds and identified 49 new HCV inhibitors. The best two hits, hydroxyzine and chlorcyclizine belonging to the family of benzhydrylpiperazine, displayed IC_{50}s in the low nanomolar range and therapeutic indexes >500. Both compounds suppress HCV entry at a late entry step prior to or concomitant with viral fusion and their inhibition efficiencies are highly dependent on virion and host cholesterol content. Both compounds are currently used in clinic for treating allergy-related symptoms and the reported *in vivo* concentration of hydroxyzine is significantly above its IC_{90} concentration against HCV, pointing to a great potential of this drug for HCV treatment.

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3.2 Introduction

Hepatitis C virus (HCV) is a positive-sense single stranded RNA enveloped virus belonging to the *Flaviviridae* family (185). After initial infection, 75-85% of the patients develop chronic hepatitis and at least one-fifth of chronically infected patients develop cirrhosis within 20 years (186). It is estimated that 3-4 million people contract HCV each year with more than 300,000 deaths attributed to HCV-end stage liver disease annually (187, 188). Until recently the unspecific antivirals - pegylated interferon alpha in combination with ribavirin - have been the only standard of care treatment (SOC) for HCV infection (189). Although the recent addition of protease inhibitors - telaprevir and boceprevir- has significantly increased the sustained virological response, improvement is accompanied by a more severe side effect profile (190-192). Moreover, both inhibitors are not very effective against patients who did not respond to SOC and monotherapy with either one results in rapid emergence of drug-resistant variants (193). Thus, compounds with novel modes of action and low toxicity are still urgently needed.

The entry of HCV into hepatocytes is a highly coordinated process involving the viral envelope glycloproteins and multiple host cell factors. Heparin sulfate glycosaminoglycans and low density receptor proteins represent the first attachment sites for HCV (194, 195). The exact sequential order of receptor engagement is still under debate, but most evidence suggests that virions first interact with scavenger receptor class B type 1 (SR-B1) followed by CD81 to form interaction complexes, which are translocated to the tight junctions gaining access to claudin-1 (CLDN1) and Occuldin (OCLD) (32). Virions are later internalized by clathrin mediated endocytosis, and upon

endosomal acidification fuse with the host membrane (196). More recently, Niemann Pick C1 Like 1 (NPC1L1), Transferrin receptor 1 (TfR1), Epidermal growth factor receptor (EGFR) and Ephrin receptor A2 (EphA2) have been added to the already extensive HCV receptor/co-factor list but their role on HCV entry remains to be elucidated (48, 96, 197).

Previously, our lab developed a cell protection assay based on an engineered hepatoma cell line – n4mBid – that undergoes apoptosis upon HCV infection (148, 198). This assay is not only highly amenable for high throughput screening but allows the identification of inhibitors at all three stages of the viral life cycle. In the current study, we screened 30,426 compounds using this cell protection assay at The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) and identified 49 compounds with previously unknown anti-HCV activity. The best two inhibitors, hydroxyzine and chlorcyclizine, belonging to the benzhydrylpiperazine family of H1-antihistamines, displayed IC_{50}s in the nanomolar range and therapeutic indexes >500. Importantly, hydroxyzine is approved by the FDA and is able to achieve plasma concentrations higher than its IC_{90} against HCVcc. Both drugs strongly suppressed HCV entry at a step immediately prior to or concomitantly with virus fusion. Moreover, inhibition was highly dependent on virion and host cholesterol content, suggesting that benzhydrylpiperazines may exert their effects through an NPC1L1-dependent pathway.

3.3 Materials and methods

3.3.1 Cells, compounds and reagents

Huh-7.5 cells were a kind gift from Dr. Charles Rice (Rockefeller University, NY). HEK 293T cells were purchased from Invitrogen (Carlsbad, CA). Hydroxyzine hydrochloride and chlorcyclizine hydrochloride were acquired from Spectrum Chemicals (New Brunswick, NJ) and Fisher Scientific (Pittsburg, PA), respectively. Bafilomycin was purchased from Axxora (San Diego, CA). Human anti-CD81 JS-81 MAb was obtained from BD Biosciences (San Jose, CA). CellTiter-Glo Luminescent Cell Viability assay kit and BioLux Gaussia Luciferase assay kit were purchased from Promega (Madison, WI) and New England Biolabs (Ipswich, MA), respectively. All mammalian cells were cultured in DMEM containing 4500 mg/l glucose, 4.0 mM L-Glutamine, and 110 mg/l sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X non-essential amino acids (Thermo Scientific HyClone, Logan, UT).

3.3.2 Production of HCVcc and pseudotyped lentiviruses

Production and titer determination of Jc1 Gluc HCVcc (150), JFH-1 (199) , and JFH-1^{G451R} (200) were carried out in Huh-7.5 cells as previously described (148). Briefly, Huh-7.5 cells were electroporated with *in vitro* transcribe viral RNA and virus-containing cell supernatant from day 3-7 was collected, pooled and stored at -80°C until use. Jc1 Gluc HCVcc contains a Gaussia luciferase gene inserted in frame between p7 and NS2 (150). For experiments requiring infection with virus containing secreted Gluc, dialyzed Jc1 Gluc HCVcc was used. To dialyze virus, 5 ml of Jc1 Gluc HCVcc

(concentrated 10-fold using a 100 kDa ultrafiltration membrane (Millipore, Billerica, MA)) was dialyzed twice in 1 l of PBS at 4 °C for 4 h and overnight, respectively. Dialyzed virus was aliquoted and stored at -80 °C until use.

Pseudotyped lentiviruses were produced by co-transfecting 293T cells with plasmids encoding HIV gag-pol (39), a provirus pTRIP-Gluc (148) and the appropriate envelope protein H77 E1E2 pcDNA3 or pVSVG (39) using the TransIT reagent (Mirus, Madison, WI). The supernatants containing the pseudoparticles were collected 48 h post transfection, filtered (0.45 μm pore size) and stored at -80 °C until use. Control pseudotyped lentivirus lacking any envelope protein (Env^-pp) was generated using the same protocol but replacing the envelope encoding plasmid with an empty vector (pCDNA3).

3.3.3 High-throughput screening

The library screening was conducted at The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB). The libraries are Prestwick (Prestwick Chemical, Washington, DC), Biomol 4 (Enzo Life Sciences, Plymouth Meeting, PA), Tocris (Tocris Bioscience, Minneapolis MN), EMD Kinase inhibitors (EMD Biosciences, San Diego, CA), SYNthesis kinase inhibitors (SYNthesis, San Diego, CA), Asinex 1 (ASINEX Corporation Winston-Salem, NC), Chembridge 3 (ChemBridge Corp., San Diego, CA), Maybridge 5 (Maybridge Ltd., England) and NCDDG7, and the total number compounds screened was 30,426. Each well of duplicate 384-well plates contains 20 μl of n4mBid cells (1.8 x 10^3 cells/well), 100 nl of the drug candidate (ranging from 2-5 mg/ml) and 20 μl of Jc1

HCVcc (MOI ~1). Each plate contained 16 wells each with nucleoside analog 2'CMA (1 μM) and DMSO solvent (0.5 %) as the positive and negative controls, respectively. The plates were incubated for 4 days at 37 °C in 5% CO_2 and the cell viability was measured using the CellTiter-Glo Kit (Promega, Madison, WI) in a Biotek Synergy HT plate reader. Values from replicate wells were averaged and drug candidates with an average percent of rescue ≥ 40% were scored as positive.

3.3.4 Secondary screening assay

Huh-7.5 cells (5.5×10^3 cells/well) were seeded in poly-L-lysine treated 384-well plates and infected with dialyzed Jc1 Gluc HCVcc (MOI 0.01) in the presence of increasing concentrations of the drugs. Supernatant Gluc activity was measured 72 h post-infection using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA), normalized to cell viability (CellTiter-Glo assay; Promega, Madison, WI) and expressed as percentage of 1% DMSO-treated cells.

3.3.5 HCV infection assay

To determine the anti-HCV activities, Huh-7.5 cells seeded in poly-L-lysine treated 96-well plates (2×10^4 cells/well) 4 to 6 h earlier were infected with dialyzed Jc1 Gluc HCVcc (MOI 0.01) in the presence of increasing concentrations of the drugs. Supernatant Gluc activity was measured 72 h post-infection using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA). Viability of the remaining cells was quantified using the CellTiter-Glo assay (Promega, Madison, WI) to gauge the drug toxicity.

To examine the effects of cellular cholesterol on the anti-HCV activity of benzhydrylpiperazines, Huh-7.5 cells were stripped of cholesterol prior to HCV infection. Briefly, Huh-7.5 cells were seeded in poly-L-lysine treated 96-well plate at 2.8 x 10^4 cells/well. The next day, these cells were incubated with 0, 1, 5 or 10 mg/ml of methyl-beta-cyclodextrin (mβcd) for 1 h at 37 °C, washed thoroughly with growth media to remove mβcd and infected with Jc1 Gluc HCVcc (MOI ~1) in the presence of the drugs for 1 h at 37 °C. Cells were washed to remove residual virus, Gluc and the drugs and replenished with fresh media. Supernatant Gluc activity was quantified 72 h post infection using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA) and normalized to cell viability gauged by the CellTiter-Glo assay (Promega, Madison, WI). Values are expressed as percentage of that from 0.015% DMSO-treated cells.

To evaluate the effect of virion cholesterol content on the anti-HCV activity of benzhydrylpiperazines, we compared the efficacy of the drugs against HCVcc with different cholesterol content. Briefly, Huh-7.5 cells were seeded in poly-L-lysine treated 48-well plates at 2 x 10^4 cells/well and incubated at 37 °C and 5% CO_2 for 5-6 hours to allow cell attachment. Cells were treated with the desired drugs for one hour prior to infection with JFH-1 WT ($HCVcc^{WT}$) or G451R clone of HCVcc ($HCVcc^{G451R}$) ($TCID_{50}$/ml 5.62 x 10^3) for 14-16 h at 37 °C and 5% CO_2. The next day, cells were washed thoroughly and replaced with 300 µl of inhibitor-containing media. Seventy-two hours post inoculation, cells were washed once with Dulbecco's phosphate-buffered saline (Thermo Scientific HyClone, Logan, UT) and underwent one freeze-thaw cycle at

80 °C before RNA extraction using an EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA). The amount of HCV RNA was quantified via TaqMan quantitative reverse transcription-PCR (qRT-PCR) (qScript One-Step Fast kit; Quanta Biosciences, Gaithersburg, MD), using previously described primers (154). As an internal control, mRNA levels of phosphoglycerate kinase 1 (PGK) (qScript One-Step SYBR Green qRT-PCR kit; Quanta Biosciences, Gaithersburg, MD) were determined for each RNA template using previously described primers (148).

3.3.6 HCVcc/pp entry assay

Huh-7.5 cells (1.8×10^4 cells/well) were seeded in poly-L-lysine treated 96-well plates and incubated at 37 °C and 5% CO_2 for 5-6 hours to allow cell attachment. Cells were treated with the desired drugs for one hour prior to infection with HCVpp (5-fold dilution), Env-pp (5-fold dilution) or VSV-Gpp (500-fold dilution) for 14-16 h at 37 °C and 5% CO_2. The next day, cells were thoroughly washed and replaced with 100 µl of fresh drug-free media. Supernatant Gluc activity was measured 48 h post washing using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA), normalized to cell viability quantified by a CellTiter Glo Luminescent Cell Viability assay kit (Promega, Madison, WI) and used as indication of viral entry.

Huh-7.5 cells (2×10^4 cells/well) seeded in poly-L-lysine treated 96-well flat bottom plates the previous day were inoculated with Jc1 Gluc HCVcc (MOI 1), HCVpp (5-fold final dilution) or Env^-pp (5-fold final dilution) in the presence of the compounds at 37 °C. Three hours post infection cells were thoroughly washed and replenished with 100 µl of fresh growth media. Supernatant Gluc activities were quantified 72 h post

infection using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA). Values are expressed as percentage of 0.015 % DMSO control.

3.3.7 Replication and virus production inhibition

Huh-7.5 cells were electroporated with Jc1 Gluc HCVcc RNA and plated in poly-L-lysine treated 48-well plates (6×10^4 cells/well). After cell attachment (4-6 h), increasing concentrations of the drugs were added to the appropriate wells. Supernatant Gluc activity was quantified 48 h post drug addition using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA), normalized to 0.15 % DMSO control and used as indication of viral replication. Concurrently, virus-containing sups from drug-treated cells were diluted 100-fold and used to infect naive Huh-7.5 cells in 96-well plates (2×10^4 cells/well) for 14-16 h. The next day, cells were thoroughly washed to remove residual virus and replenished with fresh complete growth media. Supernatant Gluc activity was quantified 48 h post washing using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA), normalized to that from DMSO-treated cells and used as indication of production inhibition.

3.3.8 Synchronized HCVcc infection assay

Huh-7.5 cells were seeded in poly-L-lysine treated 48-well plates at 4×10^4 cells/well. The next day, the cells were chilled and incubated with cold Jc1 Gluc HCVcc (MOI 5) at 4°C for 1.5 h to allow virus attachment but not internalization. Unbound virions were removed by thorough washing and internalization was initiated by transferring the plates to a 37 °C and 5% CO_2 incubator. Hydroxyzine (1 µM), chlorcyclizine (1.5 µM), bafilomycin (10 nM), JS-81 (2 µg/ml), or DMSO (0.015%) was

added to the cells at different time points post temperature shift. Cells were washed thoroughly with complete growth medium 5 h or 24 h post-drug addition and replenished with fresh media. Supernatant Gluc activity was measured at 72 h post-infection using a BioLux Gaussia luciferase assay kit (New England Biolabs, Ipswich, MA), normalized to that from the last drug time point (180 min) and used as an indication of viral infection.

3.3.9 Statistical analysis

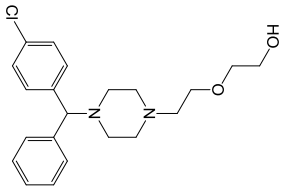
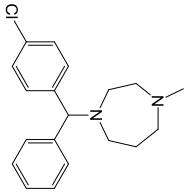
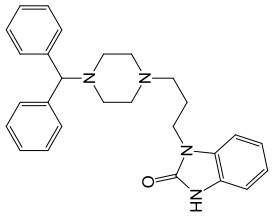
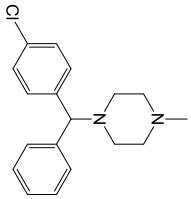
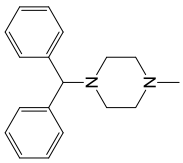
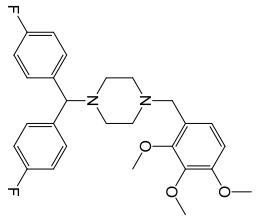
Statistical analysis was performed in Microsoft Excel by means of the Student's t test. P-values less than 0.1 were considered statistically significant.

3.4 Results

3.4.1 Identification of anti-HCV compounds using a cell protection assay

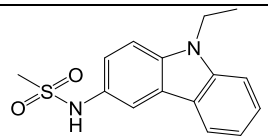
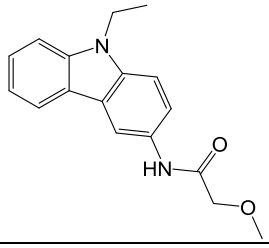
Previously, our lab engineered a human hepatoma cell line- n4m Bid – that sensitively reports HCV infection via a cell death phenotype (198). Using this reporter cell line, we screened seven libraries of known bioactive compounds, commercial compounds and natural extracts (total 30,426 compounds). The primary screening was performed in duplicate 384-well plates at a single compound dosage (ranging from 2-5 mg/ml). Nighty-seven compounds showed at least 40% cell rescue, including known HCV inhibitors: cyclosporine (201, 202), flunarizine (148), and tamoxifen (203). To reconfirm the results of the cell protection assay, hits were subjected to a secondary screening using dialyzed Jc1 Gluc HCVcc. Forty-nine of the candidates were able to reduce HCV infection by at least 40% when compared to mock treated cells (Table 3.1).

Table 3. 1 Anti-HCVcc activity of screening hits.

Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
65		11.2	88.3	92.7	89.3
64		12.9	75.3	93.7	87.1
49		11.7	73.5	88.3	100
73		14.8	70.5	90	93.1
67		16.5	66.7	87.8	100
51		10.7	49.2	79.8	87.5

Values represent mean of duplicates.

Table 3.1 Continued.

Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
13		43.3	86.3	99.4	100
30		44.3	61.25	99.4	100

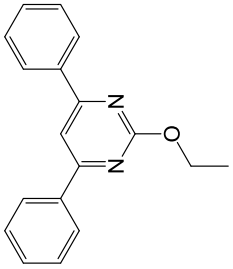
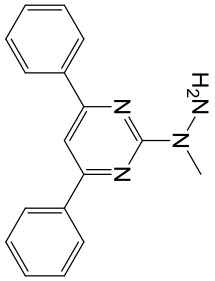
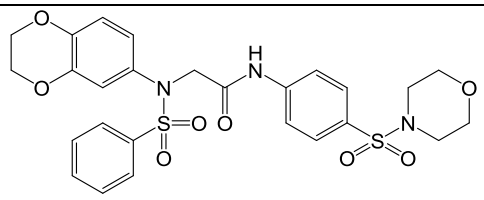
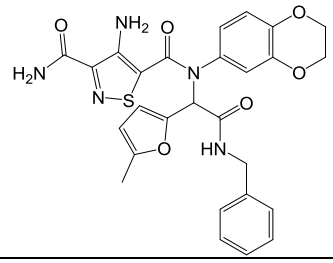
Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
20		45.2	74.5	63.2	100
4		45.2	50.2	99	100

Table 3.1 Continued.

Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
8		21.8	65.9	43.8	100
29		22.8	59.2	94.7	100

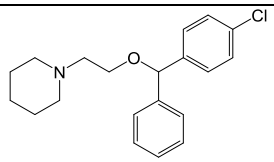
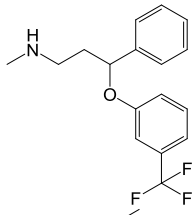
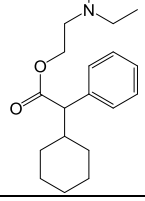
Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
76		13.6	56.4	89.5	91.5
68		14.5	52.2	93.8	68.3
75		141	49.1	80.6	98.7

Table 3.1 Continued.

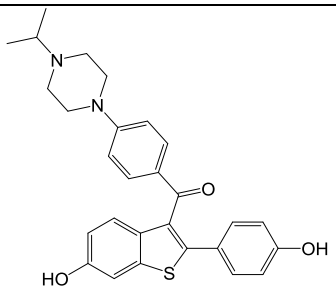
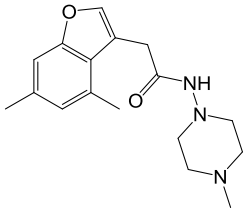
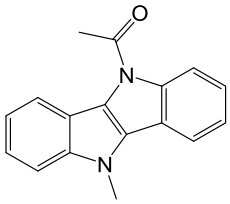
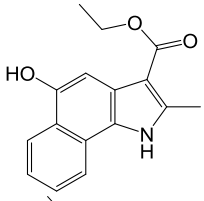
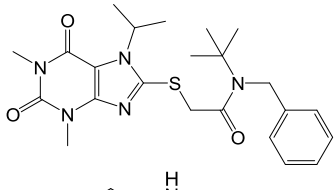
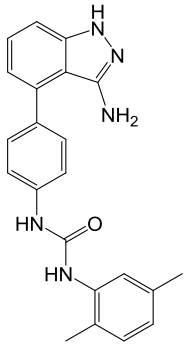
Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
97		25	58.5	99.6	89.1
10		41.5	53.4	94.1	100
2		47.6	51.8	95.5	100
32		46.4	51.6	99.6	100
31		27.3	51.5	97.8	100
89		8.3	50.5	87.5	87.9

Table 3.1 Continued.

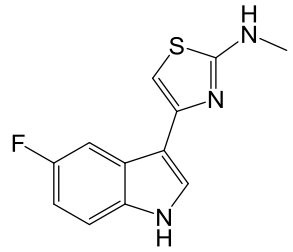
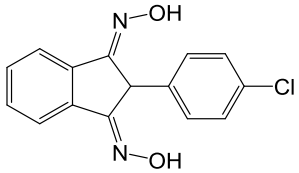
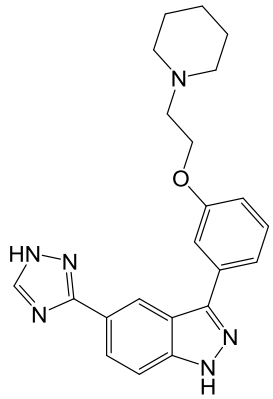
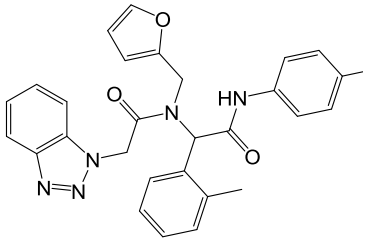
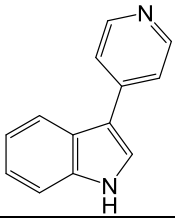
Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
35		50.5	47.4	97.6	34.7
6		43.6	45.3	98.3	40
95		25	45.3	62.9	89.3
28		25.1	45.2	98.6	100
80		8.25	42.6	43.4	100

Table 3.1 Continued.

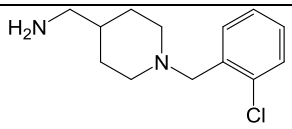
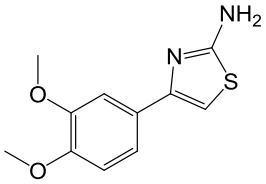
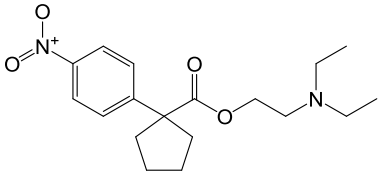
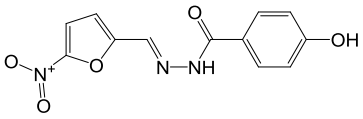
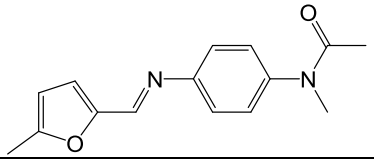
Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
11		52.4	48.7	96.8	100
26		52.9	48.1	97.3	100
77		13.5	45	83.9	95.5
70		18.2	44.6	98	34.2
3		48.8	40.2	71.4	100

Table 3.1 Continued.

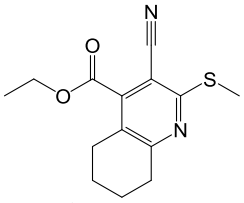
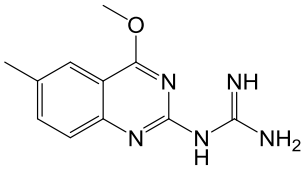
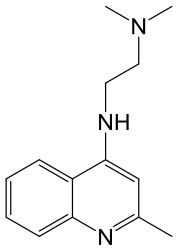
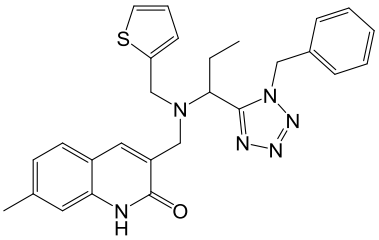
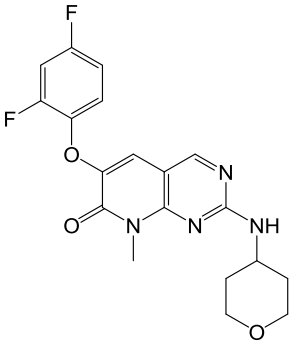
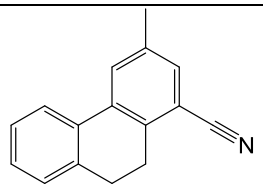
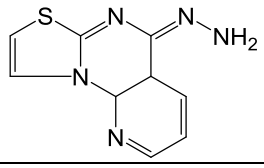
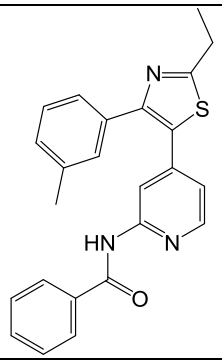
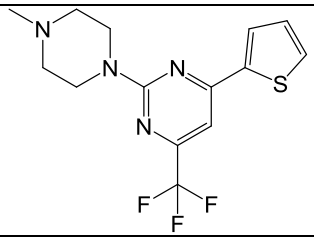
Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
14		45.2	59.4	93	100
5		54.1	49.9	96.5	100
34		54.5	43.4	98.4	96.4
27		25.8	42.9	98	100
92		25	43.5	40	99.8

Table 3.1 Continued.

Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
16		57	48.4	97.6	100
15		57.5	43.9	93.1	100

Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
93		25	47	98.6	62.8

Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
12		38.1	44.8	44.6	100

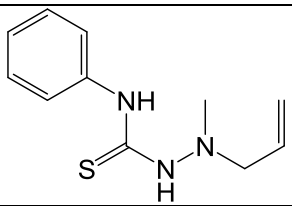
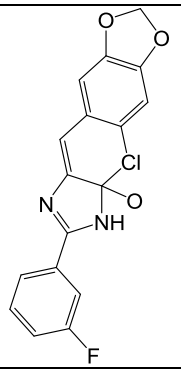
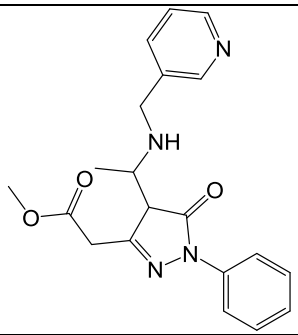
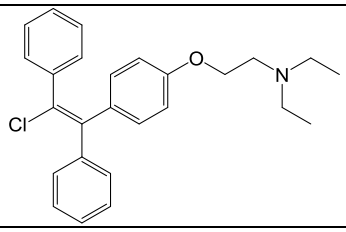
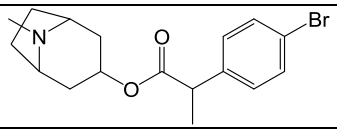
Label	Structure	Concentration (µM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
23		33.4	55.4	93.9	59.6

Table 3.1 Continued.

Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
36		36.3	49.7	84	100

Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
37		34.3	47.9	89	100

Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
69		8.4	50	86.9	96.6

Label	Structure	Concentration (μM)	% rescue (n4m assay)	% inhibition (Gluc assay)	% viability
96		25	50.1	97.9	100

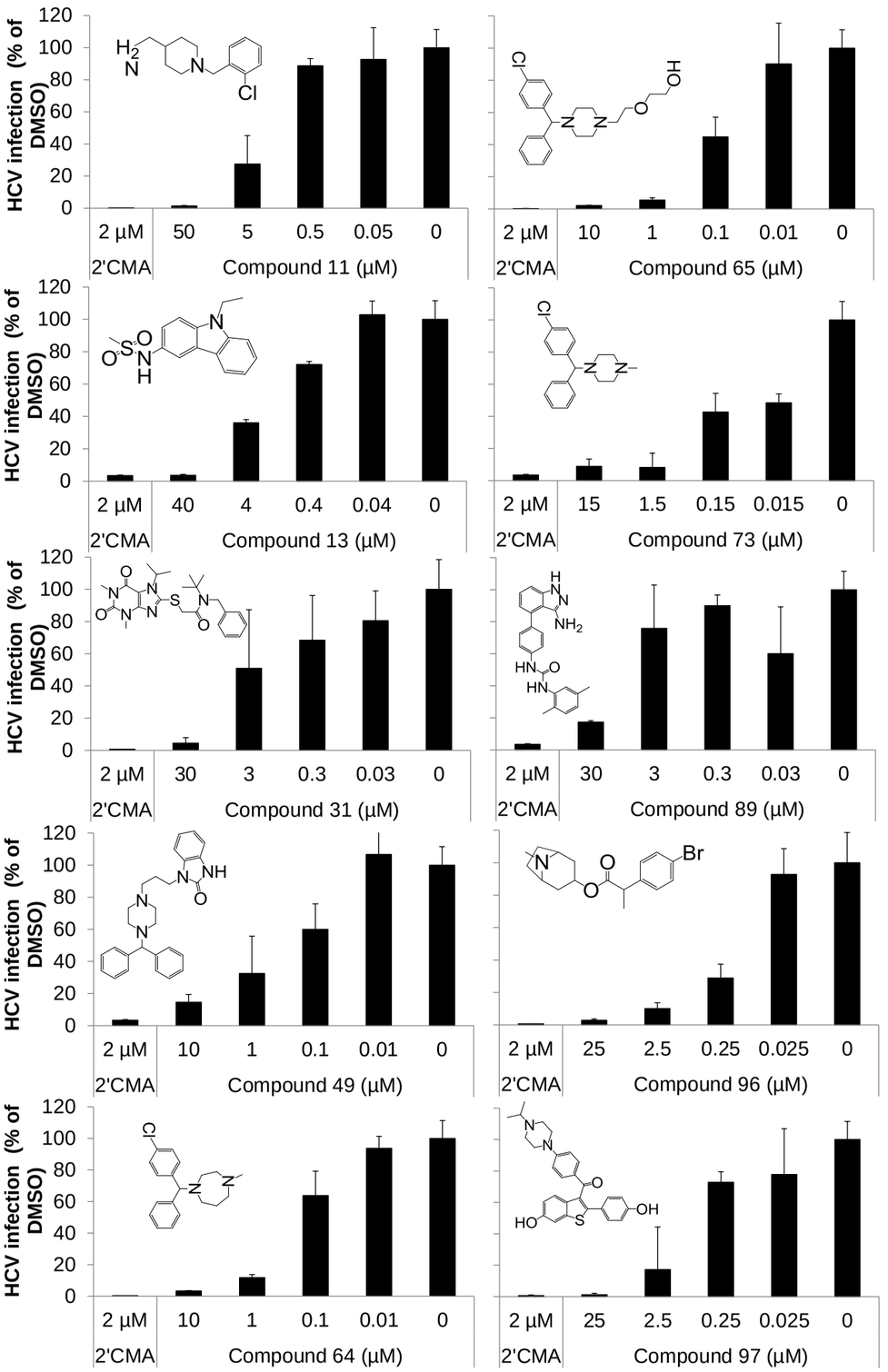


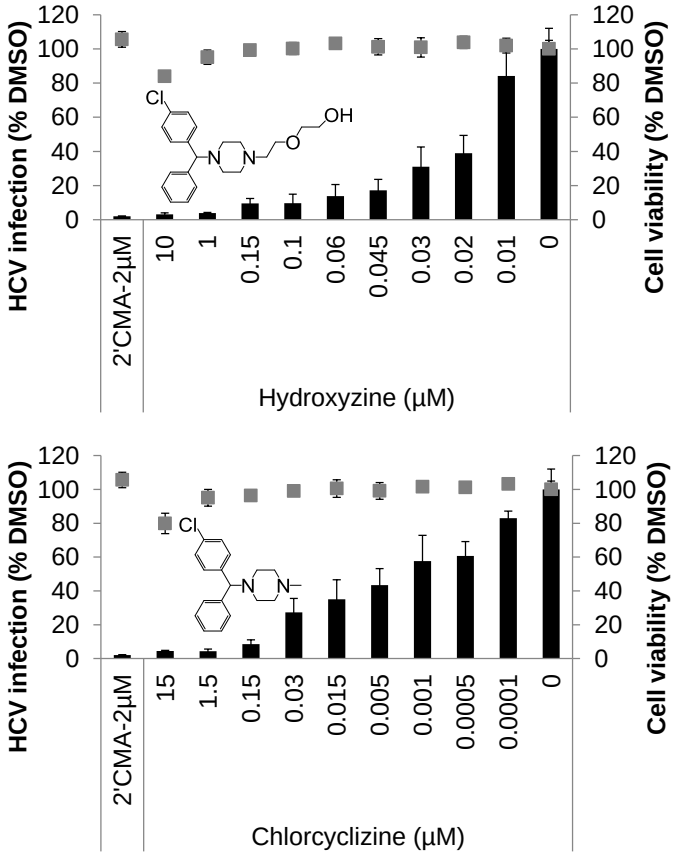
Figure 3. 1 Characterization of leading inhibitors. Huh-7.5 cells (5.5 x 10^3 cells/well) were seeded in 384-well plates and infected with Jc1 Gluc HCVcc (MOI 0.01) in the presence of screening hits. Supernatant Gluc activity was measured 72 h post infection, normalized to viable cell levels and expressed as a percentage of control cells treated with 1% DMSO. Positive control: 2µM 2'CMA. Values and error bars represent mean and standard deviation, respectively, of duplicate samples.

Table 3. 2 Antiviral activity of leading hits

Compound	IC_{50} (μM)	CC_{50} (μM)	CC_{50}/IC_{50}
11	2.4	>50μM	>20
13	1.7	>40μM	>23
31	1.4	>30μM	>21
49^{+}	0.5	>10μM	>20
64^{+}	0.17	>10μM	>58
65^{+}	0.02	>10μM	>500
73^{+}	0.002	>15μM	>6,500
89	7.8	>30μM	>3
96	0.14	>25μM	>178
97	0.52	>25μM	>48

IC_{50} values were calculated using the sigmoidal fit function in Origin Lab (OriginLab, Northampton,MA), $^{+}$ Compounds belonging to the benzhydrylpiperazine family.

A



B

Compound	IC_{50}	IC_{90}	CC_{50}	CC_{50}/IC_{50}
Hydroxyzine	19nM	64nM	>10µM	>526
Chlorcyclizine	2.3nM	394nM	>15µM	>6,521

Figure 3. 2 Benzhydrylpiperazines inhibit HCVcc infection. (A) Huh7.5-cells were infected with Jc1 Gluc HCVcc (MOI 0.01) in the presence of increasing concentrations of hydroxyzine or chlorcyclizine. Supernatant Gluc activity was measured 72 h post infection, normalized to that of 0.15% DMSO and used as indication of viral infection. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate. **(B)** $IC_{50/90}$ values of hydroxyzine and chlorcyclizine were calculated using the sigmoidal fit function in Origin Lab (OriginLab, Northampton,MA).

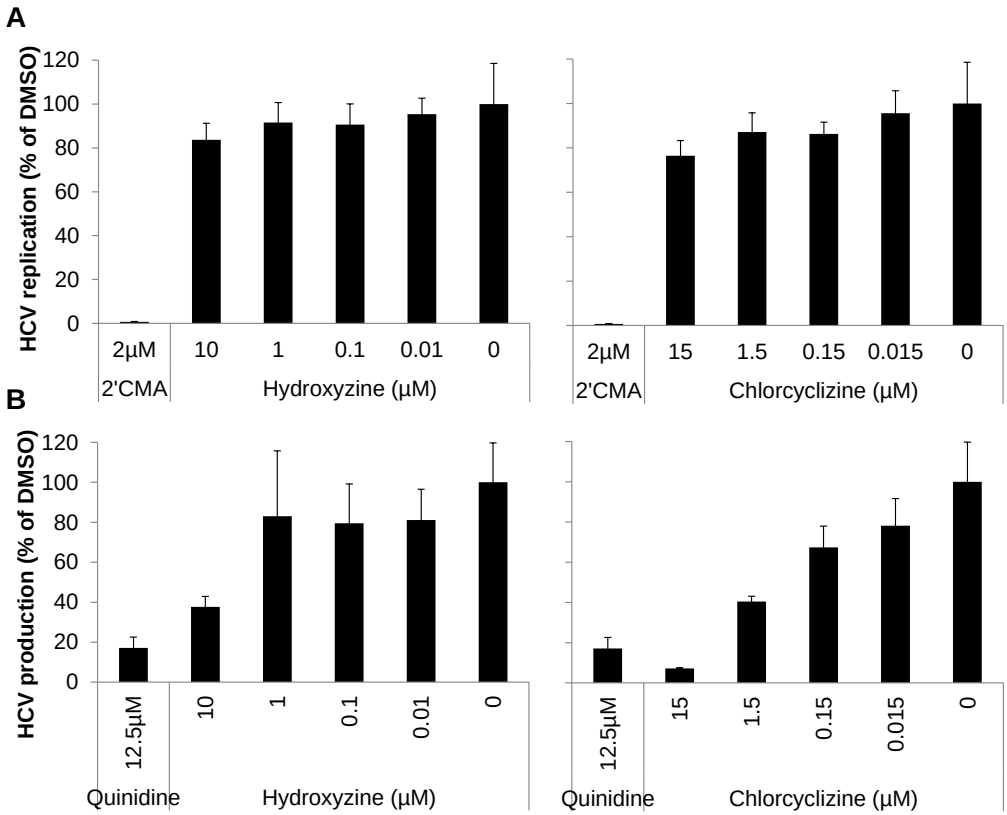


Figure 3. 3 Benzhydrylpiperazines do not inhibit HCV replication or virus production. (A) Huh-7.5 cells were electroporated with Jc1 Gluc HCVcc RNA. After cell attachment (4-6 h post seeding), cells were exposed to the indicated compounds. Supernatant Gluc activity was measured 48 h post drug addition, normalized to 0.15% DMSO treated cells and used as indication of viral replication. **(B)** Virus containing sups from (A) were diluted 100-fold in complete growth medium and used to infect naive cells. Supernatant Gluc activity was quantified 72 h post inoculation, normalized to DMSO-treated cells and used as indication of the amount of infectious virus in the supernatant. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate.

Dose responses of the top 10 hits were determined using the same assay (Figure 3.1 and Table 3.2). Among these, four compounds belong to the benzhydrylpiperazines family, including the best candidates, #65 and #73, commercially known as hydroxyzine and chlorcyclizine, respectively. The estimated IC_{50}s and CC_{50}s for hydroxyzine and chlorcyclizine are 19 and 2.3 nM and > 10 and >15 µM, resulting in drug indexes >500 and >6,500, respectively (Figure 3.2). These two drugs were selected for further characterization.

3.4.2 Benzhydrylpiperazines do not inhibit HCV replication or production

To evaluate the effect of the hydroxyzine and chlorcyclizine on HCV replication and production, Huh-7.5 cells were electroporated with Jc1 Gluc HCVcc and treated with increasing concentrations of the drugs. HCV replication levels were quantified 48 h post drug addition by measuring the supernatant Gluc activity. No inhibition was observed for either of the compounds even at the highest tested concentration (Figure 3.3A). To determine the effect of drugs on HCV production, supernatants from compound-treated cells were diluted 100-fold and used to infect naïve Huh-7.5 cells. A 3-fold and 10-fold reduction of HCV infectivity was observed at 10 µM and 15 µM of hydroxyzine and chlorcyclizine, respectively (Figure 3.3B).

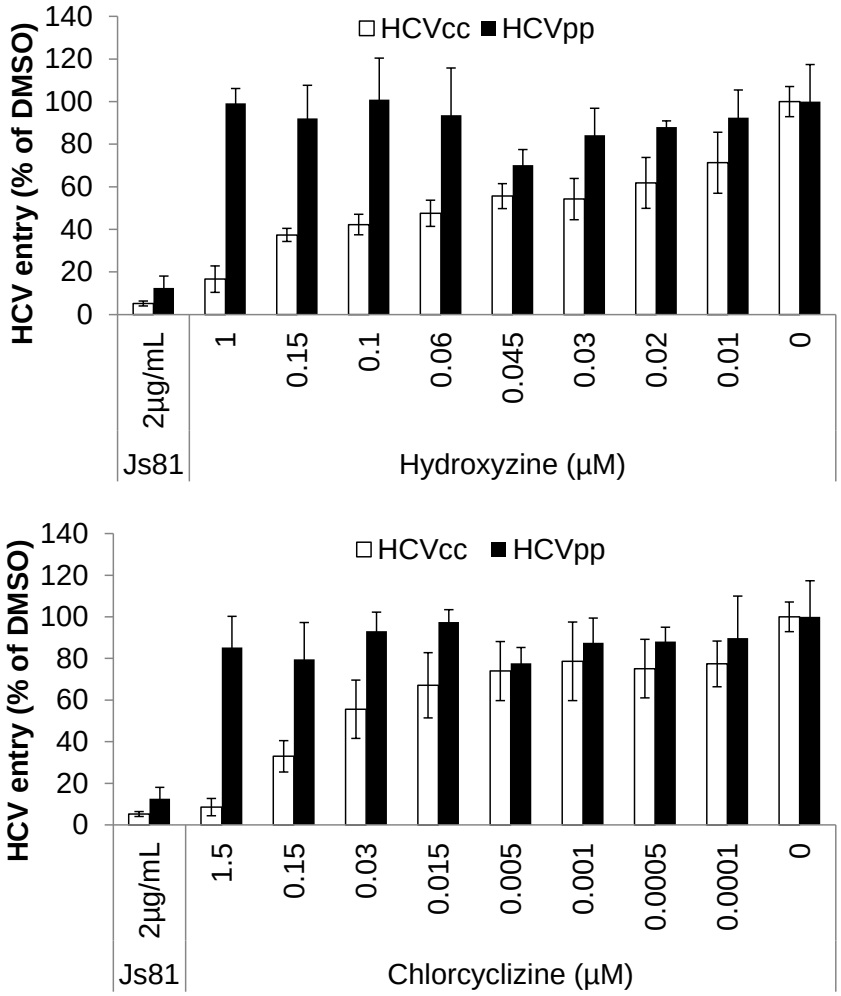


Figure 3. 4 Benzhydrylpiperazines selectively suppress HCVcc entry. Huh-7.5 cells were infected with Jc1 Gluc HCVcc (MOI 1), H77 HCVpp (5-fold diluted) or Env-pp (5-fold diluted) in the presence of compounds for 3 h at 37°C/5% $C0_2$ prior to washing to remove virus and replenishment with compound-free growth medium. Supernatant Gluc activity was measured 72 h post infection. Values are expressed as percentage of 0.015% DMSO-treated cells. For HCVpp, raw Gluc values were subtracted from Env-pp prior to DMSO normalization. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate.

However, since the effective drug concentration of hydroxyzine and chlorcyclizine in those wells during the reinfection step were 0.1 and 0.15 μM, respectively, and these concentrations were previously found to significantly inhibit HCVcc infection (Figure 3.2), we concluded that the inhibition seen in Figure 3.3B is not due to the drug effect on virus production but the inhibition of HCV infection by the residual drug. Thus, neither benzhydrylpiperazines significantly inhibits HCV production.

3.4.3 Benzhydrylpiperazines selectively suppress HCVcc entry

Since no inhibition of HCV replication or virus production was observed, we evaluated the effect of these drugs on HCV entry. Huh-7.5 cells were infected with HCVcc or HCVpp/Env$^-$pp in the presence of the drugs at 37 °C for 3 hours. Supernatant Gluc activities were quantified 72 hours later and used as indication of viral infection. The short contact time between the drugs and the cells/viruses is designed to limit the effect of the drugs to the entry step alone. Surprisingly, both compounds were able to strongly inhibit the entry of HCVcc but not HCVpp (Figure 3.4). A modest reduction of HCVpp (< 2-fold), but not VSV-Gpp, entry was only observed at 10 and 15 μM of hydroxyzine and chlorcyclizine, respectively (Figure 3.5). The ability of both benzhydrylpiperazines to selectively inhibit HCVpp but not VSV-Gpp entry suggest that cellular processes such as clathrin-mediated endocytosis and endosomes acidification are not impaired by these drugs at the tested concentrations, and that hydroxyzine and chlorcyclizine likely inhibit an HCV entry step either not or weakly depicted by the HCVpp system.

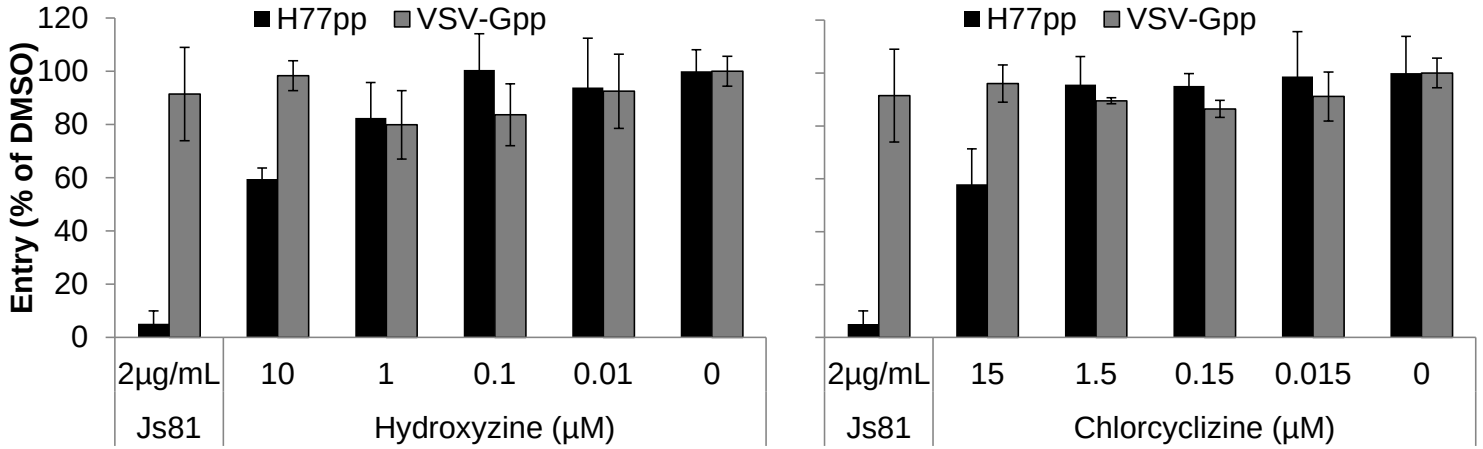


Figure 3. 5 Dose dependent effects of benzhydrylpiperazines on HCV entry. Huh-7.5 cells (1.8×10^4 cells/well) were infected with H77 HCVpp (5-fold dilution), Env-pp (5-fold dilution) or VSV-Gpp (500-fold dilution) overnight in the presence of compounds. The next day cells were thoroughly washed and replenished with complete fresh media lacking compounds. Supernatant Gluc activity was measured 48 h post washing. Gluc data from Env-pp were substracted from H77 HCVpp Gluc readings after normalization to viable cell levels and expressed as a percentage of data from negative control cells treated with DMSO (0.1-0.15%). Values and error bars represent mean and standard deviation, respectively, of at least two independent experiments carried out in duplicate.

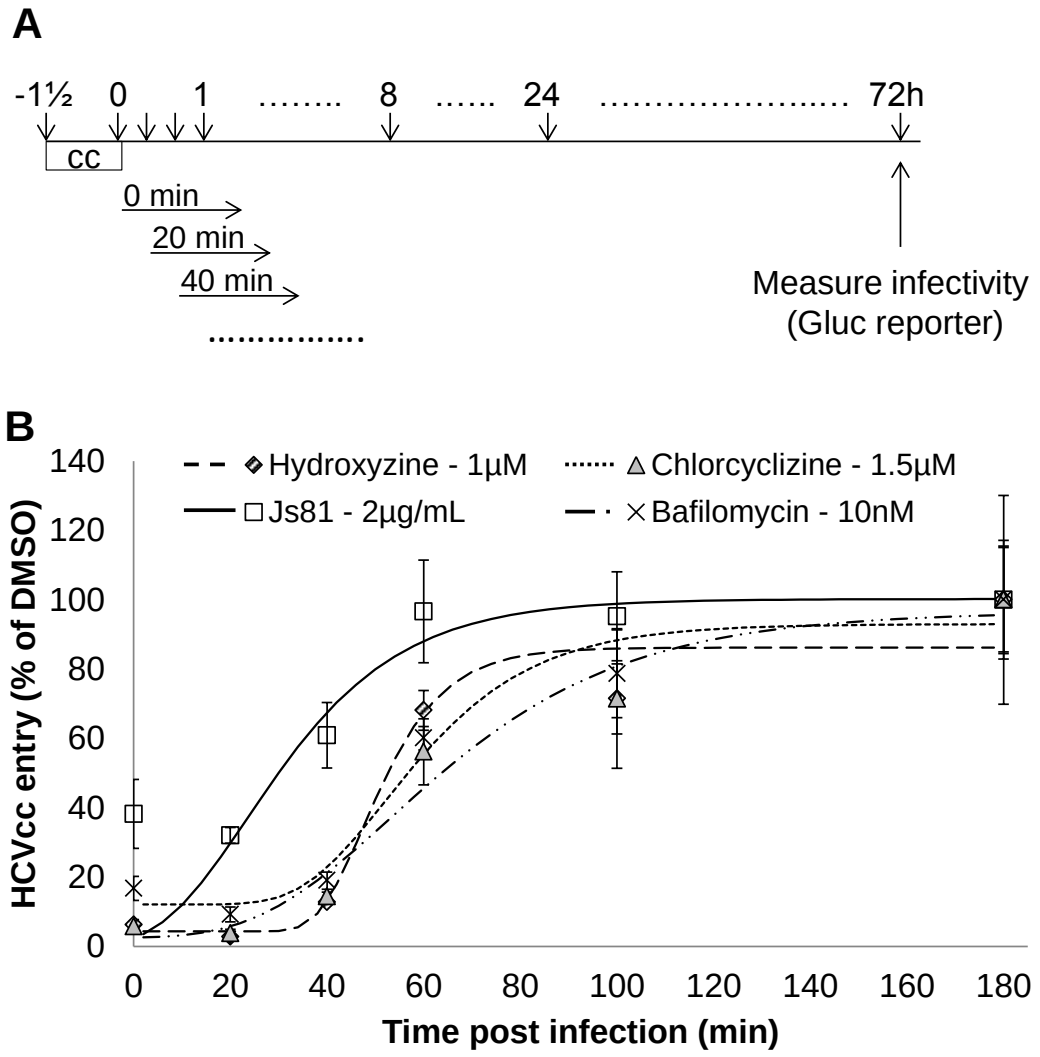


Figure 3. 6 Benzhydrylpiperazines inhibit a late HCV entry step. (A) Schematic representation of experiment. **(B)** Huh-7.5 cells were incubated with Jc1 Gluc HCVcc (MOI 5) at 4°C for 1.5 h, thoroughly washed, replenished with virus-free growth medium, and shifted to 37°C/5% CO_2. The compounds were added to the cells at the indicated time points post temperature shift and removed 5 h later. Supernatant Gluc activity was measured 72 h post temperature shift and expressed as a percentage of activity from the final compound addition time point to indicate viral entry. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate.

Next, we determined the step of entry affected by these benzhydrylpiperazines. Huh-7.5 cells were incubated with Jc1 Gluc HCVcc (MOI 5) at 4°C for 1.5 hours, thoroughly washed to remove unbound virions, and transferred to a 37 °C/5% CO_2 incubator to initiate virus entry. Drugs were added at different times post temperature shift as illustrated in Figure 3.6A. Both benzhydrylpiperazines retained inhibitory activity for up to 100 min after temperature shift to 37 °C (Hydroxyzine $t_{½}$ ~53 min, Chlorcyclizine $t_{½}$ ~63min), similar to that of endosome acidification inhibitor bafilomycin ($t_{½}$ ~57 min) (Figure 3.6B), indicating that these drugs inhibit a post binding step of HCVcc likely immediately prior to or concomitantly with the fusion process.

3.4.4 Benzhydrylpiperazine entry inhibition is cholesterol dependent

The ability of benzhydrylpiperazines to inhibit the fusion of HCVcc but not HCVpp prompted us to examine the differences between these systems. One of the major differences is their distinct cholesterol profiles. HCVpp contains 94% less cholesterol than the authentic HCVcc (48) because HCVpp is typically produced from 293T cells that do not have cholesterol-associated lipoproteins (204). On the other hand, cell culture-adapted $HCVcc^{G451R}$, containing a G451R point mutation in the viral E2 glycoprotein, was found to have ~50% more cholesterol than the wild-type HCVcc ($HCVcc^{WT}$) (48). To evaluate whether the entry inhibition by benzhyldrylpiperazines is dependent on virion cholesterol content, Huh-7.5 cells were infected with $HCVcc^{WT}$ or $HCVcc^{G451R}$ in the presence of the drugs, and the viral RNA from infected cells was quantified 48 h post infection.

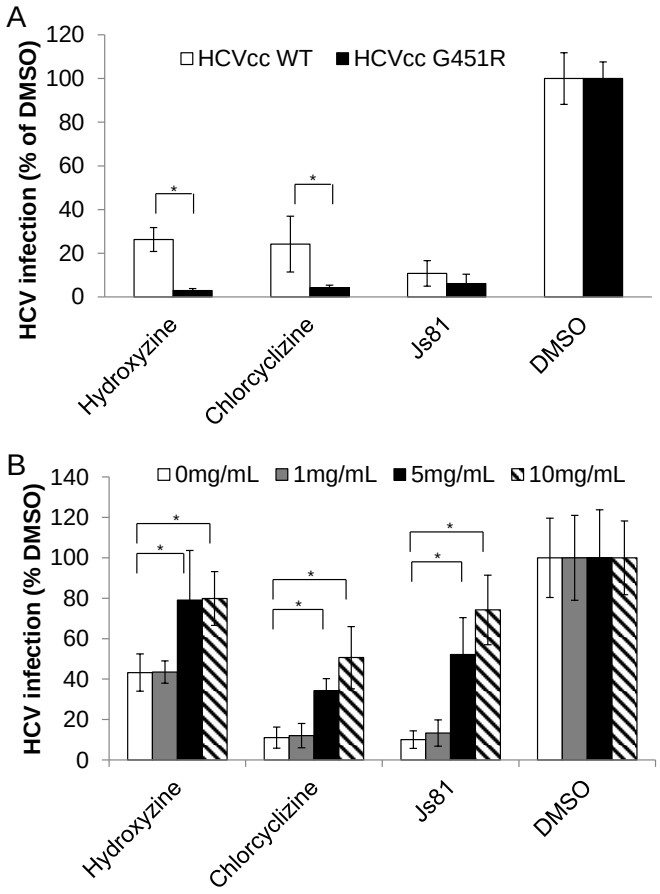


Figure 3. 7 Benzhydrylpiperazine entry inhibition is cholesterol dependent. (A) Huh-7.5 cells were infected with $HCVcc^{WT}$ or the high cholesterol-content mutant virus $HCVcc^{G451R}$ (MOI 0.04) in the presence of hydroxyzine (1 μM), chlorcyclizine (1.5 μM), JS-81 antibody (2 μg/ml) or DMSO (0.015 %) for 14-16 h. Intracellular HCV RNA was quantified 72 h post inoculation. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate. **(B)** Huh-7.5 cells were exposed to the indicated concentrations of concentrations of mβcd for 1 h at 37°C/5% CO_2. After treatment, cells were thoroughly washed and inoculated with Jc1 Gluc HCVcc (MOI 1) for 1 h at 37°C/5% CO_2 in the presence of hydroxyzine (1 μM), chlorcyclizine (1.5 μM), Js81 antibody (2 μg/ml) or DMSO (0.015 %). Residual compound and virus were removed by washing and cells were replenished with complete growth medium. Supernatant Gluc activity was quantified 72 h post infection and normalized to cell viability. Values and error bars represent the mean and standard deviation, respectively, of at least three independent experiments carried out in duplicate. Statistical significance was determined by the Student's t test (*, $p < 0.1$).

As shown in Figure 3.7A, $HCVcc^{G451R}$ was more sensitive to benzhydrylpiperazine treatment than $HCVcc^{WT}$, while the HCV entry receptor CD81 antibody JS-81 showed similar potency against both viruses, indicating that the entry inhibition of benzhyldrylpiperazines is dependent on virion cholesterol content.

Next, we examined the effect of host cholesterol content on benzhydrylpiperazines' entry inhibition. Huh-7.5 cells were treated with increasing concentrations of cholesterol depletion agent (mβcd) for 1 hour at 37 °C, washed and then infected with Jc1 Gluc HCVcc (MOI 1) for 1 hour in the presence of the drugs. HCV infection was quantified 72 h post infection via supernatant Gluc activity. Depletion of host cholesterol significantly reduced the ability of benzhydrylpiperazines to inhibit HCV entry (Figure 3.7B), suggesting that host cholesterol content also affects the potency of benzhydrylpiperazines. Similar results were obtained with JS-81, as cell surface CD81 level is affected by the host cholesterol content (166, 205).

3.5 Discussion

In this paper, we carried out a high-through screening of 30,426 compounds using an unbiased cell-based assay (198) and discovered 49 compounds with undocumented anti-HCV activity. Based on structural similarity, we categorized them into 16 groups. The best two inhibitors, hydroxyzine and chlorcyclizine, were shown to suppress HCV infection with an estimated IC_{50} of 19 and 2.3 nM and CC_{50} >10 µM and >15 µM, leading to therapeutics indexes >500 and >6,500, respectively. Hydroxyzine and chlorcyclizine are members of the first generation H_1 antihistamines belonging to the family of benzhydrylpiperazine compounds (206). Other activities of hydroxyzine

and chlorcyclizine include blockade of muscarinic, serotonin and α-adrenergic receptors (207). More recently antiviral (208), antimetabolic (209), and anticarcinogenic (210) properties were reported for chlorcyclizine. Hydroxyzine has been approved by the FDA for treating allergic disease since 1956 under the trademark name Atarax (211). The serum half-life of oral dosages of hydroxyzine (0.7 mg/kg) range from 21 to 29 h, with a peak plasma level of 72-77 ng/ml (160-171 nM) (212, 213), and an expected liver concentration of up to 1.7 μM (214). The IC_{90} concentration for hydroxyzine is 64 nM (Figure 3.2), which is well within the range of plasma drug concentration, pointing to a strong clinical potential of this compound in treating HCV infection. Moreover, hydroxyzine is currently used to alleviate dermatological adverse effects experienced by patients undergoing HCV treatment with telaprevir, as 50% of them develop cutaneous reactions within the first 4 weeks of treatment (215, 216). Chlorcyclizine (Trade name: Ahist), currently under review by the Center for Drug Evaluation and Research (FDA), is an over the counter drug for treating allergy-related symptoms.

Both hydroxyzine and chlorcyclizine showed little inhibition of HCV replication and virus production (Figure 3.3), and were found to selectively inhibit the entry of HCVcc but not HCVpp (Figure 3.4). Since HCVpp and HCVcc have vastly different virion cholesterol content (48), we further showed that the entry inhibition by these benzhydrylpiperazines is dependent on both virion and host cholesterol content (Figures 3.4 and 3.7). Previously, Sainz *et al*. identified Niemann-Pick C1-like 1 (NPC1L1), a cholesterol adsorption receptor, as a new HCV entry factor (48). NPC1L1, a homolog of Niemann-Pick 1 receptor, is a polypotic transmembrane protein important for cholesterol

adsorption (217). It is highly expressed in small intestine and human liver (46). Depending on the extracellular cholesterol concentration, NPC1L1 is localized either on the plasma membrane or in intracellular compartments (46, 218). Ezetimibe (trademark name Zetia) inhibits cholesterol adsorption (219) by blocking the internalization of NPC1L1 (220). Interestingly, ezetimibe was found to also potently inhibit the entry of HCVcc, but not HCVpp, at a step immediately prior to or concurrent to fusion, and its HCV entry inhibitory potency is also dependent on virion cholesterol content (48). However, to the best of our knowledge, clinical use of neither hydroxyzine nor chlorcyclizine was associated with reduced serum cholesterol content. Studies to explore the relationship between benzhydrylpiperazines and NPC1L1 are currently underway.

In conclusion, we screened 7 libraries of 30,426 compounds and identified 49 novel HCV inhibitors. Among those, the best two inhibitors, hydroxyzine and chlorcyclizine, have already been used in clinic for treatment of allergy-related symptoms and the achievable serum concentration of hydroxyzine is significantly higher than the effective drug concentration needed to inhibit 90% of HCV infection, pointing to the use of this drug in HCV treatment. We further showed that both compounds inhibit a post attachment step of HCVcc entry with a similar $t_{1/2}$ as HCV fusion inhibitor bafilomycin. The inhibition efficiency by both compounds is dependent on virion and host cholesterol content, similar to that of NPC1L1 inhibitor ezetimibe, indicating that benzhydrylpiperazines may also act through NPC1L1 to inhibit HCV entry.

CHAPTER IV

PD 404,182 IS A VIRUCIDAL SMALL MOLECULE THAT DISRUPTS HEPATITIS C VIRUS AND HUMAN IMMUNODEFICIENCY VIRUS[*]

4.1 Overview

We describe a virucidal small molecule, PD 404,182, that is effective against hepatitis C virus (HCV) and human immunodeficiency virus (HIV). The median 50% inhibitory concentrations (IC_{50}s) for the antiviral effect of PD 404,182 against HCV and HIV in cell culture are 11 and 1 μM, respectively. The antiviral activity of PD 404,182 is due to physical disruption of virions that is accompanied to varying degrees (depending on the virus and exposure temperature/time) by the release of viral nucleic acids into the surrounding medium. PD 404,182 does not directly lyse liposomal membranes even after extended exposure, and it shows no attenuation in antiviral activity when pre-incubated with liposomes of various lipid compositions, suggesting that the compound inactivates viruses through interaction with a non-lipid structural component of the virus. The virucidal activity of PD 404,182 appears to be virus-specific, as little to no viral inactivation was detected with the enveloped Dengue and Sindbis viruses.

Data from Table 4.1 and Figures 4.5 and 4.11 were generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay
Data from Figure 4.4B was generated at Drexel University College of Medicine by Jinhong Chang

PD 404,182 effectively inactivates a broad range of primary isolates of HIV-1 as well as HIV-2 and simian immunodeficiency virus (SIV), and it does not exhibit significant cytotoxicity with multiple human cell lines *in vitro* (50% cytotoxic concentration, >300 μM). The compound is fully active in cervical fluids, although it exhibits decreased potency in the presence of human serum, retains its full antiviral potency for 8 h when in contact with cells, and is effective against both cell-free and cell-associated HIV. These qualities make PD 404,182 an attractive candidate anti-HIV microbicide for the prevention of HIV transmission through sexual intercourse.

4.2 Introduction

Human pathogenic viruses that acquire resistance to antiviral agents by rapid evolution *in vivo* pose a serious health problem with no simple cure. Antivirals targeting features of these viruses that can be altered through changes in the viral genetic code often exhibit limited efficacy. Hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are two such viruses which cause disorders of the liver and immune system, respectively, and collectively afflict 2~3% of the world's population (221, 222). For HCV, the current interferon/ribavirin combination therapy exhibits limited efficacy, and the two recently approved small-molecule drugs, both serine protease inhibitors – telaprevir and boceprevir (223, 224) – foster the development of resistant viral strains within days when administered alone (225, 226). For HIV, there are currently more than 20 approved antiretroviral drugs, forming the basis of highly active antiretroviral therapy (HAART). Despite the availability of this large repertoire of anti-HIV drugs, drug-resistant mutant strains of HIV still emerge over time. Approximately 4 to 5 million HIV

patients are coinfected with HCV (227), and these individuals tend to exhibit a higher rate of viral persistence, increased viral load, and higher susceptibility to death compared to individuals infected with only one of these viruses (228). Thus, there is an urgent need to develop antivirals that treat and prevent infection by HCV and HIV through new modes of action.

Antiviral molecules targeting critical virus structural elements tend to be effective against several viruses and do not usually foster the emergence of drug-resistant viral isolates. One group of molecules inhibit virus-cell fusion by inducing positive membrane curvature, thus increasing the activation energy barrier for fusion with cell membranes (229-231). These molecules, which include rigid amphipathic fusion inhibitors (RAFIs) (229) and lysophosphatidylcholine (231), tend to have large hydrophilic heads and hydrophobic tails. LJ001, a recently discovered broad-spectrum small-molecule antiviral, inhibits the fusogenic activity of enveloped viruses by intercalating into the lipid membrane while leaving virion particles grossly intact (232). Alkylated porphyrins exhibit strong antiviral activity against several enveloped viruses through an unknown mechanism, perhaps by interfering with specific structures on the virus surface (233). Amphipathic peptides derived from HCV NS5A protein were shown to physically disrupt virions and were active against a variety of enveloped viruses (234, 235). Another approach to interfering with membrane elements required for virus infection is to target exposed anionic phospholipids widely expressed on infected host cells and viral envelopes, as was done with Bavituximab, a chimeric antibody which rescues mice from Pichinde virus and mouse cytomegalovirus infection (236).

Previously, we identified a small-molecule inhibitor of HCV entry, PD 404,182 (PD), from a screen of 1280 small-molecule compounds (LOPAC^{1280}) known to be pharmacologically active in a variety of cellular processes (148). Here, we report that PD, an inhibitor of bacterial 2-keto-3-deoxyctulosonic acid (KDO) 8-P synthase (237), is a virucidal compound that compromises the structural integrity of both HCV and HIV, likely by interacting with a non-lipid structural element of these viruses. *In vitro* studies revealed that PD physically disrupts variously pseudotyped lentiviruses and exposes the viral genomic RNA in a time- and temperature-dependent manner. Viral lysis is much less pronounced with cell culture-produced HCV (HCVcc), despite a clear inactivation of HCVcc infectivity on the pre-incubation of PD with viral supernatants. PD strongly inactivates multiple isolates of primary HIV-1 that utilize different coreceptors, HIV-2, and simian immunodeficiency virus (SIV), with a 50% inhibitory concentration (IC_{50}) of ~1 μM and a selectivity index (50% cytotoxic concentration [CC_{50}]/IC_{50}) of >300. A high antiviral potency and low cytotoxicity, combined with a unique mode of action, make PD a welcome addition to our current arsenal of antivirals to combat infection by HCV and HIV.

4.3 Materials and methods

4.3.1 Reagents

PD 404,182 and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). C5A was synthesized at the Scripps Research Institute. PD and C5A were dissolved in 100% dimethylsulfoxide (DMSO) to final concentrations of 30 mM and 10 mg/ml, respectively, and stored at −20°C. Unless otherwise specified, growth medium

for all cell culture work was Dulbecco's modified essential medium (DMEM) containing 4,500 mg/liter glucose, 4.0 mM L-glutamine, and 110 mg/liter sodium pyruvate (Thermo Scientific HyClone), and it was supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1× nonessential amino acids (Thermo Scientific HyClone). Conditioned complete growth medium (DMEM plus 10% FBS) was harvested on day 3 (with cells at 100% confluence) postseeding from Huh-7.5 cells initially seeded at ~20% confluence.

4.3.2 Production of HCVcc and pseudotyped lentiviruses

The production and titer determination of Jc1 HCVcc (132) was performed as previously described (148). Unless otherwise specified, all lentiviral pseudoparticles were generated from 293T cells by co-transfection of plasmids carrying HIV gag-pol, a provirus (pTRIP-Gluc, pV1-Gluc or pV1-B), and an appropriate envelope protein. For the production of murine leukemia virus (MLVpp), Sindbis virus (SINVpp), and HIV (HIVpp), plasmids encoding the viral envelope proteins pHIT456 (238), pIntron-SINV-env (239), and HIV BaL.01 (240) were used, respectively.

pV1 is a minimal HIV-1 provirus lacking most HIV genes except for all necessary *cis* acting sequences, such as Tat, Rev and Vpu open reading frames (ORF) (39, 241, 242). In pV1-B and pV1-Gluc, the Nef gene was replaced by an irrelevant peptide and the Gluc gene, respectively. The titers of vesicular stomatitis virus envelope glycoprotein (VSV-Gpp) and HIVpp harboring pV1-B or pV1-Gluc was measured on a TZM-bl indicator cell line using the *lac*Z reporter in a limiting dilution assay (243).

4.3.3 HCVcc infection assay

Jc1 Gluc HCVcc (~10^5 50% tissue culture infectious dose [$TCID_{50}$]/ml) (150) was concentrated 4-fold using an ultracentrifugation column with a 100-kDa cutoff membrane and washed twice with phenol red-free DMEM to remove any PD-inactivating molecules present in the virus supernatant. Concentrated virus was incubated with PD or DMSO at 37°C for 30 min, diluted 1,000-fold with fresh complete growth medium, and used to infect naïve Huh-7.5 cells in 24-well (10^5 cells/well) or 96-well (2.8×10^4 cells/well) plates 4 to 6 h after seeding. The control samples contain virus and PD of the same final titers/concentrations, but with the virus and PD separately diluted 1,000-fold prior to mixing. Viral infectivity was quantified by measuring the supernatant activity of the Gluc reporter or immunostaining infected cells for NS5A with 9E10 (anti-NS5A) antibody (35) 72 h post infection.

4.3.4 Spinoculation

PD-treated virus samples (HCVcc or VSV-Gpp) were cooled on ice for 5 to 10 min and added to chilled target cells seeded in 96-well plates. Spinoculation was carried out at 300 × *g* for 2 h at 4°C. After centrifugation, cells were washed 4 times with cold complete growth medium to remove any residual compound/unbound virus and returned to 37°C and 5% CO_2.

4.3.5 Viral RNA quantification

For the direct quantification of HCVcc/VSV-Gpp RNA, the total RNA from PD-treated HCVcc/VSV-Gpp and cells infected with these viruses was isolated using the EZNA viral RNA kit (Omega Bio-Tek) and total RNA kit (Omega Bio-Tek),

respectively. The amount of HCV RNA was quantified via TaqMan quantitative reverse transcription-PCR (qRT-PCR) (qScript one-step fast kit; Quanta Biosciences, Gaithersburg, MD) using previously described primers (154). The amount of lentiviral RNA was quantified using SYBR Green qRT-PCR (one-step SYBR green kit, Quanta Biosciences) with primers pV1-qPCR-F (5'- ACGGCCTCTAGAATGAGC -3) and pV1-qPCR-R (5' - ACAGCTGCTCGAGGTT -3').

Due to the large amount of residual provirus-encoding DNA present in the pseudoparticle preparations obtained from transfected 293T cells, we were not able to directly quantify the viral RNA. Instead, repackaged pseudoparticles were used in all experiments involving the direct quantification of viral RNA by qRT-PCR. Briefly, VSV-Gpp constructed from pV1-B (39, 241, 242) were used to transduce Huh-7.5 cells. Three days later, these Huh-7.5 cells were transfected with plasmids carrying HIV gag-pol and vesicular stomatitis virus (VSV)-G envelope protein to produce freshly repackaged pseudoparticles. Pseudoparticles serially repackaged in this manner at least 3 times were used in experiments requiring the direct quantification of viral RNA.

4.3.6 Gluc reporter assay

Supernatant Gluc activities were quantified 48 or 72 h postinfection with the relevant virus using a BioLux Gaussia luciferase assay kit (New England BioLabs) and normalized to viable cell levels as determined via the CellTiter-Glo luminescent cell viability assay (Promega).

4.3.7 Liposome dye release assay

Liposomes composed of 36 mg POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), 39 mg DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), 4 mg POPS (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine), and 21 mg cholesterol per 100 mg, without or with 100 mM sulforhodamine B (SulfoB; Avanti Polar Lipids, Inc.), were prepared as described previously (235) and sized via repeated extrusion through a 100-nm polycarbonate membrane filter (Avanti Polar Lipids, Inc.). Dye release assays were performed in a Gemini EM spectrofluorometer (Molecular Devices, San Francisco, CA). One μl PD (30 mM), 0.24 μl C5A (10 mg/ml), or 1 μl DMSO was added to 100 μl liposomes (100 μM; 0.06 mg/ml) in phosphate-buffered saline (PBS) in 384-well plates, and membrane disruption was gauged from the increase in SulfoB fluorescence at excitation and emission wavelength settings of 544 and 590 nm, respectively, 5 min posttreatment. The fluorescence intensity corresponding to 100% SulfoB release was obtained by liposome disruption with 0.1% Triton X-100.

4.3.8 HIV-1, HIV-2, SIV infectivity assays

TZM-bl cells (238) (100,000 cells/ml) were exposed to HIV or SIV (1 ng of p24/p27) for 4 h together with increasing concentrations of PD or DMSO control, washed, and infection was measured 48 h later by β-galactosidase activity. Primary HIV-1, HIV-2, and SIV were obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program and amplified in activated human peripheral blood mononuclear cells (PBMC; activated by phytohemagglutinin/interleukin-2 treatment). To determine the anti-HIV effect of PD in genital fluids, the same TZM cells

were exposed to HIV strain JR-CSF(1 ng of p24) for 4 h together with increasing concentrations of PD diluted in cervical fluids (pool of four donors) (244).

4.3.9 HIV-1 sedimentation assay

Purified HIV-1 (20 ng of p24 of NL4.3) was microcentrifuged for 90 min at 4°C to remove free capsid, resuspended in PBS, exposed to PD or DMSO medium control, and loaded over a 20 to 70% sucrose gradient. After ultracentrifugation at 20,000 rpm for 24 h in an SW-41 T rotor, fractions (1 ml) were collected and tested for their content of viral proteins. HIV-1 capsid was detected by p24 enzyme-linked immunosorbent assay (ELISA). Reverse transcriptase (RT) activity was measured using a polyribonucleotide template (exo-RT assay) (245). The density of each sucrose gradient fraction was determined by measuring the refractive index.

4.3.10 HIV-1 cell-to-cell transfer assay

Blood-derived immature dendritic cells (DC) were plated at 50,000 cells per well in 96-well V-bottom plates (BD Biosciences). Cells were incubated with wild-type NL4.3-eGFP (X4), NL4.3-BaL-eGFP (R5), or the single-round NL4.3ΔEnv-eGFP pseudotyped virus with NL4.3 gp160 (X4) (25 ng of p24) for 2 h at 37°C. Medium supplemented with either PD or DMSO then was added and incubated with DC for 2 h. Cells were washed three times with warm medium, and CCR5 Jurkat T cells (100,000 cells) were added. Cells were cultured in a flat-bottom 96-well plate, harvested after 3 days, and fixed in 4% paraformaldehyde-PBS, and green fluorescent protein (GFP) expression was measured by a fluorescence-activated cell sorter (FACS). The percentage of infected Jurkat T cells was selectively quantified by gating T cells using an anti-CD3

antibody. Virus isolates used in this study were pNL4.3-BaL (R5), in which wild-type NL4.3 envelope was switched for the R5 BaL envelope; pNL4.3ΔEnv, which lacks gp160; pNL4.3-eGFP (X4); and pNL4.3-BaL-eGFP (R5), which encode the GFP gene instead of the Nef gene (246).

4.4 Results

4.4.1 PD 404,182 is virucidal against HCV and pseudotyped lentiviruses

Previously, we showed that PD alleviates a HCVcc-induced cytopathic effect and inhibits the cellular entry of HIV lentivirus pseudotyped with envelope glycoproteins from the H77 isolate of HCV and vesicular stomatitis virus (VSV). In this study, we show that PD inhibits HCV infection by inactivating extracellular virions. As shown in Figure 4.1A and B, PD dose dependently inactivates cell-free HCVcc with an IC_{50} of 11 μM. To explore the possibility that the antiviral activity of PD is independent of the viral envelope protein, we incubated PD with HIV lentiviruses pseudotyped with three additional envelope proteins derived from murine leukemia virus (MLV) (238), Sindbis virus (SINV) (239), and HIV (240). PD exhibits similar antiviral activity against all of these pseudotyped lentiviruses (Figure 4.2), indicating that the antiviral activity derives from interference with a viral structural component other than the envelope proteins.

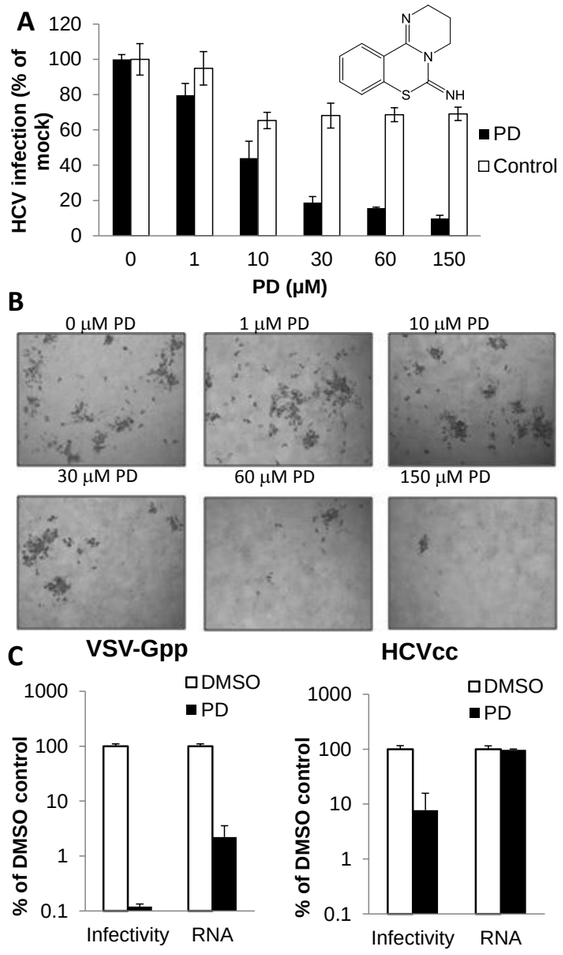


Figure 4. 1 PD is virucidal against HCVcc and pseudotyped lentivirus. (A, B) Effect on HCVcc infectivity. Jc1 Gluc HCVcc was incubated with PD or 0.5% DMSO at 37°C for 30 min, diluted 1000-fold and used to infect Huh-7.5 cells. The control samples contain virus and PD of the same final titers/concentrations, but with the virus and PD separately diluted 1000-fold prior to mixing. The infectivity was quantified by measuring the supernatant activity of the Gluc reporter 72 h post infection (A) and immunostaining for NS5A. **(B)** HCV-infected cells are brown after immunostaining. Inset in **(A)**: chemical structure of PD. **(C)** Effect on extracellular VSV-Gpp and HCVcc. VSV-Gpp (harboring pV1-B, ~10^6 $TCID_{50}$/ml; undiluted) or Jc1 HCVcc (~10^4 $TCID_{50}$/ml, 10-fold diluted) was incubated with PD (150 μM in 0.5% DMSO) or 0.5% DMSO in the presence of 7 ng/ml RNase A at 37°C for 30 min. The viral RNA levels of the virus-PD and virus-DMSO mixtures were quantified by qRT-PCR, while the infectivity of the same mixtures was determined by spinoculation of Huh-7.5 cells and quantification of intracellular viral RNA by qRT-PCR 48 h later. All data are the mean ± SD of 2 independent experiments carried out in duplicate.

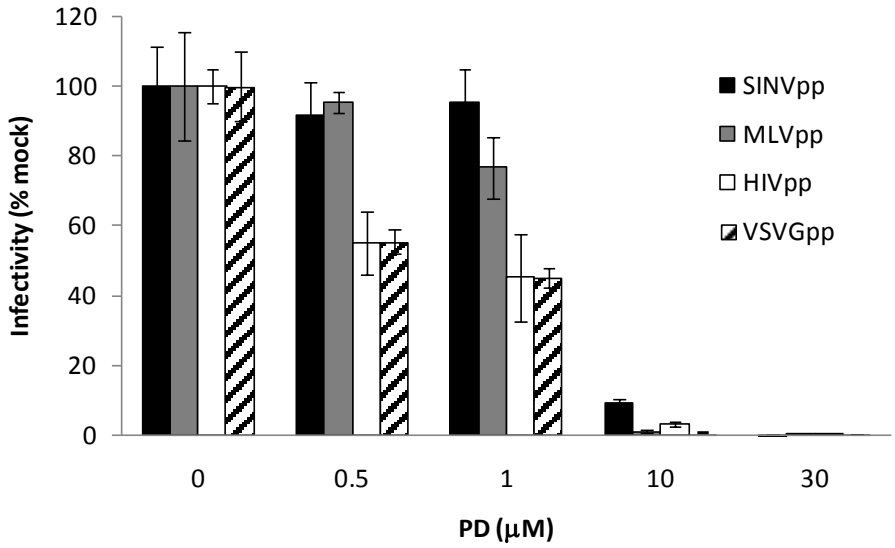


Figure 4. 2 PD inhibits infection by different pseudotyped lentiviruses. Lentiviruses (harboring pV1-Gluc provirus) pseudotyped with envelope proteins from Sindbis virus (SINVpp), murine leukemia virus (MLVpp), human immunodeficiency virus (HIVpp) and vesicular stomatitis virus (VSV-Gpp) were incubated with PD at 37°C for 30 min, and used to spinoculate BHK-J (SINVpp), Huh-7.5 (MLVpp, VSV-Gpp) and TZM-bl (HIVpp) cells at 4°C as described in *Materials and Methods*. Cells were washed 4 times with fresh medium to remove any unbound viruses and compound, and incubated at 37°C/5% CO_2. Viral infectivity was determined by measuring the supernatant activity of the Gluc reporter 48 h later. Due to differences in specific infectivity, SINVpp, MLVpp, HIVpp and VSV-Gpp virus stocks were diluted 5-, 50-, 10- and 100-fold, respectively, with fresh complete growth medium prior to compound treatment. The different virus dilutions ensured a similar final titer of the different viruses, as judged by the similar supernatant activities of the Gluc reporter after dilution. The error bars represent the mean ± SD of 2 independent experiments performed in duplicate.

We next set out to evaluate whether PD treatment causes the lysis of the virus membrane/capsid. Supernatant containing lentivirus pseudotyped with vesicular stomatitis virus envelope glycoprotein (VSV-Gpp) or HCVcc was treated with PD or with the corresponding concentration of the solvent DMSO at 37°C for 30 min in the presence of RNase A prior to the quantification of viral RNA and infectivity. As shown in Figure 4.1C, treatment with 150 μM PD induces the RNase-mediated degradation of VSV-Gpp RNA by ~30-fold (3.7% remaining) and inhibits supernatant infectivity by ~1,000-fold (0.1% remaining) relative to that of DMSO-treated virus. The significant fold difference between virus inactivation and virion lysis, which becomes more pronounced with shorter virus-PD preincubation times, suggests that virion lysis is not required for PD-mediated virus inactivation. A similar effect was observed with the virucidal peptide C5A, which showed ~100-fold more virion inactivation than lysis (235). Little to no virion lysis was observed in 150 μM PD-treated HCVcc, despite the >10-fold inhibition of virus infectivity. A low but reproducible level of HCVcc virion lysis (27%) was observed (Figure 4.3) only when a higher concentration of PD (300 μM) was used in combination with a prolonged incubation at 37°C (90 min). Since PD appears to significantly inactivate but only poorly lyse HCVcc, we asked ourselves whether PD-treated HCVcc particles that resist lysis by PD retain their ability to attach to the surface of cells. The measurement of cell surface-associated virus via qRT-PCR revealed that PD-treated HCVcc binds to cells comparably to control DMSO-treated virus, suggesting that treatment with the compound inhibits a postattachment step for virions that remain intact (Figure 4.3).

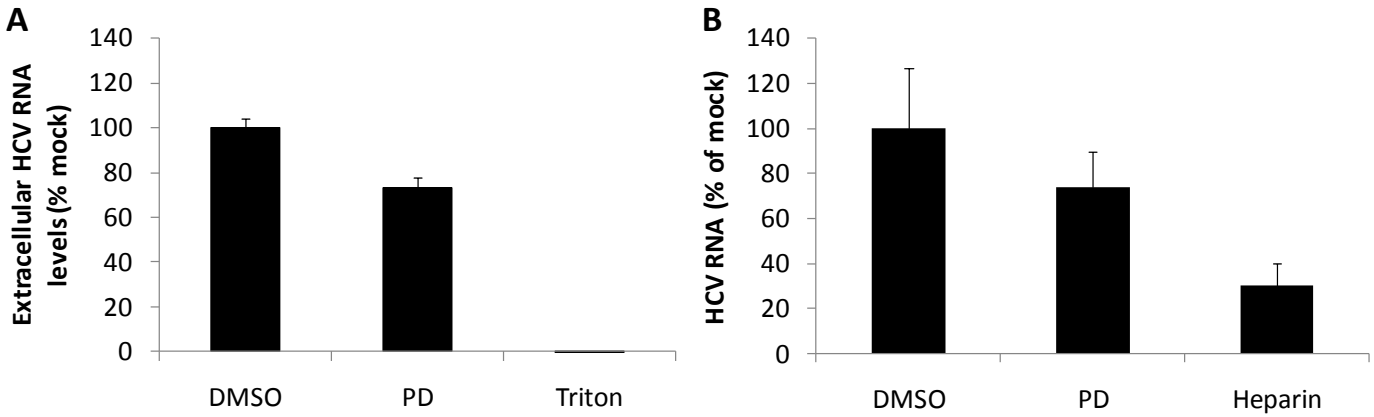


Figure 4. 3 Effect of PD on HCVcc virion integrity and attachment to cells. (A) PD only weakly disrupts HCVcc. HCVcc (10^4 $TCID_{50}$/ml) was incubated with PD (300 μM), Triton X-100 (0.1%) or 1% DMSO in the presence of 7 ng/ml RNase A at 37°C for 90 min. Isolation and quantification of viral RNA was carried out as described in *Material and Methods*. **(B)** HCVcc cell attachment assay. Jc1 HCVcc was partially clarified by four serial passages through a 300 kDa cutoff ultrafiltration membrane (Pall Life Sciences, Port Washington, NY). With each passage through the centrifugal unit, the retained virus was diluted in PBS prior to the next passage. HCVcc (10^4 $TCID_{50}$/ml) was pre-incubated with either freshly prepared heparin (1000 μg/ml; positive attachment inhibitor control) from porcine intestinal mucosa (Sigma, St. Louis, MO), 300 μM PD, or 1% DMSO at 37°C for 90 minutes under low-serum (<1%) conditions. In preparation for virus addition, Huh-7.5 cells seeded one day earlier at $3x10^5$ cells/well in 24-well plates were chilled on ice for 5 minutes. After aspirating the existing medium from the cells, 50 μl of the pre-treated HCVcc was added per well and the virus/cell mixture was incubated at 4°C for an additional 3 h. Cells were subsequently washed 5 times with complete growth medium and incubated at 37°C/5% CO_2 for an additional 2 h. Total RNA was harvested from the cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA levels of cell-bound HCVcc were determined via TaqMan qRT-PCR as described in *Materials and Methods*. Note: The observed slight decrease in attachment of PD-treated HCVcc relative to DMSO-treated virus is likely due to virion lysis (see (A)). The error bars represent the mean ± SD of 2 independent experiments done in duplicate.

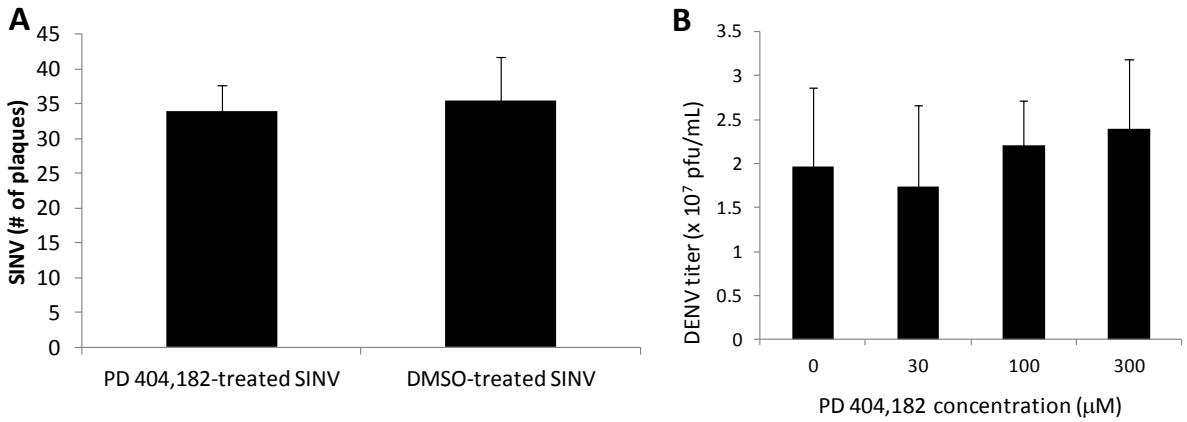


Figure 4. 4 Effect of PD on SINV and DenV. (A) Sindbis virus was produced in cell culture by electroporation of BHK-J cells with *in vitro*-transcribed viral RNA. Briefly, plasmid carrying the genome of SINV (Toto1101) (247) was linearized by digestion with *Xho*I and 1 μg of the linearized plasmid was used as a template for run-off transcription with SP6 RNA polymerase (Ampliscribe SP6 High-Yield Transcription Kit, Epicentre, Madison, WI). BHK-J cells were trypsinized, resuspended in cold DPBS to $2.8x10^7$ cells/ml and 400 μl of this cell suspension was electroporated with 3 μg of *in vitro*-transcribed viral RNA using an ECM 830 electroporator (Harvard Apparatus, Holliston, MA) using the following settings: 750 V, 5 pulses, 99 us pulse length, 1 second intervals. Virus-containing supernatant was collected 24 h post electroporation and stored at -80°C. Virus titer was determined on BHK-J cells with 10-fold serial dilutions of sample, and then plaques were visually enumerated after crystal violet staining, as previously described (248). For determination of the inhibitory effect of PD 404,182, cell culture-produced SINV was diluted 1000-fold in complete growth medium to 10^5 pfu/ml and pre-incubated with 300 μM PD 404,182 or 1% DMSO at 37°C for 1 h. Pre-incubated virus was diluted a further 2000-fold and used to inoculate BHK-J cells for enumeration of plaques. **(B)** Serotype 2 New Guinea C strain Dengue virus was propagated in Vero cells. Dengue virus serially diluted in complete medium containing 10% FBS was incubated with PD (10, 100 or 300 μM) or DMSO at 37°C for 30 min and used to infect Vero cells in a standard plaque assay. Briefly, Vero cells were seeded in 24-well plates at 10^5 cells/well and inoculated with 100 μL PD- or mock-treated Dengue virus at 37°C for 1 h. After removal of the inoculum, these cells were overlayed with 1 ml of culture medium containing 0.5% methyl cellulous. Five days later, the cells were fixed and stained with crystal violet to visualize plaques. The error bars represent the mean ± SD of 2 independent experiments done in duplicate. Data generated at Drexel University College of Medicine by Jinhong Chang.

The ability of PD to physically inactivate both VSV-Gpp and HCVcc, two very different viruses, combined with the observation that the compound does not seem to significantly distinguish between lentiviruses pseudotyped with different envelope proteins (Figure 4.2), suggests that its antiviral activity is mediated through a common non-envelope protein structural component.

To evaluate the antiviral specificity of PD, we tested the effect of the compound on two other enveloped viruses, SINV, an alphavirus (247), and Dengue virus (DenV), a flavivirus closely related to HCV (249). As shown in Figure 4.4, PD exhibits no significant inhibitory effect on the infectivity of SINV and DenV at 300 μM. Interestingly, despite the observed absence of antiviral activity against SINV, PD was found to exhibit strong antiviral activity against lentivirus pseudotyped with SINV envelope protein (Figure 4.2), underscoring the non-specific nature of the antiviral effect on pseudotyped lentiviruses. The neutrality of PD towards SINV and DenV suggests that PD may exert its antiviral effect by specifically interfering with a structural feature common to HCVcc and pseudotyped lentiviruses but not present on SINV and DenV.

4.4.2 PD inactivates a broad range of primary HIV isolates and related retroviruses

Since PD strongly inactivates all the pseudotyped lentiviruses we tested regardless of the envelope protein (Figure 4.2), we asked ourselves whether PD also inactivates primary HIV and related retroviruses.

Table 4. 1 PD 404,182 inhibits a broad spectrum of HIV and related viruses.

Primary HIV-1 isolates			PD, µM			
			DMEM[a]		Cervical Fluid[b]	
Isolate	Clade	Coreceptor usage	IC_{50} (µM)	IC_{90} (µM)	IC_{50} (µM)	IC_{90} (µM)
92RW021	A	R5	0.43 +/- 0.03	3.8 +/- 0.3	0.67 +/- 0.04	4.4 +/- 0.2
92UG029	A	X4	1.18 +/- 0.02	4.4 +/- 0.2	1.47 +/- 0.1	5.3 +/- 0.3
92TH026	B	R5	0.35 +/- 0.01	2.8 +/- 0.2	0.61 +/- 0.03	3.3 +/- 0.1
92HT599	B	X4	1.8 +/- 0.1	5.9 +/- 0.3	2.2 +/- 0.04	6.4 +/- 0.5
93IN101	C	R5	1.5 +/- 0.2	5.1 +/- 0.3	1.8 +/- 0.1	5.5 +/- 0.2
98IN017	C	X4	0.4 +/- 0.1	1.9 +/- 0.2	0.7 +/- 0.05	2.4 +/- 0.2
92UG005	D	R5	1.26 +/- 0.2	5.3 +/- 0.4	1.39 +/- 0.11	5.7 +/- 0.3
92UG024	D	X4	0.33 +/- 0.02	1.4 +/- 0.2	0.55 +/- 0.04	1.8 +/- 0.2
92TH006	E	R5	1.8 +/- 0.2	6.6 +/- 0.4	2.3 +/- 0.1	7.6 +/- 0.6
93TH053	E	X4	1.4 +/- 0.2	5.3 +/- 0.3	1.8 +/- 0.2	6.4 +/- 0.4
93BR029	F	R5	0.7 +/- 0.1	3.5 +/- 0.2	1.2 +/- 0.1	4.6 +/- 0.3
93BR020	F	X4	1.4 +/- 0.2	6.9 +/- 0.4	1.9 +/- 0.2	7.5 +/- 0.4
RU132	G	R5	0.6 +/- 0.2	3.1 +/- 02	0.9 +/- 0.2	3.9 +/- 0.5
Jv1083	G	R5	1.1 +/- 0.2	3.9 +/- 0.3	1.5 +/- 0.2	4.7 +/- 0.1
Other retroviruses						
SIVmac251 32H			1.1 +/- 0.2	6.2 +/- 0.4		
SIVsyk1.2			1.9 +/- 0.3	5.4 +/- 0.2		
HIV-2CDC310342			1.8 +/- 0.2	4.7 +/- 0.2		
HIV-27312A			2.2 +/- 0.3	6.8 +/- 0.3		

Errors represent the SD of 2 independent experiments carried out in duplicate.
[a]IC_{50} is measured with PD diluted in DMEM with 10% fetal bovine serum.
[b]IC_{50} is measured with PD diluted in cervical fluids (pool of 4 donors).
Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

Using CD4+ HeLa cells (TZM-bl cells (250)) that produce β-galactosidase in response to HIV infection, we determined the antiviral activity of PD on 14 isolates of HIV-1 which represent various subtypes and which use different coreceptors, either CCR5 (R5 viruses) or CXCR4 (X4 viruses), to infect cells, as well as isolates of other retroviruses, including HIV-2 and simian immunodeficiency virus (SIV). Viruses were added to TZM-bl cells together with PD for 4 h, cells were washed, and infection was scored 48 h later. As shown in Table 4.1, PD effectively inhibits all the tested isolates of HIV and SIV at submicromolar to low-micromolar concentrations, on par with the potency of the virucidal amphipathic peptide C5A (234, 235). Similar anti-HIV potency was observed when PD was diluted in cervical fluids (Table 4.1).

To probe the effect of PD on the structural integrity of HIV particles, we carried out a virus sedimentation assay. Purified HIV-1 (X4 NL4.3) (20 ng of p24 in PBS) was incubated in the presence or absence of PD (10 μM) for 30 min at 37°C and loaded onto a 20 to 70% sucrose gradient. Each fraction was analyzed for the amount of HIV capsid and reverse transcriptase (RT) (Figure 4.5). Untreated virus (capsid and RT proteins) sediments at a density of 1.16 g/cm^3. In contrast, viral capsid and RT relocate to the top of the gradient in PD-treated virus preparations, indicating that PD exerts its virucidal effect on HIV and retroviral particles by compromising virion integrity. This observation is consistent with the lysis of pseudotyped lentivirus shown in Figure 4.1C.

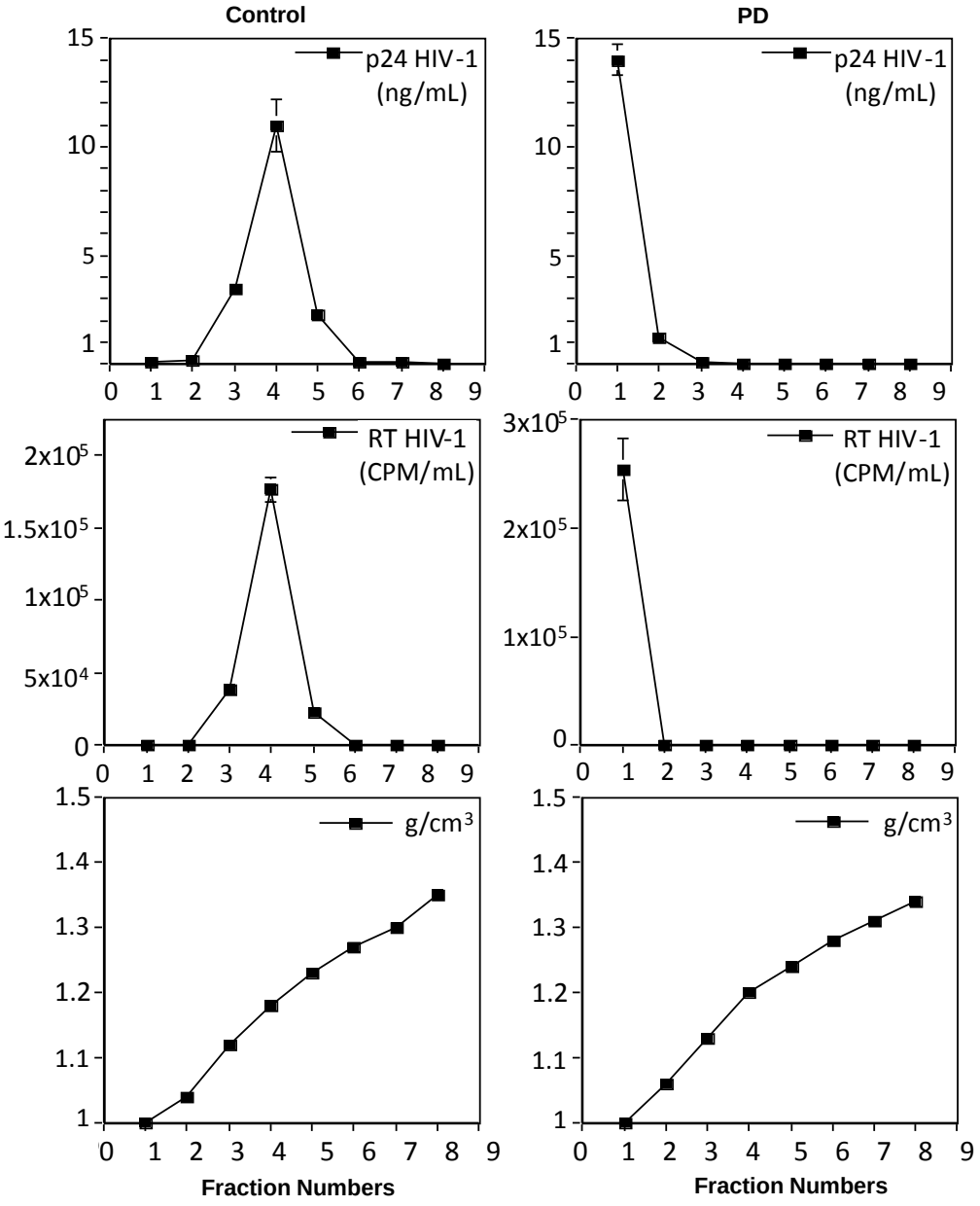


Figure 4. 5 PD destabilizes HIV-1 particles. NL4.3 virus (20 ng of p24) was incubated in the presence or absence of 10 μM PD for 30 min at 37°C and loaded over a sucrose density gradient. Quantification of HIV-1 capsid and RT proteins was conducted by p24 ELISA and exoRT assay, respectively. All data are the mean ± SD of 2 independent experiments carried out in duplicate. Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

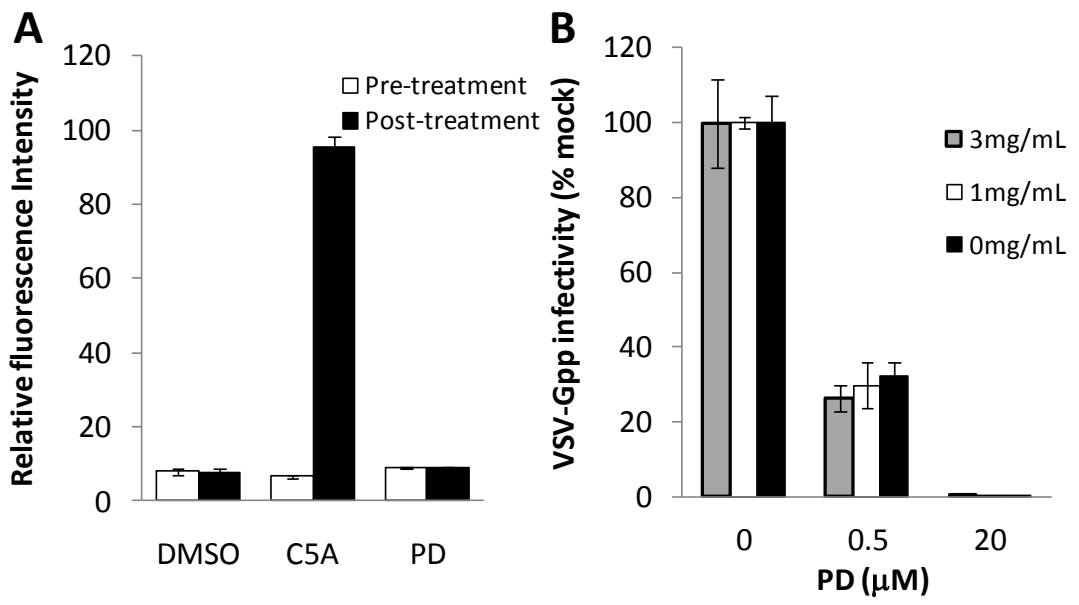


Figure 4. 6 PD does not lyse or directly interact with liposomal membranes. (A) The ability of PD (300 μM), the virucidal peptide C5A (10 μM), and solvent DMSO (1%) to permeabilize liposomes entrapping SulfoB was determined by a liposome dye release assay. A relative fluorescence intensity of 100 corresponds to SulfoB release resulting from liposome disruption with 0.1% Triton X-100. **(B)** VSV-Gpp (harboring pV1-Gluc, ~1.7 x 10^7 $TCID_{50}$/ml) was diluted 500-fold in fresh complete growth medium and pre-incubated with PD for 30 min at 37°C in the presence or absence of various concentrations of liposomes. The virus/PD/liposome mixtures were then used to spinoculate Huh-7.5 cells and infectivity was quantified by measuring the supernatant activity of the Gluc reporter 48 h post transduction. All data are the mean ± SD of 2 independent experiments carried out in triplicate.

4.4.3 PD does not lyse or interact with liposomal membranes

Because the antiviral potency of PD is virus envelope protein independent, we investigated the possibility that PD exerts its anti-viral activity via the disruption of the viral lipid membrane. Cholesterol-phospholipid liposomes entrapping the fluorescent dye sulforhodamine B (SulfoB) were incubated with PD, C5A, or DMSO. The disruption of the liposomes is accompanied by the dequenching of the fluorescent dye, and it was quantified by measuring the resultant fluorescence release. PD does not interfere with SulfoB fluorescence (data not shown). C5A, a peptide derived from HCV NS5A protein that has been shown to lyse liposomes, was used as a positive control (235). As shown in Figure 4.6A, PD is unable to permeabilize liposomes after incubation for 5 min. No significant increase in fluorescence intensity was observed even after prolonged (up to 3 h) incubation with PD (Figure 4.7A).

We next set out to determine whether PD associates with liposomal membranes without causing lysis. Since PD is not inherently fluorescent, we were unable to directly measure the interaction of PD with liposomes. Instead, we sought to determine whether the inhibitory effect of PD during infection can be reversed by the addition of liposomes. VSV-Gpp (3.4×10^4 $TCID_{50}$/ml) mixed with PD and increasing concentrations of liposomes was used to infect Huh-7.5 cells. The presence of liposomes was not able to reverse PD's antiviral effect (Figure 4.6B), suggesting that PD does not significantly interact with liposomes. In fact, liposomes of different lipid compositions were tested, but none were found to reverse the antiviral effect of PD (Figure 4.7 B and C).

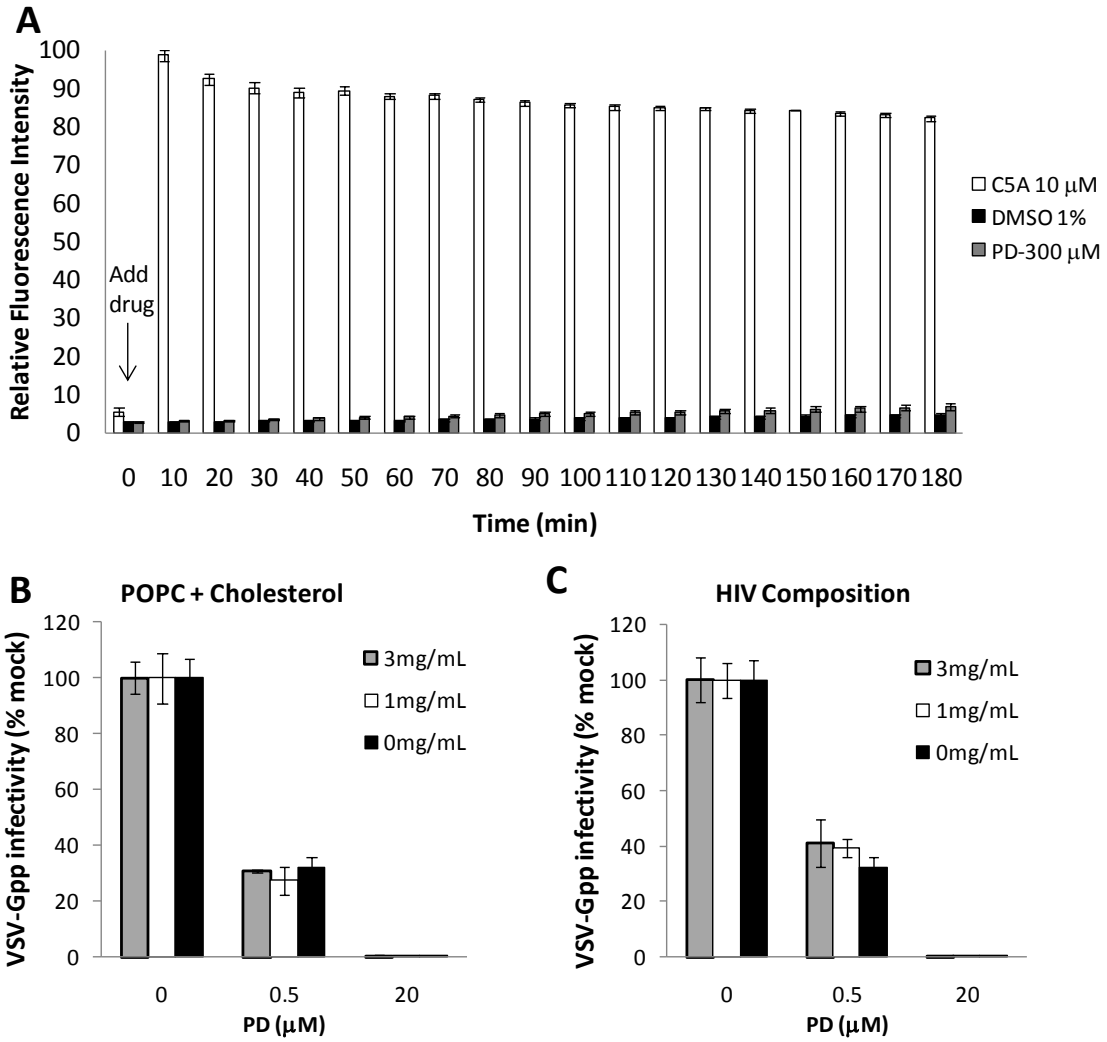


Figure 4. 7 PD does not interact with liposomal membranes. (A) No significant increase in fluorescence intensity was observed even after prolonged incubation of liposome with PD. **(B, C)** The virucidal activity of PD is not attenuated by the presence of liposomes. Liposomes composed of (B) 70 mg POPC and 30 mg cholesterol or (C) 12 mg POPC, 33 mg SM, 5 mg PE, 19 mg pl-PE, 30 mg cholesterol and 1 mg POPS (the same composition as HIV (251)) per 100 mg were incubated with PD and VSV-Gpp as described in Figure 4.6B. The error bars represent the mean ± SD of 2 independent experiments done in duplicate.
POPC: 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine; Cho: cholesterol; SM: sphingomyelin; PE: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine; POPS: 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; pl-PE: 1-alkenyl,2-acylglycerophosphoethanolamine; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine.

4.4.4 The virucidal activity of PD is temperature-, time- and virus dilution-dependent

To further elucidate the antiviral effect of PD, VSV-Gpp was incubated with PD (300 μM) or DMSO (1%) at various temperatures and for various times, and the amount of remaining viral RNA and the infectivity of the virus/compound mixtures were determined thereafter. The virion lysis activity of PD was found to be temperature dependent, as PD disrupts the virus following a 30-min incubation at 37°C but is less disruptive at 25°C and exhibits no measurable virion lysis at 4°C (Figure 4.8A), indicating that a minimum level of membrane fluidity is required for PD to lyse the virus membrane/capsid. A similar trend was observed for viral infectivity. PD rapidly inactivates pseudotyped lentivirus at 37°C as determined by the loss of viral RNA and infectivity (Figure 4.8B). More than 99.5% of the VSV-Gpp was inactivated within 5 min when in contact with 300 μM PD. However, only ~40% of the virions were compromised to the point of genomic RNA release for the same 5-min virus-PD preincubation, indicating that virion lysis is not required for virus inactivation.

The sensitivity of virus to PD is also virus dilution-dependent (Figure 4.9). The IC_{50}s of PD for cell culture-produced VSV-Gpp virus stocks diluted 5- and 500-fold in fresh complete growth medium (DMEM plus 10% FBS) are 4.6 and 0.5 μM, respectively. Similarly, the IC_{50}s for HIVpp (lentivirus pseudotyped with envelope protein from Bal.01 HIV) are 24.6 and 0.3 μM for undiluted and 100-fold-diluted virus.

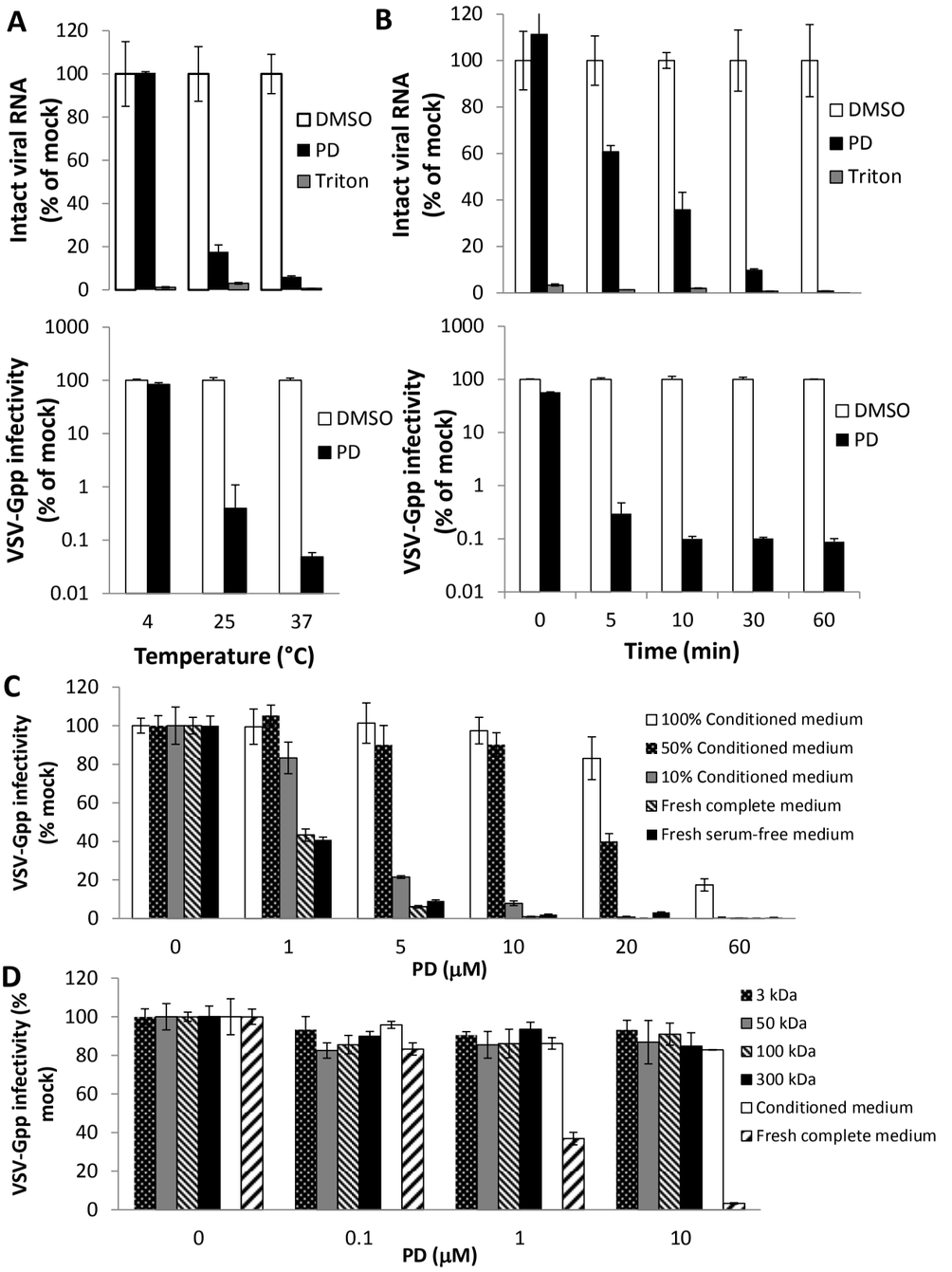


Figure 4. 8 PD exhibits virucidal activity that is temperature - and virus dilution-dependent and virus lysis activity that is time-dependent. Undiluted VSV-Gpp (harboring pV1-B or pV1-Gluc, ~5 x10^5 $TCID_{50}$/ml) was treated with 300 μM PD, 0.1% Triton X-100 or 1% DMSO in the presence of 7 ng/ml RNase A for (A) 60 minutes at different temperatures or (B) at 37°C for different times. Viral RNA was isolated thereafter and quantified using qRT-PCR. Viral infectivity was determined by measuring the supernatant Gluc reporter activity of Huh-7.5 cells 48 h post inoculation with 1000-fold diluted virus/PD mixtures. For virus dilution studies, VSV-Gpp stock (harboring pV1-Gluc, ~1.7 x 10^7 $TCID_{50}$/ml) was diluted 500-fold in (C) medium comprising different proportions of conditioned and fresh complete media (all containing 10% FBS) or fresh serum-free medium, or (D) the flow-through of conditioned medium size-fractionated through membranes with pores of the indicated size, prior to pre-treatment with PD at 37°C for 30 min. PD-treated viral samples were used to spinoculate naïve Huh-7.5 cells. Infectivity was quantified 2 days later by measuring the supernatant activity of the Gluc reporter. All data are the mean ± SD of 2 independent experiments carried out in duplicate.

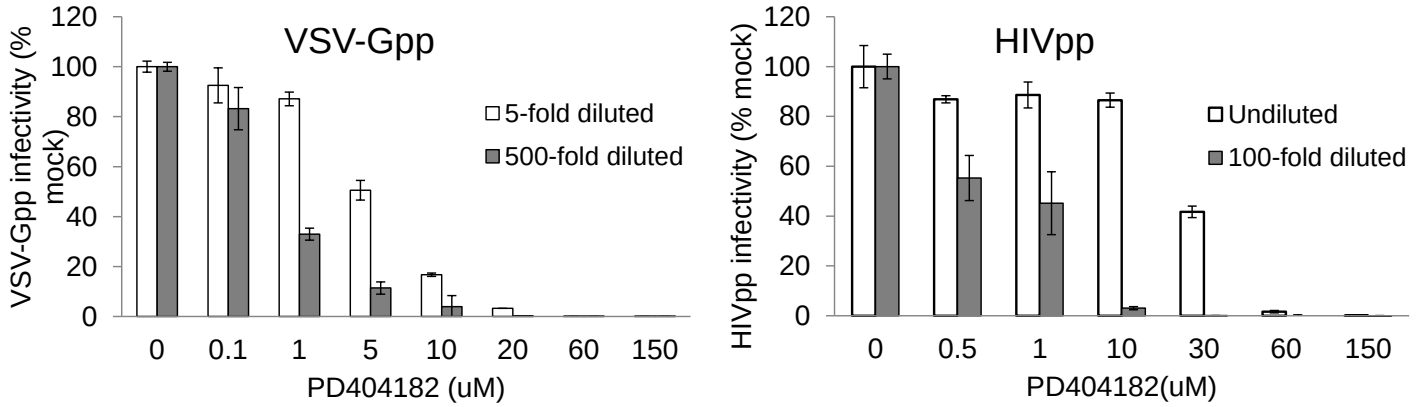


Figure 4. 9 The antiviral potency of PD against VSV-Gpp and HIVpp is virus dilution-dependent. VSV-Gpp **(A)** and HIVpp **(B)** (both harboring pV1-Gluc provirus) diluted in fresh complete growth medium and treated with PD at 37°C for 30 min were used to spinoculate Huh-7.5 and TZM-bl cells, respectively, as described in *Material and Methods*. The titers for 5-fold diluted VSV-Gpp and undiluted HIVpp were $3.4x10^6$ and $2.3x10^4$ $TCID_{50}$/ml, respectively. The error bars represent the mean ± SD of 2 independent experiments done in duplicate.

Further studies demonstrated that PD is inactivated by a molecule(s) present in conditioned cell culture medium, as virus diluted in conditioned medium is significantly less sensitive to inactivation by PD than the same virus diluted in fresh complete medium (Figure 4.8C). To gauge the approximate size of the molecule(s) responsible for neutralizing the antiviral effect of PD, we fractionated conditioned medium from Huh-7.5 cells by passage through ultrafiltration membranes with different pore sizes and found that the filtrate from a 3-kDa membrane is able to inactivate PD to the same extent as the unfiltered conditioned medium (Figure 4.8D). This result suggests that the molecule(s) responsible for neutralizing PD is relatively small (≤3 kDa). Analysis using liquid chromatography-mass spectrometry (LC-MS) demonstrated that PD is degraded (data not shown), possibly by reacting with a small molecule secreted by cells, rendering it less active against pseudotyped lentivirus. Although the presence of 10% fetal bovine serum appears to have no inhibitory effect on PD's antiviral activity (Figure 4.8C), 10% human serum yielded a significant (50-80 fold) increase in IC_{50} (Figure 4.10), suggesting the presence of PD-inhibitory factors in human serum in addition to cell culture medium conditioned by human cancer cells. It is worth noting that similar IC_{50} and IC_{90} values were obtained with PD diluted in DMEM or cervical fluids (Table 4.1), indicating that cervical fluids are free of molecule(s) that detectably inhibit the antiviral activity of PD.

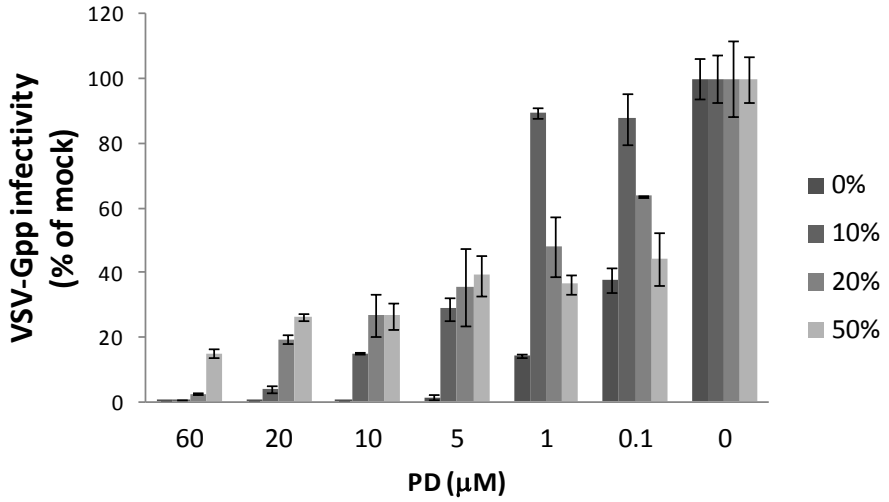


Figure 4. 10 The antiviral potency of PD is inhibited by the presence of human serum. VSV-Gpp was diluted 500-fold in medium containing different concentrations of human serum, incubated with appropriately diluted PD at 37 °C for 30 mins, and used to spinoculate Huh-7.5 cells at 4 °C as described in *Materials and Methods*. Cells were washed 4 times with fresh medium and incubated at 37°C/5% CO_2. Viral infectivity was determined by measuring the supernatant activity of the Gluc reporter 48 h later. The error bars represent the mean ± SD of 2 independent experiments done in duplicate.

4.4.5 Antiviral effect of PD before, during, and after HIV-1 exposure and on cell-to cell transmission of HIV-1

PD exhibits strong virucidal activity against pseudotyped lentivirus and primary HIV, raising the possibility of its use as a topical microbicide for preventing the sexual transmission of HIV-1. To shed light on this possibility, we investigated the antiviral effect of PD when the compound was added to cells at various time points relative to the addition of HIV-1. PD was added to TZM-bl cells at 1, 2, 4, or 16 h before the addition of HIV-1 (R5 JR-CSF) (1 ng of p24), together with the virus (time zero), and at 1, 2, 4, or 8 h after the addition of the virus, and infectivity was quantified 48 h after virus addition. As shown in Figure 4.11A, PD significantly inhibits HIV-1 infection when added together with the virus (time zero) and retains its full potency up to 8 h before the addition of the virus. However, PD loses its antiviral effect when added to cells after virus inoculation (Figure 4.11A, 1, 2, 4 and 8 h post treatment). This result suggests that PD is not able to disrupt intracellular virus. In addition, extended (>16 h) preincubation of PD with cells prior to virus inoculation also significantly reduces the compound's antiviral efficacy.

Since HIV-1 can be transmitted either as a cell-free or cell-associated virus, we examined the effect of PD on cell-to-cell transmission. Specifically, we examined the capacity of PD to prevent the dendritic cell (DC)-mediated transmission of HIV-1. We took advantage of a replication-defective virus (NL4.3ΔEnv-eGFP) which does not encode Env but which has been pseudotyped with HIV-1 Env (These pseudotyped viruses infect cells because they contain Env).

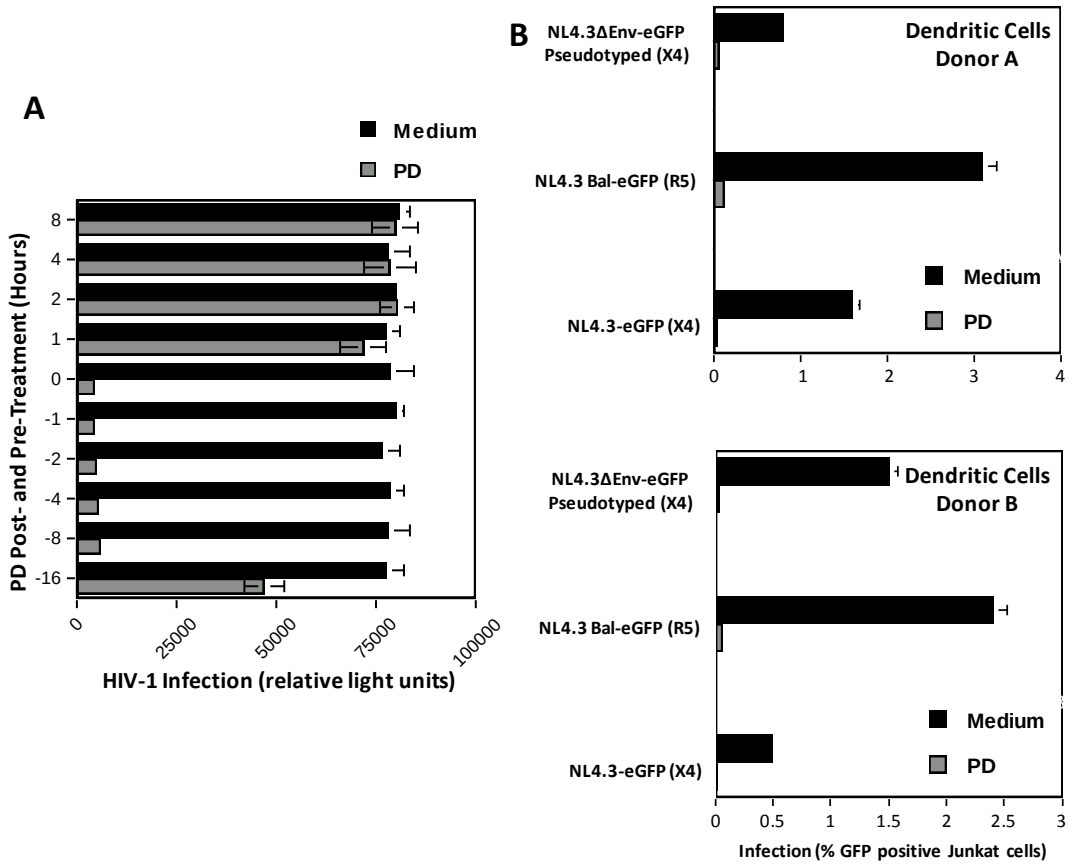


Figure 4. 11 Effect of PD on HIV-1 infection. (A) Antiviral effect of PD before, during, and after virus exposure. PD (10 μM) or just growth medium (DMSO control) was added to TZM-bl cells 1, 2, 4, 8 or 16 h before (negative values on the *y* axis), after (positive values on the *y* axis) the addition of HIV-1 (R5 JR-CSF) (1 ng of p24) or together (time zero) with the virus. Infection was quantified 48 h later via measurement of β-galactosidase activity. **(B)** DC (10^5 cells) were incubated for 2 h at 37°C with wild-type NL4.3-eGFP (X4 virus) and NL4.3-BaL-eGFP (R5 virus) viruses or with the pseudotyped NL4.3ΔEnv-eGFP/gp160 X4 Env virus (25 ng of p24). PD (10 μM) or control DMSO medium was added 2 h later. DC were washed 2 h after adding PD, Jurkat T cells (100,000 cells) were added for 3 days, and the percentage of infected Jurkat T cells (GFP+) was analyzed by flow cytometry. Error bars represent standard errors of duplicates from 2 independent experiments. Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

However, cells that have been infected by pseudotyped viruses cannot produce infectious viruses, because de novo viruses do not encode Env. Thus, the use of pseudotyped viruses allowed the analysis of the effect of PD on the transmission of infectious particles from DC to T cells independently of DC infection. DC were incubated with wild-type NL4.3-eGFP (X4 virus) and NL4.3-BaL-eGFP (R5 virus) or pseudotyped NL4.3ΔEnv-eGFP/gp160 Env viruses (25 ng of p24). Two hours later, at which time the attachment of the virus onto DC is completed (252), PD (10 μM) was added. After 2 h, DC were washed to remove both free virus and PD. To measure DC-T cell transmission, Jurkat T cells were added for 3 days, and the percentage of infected T cells (gated with an anti-CD3 antibody) was analyzed by FACS. Only pseudotyped viruses that have been rapidly transferred from DC to T cells through the virological synapse (independently of DC infection) can infect T cells. Indeed, progeny viruses from DC infected by pseudotyped viruses can no longer infect T cells because they do not encode Env. Because DC were washed before adding T cells, the T-cell infection by the pseudotyped virus observed in Figure 4.11B could arise only from pseudotyped particles that were transferred from DC to T cells. Importantly, PD added to DC prevents subsequent T-cell infection with the pseudotyped virus. This finding suggests that PD also can inactivate DC-bound virus, preventing HIV transmission from DC to T cells. These results suggest that, unlike neutralizing antibodies (246), PD blocks cell-to-cell transfer of HIV even when transmission occurs via the virological synapse.

4.5 Discussion

In this study, we report a small molecule, PD 404,182 (PD), that renders extracellular cell culture-produced HCV, pseudotyped lentiviruses, and several primary isolates of HIV and SIV noninfectious. In the case of pseudotyped lentivirus and primary HIV, the antiviral activity of PD appears to be due to the physical disruption of the virion. The antiviral action of PD is very rapid, as >99.5% of the lentivirus becomes inactivated within 5 min of contact with 300 μM PD at 37°C. However, only ~40% of the lentiviruses were lysed in the same period, indicating that PD inactivates pseudotyped lentiviruses and HIV by physical disruption that does not necessitate the complete lysis of virions. PD exposure does not appear to significantly rupture HCV or inhibit its attachment to cells, even with 90 min of exposure at 37°C (Figure 4.3), despite the inactivation of extracellular virus, suggesting a subtle disruption of virions (e.g., by irreversibly interfering with membrane fluidity or curvature) that causes the inhibition of a postattachment step, such as endocytosis or fusion with the endosomal membrane. Encouragingly, PD exhibits very low cytotoxicity in several human cell lines (CC_{50} >300 μM; Figure 4.12). The selectivity index (CC_{50}/IC_{50}) of PD is >300 for HIV and >27 for HCV. PD was originally synthesized by Birck, *el al.* as an inhibitor of bacterial KDO 8-P synthase (237) and was recently found to also affect angiogenesis (253) and mammalian circadian rhythm (254).

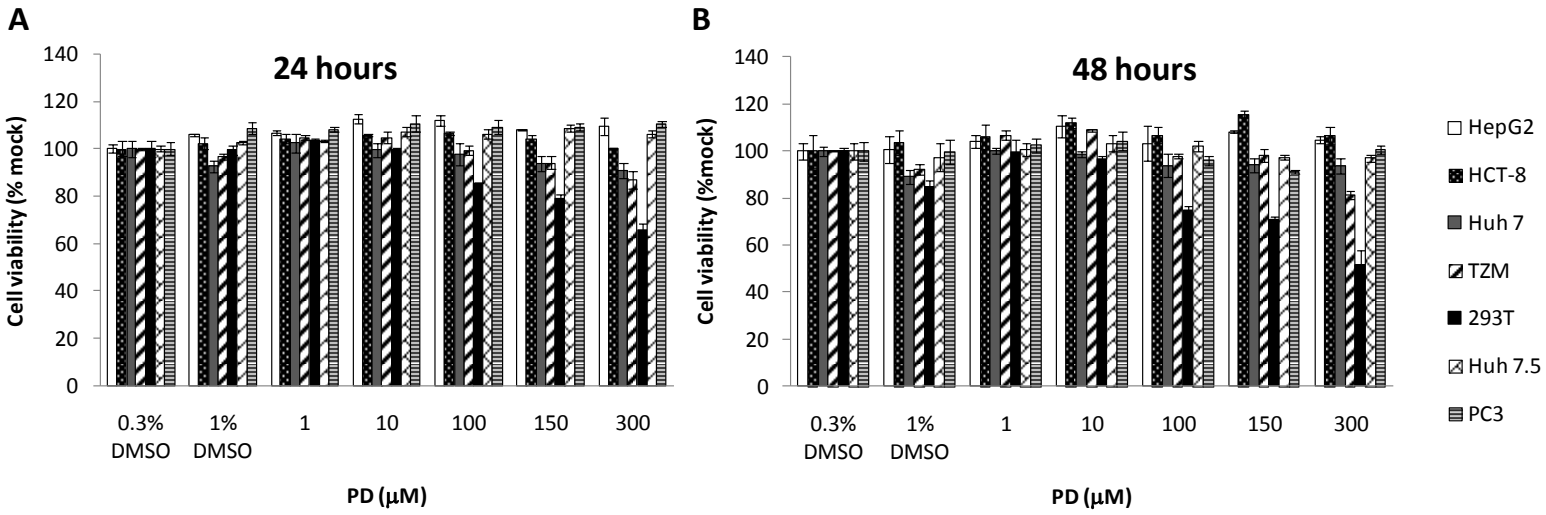


Figure 4. 12 Cytoxicity of PD 404,182 on different human cell lines. The cytotoxicity of PD was determined in the human cell lines HepG2 (hepatoma), HCT-8 (colon cancer), Huh-7 (hepatoma), Huh-7.5 (hepatoma), TZM (cervical cancer), PC3 (prostate cancer), and 293T (embryonic kidney). Cells were seeded in 96-well flat bottom tissue culture plates at 1.8 x 10^4 – 3.2 x 10^4 cells per well, where cell lines that divide faster were seeded at lower densities. After plating, cell culture supernatants were replaced with PD-containing medium, and cells were incubated at 37°C/5% CO_2. At 12 h intervals post initial treatment with compound, cell culture supernatants were removed and replaced with freshly prepared PD diluted in complete growth medium. At 24 h **(A)** and 48 h **(B)** post initial treatment with PD, cell viability was determined using CellTiter-Glo reagent. Cell culture supernatants were removed and replaced with 50 µl CellTiter-Glo reagent diluted 1:10 in ddH_2O. Microplates were then gently vortexed for 2 min and incubated at room temperature for an additional 8 min. Ten microliters of sample from each well was transferred to a white 96-well plate (Corning), and luminescence was measured in a Berthold Tristar LB 941 luminometer for 0.1 s. The error bars represent the mean ± SD of 2 independent experiments done in duplicate.

Intriguingly, despite exhibiting strong lysis of virions derived from the HIV capsid, PD does not directly lyse liposomes and shows no attenuation in antiviral activity when pre-incubated with liposomes, which is suggestive of little to no direct interaction with lipid membranes. The antiviral action of PD thus appears to be different from that reported for the amphipathic virucidal peptide C5A (234, 235), which lyses both virions and liposomal membranes, and the membrane-intercalating virucidal molecule LJ001 (232), whose antiviral effect is attenuated by pre-incubation with liposomes. It is conceivable that PD disrupts the structural integrity of virions by selectively interacting with a feature of virions that involves an interplay between two or more structural components (e.g., lipid membrane and envelope protein/capsid). We also cannot rule out the possibility that PD interferes with other virion structural components not represented in the liposome model, for example, sites that are glycosylated or phosphorylated (255-257).

The virion lysis activity of PD is temperature-dependent, suggesting that a minimal level of viral membrane fluidity may be required to sufficiently compromise virion integrity to the point of viral RNA release. PD is inactivated by human serum and medium conditioned by human cell culture, possibly by interacting with one or more small molecules/peptides secreted by humans but not bovine cells.

A striking feature of PD is its highly specific inactivation of certain viruses (only HCV, HIV and related retroviruses were found to be inactivated in this study) without strong association directly with or disruption of lipid membranes in general, as evident from our liposome studies. PD exhibited no significant antiviral effect on Dengue virus,

an enveloped flavivirus closely related to HCV, or cell culture-produced Sindbis virus, an enveloped alphavirus (Figure 4.4). Like HCV, Dengue virus acquires the viral envelope by budding into the endoplasmic reticulum lumen and is able to undergo intensive structural rearrangement in an infected cell (258). On the other hand, Sindbis virus, like HIV, buds from the plasma membrane and contains an envelope rich in cholesterol and sphingolipid molecules (259). We have yet to determine the antiviral effect of PD on other enveloped or non-enveloped viruses. The narrow target spectrum of PD as determined by our studies on a limited range of viruses, combined with the absence of non-specific lysis of or association with lipid membranes may, at least in part, account for the molecule's very low cytotoxicity.

Our studies bring to light some limitations of PD as an antiviral agent. Although PD effectively inactivates extracellular virus, it appears to be ineffective against intracellular virus, possibly due to a poor ability to enter cells and/or intracellular conversion to an inactive metabolite. Furthermore, human serum and the extended (>16 h) preincubation of PD with cells prior to virus inoculation appears to significantly reduce its antiviral efficacy. Our observation that the antiviral effect of PD is suppressed by a molecule(s) secreted by cells into the surrounding growth medium may be responsible for the latter phenomenon. Since we found that PD retains its full antiviral activity in cervical fluids, the PD-neutralizing molecule(s) present in human serum and conditioned cell culture growth medium is likely physiologically irrelevant in the case of the development of PD as a topical microbicide for the treatment/prevention of HIV infection.

It is estimated that there are approximately 4 million new incidences of HIV infection each year, mostly transmitted through heterosexual intercourse (260). The development of a vaginal (or rectal) microbicide against HIV would represent a major stride towards slowing the global spread of HIV (261). Despite the poor antiviral efficacy of PD against intracellular virus and in medium conditioned by human cell growth, this compound possesses several desirable attributes that make it an attractive candidate anti-HIV microbicide. Most notably, PD (i) exhibits antiviral activity against a broad range of primary HIV-1 isolates, HIV-2, and SIV; (ii) retains full anti-HIV potency for 8 h when in contact with cells; (iii) is effective against both cell-free and cell-associated HIV and inhibits HIV transmission from DC to T cells; (iv) retains full anti-HIV potency in cervical fluids; and (v) irreversibly inactivates HIV predominantly through virion disruption with an activity that appears to be independent of specific virus envelope proteins. Drugs that target viral proteins mediating the replication of viral nucleic acids or virus attachment to target cells often foster the emergence of escape mutants (262). The antiviral action of PD on critical components of the virus other than specific virus envelope proteins makes the development of drug-resistant mutant viruses less likely.

Several candidate anti-HIV microbicides exist (263-265), but only a handful exhibit an ability to strongly and irreversibly disrupt virions without being detrimental to cells (233, 235). PD 404,182 is an anti-HIV compound with a unique mode of action and represents a useful molecular scaffold for the generation of new anti-HIV-1 microbicides. Finally, the observation that PD 404,182 is able to inactivate both HCV

and HIV and the unique antiviral action of this small molecule justify further studies of PD 404,182 and derivatives thereof to determine antiviral effects upon other enveloped and non-enveloped viruses.

CHAPTER V

EVALUATION OF PD 404,182 AS AN ANTI-HIV AND ANTI-HSV MICROBICIDE*

5.1 Overview

PD 404,182 (PD) is a colorless and odorless synthetic compound that was previously found to compromise HIV integrity via interaction with a non-envelope protein viral structural component (Antimicrobial Agents and Chemotherapy, (2012), 56:672-81). The present study evaluates the potential of PD as an anti-HIV microbicide and establishes PD's virucidal activity towards another pathogen – herpes simplex virus (HSV). We show that the anti-HIV-1 IC_{50} of PD when diluted in seminal plasma is ~1 µM, similar to the IC_{50} determined in cell culture growth media, and that PD retains full anti-HIV-1 activity after incubation in cervical fluid at 37°C for at least 24 hours. In addition, PD is non-toxic towards vaginal commensal *Lactobacillus* species (CC_{50} > 300 µM), freshly activated human PBMC (CC_{50} ~ 200 µM) and primary CD4+ T-cells, macrophages and dendritic cells (CC50 > 300 µM). PD also exhibits high stability in PBS with little-to-no activity loss after 8 weeks at 42°C, indicating suitability to formulation for transportation and storage in developing countries. Finally, for the first

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Data from Table 5.1 and Figures 5.1, 5.2B, 5.4 and 5.5 were generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay
Data from Table 5.2 was generated at Southern Research Institute by Marie K. Mankowski and Roger G. Ptak
Data from Table 5.3 was generated at University of Pittsburg by Dr. Bernard Moncla

time, we show that PD inactivates herpes simplex virus (HSV) -1 and -2 at submicromolar concentrations. Due to the prevalence of HSV infection, the ability of PD to inactivate HSV may provide an additional incentive for use as a microbicide. The ability of PD to inactivate both HIV-1 and HSV, combined with its low toxicity, high stability and high barrier for resistance development *in vitro*, warrants additional studies for the evaluation PD's microbicidal candidacy in animals and humans.

5.2 Introduction

Since its discovery in humans in 1981, HIV, the causative agent of AIDS, has infected over 60 million people worldwide and caused more than 25 million deaths (49). Although highly active antiretroviral therapy (HAART) can significantly reduce viral load and prolong patients' life expectancy, these therapies are not curative (266). Worldwide, nearly half of all individuals living with HIV are women, most of whom acquire the virus after sexual intercourse with HIV-positive men. As receptive partners, women are twice as likely than their male counterparts to acquire HIV during sex (267). Despite the knowledge of effective prevention strategies, such as the "ABC" approach (abstinence, be faithful and use of condoms), the rate of HIV transmission remains high in developing countries (268). Moreover, many women cannot reliably negotiate safe sex practices, leaving them vulnerable to sexually transmitted infections. Thus, the development of a safe, effective and acceptable topical microbicide capable of retarding or preventing the sexual transmission of HIV could empower women to take personal responsibility to prevent HIV acquisition from their infected partners (269).

Topical microbicides are agents able to inhibit the transmission of viral infections when applied to the vagina, penis and/or lower gastrointestinal (GI) tract via the rectum. An ideal anti-HIV microbicide should fulfill most or all of the following criteria: **a**) inhibit transmission of wild type and drug-resistant virus (270); **b**) stability and potency in seminal fluids and vaginal secretions; **c**) absence of toxicity to the vaginal epithelium and commensal bacteria flora; **d**) ability to interfere with multiple transmission modes (*e.g.* as cell-free *vs.* cell-associated virus) given unknowns in the exact mode of HIV transmission *in vivo*; **e**) possess a high genetic barrier to resistance development; and **f**) preferably act through a distinct mode of action from existing therapeutics (271). The last consideration derives from the presence of rare pre-existing drug-resistant viral variants, as well as drug-resistant HIV variants from patients who underwent previous anti-retroviral treatment, that can bypass the microbicidal barrier and transmit to target cells. Most current anti-HIV microbicide candidates in clinical trials are formulated based on existing anti-retroviral drugs and target well-studied viral proteins such as HIV protease (PR), reverse transcriptase (RT) and HIV envelope protein (Env) (270-274). In the CAPRISA 004 clinical trial involving 1% tenofovir gel, HIV-1 acquisition was reduced by ~38% in all woman and by 54% in woman who used the gel 80% or more of the time (117). Interestingly and unexpectedly, in the same trial, tenofovir gel was found to also inhibit HSV-2 acquisition by 51% (117). The recently FDA-approved anti-HIV prophylactic therapeutic, Truvada ®, comprises two nucleoside analogs, tenofovir and emtricitabine (120). Truvada offered a 44% reduction in HIV transmission during initial clinical trials (120). However, since both tenofovir and emtricitabine are currently used

in the clinic for HIV treatment as part of HAART drug cocktail, concerns were raised about the potential for the spread of drug-resistant variants when the drug is used by individuals with unknown or positive HIV status. This issue becomes more significant when the drug is used on a large scale, generating an extra incentive to identify new and specific anti-HIV microbicidal compounds with unique modes of action. In addition, a recently completed comprehensive HIV prevention trial among African women known as VOICE (Vaginal and Oral Interventions to Control the Epidemic) involving tenofovir failed to provide protection against HIV, underscoring the need for additional HIV-prevention options that incentivize patient usage and adherence (270).

Recently, our laboratory discovered a synthetic small molecule – PD 404,182 (PD) – that possesses virucidal activity towards retroviruses, including HIV (156). PD is structurally and mechanistically distinct from existing HIV microbicides (270, 271, 275) and inhibits a broad range of primary isolates of HIV and SIV at submicromolar to micromolar concentrations with minimal cytotoxicity to human cells ($CC_{50}/IC_{50} > 300$). Previously, we found that PD **1**) is effective against a broad range of primary HIV-1 isolates as well as HIV-2 (IC_{50} ~1 µM) in TZM-bl cells; **2**) is fully active in cervical fluids; **3**) exhibits low toxicity in 7 different human cell lines, including cervical cancer cells ($CC_{50} > 300$ µM); **4**) is effective against both cell-free and cell-associated virus and inhibits the transmission of dendritic cell-associated HIV to T cells; **5**) retains antiviral potency in cell culture for at least 8 hours prior to the addition of HIV to the cells; and **6**) exhibits rapid antiviral action – HIV becomes >99% inactivated after a 5 min incubation with PD. In this study, we further evaluate the potential of PD as an anti-HIV

microbicide and show that PD **7**) is stable and effective at both acidic and neutral pH for at least 48 h, **8**) remains fully active in the presence of seminal plasma and after incubation in cervical fluids for at least 24 hours; **9**) retains full potency when stored in PBS under acidic pH at 42°C for at least 8 weeks; **10**) can be formulated in hydroxyethyl cellulose (HEC) gel; **11**) is non-toxic to the vaginal commensal bacteria *Lactobacilli* (CC_{50} > 300 µM) and freshly activated PBMC (CC_{50} > 200 µM), **12**) is active in PBMCs against HIV-1 clinical isolates representing different viral subtypes and tropisms (average IC_{50} = 0.55 µM); and **13**) does not foster the emergence of resistant variants when HIV-1 positive TZM-bl cells are passaged at sub-inhibitory concentrations of PD in cell culture for 60 days.

Finally, we show that PD effectively inactivates human herpes simplex virus type 1 (HSV-1) and HSV-2 at submicromolar concentrations (200 nM). In the USA alone, 16.2% of the population is estimated to be infected with HSV-2 (276). Infection with HSV-1 or -2 is an important risk factor for susceptibility to HIV-1 transmission *in vitro* (252). These new findings further underscore PD as a promising next-generation HIV microbicide. In addition, PD is a small molecule that can be easily synthesized (277) and thus can potentially be manufactured at low cost on a large scale for use in developing countries.

5.3 Materials and methods

5.3.1 Cells, media and reagents

PD 404,182 was purchased from Sigma Aldrich (St. Louis, MO). PD was dissolved in DMSO to a final concentration of 30-40 mM, aliquoted and stored at -20°C.

Dulbecco's Phosphate-Buffered Saline (DPBS) and Penicillin-Streptomycin (pen-strep) were purchased from Thermo Scientific HyClone (Logan, UT) and Lonza (Walkersville. MD), respectively. Unless otherwise stated, the complete growth media for all cell culture work was DMEM containing 4500 mg/l glucose, 4.0 mM L-Glutamine, and 110 mg/l sodium pyruvate (Thermo Scientific HyClone, Logan, UT) supplemented with 10 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1X non-essential amino acids (Thermo Scientific HyClone, Logan, UT). 293T cells were from Life Technologies (Grand Island, NY). Vero cells were obtained from ATCC (Manassas, VA). The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID , NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. ; HIV-1 isolates 92RW016, 92RW021, 92TH006, 92TH026, 93TH053, 93BR020, 93BR021, 93BR029, 98IN017, 98IN022, 92UG001, 92UG005, 92UG024, 92UG029, from The UNAIDS Network for HIV Isolation and Characterization (278); HIV-1 92HT599 from Dr. Neal Halsey; HIV-1 96USNG31 from Drs. D. Ellenberger, P. Sullivan, and R.B. Lal (279); HIV-1 RU132 from Dr. A. Bobkov and Dr. Jonathon Weber; HIV-1 93IN101 from Dr. Robert Bollinger; and HIV-1Jv1083 from Dr. Alash'le Abimiku (280). All primary HIV isolates were amplified in activated human PBMCs. NL4.3 HIV was obtained from NIH AIDS Research and Reference Program. HSV-1 (Syn 17) and HSV-2 (333) were obtained from Prof. Theo Geijtenbeek (281) and amplified and titered in Vero cells.

5.3.2 Lentiviral pseudoparticle production

Pseudotyped lentiviruses were produced by co-transfecting 293T cells with plasmids carrying HIV gag-pol (39), a provirus (pV1-B1 (156), pTRIP-Gluc (148) or NL4-3.Luc (AIDS Reagent Program)), and vesicular stomatitis virus glycoprotein (VSV-G) (39). TransIT reagent (Mirus, Madison, WI) was used to perform the transfection following the manufacturer's protocol. The supernatants containing the pseudoparticles were collected 48 h post transfection, filtered (0.45 μm pore size) and stored at -80°C until use.

5.3.3 PD stability

PD was diluted in buffered DPBS (pH 4, 6, 8, 10) or cervical fluids (pool of 3 donors, 5-fold diluted in DPBS, Lee Biosolutions, St Louis, MO) to achieve a final concentration of 30 μM. DPBS was buffered to the desired pH using hydrochloric acid or sodium hydroxide. Diluted drug was incubated at the desired temperature for 0, 8, 24 or 48 h. After the temperature incubation, the drug mixture was further diluted to 1, 0.1 and 0.05 μM in complete growth media and used to incubate with VSV-G lentiviral pseudoparticles (VSV-Gpp, harboring either pTRIP-Gluc or NL4-3.Luc viral supernatant diluted 500-fold in complete growth medium) at 37°C for 30 minutes. Huh-7.5 (2×10^4 cells/well) or 293T cells seeded 24 h earlier were inoculated with the PD-treated virus at 4°C for 2 h, thoroughly washed to remove unbound viruses and drug, replenished with complete growth media containing 1x pen-strep and returned to 37°C and 5% CO_2. Viral infectivity was quantified 48 hours later by measuring supernatant

Gluc levels using the BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA) or Firefly Luciferase assay kit.

To study the long-term stability of PD, the compound was diluted to 5 µM in DPBS buffered at pH 4 and 7 using acetic acid (0.1%) and HEPES (2.5 mM), respectively, aliquoted and incubated at 4°C, room temperature or 42°C. Each week an aliquot was removed and tested for antiviral activity as previously described above. Similar experiments were conducted with PD (5 µM) or vehicle control (0.02 % DMSO) diluted in DPBS (adjusted to pH 4) containing 1.5% HEC**.**

5.3.4 PD stability in seminal plasma

TZM-bl cells (10^5 cells/well) were seeded in a flat-bottom 96-well plate. The next day, PD dilutions were prepared at a 2X concentration in seminal plasma (pool of 10 donors, 2-fold diluted in DMEM) and 100 µl of the 2X-concentrated mixtures were added to wells. Fifty microliters of a predetermined dilution of HIV stock (X4 NL4.3, 1 ng of p24) was placed in each test well. The cultures were incubated at 37°C and 5% CO_2 for 4 hours, washed with complete growth medium to remove unbound viruses and compound, replaced with fresh growth medium, and returned to the incubator. Infection was scored 48 h later by β-galactosidase activity.

5.3.5 Anti-HIV efficacy evaluation in fresh human PBMC

Testing of PD against HIV-1 in PBMCs was performed at Southern Research Institute as described previously (282, 283). Briefly, PHA-stimulated cells from at least two normal donors were mixed together, diluted in fresh medium to a final concentration of 1 x 10^6 cells/ml, and plated in a 96 well round bottom microplate at 50 µl/well (5 x

10^4 cells/well). Test drug dilutions were prepared at a 2X concentration in microtiter tubes and 100 µl of the 2X-concentrated mixtures were added to wells. Fifty microliters of a predetermined dilution of virus stock was placed in each test well (final MOI ~ 0.1). Separate plates were prepared identically without virus for drug cytotoxicity studies. The PBMC cultures were maintained for seven days following infection at 37°C, 5% CO_2. After this period, cell-free supernatant samples were collected for analysis of reverse transcriptase activity (284), and compound cytotoxicity was measured by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; CellTiter 96 Reagent, Promega) to the separate cytotoxicity plates for determination of cell viability. Wells were also examined microscopically and any abnormalities were noted.

5.3.6 Lactobacillus toxicity testing

Reference strains of *L. crispatus and L. jensenii* were obtained from the ATCC (Manassas, VA) and cultured on Columbia blood agar plates at 35°C in air enriched with 6% CO_2. Bacterial suspensions were prepared in saline or ACES buffer (285) to a density of 2 McFarland units (~ $2x10^8$ bacteria/ml), and exposed to PD (300 µM) or DMSO (10%) for 30 minutes at 37°C. After incubation, the cells were serially diluted in ACES buffer, pH 7.0 (Sigma-Aldrich, St. Louis, MO) and plated on blood agar plates to quantify colony forming unit per ml (CFU/ml) (285).

5.3.7 Primary cells toxicity testing

Primary cells were seeded at 6 x 10^3 cells/well in flat bottom 96-well plates in triplicates in the presence of increasing concentrations of PD. We used the

permeabilization agent saponin (0.1%) as positive control. After 0, 7 and 14 days, the amounts of lactate dehydrogenase (LDH) in the cell culture media was quantified using the LDH Cytotoxicity assay kit (Cayman Chemical, Ann Arbor, Michigan).

Cell death can occur either by apoptosis or by necrosis. Necrosis is accompanied by mitochondrial swelling and increased plasma membrane permeability, whereas apoptosis involves an articulated breakdown of the cell into membrane-bound apoptotic bodies. Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors. Cayman's LDH Cytotoxicity assay kit measures LDH activity present in the culture medium using a coupled two-step reaction. In the first step, LDH catalyzes the reduction of NAD+ to NADH and H+ by oxidation of lactate to pyruvate. In the second step of the reaction, diaphorase uses the newly-formed NADH and H+ to catalyze the reduction of a tetrazolium salt (INT) to highly-colored formazan which absorbs strongly at 490-520 nm.

5.3.8 HSV infection assays

Vero cells (2 x 10^5 cells/well) were seeded in a 24 well plate. The next day, these cells were infected with increasing titers (MOI range 0.0001 – 1) of HSV-1 (Syn 17) or HSV-2 (333) in the presence of PD (2 μM and 200 nM) or control DMSO (0.01%) prepared in DMEM in the absence of serum. Two days post-inoculation, cells were harvested, fixed with 5% paraformaldehyde (PFA) in PBS, stained with antibodies

against HSV glycoprotein gD (Novus biological, Littleton, CO), and analyzed by flow cytometry (281).

5.3.9 HSV sedimentation assay

HSV was concentrated by loading 30 ml HSV-1-infected Vero cell supernatant on a 20% sucrose cushion and centrifuged in a SW28 rotor at 20,000 rpm for 1 h at 4 °C. Pelleted viruses (~20 µg/ml) were resuspended in 1 ml PBS, exposed to PD (200 nM) or DMSO (0.01%) for 30 min at 37 ˚C, and immediately loaded over a 20–70% sucrose density gradient (11 ml). After ultracentrifugation at 30,000 rpm for 24 h in a SW-41 T rotor at 4 °C, fractions of 1 ml were collected and analyzed for HSV gpB content by enzyme-linked immunosorbent assay (ELISA) using homemade rabbit polyclonal antibody. The density of each fraction from the sucrose gradient was determined by measuring the refractive index.

5.3.10 Drug resistance study

HIV-1 (NL4.3, 1 ng of p24, corresponding to approximately 1,000-5,000 infectious units) was added to TZM-bl cells (1x 10^6 cells). Fifteen minutes later, an aliquot of supernatant (50 µl) was collected for viral input normalization, and PD was added to the cells at 1, 5, or 10 µM. TZM-bl cells were split every two days for a period of 60 days. Fresh PD was added at each passage to maintain the same concentration throughout the 60 days. Before each passage an aliquot of supernatant (50 µl) was collected to determine amount of virus in cell culture via p24 ELISA (Perkin Elmer Life Sciences).

5.3.11 Statistical analysis

Statistical significance between different samples was evaluated using Student's *t*-test in Microsoft Excel. A *P* value of 0.05 was considered statistically significant.

5.4 Results

5.4.1 Efficacy of PD in seminal plasma

Previously, we showed that PD effectively inhibits several isolates of HIV-1 and SIV in TZM-bl cells at submicromolar to low micromolar concentrations (IC_{50} ~1μM) when diluted in DMEM or cervical fluid (156). It has been shown that seminal plasma can enhance HIV infectivity (286, 287) and protect HIV against the action of microbicides (124, 288, 289). We therefore sought to test the antiviral activity of PD in seminal fluids. Briefly, CD4+ CCR5+ HeLa cells (TZM-bl cells (250, 290-293)) that produce β-galactosidase in response to HIV infection were exposed to 14 different clinical and laboratory isolates of HIV-1, representing various subtypes that use either coreceptor CCR5 (R5 viruses) or CXCR4 (X4 viruses), in the presence of PD or DMSO prepared in 50% seminal plasma. After a 4 h incubation of the virus and compound with the cells, cells were washed and the infection was scored 48 h later by β-galactosidase activity. The IC_{50} and IC_{90} of PD against the tested subtypes of HIV-1 range from 0.42-1.96 μM and 1.58-7.19 μM, respectively (Table 5.1).

Table 5. 1 PD's anti-HIV potency in seminal plasma.

HIV Isolate	Clade	Coreceptor usage	IC_{50} (μM)	IC_{90} (μM)
92RW021	A	R5	0.58 +/- 0.04	4.62 +/- 0.32
92UG029	A	X4	1.33 +/- 0.02	4.71 +/- 0.26
92TH026	B	R5	0.43 +/- 0.02	2.95 +/- 0.18
92HT599	B	X4	1.93 +/- 0.03	6.31 +/- 0.52
93IN101	C	R5	1.76 +/- 0.02	5.36 +/- 0.37
98IN017	C	X4	0.45 +/- 0.05	2.09 +/- 0.19
92UG005	D	R5	1.32 +/- 0.02	5.57 +/- 0.44
92UG024	D	X4	0.42 +/- 0.03	1.58 +/- 0.20
92TH006	E	R5	1.96 +/- 0.01	6.73 +/- 0.51
93TH053	E	X4	1.67 +/- 0.02	5.56 +/- 0.48
93BR029	F	R5	0.85 +/- 0.01	3.72 +/- 0.26
93BR020	F	X4	1.61 +/- 0.02	7.19 +/- 0.62
RU132	G	R5	0.74 +/- 0.01	3.28 +/- 0.22
Jv1083	G	R5	1.27 +/- 0.01	4.22 +/- 0.39

TZM-bl cells (1×10^5 cells/ml) were exposed to the indicated HIV isolates (1 ng of p24) in the presence of PD or DMSO diluted in 50% seminal plasma. Cells were washed 4 h post inoculation and fresh growth media was added. Infection was scored 48 h later by β-galactosidase activity. Errors represent the SD of 2 independent experiments carried out in duplicate. Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

These values are consistent with those of PD's anti-HIV activity determined in DMEM (0.33-1.80 μM for IC_{50} and 1.4-6.6 μM for IC_{90}) and in cervical fluid (0.61-2.30 μM for IC_{50} and 1.80-7.50 μM for IC_{90}) (156), indicating that seminal plasma does not negatively impact PD's anti-HIV activity.

5.4.2 Efficacy and toxicity of PD evaluated using primary cells

We previously evaluated the cytotoxicity of PD on 7 different human cell lines, including human cervical cells TZM-bl (HeLa) (156). In all cases PD showed minimal cytotoxicity (CC_{50} > 300 μM), giving a therapeutic index (CC_{50}/IC_{50}) of >300 for HIV-1. In the current study, freshly activated human PBMCs pooled from multiple donors were infected with 8 HIV-1 clinical isolates representing different viral subtypes and tropisms in the presence of different concentrations of PD. The supernatant reverse transcriptase activity was determined 7 days later and used as an indication of HIV infection. The toxicity of PD was determined under identical conditions in the absence of HIV infection and replication. As shown in Table 5.2, PD exhibited antiviral activity towards all the viral isolates tested, with an average IC_{50} of 0.55 μM (ranging from 0.14 μM with HIV-1 96USNG31 to 1.18 μM with HIV-1 92UG029). A 48% reduction in cell viability was observed at the highest tested PD concentration (200 μM), resulting in a CC_{50} of ~200 μM, indicating that PD is relatively non-toxic to freshly activated human PBMC. The therapeutic index of PD ranges between 170 (for HIV-1 92USNG31) and 1,015 (for HIV-1 RU132). To evaluate the toxicity of PD against other primary cells, increasing concentrations of PD were incubated with CD4+ T-lymphocytes, macrophages and dendritic cells for up to 14 days.

Table 5. 2 Toxicity and potency of PD in PBMCs.

HIV Isolate	Clade	Coreceptor usage	IC_{50} (µM)	IC_{90} (µM)	CC_{50} (µM)	Therapeutic index
92RW016	A	R5	0.22	0.54	~ 200	916
92UG029	A	X4	1.18	4.67		170
92HT599	B	X4	0.55	1.92		364
93BR021	B	R5	0.6	4.26		334
96USNG31	C	X4/R5	0.14	1.62		1,425
98IN022	C	R5	0.4	1.48		506
92UG001	D	X4/R5	1.11	1.89		181
RU132	G	R5	0.2	0.53		1,015

Freshly activated PBMCs (5×10^4 cells/well) were infected with HIV isolates (MOI = 0.1) in the presence of different concentrations of PD. Seven days after infection, supernatants were collected and analyzed for reverse transcriptase activity. Compound toxicity was determined using a MTS assay in the parallel uninfected plates. Error bars represent standard deviation of triplicate samples from one experiment. Data generated at Southern Research Institute by Marie K. Mankowski and Roger G. Ptak.

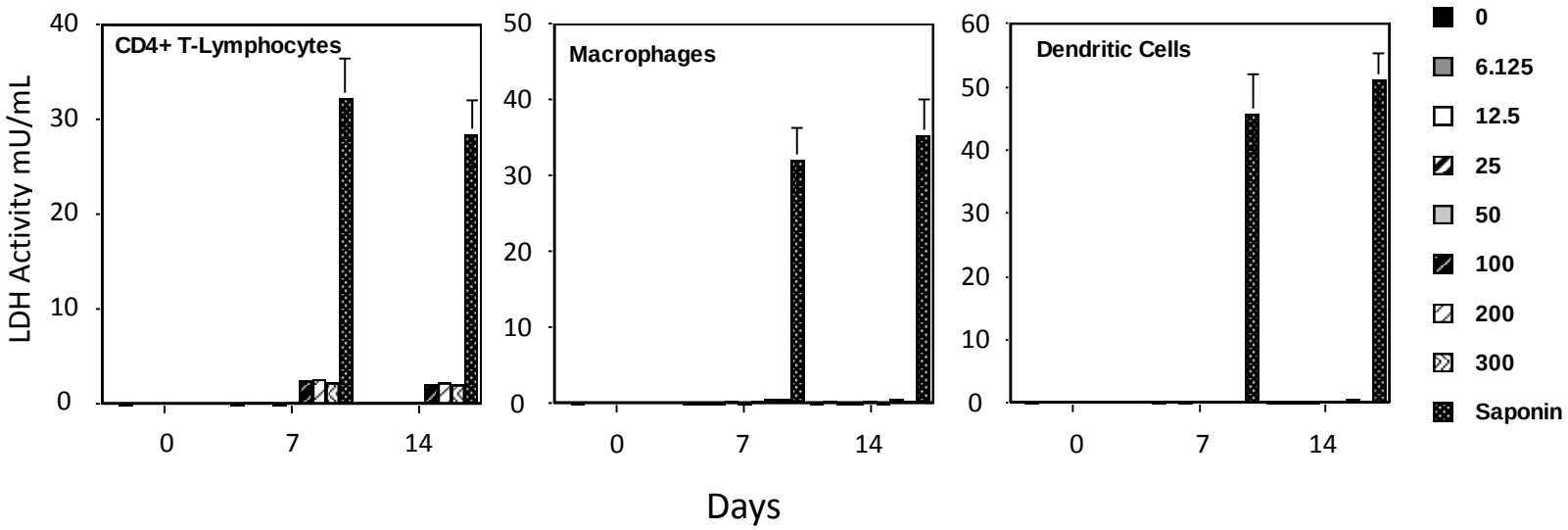

Figure 5. 1 PD exhibits minimum toxicity against primary human cells. Increasing concentrations were incubated with primary CD4+ T-lymphocytes, macrophages and dendritic cells at 37 °C and the amounts of lactate dehydrogenase (LDH) present in the culture media were quantified after 0, 7 and 14 days. Error bars represent the standard deviation from two independent experiments. Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

Table 5. 3 Toxicity of PD towards commensal bacteria *Lactobacillus*.

Bacterial Strain	Control (CFUx10^5/ml)	PD (CFU x10^5/ml)	Log(Control)-Log(PD)
Lactobacillus crispatus ATCC 33197	75.8 ± 0.19	72.6 ± 0.55	0.019
Lactobacillus jensenii ATCC 25258	99.8 ± 5.72	75.3 ± 16.8	0.122
Lactobacillus jensenii LBP 28Ab	105 ± 7.2	100 ± 0.124	0.021

Triplicate bacterial suspensions (~2x10^8 bacteria/ml) were separately exposed to PD (300 µM) or 10% DMSO for 30 minutes at 37°C. After incubation, each suspension was serially diluted and plated on blood agar plates to quantify colony forming unit per ml (CFU/ml). Errors represent standard deviations of triplicate samples. Data generated at University of Pittsburg by Dr. Bernard Moncla.

As shown in Figure 5.1, minimum toxicity was observed in all three types of primary cells (CC_{50} > 300 µM), further pointing to the extremely low cytotoxicity of PD.

5.4.3 Toxicity of PD to normal vaginal flora Lactobacillus.

Vaginal microflora is a key component of the innate immune environment and plays an important role in reducing the risk of HIV infection (294-297). The dominant bacterial species in healthy woman is *Lactobacillus* which produces lactic acid, hydrogen peroxide, bacteriocins and other antimicrobial substances that inhibit the growth of pathogenic organisms in the vagina (294-298). PD was evaluated for toxicity towards three strains of *Lactobacillus* normally found in the vagina. These strains were incubated with 300 µM PD or solvent DMSO (10%) at 37°C for 30 min and plated on blood agar plates to quantify colony forming units per ml (CFU/ml). Less than 1 log difference between the control and test CFU is considered non-toxic (285). As shown in Table 5.3, no growth inhibition was observed in all strains of bacteria after incubation with PD concentrated at 300 µM, indicating that PD is non-toxic to commensal *Lactobacillus* species.

5.4.4 PD short-term stability

We sought to determine the short-term stability of PD under conditions the compound is likely to encounter if used as a microbicide. The environment of the vagina is highly acidic (pH 3.5-4.9) due to the lactic acid produced by the commensal bacteria *Lactobacillus* (299). Exposure to seminal fluid (pH 7.2-8) (300) can raise the vaginal pH to 5.8-7.2 for several hours (301). Thus, we determined the stability of PD under different pHs at 37°C (Figure 5.2A).

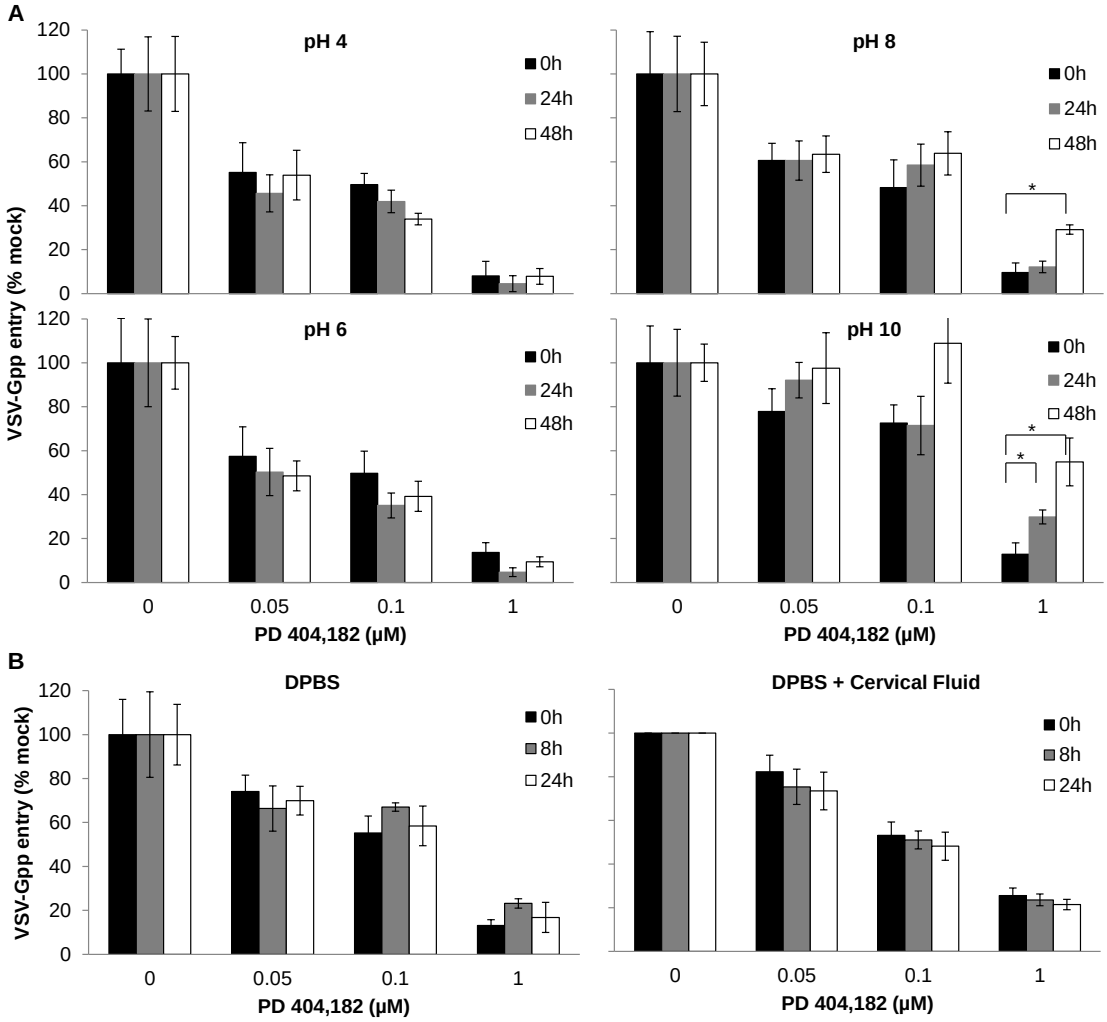

Figure 5. 2 PD is stable and fully active at acidic pH and in cervical fluid. PD (30 μM) or DMSO (10 %) was incubated at 37°C for 0, 8, 24 or 48 h in **(A)** DPBS buffered at pH 4, 6, 8 or 10, or **(B)** 20% cervical fluid (diluent was DPBS). These PD samples were then diluted to the desired concentration in complete growth medium containing VSV-Gpp (viral supernatant diluted 500-fold), and the PD/virus mixtures were incubated at 37°C for 30 min and used to inoculate naïve Huh-7.5 or 293T cells at 4°C for 2 h prior to incubation at 37°C/5% CO_2. The infectivity (virus entry into cells) was quantified by measuring the supernatant luciferase reporter activity 48 h post infection. Values and error bars represent the mean and standard deviation, respectively, of three independent experiments. Statistical significance was determined by Student's *t* test (*, $p < 0.01$). Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

PD is highly stable in acidic buffer at pH 4 or 6 at 37°C. Since PD targets a non-envelop protein HIV-1 structural component (156), we used HIV-1 pseudotyped with VSV-G (VSV-Gpp) for these studies because this virus is easy to generate in high titers and can be handled in a BSL-2 environment. Basic pHs of pH 8 or 10 were observed to compromise PD's activity, but only after extended exposure. For example, PD lost ~50% antiviral activity after incubation in pH 10 buffer for 48 h and lost ~20% activity when exposed to pH 8 for 48 h at 37°C. In contrast, no activity loss was observed for PD after 24 h exposure to pH 8 buffer and minimal (~20%) activity loss was seen after 24 h exposure to pH 10. Taken together, these results indicate that PD will likely be stable in highly acidic cervical fluid and should remain active for at least several hours upon contact with seminal fluid.

Since cervical fluid is a complex mixture, we next determined the stability of PD in cervical fluid. As shown in Figure 5.2B, no activity loss was observed after PD was incubated in 20% cervical fluid for 24 h at 37°C. These results indicate that once-a-day application of PD should be adequate to provide protection against HIV infection. The ability of PD to retain its antiviral potency at near-neutral pH suggests that PD may also be formulated as a rectal gel.

5.4.5 PD long-term stability

To evaluate long-term stability, PD was diluted in DPBS buffered at pH 4 or 7 and incubated at 4°C, room temperature or 42°C. An aliquot was taken every week for determination of antiviral activity. As shown in Figure 5.3A, PD is extremely stable when stored in pH 4 buffer and retains full antiviral potency even after 8 weeks at 42°C.

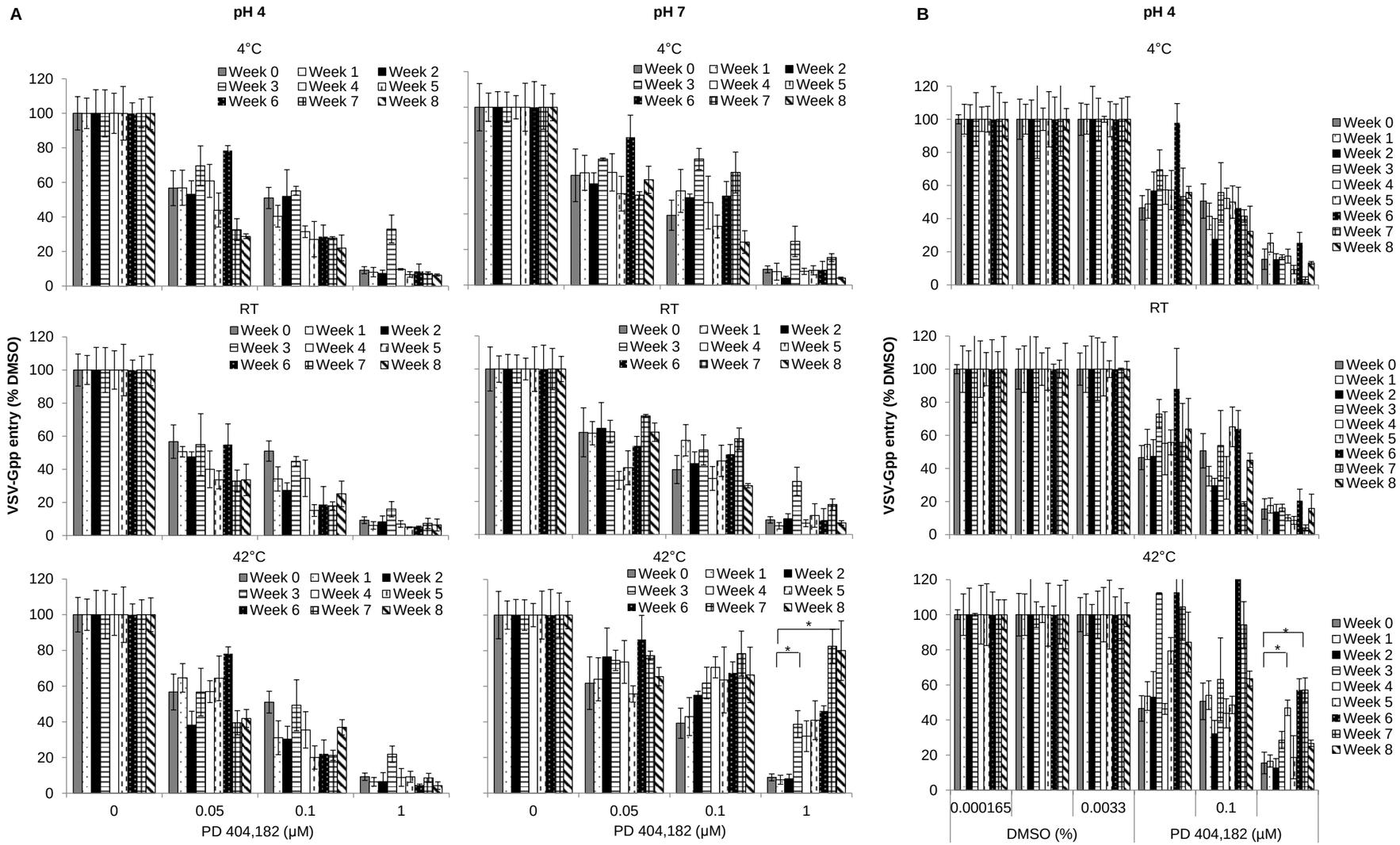

Figure 5. 3 Long-term stability of PD. PD (5 μM) or DMSO (1.67 %) were diluted in (A) pH-adjusted DPBS (pH 4 or 7) or (B) 1.5% HEC in DPBS (pH 4) and stored at the indicated temperature for 8 weeks. An aliquot was removed each week, diluted to the desired concentration in complete growth media containing VSV-Gpp (500-fold diluted), incubated at 37 °C for 30 min, and used to infect naïve Huh-7.5 cells at 4°C for 2 h prior to incubation at 37°C/5% CO_2. The viral infectivity was quantified by measuring the supernatant Gluc reporter activity 48 h post infection. Error bars represent the standard deviation of duplicate samples. Statistical significance was determined by Student's t-test (*, $p < 0.05$).

At pH 7, PD is stable only at room temperature and 4°C. Storage at 42°C and pH 7 significantly compromised PD activity after 2 weeks.

We also determined the stability of PD formulated in HEC gel at pH 4, as PD is not stable in the presence of HEC gel at pH 7 (data not shown). PD retains full potency at pH 4 in 1.5% HEC gel at 4°C and room temperature for at least 4 weeks. However, PD is not stable under same buffer conditions if stored at 42°C (Figure 5.3B) for more than 2 weeks, despite its stability in pH 4 DPBS buffer. Work is currently underway to determine other suitable formulation conditions for PD storage at high temperatures.

5.4.6 PD inactivates herpes simplex virus (HSV)-1 and -2

We investigated the ability of PD to inactivate HSV-1 and -2. Vero cells were infected with HSV-1 and HSV-2 in the presence of PD (2 μM and 0.2 μM) or DMSO (0.01%). These concentrations were selected based on their closeness to the *in vitro* IC_{50} of PD against HIV-1. Infection was quantified by the cell surface expression of HSV gD. PD was found to inhibit both HSV-1 and HSV-2 infection at low to intermediate MOI (MOI from 0.0001 to 0.1) and exhibited partial protection at MOI 1 (Figure 5.4A). Similar results were obtained for both concentrations and the data from the lowest concentration is presented.

Previously, we showed that PD compromises the integrity of retroviruses (156). We thus asked whether PD can disrupt the structure of HSV virions. Purified HSV-1 virions were resuspended in PBS and incubated with PD (0.2 μM) or DMSO (0.01%) for 30 min at 37°C.

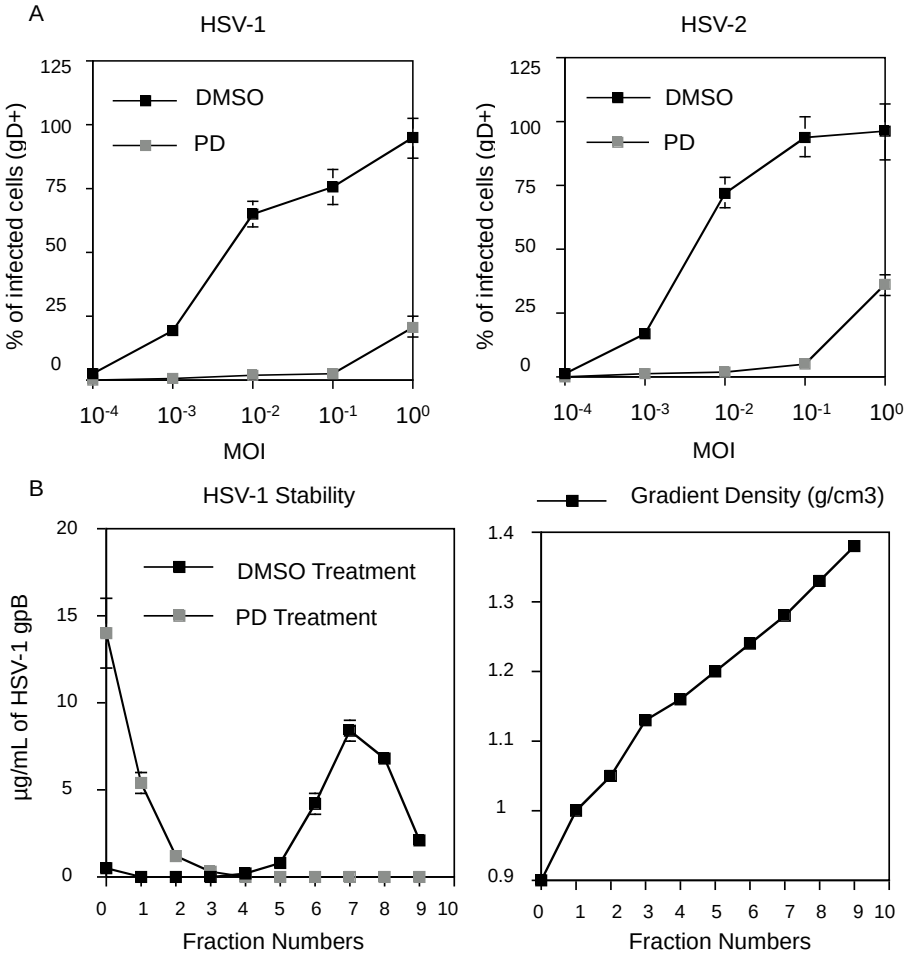

Figure 5. 4 PD is effective against HSV. (A) PD blocks HSV infection. Vero cells were seeded in a 24-well plate and infected with HSV-1 (Syn 17) or HSV-2 (333). PD (200 nM) or control DMSO (0.01 %) was added immediately to the target cells after viral inoculation. Two days later, the cells were harvested, stained with antibody against HSV glycoprotein gD and analyzed by flow cytometry. Error bars represent standard deviation of triplicate samples. Results are representative of two independent experiments. **(B)** PD Destabilizes HSV particles. Concentrated HSV (~20 µg/ml) was incubated with DMSO (0.01 %) or PD (200 nM) for 30 min at 37˚C and the mixture was immediately loaded over a 20–70% sucrose density gradient. The amount of HSV glycoprotein gpB in each fraction was analyzed by ELISA. The sucrose gradient density of each fraction was determined by measuring the refractive index. Results are representative of 2 independent experiments. Error bars represent the standard deviation of triplicate samples. Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

After the incubation, the mixture was immediately loaded over a 20-70% sucrose density gradient and centrifuged at 30,000 rpm in a SW41 T rotor for 24 hours. Each gradient fraction was analyzed for HSV glycoprotein gpB by ELISA. DMSO-treated HSV-1 sediments at a density of 1.24 g/cm^3. However, with PD-exposed HSV, all gpB distributed to the top of the gradient (Figure 5.4B). This result indicates that, like with HIV-1, PD inactivates HSV by compromising virion structural integrity.

5.4.7 HIV-1 does not acquire resistance to PD after 60 days

With the high rate of HIV mutation, an ideal microbicide should have a high threshold for viral resistance development. To gauge the ability of HIV-1 to acquire resistance to PD, HIV-1-positive TZM-bl cells were passaged in the presence of 1, 5 and 10 μM PD for 60 days. No PD-resistant variants could be detected in the course of this experiment (Figure 5.5). The inability of HIV-1 to escape PD inactivation further underscores the potential of PD as an HIV-1 microbicide. We chose TZM-bl cells for the resistance study because these cells can be passaged for an extended period and remain viable for months even in the presence of viral replication. A similar experiment was performed using freshly activated human PBMCs (2 donors) and no emergence of viral resistance was observed (data no shown). However, HIV-1 infected PBMCs were cultured for only 12 days because significant cell death was observed after this period. We also cultured HSV-1 infected Vero cells in the presence of sub-inhibitory concentrations of PD for 2 weeks and were not able to detect any viral capsid by ELISA in the supernatant (data not shown).

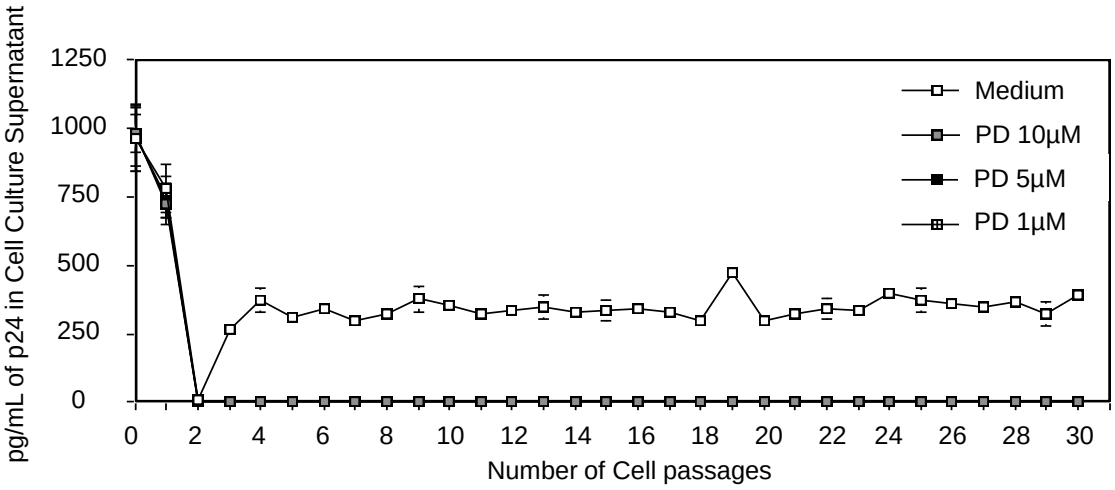

Figure 5. 5 PD does not foster the emergence of escape mutants. HIV-1 (1 ng of p24 of NL4.3) was added to TZM-bl cells (1 x 10^6 cells). Fifteen minutes later, an aliquot of supernatant was collected for viral input normalization, and PD was added to cells at the indicated concentrations. Cells were then split every two days for a period of 60 days. Fresh PD was added at each passage to maintain the same concentration throughout the 60 days. Before each passage an aliquot of supernatant was collected to determine amounts of virus in cell culture via p24 ELISA. Error bars represent standard deviations of triplicate samples. Results are representative of three independent experiments. Data generated at Scripps Research Institute by Michael Bobardt and Dr. Phillipe Gallay.

5.5 Discussion

Four major types of vaginal HIV microbicides have been developed with varying degrees of clinical success: surfactants, entry inhibitors, vaginal milieu protectors and reverse transcriptase inhibitors (270). Surfactants non-specifically disrupt membranes and were the first molecules to enter clinical trials as candidate HIV microbicides. However, these surfactants were found to be toxic to the cervicovaginal mucosa and resulted in an increased rate of HIV infection in Phase III clinical trials (302, 303). Entry inhibitors prevent HIV from binding to or entering cells and encompass a wide range of molecules, including CCR5 inhibitors (304-306) and fusion inhibitors (307, 308). Many polyanions have also been developed to inhibit HIV entry and some have been extensively tested in Phase III trials, including PRO 2000 (309), cellulose sulfate (112) and Carraguard (310). However, most of these candidates have failed to show *in vivo* efficacy in preventing HIV transmission, partly due to the complexity of the mucosal environment as well as the interference of semen (124, 311). Vaginal milieu protectors are designed to maintain or enhance the protective acidic pH of the vaginal environment through the use of strong buffering agents, such as Carbopol 974 (312), or genetically engineered *Lactobacilli* (313). An agent that is being considered for HIV microbicidal applications in clinical trials, tenofovir, is a nucleotide analogue that inhibits the reverse transcriptase of HIV (120). A 1% tenofovir gel applied before and after sexual intercourse was 39% effective overall in preventing HIV infection in women, and 54% effective among highly adherent users of the gel (117). These data are

encouraging, but nevertheless show that the efficacy of tenofovir microbicidal therapy alone is limited.

In contrast to existing anti-HIV microbicidal candidates, PD inactivates HIV via a unique, possibly novel mechanism. PD is the only non-surfactant small molecule reported to physically compromise the integrity of HIV, thus rendering the extracellular virus non-infectious (Tables 5.1 and 5.2). In addition, PD exhibits low toxicity toward several human cell lines (156), freshly activated PBMCs (Table 5.2), primary $CD4^+$ T-lymphocytes, macrophages and dendritic cells (Figure 5.1) and normal vaginal flora (Table 5.3). It should be noted, nevertheless, that we have yet to test the cytotoxicity of PD in conditions that mimic the high level of basal inflammation typically seen in developing countries. For example, it has been reported that increased levels of immune activation were observed in the genital tract of healthy young women from sub-Saharan Africa (314).

The antiviral potency of PD is not affected by the presence of seminal plasma (Table 5.1) or exposure to cervical fluid at 37°C for 24 hours (Figure 5.2B), indicating the potential for a once-a-day application of PD for HIV prophylaxis. The very high stability of PD in acidic pH at both room temperature and 42°C, and in neutral pH at room temperature (Figure 5.3A), indicate that PD can be easily formulated for convenient transportation and storage in developing countries lacking refrigeration facilities. In the current study, we evaluated the stability of PD when formulated in 1.5% HEC gel. Surprisingly, PD is not stable when formulated in HEC gel at pH 7 (data not shown), although PD formulated in HEC gel at pH 4 retains full potency after 4 weeks at

ambient temperature (Figure 5.3B). Studies are underway to investigate the reason for PD's instability in HEC gel at neutral pH. One hypothesis we are exploring is that PD reacts with the deprotonated hydroxyl group in HEC.

Genital herpes has been found to increase the vulnerability to HIV-1 infection by compromising the integrity of the mucosal barrier (315-317). Most genital herpes is caused by HSV-2 infection, although in some cases it can also be caused by HSV-1 (318-320). In one study, 50-90% of HIV-1 infected patients tested seropositive for HSV-2 (321) and HSV-2 infection was found to increase the rate of HIV-1 acquisition by 3-fold (319). Due to the synergy between HIV and HSV, a topical microbicide with dual action against both pathogens may more effectively reduce HIV transmission.

The ability of PD to inactivate HSV in addition to HIV is a big plus. Genital herpes is one of the most prevalent sexually transmitted diseases worldwide and is the common cause of genital ulcers (322). Ulcerations can disrupt the mucosal barrier and abrogate the protective barrier function of the epithelium, allowing HIV-1 to reach the sub-epithelial dendritic cells susceptible to HIV-1 infection (323) and increasing the risk of HIV acquisition (324). Currently, there is no approved vaccine for HSV and therapeutic treatment for genital herpes involves repeated dosing of antiviral drugs. Development of topical microbicides that are effective against both HIV and HSV may provide a more effective strategy to prevent HIV-1 infection/transmission. We showed that 0.2 μM PD physically compromises the integrity of extracellular HSV and effectively blocks the infection of both HSV-1 and -2 *in vitro*, in a manner similar to

PD's action on HIV-1 (Figure 5.4). It is tempting to speculate that the same target molecule(s) may be present on both viruses.

In summary, we demonstrated that the virucidal small molecule PD possesses several attributes that lend support to its use as a microbicide for combating HIV spread. These attributes include full activity and high stability in fluids and pHs encountered physiologically, lack of toxicity to freshly activated human PBMC and vaginal commensal bacteria, and activity towards another virus – HSV – that exacerbates the pathogenicity of HIV. Future studies will focus on (1) formulation of PD into a topical form that promotes high PD activity and stability, and (2) evaluation of the toxicity and efficacy of PD in animals and humans.

CHAPTER VI

CONCLUSIONS

Hepatitis C virus (HCV), Human Immunodeficiency virus (HIV) and Herpes Simplex virus (HSV) remain serious health problems lacking an effective cure. Although current therapies have played substantial roles in the fight against these pathogens, most antivirals are directed to virally encoded proteins fostering the development of resistant mutants overtime, and reducing the likelihood of viral eradication. Progress in defining the molecular mechanism of HCV, HIV and HSV entry raised the opportunity to identify entry inhibitors that target molecules indispensable for viral entry not encoded by the viral genome. Such inhibitors have the potential to prevent initial infection, limit the expansion of the infected cell reservoir, complement current antiviral therapies and in the case of HCV, reduce the extent of re-infection after liver transplantation. In line with this concept, we characterized three potential inhibitors of viral entry.

The first compounds evaluated belong to the phenothiazine family. Although phenothiazines are known for their use as antipsychotic drugs, antiviral activity has been reported for these compounds. Our studies shed light on the mechanism of action of phenothiazines as inhibitors of HCV entry and showed, for the first time, that alteration of target host cell membrane fluidity can inhibit HCV entry. Specifically phenothiazines were shown to suppress viral entry by intercalating into cholesterol rich domains of target cells and reducing viral-host fusion. Our results show that targeting an entry step independent of viral proteins may also be an effective way to retard the development of

drug resistance and inhibit HCV deletion mutants, found to reduce therapy effectiveness of chronic HCV patients.

The second candidates studied are two members of the H_1-anthistamines currently used in clinic for treatment of allergy-related symptoms. Both compounds were found to block a post attachment stage of HCV entry at a time point prior to viral fusion with the plasma membrane. The inhibition efficiency was dependent on virion and host cholesterol content similar to ezetimibe, an NPC1L1 antagonist. Most importantly, hydroxyzine can reach plasma levels higher than its IC_{90} in cell culture and is currently used to alleviate adverse dermatological effects experienced by HCV patients undergoing treatment with telaprevir, justifying further studies to evaluate this molecule for HCV therapy.

Lastly, we evaluated the antiviral activity of PD 404,182 (PD) as an alternate treatment for HCV-HIV coinfected patients as well as its potential use as an anti-HIV microbicide. PD displayed a unique mode of action by inactivating virions directly through the interaction with a structural molecule not encoded by the viral genome. For HIV and HSV, this interaction is later accompanied by irreversible disruption of the virion rendering the particle non-infectious. Moreover, PD displayed high stability in environments encountered by microbicides and no adverse effects against commensal bacteria or freshly activated PBMCs. Taken together, these attributes make PD a promising next-generation HIV microbicide.

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