Development of a Robust Virus-based Assay for Determination of 50% Effective Concentration and Analysis of Drug Resistance in the Hepatitis C Virus

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ABSTRACT

Hepatitis C virus affects 170 million people worldwide and until recently, therapeutic options have been limited. Novel and effective protease inhibitors (PI) became available in the clinic in 2011, and a plethora of new compounds and classes of compounds have been developed since. Due to the HCV polymerase lacking a proofreading mechanism, resistance to these compounds arises and decreases the success rate of treatment. Classically, drug resistance has been studied in the context of a system that does not recapitulate the entire HCV life cycle. With an established cell culture model of HCV infection in our lab we developed a novel assay for characterization of mutations conferring resistance to direct-acting antiviral compounds. We designed, improved and validated the assay with compounds stemming from different chemical classes including an NS3/4A inhibitor, NS5A inhibitors and NS5B inhibitors. Herein we present the development and implementation of the assay. With this assay in hand a more comprehensive understanding of HCV drug resistance can be realized and used as a tool for future drug development, both in HCV and in other related viruses.

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LIST OF ABBREVIATIONS AND DEFINITIONS

ΔGDD	Negative cell culture control with NS5B active site
25-HC	25- hydroxycholesterol
BSA	Bovine Serum Albumin
CD81	Cluster of differentiation 81
cDNA	Complementary deoxyribonucleic acid
cLD	Cytosolic lipid droplets
CLDN1	Claudin-1
CO_2	Carbon dioxide
DAAs	Direct-acting antivirals
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMRIE – C	1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide cholesterol liposome
DMSO	Dimethyl Sulfoxide
DMV	Double-membrane vesicle
DNA	Deoxyribonucleic acid
EC_{50}	50% Effective concentration

E1	Envelope protein 1
E2	Envelope protein 2
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
ffu	Focus-forming units
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human Immunodeficiency virus
Huh-7.5	Human hepatoma 7.5
IFNα	Interferon-α
IFNλ	Interferon-λ
IRES	Internal ribosome entry site
JFH1	Japanese fulminant hepatitis 1
$JFH1_T$	Japanese fulminant hepatitis 1 triple mutant
LD	Lipid droplet
LDL	Low-density lipoprotein
LVP	Lipoviral particle
mL	Milliliter
MOI	Multiplicity of infection
MW	Membranous web
NANBH	Non-A, non-B hepatitis
NPC1L1	Niemann-Pick C1-like 1
NS2-5B	Non-structural protein 2 through 5B (3, 4a, 4b, 5a)
UTR	Untranslated region
ORF	Open reading frame

PBS	Phosphate Buffered Saline
PEG-IFN	Pegylated interferon
PI	Protease Inhibitor
PTEN	Phosphatase and tensin homolog
QRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
RdRp	RNA-dependent, RNA-polymerase
RNA	Ribonucleic acid
SF	Serum free
SOC	Standard of care
SR-BI	Scavenger receptor B type-I
SVR	Sustained virological response
VLDL	Very low density lipoproteins

Chapter 1 Introduction

Overview

Estimates of the global disease burden of hepatitis C virus (HCV) vary widely, but comprehensively approximate the global infection to be between 64 and 185 million individuals[1–4]. The prevalence of infection varies greatly by country worldwide. In Canada the infection rate is estimated to be 0.8%[5], conversely in Egypt the prevalence is approximately 20% due to the negligence of sterility during parenteral antischistosomal therapy [6].

A blood-borne virus, HCV is commonly spread through injection drug use and associated risk behaviours in the modern world[7]. Preceding the advent of serological and nucleic acid testing[8] the primary route of viral transmission was blood and blood product transfusions (as reviewed in [9]).

Acute infection with HCV is often asymptomatic and difficult to diagnose, leading to a large proportion of those infected to develop chronic infection[10]. Chronic HCV infection leads to the development of liver fibrosis, cirrhosis and an increased risk of hepatocellular carcinoma (HCC). With all of the preceding hepatic sequelae it is no surprise that chronic HCV infection is quickly becoming the leading cause of liver-related deaths worldwide[11–13].

In recent years there has been a rapid evolution of pharmaceutical interventions available to treat HCV-infected individuals (As reviewed in [14]). In the era of these new treatment options, clearance of the virus, also known as a sustained virological response (SVR), is becoming more and more common and leading to a wide range of health, social and economic benefits[15].

Interestingly, companies have begun to subsidize HCV treatments in an effort to provide treatment globally[16]. However, the pressure of the HCV epidemic on the North American healthcare systems will not be relieved until the controversially expensive and seemingly monopolized treatment options become widely available and affordable, and active screening and social programs preventing novel infections are in place[17,18]

Discovery of the Virus

HCV was originally identified in 1975 as non-A non-B hepatitis (NANBH)[19,20]. Termed NANBH because some believed this form of hepatitis could be caused by more than a single infectious agent and should therefore not be referred to as the proposed "Type C"[21,22] until the etiological agent(s) responsible for this form of hepatitis was explicitly identified[20]. Soon after the speculation of a third form of hepatitis, the mode of transmission was investigated with both human samples and chimpanzee inoculations[23,24]; evidence suggested that the infection was likely passed from chronic carriers through blood sera.

The infectious agent was suspected to be a virus and was titrated in chimpanzees, a high titer strain identified as Strain H became the staple for future analyses[25]. A major structural determinant of a virus is the presence or absence of a lipid envelope, to determine if an enveloped virus was responsible for NANBH, Strain H preparations were extracted with a common lipid solvent[26]. Using a convincing series of well-controlled experiments in chimpanzees, a NANBH infectious agent was shown to be sensitive to detergents and thus containing a lipid component necessary for transmission of infection[26]. To further characterize

the suspected virus, aliquots of Strain H were passed through filters ranging in pore size from 100nm to 30nm[27], the infectivity of the filtrates reflected a virus approximately 30-60nm in diameter.

Despite valiant research efforts, the virus that caused NANBH remained a mystery until 1989 [28]. The discovery of the virus came in the form of an ingenious twist on a cloning protocol developed to isolate DNA encoding unknown proteins[29]. By modifying the protocol to identify clones created with antibodies from NANBH patients or chimpanzee sera it became possible to isolate and characterize the unknown viral genome. This seminal paper represented the identification of the virus as an enveloped, positive-stranded RNA virus which the authors speculated in the discussion belonged to the *Flaviviridae* family of viruses; HCV was explicitly identified.

Natural History of Infection

The progression of HCV infection is influenced by multiple host and viral factors and notably variable in the infected population (as reviewed in [30]). Interestingly, only a small minority of those infected with HCV will present with symptoms during the acute stage of infection; decreasing rates of acute stage diagnosis [31,32]. Of those infected approximately 15-25% will spontaneously clear the virus over a variable timeframe[33–35], the remainder will progress to chronic infection leading to extrahepatic manifestations, fibrosis, cirrhosis and possibly HCC[10–13,36,37]. Figure 1.1 is a flow chart representing the natural history of infections.

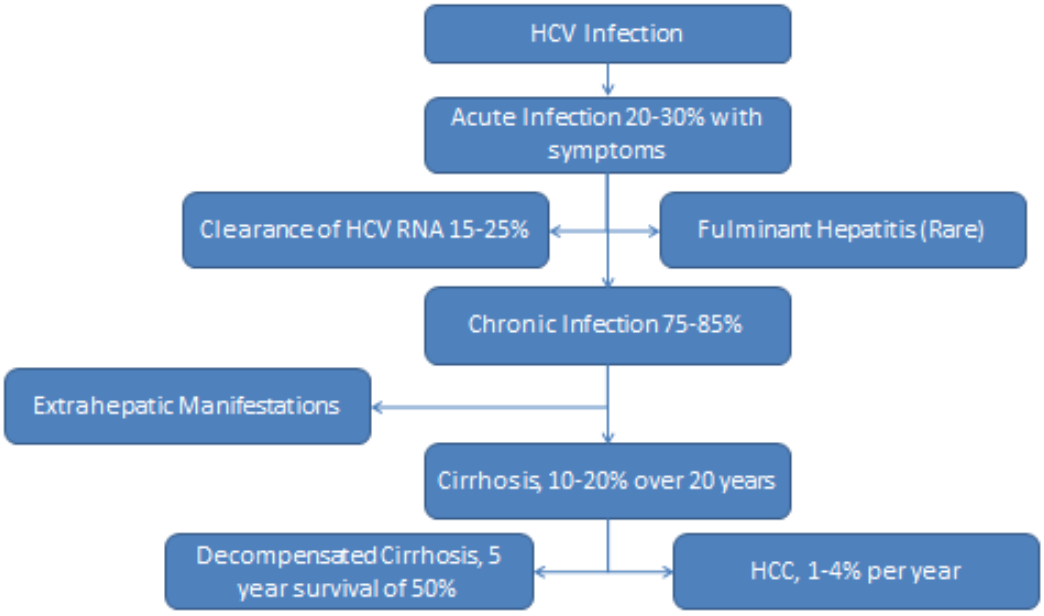


Figure 1.1 Flow Chart of Viral Infection and Related Outcomes (modified from [36]). The asymptomatic nature of acute infection leads to a great number of infections being diagnosed during chronic stages, leading to high rates of liver disease.

Viral Genome

HCV is a 9.6 kilobase positive-polarity RNA virus belonging to the family *Flaviviridae*[28] and until recently was only accompanied by GB-virus B in the *Hepacivirus* genus (GB-virus B reviewed in [38]). The novel viruses in the genus have been termed nonprimate hepaciviruses, directly reflecting their eclectic infectious origins in rodents[39,40], canines[41,42], horses[43], and bats[44].

HCV exists as seven primary genotypes (1-7) with each genotypic group containing several subtypes (a, b, c etc.)[45–47]. Interestingly, although patients are diagnosed with a specific genotype, HCV circulates within each individual as a dynamic series of divergent sequences due to the error-prone nature of the viral polymerase[48,49]; this is referred to as a quasispecies. Considering the estimated viral production rate of 10^{12} virions per day[50], and the estimated error rate of the polymerase, it has been mathematically determined that each mutation and combination of mutations can transiently exist in every patient every day[51].

HCV has a single open reading frame (ORF) between conserved 5'- and 3'-untranslated regions (UTRs) and initiates translation with an internal ribosomal entry site (IRES)[52,53] located within the 5' UTR to produce an approximately 3000 amino acid polypeptide. Interestingly, a liver specific microRNA, miR-122 has been shown to directly interact with the IRES and promote viral replication, further explaining the hepatotropic nature of HCV[54]. On the other end, the 3' UTR contains a variable region, a polyU-polyUC tract and a highly structured terminal region

referred to as the 3'X tail. A pair of mutational studies highlighted the importance of each region and concluded that the 3'X tail was of the utmost importance for replication[55,56].

The polypeptide produced from the IRES-mediated translation of the HCV ORF follows a common *Flaviviridae* organizational pattern with structural proteins preceding non-structural[28]. The polypeptide is processed by viral and cellular proteases to produce 10 proteins, in order from 5' to 3', Core, Envelope 1 (E1), E2, p7, Non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B[57]. Figure 1.2 depicts the viral genome organization.

Viral Life Cycle

Virion Structure, Receptor Binding, Entry and Fusion

The HCV virion consists of a nucleocapsid comprised of oligomerized core protein and a single copy of the RNA genome, the nucleocapsid is surrounded by a lipid envelope, viral envelope proteins E1 and E2, and host Low-Density Lipoproteins (LDL), as represented in Figure 1.3 (as reviewed in [58]).The inclusion of serum lipoproteins in the virion membrane leads to an unusually low buoyant density, and redefines the virion as a lipoviral particle (LVP)[59–61]. The presence of LDL as part of the virion is actually quite common in *Flaviviruses*, and promotes endocytosis through interaction with LDL receptors on target host cells[62]. Interestingly, imaging studies have revealed that the virion is pleiomorphic and does not closely resemble any other *Flaviviridae* virion structures[63].

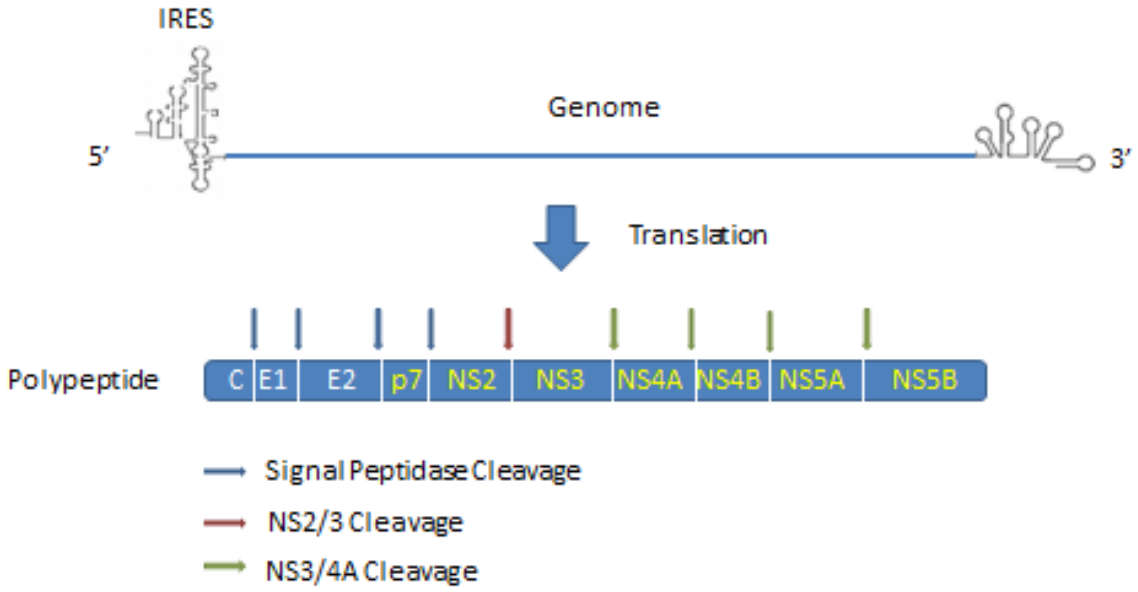


Figure 1.2 The Viral Genome, Polypeptide and Cleavage Sites (modified from[64]). HCV has a positive polarity RNA genome that is translated via an internal ribosomal entry site (IRES) in the 5' non-coding region. The resultant polypeptide is then cleaved by host and viral proteases as indicated by the coloured arrows. Of note, core is also processed by signal peptide peptidase. Structural proteins core (C), envelope 1 (E1) and envelope 2 (E2) are indicated with white font and non-structural proteins are indicated in yellow. For the purposes of this figure p7 was included as a non-structural protein but has truly yet to be designated as structural or non-structural.

The first host receptor identified to be necessary for HCV infection was Cluster of Differention 81 (CD81), a nearly ubiquitously expressed tetraspanin that was proposed to bind E2 at an initial entry step[65]. Soon after, it was discovered that the LDLs present on the LVP play a major role in promoting HCV entry through the LDL receptor, and glycosaminoglycan receptors act at a recognition step preceding infection[62,66]. Within a few years another candidate receptor was identified when E2 interactions leading to entry were shown to be cell specific. The human Scavenger Receptor Class B Type 1 (SR-BI) was suggested to act as a co-receptor bound by E2[67].

A tight junction protein, claudin-1 (CLDN1) was shown to also play a key role in mediating the late steps of entry, and could be used to make non-hepatic cell lines susceptible to HCV infection[68]. Interestingly, within two years another tight junction protein, namely, occludin, was identified and reiterated the importance of HCV entering through the tight junction[69]. Finally, a cholesterol uptake receptor, Niemann-Pick C1-like 1 (NPC1L1), was identified and suggested as a convenient antiviral target due to the availability of previously NPC1L1 targeted FDA-approved compounds[70]. All of these receptors play key roles in mitigating viral recognition and entry and the complete process is reviewed in [58] and represented in Figure 1.4.

HCV enters the cell via clathrin-dependent endocytosis following the binding of host receptors and requires a low-pH step within the early endosome to mediate complete entry and fusion of the virion with the endosome[71–74]. The exact mechanism of fusion remains poorly understood but modern work suggests that several of the host receptors identified play key roles in the conformational changes of the envelope glycoproteins in the acidic endosomal compartment

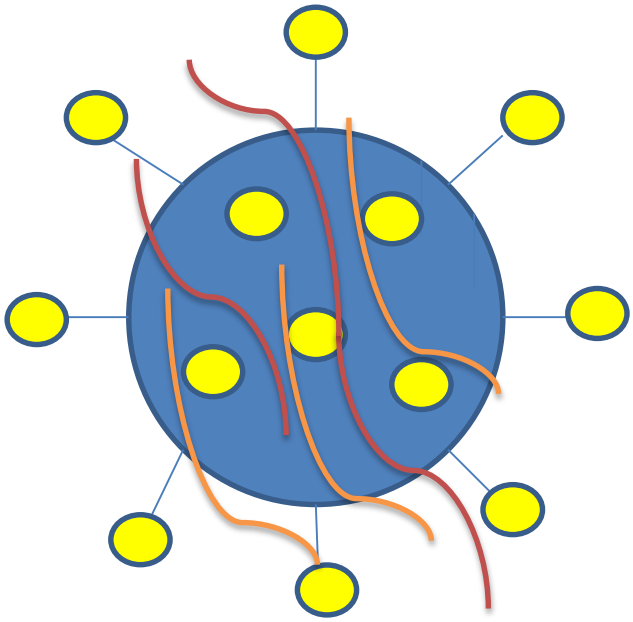


Figure 1.3 Structure of the Hepatitis C Virion. The hepatitis C virion is comprised of a nucleocapsid consisting of core protein, not depicted in this figure, a host lipid membrane depicted in blue, E1 and E2 heterodimers depicted in yellow and host lipoproteins depicted in red and orange. The structure of the virion is pleomorphic and does not closely resemble any other member of the *Flaviviridae* family.

leading to fusion and release of the nucleoscapsid[75]. E2 was primarily suggested to be a class II fusion protein responsible for the release of the nucleocapsid and the viral genome[76]. Conversely, recent data on pestiviral entry has led to the development of hypotheses that E1 is actually functioning as a novel form of fusogen[77,78]. Figure 1.4 represents virion entry into hepatocytes via cellular receptors, endocytosis and fusion.

Translation and Polyprotein Processing

Translation of the RNA genome is initiated through the action of the IRES at the 5' terminus[52,53]. Translation results in an approximately 3000 amino acid polyprotein that is co- and post-translationally cleaved by host and viral proteases[57,79]. The structural region of the polyprotein is cleaved by host proteases[80] notably associated with the endoplasmic reticulum (ER), the NS proteins are cleaved by the actions of NS2-NS3 to liberate themselves, and NS3/4A[81] acting on the remainder of the polyprotein cleavage sites[82]. Figure 1.2 depicts the proteases responsible for cleavage and their proteolytic target sites.

Replication

HCV has a unique approach to replication when compared to other viruses within the Flavivirus genus[83]. Replication of HCV takes place in multiprotein complexes associated with a virally-induced rearrangement of the ER termed the membranous web (MW)[84]. Interestingly, the multiprotein complexes responsible for replication are housed in double membrane vesicles (DMV), and it is hypothesized that eventually the stress of replication leads to the formation of multiple membrane vesicles[85]. By rearranging the host ER into a series of membranous

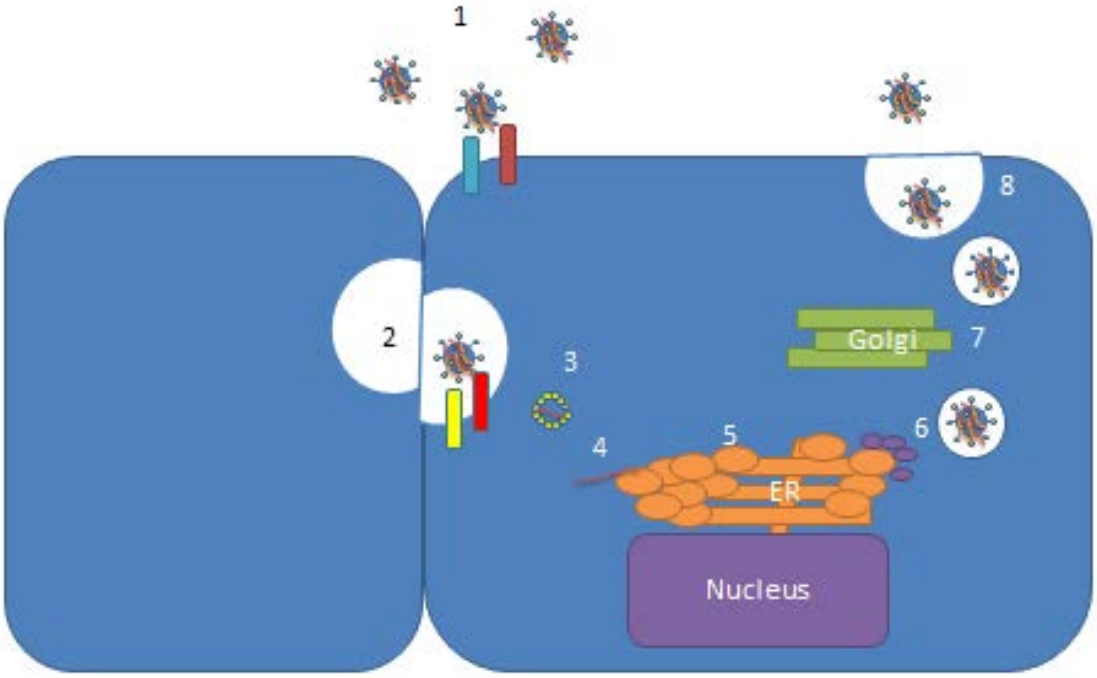


Figure 1.4 The HCV Life Cycle (modified from[14]). The viral life cycle, starting with free virus encountering a hepatocyte. 1) HCV interacts with the host hepatocyte via interactions between E1/E2 and CD81 and SR-BI 2)The virion is moved to the tight junction between hepatocytes, interacting further with tight junction proteins claudin and occludin 3) Following fusion the capsid is released, uncoated and free RNA is released 4) RNA is translated at the ER via IRES-mediated translation 5) The translated polypeptide is processed and mature proteins begin to form replication complexes within the virally-induced membranous web 6) Lipid droplets (purple) associated with the membranous web are used to load core and the viral genome to assemble viruses within the ER lumen, where they are eventually transported to the Golgi apparatus 7) Nascent particles are trafficked through the Golgi 8) Virus is exocytosed and released into the extracellular environment. Of note, HCV has been proposed to undergo direct cell-to-cell transfer and be infectious as RNA transferred via exosomes.

replication complexes local concentrations of metabolites are increased, viral proteins and RNA are hidden from intracellular immune surveillance, and enzymatic interactions are tightly regulated (as reviewed in [86]).

The replication complex consists of NS3/4A, NS4B, NS5A and NS5B coordinated with host proteins in the DMVs (as reviewed in [87]). It is the concerted action of these proteins that make both the replication complex and replication itself possible. Replication occurs in two steps, both mediated primarily by the RNA-dependent RNA polymerase (RdRp) NS5B[88–91]. The first step requires polymerization of a negative strand of RNA mediated by using the positive strand as a template; the second step produces viral genomes for packaging by using the nascent negative strand produced in the first step. There is an approximately one log-10 ratio of positive strand RNA being produced to negative strand (as reviewed in [92]). The circulating viral heterogeneity originates with the propensity of NS5B to make errors during these replication steps[48,49].

Assembly and Egress

Assembly of the HCV virion requires the consolidation of the structural proteins, a nascent RNA genome and several host proteins responsible for the low density of the LVP (as reviewed in [58]). An important component of this process is the trafficking of the homodimerized core protein to cytosolic lipid droplets (cLDs), which are thought to act as a platform for nucleocapsid formation[93,94]. The nature of the core proteins' RNA binding and lipophilic regions[95,96] and the spatial organization of its localization on the membranes of cLDs prime assembly of a nucleocapsid harbouring a genome within premature viruses[97]. The envelope glycoproteins E1

and E2 are translated and remain associated with the budding endoplasmic reticular membrane as non-covalent heterodimers during this process[98]. Notably, this process is highly regulated by host and viral proteins and the concerted action of these factors leads to the transfer of core protein to the cLDs and eventually to the nascent virus budding in the ER[99–102].

In tandem with structural and host proteins, the non-structural proteins each have pivotal functions during assembly. NS2 and p7 both play critical roles in the viral assembly process (as reviewed in [64]). NS2 has been reported to orchestrate the interaction of envelope proteins and the non-structural proteins involved in assembly, acting as a keystone during the initial steps of this process[103–105]. The enigmatic p7 protein and its actions during the viral life cycle remain to be fully characterized, but mutational studies suggest a critical role along with NS2 during early morphogenesis[106]. p7 has further been speculated to act as a viroporin[107], preventing premature degradation of the envelope glycoproteins during morphogenesis[108].

A great deal of genetic evidence has been gathered reflecting the importance of the helicase domain in NS3 and the NS4A cofactor in the process of assembly[58]. The NS3 helicase has been shown repeatedly to play a significant role in the process of assembly through mutation, deletion and adaptive studies[109–111]. NS4A has been linked to interactions with NS3 in assembly; an NS4A mutant that was shown to be assembly-defective can be rescued by an NS3 mutation outside of the protease domain, providing evidence that these non-structural proteins interact during the assembly process[112].

As mentioned previously, NS5A serves a plethora of purposes within the viral life cycle and interacts with numerous host and viral proteins[58]. A critical phosphorylation state in domain III

of NS5A has been hypothesized to bridge the gap between the replication complex and the initial stages of assembly[113]. Furthermore, it has been observed that domain III of NS5A has an intimate connection with the unloading of core protein from lipid droplets[114], and this aligns well with data suggesting that the interaction between these two proteins correlates with the efficiency of virus production[115]. Taken together, assembly is a complex and intricately organized process that remains to be fully elucidated.

Host microRNAs and Viral Regulation

As mentioned previously, miRNA-122 plays a significant role in the HCV life cycle, but beyond this direct interaction with the RNA genome host miRNAs perform a variety of functions in metabolic regulation and immunomodulation during infection (as reviewed in[116]). IFN-β has been previously shown to activate several miRNAs with anti-viral effects in the HCV life cycle[117], and although several miRNAs were predicted to bind to HCV directly, host pathway modulation may also play a role during infection. Recently, two miRNAs were implicated in the association of a single nucleotide polymorphism in the IFN-λ3 (*IFNL3*) gene with a decreased response to exogenous IFN therapy[118]. The deleterious polymorphism conveyed an increased susceptibility of host mRNAs to AU-rich element-mediated decay and degradation by miRNAs induced by HCV infection.

HCV induces modulation of cellular lipid metabolism to create an environment conducive to viral production[14,116], one way in which the virus does this is through induction of miRNA pathways. Changes to host lipid metabolism can often lead to a form of lipid accumulation termed steatosis[119]. HCV genotype 3 infections have a strong association with steatosis and

genotypic comparisons *in vivo* and *in vitro* provided the means to identify the downregulation of phosphatase and tensin homolog (PTEN) in a post-transcriptional manner[120], mimicking a similar mechanism in non-alcoholic steatohepatitis. Furthermore, a comparative study analyzing miRNA expression in either genotype 3a or 1b core-expressing Huh-7 cells implicated several miRNAs involved in cellular metabolism and growth[121].

These are just a few examples of miRNA modulation in HCV infection. Recently, two more dysregulated miRNAs were shown to intimately link immunomodulation and lipid metabolism by collaborative work with colleagues at the University of Ottawa that was subsequently published in Nature Chemical Biology[122]. Recent work has identified an interferon-stimulated gene product 25-hydroxycholesterol (25-HC) as a mediator of antiviral activity in several viruses, including HCV[123–125]. This lipid effector has been linked to membrane modification inhibiting viral entry and intracellular metabolic pathway regulation. Two intracellular metabolic pathways regulated by both 25-HC and HCV infection are the liver X receptor α signalling pathway[126–128] and sterol element-binding protein processing[125,129–131]. Considering the role of 25-HC in membrane modifications, a control oxysterol that could not act on intracellular pathways was tested against HCV and had no antiviral effect, implicating metabolic regulation as the route of antiviral efficacy. miRNA profiling was performed and two miRNAs of interest were identified and characterized further. miRNA-130b and miRNA-185 both regulate hepatic lipid metabolism and are influenced by 25-HC and HCV infection. As part of our collaboration we analyzed the effects of knocking down these miRNAs during an *in vitro* viral infection (Appendix 1). The regulatory cycle identified with this work is outlined in Figure 1.6.

Model Systems

The field of HCV research has experienced a dramatic evolution of laboratory techniques since the identification of NANBH. Over the years there have been several technical breakthroughs, but for the purposes of this thesis, I will focus on the four major developments that exponentially increased the potential for HCV research. As mentioned previously, when research began on NANBH, chimpanzee infection was the primary model of disease spread and virological characterization. This led to the initial characterization of the infectious agent, which undoubtedly supported the eventual discovery of HCV. The chimpanzee model of infection remains a physiologically relevant model for analysis of innate and adaptive immunology and pathogenesis (animal models reviewed in [132]).

Ten years after the discovery of the virus, a great leap forward came in the form of the replicon system[133]. The authors of this seminal paper originally attempted to grow fully infectious virus with full length clones of a patient isolate. Following failure of this approach, they engineered bicistronic constructs with an antibiotic resistance gene to select for cells harbouring replicating virus. Similar bicistronic constructs had been previously established in flavi [134] and pestivirus [135] systems. These constructs were commonly referred to as subgenomic replicons. These replicons only express the non-structural proteins and represented a major breakthrough for the study of replication and the development of antiviral compounds.

One of the major limitations of the replicon system is the absence of structural proteins and the inability of the replicon to generate virus particles. To address this, in 2003, Bartosch et. al.

established what was termed a pseudoparticle system[136] and within months Hsu et. al. published a similar system[137]. These systems revolved around expressing the viral packaging machinery of HIV and the HCV envelope proteins to create chimeric pseudoparticles. Using either GFP[136] or luciferase[137] markers of infection, these systems provided the means to characterize the HCV envelope proteins and the process of viral entry.

What both of these previous systems failed to recapitulate was the entire viral life cycle of HCV. In 2005 a system was discovered based on a patient sequence that required no adaptation to grow in cell culture, the sequence came from a Japanese patient with fulminant hepatitis and was aptly abbreviated JFH1[138,139]. Quickly following this discovery chimeric viruses were designed and the doors swung open to widely applicable mutagenesis studies [140].

The original viral strain replicated in cell culture albeit at low levels, but with its discovery came the possibility of viral adaptation to cell culture conditions. One adaptive strain of particular interest for the work to be presented herein is the $JFH1_T$ virus[108,110,141]. $JFH1_T$ contains three adaptive mutations, one each in E2, NS2 and p7, these mutations increase the production of virus and make it possible to analyze highly relevant mutations that would otherwise be lethal in culture. All of the experimental work for this thesis was done with this adapted viral strain.

Finally, the development of a convenient and relevant mouse model has eluded the scientific community for decades. A complicated transplant mouse model[142] served as the only small animal system until very recently when transient[143] and eventually stable expression[144] of viral entry receptors in mice facilitated viral replication. This novel approach developed by Ploss et.al. may serve to be the breakthrough necessary to facilitate *in vivo* analyses of viral infection

and pathogenesis, but the model has a low production of virus and the efficacy of the system in multiple studies is yet to be determined.

Treatment and the Evolution of Standard of Care

Recently, there has been a massive expansion of available compounds for the treatment of HCV, but the history of HCV care precedes its discovery (evolution of care reviewed in [14]). The treatment of HCV has evolved from the use of modified cytokines and broad spectrum antivirals to the use of specifically-acting compounds targeting both host and viral factors during infection. This dramatic transformation of therapeutic options has been the result of the evolution of scientific tools and industrial approaches to drug development. Despite all of the progress to date on treatment issues such as resistance, patient comorbidities and pharmaceutical financial discrepancies still hinder the real world efficacy of these compounds[145,146].

Interferon and Ribavirin

As early as 1986 recombinant alpha interferon was being investigated to control the hepatic alterations caused by NANBH. Primary outcomes were assessed based on liver enzyme tests and histological analysis[147]. By 1990 several randomized controlled trials had been performed with interferon alpha and immediate positive outcomes in a minority of patients had become apparent[148]. In 1998 an addition to interferon monotherapy came in the form of Ribavirin[149] and approximately doubled the response rate to treatment but came with additional side effects. The final breakthrough impacting the classical standard of care came in 2001 with the

approval of polyethylene glycol-conjugated interferon alpha (PEG-IFN). By addition of these chemical groups the half-life of the protein *in vivo* was increased drastically and PEG-IFN became the recommendation of choice for treatment in combination with ribavirin[150–154]. In 2009 a major clinical advance came in the identification of the correlation between host *IL28B* alleles and success of treatment with PEG-IFN[155,156]. This observation led to the optimization of standard treatment algorithms preceding the approval of any specific antiviral compounds (as reviewed in [157]).

Host-Targeting Antivirals

The first host-targeting antivirals to move into clinical development were cyclosporine A and analogues of cyclosporine A, all of which suppress the activity of cyclophilin A[158]. The most promising of this group of compounds was termed alisporivir[159,160]. Cyclophilin A is a host enzyme with isomerase activity that has been shown to interact with NS5A to promote replication[161]. Work with these inhibitors and cell culture systems reiterated the importance of this interaction and demonstrated that resistance mutations reduced the dependence of the viral replication cycle on the enzymatic actions of cyclophilin A[162]. Although these compounds have shown great promise in clinical trials alone, the most advanced of the compounds in combination with the classical standard of care has led to the development of pancreatitis in isolated cases and is now on clinical hold[163]. Considering the severity of some adverse events it has been suggested that these compounds are better suited for interferon-free combination therapy (reviewed in [14]).

In 2008, a modified oligonucleotide termed SPC3649 was identified as the result of a large screen of similar compounds designed to inhibit miR-122[164]. This compound was shown to reduce cholesterol levels *in vivo* and similarly inhibit HCV infection *in vitro.* As mentioned previously, miR-122 plays a significant role in viral replication and has been implicated in protecting the viral genome from host nuclease digestion as well as enhancing viral translation[54,165,166]. The discovery of this inhibitor, now termed miravirsen, represents a significant leap forward in therapeutics as miravirsen has the potential to be the first approved therapy of its kind (as reviewed in [167]). Miravirsen is currently in Phase 2 trials and seems to be a promising candidate for future treatment regimens.

Both miravirsen and alisporivir have no genotype-specific targets and thus provide pan-genotypic additions to approved therapeutic regimens. Considering the these compounds target host proteins, it comes as no surprise that there has been no viral resistance observed to date *in vivo*. The host-acting antivirals described here will no doubt play a role in interferon-free therapy and significantly impact pan-genotypic treatment options (as reviewed in [14]).

Direct-Acting Antivirals

A plethora of direct-acting antivirals (DAA) targeting multiple points during the hepatitis C viral replication cycle have become available over the last 4 years[14]. Protease Inhibitor (PI) development showed the first signs of success in 2003 with the compound BILN-2061, a revolutionary protease inhibitor that opened the door to subsequent successful therapies[168].

The first approved PIs came in 2011 with telaprevir and boceprevir in 2011, both NS3/4A PIs were the pioneering compounds in the DAA field and were quickly integrated into standard of care for genotype-1 patients along with PEG-IFN and ribavirin[169–171].. Both boceprevir and telaprevir are linear peptidomimetic compounds that act on the proteolytic active site of NS3. These compounds stemmed from a rational design approach to mimic the natural substrate of the viral protease (as reviewed in [172]). Additionally, a second wave of protease inhibitors with similar designs to the first wave and a second generation of optimized compounds are in clinical development (as reviewed in [173]). As recently as 2013 a macrocyclic protease inhibitor termed Simeprevir has been FDA-approved for combination therapy with PEG-IFN and ribavirin[174,175]. Protease inhibitors have a low barrier to resistance, meaning that mutations conferring resistance to this class of inhibitors arise early and can be sustained in the viral quasispecies[176,177]. Several variably fit mutations repeatedly arose during clinical trials[172]. The fitness of a mutant is defined as the replicative capacity, and often in terms of HCV is defined as the ability of mutant viruses to replicate in the presence of wild-type virus, reflective of the effects of mutations in a quasispecies[172], that is to say a highly fit virus has a high replication capacity and can be maintained in a quasispecies. The approval of these compounds symbolize a major breakthrough in viral therapeutics as they are the first specifically designed inhibitors with the ability to cure a chronic infection and have significantly improved SVR rates in genotype-1 infected populations[14].

Another major pharmaceutical advance came in the form of Sofosbuvir in 2010[178] and it's FDA approval in 2013[179]. A markedly different inhibitor, this compound acts as a nucleotide analogue, inhibiting polymerization after incorporation into a nascent viral RNA strand. This was

the first inhibitor to be paired with Ribavirin in an interferon-free regimen and illustrated the plausibility of an interferon-free therapeutic landscape[180]. Sofosbuvir significantly improves SVR rates in triple therapy, and due to the compound's mechanism of action has a pan-enotypic potential[181]. A single resistance mutation has been observed with Sofosbuvir treatment, S282T. S282T rarely arose in clinical trials[182] but patients being excluded from clinical trials may represent a more complicated population to treat[183]. Several allosteric NS5B inhibitors are in development but none have met FDA approval and this class is riddled with highly genotype-specific compounds that are speculated to only be useful in DAA combination therapies (Detailed review of all NS5B compounds[184])

Over the last year, a potent class of NS5A inhibitors, including Daclatasvir and Ledipasvir, has become a prioritized focus for companies interested in developing combination therapies[185–191]. The mechanism of action of this class of inhibitor remains ambiguous, but resistance data maps the interactions to domain I of NS5A at the homodimer interface[190] and data suggests that these compounds inhibit both replication and assembly in temporally distinct manners[192]. These discoveries and approvals serve as examples of the dramatic therapeutic reform taking place in the field of hepatitis C.

Project Design and Hypotheses

The field of HCV antiviral resistance mapping, phenotyping and compound discovery has primarily been advanced with the replicon system[14]. Analysis of compounds in replicons provides early data and plausible resistance mapping, that is to say that genomic positions containing resistance mutations offer insight into any given compounds mechanism of action. Although it is an excellent tool, mutations arising in the replicon system could be due to a lack of genetic pressure on portions of the genome essential for assembly, and thus represent artifacts rather than *bona fide* resistance mutations. Considering the size of the HCV genome, it comes as no surprise that the virus has evolved with polyfunctional non-structural proteins. Thus the HCV life cycle is inherently complex and regulated by a labyrinth of interactions amongst both viral and host proteins. To fully comprehend the efficacy of antiviral compounds and/or the prominence of resistance *in vitro*, a fully infectious system needs to be implemented when dealing with compounds inhibiting proteins acting at points outside of the replication cycle. Several groups have used cell culture systems to analyze resistance[192–195], but these assays have yet to be fully embraced and implemented due to technical or theoretical caveats. With this in mind we set out to develop an assay in a fully infectious viral system that was easy to use and best recapitulated the *in vivo* setting.

To begin we searched the literature for PI-resistant mutations that were prominently represented *in vivo* and *in vitro*. We further specified our selection criteria by choosing telaprevir and boceprevir cross-resistant mutations that spanned the entire 180 a.a. protease domain of NS3. To increase the potential for future analyses, we selected mutations that arose

together or individually in the literature. This led us to select four mutations to introduce into our adapted virus. The virus used for all of this work is a genotype-2a construct with three adaptive mutations, one each in E2, p7 and NS2[108,110,141]. Notably, there is no adaptive mutation in the NS3 protein to be manipulated. We hypothesized that these mutations would reduce the fitness of the virus as measured by a focus forming unit (ffu) assay and exhibit a PI-resistant phenotype. We confirmed our results with one mutation in a competition assay and assessed the potential for select mutants to revert to wild-type in a long-term passage. We were also interested in the correlation between fitness and the frequency of the mutations observed in the literature, presumably the more often the mutation is observed the more fit it would be in our system. That being said, the fitness is a reflection of a much greater number of factors *in vivo* than *in vitro,* and the majority of sequences identified in the clinic stemmed from HCV genotype-1 infected patients.

Following our initial analyses of potential drug resistant mutant fitness, we went on to develop and optimize an assay for evaluating compound efficacy. We hypothesized that compounds tested would inhibit wild-type virus at concentrations similar to those reported in the literature. The objective of this development was to provide a tool to the scientific community for evaluation of novel compounds in the context of a complete viral replication cycle. As secondary objectives, we designed the assay to be increased in throughput, and to be easily replicated in other labs. With this in mind, we began with 6-well plates, we used commercially available quantitative reverse-transcription polymerase chain reaction (QRT-PCR) reagents and harnessed the high dynamic range of the $JFH1_T$ virus mentioned previously (Up to 10^6 ffu/ml).

Project Aims

i. Assess the fitness of common protease inhibitor resistance mutations in the context of $JFH1_T$, and confirm any correlation between mutation frequency in patients and fitness in our construct.

ii. Develop and optimize an assay to phenotype resistance mutations in the context of a complete life cycle and confirm their potential resistance in a genotype-2a construct.

iii. Test novel compounds as a proof-of-principle that the assay is functional with multiple drug classes.

Chapter 2 Materials and Methods

Plasmids and cloning

$JFH1_T$ contains three amino acid substitutions encoded by one mutation each. There is one mutation each in E2, p7 and NS2. The $JFH1_T$ construct is derived from a cell culture-adapted version of JFH1, with mutations leading to greater infectious virus production[141]. Mutations in the protease: L36M, T54A, R155K, and I170A were all introduced using the Quikchange II XL site-directed mutagenesis kit (Agilent Technologies). Forward primers were as follows from 5' to 3': L36M: GGGGAAGTCCAAATCATGTCCACAGTCTCTCAG, T54A:

GGGGTTTTGTGGGCAGTTTACCACGGAGCTGGC, R155K: GTCGTTGGGCTCTTCAAGGCAGCTGTGTGC, I170A: GCCAAATCCATCGATTTCGCCCCCGTTGAG ACACTCGAC. All reverse primers were complementary to forward primers listed. Primers were ordered as standard oligos (Life Technologies).

Cell Culture

Human Hepatoma Huh-7.5 cells were propagated in Dulbeccos Modified Eagles Medium supplemented with High Glucose, L-glutamine, phenol red and sodium pyruvate (DMEM; Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco) and 1% Penicillin/Streptomycin (Sigma) to make complete DMEM ($DMEM_{(comp)}$). Cells were maintained at 37°C and 5% CO_2.

Antibodies

The following antibodies were used in this study: Primary Mouse anti-HCV core monoclonal B2 antibody (Anogen), and secondary Alexafluor 488 goat anti-mouse (Life Technologies). The core antibody was diluted 1:200 in 5% BSA in PBS and the Alexafluor 488 was diluted 1:500 in PBS.

In vitro transcription and RNA transfection

Plasmid DNA coding for $JFH1_T$ or the protease mutants of $JFH1_T$ was linearized by digestion with XBaI for 2 hours at 37° C. Linearized DNA was extracted from the digestion solution with UltraPure Phenol:Chloroform:Isomyl alcohol (Invitrogen) followed by ethanol precipitation. Linearized DNA was either stored at -20°C or used directly for transcription. Twenty-four hours preceding transfection Huh-7.5 cells were seeded in 10cm dishes at a density of 1-1.5 x 10^6 cells per dish. On the day of transfection 1 μg of linearized DNA was transcribed using the T7

RiboMAX Express large-scale RNA production system (Promega). Plate medium was removed and replaced with 2 ml of serum-free (SF) medium/plate. 500 µl of SF medium, 50 µl of DMRIE-C transfection reagent (Invitrogen) and 4 µl of RNA transcripts were mixed lightly. Transfection mixtures were then added to cells and incubated at 37°C and 5% CO_2 for 4 hours. Following incubation, SF medium was removed and replaced with 7 ml of $DMEM_{(comp)}$. Transfected cells were incubated for 72 hours before supernatants were collected and clarified.

For QRT-PCR standards, linearized DNA was transcribed as described above. The resulting solution was subjected to RQ1 DNase digestion (Promega) and RNA was extracted using Trizol LS (Invitrogen). The resulting RNA concentration was determined by spectrophotometer readings on a Biophotometer Plus (Eppendorf). HCV genome copies in the resulting concentration were determined by using the molar mass of a genome. Once the genome copy was determined the solution was 10-fold serially diluted on ice until the concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 per 2 µl were achieved. These standards were frozen at -80°C until needed.

Infectious HCV titer determination

Twenty-four hours preceding infection 8-well chamber slides (LabTek) were seeded with 5 x 10^4 cells in 400 µl per well and incubated at 37°C and 5% CO_2. 72 hours post-transfection transfection media were collected and clarified by centrifugation at 1800 x *g* for 3 minutes. The clarified supernatants were aliquoted and frozen at -80°C before use. Supernatants containing virus were then thawed and serially diluted 10-fold in triplicate in $DMEM_{(comp)}$. 100 µl from each dilution were used to infect each well of the 8-well chamber slides. Infections were incubated,

rocking every hour, at 37°C and 5% CO_2 for four hours. Following incubation inocula were removed and replaced with 400 µl of $DMEM_{(comp)}$. 72 hours post-infection cells were fixed with acetone and stained with mouse anti-core and goat anti-mouse Alexafluor 488. Viral titers are expressed as focus forming units per millilitre (ffu/ml).

Infection of 6-well plates: For the EC_{50} assay, 24 hours preceding infection, 6-well plates (Corning) were seeded with 1.5 x 10^5 cells per well. Cells were infected at an MOI of 0.1 (defined as 15000 FFU) in 1 ml of $DMEM_{(comp)}$ and incubated for four hours before infectious media was removed and replaced with 3 ml of $DMEM_{(comp)}$ containing serial dilutions of the approved drug or the unapproved test compound of interest, the highest concentration of DMSO remained below 0.1%.. 72 hours post-infection medium was removed and clarified by centrifugation. Clarified supernatants were then stored at -80°C preceding use.

RNA extraction, QRT-PCR and EC_{50} determination

Following clarification of supernatants RNA extraction was performed using the QIAamp Viral RNA Mini kit (Qiagen) and extracts were stored at -80°C preceding use. QRT-PCR was performed on a 7500 Fast Real-Time PCR (Applied BioSystems) with a 5' UTR primer and probe (ABI cat#: 4331182) and the Taqman Fast Virus 1-Step Master Mix. Standards and samples were run in triplicate. The primers and probe were previously designed by colleagues within the lab, namely Dr. Ali Atoom and Dr. Rodney Russell. Once designed the primers and probe designs became publically available through ABI. Genome copies/ml were used to plot the EC_{50} graphs in GraphPad Prism 5 (GraphPad) using a three-parameter dose response curve. Zero percent

inhibition was defined as the highest genome copy value in the data set; all other data points were transformed into a percentage of this value.

Chapter 3 Fitness Analysis of Common Protease-Inhibitor Resistant Viruses

Viral Production and Spread

We selected four common PI resistant mutations to analyze in the context of our robustly replicating viral construct. The four selected mutations were: L36M, T54A, R155K and I170A. The rationale for the selection of these mutants is outlined in detail in the project design section of this thesis. We introduced these four mutations into the viral genome encoding portion of the plasmid independently to create four separate constructs via site-directed mutagenesis with custom primers. All mutated plasmid constructs were validated by Sanger sequencing at The Center for Applied Genomics in Toronto, Ontario, Canada.We transfected RNA stemming from mutated plasmids into Huh-7.5 cells to produce viral stocks. These stocks were then used to infect cells in 8-well chamber slides which were subsequently used for immunofluorescent analysis of the presence of viral core protein. Using this assay we can quantify virus production and qualitatively assess viral spread. To quantify, we followed a limiting dilution viral titer assay. Qualitative assessment of the size of each viral foci gave us indication of viral spread from cell to cell. Detailed description of these quantitative and qualitative viral techniques can be found in a seminal HCV paper published from Dr. Francis Chisari's lab in 2005[138] The least conservative mutant in terms of amino acid substitution,

I170A, showed the greatest reduction in virus production. Aside from I170A minimal reductions in virus production were observed. The replication negative control, ΔGDD, which contains a three amino acid deletion within the active site of the viral polymerase, had no observable foci (Figure 3.1). Virus spread was similar amongst mutants and wild-type (Figure 3.2). Taken together these data indicate that the resistant mutations in our panel stemming from conservative amino acid changes produced virus within approximately one log of the wild-type construct and spread was minimally effected in each conservative construct.

Confirmation of R155K Fitness Deficit and Analysis of the Potential for Reversion in T54A and R155K

To confirm mutant viral fitness was indeed compromised we selected the mutations with the highest clinical frequency to analyze in a competition assay. This assay consisted of co-culturing the mutant with the wild-type virus at increasing ratios of mutant:wild-type. RNA was then extracted from co-culture supernatant and subjected to cDNA synthesis. Following cDNA synthesis, a PCR protocol was implemented to amplify the protease region of interest. PCR products were subjected to population sequencing and the codon of interest was identified as either mutant or wild-type for each ratio. We observed that at any ratio cultured except for 1:1 the R155K codon remained dominant when competing with wild-type virus (Table 3.1). This confirmed the results of our previous experiment in which R155K was indeed less fit than wild-type.

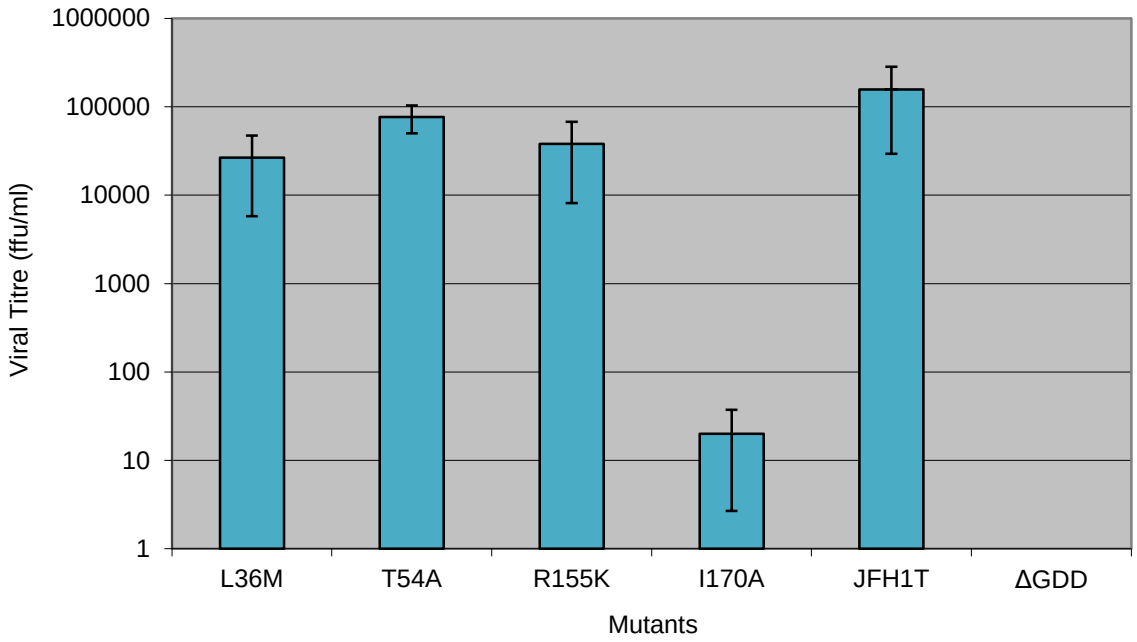


Figure 3.1 The Effect of Protease Inhibitor Resistant Mutations on Viral Production. Mutated viral plasmids were transcribed and RNA was subsequently transfected into Huh-7.5 cells at a density of 1 x 10^6 per 10 cm plate. Supernatants were harvested 72 hours post-transfection and serially diluted before infecting Huh-7.5 cells at a density of 50 000 cells per well in 8-well chamber slides. 8-well chamber infections were incubated for 72 hours before media was removed, cells were fixed with acetone and stained for core protein. Foci were counted in the highest positive well of each dilution series and focus forming units (ffu)/ml was calculated accordingly. All mutants were tested in triplicate wells and the experiment was repeated three times. The ΔGDD construct is a replication negative control and the lower limit of detection is 10 ffu/ml[141]. Bars represent the mean of triplicate values.

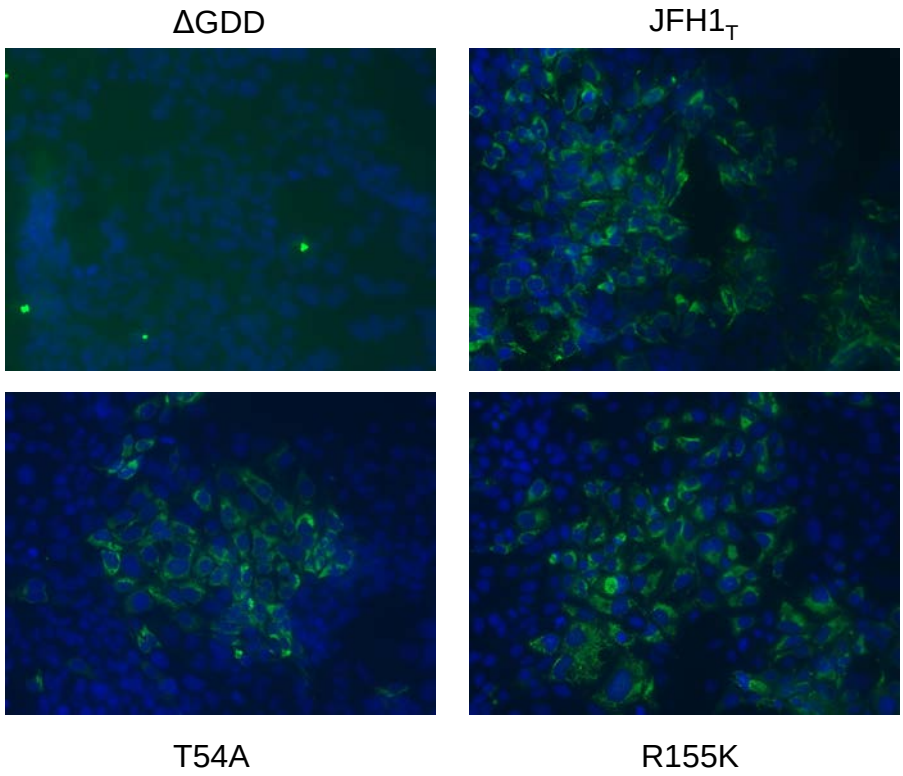


Figure 3.2 The Effect of Protease Inhibitor Resistant Mutations on Viral Spread. Mutated viral plasmids were transcribed and RNA was subsequently transfected into Huh-7.5 cells at a density of 1 x 10^6 cells per 10cm plate. Supernatants were harvested 72 hours post transfection and serially diluted before infecting Huh-7.5 cells at a density of 50 000 cells per well in 8-well chamber slides. 8-well chamber infections were incubated for 72 hours before media was removed; cells were fixed with acetone and stained for core. Nuclei were visualized by staining with DAPI. Shown here are representative fields of view from representative mutants viewed with a 20X objective lens. No observable difference in viral spread was noticed in any construct.

Following up on work done by a previous lab member, the two most common mutations, T54A and R155K, were subjected to long-term passage in order to analyze the preferential genetic pathways to amino acid substitutions. This assay was designed to quantify the contribution of mutational bias to genetic barrier and test the hypothesis that mutations arise through energetically favourable means. Genetic barrier is defined as the number of mutations necessary to overcome selective drug pressure, the nature of the mutations (i.e. transition or transversion) and their combined impact on viral fitness[172]. Mutational bias refers to the strong preference the polymerase displays for transitions (*e.g.* guanine to adenine) rather than transversions (*e.g.* guanine to cytosine or uracil) when making errors[196]. By passaging the mutant viruses for 15 days or more they are given ample opportunity to generate mutations that encode f the wild-type NS3 protein. If a wild-type protein coding sequence arises in culture it will out-compete the mutant virus and become the dominant sequence in the quasispecies. To assess the reversion characteristics of the T54A and R155K mutants they were cultured between 15-45 days and, similar to the competition assay, RNA was reverse transcribed, amplified and sequenced. In both mutant viruses no reversion was observed, this further emphasizes the fitness of the mutants in our system. Considering the negative nature of this result, no table is provided.

Table 3.1 Competitive Fitness of R155K. 10cm culture plates were seeded with Huh-7.5 cells at a density of 1 x 10^6 cells/plate and incubated for 24 hours preceding infections. Mutant and wild-type virus stocks were used to infect Huh-7.5 cells at increasing ratios of mutant to wild-type. A total multiplicity of infection (MOI) of 0.1 was implemented for every ratio and a plate with mutant virus containing no wild-type was used as a control. Infections were incubated for 72 hours before supernatant was removed, RNA extracted, cDNA synthesized and PCR amplified for sequencing.

Ratio	**Codon**	**Dominant Virus**
1 to 1	CGA	Wild-type
10 to 1	AAG	Mutant (R155K)
100 to 1	AAG	Mutant (R155K)
Control	AAG	Mutant (R155K)

Chapter 4 Development of a Robust Virus-based Assay for Determination of Antiviral 50% Effective Concentration

Note: The results in this chapter are currently being compiled into a manuscript that will be submitted to the journal *Antimicrobial Agents and Chemotherapy*.

Confirmation of Resistant Phenotype in Common PI-resistant Mutants

The conventional means to determine an EC_{50} in the HCV field is through the use of high throughput replicon systems[14,172]. The replicon system does not recapitulate the entire life cycle of HCV and so we sought to improve the accuracy of compound analysis by using the entire HCV life cycle. To establish our assay we began with the two most common protease inhbitor resistant mutations and a wild-type control. To measure the quantity of virus released into the supernatant we implemented a commercially available QRT-PCR protocol. We used a custom calculation of viral genomes and we tested the probe and the design of our standard RNA concentrations by a simple comparison of infected and uninfected wells (Figure. 4.1). We assessed the growth rates of the mutants and the wild-type virus in 6-well plates over 4 day cultures to assess whether growth would peak on day 3 or 4, commonly transfections or infections are incubated for 3 days in our lab for optimal yields, but because we were working with a new quantitation method, we expanded our curves to incorporate a 4-day incubation (Figure 4.2). We set up a protocol using an MOI of 0.1 for 4 day culture based on the results of our growth curve. Although the growth curve gave us insight concerning the optimum day to harvest we could not help but notice the high background levels observed with the replication negative control. With no increase in copy number in the control we believed the curves were

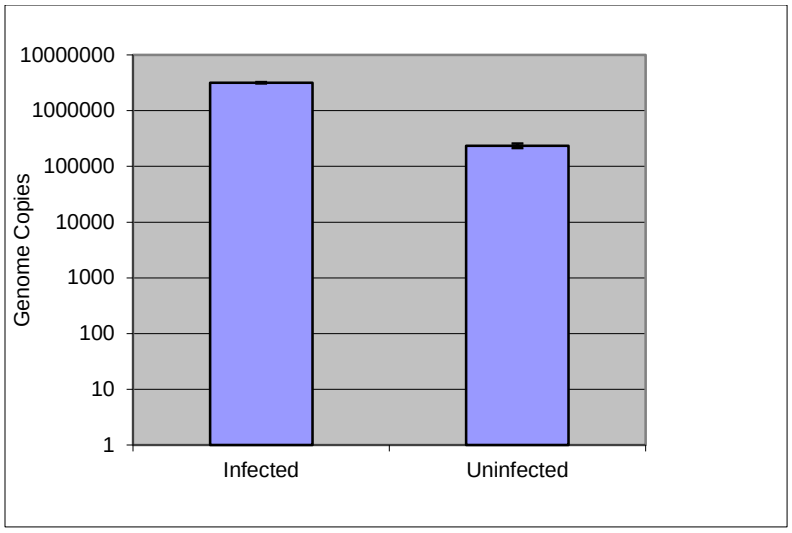


Figure 4.1 Initial Test of QPCR Assay with Primers and Probe. Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 or uninfected and incubated for 3 days. All samples were subjected to RNA extraction and QRT-PCR quantification on the same day following completion of the experiment**.** Samples were quantified in duplicate and black bars represent standard deviation.

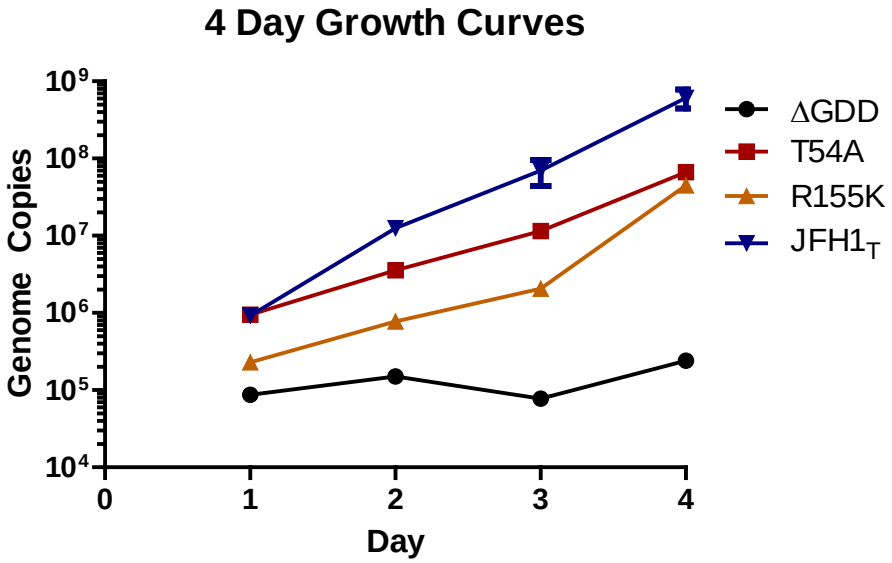


Figure 4.2 Analysis of Peak Viral Growth in 6-Well Cultures. Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 and incubated for 4 days. Supernatant was sampled every 24 hours, clarified and frozen at -80° C. All samples were subjected to RNA extraction and QRT-PCR quantification on the same day following completion of the experiment. Δ GDD served as a replication negative control. All samples were quantified in duplicate and bars represent standard deviation.

accurately representing growth qualitatively but may be overrepresented quantitatively. Our initial standard design was based on an *in* vitro transcription followed by concentration analysis by a spectrophotometer and then genome calculation based on the molecular weight of one HCV genome. To further increase the accuracy of our quantitation and better design our standards we followed the *in vitro* transcription step with DNA digestion with RQ DNase and then RNA extraction with the TRIzol reagent. We then followed the remainder of the protocol as specified previously and saw a dramatic decrease in total copy number leading us to believe that the previous standards were misrepresenting the data due to the presence of input template DNA, this reduction in copy number can be observed in Figure 4.3. Before testing any compounds we implemented a serial dilution of DMSO on the 6-well growth assay to assess the impact of the solvent and to identify a concentration that will have little to no effect on the yield of virus (Figure 4.3). Of note, in the 10% DMSO condition, cells showed a noticeably diminished ability to grow when observed under the light microscope, all other conditions phenotypically resembled no DMSO when observed similarly. The 10% DMSO was also the only condition to significantly vary from the no DMSO control condition when analyzed by QRT-PCR (Figure 4.3). Following this assay all compounds were resuspended to be implemented at a concentration of no more than 0.1% DMSO in complete media to minimize any impact of DMSO on virus replication and/or cell viability. To calculate the EC_{50} of telaprevir in the context of $JFH1_T$ we tested the production of the virus in serial dilutions of telaprevir. Our data analyses led us to compare two types of constraints on the calculation of effective concentration. Upon constraining the maximum and minimum asymptotes to 100% and 0% respectively we saw a

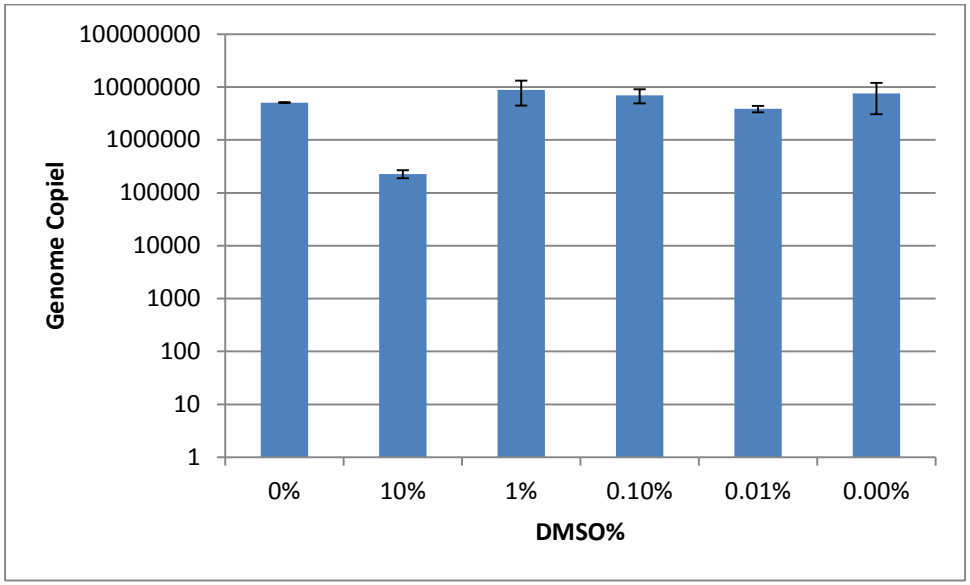


Figure 4.3 Optimization of DMSO concentration in Huh-7.5 cells . Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 and incubated for 4 days with no DMSO or a serial dilution represented as a percentage of $DMEM_{(comp)}$. Samples were quantified in duplicate and black bars represent standard deviation.

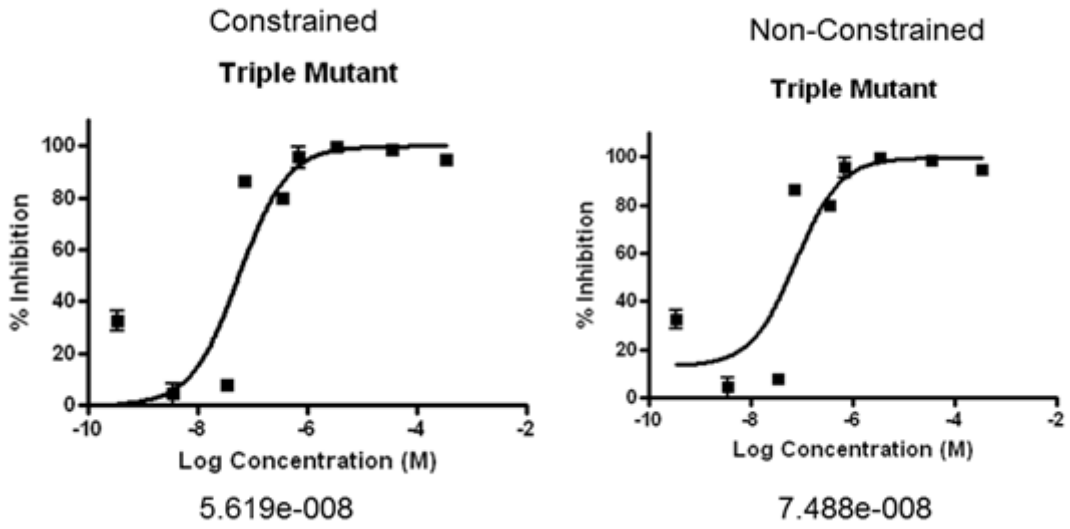


Figure 4.4 Comparison of Constrained and Non-Constrained Calculations. Representative EC_{50} graphs and corresponding effective concentrations. Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 and treated with 10-fold logarithmic dilutions of telaprevir. The treated infections were incubated for 4 days. On day 4 supernatant was clarified and subjected to RNA extraction and QRT-PCR. EC_{50} analyses were carried out with Graphpad Prism software. All points were quantified in duplicate. Constrained calculations were performed by restricting the maximum and minimum asymptotes to 100% and 0% respectively. Non-constrained had no such restrictions. Numbers below graphs represent EC_{50} values expressed in Moles/L.

slight shift in effective concentration as visualized in Figure 4.4. The data curves were better fit, and therefore the data and effective concentrations were better represented when these constraints were relieved and therefore all subsequent calculations were performed without constraints. Finally, to confirm that the mutations chosen were indeed *bona fide* resistance mutations in our system we tested their replication capacity in the presence of increasing concentrations of telaprevir, one of the two clinically approved inhibitors of NS3[171]. Both T54A and R155K showed an approximately one log increase in the concentration necessary to inhibit viral growth by 50% (EC_{50}), when compared to wild-type (Figure 4.5). This result confirms the resistant phenotype of the selected mutants in our system and serves as a proof of principle that our assay is functional for resistance analysis.

Analysis of Novel Anti-HCV compounds

Once the assay had been established with telaprevir and common resistant mutations, we received novel compounds developed by Dr. Raymond Schinazi's laboratory at Emory University. These five compounds represented two additional distinct antiviral classes. RSRR-1 and RSRR-2 are nucleoside analogues that were selected due to their interesting metabolic characteristics. RSRR-3, RSRR-4 and RSRR-5 represent NS5A targeting compounds and were selected for their antiviral potency and novel designs. Unfortunately, we are not permitted to share the chemical structure or specifics of each compound, but for the purposes of our work, knowledge of the chemical class suffices. We initially tested RSRR-1, 3, 4 and 5 at the suggestion of our collaborator (Figure 4.6). We observed EC_{50} values in the nanomolar range with the NS5B targeting compounds and EC_{50} values in the picomolar range with the NS5A targeting

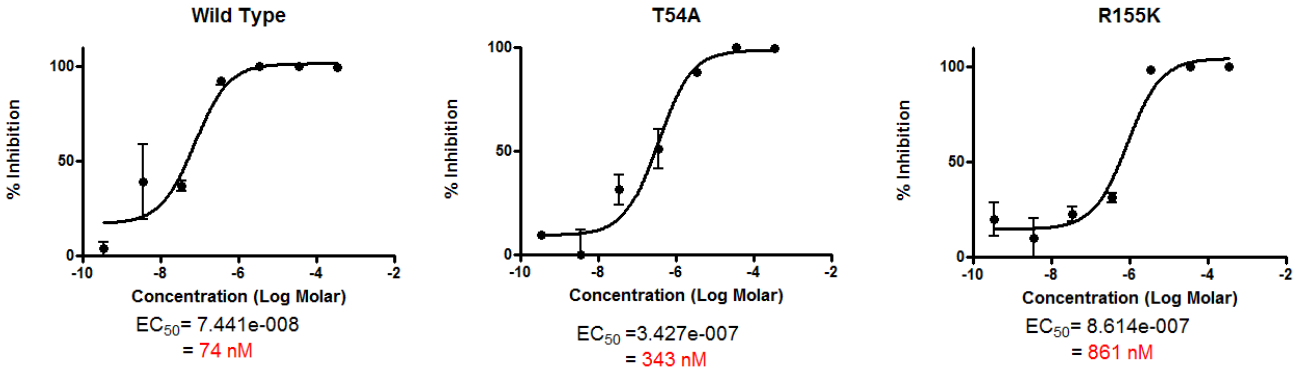
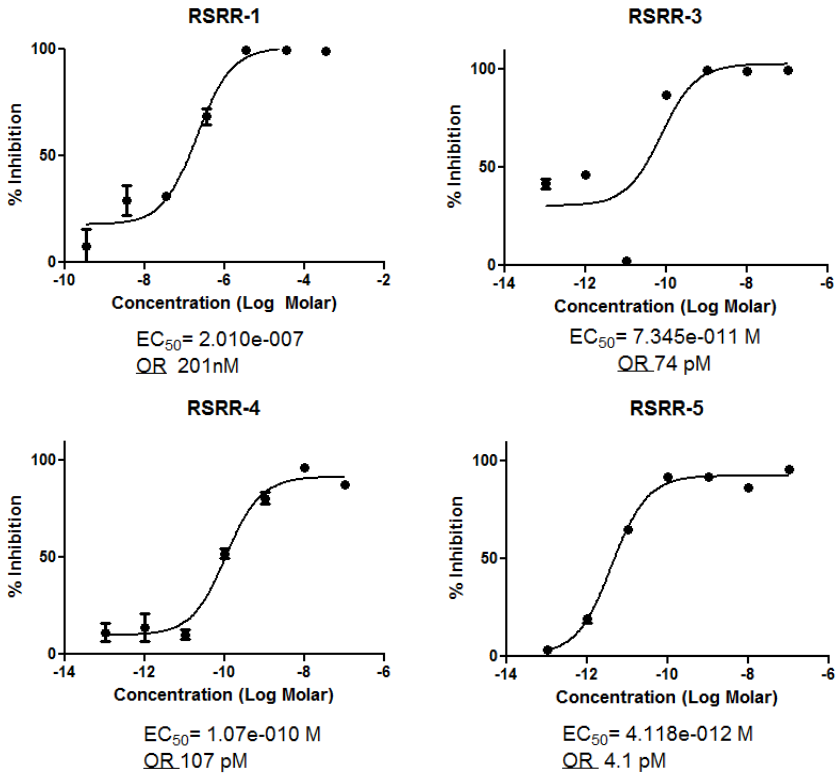


Figure 4.5 Demonstration of Telaprevir-Resistant Phenotype. Representative EC_{50} graphs and corresponding effective concentrations. Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 and treated with 10-fold logarithmic dilutions of telaprevir. The treated infections were incubated for 4 days. On day 4 supernatant was clarified and subjected to RNA extraction and QRT-PCR. EC_{50} analyses were carried out with Graphpad Prism software. All points were quantified in triplicate.



F**igure 4.6 Demonstration of Assay Utility in Novel Compounds.** Representative EC_{50} graphs and corresponding effective concentrations. Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 and treated with 10-fold logarithmic dilutions of each compound. The treated infections were incubated for 4 days. On day 4 supernatant was clarified and subjected to RNA extraction and QRT-PCR. EC_{50} analyses were carried out with Graphpad Prism software. All points were quantified in triplicate.

compounds. This further confirmed the efficacy of our system as these ranges were commonly observed in the literature for these respective classes of compounds[14]

Improvement of the EC_{50} Assay with a Novel Compound

To increase the accuracy and validity of our assay we increased the number of dilutions used to test each compound. This strategy increased the total number of data points per graph and provided a better potential for curve-fitting. We decided to test the final compound of the five provided by Dr. Schinazi's group in this fashion. RSRR-2 was 10-fold diluted 12 times and tested in the same fashion as the previous compounds (Figure 4.7. To further increase the accuracy of our curve, we analyzed the data points with both the standard fixed slope three-parameter (shown in red) and the variable slope four-parameter (shown in blue). The fixed slope parameters set the slope of the curve automatically to 1 or -1 and fits the data accordingly. The variable slope parameter uses any slope possible to fit the data. We were interested in exploring both possibilities and analyzing the data thoroughly to ensure the most accurate method was used, with that in mind we sought to increase the number of data points and vary the slope to create a well fit curve over more concentrations, increasing the accuracy of the interpretation. Interestingly, the EC_{50} calculations were within 4 nM of each other.

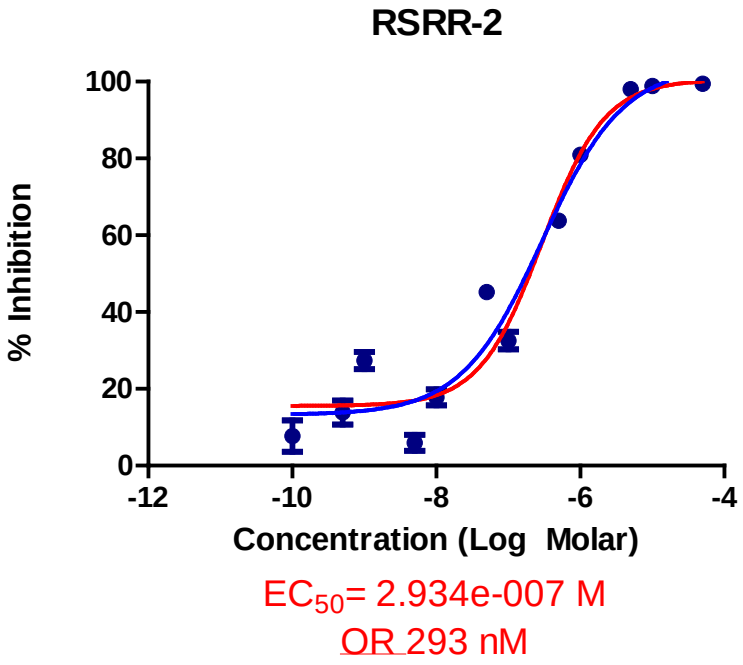


Figure 4.7 Increased Number of Dilutions Increases Accuracy of EC_{50} Determination. Representative EC_{50} graph and corresponding effective concentration. Huh-7.5 cells were seeded at 1.5 x 10^5 cells/well in 6-well dishes. Cells were infected at an MOI of 0.1 and treated with 12 10-fold dilutions of RSRR-2. The treated infections were incubated for 4 days. On day 4 supernatant was clarified and subjected to RNA extraction and QRT-PCR. EC_{50} analyses were carried out with Graphpad Prism software. All points were quantified in triplicate. The red curve represents a fixed slope analysis and the blue curve represents a variable slope analysis.

Chapter 5 Discussion

The field of hepatitis C treatment has recently been revolutionized by the discovery and development of several classes of highly effective antiviral compounds[14]. These drugs were discovered primarily through high-throughput screens using the replicon model *in vitro*. While this is excellent for discovery and early characterization of compound efficacy and viral resistance, this model does not reflect the complex interactions of non-structural proteins, host factors and structural proteins throughout the complete viral life cycle. With that in mind, mutations arising during *in vitro* characterization may indeed be artifacts of this caveat.

While this may not seem important for early analyses of compound efficacy and mechanisms of action, the downstream application of these drugs, especially in combination may be hindered by an incomplete understanding of the viral response to drug pressures. For instance, a 'drug resistant' mutation in the replicon may have lethal effects in a fully infectious system due to the polyfunctional nature of the target protein. One paper demonstrating this phenomenon analyzed NS4A mutations, some of which caused little to no decrease in RNA replication but had severe impacts on assembly and release of viral particles[112]. A resistance mutation identified in the replicon may indeed be resistant in the context of replication, but when paired with the life cycle may severely inhibit downstream viral machinery. Considering the ever increasing need for optimization with novel compounds and combinations, a fully infectious viral assay may be the tool necessary to completely characterize viral resistance and

recommend novel treatment regimens. With this in mind, we set out to develop an assay to analyze compound efficacy in the context of a complete viral life cycle.

To begin we chose several common protease inhibitor mutations to characterize, namely: L36M, T54A, R155K and I170A. Interestingly, the three conservative amino acid substitutions showed less than a 1 log decrease in viral fitness as measured by a viral titration assay (Figure 3.1). This seemed rather intuitive and the striking reduction of fitness when the Isoleucine at position 170 was mutated to an alanine reflected the non-conservative nature of this substitution and it's relatively low frequency of observation clinically and in the literature. The panel of mutants selected were all viable and, with the exception of I170A, highly productive in the context of our genotype-2a construct. This reflected similar observations in genotype-1[172].

To further confirm our preceding data concerning the fitness of the mutants we decided to focus on the most clinically relevant of the four in a competition assay (Table 3.1). R155K was of significant interest during the period of these experiments and remains a fascinatingly fit mutant in the context of drug resistance. This mutation was of particular interest due to its high fitness *in vitro* and *in vivo*, its ability to persist in a quasispecies containing wild-type sequences and its potential for harbouring multiple resistance mutations (most likely due to its low impact on fitness)[176,177]. In the competition assay, we observed that even in the context of a quasispecies harbouring 1 wild-type virus for every 10 R155K mutants, the R155K mutation maintained dominance over a 3 day culture. This confirmed our titration data and was further validated by the existing literature[176,177].

Recently, a colleague from the laboratory developed an assay for analysis of genetic barrier (Morris, Taylor et al, manuscript in preparation). In the interest of a thorough analysis of fitness we subjected T54A and R155K to a similar long term culture period to evaluate the genetic pathway to a wild-type sequence. This assay assumes the less-fit mutant virus will always mutate towards the more-fit wild-type sequence when no drug pressure is applied. This assumption is rather intuitive considering the nature of the wild-type sequence to be more fit than the mutant and often competes to dominate the quasispecies. It has further been observed that *in vivo* the viral polymerase causes mutations through nucleotide transitions (pyrimidine to pyrimidine, purine to purine) more often than transversions (purine to pyrimidine or vice versa)[196]. We were interested to see how this would apply to highly fit antiviral resistance mutations, and sought to further understand the mechanisms of resistance mutation. Intriguingly, after culturing for 30 days or more, neither T54A nor R155K reverted to the wild-type sequence, providing further evidence that these mutants are highly fit in the context of our virus and can remain in the quasispecies for extended periods of time, similar to observations of maintenance in patient quasispecies[176]

Finally, to complete the analysis of our panel of mutations we sought to develop an assay to assess the efficacy of compounds in the face of resistance mutations. To do this, we began by assessing viral growth in 6-well plates (Figure 4.2) and optimizing our collection of samples based on a 4-day peak in viral growth. We tested telaprevir at 10-fold logarithmic dilutions and analyzed the data using a three-parameter dose response curve. Our results in Figure 4.5 indicated that the mutations introduced into our adapted virus were indeed *bona fide* resistance mutations in our system. Both T54A and R155K showed a 10-fold logarithmic

increase in the drug concentration necessary to inhibit virus propagation by 50%. Consistent with previously established EC_{50}s with telaprevir, all of the viruses tested were in the nanomolar range[172]. This established our assay and confirmed the resistant phenotype of T54A and R155K in a genotype-2 adapted virus strain. Two caveats to our design at this stage were the minimal number of data points for curve-fitting and the requirement for viruses with titers of 15000 ffu/ml or more to accommodate an MOI of 0.1. A common criticism of our work among experts is the genotype-2 nature of our viral strain. Considering the primary target of protease-inhibitor therapy is HCV genotype-1 infected patients, it is a common misconception that these drugs have little to no effect on genotype-2, or that due to the high efficacy of the classical SOC in achieving SVRs in genotype-2 there is no need to research the topic further. In stark contrast to these points, it has still been suggested that the original PIs improve the efficacy of SOC and can be used for hard to treat genotype-2 patients[197–201]. Even beyond the scope of specifically testing for genotype 2, our assay has been shown effective to analyze mutations, and confirm their resistant phenotype. Future studies in our laboratory could include passaging the virus in the presence of drug and confirming resistance in the EC_{50} assay. Regardless of genotype the mechanism of viral mutation during replication remains the same. Our system still provides a legitimate map of interactions and plausibly more accurately defines the resistance profile due to the intact downstream interactions of NS proteins.

Once the assay had been developed and implemented with resistant mutations we moved on to analyze a collaborators compounds using the same protocol. Compounds representing two distinct classes of antivirals, NS5B and NS5A inhibitors, were tested at 10-fold logarithmic dilutions with our wild-type strain of virus (Figure 4.6. Consistent with the observed EC_{50}s in the

literature[14], NS5B inhibitors showed nanomolar EC_{50}s while the NS5A inhibitors required picomolar amounts to inhibit viral growth by 50%. This further validated our system and was the first assessment of these novel compounds in a fully infectious system.

To finalize our analysis we chose an NS5B inhibitor (RSRR-2) to use in an optimized protocol, increasing data points and providing the basis for more accurate EC_{50} determination (Figure 4.7. We increased the number of data points from 7 to 12 and used both three-parameter and four-parameter dose-response calculations to evaluate the compounds efficacy. Interestingly, we saw that both analyses results in similar EC_{50}s. That being said, the increase in data points still provided a more accurate assessment of the necessary concentration to inhibit viral production. To increase the assays utility, it would still have to be scaled up to increase its throughput and optimized with lower requirements of virus for both resistance and compound analysis.

We present here, for the first time, the analysis of drug resistant mutations in our adapted viral strain, the development of an assay for compound analysis and the results of analyses with novel compounds from multiple antiviral drug classes. Several groups have provided similar insights, with similar systems[192–195], but we strongly believe that our system has the most potential for accurate and high throughput analysis of compounds and resistance mutations due to the wide dynamic range of our naturally fit wild-type strain, the ease of manipulation following treatment and the commercial nature of our QRT-PCR analyses. Potential future studies could include the identification of key residues involved in compound mechanisms of action. By selecting and substituting key residues in NS proteins, and analyzing the resultant

EC_{50}s we could further probe the mechanisms of actions and improve the efficacy of novel compounds. Due to the availability of this assay in our lab, Dr. Russell has recently received funding from Merck Sharpe & Dohme Corp to evaluate a number of compounds they have in development and for which they do not currently know the mechanism of action. The assay is also being used in our lab to evaluate potential antiviral efficacy of 70 nucleoside analog-like compounds received from Dr. Paris Georghiou in the Department of Chemistry here at Memorial. We have also created the potential to optimize treatment regimens by passaging the virus in the presence of multiple drugs, individually or in combination. Following passage we could analyze the sequences, clone them into our construct and assess antiviral efficacy against the mutants. This process would increase the stringency of resistance analysis and provide insights into viable resistance mutations arising from combination therapy. This type of *in vitro* analysis remains to be done and would provide important information in therapeutic design. We have developed a tool for compound analysis that has a wide range of applications in the field of HCV compound development that could easily be adapted to facilitate evaluation of putative antiviral agents targeting other viruses in similar systems.

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Appendix

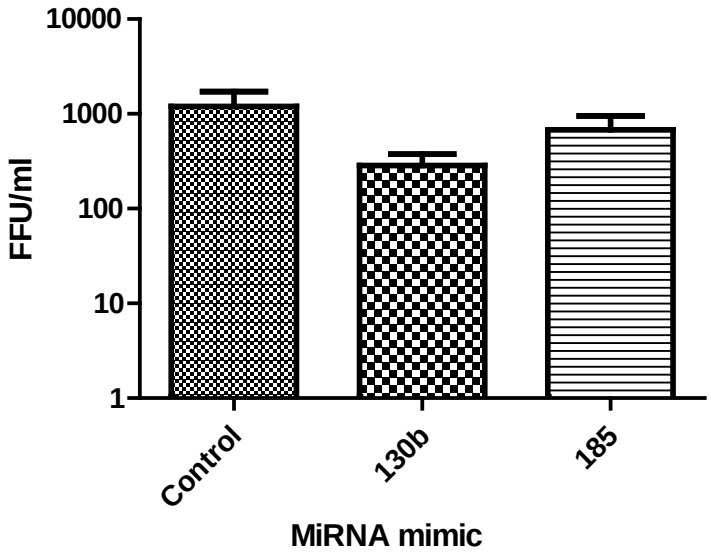

Figure A1. miRNA Mimics Downregulate Infectious Virus Production. miRNA mimics were transfected into Huh-7.5 cells and infected with $JFH1_T$ at an MOI of 0.1 24 hours post-transfection. 48 hours post-infection supernatants were collected, filtered and subjected to an FFU assay, as described in Chapter 2.