Mechanisms of Action and Resistance to Novel Inhibitors of the Hepatitis C Virus RNA-Dependent RNA Polymerase

by

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To my loving Anna & Ajji,

To my Baba, Mamma & Aniket,

To my Mihir,

For their unconditional support and strength

I love you all from the bottom of my heart

ABSTRACT

The hepatitis C virus (HCV) is a positive-sense RNA virus that encodes a non-structural protein, NS5B, which is an RNA-dependent RNA polymerase. NS5B is required for replication of the viral genome, making it an attractive target for antiviral drug development efforts. NS5B inhibitors are classified into two major categories: nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs). NIs bind to the enzyme active site and compete with the natural NTP for incorporation. In contrast, NNIs bind away from the active site and inhibit the polymerase activity allosterically, specifically during initiation of RNA synthesis by introducing conformational changes in the polymerase.

The NS5B polymerase lacks proofreading ability, which results in a high rate of mutations during replication. This error prone nature of the HCV polymerase has given rise to a number of variants of the virus which are classified into different genotypes and subgenotypes. Different HCV genotypes show variations in drug susceptibility, particularly to NNIs, which limits their clinical utility. At least four distinct binding sites for NNIs have been identified on the NS5B protein and these binding sites are not necessarily conserved across the genotypes. This provides a possible explanation for the observed variations in drug susceptibility. As part of the first study described in this thesis, we tested the inhibitory activity of acyl pyrrolidine and 1,5-benzodiazepine against purified NS5B enzymes that represent the major HCV genotypes. We identified natural amino acid substitutions that contribute to resistance to the NNIs.

In the second study described herein, we turned our attention to the mechanisms of action and resistance to NIs, with focus on sofosbuvir, which is part of the current standard of care in anti-HCV therapy. Previous crystallographic data of HCV NS5B in complex with an RNA

primer-template has provided important information on the protein-nucleic acid interface during the elongation process. The structure points to specific interactions between residues of the nucleic acid binding channel and the 2'-hydroxyl group of the bound RNA substrate. Using templates with strategically engineered DNA-like residues, we examined the role of the 2'-hydroxyl group of the template strand in nucleotide incorporation. Our biochemical findings suggest that this 2'-hydroxyl group plays an essential role in establishing resistance to NIs, and this process may be mediated by the signature S282T mutation, which is been known to cause resistance to sofosbuvir.

In the third study, we further evaluated the efficacy of novel NIs against known NI resistance-conferring mutations in NS5B. The compounds 2'F-2'-C-Me-UTP (sofosbuvir), 2'-C-Me-2'-NH_2-UTP, 2'-C-Me-UTP and 2'-C-Me-(1-Thio)UTP, which were modified derivatives of sofosbuvir, were tested against mutations S282T, L159F, C316N and L320F. It was observed that S282T showed resistance to all the modified compounds. Mutations L159F, C316N and L320F showed sensitivity towards 2'-C-Me-UTP. WT NS5B, L159F, C316N and L320F showed increased IC_{50} values towards 2'-C-Me-2'-NH_2-UTP and 2'-C-Me-(1-Thio) UTP suggesting that addition of NH_2 and Thio groups do not increase the efficacy of the compounds.

Overall, these studies provide a more detailed understanding of mechanisms of action and resistance to Nucleoside and Non-nucleoside inhibitors of the HCV polymerase. Our findings offer new avenues in current efforts to develop new viral polymerase inhibitors.

RÉSUMÉ

Le virus de l'hépatite C (VHC) est un virus à ARN positif qui exprime une protéine virale non-structurale, l'ARN polymérase ARN-dépendante NS5B. Cette polymérase est requise pour la réplication du génome viral, ce qui en fait une cible attrayante pour les efforts de développement de médicaments antiviraux. Les inhibiteurs de NS5B sont classés en deux grandes catégories: les inhibiteurs nucléosidiques (INs) et les inhibiteurs non-nucléosidiques (INNs). Les INs se lient au site actif de l'enzyme et compétitionnent avec le NTP naturel pour incorporation. En revanche, les INNs se lient hors du site actif et inhibent l'activité de la polymérase par allostérie, spécifiquement lors de la phase d'initiation de la synthèse de l'ARN en provoquant des changements de conformation dans la polymérase.

NS5B est dépourvue d'activité de relecture, ce qui résulte en un taux élevé de mutations lors de la réplication. Cette nature sujette aux erreurs de la polymérase du VHC a généré nombres de variantes du virus qui sont classées en différents génotypes et sous-génotypes. Les différents génotypes du VHC présentent des variations dans leur sensibilité aux médicaments, particulièrement avec les INNs, ce qui limite leur utilité clinique. Au moins quatre sites de liaison des INNs ont été identifiés sur la protéine NS5B et ces sites ne sont pas nécessairement conservés à travers les génotypes. Cela représente un possible mécanisme pour expliquer les variations observées dans la sensibilité aux médicaments. Dans le cadre de la première étude décrite dans cette thèse, nous avons testé l'activité inhibitrice de l'acyl pyrrolidine et du 1,5-benzodiazépine contre des enzymes NS5B purifiées représentant les principaux génotypes du VHC. Nous avons ainsi identifié des substitutions naturelles d'acides aminés qui contribuent à la résistance aux INNs.

Dans notre seconde étude, nous nous sommes intéressés aux mécanismes d'action et de résistance aux INs, en particulier pour le sofosbuvir, qui fait partie de la norme actuelle de soins dans le traitement du VHC. Les précédentes données cristallographiques de la protéine NS5B du VHC en complexe avec une amorce-matrice d'ARN fournissent des informations importantes sur l'interface protéine-acide nucléique lors de la phase d'élongation. Cette structure indique des interactions spécifiques entre des résidus du canal de liaison à l'acide nucléique et le groupe 2'-hydroxyle du substrat ARN lié. En utilisant des matrices stratégiquement conçues avec des résidus semblables à l'ADN, nous avons examiné le rôle du groupe 2'-hydroxyle du brin matrice dans l'incorporation des nucléotides. Nos données biochimiques suggèrent que ce groupe 2'-hydroxyle joue un rôle essentiel dans l'établissement de la résistance aux INs et que ce processus pourrait être médié par la mutation signature S282T, connue pour causer de la résistance au sofosbuvir.

Dans la troisième étude, nous avons examiné de manière plus approfondie l'efficacité de nouveaux INs contre des mutations dans la protéine NS5B connues comme conférant une résistance aux INs. Le 2'F-2'C-Me UTP (sofosbuvir), le 2'-C-Me-2'-NH_2-UTP, le 2'-C-Me-UTP et le 2'-C-Me-(1-Thio)UTP, qui sont des dérivés modifiés du sofosbuvir, ont été testés contre les mutations S282T, L159F, C316N et L320F. La mutation S282T a montré de la résistance à l'ensemble des composés modifiés. Les mutations L159F, C316N et L320F ont montré de la sensibilité envers le 2'-C-Me-UTP. De plus, NS5B WT, L159F, C316N et L320F ont montré une augmentation des valeurs IC_{50} pour le 2'-C-Me-2'-NH_2-UTP et le 2'-C-Me-(1-Thio) UTP, suggérant que l'addition du NH_2 et du groupe Thio n'augmente pas l'efficacité de ces composés.

En conclusion, ces études fournissent une compréhension plus détaillée des mécanismes d'action et de résistance aux INs et aux INNs de la polymérase du VHC. Nos résultats offrent de

nouvelles avenues dans les efforts actuels pour développer de nouveaux inhibiteurs de la polymérase virale.

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PREFACE

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation'. The candidate has chosen to present in a "Manuscript-based thesis" format following these recommendations:

"Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin size and must be bound together as an integral part of the thesis".

The information with regard to the submitted and published articles, such as journal name and date of publication, appears on the title page of each chapter. The candidate is the first author of all three manuscripts and the contributions of all authors to each article are stated in the contributions of authors section. The publications to which the candidate had a significant contribution are listed below:

Manuscripts presented in this thesis:

- **Anupriya Kulkarni**, Jean Bernatchez, Matthias Götte. Biochemical Characterization of Genotype Dependent, Natural Resistance to Non-Nucleoside Inhibitors (NNIs) of HCV NS5B. *In preparation.*
- **Anupriya S. Kulkarni,** Masad J. Damha, Raymond F. Schinazi, Hongmei Mo, Brian Doehle, Selena M. Sagan and Matthias Götte. Complex interactions between S282 and G283 of HCV NS5B with the template strand affect susceptibility to Sofosbuvir and Ribavirin. *In preparation.*
- **Anupriya S. Kulkarni**, Matthias Götte. Effect of Mutations S282T, L159F, C316N and L320F in HCV NS5B on Inhibition of RNA Synthesis by 2'-C-methylated Nucleotides. *In preparation.*

Other manuscripts not included in this thesis, but to which a significant contribution was made by the candidate, are listed as follows:

- Martínez-Montero S, Deleavey GF, Kulkarni AS, Martín-Pintado N, Lindovska P, Thomson M, González C, Götte M, Damha MJ. Rigid 2',4'-difluororibonucleosides: synthesis, conformational analysis, and incorporation into nascent RNA by HCV polymerase. Journal of Organic Chemistry. 2014

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Chapter 2:

Biochemical Characterization of Genotype Dependent, Natural Resistance to Non-Nucleoside Inhibitors (NNIs) of HCV NS5B. Anupriya Kulkarni, Jean Bernatchez, Matthias Götte.

- *A.S. Kulkarni conducted all the experiments and wrote the manuscript under supervision of M. Götte. J. Bernatchez provided modeling figures.*

Chapter 3:

Complex interactions between S282 and G283 of HCV NS5B with the template strand affect susceptibility to Sofosbuvir and Ribavirin. Anupriya S. Kulkarni, Masad J. Damha, Raymond F. Schinazi, Hongmei Mo, Brian Doehle, Selena M. Sagan and Matthias Götte.

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Chapter 4:

Effect of Mutations S282T, L159F, C316N and L320F in HCV NS5B on Inhibition of RNA Synthesis by 2'-C-methylated Nucleotides**. Anupriya S. Kulkarni**, Matthias Götte.

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List Of Abbreviations

AH	amphipathic helix
ALT	alanine transaminase
cDNA	complementary DNA
CLDN-1	claudin-1
C	core
Cyp A	cyclophilin A
DAA	direct acting antiviral
DC	dendritic cells
DC-SIGN	dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DNA	deoxyribonucleic acid
ds	double-stranded
E1	envelope protein 1
E2	envelope protein 2
ECMV	encephalomyocarditis virus
ER	endoplasmic reticulum
FDA	Food and Drug Administration
GBV-C	GB-virus type C
GDD	glycine-aspartic acid-aspartic acid
GFP	green fluorescent protein
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus

HCVcc	infectious cell culture-produced HCV
HIV-1	human immunodeficiency virus-1
hnRNP	heterogeneous nuclear ribonucleoprotein
Huh-7	Human hepatoma 7
IMPDH	inosine monophosphate dehydrogenase
IRES	internal ribosomal entry site
JFH	Japanese fulminant hepatitis
kDa	kilodaltons
LDL	low density lipoprotein
LNA	locked nucleic acid
L-SIGN	liver-specific intercellular adhesion molecule 3-grabbing nonintegrin
miRNA	microRNA
NANB	non A non B
NS	non-structural
NS5B	non-structural protein 5B
NI	nucleoside inhibitor
NNI	non-nucleoside inhibitor
NTP	nucleotide triphosphate
OCLN	occluding
ORF	open reading frame
PT	post-transfusion
PPi	pyrophosphate
PPI	peptidyl-prolyl *cis-trans* isomerase
PEG	polyethelyne glycol

RC	Replication Complex
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RMP	ribavirin monophosphate
RDP	ribavirin diphosphate
RT	reverse transcriptase
RTP	ribavirin triphosphate
ss	single-stranded
SR-B1	scavenger receptor class B type 1
SVR	sustained virological response
UTR	untranslated region
VLDL	very low density lipoprotein

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Hepatitis C virus (HCV) is a major global health concern that was identified 25 years ago (1). It infects more than 170 million people worldwide (3% of the global population), including more than 250,000 Canadians (2-4). The virus has a significant impact on global health with an estimated 4 million new cases of HCV infection occurring each year (1, 5). Infection is often asymptomatic in the acute phase but once established, 75-85% of individuals are at a risk of developing chronic hepatitis and serious liver complications such as cirrhosis and hepatocellular carcinoma (6, 7). HCV infection is also the leading cause of liver transplant in developed countries (7). While there is no prophylactic vaccine for prevention of Hepatitis C, a number of advances have been made towards providing effective treatment options. In this chapter, I provide a comprehensive review of HCV that describes its discovery, epidemiology, life cycle and molecular virology, model systems and advances in therapy and drug targets. The major focus will be on the HCV non-structural protein 5B (NS5B) and the antiviral compounds that target this viral protein, which is the major theme of the thesis research.

1.1 Discovery and Epidemiology of HCV

With the development of diagnostic assays for hepatitis A virus (HAV) and hepatitis B virus (HBV) in 1970, it was recognized that there was an additional viral hepatitis of unknown cause, which was designated non-A, non-B (NANB) hepatitis (8). The majority of post-transfusion (PT) hepatitis in the 1970s was NANB type and the etiological agent of PT – NANB remained elusive until the late 1980s (1). With the development of powerful molecular techniques, the causative

agent of PT–NANB hepatitis was identified (9). Investigation of the biophysical properties of the responsible viral agent included sensitivity to organic solvents and passage through filters with 80 nm pore size, suggesting that PT–NANB hepatitis was caused by a small enveloped virus (10, 11). The major breakthrough in the identification of the elusive virus occurred in 1989 when Choo and colleagues used antibodies from an infected patient to detect virus-specific proteins *in vitro* (2). Nucleic acid was extracted from the plasma of chimpanzees experimentally infected with NANB hepatitis and subjected to reverse transcription for the production of complementary DNA (cDNA). Restriction enzyme fragments of the cDNA were inserted into a bacteriophage expression vector (gt-11) and the expressed proteins were analyzed by comparing with proteins expressed from serum obtained from HCV-infected patients (2). As a result, the etiological agent of PT–NANB hepatitis was identified and named hepatitis C virus (HCV). The HCV genome was sequenced and was classified as a member of the *Flaviviridae* family. This family also includes blood-borne viruses such as yellow fever virus, West Nile virus, dengue virus, tick-borne encephalitis virus, bovine diarrhea virus, and GB-virus C (12). Vaccines exist for some members of the *Flaviviridae* family like yellow fever virus and tick-borne encephalitis virus; however, in the case of HCV, the multiple genotypes/subtypes and the hyper-mutability of the virus have hampered vaccine development (13). Risk factors for HCV infection include intravenous drug use, receiving blood transfusion prior to 1992, receiving organ transplantation prior to 1987, body tattoos, intranasal cocaine use, and unprotected sexual activity (14).

HCV exhibits a high degree of genetic diversity, and there are seven major genotypes and several subtypes of the virus (15). HCV genotypes differ 30-35% at the nucleotide level, and differences between subtypes within a single genotype are approximately 15% (15, 16).

Distribution of the HCV genotypes varies worldwide (17) (Figure 1.1). Genotypes 1, 2, and 3 show relatively broad geographical distribution, whereas genotypes 4, 5, 6 and 7 are more confined to specific geographic regions (17). Genotype 1 is the most common variant worldwide, accounting for ~46% of all HCV cases (17). Genotype 3 is the second most prevalent accounting for ~30% of cases and is highly represented in South Asia (18). Genotypes 2, 4, and 6 account for ~22% of cases, and genotype 5 accounts for <1% of cases. HCV genotype 4 is localized mainly in Africa and the Middle East. In Africa, the highest prevalence of HCV infection has been reported in Egypt and Cameroon (19, 20). HCV infection rates in Egypt are about 10-13% of the total population due to improper sterilization techniques used during anti-schistosomal immunization campaigns that took place between 1960 to 1987 (4, 21). Genotype 5 is localized almost exclusively to South Africa, and genotype 6 is endemic to South East and East Asia. Elevated HCV prevalence is also documented in the elderly Italian population, which can be linked to the routine use of glass syringes for medical treatment in Italy until the late 1970s (22).

In HCV-infected patients, the virus exists as a population of non-identical but closely related quasispecies that arise from mutations accumulating over time, but are not sufficient to be considered different subtypes. These variants arise due to the error-prone nature of the HCV polymerase which lacks proof-reading activity (23). Furthermore, selective pressure exerted by antiviral therapy can influence the presence of these quasispecies, and the composition of the quasispecies within a given patient may also contribute to resistance to therapy. It has been observed that a low diversity in the quasispecies is associated with an increase in the rate of viral clearance (23).

Response to treatment is highly dependent on the genotype of the infecting virus (24). Hence, development of pan-genotypic anti-HCV therapy is a major priority in research. Advancements in HCV research have helped to elucidate the role of the HCV proteins and identified specific proteins as potential targets for antiviral intervention. The following sections examine the role of each component of the viral genome.

1.2 Molecular Virology of HCV

1.2.1 HCV Genome Organization and Viral Proteins

Genome Organization. The HCV genome is a 9.6 kb and is comprised of a 5'-UTR, a single long (~3000 amino acid) open reading frame (ORF), and a 3'-UTR (25). The HCV genome is exposed within the cytoplasm as a positive-sense RNA virus, the genome is directly translated by the ribosome yielding the mature structural (C, E1, E2, p7) and NS (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins (Figure 1.2) (26-30).

5'UTR and 3'UTR. The 5' UTR is highly conserved among different HCV isolates and contains an internal ribosomal entry site (IRES) which is essential for cap-independent translation of the viral RNA (31). The complete 5' UTR consists of approximately 342 nucleotides and is one of the most conserved regions of the genome across the different HCV genotypes (32). The 5' UTR contains the IRES which plays an important role in initiation of translation of the HCV genome. The 5' UTR is composed of four highly ordered domains numbered I through IV (25, 33). Domain I is required for RNA replication, domains III and IV are required for protein

translation, while domain II is involved in both of these processes (34). The HCV IRES can recruit ribosomes and initiate translation independently of the ribosome scanning process involved in canonical translation initiation (35). The 60S ribosomal subunit associates with the 40S subunit to form the 80S complex that translates the ORF into a single polyprotein of ~3,000 amino acids in length (36, 37). Furthermore, it has also been shown that an abundant liver-specific microRNA (miRNA), miR-122, binds to the 5' UTR and enhances viral RNA accumulation in cell culture and in the liver of infected patients (38, 39).

The 3' UTR contains a variable region, a poly-U/UC tract, and a highly conserved 98 nucleotide X-tail (40). The variable region is approximately 40 nucleotides in length and is poorly conserved across HCV isolates (41, 42). It is predicted to form two stem-loop structures that promote efficient replication since deletion of this region leads to impaired RNA replication (43, 44). The poly-U/UC tract consists of a homopolyuridine stretch interspersed by single cytosine residues (45). It is hypothesized to provide a platform for recruitment of host or viral proteins essential for viral RNA replication (43, 46). The 98 nucleotide X-tail contains 3 stem-loop structures essential for HCV RNA replication (43, 44). The X-tail is the main *cis*-acting RNA element required for negative-strand synthesis and is highly conserved across HCV isolates (42, 45).

Core (C) Protein. Core is the first structural protein encoded by the HCV ORF and forms the viral nucleocapsid (47). It is a multi-functional acidic protein implicated in virus-mediated pathogenesis. The core protein is localized to the endoplasmic reticulum (ER) membrane, in the membranous web and on the surface of lipid droplets (48). The membranous web is a structure

formed by the HCV viral proteins in conjunction with the host factors, and is composed of single-, double-, and multi-membraned vesicles as well as lipid droplets (49). Based on a multitude of cellular ligands, the core protein is associated with various processes beyond nucleocapsid formation and viral morphogenesis, such as interference with lipid metabolism, suppression of the host immune response, and modulation of cellular gene transcription, proliferation, and cell death (50-53).

Envelope glycoproteins (E1 and E2). HCV encodes two envelope glycoproteins, E1 and E2, which are processed by the host signal peptidase and serve as the building blocks for the viral envelope (54). Both E1 and E2 are glycoproteins and contain 4-5 and 11-12 glycosylation sites on their N-termini, respectively (55). When expressed together, E1 and E2 are retained in the ER and form non-covalent heterodimers (56). The E2 protein contains two hypervariable regions that serve as mutational hotspots and have been found to contain extreme sequence variability (57). Epitopes localized to these regions evolve rapidly, and while they are recognized by the adaptive immune system, the mutant epitopes help HCV to evade the host immune response (58). The E1, E2-heterodimer is important for viral entry and mediates interactions with the cellular receptors like CD81 and SR-B1. Claudin and Occludin are also known to be critical for HCV entry. (59-61). In addition to their role in viral entry, assembly, and morphogenesis, both E1 and E2 have been extensively investigated in order to understand their antigenic variation, immune evasion capacity, as well as for their potential as vaccine candidates (62).

p7. The p7 protein is a hydrophobic peptide that is often incompletely cleaved from the E2 protein by signal peptidase (63). It has two transmembrane segments connected by a short cytoplasmic loop, and its N- and C-termini are oriented towards the ER lumen (64). Although not required for RNA replication, p7 is essential for the production of infectious virions both *in vitro* and *in vivo* (65). Structural and membrane-permeability evidence indicate that p7 has cationic ion channel activity and belongs to the viroporin family (66). With its crucial function in virion maturation and release, p7 may serve as a novel target for anti-HCV therapy.

NS2-3 protease. The NS2 protein is a cysteine protease that forms a complex with the NS3 protein, to form an autoprotease that cleaves the NS2/3 junction (67-69). The catalytic activity of the NS2-3 protease resides in the C-terminal half of the NS2 and the N-terminal one-third of the NS3 protein (69, 70). Site-directed mutagenesis has revealed that amino acids His143, Glu163, and Cys184 are essential for NS2's proteolytic activity (69). NS2-3 is not required for replication of subgenomic replicons, but it is important for the complete life cycle in order to release the NS3 serine protease. Therefore, the NS2/3 junction cleavage represents a pivotal step in HCV replication by allowing downstream polyprotein processing to occur. NS2 has also been shown to interact with the envelope proteins and play an important role in virion assembly (71).

NS3-4A protease and helicase. NS3 is a multifunctional protein, with a serine protease located in the N-terminal one-third and a RNA helicase/NTPase located in the C-terminal two-thirds of the protein (72). Both of these enzymes have been well-characterized and high resolution structures have been solved (73). The NS3 serine protease domain (N-terminal 189 amino acids)

is responsible for the downstream cleavage of the subsequent NS proteins (NS3/4A, 4A/4B, 4B/5A, 5A/5B junctions) (74-76). NS3 associates with NS4A, which is a cofactor (8 kDa) that stabilizes and activates the protease (77, 78). In addition, crystal structures of the NS3-4A complex revealed that NS4A is an important part of the core enzyme and helps in directing and anchoring NS3 to the ER membrane via an N-terminal transmembrane domain (79). NS3 also inhibits innate immune signaling in infected cells via cleavage of mitochondrial antiviral signaling protein (MAVS) and TIR-domain-containing adapter-inducing interferon-β (TRIF) which are downstream effectors in the retinoic acid inducible gene-I and toll-like receptor-3 pathways, respectively (80-82). This leads to attenuation of the cellular interferon response (83). Thus, since the NS3 protein has a major influence on both the virus and the host, it is an attractive drug target for antiviral development.

The crystal structure of the NS3/4A protein revealed that the NTPase/helicase domain is a member of the 2DExH/D box helicase superfamily (84). This enzyme displays several functions including: unwinding double-stranded (ds) RNA replication intermediates, unwinding single-stranded (ss) RNA with extensive secondary structures, and separation of the viral RNA genome from RNA binding proteins (84). The NS3/4A is thus an essential component of the HCV replication complex (RC) (85).

NS4B. NS4B has been difficult to study due to its high hydrophobicity (86). NS4B harbors an amphipathic helix at its N-terminus, which targets the protein to the ER membrane. The NS4B protein is an essential component of the HCV RC (87) and contains a functional nucleotide binding motif that binds to and hydrolyzes GTP (88). Although the functions of this activity are

not clear, mutations that abrogate GTP binding as well as GTPase activity have been found to dramatically reduce HCV replication (88, 89).

NS5A. NS5A is a membrane-associated zinc metalloprotein whose function in the viral life cycle is not well understood (90). Amino acids in the zinc binding motif of NS5A chelate zinc and allow the protein to form homo- and heterodimers, and also to bind RNA (91). It is found in two forms: basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) (92). The phosphorylation state of NS5A is suggested to mediate HCV replication efficiency, presumably via interactions with host regulatory factors or other HCV NS proteins (93-96). NS5A is tethered to the membrane via an amphipathic helix localized to domain I of its N-terminal region (97-99). Apart from domain I, two other structurally distinct domains (II and III), are found in NS5A; however, their functions are unknown (100). Despite its lack of enzymatic activity, NS5A appears to be a promising target for antiviral intervention and potent inhibitors with a high genetic barrier to resistance are currently in clinical trials (discussed in more detail in section 1.4.4 HCV therapy) (101).

NS5B. NS5B is the RNA-dependent RNA-polymerase (RdRp) of HCV. It is a 68 kDa protein which is crucial for HCV genome replication (25, 33). The NS5B sequence is highly conserved among all HCV isolates, as well as other members of the *Flaviviridae* family (102). This protein is present in the cytosol and is anchored to the cytosolic side of the ER membrane via its C-terminal 21 amino acid transmembrane helix (103). NS5B serves as the catalytic core of the HCV RC, and due to its crucial role in RNA replication, it serves as an attractive target for drug

development. As the NS5B protein is the major focus of this work, the structural features, expression, enzymatic activity, and interaction with other viral and host factors as well as efficacy of anti-NS5B compounds is discussed in more detail in subsequent sections (Section 1.5 Inhibitors of HCV NS5B).

1.2.2 HCV Virion Structure and Life Cycle

Virion Structure. Electron microscopy and ultrafiltration studies have demonstrated that the virus is about 60 nm in diameter and spherical, with a lipid envelope and fine peplomers, containing the two viral envelope glycoproteins, E1 and E2 (104). The envelope surrounds the viral nucleocapsid, which assumes an icosahedral shape composed of HCV core protein units. The viral nucleocapsid contains the positive-sense, single-stranded viral RNA genome (105).

Attachment and Viral Entry. Attachment is the first step of the viral life cycle, which is facilitated by receptor mediated endocytosis (Figure 1.3-1). The virus circulates within the infected host as a lipoviral particle in association with low density lipoprotein (LDL) or very low density lipoprotein (VLDL), or as naked virions (106, 107). HCV infection primarily occurs in hepatocytes, but infection has been described in other cell types such as B cells and dendritic cells (DC) (108, 109). Data showing productive infection of DC cells is lacking; however, productive infection of the B cells has been demonstrated through detection of negative-strand HCV RNA, indicative of active HCV replication (110, 111).

The virions are thought to be captured from the plasma by two lectins, DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin) and the related L-SIGN (liver-specific intercellular adhesion molecule 3-grabbing non-integrin), on the liver sinusoidal endothelial cells, and guided towards the hepatocytes (112, 113). The glycosaminoglycan (GAG), heparan sulfate, then acts in an initial attachment step along with the LDL receptor (114, 115). HCV entry into host hepatocytes requires interactions with several additional cellular receptors which include scavenger receptor SR-B1, the tertaspanin CD81, the tight junction protein claudin-1 (CLDN-1), and occludin (OCLN) (114, 116-118). Binding of E1 and E2 to the hepatocyte triggers clathrin-mediated endocytosis and allows the virion to enter the host cell (Figure 1.3-2) (119). The acidic environment of the endosome leads to membrane fusion by exposing new epitopes on the E2 protein which favors membrane fusion and releases the viral RNA genome into the cytoplasm (Figure 1.3-3) (119, 120).

Viral Translation and Polyprotein Processing.

The viral nucleocapsid liberates the positive-strand genomic RNA into the cell cytoplasm, where it serves as mRNA for synthesis of the HCV polyprotein. Translation of the HCV ORF yields a polyprotein precursor that is processed by cellular and viral proteases into the mature structural and non-structural proteins, respectively (Figure 1.3-4). The structural proteins include the highly basic C protein, the two glycoproteins (E1 and E2) and small integral membrane protein, p7. The cellular signal peptidase embedded in the endoplasmic reticulum (ER) membrane cleaves the junctions between core-E1, E1-E2, and E2-p7, while further cleavage by signal peptide peptidase is required for cleavage at the core-E1 and p7-NS2 protein

junction (121). The remainder of the polyprotein constitutes the NS proteins: NS2, NS3, NS4A, NS4B, NS5A and NS5B. The NS proteins are processed by two viral proteases: the NS2-3 cysteine protease cleaves the NS2-3 boundary and the NS3-4A serine protease, which cleaves the boundaries between all of the remaining NS proteins (28).

Viral RNA Replication: The positive-strand viral RNA genome serves as a template for negative-strand synthesis, which in turn serves as a template for synthesis of positive-strand viral RNA genomes (Figure 1.3-5). The HCV RC is composed of the NS3/4A, NS4B, NS5A, and NS5B proteins, as well as several host factors (122). NS5B is the RdRp responsible for RNA synthesis and replication of the HCV genome.

Initiation of RNA replication is thought to be dependent on the RNA elements found in the 3'UTR of the HCV genome (123). These elements include the three stem-loops (SL) of the X-tail (SL1, SL2, and SL3) and RNA elements in the NS5B coding region. Chemical and enzymatic probing combined with secondary structure prediction has demonstrated that the NS5B coding region consists of six SL structures: 5BSL1, 5BSL2 and 5BSL3 further divided into four structures (5BSL3.1, 5BSL3.2, 5BSL3.3, 5BSL3.4) (124-126). 5BSL3.2 is known to be essential for RNA replication (126). The upper loop of 5BSL3.2 contains a highly conserved CACAGC nucleotide sequence which is able to interact with the loop region of SL2 in the X-tail (127, 128). This results in the formation of a pseudoknot, also known as the "kissing-loop" interaction, allowing the 3' end of the genome to form a closed loop (127). This interaction has been demonstrated to be important for RNA replication as mutagenesis of the 3' UTR abrogated RNA replication (127). This interaction has been hypothesized to facilitate NS5B binding to the

3' end, which allows initiation of the negative-strand RNA synthesis (123). In addition, a number of host factors including Cyclophilin, miR-122, and lipoproteins also play a role in HCV replication (129).

Virus Assembly and Release. Little is known about the final step of the viral life cycle (Figure 1.3-6,7). Core, p7, NS2 and possibly other NS proteins are involved in this process (71). Within the intracellular vesicles, virions undergo morphogenesis and maturation. The virions are then secreted from the ER via utilization of the VLDL assembly pathway (130). The secretory vesicles containing the mature virion fuse with the plasma membrane releasing the mature virions from the cell (106). Much remains to be determined regarding the early (entry and uncoating) as well as the late stages (assembly and budding) of the viral life cycle; however, with the development of HCV model systems, every step of the viral life cycle can now be studied in detail.

1.2.3 HCV Model Systems

Previously, the lack of cell culture-based and small animal model systems was a major limitation in hepatitis C research. However, the emergence of cell culture-based replicon systems in 1999 enabled the study of viral replication, and the pseudoparticle systems enabled the study of viral entry (131). Furthermore, the Japanese fulminant hepatitis (JFH)-1 system, which is fully infectious, enabled study of the entire viral life cycle in cell culture (132). Recently, advances have also been made in genetically modified mouse models that hold significant promise for large scale *in vivo* studies (133, 134)

Cell culture systems for the study of HCV.

Hepatocytes are the primary target of HCV *in vivo*. However, *in vitro* infection of hepatocytes support only a low level of viral replication making it difficult to study the viral life cycle (135). This limitation was conquered by the development of subgenomic replicon systems for HCV in 1999, which supported measurable levels of replication (131). These subgenomic replicons lack the viral C, envelope (E1 and E2), p7, and NS2 proteins and thus are unable to produce virions, but can be translated; and, since they contain all the essential proteins of the RC, are able to undergo viral RNA replication in cell culture. The first replicon to be studied was the Con1 isolate (genotype 1b) (131). In this system, the C, E1 and E2, p7 and NS2 genes of the virus were replaced by a neomycin phosphotransferase gene which confers resistance to the antibiotic G418, and acts as a selectable marker (131). In this replicon, translation of the neomycin phosphotransferase gene is regulated by the HCV IRES, which is located the 5'-untranslated region (UTR) of HCV (Figure 1.4). Translation of the HCV non-structural (NS) genes is under control of the encephalomyocarditis virus (ECMV) IRES. Reporter genes such as luciferase are also included in the replicon (Figure 1.4 A, B, C) (131). Replicon activity is also enhanced by cell culture adaptive mutations (136).

Although the replicon system is effective in studying HCV replication, it does not recapitulate all the stages of the viral life cycle and hence is not useful for studying viral entry or packaging since it lacks the structural proteins that make up the HCV particle. In 2005, Wakita *et al.* developed a genotype 2a replicon (JFH-1) (132). Full-length JFH-1 RNA sequences were shown to stably replicate to high titres and generate infectious progeny in cell culture (HCVcc) when introduced into Human hepatoma 7 (Huh-7) cells (132). HCVcc was demonstrated to be infectious in chimpanzees and in mice with chimeric human livers (137). Together, these

systems have greatly enhanced our understanding of the HCV replication and pathogenesis. Efforts are now being focused on developing infectious cell culture systems for additional HCV genotypes in order to better understand the effects of antiviral treatments across HCV genotypes.

Animal models for the study of HCV.

Chimpanzees were the first model organisms used to study HCV infection, and played an important role in the discovery of the virus (138). HCV infection in chimpanzees physiologically mimics infection in humans, which makes them a useful model for HCV infection and disease pathogenesis (139). A major limitation to the use of chimpanzees as a model organism is the high cost of these studies, as well as the infrastructure required for maintaining primate research facilities (140). Ethical issues with the use of non-human primates in research is also a concern, and recent regulatory decisions in this regard have limited the use of chimpanzees to vaccine trials (141).

Significant advances have been made in small animal-based model systems for HCV. The most significant of these is the development of humanized mouse models for HCV infection. A major advantage with the use of murine models is that they are relatively inexpensive, highly scalable, and also open to genetic manipulation (134). Although mouse models have been extensively used to study human disease, HCV does not infect mice, thus severely limiting their use in HCV research. Subsequently, immunocompromised mice, such as the severe combined immunodeficient (SCID) mice, are extensively used in research, particularly in xenotransplantation studies where human cells and tissues are grown in mice (142). In 2001, it was shown that human hepatocytes could be engrafted in SCID mice carrying a plasminogen

activator transgene (*Alb-uPA*) (143). These mice had chimeric human/mouse livers, and could be infected with HCV (144). This was the first murine model which could support the entire HCV life cycle. However, a major limitation of this model is that the mice lack an effective immune response. This is significant since HCV pathology is closely associated to the immune response to the virus in the liver, which leads to chronic hepatitis, steatosis, cirrhosis and hepatocellular carcinoma.

Immunocompromised mice, which can be engrafted with human hepatocytes, have enabled the study of HCV entry in small animal models. (134). Recently, it was shown that immunocompetent transgenic mice expressing human CD81, and Occludin could be infected with HCV (133). Hence, the complete HCV life cycle could be recapitulated in this mouse model, and these mice remained infectious for 12 months post inoculation (145). This mouse model mimics human HCV infection and pathology, including HCV-associated fibrosis and cirrhosis. Thus, this humanized mouse model may provide an attractive alternative to the use of chimpanzees for *in vivo* testing of anti-HCV inhibitors. Together, these model systems have played an important role in elucidating functions of the HCV proteins in the viral life cycle, and have also helped in antiviral discovery efforts (discussed in more detail below).

1.3 HCV NS5B

1.3.1 NS5B is an RNA-dependent RNA polymerase.

Among the HCV-encoded proteins, NS5B has been characterized as the viral RdRp, which is a key enzyme for RNA synthesis and genome replication (146). In its active site, it contains the

glycine-aspartic acid-aspartic acid (GDD) sequence motif which is fully conserved in all HCV isolates as well as in polymerases of the *flaviviruses*, poliovirus and tobacco mosaic virus (147). *In vitro* biochemical assays have shown that NS5B functions processively on RNA templates during synthesis of the RNA transcript(s) (146). It does not contain DNA-dependent RNA polymerase or RNA-dependent DNA polymerase (reverse transcriptase) activity (148, 149). However, NS5B can incorporate nucleotides onto chimeric RNA-DNA primer templates (148, 149).

1.3.2 Structural Features of NS5B.

The three-dimensional crystal structure of NS5B has been solved independently by three groups (150-152). Like many other viral polymerases, NS5B resembles a typical right hand with palm, finger and thumb sub-domains (153, 154). A groove is present between the fingers and the thumb domain where the modeled primer-template duplex sits (152). The incoming nucleoside triphosphates (NTPs) enter into the active site located in the palm sub-domain via a specific NTP tunnel (155) (Figure 1.5).

The palm sub-domain contains the catalytic GDD motif which is highly conserved across all HCV isolates (152, 156). During polymerization, the aspartic acid residues are involved in the nucleotidyl transfer reaction. These residues interact with two divalent metal ions, and aid in nucleophilic attack of the 3'-hydroxyl group of the primer terminus or priming nucleotide in the α-phosphate of the incoming nucleotide. These metal ions may also be involved in the release of pyrophosphate (PPi) (157). Many polymerases like HIV-1 RT (human immunodeficiency virus-1 reverse transcriptase) have an open structure while the HCV NS5B adopts a compact globular

shape encircling the active site in the palm domain due to strong interactions between the fingers and thumb sub-domains (152). Other viruses, like rhinovirus, bacteriophage ϕ6, rabbit hemorrhagic disease virus, and bovine viral diarrhea virus (BVDV), contain a polymerase that has an enclosed active site (150-152, 158). The structure of both the free NS5B and the enzyme associated with short RNA substrates, suggests that NS5B must undergo substantial conformational changes to accommodate the newly synthesized dsRNA (150, 151, 159). The Δ1-loop of the finger domain is thought to facilitate the closed conformation of NS5B (155). This loop aids in making several contacts between the finger and thumb sub-domains. This interaction helps the two domains to collaborate with each other during RNA template translocation (151). Experimental data has suggested that substitution of leucine-30 in the Δ1-loop with serine or arginine prevents the thumb from forming the closed conformation and results in a nonfunctional enzyme (155).

Another important feature of the NS5B polymerase is the β-hairpin loop in the thumb sub-domain that protrudes towards the active site (Figure 1.5). This 12 amino acid sequence causes steric hindrance and interferes with binding to dsRNA (160). The position of the β-hairpin loop appears to be important for RNA binding, elongation, as well as the processivity of NS5B (161). While experimental data has elucidated the role of the various domains and amino acid residues of NS5B in the RdRp activity of the enzyme, it is important to further evaluate these interactions in the light of their implications on the development of HCV antivirals.

1.3.3 Recombinant Expression of NS5B

Recombinant protein expression is a practical method to study the activity of the NS5B polymerase. The first HCV polymerase to be expressed was an untagged full-length protein of HCV genotype 1b (146). It was expressed in *Spodoptera frugiperda* insect cells using the baculovirus expression system (162). The expressed and purified NS5B was found to catalyze RNA synthesis from both genomic and homopolymeric RNA templates (163).

Purification techniques were further improved using affinity chromatography by using *Escherichia coli* (*E.coli*) bacterial cells and histidine or glutathione-S-transferase affinity tags (149, 164). The histidine-tag was attached to either the N- or C-terminus of the protein; however, it was observed that attaching the histidine-tag to the N-terminus decreased the RdRp activity of NS5B compared to those with C-terminal tags (163, 165). Truncation of the C-terminus transmembrane tail helps to solubilize the protein and also permits better expression compared with full-length NS5B (149, 165). Truncated versions of 21 (NS5BΔ21) and 55 (NS5BΔ55) amino acids from the C-terminus are commonly used in biochemical assays for NS5B activity (166).

1.3.4 Standard NS5B RdRp Assay

In vitro biochemical assays have helped us to understand and study the different properties of NS5B. Once expressed and purified, enzymatic activity can be measured with the incorporation of nucleotides onto an RNA template in the presence of divalent metal ions in the presence or absence of complementary primers. Initially it was thought that NS5B could conduct RNA synthesis only via elongation of a nucleotide primer by addition of NTPs to the 3'-

hydroxyl group of the primer (146). The 3'–end of the template can fold back and anneal to form a hairpin like structure, which can then be used as a primer for synthesis of the transcript (146). Subsequent experiments showed that like other positive-strand viral RNA polymerases, such as BVDV and bacteriophage ϕ6, NS5B is able to conduct *de novo* RNA initiation in a primer-independent manner (163, 167-169). *De novo* initiation of RNA synthesis starts with the use of a single nucleotide as a primer (153). Two NTP binding sites are used in the catalytic pocket: the first site is called the "I site", which recognizes the initiating NTP (NTP_i); and the second is called I+1 (NTP_{i+1}) site, where the nucleotide complementary to the second template nucleotide gets incorporated. *Flaviviral* RdRps prefer GTP as the initiating NTP with the 3'-hydroxyl end of the template contains a cytidine (168-171). Because of the presence of this cytidine at the 3'hydroxyl end, increased NS5B activity is observed with an increase in concentration of GTP as the NTP_i (159, 172-175).

RNA synthesis can be initiated by using short oligonucleotides that can replace the requirement for an initiating NTP_i (169). Dinucleotide (5'-GG-3') and trinucleotide (5'-GGG-3') primers have been used to study initiation and elongation during HCV as well as BVDV RNA synthesis (176). Studies have shown that *de novo* and short-primer based initiation have different nucleotide requirements (165). For *de novo* RNA synthesis, high concentrations of the first three incorporated NTPs are required, while 10^3-10^4-fold less of these NTPs are required for primer-dependent extension in the presence of di- or tri-nucleotides (165).

Besides template and NTP specifications, NS5B is sensitive to other experimental conditions. Polymerases require divalent metal ions for activity (177). Both Mn^{++} and Mg^{++} divalent metal cations support HCV RNA synthesis, which is increased by 4-20 fold when Mn^{++} is present in the reaction compared to that with Mg^{++} alone (165, 168, 169, 175). Other divalent

metal ions like Co^{++}, Cu^{++}, Ni^{++} and Zn^{++} do not support HCV RdRp activity (168, 175). It has also been shown that Fe^{++} is able to bind specifically to the Mg^{++} binding site of NS5B, thereby inhibiting RNA synthesis (178). Mn^{++} appears to improve the efficiency more specifically towards *de novo* RNA synthesis, while Mg^{++} improves the efficiency to primer-dependent RNA synthesis (175). Other conditions, such as a neutral pH environment and temperatures between 22ºC - 25ºC are preferred for the activity of HCV NS5B (179). When tested at 37ºC the enzyme seems to significantly lose activity (180). These conditions may not be reflected *in vivo* as Mg^{++} is more physiologically relevant compared to Mn^{++}, and the temperature in the host is 37ºC (175). In addition, although recombinant NS5B is capable of carrying out RNA synthesis, the enzyme added to the reaction is not 100% processive as less than 1% of the purified protein is active (181). This suggests that other viral and host factors may be required for efficient replication under physiological conditions.

1.3.5 Interaction of NS5B with Other Proteins

The HCV RC is thought to contain both host and viral proteins. HCV NS5B can form dimers and oligomers in solutions which cooperate to catalyze RNA synthesis (182, 183). Glutamic acid-18 present in the Λ1-loop and histidine-502 present in the thumb domain are found to be critical for NS5B oligomerization (182, 183). This formation of oligomeric sheets is similar to what is found in other viral polymerases such as those from poliovirus and rhinovirus (184). Pull-down experiments, co-localization, and yeast two-hybrids have demonstrated several interactions between NS5B and other HCV NS proteins (185). For example, NS5B binds to NS3 via the protease domain and this interaction increases the helicase activity of the protein,

suggesting that the HCV RdRp regulates the functions of NS3 during viral replication (186). Additionally, the NS4B protein is known to play an important role in formation of the membranous web at the ER, which is essential for formation of the RC (187). An interaction between NS5A with NS5B has also been shown to be essential for modulation of RdRp activity (96). Together these findings highlight the importance of the interaction of NS5B with other NS proteins for RNA synthesis and formation of the RC (175).

Several host proteins are involved in the RC and are vital for efficient viral replication (188). Studies have shown that cyclophilin A (Cyp A) functions as a cofactor for HCV infection with the peptidyl-prolyl *cis-trans* isomerase activity of CypA playing a key role in viral replication (189, 190). Heterogeneous nuclear ribonucleoproteins (hnRNPs) also interact with scaffold protein septin 6 and NS5B to form a complex playing a key role in viral replication (191). Several other host factors, such as nucleolin, RNA helicase p68, sphingomyelin and vesicle-associated membrane protein (VAMP)-associated proteins (VAP) subtype A and B directly or indirectly interact with NS5B to regulate viral RNA replication (192). Together, this suggests a key role for host factors in the life cycle of HCV. Targeting host factors with antiviral compounds may reduce the likelihood of occurrence of resistance mutations. However, on the other hand, targeting host factors may cause toxicity by interfering with normal cellular processes. Thus, expanding our knowledge of host-virus interactions and HCV pathogenesis is likely to better inform the development of new antivirals in future.

1.4 HCV Therapy

Since the identification of the virus, a number of therapeutics have been evaluated for treatment of chronic HCV infection. Acyclovir, a synthetic purine analog inhibitor, was the first antiviral agent to be tested for treatment of Hepatitis C in the clinic. However, this drug failed to show significant patient response (193). Subsequently, biologics such as Interferon-alpha, and drugs such as nucleoside analogues, protease inhibitors, as well as direct acting antivirals (DAAs) targeting other components of the HCV replication machinery have been used with varying levels of success in either mono- or combination therapy.

1.4.1 Interferon (IFN)

A beneficial role for recombinant IFN-alpha was first reported in a pilot study in 1986 (194). Subsequently, a number of clinical trials confirmed this finding (195-197). IFN is a cytokine that is naturally produced by cells, and plays an important role in the antiviral response. IFN-alpha has historically been used for treatment of a number of viral infections, including HBV and Herpes Simplex Virus (HSV) (198). In a series of studies published in 1989, treatment for 24 weeks with IFN-alpha was shown to be effective in treatment of chronic hepatitis C (195, 199). Although rates of relapse were shown to be relatively high in these studies (~50%), IFN-alpha was the only contemporary treatment available at the time that demonstrated efficacy against chronic HCV infection. These findings, along with various subsequent studies, led to the establishment of IFN-alpha as a base for all anti-HCV therapies and it remained a part of the standard of care for chronic HCV infection until recently (195, 199).

One of the limitations associated with the use of IFN-alpha was its short half-life (200). IFN-alpha has a half-life of 5-8 hours *in vivo*, and this necessitated frequent dosing and limited

the efficacy of the therapeutic (201). The covalent attachment of polyethylene glycol (PEG) to IFN-alpha was found to significantly enhance its stability and absorption. PEG-IFN-alpha was found to be significantly more effective than IFN-alpha alone in treating patients, as it increased the rate of sustained virological response (SVR) and required less frequent administration (202). SVR is defined as aviremia 24 weeks after completion of antiviral therapy for chronic HCV infection. These findings led to the replacement of IFN with PEG-IFN-alpha in the clinic (203). PEG-IFN-alpha monotherapy for 48 weeks led to SVR rates in approximately 39% in patients (204). Both IFN and PEG/IFN showed significant side effects which include headache, myalgia, arthralgia, depression, autoimmune diseases, and anemia. While the overall safety profile of IFN vs PEG-IFN was similar, some adverse effects such as influenza-like symptoms, pyrexia, and myalgia were less severe in patients treated with PEG-IFN (205). These side effects resulted in compliance issues with many patients not completing therapy. Thus, there was a pressing need for the development of less toxic and more effective HCV treatments.

1.4.2 PEG-IFN-alpha and Ribavirin.

Due to the lower SVR rates of viral clearance with PEG-IFN-alpha alone, the nucleoside inhibitor ribavirin was introduced in combination with PEG-IFN-alpha for treatment of chronic HCV (206). While ribavirin monotherapy failed to show benefits in the clinic (207), the combination of ribavirin with PEG-IFN-alpha was shown to cause significantly increased SVR rates, and 56% of patients treated with combination therapy (207). Ribavirin is a guanosine analog that can be incorporated by the NS5B into the viral RNA transcript (208). Although multiple mechanisms of action of ribavirin have been proposed (209); it is thought to act predominantly through an ‘error catastrophe’ mechanism whereby it acts as a viral mutagen

(210). Incorporation of ribavirin into viral transcripts increases the frequency of G to A mutations in the HCV genome resulting in non-functional viral transcripts (211). The combination of PEG-IFN-alpha and ribavirin was approved as the standard of care for chronic hepatitis C in 2002, and ribavirin remained a crucial part of the standard of care until recently with the advent of DAAs (212, 213). Despite the clinical benefits of the treatment, there were various limitations associated with IFN/ribavirin-based therapy. One limitation was the limited efficacy against genotype 1 infection (214). Only approximately 40% to 50% of genotype 1 patients responded to this therapy compared with 70% to 80% of genotype 2 or 3 patients (215). Moreover, ribavirin was associated with a number of side effects on top of those associated with IFN alone, including chest pain, nausea, and hemolytic anemia (216). Thus, the shortcomings of this treatment emphasized the need for the development of pan-genotypic HCV treatment options.

1.4.3 NS3/4A Protease Inhibitors.

Because of the severe side effects associated with the use of IFN, it was considered essential to develop IFN-free therapy for chronic HCV infection. The establishment of HCV subgenomic replicons for genotype 1 (Con1), and genotype 2 (JFH1) led to significant advances in the development of DAAs for HCV (213). Protease inhibitors, which target the NS3/4A protease have shown significant promise in treating HCV infection (217). In 2003, BILN 2061, also called Ciluprevir, was one of the first NS3 protease inhibitors and showed promising results in Phase II clinical trials by reducing HCV RNA plasma levels when given to genotype 1 patients (218). This drug was further discontinued as it showed cardiac toxicity in animal studies

(219). In 2011, the first generation NS3-4A protease inhibitors, boceprevir and telaprevir, became the first DAAs to be approved for treatment of chronic hepatitis C (220, 221). These protease inhibitors were used in combination therapy with PEG-IFN-alpha and ribavirin, and treatment was limited to genotype 1 patients as they did not provide significant benefit over ribavirin/PEG-IFN alone in the other genotypes (222). The addition of protease inhibitors to PEG-IFN-alpha and ribavirin led to significant improvements in SVR rates in patients. In a clinical trial, the addition of boceprevir to PEG-IFN-alpha/ribavirin increased SVR rates to 66% compared with 29% in the control group (223). Telaprevir administered in combination with PEG-IFN-alpha/ribavirin increased SVR rates in patients to 75% compared with 44% in those treated with PEG-IFN-alpha/ribavirin alone (220). However, it was necessary to administer the protease inhibitors in combination with PEG-IFN-alpha and ribavirin since protease inhibitor monotherapy leads to the rapid development of viral resistance (224). Additionally, telaprevir and boceprevir are both associated with adverse effects such as rash, flu-like symptoms, fatigue, nausea, anemia, and dysguesia (225).

The success of telaprevir and boceprevir led to the subsequent evaluation of second generation protease inhibitors: simeprevir, danoprevir, paritaprevir, and faldaprevir (226). In clinical trials, simeprevir showed high SVR rates in genotype 1 patients when added to PEG-IFN-alpha/ribavirin, and 80% of patients treated with this therapy showed SVR compared to 50% of those treated with PEG-IFN-alpha/ribavirin alone (227). In addition, simeprevir was associated with better quality of life and fewer side effects compared to telaprevir and boceprevir; making it preferable over the first generation protease inhibitors (226). Danoprevir also showed high rates of SVR in genotype 1 patients when administered in combination with PEG-IFN-alpha/ribavirin, and 76% of patients treated with this therapy showed response

compared to 42% of those treated with PEG-IFN-alpha/ribavirin (228). However, the high doses required for efficacy resulted in significantly elevated serum alanine aminotransferase (ALT) levels, which led to discontinuation of therapy. Faldaprevir also showed high SVR rates (79%) in genotype 1-infected patients in triple therapy with PEG-IFN-alpha/ribavirin, combined with a favorable safety profile (229). However, faldaprevir was withdrawn by the manufacturer from the Food and Drug Administration (FDA) approval process due to the advent of IFN-free regimens, and the necessity of faldaprevir to be administered in an IFN-based regimen.

One of the main objectives behind the development of DAAs is the potential to use them in IFN-free combinations. The NS3/4A inhibitor paritaprevir has been successfully used in IFN-free combination therapy (230). Administration of paritaprevir with ritonavir, ombitasvir, and dasabuvir led to SVR rates of up to 100% in patients infected with genotype 1b HCV infection (231). Ombitasvir and dasabuvir are NS5A and NS5B inhibitors, respectively, that will be discussed further below; and ritonavir is a cytochrome P450 inhibitor which increases the bioavailability of paritaprevir. Currently, telaprevir, boceprevir, simeprevir, and paritaprevir are the only FDA approved NS3/4A protease inhibitors for treatment of chronic hepatitis C.

1.4.4 NS5A Inhibitors

The NS5A protein plays an essential role in HCV viral replication as well as assembly/release of infectious particles, and thus it represents an important target for development of DAAs (232). A number of NS5A inhibitors have shown promise in the clinic. The NS5A inhibitor ombitasvir, when used in combination therapy with the NS3/4A protease inhibitor paritaprevir, the NS5B non-nucleoside inhibitor dasabuvir, and ribavirin, resulted in

96% SVR in genotype 1 patients (233). The contribution of ombitasvir was further highlighted in this therapy when elimination of the drug from the treatment reduced the SVR to 83% (233). Another NS5A inhibitor, ledipasvir, has shown SVR rates approaching 100% when used in combination with the NS5B inhibitor sofosbuvir (discussed further below) in genotype 1 patients (234). Daclatasvir, an additional NS5A inhibitor, has demonstrated SVR rates of ~96% in genotype 3 patients when used in combination with sofosbuvir in clinical trials (235). For patients infected with genotype 1b, daclatasvir has demonstrated SVR rates of 90% in treatment-naïve patients, and 86% in treatment experienced patients in combination with the NS3/4A protease inhibitor asunaprevir (236). These findings indicate that NS5A inhibitors hold significant promise in treatment of HCV, especially in IFN-free therapy when used in combination with other DAAs. Ledipasvir and ombitasvir were approved by the FDA for treatment of hepatitis C in 2015 (237). Daclatasvir was approved in Europe in 2014, and is currently pending approval by the FDA (372).

1.4.5 Host targeting therapies

As a number of host factors are known to play an important role in HCV infection, these have also been evaluated as targets for antiviral therapy. The highly abundant, liver-specific microRNA, miR-122, plays an important role HCV RNA accumulation through a direct interaction with the 5' UTR of the HCV genome in cultured hepatocytes and the liver of HCV-infected individuals (39). The antisense locked nucleic acid (LNA) drug, miravirsen, is being evaluated in the clinic for HCV treatment. A phase 2a study showed dose-dependent decreases in HCV RNA levels in treatment-naïve patients in response to miravirsen administration (39).

Further evaluation of miravirsen in treatment of chronic hepatitis C is currently underway [Clinical trial: NCT02031133].

Another host protein, Cyp A has been demonstrated to be crucial for HCV RNA replication (189). Cyp A interacts with the HCV NS5B protein, and the proteins peptidyl-prolyl isomerase activity is important for maintaining the integrity and function of the HCV RC (190). The Cyp A inhibitor SCY-635 was shown to inhibit replication of HCV in replicon-based assays (238). In clinical trials, the Cyp A inhibitor, alisporivir, has been shown to enhance efficacy of PEG-IFN-alpha/ribavirin (239). Further evaluation of Cyp A inhibitors, as well as other host targets such as miR-122, in combination with new DAAs is necessary in order to incorporate host-targeted therapies into HCV antiviral treatment.

1.5 Inhibitors of HCV NS5B

In the discovery and development of anti-HCV therapeutics, structural, biochemical and replicon-based approaches have provided important pre-clinical information. NS5B has been extensively studied as a target for drug development due to its essential role in viral RNA synthesis and due to the clinical success of the other viral polymerase inhibitors such as HIV and HBV RT (240, 241). HCV polymerase inhibitors are divided into three categories based on their molecular structure and mechanism of action: (i) nucleoside inhibitors (NIs), (ii) non-nucleoside inhibitors (NNIs), and (iii) pyrophosphate (PPi) analogues. NIs bind to the enzyme active site and compete with the natural NTP for incorporation. In contrast, NNIs bind away from the active site and inhibit the polymerase activity allosterically, specifically during initiation (242-244). Research has also been done on PPi analogues which mimic the natural PPi released during the

NTP incorporation reaction (245). Several NIs and NNIs are currently in clinical trials (discussed further below).

1.5.1 Nucleoside Inhibitors (NIs) of HCV NS5B

NIs are modified purines or pyrimidines that mimic the native ribonucleotides, and are recognized by the RdRp (examples of the chemical structure of NIs are shown in Figure 1.6). Cellular RNA polymerases show a high degree of resistance to NIs due to their intrinsic proofreading ability which can lead to excision of incorporated NIs (246). Hence, NIs typically do not interfere with endogenous cellular polymerases.

NIs are typically administered in a non-phosphorylated or 'prodrug' form, which are able to permeate cellular membranes (247). Phosphorylation of the nucleotide inhibitor is necessary for incorporation, as the RdRp incorporates NTPs into the elongating RNA chain. The NI prodrugs are then converted into active NTPs by endogenous cellular kinases. Following phosphorylation, the NIs are incorporated by NS5B into the growing RNA chain. NIs that act as chain terminators carry modifications normally at the 2' or 4' positions which prevent incorporation of the subsequent NTP, thus terminating RNA synthesis.

NIs can be obligate or non-obligate chain terminators. Obligate chain terminators lack a 3'OH group which prevents formation of a covalent bond with the alpha-phosphate of the incoming NTP, necessary for incorporation (e.g. 3'deoxycytidine triphosphate, Figure 1.6) (248). Due to the requirement of the 3'OH group for phosphorylation by cellular kinases, obligate chain terminators have not been developed for the clinic. In contrast, non-obligate chain terminators contain a 3'OH group, and thus can be phosphorylated into NTPs. However, they carry

additional modifications that enable them to act as chain terminators. For example, 2'C-methylcytidine (Figure 1.6) acts as a non-obligate chain terminator; and, once incorporated, it inhibits incorporation of subsequent NTPs via a steric conflict of the 2'methyl group with the ribose of the incoming NTP (249). While 2'C-methylcytidine is capable of inhibiting NS5B through non-obligate chain termination, its low oral bioavailability is a major hurdle to therapeutic use of this compound (250). To bypass this issue, NM283, which is a 3'-O-l-valinyl ester derivative of 2'C-methylcytidine was developed (250). The 3'-O-l-valinyl ester modification improves uptake of the drug via peptide transporters. However, this derivative was discontinued in phase II trials due to gastrointestinal toxicity (251).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Figure 1.6), which has been widely used in treatment of NS5B is a modified guanosine analog, also acts as a prodrug (252). This prodrug is metabolized in the cell into ribavirin monophosphate (RMP), ribavirin diphosphate (RDP), and ribavirin triphosphate (RTP) forms, with RTP being the major metabolite *in vivo* (253). RTP can be incorporated into the elongating HCV transcript by NS5B, and it has been suggested that this leads to error catastrophe in the virus through the introduction of mutations (252). Additionally, RMP can also act as an inhibitor of inosine monophosphate dehydrogenase (IMPDH) which leads to depletion of cellular GTP levels thus inhibiting viral RNA synthesis (253). Ribavirin has also been proposed to act through immunomodulation by enhancing T-cell response, and also by increasing the natural killer (NK) cell response to IFN increasing immune surveillance to HCV-infected cells (254, 255).

Mericitabine is another NI that is a di-isobutyl ester prodrug of RO-5855 (β-D-2'-deoxy-2'-fluoro-2'-C-methylcytidine, Figure 1.6) (256). Following oral administration, mericitabine is converted into RO-5855 in the plasma, which is subsequently converted to RO5855-TP, which is

a CTP analogue, and RO2433-TP, which is a UTP analogue (256). Both the analogues have been shown to be active, with the CTP analogue being predominant *in vivo* (257). In phase II clinical trials, mericitabine, when administered in combination with PEG-IFN-alpha and ribavirin, has been shown to be more effective in improving SVR in patients in a 24 week course, compared to PEG-IFN-alpha/ribavirin in 48 week therapy (258). Although mericitabine has been tested in combination with ritonavir and danoprevir in IFN-free therapy, this therapy showed low SVR rates, indicating that it may be necessary to administer IFN for mericitabine to be effective (259). Subsequent trials have shown that quadruple administration of ritonavir, danoprevir, mericitabine, and PEG-IFN-alpha show significantly improved SVR rates (260).

Sofosbuvir is a second generation NI that has shown high efficacy in treatment of HCV (Figure 1.6). Sofosbuvir is a prodrug that undergoes intracellular processing to the 2′-deoxy-2′-fluoro-2′-C-methyluridine monosphate, and subsequent phosphorylation to the UTP analogue 2′-deoxy-2′-fluoro-2′-C-methyluridine triphosphate (2′F-2′C-Me-UTP) (261). The active metabolite of sofosbuvir (2′F-2′C-Me-UTP) competes with the endogenous UTP for incorporation into the HCV transcript, and once incorporated, acts as a non-obligate chain terminator to prevent RNA synthesis. Importantly, 2′F-2′C-Me-UTP has very low binding affinity for cellular DNA and RNA polymerases, which leads to low toxicity (261). The low levels of toxicity associated with sofosbuvir further enables its use in combination therapies with other drugs.

Sofosbuvir has been shown to be effective in IFN-free therapy in clinical trials, and has since become part of the standard of care for chronic hepatitis C (262). When administered in combination with ribavirin, sofosbuvir shows high SVR rates in patients infected with all major HCV genotypes (263). Sofosbuvir has also been evaluated in combination with other DAAs. In patients infected with HCV genotype 1, sofosbuvir in combination with the NS3/4A protease

inhibitor, simeprevir, showed high rates of SVR (~95%) (263). In combination with the NS5A inhibitor, daclatasvir, SVR rates approached 100% in patients infected with genotypes 1, 2, or 3 (264). Combination therapy of sofosbuvir with another NS5A inhibitor, ledipasvir, also showed high rates of SVR (94-99%) in patients infected with genotype 1 (265).

1.5.2 Non-Nucleoside Inhibitors (NNIs) of HCV NS5B

In contrast to NIs, NNIs do not compete with the natural NTPs. They bind away from the enzyme active site and inhibit the polymerase activity allosterically prior to RNA elongation (Figure 1.7) (242-244, 266). Detailed mechanisms of inhibitory action of NNIs have been determined with the help of structural and biochemical approaches as well as cell culture models (267). Unlike HIV RT, which uses the same allosteric binding site for all its known NNIs, HCV NS5B has at least four binding sites for NNIs (Figure 1.7A) (268). Two sites are located on the thumb domain and the other two sites are located on the palm domain close to the active site. NNI-1s binds to the thumb 1 site, NNI-2s bind to the thumb 2 site, NNI-3s bind to the palm 1 site, and NNI-4s binds to the palm 2 site which is close to the active site of NS5B (Figure 1.7A). Examples of NNIs currently in clinical development are shown in Figure 1.7B.

NNI-1*: Benzimidazoles and Indoles*

NNI-1 inhibitors target the upper region of the thumb domain approximately 30Å from the active site at the junction of the thumb and finger loops (Figure 1.7A) (269). Compounds that bind to the NNI-1 site are derivatives of benzimidazoles and indoles that were initially designed and synthesized by Boehringer Ingelheim and Japan Tobacco (270, 271). Several NNI-1s have

been investigated in the clinic, including: deleobuvir, tegobuvir, and beclabuvir (272). In clinical trials, oral administration of deleobuvir was shown to result in high rates of SVR (~94%) in patients infected with genotype 1 when used in combination with the NS3/4A protease inhibitor, faldaprevir, and ribavirin (273). Combination therapy of tegobuvir with ledipasvir, vedroprevir, and ribavirin resulted in SVR rates of 63% in patients in an IFN-free regimen (274). However, tegobuvir and deleobuvir were discontinued in clinical trials as they did not show sufficient efficacy compared to other interferon-free therapies in development. Beclabuvir (Figure 1.7B), when used in combination therapy with daclatasvir and asunaprevir, showed SVR rates of 92% after 12 weeks of treatment in an IFN-free regimen in genotype 1 patients (275).

NNI-2*: Thiopenes, Phenylalanines, and Pyranones*

NNI-2s bind to a hydrophobic cavity at the base of the thumb domain (Figure 1.7A) (276). This site is located 35 Å away from the active site of NS5B, and the enzyme-inhibitor complex is stabilized via hydrogen bonding and van der Waals interactions (277). Crystal structures have indicated that this interaction switches the enzyme from an active conformation to an inactive conformation (277). Lomibuvir (Figure 1.7B) is an NNI-2 currently under investigation in the clinic. In a Phase II trial, combination of Lomibuvir with PEG-IFN-alpha/Ribavirin and Telaprevir has shown SVR rates of 90% in genotype 1 patients (278).

NNI-3*: Benzothiadiazines and Acyl pyrrolidines*

NNI-3s bind to the palm I site located at the junction of the palm and thumb domain of NS5B close to the catalytic site (Figure 1.7A) (279). NNI-3s currently under evaluation are setrobuvir, dasabuvir and ABT-072 (Figure 1.7B) (280). Dasabuvir has been approved for use in combination therapy with ombitasvir, paritaprevir, and ritonavir (281). This combination therapy has resulted in very high SVR rates of 97-100% in genotype 1 patients (231). In phase II trials, ABT-072 has shown significant promise when administered in combination therapy with paritaprevir, resulting in SVR rates of 91% in genotype 1 patients (282).

NNI-4*: Benzofurans*

NNI-4s bind to the palm II site located proximal to the junction between the palm and thumb domain (Figure 1.7A) (283). HCV-796 (Figure 1.7B) was an NNI-4 under clinical development; however, this drug was discontinued due to elevated liver enzyme levels in human subjects (284). Another palm II inhibitor, IDX-375 was also discontinued after phase I trials due to hepatotoxicity (285).

1.5.3 Antiviral Resistance

Resistance to DAAs is one of the major challenges to their use in treatment of chronic hepatitis C. The error rate for the HCV RdRp is 10^{-4} substitutions per base, which is approximately 100-fold higher than that of HIV, which makes the emergence of resistance

variants much more likely (286). A number of resistance-conferring mutations have been identified in the clinic.

Resistance to NS3/4A protease inhibitors

Crystal structures of the NS3/4A protease have revealed a relatively conserved consensus site where protease inhibitors bind (287). Mutations that confer resistance to protease inhibitors are typically located at the site on the protease where the inhibitors protrude from the substrate envelope (Figure 1.8) (286). Monotherapy with the protease inhibitor telaprevir has been shown to cause emergence of resistance-causing variants within the first week of treatment in patients (224). The R155K/T/I and A156S/V/T mutations are commonly associated with resistance to telaprevir (224). Interestingly, the R155K mutation has been shown to be naturally occurring in a minority of treatment-naïve patients (288). These mutations cause resistance by inducing a conformational change in the S4 and S2 binding pocket of the protease (289). The V36M/A, T54A/S, R155K/T, A156S, V170A mutations are known to confer resistance to boceprevir (290) and the Q80K mutation has been shown to confer resistance to simeprevir. The Q80K mutation was detected in 30% of patients infected with genotype 1a when treated with PEG-IFN-alpha/ribavirin/simeprevir combination therapy (291).

Resistance to NS5A inhibitors

The Q30R/E, L31V/M, and Y93N/H mutations confer resistance to the NS5A inhibitor daclatasvir; and L31M, Y93C, M28V, and M28T mutations confer resistance to ledipasvir

(Figure 1.9) (292, 293). The L31V and Y93H mutations confer 24- and 28-fold resistance to daclatasvir (292). However, the L31V/Y93H double mutation causes 15,000-fold resistance to the drug, indicating that these substitutions may act synergistically to induce resistance (292). In biochemical studies, the Y93H mutant has been shown to reduce ledipasvir binding, indicating that this may be the underlying mechanism of resistance to the inhibitor (Figure 1.9) (294).

Resistance to NS5B inhibitors

NIs bind to the active site of NS5B, and have a medium to high barrier to resistance, since the active site is highly conserved across variants (295). Mutations close to the active site of the enzyme, such as S282T (Figure 1.10), can cause resistance to NIs. In replicon studies, the S282T mutation was shown to cause resistance to the NI sofosbuvir (296). Subsequently, emergence of this mutation was detected in a patient in clinical trials upon sofosbuvir monotherapy and was associated with relapse(296). The S282T mutation was shown to reduce sensitivity to sofosbuvir by 13.5-fold (297). Deep sequencing analysis has indicated that this mutation may be preexisting at low levels in patients, and is subsequently selected for in response to treatment with sofosbuvir (298). Interestingly, a patient who carried the S282T mutation, and was refractory to sofosbuvir therapy was subsequently treated with sofosbuvir/ribavirin combination therapy and was able to achieve a SVR. The S282T mutation also confers resistance to mericitabine (299). In addition, the L159F/L320F double mutation has been detected in patients and confers low levels of resistance to both sofosbuvir and mericitabine (300). The C316N and V321A mutations have also been detected in 2-4% of patients who failed sofosbuvir treatment (301).

Mutations that confer resistance to NNIs have also been identified in the clinic (302). Substitutions at P495, P496, and V499 have been shown to confer high levels of resistance to NNI-1s (Figure 1.10) (269). This resistance is thought to arise through a decrease in binding affinity for the drugs (269). Mutations at P495, which have been shown to cause resistance to deleobuvir, were identified in 9 out of 59 patients treated with the drug in one study (303). The P495L mutation reduces sensitivity to deleobuvir by 120- to 310-fold (303). In preclinical studies, the P495A/S/L/T mutation has been shown to confer resistance to BMS791325, which is another NNI-1.

Mutations at M423, R422, M426, and I482 sites are known to confer resistance to NNI-2s (Figure 1.10) (304). The M423I/T/V mutations have been established to be the predominant mechanism of resistance to the NNI-2 filibuvir in the clinic. These mutations arise in response to therapy; however, as the mutants have lowered replicative fitness, they are rapidly eliminated upon cessation of therapy (302). Mutations at R422 and M426 have also been identified to cause resistance to filibuvir in a minority of patients. The M423T substitution has been shown to cause a 250-fold decrease in binding to the inhibitor (305). In the replicon system, the I482L substitution was also shown to cause moderate resistance to filibuvir (305).

The C316Y, M414T, Y448C, Y448H, and S556G mutations are known to cause resistance to NNI-3s (Figure 1.10) (306). In replicon studies, treatment of subgenomic replicons from genotypes 1a and 1b with the NNI-3 inhibitor dasabuvir led to the emergence of these resistance-causing mutations (306). These mutations map to the palm I site of the polymerase which is the binding site for the NNI-3s.

Finally, mutations at L314, C316, I363, S365, and M414 are known to confer resistance to NNI-4s (Figure 1.10) (307). Additionally, C316Y, C316N, C445F, Y448H, and Y452H mutations confer resistance to tegobuvir (308).

1.5.4 Overcoming Resistance

Combination therapy can be effective in preventing and circumventing the emergence of resistance causing variants of HCV. As telaprevir monotherapy previously led to the emergence of resistance causing variants, the first generation protease inhibitors (telaprevir and boceprevir) were administered as part of combination therapy with PEG-IFN-alpha/ribavirin to prevent the emergence of resistant variants (309). Similarly, simeprevir is also administered in combination with sofosbuvir in IFN-free regimens (310). As NS5A inhibitors do not show cross-resistance with any other DAAs, they are ideal for use in combination therapy and this strategy helps in circumventing resistance to NS5A inhibitors (310). Similarly, as NNIs have a relatively low barrier to resistance, and NIs have a high barrier to resistance, the NS5A inhibitor ombitasvir is administered as part of combination therapy with the protease inhibitor ABT450/r, and NNI dasabuvir (311). Clinical data suggests that although resistance to sofosbuvir is rare, the resistance-conferring mutation S282T can arise at low frequencies, and can be selected for after treatment with sofosbuvir (234). Hence, the possibility that S282T mediated resistance to sofosbuvir may become a challenge in anti-HCV therapy needs to be considered, and the underlying mechanism of action of the S282T mutation needs to be elucidated, along with developing ways of bypassing this resistance.

1.6 Thesis Objectives

Recent advances in HCV research have led to the development of DAAs that target various HCV proteins. In order to successfully treat the infection, it is important to identify the underlying mechanisms by which these inhibitors target HCV. Furthermore, it is important to identify mechanisms that are associated with the development of resistance to these drugs. Together, this information can be used to improve therapy for chronic hepatitis C.

NNIs of NS5B have generated significant interest, and are an area of active research in the clinic, particularly for use in combination therapy. In Chapter 2, we investigate the efficacy of two NNI-3s, and identify mechanisms through which HCV develops resistance to these inhibitors. This information can be used towards the development of improved NNIs, as well as designing NNI-based combination therapy for chronic hepatitis C.

NS5B is an attractive therapeutic target, as it is essential for viral replication. A number of NS5B inhibitors such as the NI sofosbuvir have shown promise in the clinic. Although sofosbuvir has shown great success in treating chronic hepatitis C infection, a remaining concern is the emergence of resistance mutations. However, the underlying mechanism of resistance caused by the signature mutation S282T is still unknown and is addressed in Chapter 3. In Chapter 4, we extend these studies to test and compare efficacies of novel nucleotide inhibitors in the context of NS5B containing resistance-associated mutations S282T, and the more recently discovered L159F, C316N and L320F mutations.

Overall, these studies aim to provide a more detailed understanding of mechanisms of inhibition of the NS5B polymerase and the development of antiviral resistance. In Chapter 5, I summarize and discuss our findings and provide future directions for the development of

effective therapeutics against chronic hepatitis C, which is one of the most common causes of chronic liver disease worldwide.

Figures for Chapter 1.

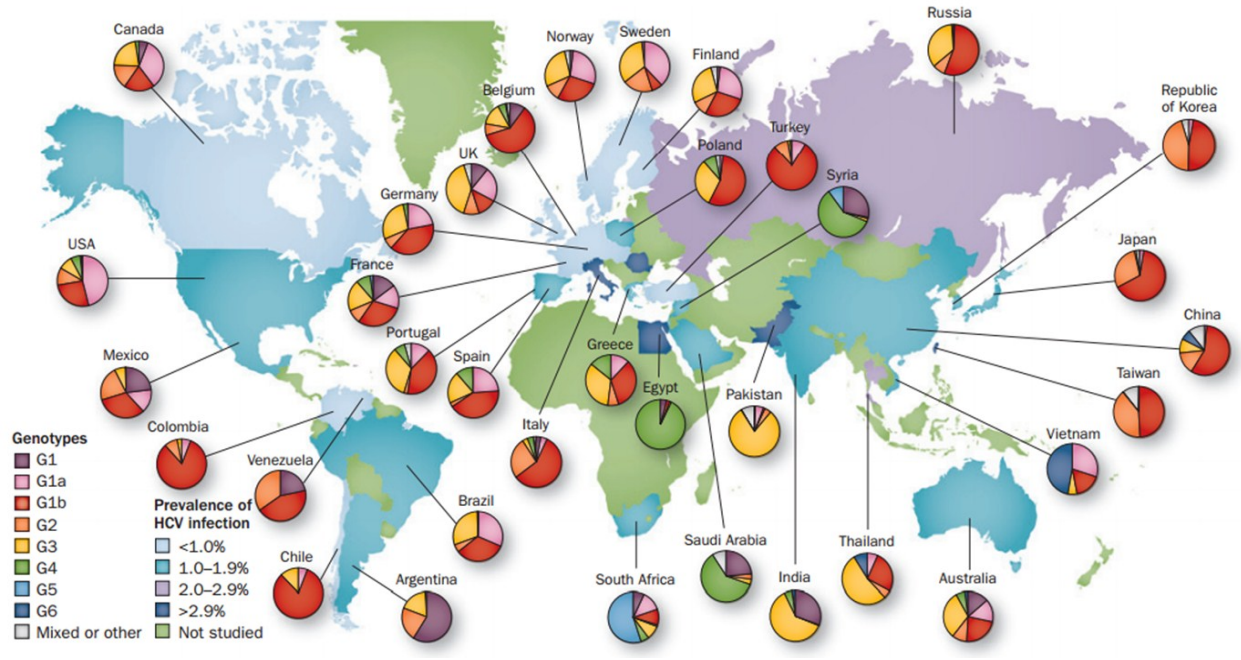


Figure 1.1: Global distribution of HCV genotypes. Worldwide distribution of HCV infection and genotypes 1-6. Genotypes 1, 2 and 3 have a worldwide distribution while genotypes 4, 5 and 6 are restricted to specific geographic regions. *Figure adapted from Hajarizadeh et al, Nature Reviews, 2013. (18)*

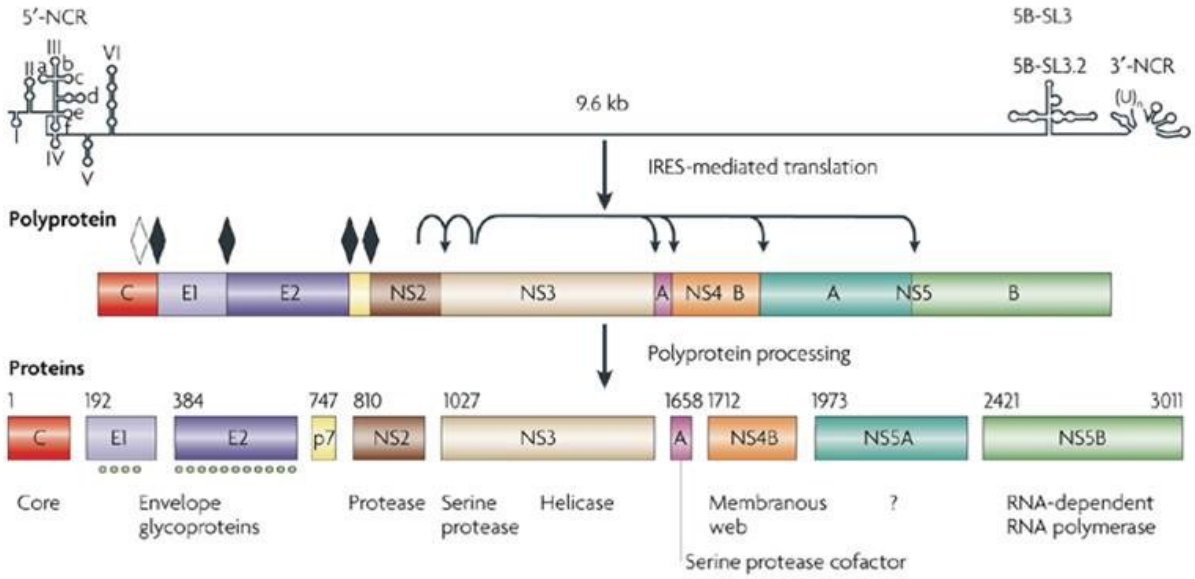


Figure 1.2: HCV genome organization. Schematic illustration of the 9.6 kb positive-strand HCV RNA genome. The genome consists of structured 5' and 3'UTRs and a central coding region. The 5'UTR contains an internal ribosomal entry site (IRES) which directs translation of the viral polyprotein that is processed into the mature structural and non-structural proteins by host and viral proteases. The open diamond signifies C-terminal cleavage of the core protein by signal peptide peptidase. Solid diamonds indicate cleavage sites by ER signal peptidase. Curved arrows represent viral NS2-3 and NS3-4A protease cleavage sites. Amino acid numbers are shown above each protein. Open circles indicate glycosylation sites. *Figure adapted from Moradpour et al, Nature Reviews, 2007. (25)*

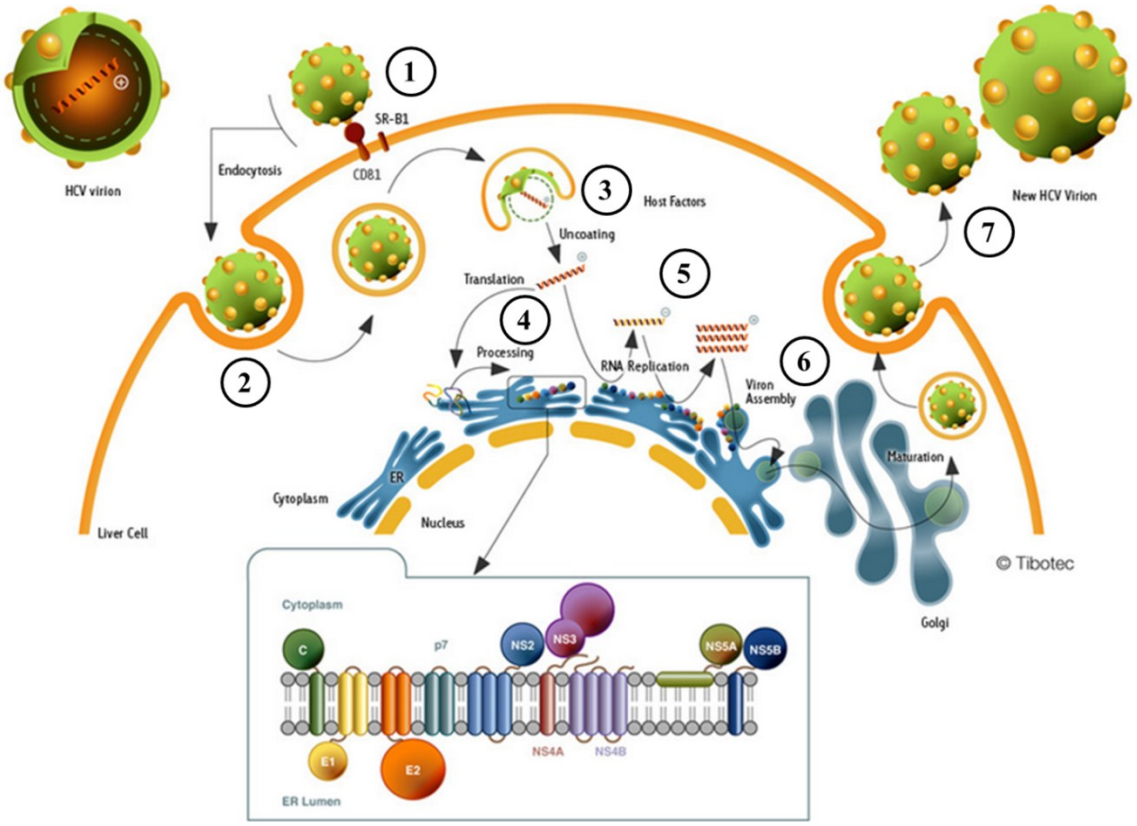


Figure 1.3: HCV life cycle. The HCV life cycle can be summarized as follows: (1) receptor recognition and attachment; (2) cell entry by endocytosis; (3) pH-dependent membrane fusion and release of the single-stranded viral RNA genome into the cell cytoplasm; (4) translation and polyprotein processing; (5) viral RNA replication; (6) virion assembly; and (7) viral release. *Figure adapted from Tibotec Pharmaceuticals, 2009.*

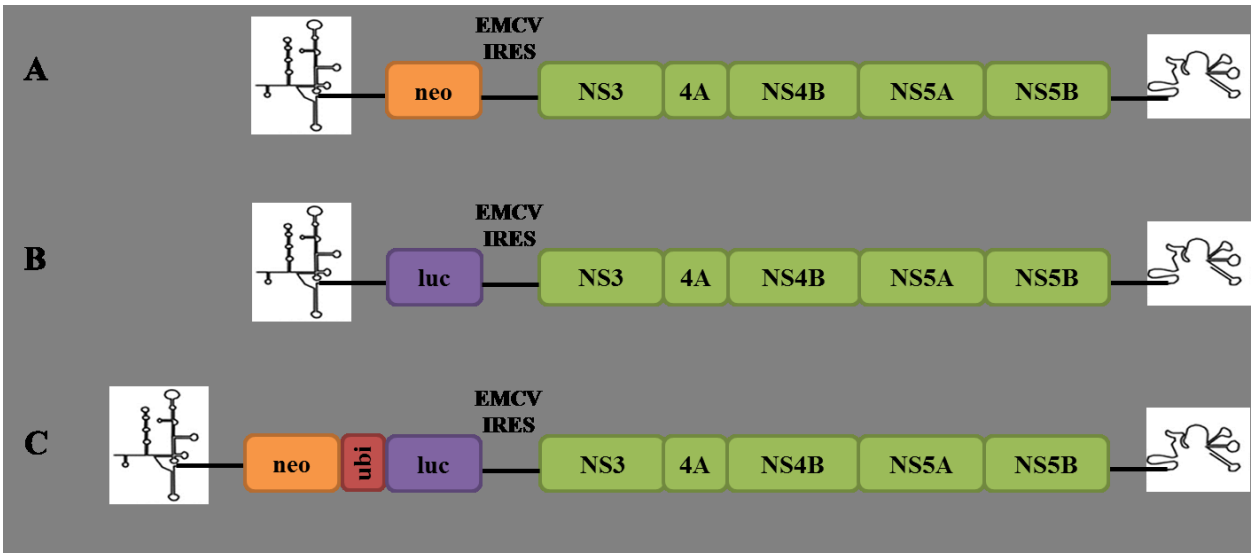


Figure 1.4: HCV replicons. (A) Schematic of bicistronic selectable subgenomic HCV replicon. Translation of neomycin phosphotransferase (neo) is directed by the HCV IRES while translation of the structural genes NS3-NS5B are directed by the EMCV IRES. (B) Structure of a subgenomic luciferase (luc) reporter replicon used for transient replication assays. (C) Structure of a selectable reporter replicon. This construct encodes a neomycin phosphotransferase-ubiquitin-luciferase fusion protein in the first cistron. The RNA supports stable expression of luciferase; ubiquitin (ubi) is used to trigger proteolytic removal of the neomycin phosphotransferase (neo) from the fusion protein. *Figure adapted from Woerz et al, Journal of Viral Hepatitis, 2009 and Cheng et al, Antimicrobial Agents in Chemotherapy, 2011. (312, 313)*

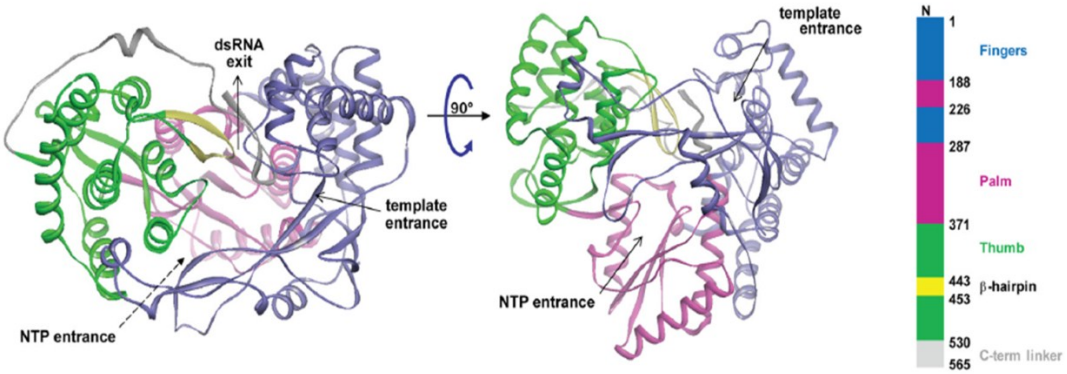


Figure 1.5: Structure of HCV NS5B polymerase. Crystal structure of HCV NS5B with the finger (purple/blue) palm (pink), thumb (green), and C-terminal linker domain (gray). The β-hairpin loop (yellow) descends towards the palm domain partially blocking the exit of the dsRNA. *Figure adapted from Mosley et al, Journal of Virology, 2012. (314)*

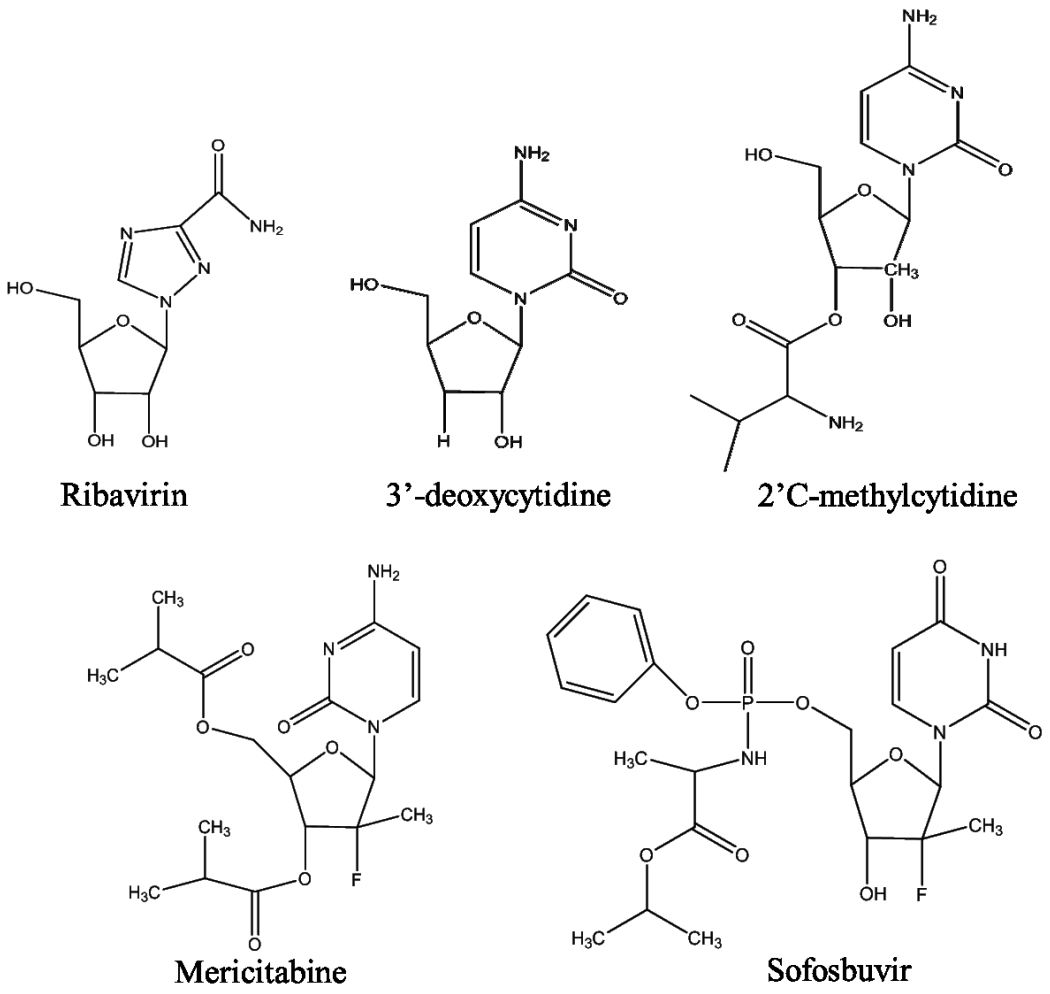
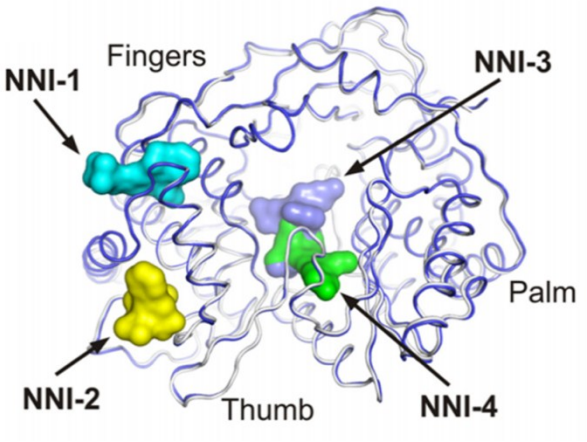


Figure 1.6: Structures of HCV nucleoside inhibitors. Examples of nucleoside analogue inhibitors: ribavirin, 3'-deoxycytidine, 2'C-methylcytidine, Mericitabine, and Sofosbuvir.

A



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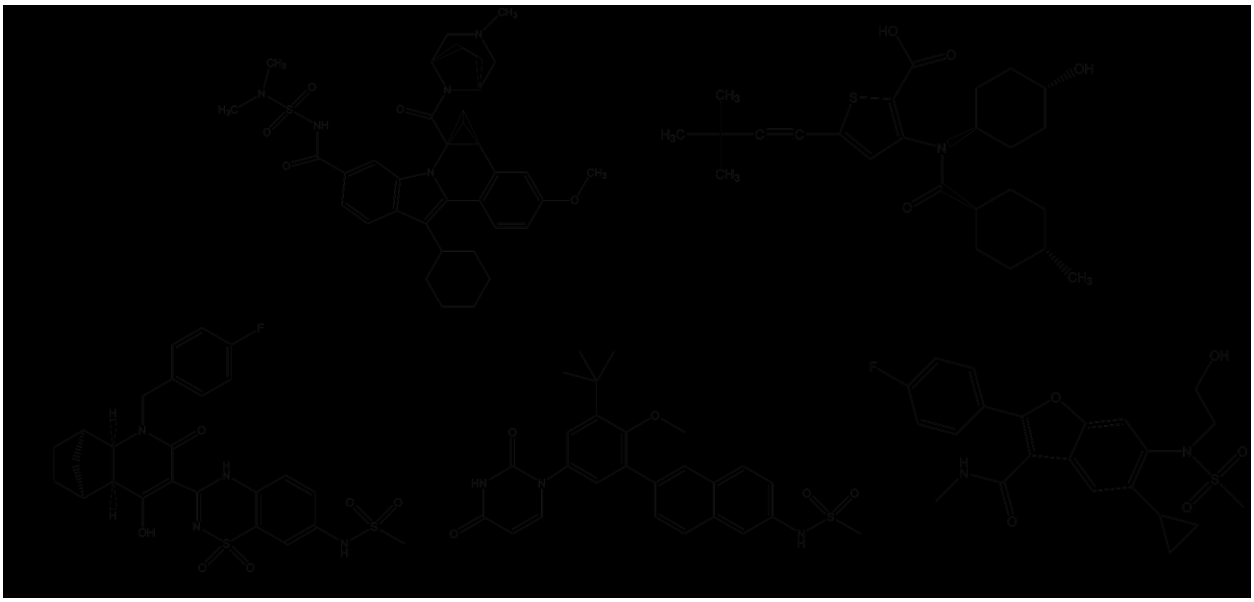

Figure 1.7: Structures of HCV non-nucleoside inhibitors and their binding sites on NS5B. (A) Binding sites of NNI-1 (cyan), NNI-2 (yellow), NNI-3 (blue), and NNI-4 (green). (B) Examples of representative NNIs: NNI-1, Beclabuvir; NNI-2, Lomibuvir; NNI-3s, Setrobuvir and Dasabuvir; and NNI-4, HCV-796. *Figure adapted from Nyanguile et al, Journal of Virology, 2010. (315)*

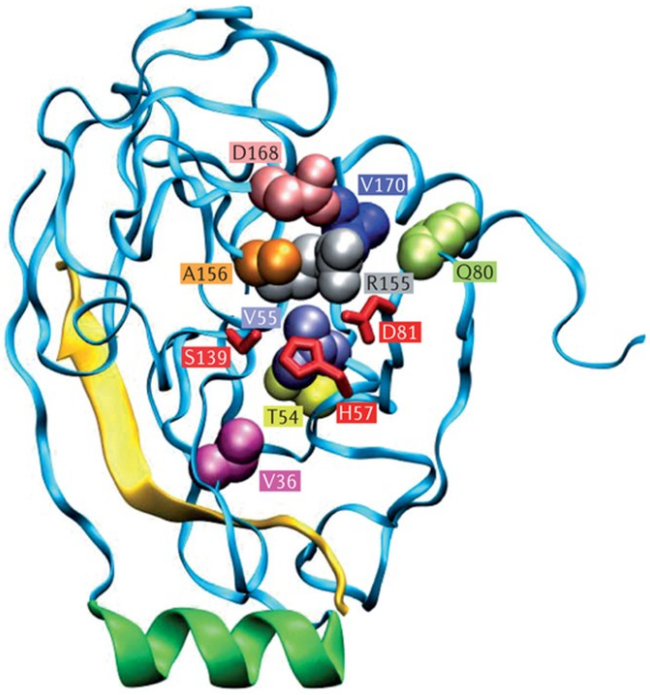

Figure 1.8: Mutations conferring resistance to NS3/4A protease inhibitors. Structure of the NS3 protease domain with the central NS4A activation domain (yellow). The catalytic triad (H57, D81 and S139) is indicated (red). Mutation at amino acid positions V36, T54, R155, A156 and V170 confer resistance to Boceprevir. Mutations at amino acid positions R155 and A156 confer resistance to Telaprevir. Mutations at Q80 confer resistance to Simeprevir. *Figure adapted from Bartenschlager et al, Nature Reviews, 2013. (49)*

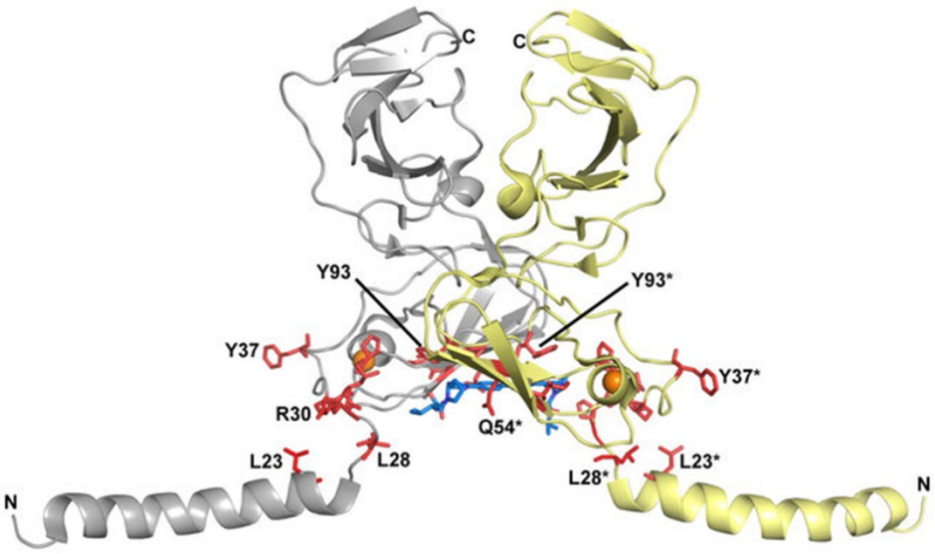

Figure 1.9: Mutations conferring resistance to NS5A inhibitors. Computational docking of Daclatasvir (blue) to the channel formed by the NS5A dimer showing interactions with amino acid residues Q30R, L31V and Y93N that confer resistance. *Figure adapted from Ascher et al, Scientific Reports, 2014. (316)*

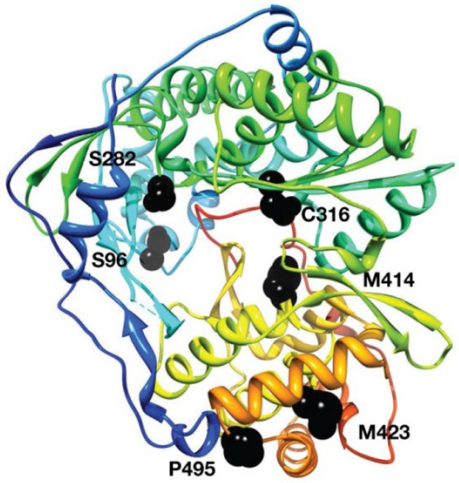

Figure 1.10: Mutations conferring resistance to NIs and NNIs. Structure of the NS5B polymerase showing key residues associated with resistance to NS5B inhibitors. Mutations at S282 and S96 are associated with resistance to NIs; while mutations at P495 confer resistance towards NNI-1s. Mutations at M423 confer resistance to NNI-2s. M414 mutations confer resistance to NNI-3s; and C316 mutations confer resistance to NNI-4s. *Figure adapted from Aloia et al, Antiviral Therapy, 2012. (307)*

CHAPTER 2

Biochemical Characterization of Genotype Dependent, Natural Resistance to Non-Nucleoside Inhibitors (NNIs) of HCV NS5B

Anupriya Kulkarni, Jean Bernatchez, Selena M. Sagan and Matthias Götte

2.1 Preface to Chapter 2

In *Chapter 1*, we discussed the potential advantages in using NNIs in anti-HCV therapy. Preclinical characterization of such inhibitors involves biochemical testing of their efficacy against the NS5B polymerase as well as identifying potential mechanisms of action and resistance. As substantial amino acid variation exists in the NNI binding sites across the HCV genotypes, variations in drug susceptibility are observed for each genotype. Herein, we characterized the inhibitory activity of two different Class 3 NNIs, acyl pyrrolidine and 1,5-benzodiazepine, with purified NS5B enzymes representative of the major HCV genotypes. We identified novel amino acid substitutions that contribute to natural resistance towards these NNIs.

2.2 Abstract

NS5B is the RNA-dependent RNA polymerase (RdRP) responsible for HCV replication and is an attractive target for anti-HCV therapy. NS5B inhibitors are classified into nucleoside inhibitors (NIs), which act as chain terminators and bind to the active site of the polymerase, and non-nucleoside inhibitors (NNIs), which bind to allosteric binding sites. While NIs commonly show pan-genotypic activity, different HCV genotypes show variations in susceptibility to NNIs. Genotype-dependent amino acid changes in the NNI binding sites help to explain this observation, although the detailed underlying mechanisms remain elusive. As part of our study, we tested the inhibitory activity of two different NNI-3s, acyl pyrrolidine and 1,5-benzodiazepine with purified NS5B enzymes that represent the major genotypes and identified specific amino acid substitutions that contribute to resistance towards these NNI-3s. In agreement with previous cell-based susceptibility studies, our biochemical studies demonstrate that most NNIs have potent inhibitory activities towards NS5B of genotype 1b, while genotypes 2a, 3a, and 5a show varying degrees of resistance. Using *in silico* docking analysis we identified several amino acid residues within 5Å of the NNI binding sites that were different in the resistant genotypes. When introduced in NS5B 1b, these mutations showed decreased sensitivity when compared with the wild-type. Specifically, we find that mutants A218S and V405I showed significant resistance to both acyl pyrrolidine and 1,5-benzodiazepine. The identification of such residues that confer resistance may contribute to the rational design of novel NNIs with increased efficacy across all genotypes.

2.3 Introduction

The high tendency of the Hepatitis C virus (HCV) to mutate poses a major challenge in the development of new therapies. The HCV NS5B RNA-dependent RNA polymerase (RdRp) lacks proofreading activity, and hence generates numerous viral variants with a high degree of genetic heterogeneity, which complicates the development of effective antiviral therapies. Seven different genotypes and several subtypes of the HCV have been characterized (15). These have distinct geographic distribution, with genotype 1 being the most common, accounting for 45-60% of infections globally (15, 17, 317). In 2011, the NS3/4A protease inhibitors, telaprevir and boceprevir, were approved by the Food and Drug Administration (FDA) for use in combination therapy with pegylated Interferon (PEG-IFN) and Ribavirin for patients infected with genotype 1, thus representing a significant advance in the use of Direct Acting Antivirals (DAAs) in anti-HCV therapy (318-320). Although inclusion of protease inhibitors in combination therapy led to improved patient response, these compounds show a low genetic barrier to resistance (321, 322).

NS5B has a typical polymerase-like right-hand structure that includes palm, finger, and thumb sub-domains (103). As it is responsible for HCV replication, NS5B represents a logical therapeutic target (152). Approved NS5B inhibitors include both nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (279). NIs inhibit viral RNA synthesis following incorporation during the elongation stage of RNA synthesis. Members of this family of compounds typically display pan-genotypic activity. Sofosbuvir is a nucleotide-based prodrug that was approved by the FDA in 2013 for the treatment of chronic HCV infection in combination therapy (323, 324). While NIs bind to the active site of NS5B, NNIs bind to distinct allosteric binding pockets and inhibit polymerase activity specifically during initiation (325). NNI-1 includes benzimidazole

and indol derivatives which bind to the thumb-1 pocket of the polymerase (269, 326). NNI-2 includes derivatives of thiophenes, phenylalanines, hydroxypyranones and pyranoindoles which bind to the adjacent thumb-2 pocket (327, 328). Closer to the active site, in the palm-1 domain is the NNI-3 binding site, where benzothiadiazines, acylpyrrolidines and 1,5-benzodiazepines bind (329). Finally, NNI-4 benzofuran derivatives bind to the palm-4 subdomain adjacent to the NNI-3 binding site (330, 331). NNIs currently under evaluation in the clinic include the Class-1 NNI BI207127 and Class-3 NNI ABT-333 which are in Phase III clinical trials, as well as Class-1 NNIs TMC647055 and BMS-791325, Class-2 NNIs VX-222 and GS-9969, Class-3 NNI ABT-072, and class-4 NNI IDX-375 which are in Phase II clinical trials (279, 295).

NNIs have a lower genetic barrier to resistance (defined as the number and type of mutations required to acquire drug resistance) compared to NIs, and varying efficacy against different genotypes of the virus (279, 295). This differential efficacy is likely due to variations in amino acid residues in the NNI binding pockets (315, 332). Substitutions at P495, P496, and V499 have been shown to confer high levels of resistance to thumb-1 inhibitors (269). Similarly, substitutions at M423, R422, M426, and I482 sites are known to confer resistance to thumb-2, and the mutations C316Y, M414T, Y448C, Y448H, and S556G are known to cause resistance to palm-1 and palm-2 inhibitors (304) Here, we employed a biochemical approach to investigate potential mechanisms that contribute to the genotype-specific variations in drug susceptibility. In this study, we tested the inhibitory activity of two class 3 NNIs, Acyl pyrrolidine (Figure. 2.1 A) and 1,5 Benzodiazepine (Figure 2.1 B), across the major HCV genotypes, and identified amino acid residues contributing to resistance to the NNIs. As class-3 NNIs, these inhibitors bind to NS5B adjacent to the active site (Figure 2.1 C) and our analyses revealed novel pre-existing amino acid residues contributing to decreased sensitivity against this class of inhibitors.

2.4 Materials and Methods

Nucleic acids, nucleotides and inhibitors. Nucleic acids and nucleotides used in this study were purchased from Trilink unless otherwise indicated. We devised a model RNA template with the following sequence: 5' – AACAGUUUCCUUUUCUCUCC – 3', and the 5' – GG – 3' dinucleotide was used as a primer. The 5' end of the dinucleotide primer was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Thermo Scientific). For detection of *de novo* RNA synthesis, we utilized a 5' – biotinylated RNA template with the following sequence: 5' – AUCCGUUUCCUUUUCUCUCC – 3'. Nucleoside triphosphates (NTPs) were purchased from Thermo Scientific. The NNI-3 inhibitors, acyl pyrrolidine and 1,5-benzodiazepine, were kindly provided by Tibotec (Belgium).

Expression and purification of HCV NS5B. The NS5B sequence from genotypes 1b, 2a (JFH1), 3a, and 5a were inserted into the expression vector pET-21 (Novagen). The 21 amino acid C-terminal hydrophobic membrane anchor was deleted and replaced by a C-terminal His-tag to facilitate protein purification with Ni-chelating sepharose. Plasmids encoding the C-terminal truncated enzyme (Δ21) were transformed into *Escherichia coli* BL21 (DE3) cells. Protein expression was induced with the addition of 0.25 mM isopropyl-β-$_D$-thiogalactopyranoside at 25ºC. Mutations A218S, V405I, A450S and C451T were generated in genotype 1b NS5B by Stratagene Quik-Change kit, according to the manufacturer's instructions (248). All sequences were confirmed by sequencing at the McGill University Genome Quebec Innovation Center.

Primer-dependent RNA synthesis. The standard reaction mixture contained 500 nM of RNA template, 1 μM of purified HCV NS5B (genotype 1b, 2a, 3a or 5a), 0.2 μM radiolabeled GG primer, and 5 μM of NTPs. Reactions were carried out in a buffer containing 40 mM HEPES (pH 8.0), 15 mM NaCl, 1 mM dithiothreitol (DTT), and 0.5 mM EDTA. The reactions were started using 6 mM $MgCl_2$, and monitored at room temperature and stopped at specific time points by the addition of 0.5 M EDTA in 95% formamide containing xylene cyanol and bromophenol blue. Samples were then heat denatured at 95 ºC for 5 min, the reaction products were resolved on a 20% polyacrylamide-7 M urea gel, and visualized using a Bio-Rad Phosphorimager. In efforts to compare NNI-mediated inhibition of RNA synthesis with enzymes derived from different genotypes, we normalized activities in the absence of inhibitor. For testing NNIs, 5 μM of GTP, ATP and 3'dCTP with increasing concentrations of inhibitor were included in to the reaction mixture. The canonical chain-terminator 3'dCTP was used to yield a specific reaction product during elongation. Chain-termination was monitored in a control experiment with 5 μM of each of GTP, ATP, UTP, 0.1 μM of the competing nucleotide (CTP), and increasing concentrations of 3' dCTP. The reactions were allowed to proceed for 45 min. The concentration of inhibitor required to inhibit 50% of full-length product formation (IC_{50}) was calculated using Prism software (GraphPad, Inc). All assays were repeated a minimum of three times.

De novo **RNA synthesis and scintillation proximity assay (SPA)**. The standard reaction mixture contained 500 nM of 5' – biotinylated RNA template, 1 μM of purified HCV NS5B, 500 nM of [^{3}H] ATP, and 100 μM of CTP, UTP and GTP. Reactions were carried out in 96-well flat-bottom, white polystyrene plates (Costar) in a buffer containing 40 mM HEPES (pH 8.0), 8.8

mM NaCl, 1 mM DTT, 0.5 mM EDTA, and 1.25 mM of $MgCl_2$ and $MnCl_2$, respectively (168, 180, 333). All reactions were started by addition of the NS5B enzyme, and were allowed to incubate at room temperature for 20 min. Reactions were stopped using 1 mg/ml streptavidin coated beads and 0.5M EDTA (Perkin Elmer; catalog no. RPNQ0006), and the 96-well plates were subjected to scintillation count by scintillation proximity analysis on MicroBeta liquid scintillation and luminescence counter (Perkin Elmer). All assays were repeated a minimum of three times.

Molecular modeling and bioinformatics. Amino acid sequences of NS5B from genotypes 2a, 3a and 5a were submitted to iTasser model prediction web service, and the crystal structure of HCV NS5B Con 1b genotype (PDB: 3FQL) was used as a scaffold for the generation of structural models (334-336). The models for 2a, 3a, and 5a genotypes were superimposed to genotype 1b using the Matchmaker tool in the UCSF Chimera software package (337). Following structural superposition, the Match – Align tool was used in Chimera to generate a sequence alignment of NS5B from genotypes 1b, 2a, 3a and 5a.

The structures of the acyl pyrrolidine and 1,5-benzodiazipine were created in ChemDrawUltra software (CambridgeSoft) and then were exported to Chimera, where they were subjected to 100 cycles of energy minimization using default settings, and saved as PDB files. Using AutoDockTools version 1.5.6 (338), protonation states and torsion angles were assigned to the two small molecules and the crystal structure of HCV NS5B Con 1b genotype (PDB: 3FQL); the files were saved in .pdbqt format. The acyl pyrrolidine and 1,5-benzodiazipine NNIs were then individually docked to NS5B genotype 1b using AutoDock Vina (339). For both docking

runs, the grid box was centered at x = 121.497, y = 100.691, z = 51.032, with size of x = 20, y = 30, z = 22, with the grid spacing being 1Å. The exhaustiveness was set to 100. The top pose from each run was selected and exported to UCSF Chimera and all figures were generated with this program.

Residues in HCV NS5B Con 1b genotype located within 5Å of the best docked NNI poses for each compound were annotated on the structure-based sequence alignment for NS5B genotypes 1b, 2a, 3a and 5a; polymorphisms that appeared in the different genotypes at these positions were introduced into the 1b backbone using the swap amino acid tool in UCSF Chimera and superimposed onto the docked poses of the acyl pyrrolidine and 1,5-benzodiazipine in unmodified 1b to validate the docking study.

2.5 Results

Activity of NS5B from genotypes 1b, 2a, 3a and 5a. HCV NS5B is capable of initiating RNA synthesis either *de novo* or through primer extension by short dinucleotide RNA primers (167). In this study, we purified HCV NS5B from genotypes 1b, 2a, 3a and 5a and examined NS5B activity using both primer-dependent and *de novo* RNA synthesis. The amount of full-length RNA synthesis varied between genotypes, indicating that the polymerases have different activities. Relative enzyme activities were compared to genotype 1b activity, which served as a well-characterized reference genotype. In assays that measure primer-dependent and *de novo* RNA synthesis, respectively, NS5B of genotype 2a showed the highest activity among the four enzymes tested. The activity of NS5B 2a is approximately 3-fold higher when compared with

NS5B 1b, while NS5B 3a and NS5B 5a show subtle reductions in enzymatic activity (340) (Figure 2.2).

Inhibition of RNA synthesis by the class-3 NNIs acyl pyrrolidine and 1,5-benzodiazepine and the NI 3'dCTP. For both acyl pyrrolidine and 1,5-benzodiazepine, increasing concentrations of the inhibitors resulted in a decrease in full-length RNA synthesis using NS5B genotype 1b, but the inhibitors were not able to efficiently inhibit NS5B of the other genotypes (2a, 3a and 5a) in a primer extension assay (Figure 2.3 A). NS5B genotype 1b was sensitive to both Acyl pyrrolidine and 1,5-benzodiazepine showing an IC_{50} of 0.06 μM and 0.13 μM, respectively. Genotype 3a was moderately sensitive to acyl pyrrolidine showing an IC_{50} value of 1.2 μM, while 1,5-benzodiazepine shows no inhibition. NS5B from genotypes 2a and 5a were not inhibited (Table 1). The results from *de novo* RNA synthesis were consistent with these findings (Table 2).

To confirm that the variation in sensitivity is specific to NNIs, we tested RNA synthesis in the presence of a nucleoside inhibitor, 3'dCTP (Figure 2.3 B). 3'dCTP lacks a 3'-hydroxyl group and hence acts as a chain terminator. With increasing concentrations of 3' dCTP, chain termination occurred at position +16 of the RNA template and there was a decrease in full-length product formation at position +20 (Figure 2.3 B); and this inhibition was similar across all genotypes showing IC_{50} values ranging from 4 μM to 12 μM (Table 1).

Identification of genotype-specific determinants of NNI resistance. In order to identify specific amino acids which govern resistance and susceptibility to Class 3 NNIs across HCV

genotypes, we generated structural models of NS5B from the amino acid sequences of genotypes 2a, 3a and 5a using the i-Tasser structure prediction web service. The crystal structure of NS5B genotype 1b, Con1 isolate (PDB: 3FQL) was used as a scaffold for model generation. The models were aligned to the structure of NS5B 1b, and a primary sequence alignment based on structural superposition was generated using the UCSF Chimera software.

The acyl pyrrolidine and 1,5-benzodiazepine NNIs were docked *in silico* to the structure of NS5B 1b using AutoDock Vina software. Residues located within 5 Å of each of the docking results for the NNIs (A218, V405, A450 and C451) were retained (Figure 2.4). These positions were then examined in the context of the structure-based sequence alignment generated for the genotypes studied. Polymorphisms in genotypes 2a, 3a, and 5a, which differed from the genotype 1b reference at these selected residue positions, were collated and are shown (Figure 2.4 C). At position 218, the genotype 1b NS5B contains an alanine, while genotypes 2a, 3a and 5a contain a serine. At position 450, the genotype 1b and 3a NS5Bs contain alanine while genotypes 2a and 5a contain serine. At position 405, genotype 1b, 3a and 5a all contain a valine while only genotype 2a has an isoleucine (Figure 2.4 C). Finally, at position 451 genotype 1b contains a cysteine while genotypes 2a and 5a contain valine and genotype 3a contains a threonine (Figure 2.4 C). The biochemical relevance of these residues was further analyzed by inserting these natural alterations (A218S, V405I, A450S, C451T and C451V) against the genotype 1b NS5B and performing inhibition assays.

Characterization of mutant enzymes. We monitored full-length RNA synthesis by each of the mutants in the presence of the inhibitors using both the primer-dependent and *de novo* methods

(Figure 2.5). Variation was seen in the baseline polymerase activity of the mutants with respect to wild-type (WT) genotype 1b NS5B (Figure 2.5). The A218S mutant was found to be less active whereas V405I was significantly more active than WT NS5B. The activities of A450S and C451T were similar to that of WT NS5B 1b (Figure 2.5). Mutation C451V showed no activity. All of the mutant enzyme activities were normalized to WT before testing inhibition of RNA synthesis with the NNI-3 inhibitors acyl pyrrolidine and 1,5-benzodiazepine.

The A218S and V405I mutants displayed significantly reduced sensitivity to acyl pyrrolidine and 1,5-benzodiazepine showing a 16-fold increases in IC_{50} values compared to WT NS5B, while A450S and C451T were similar to WT (Table 2.3). When tested in the *de novo* RNA synthesis assay, the A218S and V405I mutants were consistent with what was seen in primer-dependent RNA synthesis assays (Table 2.4), and a slight increase in IC_{50} was seen in the A450S and C451T mutants with acyl pyrrolidine and 1,5-benzodiazepine (Table 2.4).

To test the specificity of the NNI-3 acyl pyrrolidine and 1,5-benzodiazepine, which both bind to the palm sub-domain of the polymerase, their inhibitory activity was tested with mutant enzymes that are known to cause resistance to the different classes of polymerase inhibitors. The mutations tested were: S282T, which confers resistance to NIs; V499A, which confers resistance to thumb-1 inhibitors (NNI-1); M423I which confers resistance to thumb-2 inhibitors (NNI-2); and finally M414Q which confers resistance to both palm-1 (NNI-3) and palm-2 (NNI-4) inhibitors (279). Each of these mutations were generated in NS5B genotype 1b and RNA synthesis was monitored in the presence of the inhibitors. Significant variation in polymerase activity was seen with each of these mutations, with S282T being the least active, V499A and M423I having similar activity, and the M414Q was more active when compared to WT NS5B (Figure 2.6 A, B). Hence, all mutant enzyme activities were normalized to WT before testing

inhibition of RNA synthesis with acyl pyrrolidine and 1,5-benzodiazepine. As anticipated, we found that S282T, V499A and M423I showed sensitivity to both inhibitors showing IC_{50} values ranging from 0.06 μM to 0.09 μM, while M414Q, which is known to confer resistance to NNI-3, showed resistance to both inhibitors with almost a 10-fold increase in the IC_{50} values (Table 2.5). Similar results were obtained in *de novo* RNA synthesis assays (Table 2.6). These results indicate that both acyl pyrrolidine and 1,5-benzodiazepine are Class-3 NNIs that bind to the palm 1 domain of HCV NS5B.

2.6 Discussion

NNIs currently under evaluation in clinical trials are: NNI-1 inhibitor: BI-207127; NNI-2 inhibitors: GS-9669, Filibuvir, and VX-222; NNI-3 inhibitors: ABT-333, ABT-072, and Setrobuvir; and NNI-4 inhibitor: Tegobuvir (341). The genotype of HCV is known to play an important role in predicting patient response to therapy (342). Since the NNI binding sites are more variable than the highly conserved NS5B active site, the responses to NNIs are genotype-specific (343).

In this study, we employed a biochemical approach to identify amino acid polymorphisms responsible for changes in drug susceptibility. Based on our *in silico* docking analysis, we hypothesized that the resistance of NS5B from genotypes 2a, 3a, and 5a to palm 1 inhibitors (NNI-3) is mediated by specific pre-existing amino acid polymorphisms within 5 Å of the NNI-3 binding site. When we introduced these polymorphisms in genotype 1b, which is normally sensitive to the inhibitors, we found that the mutations induced resistance towards both

acyl pyrrolidine and 1,5-benzodiazepine, supporting our hypothesis. These modifications may cause interference in the interaction of the class 3 NNIs with their binding sites.

Of all the mutants generated in our experiments, the substitution of alanine to serine at position 218 (A218S) led to greatest decrease in sensitivity to acyl pyrrolidine and 1,5-benzodiazepine when introduced into genotype 1b NS5B. The Serine-218 polymorphism is present in NS5B from genotypes 2a, 3a, and 5a, all of which are known to be resistant to palm 1 inhibitors (NNI-3). Our model suggests that the serine side chain contains an additional hydroxyl group compared to alanine, which may lead to disruption of the hydrophobic environment in the palm 1 binding pocket. This in turn could affect the interaction of palm 1 inhibitors (NNI-3) with the enzyme (Figure 2.7 A, B).

The isoleucine-405 polymorphism is present in genotype 2a NS5B while 1b, 3a, and 5a contain a valine at this position (Figure 2.4 C). Substitution of isoleucine for valine at position 405 (V405I) conferred considerable resistance to acyl pyrrolidine and 1,5-benzodiazepine when introduced into genotype 1b NS5B. Our model also suggests that the isoleucine introduces an additional methyl group at position 405, which may result in a steric conflict with the inhibitors, thereby contributing to resistance (Figure 2.7). Furthermore, we found that the V405I mutation lead to a significant increase in activity of genotype 1b NS5B. These findings are consistent with earlier reports where introduction of isoleucine at position 405 in genotype 2a was shown to stabilize a more closed conformation of the thumb domain of NS5B, leading to increased RdRP activity (340). Hence, the increased activity of genotype 2a NS5B compared with the other HCV genotypes may be attributed partially to the presence of isoleucine at position 405. This mechanism may also explain the increased activity of the isoleucine-405 mutation when introduced into NS5B of genotype 1b (Figure 2.2, 2.5).

In addition, substitution of alanine to serine at position 450 (A450S) lead to a modest increase in resistance to acyl pyrrolidine in *de novo* RNA synthesis (Table 2.4). The serine at position 450 is present in genotypes 2a and 5a, and has been previously shown to be responsible for increased activity of the enzyme through a mechanism that involves formation of a hydrogen bond with isoleucine-560 in the linker region of NS5B (340). The formation of this additional hydrogen bond by the hydroxyl group in the serine side chain is hypothesized to result in a closed conformation of the polymerase. This conformation could also prevent NNI-3 association with the binding pocket.

Position 451 was the most variable position among the genotypes tested. While genotype 1b contains a cysteine, genotypes 2a and 5a contain a valine, and genotype 3a contains a threonine. Substitution of cysteine to valine at position 451 (C451V) resulted in an inactive enzyme, which could be due to the non-polar nature of the valine side chain. However, substitution of cysteine to threonine at position 451 (C451T) resulted in a functional enzyme and resulted in a minor increase in resistance to acyl pyrrolidine and 1,5-benzodiazepine in *de novo* RNA synthesis assays showing a 3-4 fold increase in the IC_{50} values (Table 2.4).

While we have shown that individual mutations introduced in genotype 1b can lead to significant resistance to class 3 NNIs, it is likely that multiple polymorphisms present in other HCV genotypes act collectively and synergistically to establish a resistant phenotype. Treatment with class-3 NNIs have also been shown to generate the alanine to serine mutation at position 218 (A218S) in genotype 1b, and the valine to isoleucine mutation at position 405 (V405I) in genotypes 1b, 3a, and 5a (340). This may be due to the selective pressure exerted on the virus due to NNI treatment. Improved understanding of the interactions of class-3 NNIs with their binding site, and the mechanisms of resistance of these natural variants may aid in the

development of compounds that bypass the natural resistance caused by genotype-specific amino acid polymorphisms. Together, our findings provide insight into the interactions of two class-3 NNIs, acyl pyrrolidine and 1,5-benzodiazepine, with their NS5B binding pockets, and suggest mechanisms of natural resistance to these NNIs. Understanding these mechanisms will inform the rational design of novel NNIs with improved activity and may also aid in the development of NNI-based combination therapies for treatment of chronic HCV infection.

2.7 Acknowledgements

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Figures for Chapter 2.

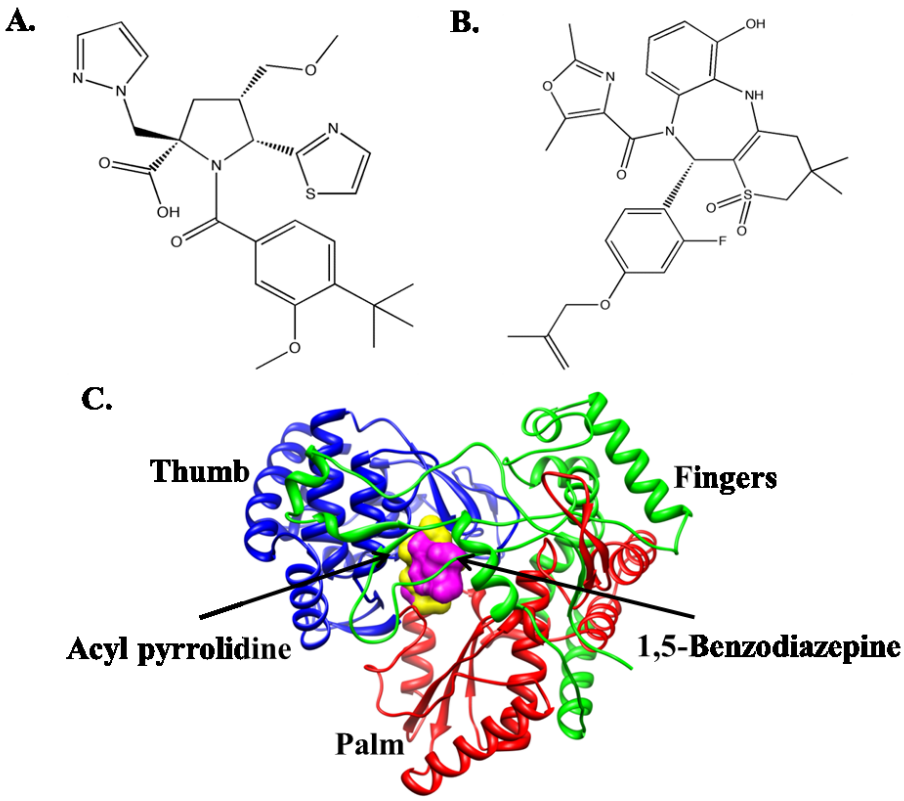

Figure 2.1: Acyl pyrrolidine and 1,5-Benzodiazepine. (A) Chemical structure of Acyl pyrrolidine. (B) Chemical structure of 1,5-Benzodiazepine. (C) Structural representation of HCV NS5B polymerase from genotype 1b showing palm (red), fingers (green), and thumb (blue) domains. The spheres colored in yellow and magenta represent Acyl pyrrolidine and 1,5-Benzodiazepine bound adjacent to the NS5B active site respectively.

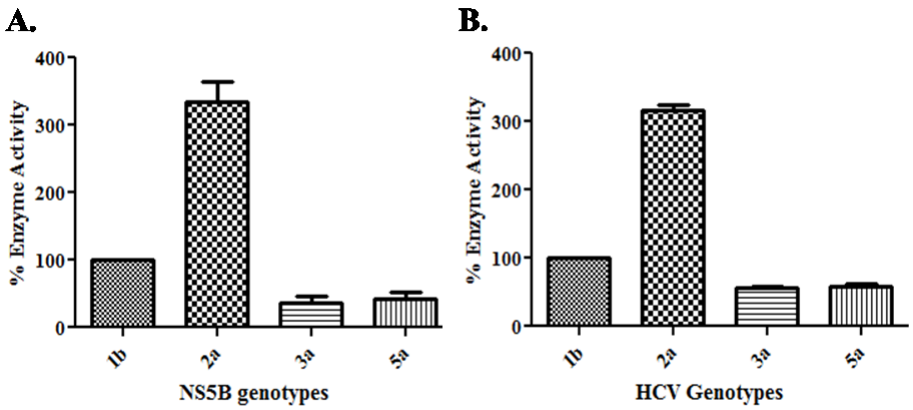

Figure 2.2: Activity of NS5B from different genotypes compared to genotype 1b. (A) Relative activity of NS5B from genotypes 1b, 2a, 3a and 5a measured using primer extension in an *in vitro* RNA synthesis assay. (B) Relative activity of NS5B from genotypes 1b, 2a, 3a and 5a tested *de novo* using scintillation proximity assay.

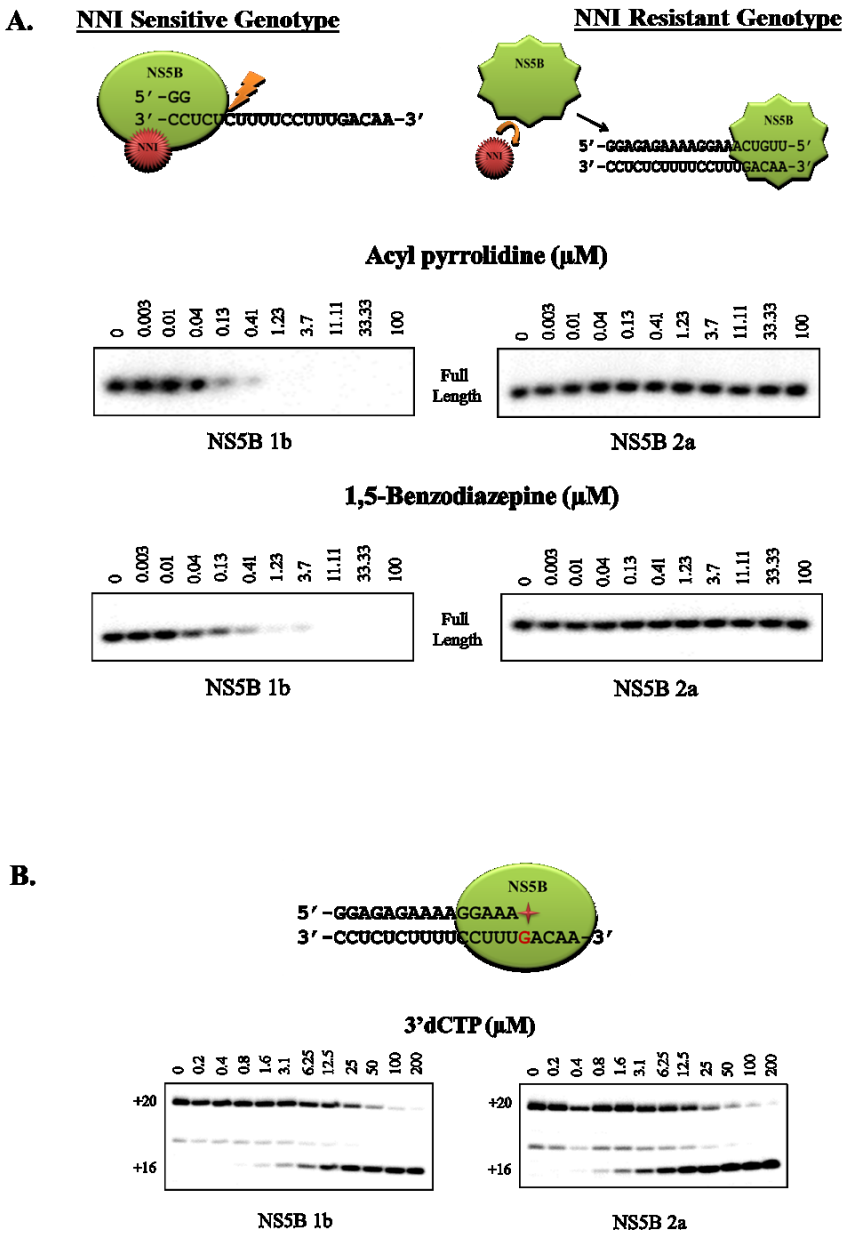

Figure 2.3: Full length RNA synthesis in the presence of the inhibitors Acyl pyrrolidine, 1,5-Benzodiazepine and 3'dCTP. RNA synthesis by NS5B from genotypes 1b and 2a in presence of increasing concentrations of (A) Acyl pyrrolidine and 1,5-Benzodiazepine and (B) 3'dCTP.

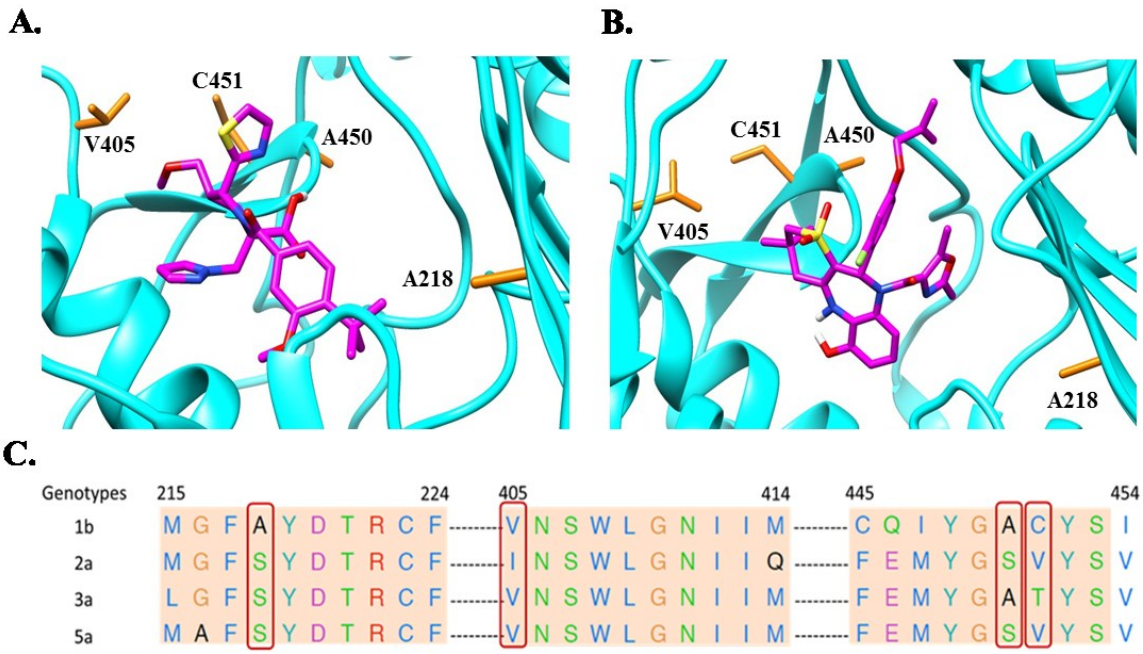

Figure 2.4: **Molecular Modeling of Acyl pyrrolidine and 1,5-Benzodiazepine and structural alignment across genotypes.** (A) Binding site of Acyl pyrrolidine in genotype 1b NS5B with residues within 5 Å of the inhibitor highlighted in orange. (B) Binding site of 1,5-Benzodiazepine in genotype 1b NS5B with residues within 5 Å of the inhibitor highlighted in orange. (C) Structural alignment of NS5B from different genotypes of HCV with amino acid residues highlighted in the NNI binding site. The red boxes indicate positions that are within 5 Å of the inhibitor that are variable across genotypes.

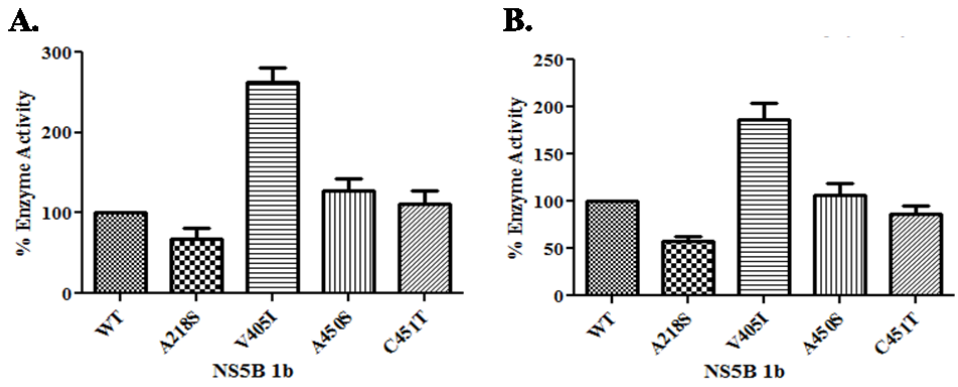

Figure 2.5: Activity of mutants generated in NS5B 1b compared to WT. (A) Relative activity of NNI-3 binding site mutants measured using primer extension in an *in vitro* RNA synthesis assay. (B) Relative activity of novel mutants tested *de novo* using scintillation proximity assay.

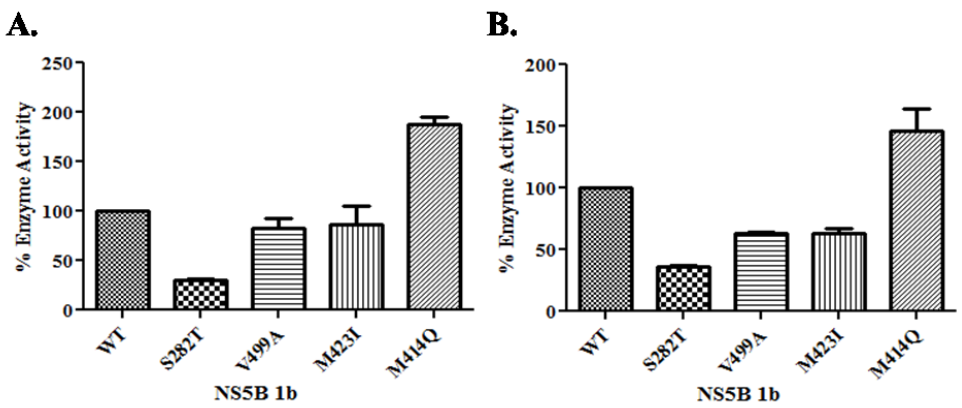

Figure 2.6: Activity of control mutants generated in NS5B 1b compared to WT. (A) Relative activity of control mutants measured using primer extension in an *in vitro* RNA synthesis assay. (B) Relative activity of control mutants tested *de novo* using scintillation proximity assay (SPA).

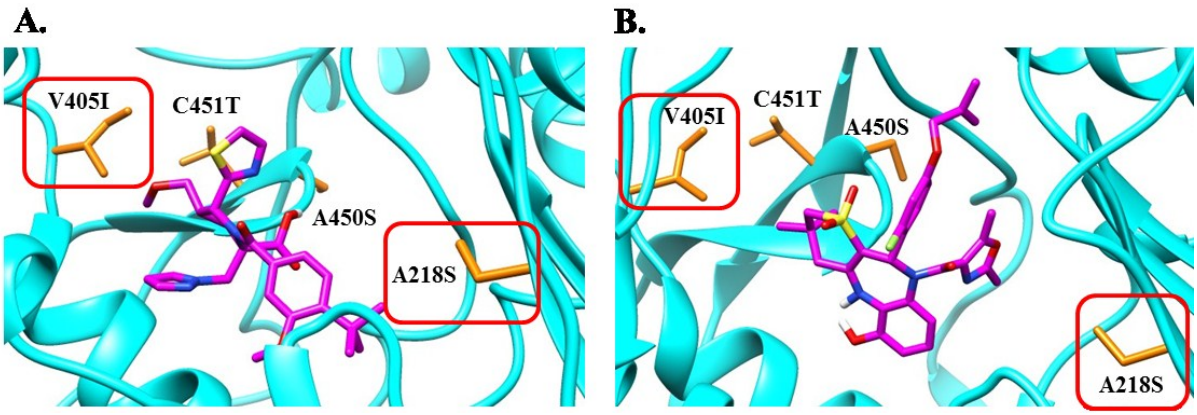

Figure 2.7: Mechanisms of resistance towards Palm I binding NNIs. Molecular docking of (A) Acyl pyrrolidine and (B) 1.5–Benzodiazepine to NS5B 1b harboring mutations within the binding pocket.

Tables for Chapter 2.

Table 2.1: IC_{50} values for Acyl-pyrrolidine and 1, 5-Benzodiazepine against NS5B from different genotypes by primer extension assay

Genotype	Acyl pyrrolidine	1,5-Benzodiazepine	3'dCTP
	IC_{50}[a] (μM)	IC_{50}[a] (μM)	IC_{50}[a] (μM)
1b	0.06 ± 0.003	0.1 ± 0.01	11.9 ± 1.6
2a	>100	>100	4.7 ± 0.04
3a	1.2 ± 0.15	>100	10.1 ± 0.7
5a	>100	>100	5.3 ± 0.2

[a] - IC_{50} is the inhibitory concentration of Acyl-pyrrolidine and 1, 5-Benzodiazepine that inhibits 50% of the enzyme. Values were calculated by fitting 10 data points to a sigmoidal dose-response equation using GraphPad Prism (version5.0). S.D. values were determined on the basis of three independent experiments.

Table 2.2: IC_{50} values for Acyl-pyrrolidine and 1, 5-Benzodiazepine against NS5B from different genotypes by *de novo* RNA synthesis assay

Genotype	Acyl pyrrolidine	1,5-Benzodiazepine
	IC_{50}[a] (μM)	IC_{50}[a] (μM)
1b	0.05 ± 0.001	0.2 ± 0.02
2a	>100	>100
3a	1.2 ± 0.11	>100
5a	>100	>100

[a] - IC_{50} is the inhibitory concentration of Acyl-pyrrolidine and 1, 5-Benzodiazepine that inhibits 50% of the enzyme. Values were calculated by fitting 10 data points to a sigmoidal dose-response equation using GraphPad Prism (version5.0). S.D. values were determined on the basis of three independent experiments.

Table 2.3: IC_{50} values for Acyl-pyrrolidine and 1, 5-Benzodiazepine against novel mutants of NS5B 1b by primer extension assay

Enzyme	Acyl pyrrolidine		1,5-Benzodiazepine	
	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]
WT	0.06 ± 0.003		0.1 ± 0.01	
A218S	1.0 ± 0.05	16.8	1.1 ± 0.1	8.5
V405I	0.7 ± 0.03	10.2	1.7 ± 0.11	13.2
A450S	0.04 ± 0.006	0.7	0.08 ± 0.01	0.6
C451T	0.06 ± 0.003	0.9	0.09 ± 0.01	0.7

[a] IC_{50} is the inhibitory concentration of Acyl-pyrrolidine and 1, 5-Benzodiazepine that inhibits 50% of the enzyme. Values were calculated by fitting 10 data points to a sigmoidal dose-response equation using GraphPad Prism (version5.0). S.D. values were determined on the basis of three independent experiments.

[b] -Fold increase is calculated as a ratio of the IC_{50} value of the mutant enzyme as compared with the wild-type enzyme.

Table 2.4: IC_{50} values for Acyl-pyrrolidine and 1, 5-Benzodiazepine against novel mutants of NS5B 1b by *de novo* RNA synthesis assay

Enzyme	Acyl pyrrolidine		1,5-Benzodiazepine	
	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]
WT	0.05 ± 0.001		0.2 ± 0.02	
A218S	1.0 ± 0.02	18.5	1.5 ± 0.1	7.2
V405I	0.7 ± 0.08	12	1.9 ± 0.2	8.6
A450S	0.3 ± 0.02	4.3	0.6 ± 0.03	2.7
C451T	0.2 ± 0.03	3.4	0.9 ± 0.02	4.3

[a] IC_{50} is the inhibitory concentration of Acyl-pyrrolidine and 1, 5-Benzodiazepine that inhibits 50% of the enzyme. Values were calculated by fitting 10 data points to a sigmoidal dose-response equation using GraphPad Prism (version5.0). S.D. values were determined on the basis of three independent experiments.

[b] -Fold increase is calculated as a ratio of the IC_{50} value of the mutant enzyme as compared with the wild-type enzyme.

Table 2.5: IC_{50} values for Acyl-pyrrolidine and 1, 5-Benzodiazepine against control mutants of NS5B 1b by primer extension

Enzyme	Acyl pyrrolidine		1,5-Benzodiazepine	
	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]
WT	0.06 ± 0.003		0.1 ± 0.01	
S282T	0.08 ± 0.01	1.3	0.06 ± 0.01	0.5
V499A	0.07 ± 0.01	1.1	0.08 ± 0.01	0.6
M423I	0.09 ± 0.01	1.3	0.06 ± 0.01	0.5
M414Q	0.6 ± 0.07	9.8	1.2 ± 0.07	8.9

[a] IC_{50} is the inhibitory concentration of Acyl-pyrrolidine and 1, 5-Benzodiazepine that inhibits 50% of the enzyme. Values were calculated by fitting 10 data points to a sigmoidal dose-response equation using GraphPad Prism (version5.0). S.D. values were determined on the basis of three independent experiments.

[b] -Fold increase is calculated as a ratio of the IC_{50} value of the mutant enzyme as compared with the wild-type enzyme.

Table 2.6: IC_{50} values for Acyl-pyrrolidine and 1, 5-Benzodiazepine against novel mutants of NS5B 1b by *de novo* RNA synthesis assay

Enzyme	Acyl pyrrolidine		1,5-Benzodiazepine	
	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]
WT	0.05 ± 0.001		0.2 ± 0.02	
S282T	0.07 ± 0.01	1.3	0.2 ± 0.04	0.7
V499A	0.07 ± 0.01	1.3	0.2 ± 0.03	1.1
M423I	0.1 ± 0.01	2	0.3 ± 0.002	1.4
M414Q	0.7 ± 0.03	12	1.3 ± 0.3	6.3

[a] IC_{50} is the inhibitory concentration of Acyl-pyrrolidine and 1, 5-Benzodiazepine that inhibits 50% of the enzyme. Values were calculated by fitting 10 data points to a sigmoidal dose-response equation using GraphPad Prism (version5.0). S.D. values were determined on the basis of three independent experiments.

[b] -Fold increase is calculated as a ratio of the IC_{50} value of the mutant enzyme as compared with the wild-type enzyme.

CHAPTER 3

Complex Interactions between S282 and G283 of HCV NS5B and the Template Strand Affect Susceptibility to Sofosbuvir and Ribavirin

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3.1 Preface to Chapter 3

As discussed in *Chapters 1* and *2*, NNIs have a tendency to show genotype-specific inhibitory activity. In contrast, NIs typically have pan-genotypic activity. One such NI, sofosbuvir, has shown great success in the treatment of HCV in the clinic in IFN-free regimens. The S282T mutation has been isolated in the clinic and this substitution confers decreased susceptibility to 2'-C-methylated compounds, including sofosbuvir. In this *Chapter* we have investigated the mechanism with which S282T shows resistance to sofosbuvir and other 2'C-methylated compounds. Furthermore, we have revealed that the S282T mutation sensitizes NS5B to ribavirin. Our results suggest that the emergence of resistance could be suppressed by the use of sofosbuvir/ribavirin combination therapy in the clinic.

3.2 Abstract

The HCV RNA-dependent RNA-polymerase NS5B is essentially required for viral replication and serves as a logical drug target. The NS5B inhibitor sofosbuvir is a nucleotide analogue that has been approved for HCV treatment in combination with ribavirin. Although the emergence of resistance to sofosbuvir is rarely seen in the clinic, the S282T mutation was shown to decrease susceptibility to this drug. S282T was also shown to confer hypersusceptibility to ribavirin, which is of potential clinical benefit. Here we devised a biochemical approach to elucidate the underlying mechanisms. Recent crystallographic data revealed a hydrogen bond between S282 and the 2′-hydroxyl of the bound nucleotide, while the adjacent G283 forms a hydrogen bond with the 2′-hydroxyl of the residue of the template that base pairs with the nucleotide substrate. We show that DNA-like modifications of the template that disrupt hydrogen bonding with G283 cause enzyme pausing with natural nucleotides. However, the specifically introduced DNA residue of the template re-establishes binding and incorporation of sofosbuvir in the context of S282T. Moreover, the DNA-like modifications of the template prevent the incorporation of ribavirin in the context of the wild-type enzyme, while the S282T mutant enables binding and incorporation of ribavirin under the same conditions. Together these findings provide strong evidence to show that susceptibility to sofosbuvir and ribavirin depends crucially on a network of interdependent hydrogen bonds that involve the adjacent residues S282 and G283 and their interactions with the incoming nucleotide and complementary template residue, respectively.

3.3 Introduction

Hepatitis C Virus (HCV) has a single-stranded RNA genome of positive polarity and belongs to the *Flaviviridae* family (344). Chronic HCV infection is associated with severe liver disease, including cirrhosis, and an increased risk of hepatocellular carcinoma (345). If adequately treated, HCV can be cured. Previously, treatment options for HCV infected persons were limited to combination therapy with pegylated interferon α (IFN-α) and ribavirin (346). Ribavirin is a nucleoside analog, with a guanine-like base moiety, that can be incorporated by the HCV RNA-dependent RNA polymerase (RdRp) opposite cytosine or uracil, although the detailed mechanism of action remains elusive (347). In 2011, the first direct-acting antivirals (DAAs) targeting the viral protease, non-structural protein 3 (NS3), were approved as a component of interferon-based therapies (348); however, the clinical use of the first generation protease inhibitors boceprevir and telaprevir is limited by a narrow coverage of HCV genotypes and a low barrier to the selection of resistance (349). Nucleoside or nucleotide inhibitors (NIs) that target the HCV RdRp, or non-structural protein 5B (NS5B), address these weaknesses (350). In 2013, sofosbuvir, a nucleotide prodrug of 2′-deoxy-2′-α-fluoro-β-*C*-methyluridine, was approved in IFN-free combination therapies for chronic HCV infection (323, 324).

NIs compete with the natural ribonucleoside 5′-triphosphates (NTP) for binding to the highly conserved HCV polymerase active site, and, once incorporated, interfere with subsequent nucleotide additions (351). These compounds generally show pan-genotypic activity and exhibit a high barrier to the development of resistance (352). HCV NS5B is capable of initiating RNA synthesis *de novo* or in the presence of a dinucleotide primer that represents the initial product following phosphodiester bond formation (168, 353). This stage of RNA synthesis is fragile and accompanied by frequent dissociation events (168, 353). Conversely, RNA synthesis is highly

processive following the incorporation of two to three nucleotides, which defines the elongation stage (164). As the HCV RNA genome contains approximately 9600 residues, the elongation stage provides theoretically thousands of opportunities for NIs to incorporate and inhibit viral RNA synthesis (354).

The crystal structure of the HCV NS5B polymerase has been determined in the absence and presence of nucleic acid substrates, with and without a bound nucleotide (152, 314, 355). The fold is reminiscent of a right hand with fingers, palm, and thumb subdomains that form a fist-like conformation without sufficient space for the RNA primer-template to bind (154). The HCV NS5B polymerase contains a β-hairpin loop that appears to interfere with binding to double-stranded RNA (160). Recent structures of NS5B with a bound primer-template show that the thumb domain opens relative to the fingers, which helps to accommodate the nucleic acid substrate (314). Structures of NS5B that have a bound nucleotide and mimic the elongation phase shed light on binding of nucleotides and nucleotide analogues (355). The 2′-hydroxyl group of the ribonucleotide substrate is involved in a hydrogen bonding network that includes position S282 that is associated with resistance to sofosbuvir (356). The S282T mutation confers decreased susceptibility to 2′-C-methylated compounds, including sofosbuvir (357). This mutation is rarely seen in the clinic, which can be ascribed to a fitness deficit and a relatively high genetic barrier (354, 358). It has been suggested that this mutation may discriminate against the inhibitor, causing a steric clash with the 2′-C-methyl motif; however, the detailed mechanism of resistance has yet to be elucidated (358). The S282T mutation was also shown to confer hypersusceptibility to ribavirin, which adds another layer of complexity to this problem (32, 33). Here, we devised a biochemical approach and identified complex interactions between the

template strand and the adjacent residues S282 and G283 that affect susceptibility to sofosbuvir and ribavirin.

3.4 Materials and Methods

Nucleic acids, nucleotides and inhibitors. The following 20-mer heteropolymeric RNA template sequences were used in this study: 5′ – AACAGUXUCCUUUUCUCUCC – 3′ (T20-X14) where the base X represents uridine (U, T20-U14), thymidine (dT, T20-dT14), deoxyguanosine (dG, T20-dG14), 2′-deoxyuridine (dU, T20-dU14) and 2′-fluorouridine (2′-FU, T20-2'F-U14) (Trilink); 5′ – AACAGUUUCCUUUUCUCUCC – 3′ (T20-G16) where the base G represents guanine (G, T20-G16), deoxyguanosine (dG, T20-dG16), 2′-deoxy-2′-fluoro-guanosine (2′-FG, T20-2'F-G16) and 2′-*O*-methyl-guanosine (2′-O-Me-G, T20-2'OMe-G16). The modified nucleic acids were provided by Dr. Masad Damha (McGill University); 5′ – CUCGAUUUCCUUUUCUCUCC – 3′ (T20-A16) where the base A was modified to adenosine (A, T20-A16) or deoxyadenosine (dA, T20-dA16) (Trilink); and 5′ – AAAUCGAGAAGGAGAAAGCC – 3′ (T20-C16) where the base C was modified to cytosine (C, T20-C16) or 2′-deoxycytidine (dC, T20-dC16) (Trilink). All the templates were polyacrylamide gel electrophoresis (PAGE) purified. The 5′ – GG – 3′ dinucleotide (Trilink) was used as a primer. Labeling of the 5′ end of the dinucleotide primer with [γ-^{32}P] ATP was carried out with T4 polynucleotide kinase (Thermo Scientific). NTPs were purchased from Thermo Scientific. The nucleotide analogue 2′-C-methyl-cytidine triphosphate (2′-C-methyl-CTP) was synthesized by Dr. Raymond Schinazi (Emory University); the triphosphate form of the active

metabolite of sofosbuvir (sofosbuvir-TP) was provided by Gilead, and ribavirin-TP was purchased from Jena Bioscience (Jena, Germany).

Expression and purification of HCV NS5B. The HCV NS5B sequence derived from genotype 1b (Con-1) without the C-terminal, hydrophobic tail of 21 amino acids was inserted into the expression vector pET-21 (Novagen). The plasmid encoding the truncated enzyme with a C-terminally added His-tag was transformed into *Escherichia coli* BL21 (DE3) cells. The protein was purified using a Ni-chelating Sepharose column. Mutant enzymes were generated by site-directed mutagenesis using the Stratagene Quik-Change kit, according to the manufacturer's instructions (248). All mutations were confirmed by sequencing at the Genome Quebec Innovation Center.

Full-length RNA synthesis. The standard reaction mixture contained 500 nM of RNA template (T20-X14), 1 μM of HCV NS5B, 0.2 μM radiolabeled 5′-GG-3′ primer, and 5 μM NTPs. Reactions were carried out in a buffer containing 40 mM HEPES (pH 8.0), 15 mM NaCl, 1 mM dithiothreitol (DTT), and 0.5 mM EDTA. RNA synthesis was initiated at room temperature using 6 mM $MgCl_2$, and stopped at specific time points by the addition of 0.5 M EDTA in 95% formamide containing xylene cyanol and bromophenol blue. The samples were then heat denatured at 95ºC for 5 min and resolved on a 20% polyacrylamide-7M urea gel. Products were visualized using a Bio-Rad Phosphorimager.

Dose-dependent incorporation of nucleotides and nucleotide analogues. Five hundred nM of RNA template (T20-G16, T20-dG16, T20-2'OMe-G16), 1 μM of HCV NS5B, and 5 μM of ATP and GTP were incubated for 45 min to halt RNA synthesis at position +15. Increasing concentrations of CTP were then added to determine the incorporation efficiency against G, dG, and 2′-O-Me-G at position +16. Reactions were allowed to proceed for 1 min. For 2′C-Methyl-CTP, the standard reaction mixture contained RNA template (T20-G16, T20-dG16, T20-2'F-G16), 5 μM of GTP, ATP, UTP and 0.5 μM of the competing nucleotide CTP with increasing concentrations of 2′-C-methyl-CTP. For the active metabolite of sofosbuvir-TP, the standard reaction mixture contained RNA template (T20-A16, T20-dA16) and 5 μM of GTP, ATP, CTP and 0.5 μM of the competing nucleotide UTP, with increasing concentrations of sofosbuvir-TP. The reactions were allowed to proceed at room temperature for 45 min. The concentration of 2′-C-methyl-CTP or 2′-C-methyl-2′-fluoro-UTP required to inhibit 50% of full-length product formation (IC_{50}) was calculated using Prism software (GraphPad, Inc). For Ribavirin assays, the standard reaction mixture contained RNA template (T20-C16, T20-dC16), and 5 μM of CTP and UTP. Reactions were allowed to proceed at room temperature for 45 min up to position +15. Increasing concentrations of GTP or ribavirin were then added to determine incorporation efficiency against C or dC at position +16. GTP was allowed to incorporate for 1 min and ribavirin was allowed to incorporate for 5 min before the reactions were stopped. Samples were analyzed as described above. All assays were repeated a minimum of three times.

3.5 Results

Experimental strategy. The recent structures of binary and ternary complexes of HCV NS5B revealed that the side chain of S282 is involved in a hydrogen bond network that involves the 2′-hydroxyl of the bound nucleotide (Figure 3.1 A). The structures further show that the complementary residue of the template forms a hydrogen bond with its 2′-hydroxyl group and the backbone of the conserved G283 (Figure 3.1 B). In light of these structural observations, it is tempting to hypothesize that the precise arrangement between the ultimate base pair and the adjacent residues S282 and G283 is crucial for nucleotide binding and/or incorporation. The functional role of the hydrogen bond between G283 and the 2′ hydroxyl of the template is of potential relevance in this regard and remains to be elucidated. To address this problem, we studied NS5B-mediated RNA synthesis with chimeric templates with strategically engineered modifications at the site of incorporation.

RNA synthesis by wild-type NS5B with RNA and RNA-DNA chimeric templates. Previous studies have shown that HCV NS5B is able to utilize DNA templates, albeit with reduced efficiency (170). The reduction in RNA synthesis can be ascribed to missing hydrogen bonds between the template and specific amino acids of the RNA binding channel, including G283. To better understand the specific contribution of G283, we synthesized chimeric RNA-DNA templates with chemical modification introduced at the site of incorporation opposite to the incoming nucleotide substrate. The modifications are strategically engineered at position +14 of a model 20-mer heteropolymeric template (T20X14) (Figure 3.2 A). Modifications at position +14 include U, dT, dG, dU or 2′-F-U (Figure 3.2 B). Hence, the lack of the 2′-hydroxl group is

studied in the context of different bases dT, dG, and dU. In 2′F-U the 2′-hydroxyl group is substituted with the bioisosteric 2′-fluoro, which locks the sugar pucker in an RNA-like North conformation.

RNA synthesis with the regular RNA template shows full-length product formation in the absence of specific pausing sites. The replacement of U by dT or dU resulted in pausing at position +13 (Figure 3.2 C). This could either be due to the lack of 2′-hydroxyl group at template position +14, when RNA is replaced by DNA, or to potential conformational differences in the sugar pucker. The DNA residue of the template may adopt the South conformation that is typical for DNA residues (359). However, pausing is also evident with 2′-F-U at position +14, which suggests that the lack of the 2′-hydroxyl group rather than putative differences in the sugar conformation causes pausing of the polymerase. The nature of the base seems to play an additional role in this regard, given that pausing is not seen with a dG modification under the same reaction conditions.

CTP dose-dependent elongation by WT NS5B. To further assess the importance of the 2′-hydroxyl group of the template opposite the incoming nucleotide, we included the 2′-O-Me-G and compared the efficiency of CTP incorporation with the regular RNA template (G) and the DNA chimeric variant (dG) that showed no significant pausing. Pre-elongation by NS5B was conducted up to position +15 by the omission of CTP and UTP from the reaction mixture. The natural CTP substrate, along with UTP, was then added at increasing concentrations to monitor the efficiency of nucleotide incorporation at the modified template position (Figure 3.3 A). Templates containing G and dG gave rise to full-length product formation with similar efficiencies (Figure 3.3 B). However, templates containing the 2′-O-Me-G modification required

markedly higher substrate concentrations for synthesis of the full-length product (Figure 3.3 B). The bulky nature of the 2′-O-Me group can interfere with several processes including nucleotide binding, nucleotide incorporation, and/or translocation of NS5B relative to the bound nucleic acid. Formation of the post-translocated conformation is required for nucleotide binding; hence, a bias to the pre-translocated state of the complex would also reduce access to the nucleotide binding site (248).

Incorporation of 2′-C-methyl-CTP and 2′-C-methyl-2′-fluoro-UTP by WT NS5B and resistance-conferring mutants. We next evaluated the potential impact of changes of the interaction between G283 and the 2′-hydroxyl group of the RNA template with respect to the incorporation of NIs. For this purpose, we compared the efficiency of incorporation of 2′-C-methyl-CTP by WT NS5B with templates containing G, dG or 2′-F-G at position +16 (Figure 3.4 A). Full-length RNA synthesis by WT NS5B with the natural template was sensitive to inhibition by 2′-C-Me-CTP, which causes chain-termination at position +16 (Figure 3.4 B). The modifications of the template had now a significant effect on the inhibition pattern. The S282T mutant enzyme was unable to efficiently incorporate the inhibitor under these conditions, which is consistent with the expected, resistant phenotype (Figure 3.4 C). WT NS5B showed an IC_{50} of approximately 12 μM, while the S282T mutant prevented incorporation of the inhibitor under these conditions (Table 3.1). However, when tested with a template containing dG at position +16, both WT NS5B and S282T efficiently incorporated the inhibitor with IC_{50} values of ~10 μM. Similar results were obtained with templates containing 2′-F-G at position +16. WT NS5B and S282T are inhibited by 2′-C-Me-CTP with IC_{50} values of 10.6 μM and 21.5 μM, respectively (Figure 3.4 B, C and Table 3.1). Together, these data provide strong evidence to suggest that the

2′-hydroxyl group of the template nucleotide also plays an important role in establishing the resistant phenotype.

Very similar patterns are seen with the uridine analog 2′-C-Me-2′-F-UTP. WT NS5B was sensitive to 2′-C-Me-2′-F-UTP, while S282T had reduced rates of incorporation of this inhibitor (Figure 3.5 B and Table 3.2). With WT NS5B and a template containing A at position +16, we measured an IC_{50} value of 2 μM, while S282T had an IC_{50} value of 19.5 μM. When tested with a template containing dA at position +16, S282T showed sensitivity to sofosbuvir, resulting in a lower IC_{50} value of 2 μM as measured with WT NS5B (Table 3.2). The lack of a hydrogen bond between G283 and the template at the site of incorporation seems to neutralize the effect of the 2′-C-Me group, which is a common structural feature of 2′-C-Me-2′-F-UTP and 2′-C-Me-CTP.

Incorporation of Ribavirin by WT NS5B and S282T. Finally, we measured the efficiency of incorporation of ribavirin-TP by WT NS5B and S282T (Figure 3.6). Pre-elongation by WT NS5B or S282T was conducted up to position +15, followed by the addition of ribavirin in a dose-dependent manner to monitor incorporation opposite C or dC at position +16 (Figure 3.6 A). When tested with the natural RNA template, we measured higher levels of RNA synthesis with the S282T mutant when compared with WT NS5B (Figure 3.6 B, C). Note that both template positions +16 (C) and +17 (U) allow the incorporation of ribavirin, which together with the pre-existing NTP pool yields the full-length product +20. The incorporation of ribavirin-TP is negligible when tested with a template containing dC, indicating that the loss of the hydrogen bond between G283 and the sugar moiety of the template prevents the reaction in this context. However, modest levels of ribavirin incorporation are seen with the S282T mutant enzyme, which supports the trend seen with the natural RNA template.

3.6 Discussion

Sofosbuvir and ribavirin are currently the only FDA-approved NIs for HCV treatment, although ribavirin is not classified as a DAA (360). Clinical trials revealed sustained virological response (SVR) rates of >90% in the context combination therapies with the two drugs (361). The emergence of resistance to regimens containing sofosbuvir is rare, although a few cases are documented (297). The NS5B S282T variant can emerge *in vitro* and *in vivo* under the selective pressure of 2′-C-methylated nucleotides, including sofosbuvir. Specific mutations conferring resistance to ribavirin have not been identified (249). However, Svarovskaia and colleagues reported subtle increases in susceptibility to ribavirin in the context of the S282T mutation (32, 33). Such a pattern may translate in clinical benefits. Moreover, the observation that a mutation in close proximity to the active site of HCV NS5B can affect susceptibility to ribavirin, suggests that the viral RdRp is the drug target. Potential underlying mechanisms include competitive inhibition with cellular nucleotide pools, reductions in nucleotide incorporations following to ribavirin-terminated primers, or decreases in fidelity; however, the mechanism of action of ribavirin remains elusive and indirect effects are likewise considered. In contrast, biochemical data have shown that sofosbuvir acts as a non-obligate chain terminator that prevents further incorporation events (325, 362). Modeling studies suggested that that S282T may cause a steric conflict with the 2′C-methyl group, which provides a possible mechanism for resistance. Based on structures of NS5B with a bound primer/template, Mosley and colleagues pointed to more complex mechanisms that can involve adjacent amino acids such as G283, which forms a hydrogen bond with the 2′-hydroxyl of the template (314). Here we utilized templates with DNA-like modifications to disrupt this hydrogen bond and studied the consequences on rates of incorporation of sofosbuvir and ribavirin, respectively. The data provides a model that helps to

explain the effect of S282T on changes in susceptibility to nucleotide analogue inhibitors (Figure 3.7).

The model is derived from Figure 3.1 and depicts the relevant interactions between the three components that form the ternary NS5B-primer/template/NTP complex (Figure 3.7). The structural data suggest that the covalently linked residues S282 and G283 facilitate binding and proper positioning of the NTP substrate and template (Figure 3.1). The loss of the hydrogen bond between G283 and a DNA template causes in some cases enzyme pausing, which supports this notion. Pausing is evident with dT, dU and 2'F-U modifications in the template strand, while pausing is not seen with the dG modification. The additional hydrogen bond in GC versus AT or AU base pairs may at least partially compensate for the loss of the hydrogen bond between G283 and the 2'-deoxy residue in the template.

S282T diminishes binding and/or incorporation of 2′C-methylated compounds. The putative steric clash between S282T and the 2'C-methyl group of the inhibitor can diminish the interaction between the side chain of residue 282 and the sugar moiety of the nucleotide analogue. The altered interaction may in turn affect proper base pairing (Figure 3.7 A). As a result the compound is not in a favorable position for incorporation. However, a DNA residue in the template appears to neutralize this effect. A possible explanation is that the lack of the hydrogen bond between G283 and the 2'-OH of the template causes an increase in flexibility at the nucleotide binding site that permits incorporation of the analogue. It is therefore conceivable that the postulated constraints introduced by the S282T mutation are relieved through a more favorable positioning of the template for base pairing with the inhibitor (Figure 3.7 B). The total

available space for productive inhibitor binding is likely increased. This model is consistent with an increase in rates of incorporation of ribavirin in the context of S282T. Ribavirin is a poor substrate for NS5B, which is likely due to weak base pairing between the pseudo base of ribavirin and the template. The DNA residue of the template further enhances this effect, which helps to explain the lack of activity in this context (Figure 3.7 C). The ability to incorporate ribavirin is partially restored with the S282T mutant. This suggests an improvement in inhibitor binding, although the nature of the altered interaction remains to be characterized (Figure 3.7 D).

Taken together, our findings provide strong evidence to show that susceptibility to nucleotides and nucleotide analogs depends crucially on a network of interdependent hydrogen bonds that involve the adjacent residues S282 and G283 and their interactions with the incoming nucleotide and complementary template residue, respectively. Changes in this ring-like structure can affect substrate binding and/or the precise alignment of substrate and primer for catalysis. However, the inherently flexible nature of this hydrogen bonding network may also be exploited in drug development efforts. The data provide a rationale and the experimental tools for the design of inhibitors that are simultaneously modified at their base and sugar moieties in attempts to maximize inhibitor binding and rates of incorporation.

3.7 Acknowledgements

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Figures for Chapter 3.

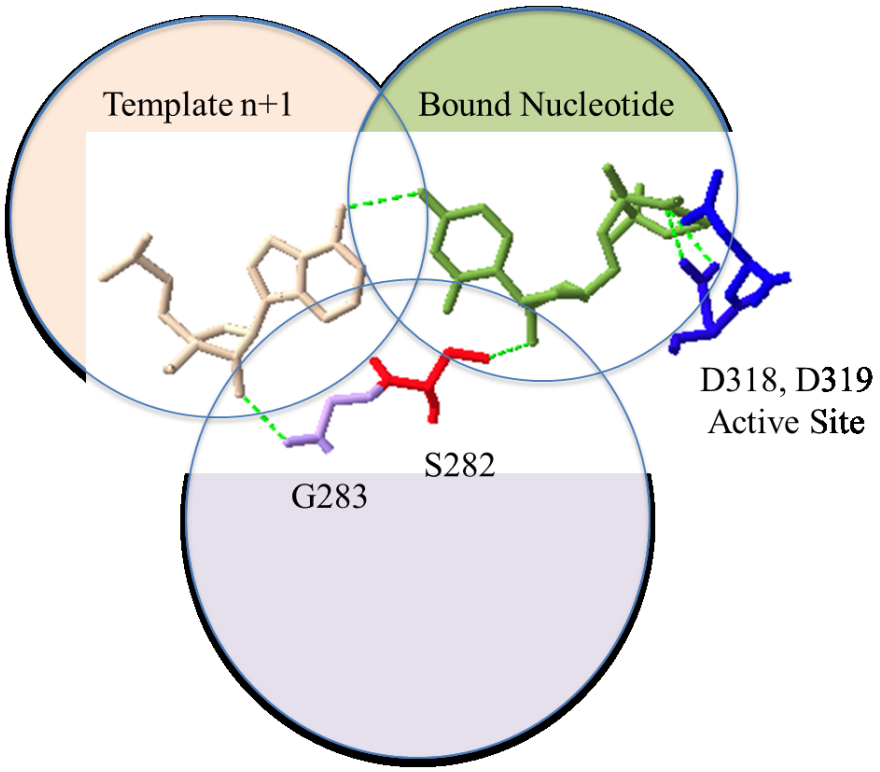

Figure 3.1: Hydrogen bonding network at the active site of NS5B. The network involves three components of a ternary complex of NS5B, primer/template, and bound nucleotide. The nucleotide (UDP) is shown in green, and the complementary residue of the template is shown in beige. The 2′-hydroxyl of the template forms a hydrogen bond with backbone oxygen of G283 (purple), and the covalently linked S282 (red) forms a hydrogen bond with the 2′-hydroxyl of the bound nucleotide. Active site residues D318 and D319 (blue), and diphosphate moiety of the bound nucleotide interact with the catalytic metal ions. The figure was generated with SwissPdbViewer software (PDB: 4WTA).

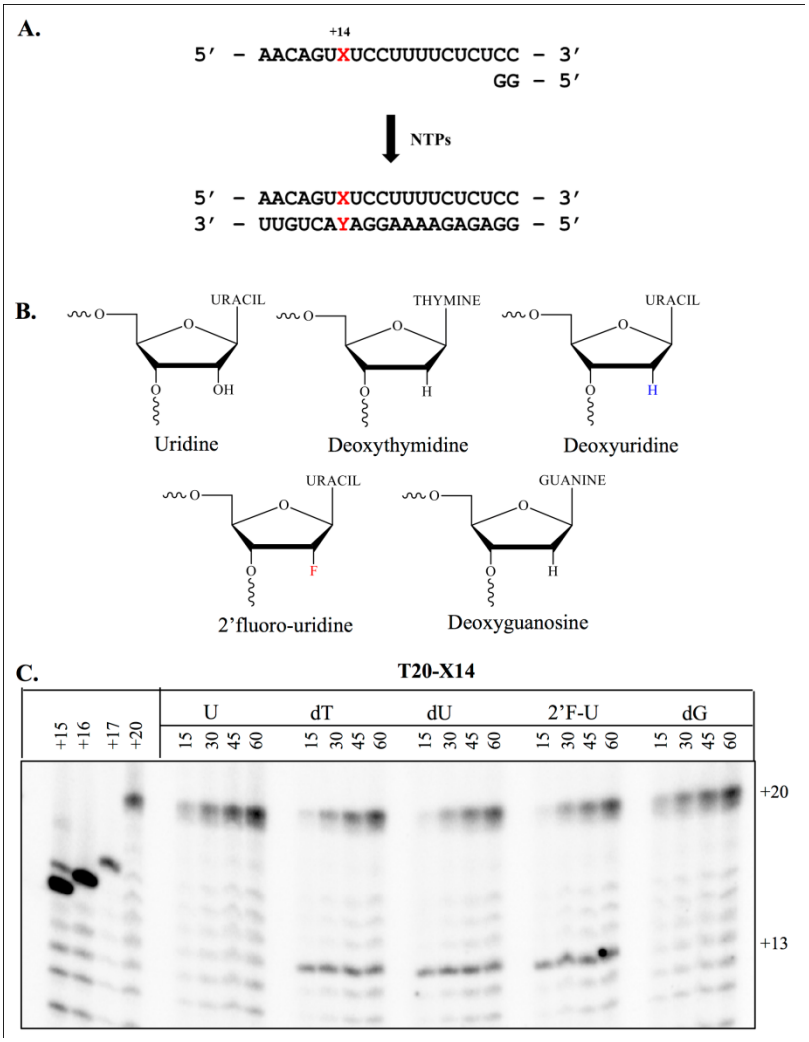

Figure 3.2: NS5B-mediated RNA synthesis and effects of DNA-like modifications on the template. (A) Experimental design for RNA synthesis with a short model template. The modification (X) in the template and its complementary residue Y of the newly synthesized RNA are shown in red. The reaction is initiated with a dinucleotide primer and yields a 20mer full-length product following exposure to all four NTPs. (B) Structures of modifications at template position +14: dT, dG, dU (South sugar pucker conformation), and U, 2′F-U (80-90% North sugar pucker conformation). (C) RNA synthesis with modified templates as described under B with RNA length markers of positions +15, +16, +17 and +20 are shown on the left. Enzyme pausing is seen at position +13.

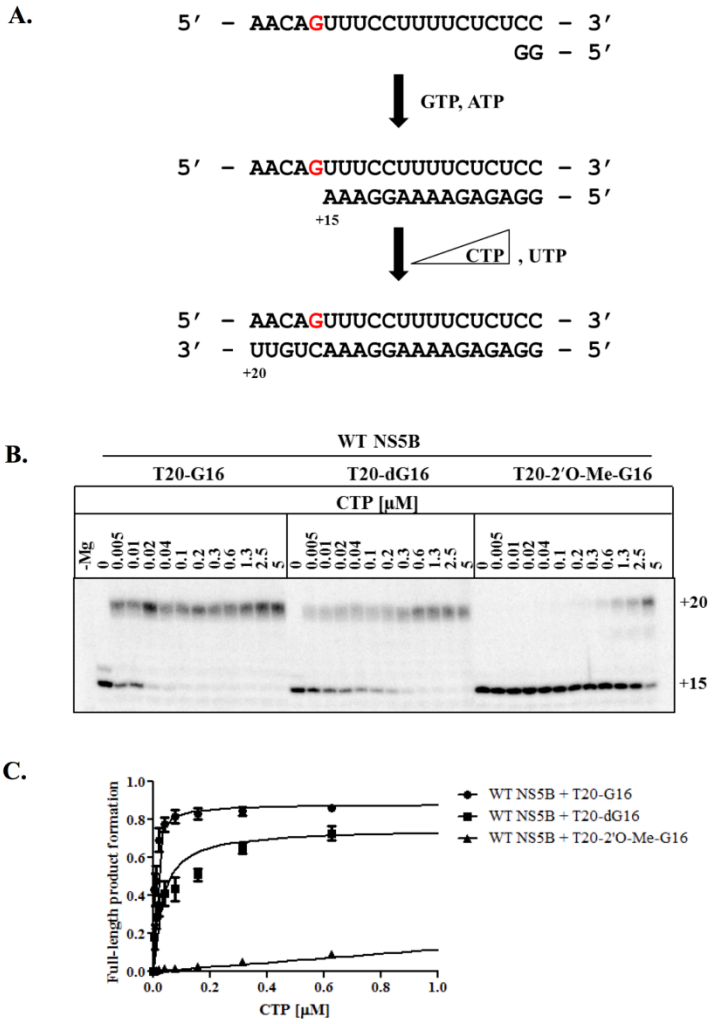

Figure 3.3: Incorporation efficiency on modified templates. (A) Schematic of NS5B elongation on T20-G16 templates modified at position +16 (red). The addition of GTP and ATP to the reaction mixture leads to a 15mer RNA product, and the subsequent addition of UTP and increasing concentrations of CTP yields a 20mer full-length product. (B) WT NS5B-mediated RNA synthesis performed with increasing concentrations of CTP in the presence of RNA templates containing G, dG, and 2′-O-Me-G at position +16. (C) Graphical representation of data shown under B.

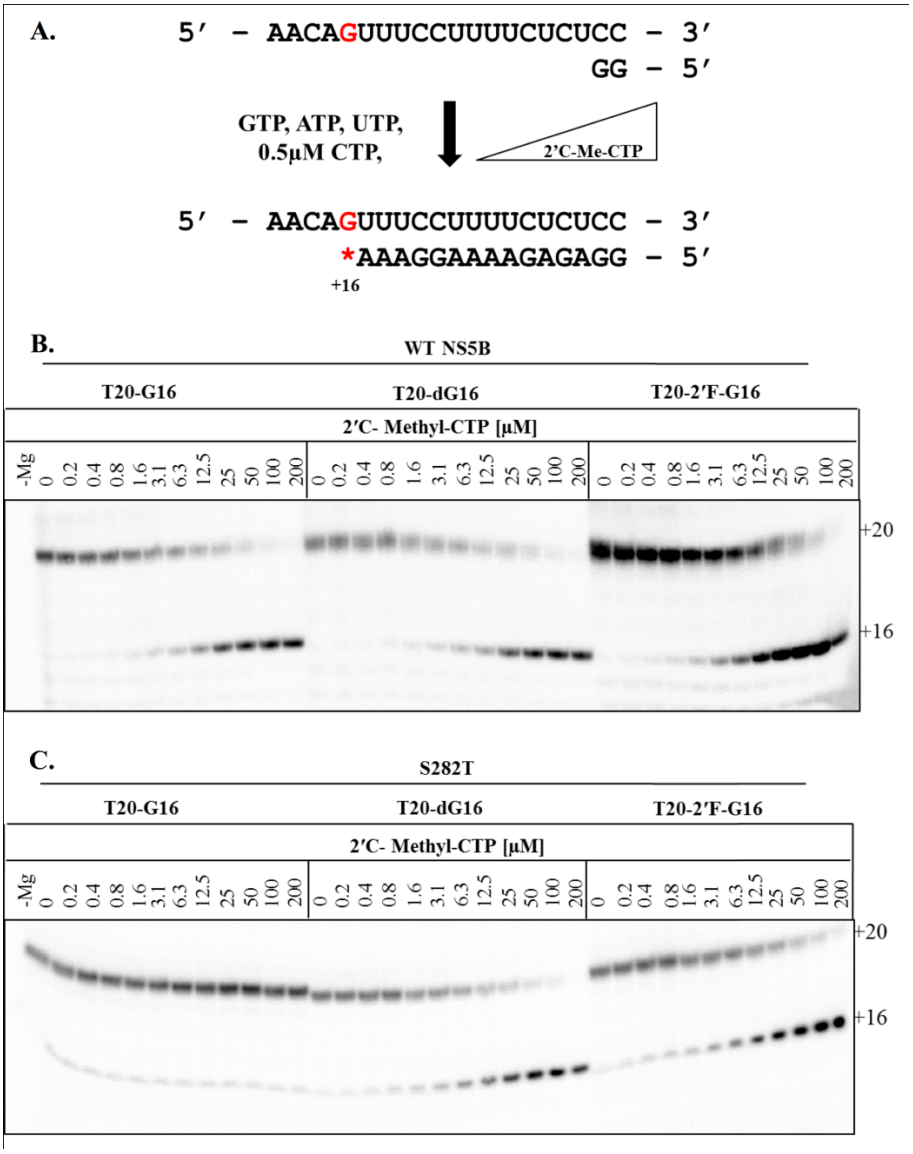

Figure 3.4: RNA synthesis performed in the presence of increasing concentrations of 2'C-Methyl-CTP. (A) Schematic of chain termination by 2′-C-methyl-CTP on T20-G16 modified at position +16 to G, dG, and 2′-F-G (red). RNA synthesis performed in the presence of increasing concentrations of 2′-C-methyl-CTP with (B) WT NS5B and (C) the S282T mutant enzyme.

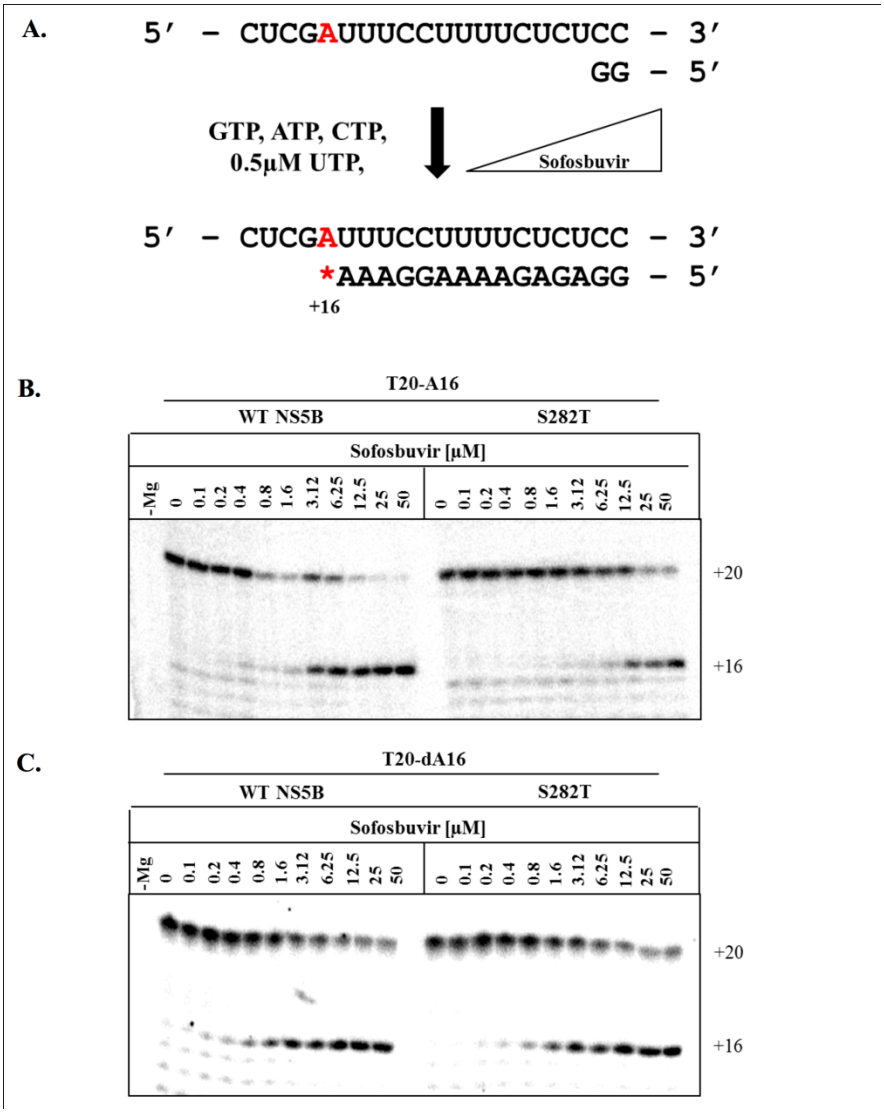

Figure 3.5: RNA synthesis performed in the presence of increasing concentrations of sofosbuvir-TP. (A) Schematic of chain termination by 2′-C-Me-2′-F-UTP on T20-A16 modified at position +16 to A or dA (red). RNA synthesis performed in presence of increasing concentrations of 2′-C-Me-2′-F-UTP with WT NS5B and the S282T mutant on the natural template (B) T20-AU16 and the (C) modified template T20-dA16.

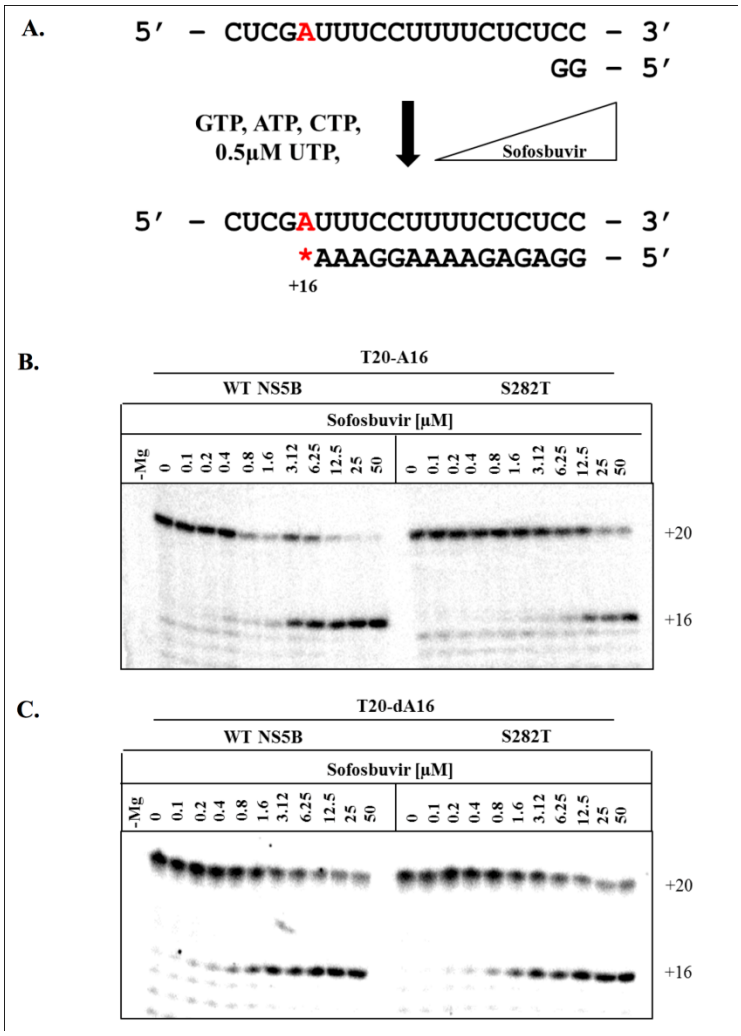

Figure 3.6: RNA synthesis performed in the presence of increasing concentrations of ribavirin-TP. (A) Chemical structure of ribavirin-5′-triphosphate (B) The scheme depicts incorporation of ribavirin (R), shown in red, on T20-C16 templates modified at position +16. Note that ribavirin can get incorporated opposite C and U. (C) RNA synthesis performed by WT NS5B and S282T with increasing concentrations of ribavirin on T20-C16 and T20-dC16. (D) Graphical representation of data shown under C.

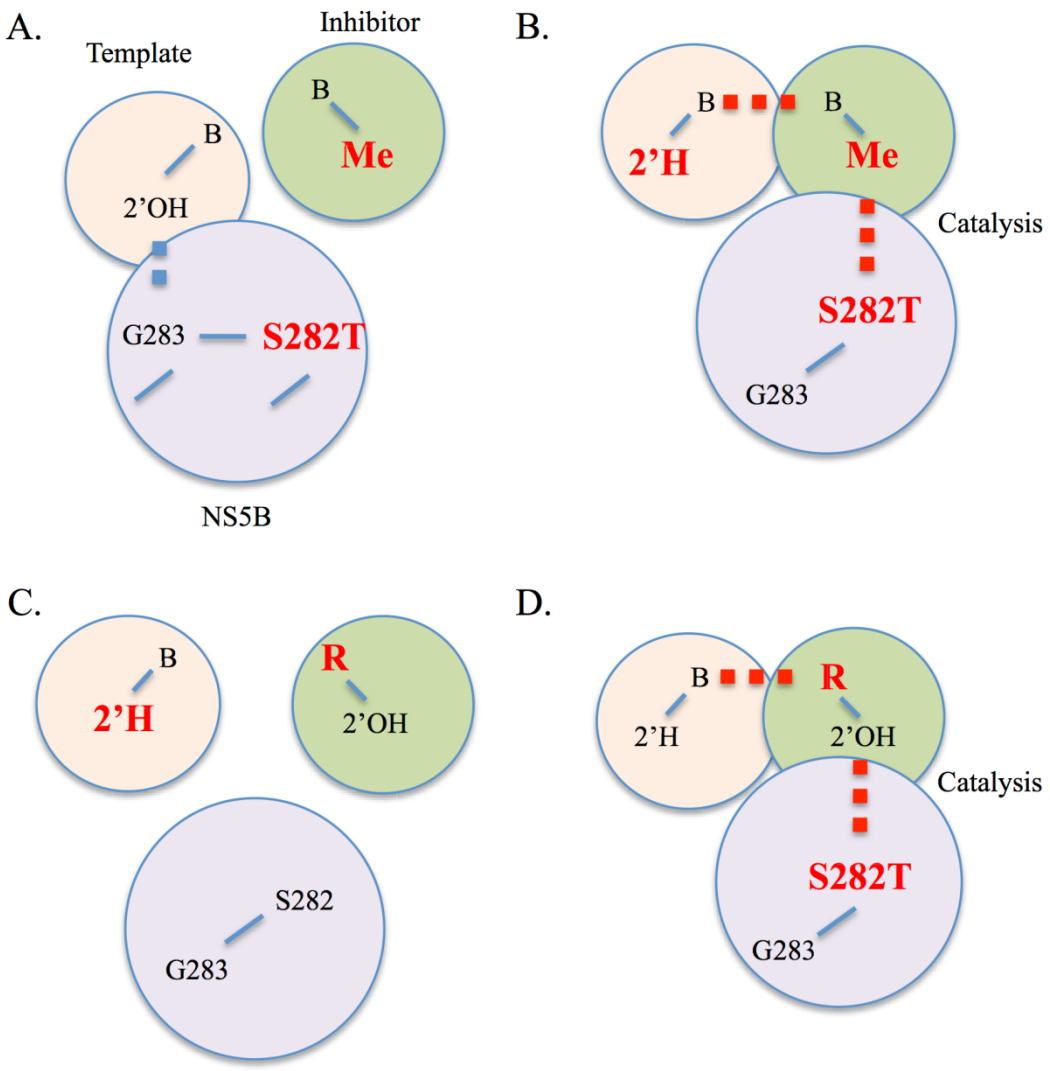

Figure 3.7: Model of interdependent hydrogen bonding affects susceptibility to 2'C-methylated compounds and ribavirin. (A) S282T prevents binding of 2′-C-methyl modified nucleotides. (B) The loss of a hydrogen bond between G283 and a DNA template increases the flexibility at S282T and facilitates binding the inhibitor. (C) The loss of a hydrogen bond between G283 and a DNA template prevents binding of ribavirin (R) that shows per se weak base pairing. (D) S282T can partially compensate for this deficiency and facilitates binding and/or incorporation of ribavirin.

Tables for Chapter 3.

Table 3.1: IC_{50} values for 2′C-Me-CTP incorporation by WT NS5B and S282T on modified templates.

Template	NS5B WT	S282T	
	IC_{50}[a] (μM)	IC_{50}[a] (μM)	Fold Change[b]
T20-G16	11.3 ± 1.3	>200	N/A[c]
T20-dG16	10.3 ± 1.7	8.0 ± 1.4	0.77
T20-2′F-G16	10.6 ± 1.1	21.5 ± 2.9	2

[a] IC_{50} is the inhibitory concentration of 2′-C-Methyl-CTP that inhibits 50% of the enzyme. Values were calculated by fitting 12 data points to a sigmoidal dose-response equation using GraphPad Prism (version 5.0). S.D. values were determined on the basis of three independent experiments.

[b] Fold change is calculated as a ratio of the IC_{50} value of the mutant enzyme as compared to the wild-type enzyme.

[c] N/A, not applicable; unable to calculate

Table 3.2: IC_{50} values for Sofosbuvir incorporation by WT NS5B and the S282T mutant on T20-A16 and T20-dA16.

Enzyme	T20-A16		T20-dA16	
	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]
WT NS5B	2.1 ± 0.1	-	1.4 ± 0.2	0.6
S282T	19.5 ± 2	9.2	2.1 ± 0.5	9.2

[a] IC_{50} is the inhibitory concentration of 2′-C-Me-2′-F-UTP that inhibits 50% of the enzyme. Values were calculated by fitting 12 data points to a sigmoidal dose-response equation using GraphPad Prism (version 5.0). S.D. values were determined on the basis of three independent experiments.

[b] Fold increase is calculated as a ratio of the IC_{50} value of the mutant enzyme compared with the wild-type enzyme.

CHAPTER 4

Effect of Mutations S282T, L159F, C316N and L320F in HCV NS5B on Inhibition of RNA Synthesis by 2'-C-methylated Nucleotides

Anupriya S. Kulkarni and Matthias Götte

4.1 Preface to Chapter 4

In *Chapter 3*, we investigated the mechanism by which the S282T mutation confers resistance to sofosbuvir and other 2'C-methylated compounds. In this *Chapter* we focus on testing various 2'C-methylated nucleotides in the context of mutations S282T, L159F, C316N and L320F.

4.2 Abstract

Recent advances in direct acting antivirals (DAAs) have led to significant improvement in anti-HCV therapy. Specific mutations in the NS5B polymerase, including S282T, L159F, C316N and L320F have been associated with resistance to 2'-C-methylated compounds. In this study, we evaluate the antiviral efficacy of novel NS5B nucleoside analogue inhibitors against these mutations in biochemical assays.

4.3 Results and Discussion

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and causes acute hepatitis, which may progress to chronic hepatitis that is associated with cirrhosis and hepatocellular carcinoma (363). Historically, HCV treatment comprised a combination of ribavirin and pegylated interferon (IFN)-alpha (364). However, this treatment resulted in serious side effects and poor response rates, especially in the context of infections with genotype 1, as only approximately 40% of genotype 1 patients responded to this therapy (364). In 2011, the first direct-acting antivirals (DAAs) were approved (365). The NS3 inhibitors (telaprevir or boceprevir) were administered in combination with ribavirin and IFN to chronic HCV-infected patients with genotype 1 (365). Although the introduction of the NS3 protease inhibitors improved response rates to approximately 80% (366), this treatment was still associated with adverse side effects and the rapid emergence of resistant HCV variants, posing additional challenges in antiviral therapy (367). In 2013, a prodrug of the nucleotide 2'-deoxy-2'-fluoro-2'-C-methyluridine (sofosbuvir), was approved in IFN-free combination therapies for chronic HCV infection (323, 324). Nucleotide inhibitors (NIs) compete with the natural ribonucleoside triphosphates (NTP) for binding to the highly conserved active site of HCV RNA-dependent RNA polymerase (NS5B). These compounds generally show pan-genotypic activity and exhibit a high barrier to the development of resistance (352).

In spite of the significant progress made due to the development of these drugs, the resistance profile associated with NIs is not fully characterized. The S282T substitution confers decreased susceptibility to 2'-C-methylated compounds (358). It has been suggested that this mutation may discriminate against the inhibitor, causing a steric clash with the modification at the 2'-C position (358). While S282T is a well described amino acid substitution in this context,

other more recently identified mutations, including L159F, C316N and L320F have also been associated with decreases in susceptibility to this class of compounds (301). Here we compared the effects of various modifications at the 2'-C position of the sugar on inhibition of RNA synthesis by WT NS5B and mutant enzymes containing the S282T, L159F, C316N and L320F mutations.

A series of NI analogues 2'F-2'C-Me UTP (sofosbuvir), 2'-C-Me-2'-NH_2-UTP, 2'-C-Me-UTP and 2'-C-Me-(1-Thio) UTP were synthesized by Medivir Pharmaceuticals (Sweden) (Figure 4.1). These compounds were then tested against WT NS5B and mutations known to confer resistance to sofosbuvir, including S282T, L159F, C316N and L320F (301). C316N and L320F mutations were tested as they show resistance to sofosbuvir in combination with S282T and L159F (301).

To test the effect of these inhibitors, full-length RNA synthesis was monitored on a template containing an A at position +16 for U analogues (T20-U16) (Figure 4.2A). With increasing concentrations of NIs, chain termination occurred at position +16 of the RNA template and there was a decrease in full-length product formation at position +20 (Figure 4.2A). It was observed that WT NS5B, C316N and L320F showed sensitivity to 2'F-2'C-Me UTP (sofosbuvir) with an IC_{50} value of 2.7 μM, 1.1 μM and 2.3 μM respectively, while S282T and L159F showed resistance with higher IC_{50} values of 16.2 μM and 9.8 μM, respectively (Figure 4.2B and Table 4.1).

In the case of 2'-C-Me-2'-NH_2-UTP and 2'-C-Me-(1-Thio) UTP, WT NS5B, L159F, C316N and L320F show increased IC_{50} values compared to 2'F-2'C-Me UTP (sofosbuvir). With S282T, 2'-C-Me-2'-NH_2-UTP and 2'-C-Me-(1-Thio) UTP were incapable of inhibiting the

activity of the enzyme as it did not allow incorporation of these compounds, even at high concentrations (Figure 4.2C,D and Table 4.1). These results suggest that the substitution of the fluoride group with NH_2 at the 2'-C position (2'-C-Me-2'-NH_2-UTP) or the addition of a Thio group at the α-phosphate (2'-C-Me-(1-Thio) UTP) fail to increase the inhibitory activity of the compounds. 2'-C-Me-UTP led to decreased IC_{50} values for WT NS5B, L159F, C316N and L320F, but S282T continued to demonstrate resistance to this compound (Figure 4.2E and Table 4.1).

In conclusion, S282T diminishes the inhibitory effects of 2'-C-methytlated compounds and L159F shows a subtle reduction in the inhibitory effect of sofosbuvir. The addition of NH_2 at the 2'-C position (2'-C-Me-2'-NH_2-UTP), or the addition of the thio group at the α-phosphate (2'-C-Me-(1-Thio)-UTP) led to increases in IC_{50} values also against WT NS5B, suggesting that these modifications do not increase potency in this context.

4.4 Acknowledgements

We would like to thank Medivir for kindly providing us with the nucleoside inhibitors. We are also very thankful to Dr. Selena Sagan for revising this paper. We would also like to thank Genome Quebec Innovation Center for sequencing our mutagenesis samples. A.S.K would like to thank the National CIHR Research Training Program in Hepatitis C (NCRTP) for a graduate student fellowship. This study was sponsored by the Canadian Institutes of Health Research (CIHR) and Medivir Pharmaceuticals.

Figures for Chapter 4.

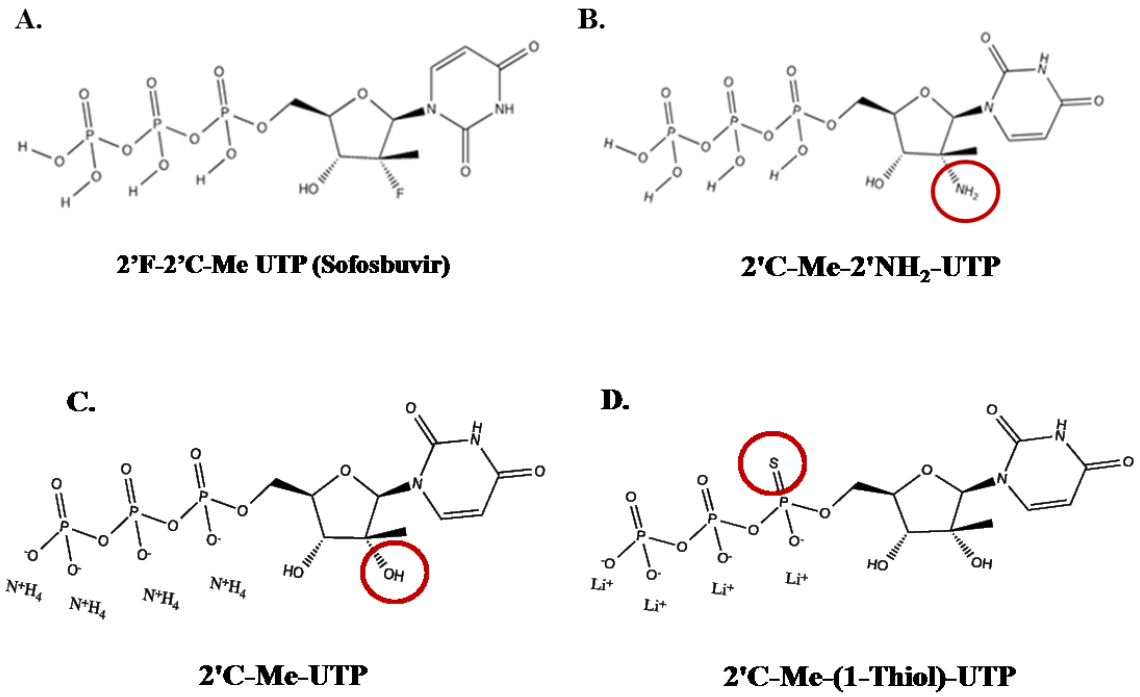

Figure 4.1: Structures of modified derivatives of Sofosbuvir. Chemical structures of (A) 2'F-2'C-Me UTP (sofosbuvir) (B) 2'C-Me-2'NH_2-UTP; (C) 2'C-Me-UTP; (D) 2'C-Me-(1-Thiol)-UTP

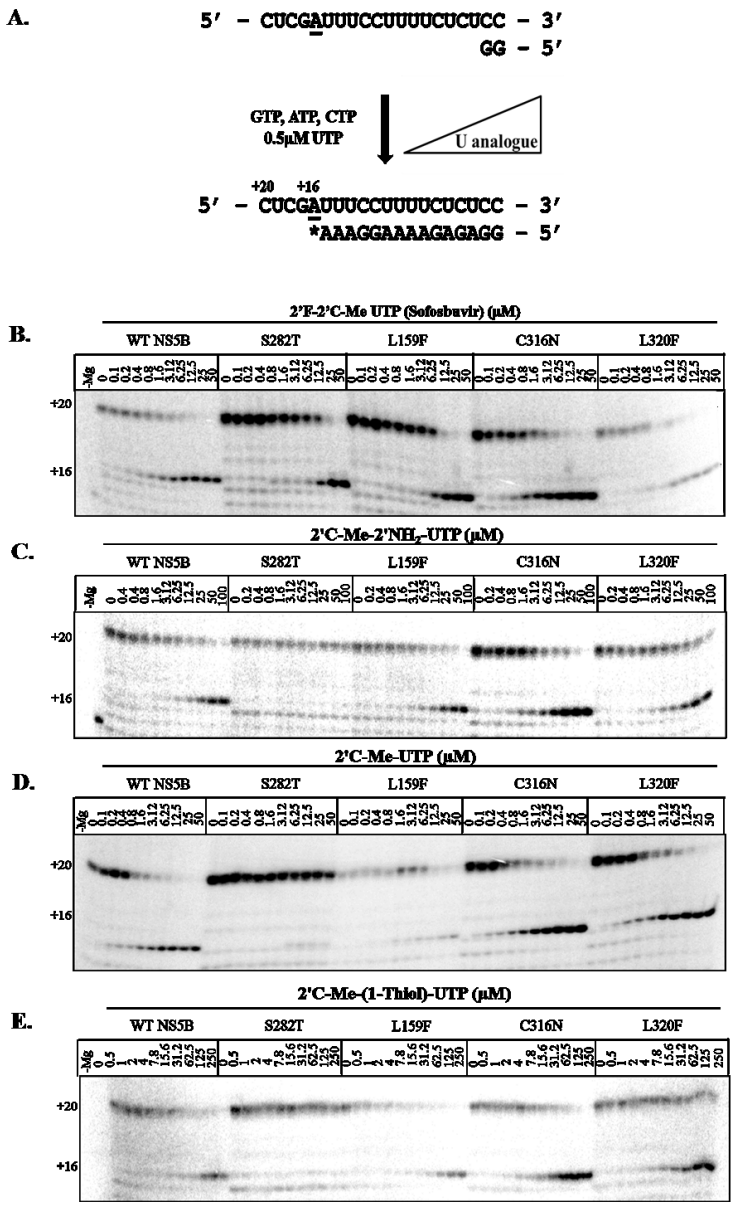

Figure 4.2: RNA synthesis performed in the presence of 2'F-2'C-Me UTP (sofosbuvir); 2'C-Me-2'NH_2-UTP; 2'C-Me-UTP; 2'C-Me-(1-Thiol)-UTP. (A) Schematic of chain termination by U analogues on RNA template T20-U16. RNA synthesis performed in presence of increasing concentrations of (B) 2'F-2'C-Me UTP (sofosbuvir); (C) 2'C-Me-2'NH_2-UTP; (D) 2'C-Me-UTP; (E) 2'C-Me-(1-Thiol)-UTP with WT NS5B, S282T, L159F, C316N, and L320F. Chain termination seen at position +16 verses full length at position +20.

Tables for Chapter 4.

Table 4.1. IC_{50} values for modified nucleoside inhibitors by WT NS5B and sofosbuvir resistant mutations S282T, L159F, C316N and L320F.

Compound	T20-U16 (A)								
	WT NS5B	S282T		L159F		C316N		L320F	
	IC_{50}[a] (μM)	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]	IC_{50}[a] (μM)	Fold Change[b]
U analogues									
2'F-2'C-Me UTP	2.7 ± 1.3	16.2 ± 1.4	6	9.8 ± 1.7	3.6	1.1 ± 0.1	0.4	2.3 ± 0.9	0.8
2'-C-Me-2'-NH2-UTP	19.3 ± 5.11	>100	N/A[c]	14.8 ± 1.7	0.7	8.4 ± 2	0.4	11.8 ± 2.9	0.6
2'-C-Me-UTP	1.4 ± 0.34	>50	N/A[c]	1.2 ± 0.1	0.8	1 ± 0.1	0.7	1.3 ± 0.3	0.9
2'-C-Me-(1-Thio)UTP	31.5 ± 2.4	>250	N/A[c]	20.7 ± 3.4	0.6	10.6 ± 2.4	0.03	40.8 ± 3.8	1.2

[a] IC_{50} is the inhibitory concentration of 2'-C-Methyl-CTP that inhibits 50% of the enzyme. Values were calculated by fitting 12 data points to a sigmoidal dose-response equation using GraphPad Prism (version 5.0). S.D. values were determined on the basis of three independent experiments.

[b] Fold change is calculated as a ratio of the IC_{50} value of the mutant enzyme as compared to the wild-type enzyme.

[c] N/A, not applicable; unable to calculate

CHAPTER 5

DISCUSSION

5.1 Preface to Chapter 5

HCV represents a significant public health burden; however, progress has been made in the development of new therapies with improved potency and tolerance in HCV-infected patients (368, 369). The genetic diversity and variability of HCV has proven to be a considerable obstacle in developing broadly effective antivirals. HCV can be divided into seven major genotypes and several subtypes based on sequence homology. The difference between genotypes at the nucleotide level is about 35-40% and between subtypes about 25-30% (370). The HCV RdRp NS5B is error-prone and lacks proofreading activity, thus creating diverse populations from which resistant variants can emerge under selective drug pressure (371, 372). Resistance to inhibitors is a concern in anti-HCV therapy. Although resistance to the new generation of nucleoside inhibitors is not a major concern, non-nucleoside inhibitors of NS5B, and NS3/4A protease inhibitors are known to be particularly prone to the development of resistance. Hence, it is necessary to evaluate additional strategies for treatment of hepatitis C.

Drug discovery and development efforts have focused mainly on the viral NS3 protease and NS5B polymerase, though progress is rapidly being made with other viral and cellular targets (373). The NS5A protein plays an essential role in HCV viral replication as well as assembly/release of infectious particles, and thus it represents an important target for development of DAAs (232). NS5A inhibitors ledipasvir, ombitasvir and daclatasvir have been approved for hepatitis C treatment (237). HCV NS5B has been extensively studied as a target for drug development due to its essential role in viral RNA synthesis (240, 241). Inhibitors of HCV NS5B are divided into two classes, namely the NIs and the NNIs (279). Herein, we describe the inhibitory activity of NI and NNIs on NS5B. The findings of this study provide insight into the

mechanisms of action NIs and NNIs, and highlight mechanisms through which the NS5B polymerase develops resistance to these drugs.

5.2 Genotype-specific inhibitory activities of the class 3 NNIs

Unlike NIs, which target the highly conserved NS5B active site, NNIs bind to allosteric sites that are less conversed and have high variability across the different HCV genotypes. As such, different genotypes have disparate susceptibility to NNIs. In *Chapter 2*, we tested the efficacy of NNI-3s against different genotypes of NS5B, and identified amino acid residues that confer resistance to these NNIs. NNI-3 dasabuvir, has already advanced in clinical trials as part of combination therapies for HCV genotype 1 infection (374, 375). This study will improve our understanding of the interactions of NNI-3s with their binding site, and the mechanisms of resistance conferred by natural amino acid substitutions in NS5B of different genotypes.

We characterized the NNI-3s, acyl pyrrolidine and 1,5-benzodiazepine, and studied their efficacy against NS5B from different HCV genotypes. Acyl pyrrolidine was first identified as an NS5B inhibitor in a high-throughput screen of compound libraries using the *in vitro* RNA-dependent RNA synthesis assay (376). Subsequent studies involving docking experiments, and testing of known resistance causing mutants eventually led to the identification of the binding site of this drug, and established it as a class 3 NNI (332). Similarly, 1,5-Benzodiazepine was also identified in a high-throughput screen and subsequent studies using crystallography and cell-based replication assays led to the establishment of this compound as an NNI-3 (377).

As HCV NS5B is capable of initiating RNA synthesis either *de novo* or through dinucleotide primer extension, both methods were employed to test the activity of the enzyme

and the inhibitory effect of acyl pyrrolidine and 1,5-benzodiazepine on RNA synthesis (167). We observed that genotype 2a NS5B was more active when compared with NS5B of genotypes 1b, 3a or 5a. The high activity of genotype 2a may at least partially be due to the isoleucine-405 (I405) polymorphism, which is present only in NS5B from genotype 2a; while genotypes 1b, 3a, and 5a all contain a valine at this position (340). The I405 residue of genotype 2a stabilizes the closed conformation of the thumb domain of NS5B, leading to an increase in RdRP activity (340). Furthermore, the mutations Y561F and L571S, synergistically enhance the effect of the V405I mutation. Although we did not include genotype 1a in our study, this genotype also contains an isoleucine at position 405; however, it is not known whether this residue plays a similar role in this genotype due to the high degree of amino acid variation between these genotypes. Genotypes 4a, 6a and 7a were also not included in our study, but similarly to 1b, 3a and 5a, they contain a valine at this position. In addition, a serine residue at position 450 is present in genotypes 2a and 5a, and has been previously shown to be responsible for an increase in activity of the enzyme through a mechanism that involves formation of a hydrogen bond with isoleucine-560 (I560) in the NS5B linker region (340). In the case of genotypes 4a, 6a and 7a, a valine is present at position 450, and hence it is unlikely to aid in coordination of the linker region.

Consistent with the fact that different genotypes show differential sensitivity towards NNIs, we observed that NS5B from genotype 1b demonstrated sensitivity to both acyl pyrrolidine and 1,5-benzodiazepine; while genotypes 2a, 3a and 5a demonstrated significantly reduced sensitivity. Both acyl pyrrolidine and 1,5-benzodiazepine have comparable levels of inhibitory activity towards NS5B from genotype 1b, suggesting similar levels of efficacy of both these inhibitors. In contrast, all of the genotypes were similarly inhibited by the NI 3'dCTP.

Docking experiments with both acyl pyrrolidine and 1,5-benzodiazepine revealed that residues A218, V405, A450 and C451 were present within 5 Å of both the inhibitors (Figure 2.4 A,B). Structure-based sequence alignment has revealed that amino acid polymorphisms are present at positions 218, 405, 450, and 451 of genotypes 2a, 3a, and 5a. We introduced these naturally occurring amino acid polymorphisms into NS5B from genotype 1b to further study the contribution of each polymorphism on enzymatic activity and resistance to acyl pyrrolidine and 1,5-benzodiazepine.

When generated in NS5B 1b, both the A218S and V405I mutants showed significant resistance to acyl pyrrolidine and 1,5-benzodiazepine, while A450S and C451T had similar sensitivities to the WT enzyme. Both *de novo* and primer-dependent RNA synthesis assays were consistent for A218S and V405I mutant; however, a slight increase in IC_{50} was seen in the A450S and C451T mutants with acyl pyrrolidine and 1,5-benzodiazepine in *de novo* RNA synthesis. The A218S mutation conferred the highest level of resistance to acyl pyrrolidine and 1,5-benzodiazepine when introduced into genotype 1b NS5B. It is conceivable that the additional hydroxyl group on the serine side chain (compared with alanine) leads to disruption of the hydrophobic environment in the NNI binding pocket. In turn, this may affect the interaction of the NNI-3 with the enzyme. In addition, V405I conferred considerably reduced sensitivity to acyl pyrrolidine and 1,5-benzodiazepine when introduced into genotype 1b NS5B. Isoleucine introduces an additional methyl group at position 405, which may result in a steric conflict with the inhibitor(s), thereby contributing to resistance. The A450S mutation leads to a modest decrease in sensitivity to acyl pyrrolidine in *de novo* RNA synthesis. As discussed above, the serine at position 450 forms a hydrogen bond with isoleucine-560 in the linker region of NS5B,

resulting in a closed conformation of the polymerase. This conformation may also result in a reduction in the NNI-3's association with the binding pocket.

While we have shown that individual mutations introduced in genotype 1b can lead to significantly reduced sensitivity to NNI-3s, it is likely that multiple polymorphisms act collectively and synergistically to establish a resistant phenotype to both acyl pyrrolidine and 1,5-benzodiazepine. Since acyl pyrrolidine and 1,5-benzodiazepine are different class 3 NNIs, yet show comparable efficacies, these findings can potentially be extrapolated to other compounds belonging to this class for NS5B of genotypes 1b, 2a, and 5a. However, NS5B of genotype 3a was sensitive to acyl pyrrolidine but demonstrated a strong resistance to 1,5-benzodiazepine, making it hard to predict the efficacy of other class 3 NNIs towards this genotype.

Our findings provide insight into the interactions of two class-3 NNIs, acyl pyrrolidine and 1,5-benzodiazepine, with their NS5B binding pockets, and suggests mechanisms of natural resistance to these NNIs. Understanding these mechanisms will inform the rational design of novel NNIs with improved activity and may also aid in the development of NNI-based combination therapies for treatment of chronic HCV infection. Furthermore, the approach detailed in our study can be utilized to study the efficacy of other NNI-3s, and identify potential resistance-causing mechanisms that confer resistance to these inhibitors. The efficacy of NNIs against other HCV genotypes could also be studied in a similar manner and resistance-causing polymorphisms could be mapped accordingly. Additionally, this approach could be extrapolated to characterize NNIs of other classes, and could help to map resistance-causing mutations to these inhibitors. An additional factor that should be considered is the existence of multiple HCV variants *in vivo*. Since HCV exists as quasispecies, the existence of various amino acid

polymorphisms may act synergistically in *trans* to affect the efficacy of the NNIs in patients, and this possibility should be tested in cell culture and *in vivo* systems. Importantly, the NNI-3 dasabuvir, has already been approved in the clinic as part of combination therapies for HCV genotype 1 infection (374, 375). Thus, the work described herein supports an improved understanding of the interactions of NNI-3s with their binding site, and the mechanisms of resistance conferred by natural amino acid substitutions in NS5B derived from different HCV genotypes. In future, this information is likely to aid in the development of inhibitors that bypass the natural resistance caused by genotype-specific amino acid polymorphisms.

5.3 S282T mediated resistance and susceptibility to NIs of NS5B

Although sofosbuvir has shown significant promise in treating HCV infection, certain resistance-causing mutations have been observed in the clinic in a small number of patients in response to sofosbuvir treatment (298). In *Chapter 3*, we used chimeric RNA-DNA templates to investigate the interactions between the S282 and G283 residues of NS5B with template RNA, and we tested the effect of WT and the S282T mutation containing NS5B on sofosbuvir and ribavirin to elucidate the underlying mechanisms. Our findings elucidated the mechanism of interaction of sofosbuvir and ribavirin with NS5B. Our data suggests that the S282T mutation causes a shift in the backbone of G283, moving it closer to the template strand, which prevents efficient incorporation of sofosbuvir. Surprisingly, this shift resulted in a more efficient incorporation of ribavirin. These results may explain the underlying mechanism of resistance of S282T towards sofosbuvir and the heightened susceptibility of NS5B containing this mutation to ribavirin.

It is known that the S282T mutation decreases the affinity for 2'C-methyl substituted nucleotides (296, 358), such as sofosbuvir. Incorporation of 2'C-methyl modified nucleotides in the primer position may result in steric hindrance between the methyl group and the ribose of the incoming NTP through which 2'C-methylated analogues could affect chain termination. In recent crystal structures, the backbone oxygen of G283, which is located in close proximity to S282, was shown to form a hydrogen bond with the 2' hydroxyl group of the template strand RNA residue present at the site of incorporation (Figure 5.1). We hypothesized that the resistance conferring mutation S282T may shift the position of the G283 residue resulting in perturbation of the interaction with the template strand 2' hydroxyl group. To test this hypothesis, we monitored RNA synthesis and inhibitor incorporation by WT NS5B and S282T on RNA-DNA chimeric templates. We took advantage of the fact that NS5B is able to utilize chimeric DNA-RNA templates and found that introduction of a single deoxynucleotide in the RNA template leads to significant pausing of the WT NS5B polymerase (170). The main differences attributed to the interaction of RNA and DNA templates with the polymerase are the absence of the 2′ hydroxyl and the south sugar pucker orientation, relative to the north sugar pucker orientation of a ribonucleotide (359). By monitoring RNA synthesis on these templates, we found that the lack of 2' hydroxyl group, rather than the sugar pucker, was responsible for NS5B polymerase pausing. Moreover, monitoring dose-dependent CTP elongation on templates modified with G, dG and 2'O-Me-G led us to conclude that the structural integrity around the 2' hydroxyl group was important for nucleotide incorporation.

To further understand the role played by the 2' hydroxyl group and to determine the underlying mechanism of S282T resistance to 2'C-methylated compounds, we tested the incorporation of the nucleoside inhibitors, 2'C-methyl-CTP and sofosbuvir, by WT NS5B and

the resistance-conferring mutant S282T on modified templates. The S282T mutation caused decreased sensitivity to 2'C-methyl-CTP, but was fully sensitive when incorporated against dGTP. S282T also showed sensitivity when incorporated against 2'-F-GTP, confirming that the change in phenotype was observed because of the lack of 2' hydroxyl group, and was not due to the difference in sugar pucker orientation. Similarly, with sofosbuvir, S282T showed reduced sensitivity when incorporated opposite to A; but when replaced by a dA, a change to a sensitive phenotype was observed.

The crystal structure of WT NS5B in combination with RNA primer-template demonstrates that the 2' hydroxyl group of the template strand (+1) RNA normally forms a hydrogen bond with the backbone of G283 (314). WT NS5B can incorporate 2'C-methylated compounds, but likely the steric conflict caused by S282T prevents this incorporation in the mutant enzyme. When replaced by a denoxynucleotide, the lack of a 2' hydroxyl group at this position may increase the flexibility of S282T at the priming site, which in turn helps to accommodate the modified nucleotide (2'C-methyl-CTP or sofosbuvir).

In the case of ribavirin, S282T can incorporate ribavirin more efficiently compared with WT NS5B since the S282T mutation shifts the backbone of G283 towards the template strand. With the natural nucleotide, there is not enough room and this causes a steric conflict; but, as ribavirin has a small chemical moiety replacing the nucleobase, it gets incorporated with increased efficiency. Our model suggests that S282T and the hydrogen bonding between the 2' hydroxyl group and backbone of G283 causes the template strand to move closer to the primer causing additional interaction and this allows the efficient incorporation of ribavirin. Hence, our results suggest that the 2' hydroxyl group of the template nucleotide plays an important role in establishing the S282T-associated resistant and sensitive phenotypes.

Our findings are significant since sofosbuvir is frequently used in treatment of chronic HCV infection. Although S282T shows a reduced replicative fitness compared with WT NS5B, and this could prevent the mutant variant from gaining a selective advantage, S282T variants have been selected for two sofosbuvir-treated patients. Our finding that the sofosbuvir resistant-conferring mutation, S282T, shows increased sensitivity to ribavirin therefore has significant clinical implications. This increased sensitivity can be exploited in the clinic by using ribavirin/sofosbuvir combination therapy to prevent the emergence of resistant variants. Additionally, ribavirin can potentially be used as a second line of treatment for patients that have developed S282T-mediated resistance to sofosbuvir treatment. Finally, the mechanistic understanding of sofosbuvir resistance obtained from our research may also aid in the development of new drugs with improved efficacy against the HCV polymerase.

5.4 2'-C-methylated compounds and analysis of their efficacy against resistance-conferring mutations

The results from *Chapter 3* highlighted a potential mechanism through which NS5B mutations may confer resistance to sofosbuvir. These findings suggested that combination therapy of ribavirin/sofosbuvir may reduce selection of sofosbuvir resistance-conferring mutations (360). An alternative approach, which could avoid the use and the toxicity associated with ribavirin, is the development of inhibitors with modifications on the sofosbuvir backbone, that have improved efficacy against NS5B resistance-conferring mutations. This is a particularly attractive strategy since sofosbuvir is well tolerated and demonstrates a high level of efficacy in

treatment of chronic HCV in the clinic (323). The emergence of a low frequency of resistance-conferring mutations is one of the few limitations to the use of this drug (301).

In *Chapter 4*, we evaluated the effect of different nucleotide analogue inhibitors on WT NS5B and NI resistance-associated mutations: S282T, L159F, C316N and L320F (301). The analogues tested were 2'F-2'C-methyl-UTP (sofosbuvir), 2'C-Me-2'NH_2-UTP, 2'C-Me-UTP, 2'C-Me-(1-Thiol)-UTP (Figure 4.1). 2'C-Me-2'NH_2-UTP carries an amino group at the 2'-C position instead of fluoride (Figure 4.1B), 2'C-Me-UTP carries a hydroxyl group at the 2' position (Figure 4.1C), and 2'C-Me-(1-Thiol)-UTP carries a hydroxyl group at the 2'-C as well as a thiol group at the α-phosphate position (Figure 4.1D).

We found that S282T and L159F showed reduced inhibition of 2'F-2'C-methyl-UTP (sofosbuvir) compared with WT NS5B (Figure 4.2B; Table 4.1). C316N and L320F showed sensitivity to it (Figure 4.2B; Table 4.1). However, both 2'C-Me-2'NH_2-UTP, and 2'C-Me-(1-Thiol)-UTP showed less inhibition against WT NS5B or the S282T, L159F, C316N, and L320F mutations (Figure 4.2C, D; Table 4.1). 2'C-Me-UTP showed significantly improved inhibition against WT NS5B and all the mutants tested with the exception of S282T (Figure 4.2E; Table 4.1). The S282T mutation showed resistance to all of the tested derivatives.

Addition of NH_2 at the 2'-C position of 2'-C-Me-2'-NH_2-UTP and Thio group at the α-phosphate of 2'-C-Me-(1-Thio) UTP led to increase in WT NS5B, L159F, C316N and L320F IC_{50} values suggesting that NH_2 and Thio group do not increase the sensitivity of the compounds. This study gives us insights into potential modification that can be made to improve the efficacy of sofosbuvir.

5.5 Significance and future directions

We have identified specific amino acid mutations that are preexisting across non-1a/1b HCV genotypes that interact with class 3 NNIs and confer resistance to the drugs. This work will help to provide insight into the potential mechanisms of resistance to class 3 NNIs. A limitation of our study was that it was restricted to NS5B purified from HCV genotypes 1b, 2a, 3a, and 5a. In order for our findings to have broader applicability, this analysis should also be extended to NS5B from genotypes 1a, 4a, and 6a, and the recently classified genotype 7a (15). Also, our biochemical findings can be further validated in cell-based replicon assays, which may provide us information about the potential *in vivo* relevance of our study.

Class 3 NNIs are currently being evaluated in the clinic, and Dasabuvir is a benzodiazepine based NNI-3 which has already received FDA approval and is used in combination therapy (311). Although clinical evidence for the emergence of resistance to dasabuvir is lacking, *in vitro* studies using patient derive chimeric replicons have demonstrated the potential for dasabuvir resistant variants to emerge under selective drug pressure (306). The information obtained from our work may help elucidate mechanisms of resistance to class 3 NNIs such as dasabuvir, and assist in the design and study of NNI-3s with increased potency.

NI-based anti-HCV therapy has shown very promising results in the clinic, and sofosbuvir in particular has emerged as a backbone of hepatitis C treatment. Although sofosbuvir has a very high barrier to resistance, mutations such as S282T and L159F have been identified which cause resistance to this drug. Although these variants have only been detected in a minority of patients, and have not posed a public health problem thus far, the evolution of these variants in a population and their subsequent challenge to the efficacy of sofosbuvir treatment

cannot be ruled out. Our study helps elucidate the mechanism of resistance to sofosbuvir caused by the S282T mutation. This information can be used to enhance the effectiveness of sofosbuvir derivatives, which may show increased efficacy against these variants. Furthermore, our finding that the S282T variant shows increased sensitivity to ribavirin provides a rationale for the use of sofosbuvir/ribavirin combination therapy in the clinic. The approach detailed in our study may be further utilized to study the mechanism of resistance of the L159F to sofosbuvir.

In the last part of our study we have tested the effect of different modifications made to the sofosbuvir backbone against mutations that confer resistance to sofosbuvir. This study provides us information about the specific modifications at particular positions on sofosbuvir that may improve its effectiveness against resistance-conferring mutations. However, we found that S282T showed resistance to all the sofosbuvir derivatives tested (Figure 4.1 and Table 4.1). Hence, we propose expanding this study by trying to identify the mechanism by which S282T confers resistance to these compounds. For this purpose, we could use a similar approach used herein by taking advantage of modified templates, as described in *Chapter 4*. Also, additional modifications can be introduced either individually or in combination to the sofosbuvir backbone to enhance its efficacy against these mutations.

5.6 Concluding Remarks

Significant progress has been made in hepatitis C-targeted therapy in recent years, and the work presented herein contributes to understanding mechanisms of resistance to new HCV therapies. Improvements in HCV model systems have contributed significantly to antiviral chemotherapy and vaccine development. Various HCV proteins are being studied as candidate drug targets, and numerous small molecules targeting these proteins are under evaluation as therapeutics. Out of these, NS5B targeting inhibitors are a particularly promising subset.

In the work presented in this thesis, we elucidated aspects of NS5B targeted therapy including:

- The mechanism with which NNIs target the HCV NS5B polymerase, and the underlying reasons why major genotypes of HCV are refractory to these NNIs. To achieve this, we mapped the NNI-3 binding site on the polymerase belonging to the major HCV genotypes (1b, 2a, 3a and 5a), and came across novel preexisting amino acid polymorphisms that lead to resistance towards NNI-3s. These findings will contribute towards the development of improved NNI-3s for HCV therapy.

- The underlying mechanism of S282T-mediated resistance towards 2'C-methylated compounds including, sofosbuvir and the S282T-conferred sensitivity towards ribavirin. Our findings highlight the importance of 2' hydroxyl group of the template strand in creating this phenotype. This finding suggests that the use of sofosbuvir/ribavirin combination therapy may reduce the incidence of resistance to sofosbuvir in the clinic.

- Finally, we evaluated derivatives of sofosbuvir on NS5B mutants that are known to confer resistance to sofosbuvir. We identified specific modifications which could increase the sensitivity of sofosbuvir against such mutations.

Improving our understanding of enzyme-inhibitor interactions may be an effective way of improving current therapies and reducing the emergence of resistance to therapy. Taken together, the work presented herein will help widen our understanding of resistance and susceptibility to inhibitors of HCV NS5B, thus helping in the development of improved therapeutics for chronic HCV infection.

Figures for Chapter 5.

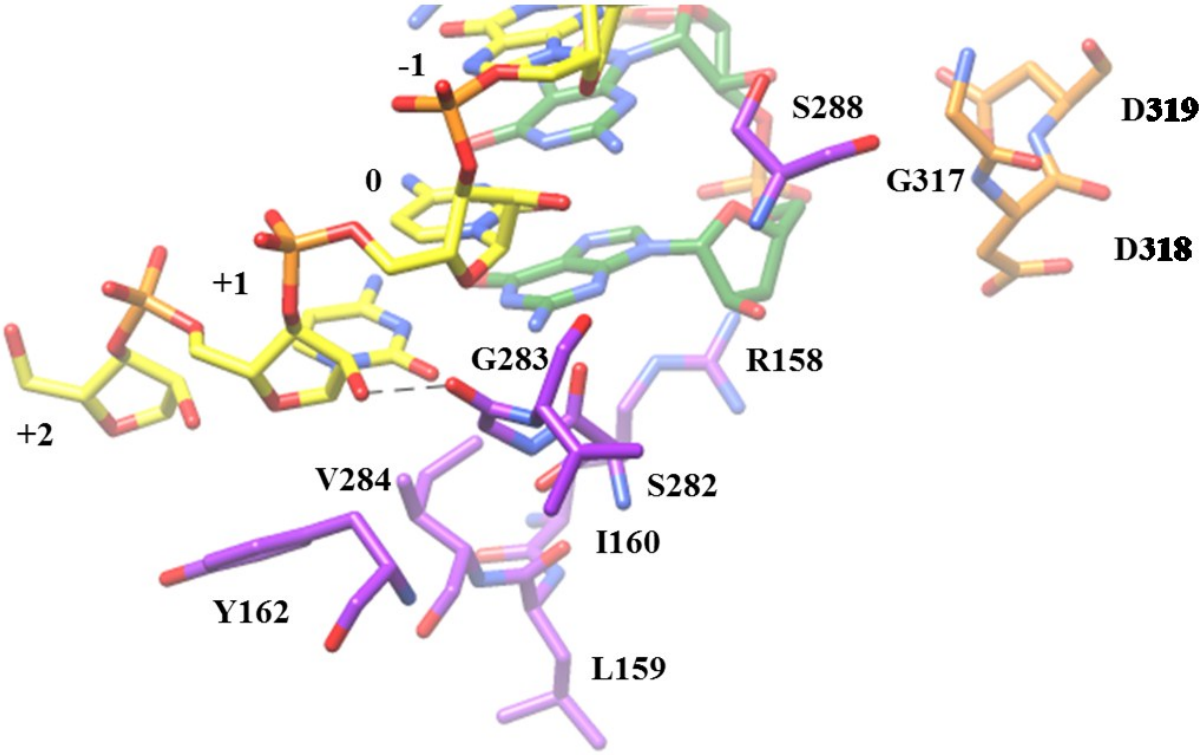

Figure 5.1. Primer-template interaction with the HCV polymerase active site. Graphical representation of primer template recognition by NS5B showing template strand (yellow), primer strand (green), and amino acid residues present in the active site (magenta) and active site residues (orange). The 2'OH at the site of incorporation (position +1 on template strand) interacts with backbone oxygen of G283 which is in close proximity to S282 (implicated in Sofosbuvir resistance). *Figure adapted from Mosley et al, Journal of Virology, 2012. (314)*

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