Université de Montréal

# Characterization of a Novel Class of Anti-HCV Agents Targeting Protein-Protein Interactions

par Alex Park

Programme de microbiologie et immunologie
Faculté de Médecine

Mémoire présenté à la Faculté de Médecine
en vue de l'obtention du grade de maîtrise en sciences (M.Sc.)
en microbiologie et immunologie
option générale

Septembre, 2016

© Alex Park, 2016

# Résumé

Le virus de l'hépatite C (VHC) est un agent causateur de maladies du foie important responsable d'une pandémie affectant près de 180 millions d'individus mondialement. L'absence de symptômes dans les premières années d'infection entraîne des diagnostics tardifs qui empêchent la prise en charge rapide des patients avant l'apparition d'une fibrose et, dans près de 16 % des cas d'infection, d'une cirrhose.

En exploitant les interactions protéine-protéine membranaires, des essais utilisant la technologie BRET, dans les cellules vivantes, ont été précédemment optimisés afin d'établir le réseau complet des interactions du VHC. En utilisant les fondements de cette étude, un essai à haut débit dans les cellules vivantes a été réalisé pour identifier de nouveaux composés anti-VHC ciblant une nouvelle interaction NS3/4A-NS3/4A. Approximativement 110,000 petites molécules ont été criblées pour leurs effets sur l'homodimérization de NS3/4A et ont été classées par rapport à leur spécificité et à leur puissance contre le VHC. Au terme de cette étude, UM42811 a été identifié comme un activateur potentiel de l'interaction NS3/4A-NS3/4A offrant une activité antivirale prometteuse dotant une excellente fenêtre thérapeutique. Par la suite, un séquençage exhaustif des virus, soumis à un traitement de UM42811, a permis d'établir le profil de résistance du VHC contre ce composé. Grâce à cette fine cartographie, il a été possible d'identifier un nouveau mécanisme d'inhibition de NS3/4A qui est indépendant de son activité protéase.

En utilisant les données de notre groupe sur les interactions VHC-hôte, il a été possible de continuer la caractérisation fonctionnelle du composé UM42811 en étudiant son effet sur les interactions potentiellement bénéfiques à la persistance virale. Pour ce faire, les protéines associées au transport nucléaire et mitochondriale qui sont des interactants de choix de NS3/4A ont été priorisées. Parmi ces facteurs de l'hôte, l'étude de karyopherin subunit beta 1 (KPNB1) et de heat shock protein 60 (HSP60) a été priorisée. De façon intéressante, les expériences de co-immunoprécipitation ont démontré que UM42811 était capable de prévenir l'interaction KPNB1-NS3/4A ainsi que l'interaction HSP60-NS3/4A. De plus, les études fonctionnelles et les analyses d'immunobuvardage de type western ont démontré que l'interaction KPNB1-NS3/4A avait des effets délétères sur l'induction des gènes stimulés par l'interféron (ISG). Finalement, il a été démontré que KPNB1 est possiblement clivé par NS3/4A suggérant la présence potentielle d'un mécanisme de subversion ou d'échappement.

En bref, cette étude démontre la puissance des stratégies impliquant les interactions protéine-protéine dans les cellules vivantes pour l'identification de nouveaux composés inhibiteurs, caractérise un nouveau mécanisme d'inhibition anti-VHC et révèle la possibilité d'un nouveau mécanisme d'évasion du système immunitaire.

**Mots-clés** : virus de l'hépatite C, VHC, antiviraux à action directe, ADD, criblage à haut débit, BRET, interaction protéine-protéine, résistance

# Abstract

Hepatitis C virus (HCV) is an important causative agent for liver diseases and is responsible for a worldwide pandemic affecting roughly 180 million individuals worldwide. Late diagnosis following the progression to fibrosis and to cirrhosis, in nearly 16% of chronic infections, is attributed to the absence of symptoms in the first years of infection.

By exploiting membrane protein-protein interactions (PPI), live cell assays using bioluminescence resonance energy transfer (BRET) technology have previously been optimized to complete a comprehensive hepatitis C virus (HCV) protein interaction network. Using the groundwork laid by this network study, a high-throughput assay (HTS) cell-based assay was implemented to identify novel inhibitory compounds targeting an unreported NS3/4A-NS3/4A interaction. Approximately 110,000 compounds from a small-molecule collection were screened to monitor modulation of NS3/4A homodimerization and were discriminated based on specificity and potency. UM42811 was identified as a potential NS3/4A-NS3/4A interaction activator and found to have a promising antiviral activity boasting an excellent therapeutic window. Combined deep sequencing and mutation mapping have yielded a resistance profile based on statistical and functional probability pointing towards a novel inhibitory mechanism targeting the HCV NS3/4A independent from protease activity inhibition.

Data from an HCV to host protein interaction network generated by our group was used to analyze alternative effects of UM42811 on interactions which potentially benefit viral persistence. NS3/4A-specific host interactors were heavily associated with nuclear and mitochondrial transport based on Gene Ontology (GO). Among these specific interactors, karyopherin subunit beta 1 (KPNB1) and heat shock protein 60 (HSP60) were selected for further study. Interestingly, co-immunoprecipitation experiments revealed that UM42811 was able to prevent both KPNB1-NS3/4A and HSP60-NS3/4A interactions. Moreover, functional and western analysis revealed the KPNB1-NS3/4A interaction to have deleterious effects on interferon stimulated gene (ISG) induction. Unexpectedly, analysis revealed a putative NS3/4A mediated cleavage of KPNB1.

Overall, this study demonstrates the strength of cell-based PPI strategies in the identification of novel HCV antiviral compounds, characterizes a novel inhibitory mechanism for HCV and reveals a potentially novel viral immune evasion mechanism.

**Keywords** : Hepatitis C virus, HCV, direct-acting antivirals, DAA, high-throughput screening, HTS, bioluminescence resonance energy transfer, BRET, protein-protein interaction, PPI, resistance

# Table of Contents

Résumé ..... i

Abstract ..... iii

Table of Contents ..... v

List of Tables ..... viii

List of Figures ..... ix

List of Abbreviations & Acronyms ..... x

Acknowledgements ..... xiv

Introduction ..... 1

1. Global Significance ..... 2
    1.1. Virus Discovery ..... 2
    1.2. Worlwide Prevalence ..... 3
    1.3. Genetic Variance, Genotypic Distribution and Origins ..... 5
2. HCV Lifecycle ..... 8
    2.1. Viral Entry ..... 8
    2.2. Genome Translation and Replication ..... 9
    2.3. Assembly and Release ..... 11
    2.4. Innate Response and Immune Evasion ..... 12
3. Treatment Evolution ..... 14
    3.1. Direct Acting Antiviral (DAA) – Design Strategies ..... 16
        3.1.1. NS3/4A Serine Protease ..... 16
        3.1.2. NS5B RNA-Dependent RNA Polymerase ..... 20
        3.1.3. NS5A Phosphoprotein ..... 22
    3.2. Host-Directed HCV Inhibitors – Design Strategies ..... 25
        3.2.1. RNA-Based Inhibitors ..... 25
        3.2.2. Cyclophilin A Inhibitors ..... 25
        3.2.3. Entry Inhibitors ..... 26

    3.2.3. Immunomodulators ........ 27
  3.3. Current Combination Therapies ........ 28
4. Models for the Study of HCV ........ 31
  4.1. Animal Models ........ 31
  4.2. Cell Culture Based Systems ........ 32
5. Bioluminescence Resonance Energy Transfer (BRET) ........ 35
Hypothesis & Objectives ........ 37
Experimental Procedures ........ 38
  Cell culture ........ 38
  Expression vectors ........ 38
  BRET assays ........ 38
  HCV replication ........ 39
  $EC_{50}$ and $CC_{50}$ assays ........ 39
  Selection of HCV replicon escape resistant variants ........ 40
  Production of mutated HCV enzymes ........ 40
  shRNA gene silencing ........ 41
  Functional firefly luciferase assays ........ 41
  Western blot analysis ........ 42
  Co-immunoprecipitation ........ 42
Results ........ 43
  1. Investigation of pairwise interactions between HCV proteins ........ 43
  2. Homodimerization of the NS3/4A heterodimer ........ 46
  3. Implementation of a BRET HTS assay ........ 48
  4. Validation and antiviral characterization of lead compound UM42811 ........ 52
  5. Characterization of UM42811 resistance profile reveals mutations located at the surface of the C-terminal NS3 helicase subdomain ........ 55
  6. Generation of NS3/4A mutant fusion proteins and of mutant HCV replicon DNA precursors ........ 58
  7. UM42811 affects the dimer/oligomer conformation of NS3/4A heterodimers ........ 62

8. Functional consequences of a UM42811/BILN2061-sensitive NS3/4A-KPNB1 interaction ........ 65
9. Differential HSP60-IRF3-NS3/4A interaction configurations under UM42811 or BILN2061 treatment ........ 70

Discussion ........ 75

Perspectives ........ 81

Conclusion ........ 83

Bibliographie ........ i

Annex 1: Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response. ........ i

Annex 2: HCV NS3/4A Protease Inhibitors and the Road to Effective Direct-Acting Antiviral Therapies. ........ ii

# List of Tables

**Table I.** Generation of single point mutations within HCV NS3 ............................................ 59

# List of Figures

**Figure 1.** Worldwide estimated prevalence of HCV and genotype distribution .................. 7

**Figure 2.** HCV lifecycle – Potential points of intervention................................................ 30

**Figure 3.** Overview of cell culture based systems for the study of HCV............................ 34

**Figure 4.** Bioluminescence resonance energy transfer identifies novel HCV protein-protein interactions........................................................................................................... 45

**Figure 5.** Specific interaction between NS3/4A heterodimers ............................................ 47

**Figure 6.** Identification of potential NS3/4A PPI modulators through a BRET HTS assay 50

**Figure 7.** Validation and antiviral characterization of lead compound UM42811............... 54

**Figure 8.** Characterization of UM42811 resistance profile reveals mutations located at the surface of the C-terminal NS3 helicase subdomain ................................................................ 57

**Figure 9.** Mutated NS3/4A expression vectors and mutated replicon sequences ................ 60

**Figure 10.** Point mutations at putative resistant sites of UM42811 may affect the NS3/4A heterodimeric homodimers - UM42811binding reduces precursor interaction induced by BILN2061…… .......................................................................................................... 64

**Figure 11.** NS3/4A interacts with KPNB1, but is disrupted by both UM42811 and the protease inhibitor BILN2061. ........................................................................................ 68

**Figure 12.** NS3/4A protease activity is required for the inhibition of KPNB1-mediated ISG56 induction following IFN-α 2A stimulation................................................................. 69

**Figure 13.** NS3/4A interacts with HSP60, but is disrupted by UM42811. ........................ 72

**Figure 14.** Quantitative changes in HSP60 expression have no effect on IFN-$\beta$ induction following SeV infection.......................................................................................... 73

**Figure 15.** Hypothesized interaction configuration between NS3, HSP60 and IRF3 in the context of UM42811 and BILN2061 treatment....................................................... 74

# List of Abbreviations & Acronyms

| | |
|---|---|
| ACCA | Aminocyclopropane carboxylic acid |
| ACSL3 | Acyl-CoA synthethase 3 |
| ALT | Alanine aminotransferase |
| ApoE | Apolipoprotein E |
| ARFGAP1 | ADP Ribosylation Factor GTPase Activating Protein 1 |
| BRET | Bioluminescence resonance energy transfer |
| CD81 | Cluster of differentiation 81 |
| cLD | Cytosolic lipid droplet |
| CLDN1 | Claudin-1 |
| CypA | Cyclophilin A |
| DAA | Direct-acting antiviral |
| DGAT1 | Diacylglycerol acyltransferase-1 |
| DMV | Double-membrane vesicle |
| DNA | Deoxyribonucleic acid |
| dsRNA | Double-stranded RNA |
| EGFR | Epidermal growth factor receptor |
| EMCV | Encephalomyocarditis virus |
| EphA2 | Ephrin receptor A2 |
| ER | Endoplasmic reticulum |
| ESCRT | Endosomal-sorting complex required for transport |
| eYFP | enhanced Yellow fluorescent protein |
| Fluc | Firefly luciferase |
| GAG | Glycosaminoglycan |
| GO | Gene ontology |
| GFP | Green fluorescent protein |
| IDU | Injection drug user |
| IRIC | Institute of Research in Immunology and Cancer |
| HAV | Hepatitis A virus |

| | |
|---|---|
| HBV | Hepatitis B virus |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| HNF4α | Hepatocyte nuclear factor 4α |
| HSP60 | Heat shock protein 60 |
| HTA | Host-targeted agents |
| HTS | High throughput screening |
| IFN | Interferon |
| IFN-λR1 | Interferon-λ receptor chain 1 |
| IFNAR1 | Interferon-α/β receptor alpha chain |
| IFNAR2 | Interferon- α/β receptor beta chain |
| IKK-α | IκB kinase-α |
| IL-10R2 | Interleukin-10 receptor chain 2 |
| IRES | Internal ribosome entry site |
| IRF3 | Interferon regulatory factor 3 |
| IRF7 | Interferon regulatory factor 7 |
| ISG | Interferon-stimulated gene |
| ISRE | IFN-stimulated response element |
| JAK1 | Janus kinase 1 |
| JFH-1 | Japanese patient with fulminant hepatitis |
| kDa | kiloDalton |
| KPNA1 | Karyopherin (importin) subunit alpha 1 |
| KPNB1 | Karyopherin (importin) subunit beta 1 |
| LD | Lipid droplet |
| LDLR | Low-density lipoprotein receptor (LDLR) |
| LuLD | Luminal lipid droplet |
| MAPK | Mitogen-activated protein kinase |
| MAVS | Mitochondrial antiviral signalling |
| miR-122 | microRNA 122 |
| mRNA | messenger RNA |

| | |
|---|---|
| MTP | Microsomal triglyceride transfer protein |
| Myd88 | Myeloid differentiation primary response 88 |
| NANBH | Non-A Non-B hepatitis |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NI | Nucleoside inhibitor |
| NNI | Non-nucleoside inhibitor |
| NPC1L1 | Niemann-Pick C1-like 1 |
| NS | Non-structural |
| NTR | Non-translated region |
| OAS | Oligoadenylate synthethase |
| OCLN | Occludin |
| PAMP | Pathogen-associated molecular pattern |
| PEG-IFN | Pegylated interferon |
| PI4KIIIα | Phosphatidylinositol 4-kinase III α |
| PI4P | Phosphatidylinositol-4-phosphate |
| PIP | Pipecolinic acid |
| PKR | Protein kinase R |
| PLA2G4 | MAPK-regulated cytosolic phospholipase A2 |
| PPI | Protein-protein interaction |
| PRR | Pattern-recognition receptor |
| qPCR | Real-time polymerase chain reaction |
| RBV | Ribavirin |
| RdRp | RNA-dependent RNA-polymerase |
| RIG-I | Retinoic acid-inducible gene 1 |
| RLR | RIG-I-like receptor |
| Rluc | Renilla luciferase |
| RNA | Ribonucleic acid |
| SeV | Sendai virus |
| shRNA | short hairpin RNA |
| SOC | Standard of care |
| SRB1 | Scavenger receptors class B type 1 |

| | |
|---|---|
| ssRNA | Single-stranded RNA |
| STAT | Signal transducer and activator of transcription |
| SVR | Sustained virologic response |
| TAP1 | Transporter associated with Antigen Processing 1 |
| TfR1 | Transferrin Receptor 1 |
| TG | Triglyceride |
| TIC | Tetrahydroisoquinoline-3-carboxylic acid |
| TIR | Toll-interleukin receptor |
| TLR | Toll-like receptor |
| TRIF | TIR-domain-containing adapter-inducing interferon-β |
| UTR | Untranslated region |
| VAP-A | Vesicle-associated membrane protein-associated protein A |
| VAP-B | Vesicle-associated membrane protein-associated protein B |
| VLDL | Very low density lipoprotein |
| WHO | World Health Organization |
| YB-1 | Y-box-binding protein 1 |
| YFP | Yellow fluorescent protein |

# Acknowledgements

Having started as summer undergraduate intern, I am very grateful that Dr. Daniel Lamarre took a chance with my inexperience and provided me with numerous resources available in his laboratory to encourage my growth as an independent and critical thinker. The tools and techniques used in his laboratory stretch far and wide and provide hands-on experience in a multitude of disciplines great for any aspiring researcher. Dr. Lamarre's experience and vast knowledge has never failed to be insightful and has definitely been an example to follow in the refinement of my critical thinking ability and ambition. It is without question that his guidance and support have been instrumental in making my journey possible.

I would also like to thank Martin Baril, former research associate in Dr. Lamarre's lab, for being an inspiring mentor during much of my early days as a research intern and eventual M.Sc student. Research can be tough at times, but Martin never failed to provide the much needed boost in moral with his advice and optimism. His outlook and clear-headedness have left an imprint on my own attitude when faced with complex situations and his positivism and support have been missed dearly since his departure.

To past and current student colleagues of mine, Bridget Gagné, Michael Meloche, Salwa Es-Saad, and Bassim Mohamed, I will say that I have truly appreciated the sense of community you have all brought to this relatively small laboratory and I am grateful to have shared my experience with individuals with such ambition and resolve. My relationship with you all will be cherished. To Nicolas Tremblay, secretly master barista and O' Wise One of R9.200 whose experiences surprisingly surpass expectation, I say: "thank you". Thank you for your advice in all things regarding life and science in general. Thank you for making work days enjoyable with your quirky personality and impromptu adventures. Finally, thank you for taking all this time out of your own despite being under no obligation do so.

I cannot end my acknowledgements without thanking my family, friends and significant other whose support made difficult situations all the more bearable and without which any of this would be possible. Whether my future leads me towards a path in research or a diverging one, my appreciation for the scientific method and its virtues have increased exponentially and I would like to thank everyone for this experience.

# Introduction

# 1. Global Significance

## 1.1. Virus Discovery

The historical background surrounding the discovery of Hepatitis Cvirus (HCV) is quite interesting and is undeniably important to fully appreciate the global significance of the virus. In the early 1960s, Hepatitis A virus (HAV) and Hepatitis B virus (HBV) were the only established causative agents of viral hepatitis though other viral agents such as cytomegalovirus and Epstein-Barr virus were known to cause liver damage as a generalized infection characteristic(1).

Before the advent of specific serological assays, clinical and epidemiological features distinguished type A from type B hepatitis; while hepatitis A was characterized by an acute infection of short incubation period transmitted via the oral-fecal route, hepatitis B was defined by a blood-borne infection of long incubation period (2). It was only after the development of specific antigen and antibody testing and the subsequent findings of patients with viral hepatitis lacking positive serologies for hepatitis A and B that, in the mid-70s, a Non-A Non-B hepatitis (NANBH) was described (1, 2). Though the identification of NANBH-specific antibodies did not occur until 1985 (3), standard HAV and HBV testing along with the use of elevated serum alanine aminotransferase (ALT) levels as a specific marker for hepatitis quickly linked patient onset of NANBH with contaminated blood transfusions (4, 5).

In 1989, following the long-awaited full isolation of a complementary DNA (cDNA) clone derived from a NANBH genome along with the discovery of a positive-stranded RNA virus of 10 000 nucleotides, hepatitis C virus was, for the first time, used in place of NANBH (6).

## 1.2. Worlwide Prevalence

Following the official discovery of HCV in 1989, the World Health Organization (WHO) released a report attributing over 90% of NANBH cases to the virus (7). It was also estimated, at the time, that approximately 100 million people worldwide were chronically infected (7). The global prevalence estimates have since increased, but have not changed significantly over recent years, partly due to the lack of new and more accurate data. As of late, assessments are still approximating a global prevalence of 235:10,000 or roughly 160 million chronically infected individuals (8).

Many cases of HCV infections go undiagnosed as acute infections are symptomatic in only an estimated 15-30% of cases (9) and as nearly a quarter of all acute infections are spontaneous cleared (10). Left untreated, chronically infected individuals are at risk of developing cirrhosis with an estimated probability of 16% after 20 years and that probability increases exponentially with prolonged infection (11). Similarly, hepatocellular carcinoma (HCC) develops in 1-3% of these cases 30 years post-infection (12). Looking at its contribution in annual deaths, in 2010, about half a million worldwide deaths were attributed to HCV infection representing, but not restricted to, 28% of all cirrhosis-related and 26% of all HCC-related deaths (13).

While the global burden of HCV-associated advanced liver disease is useful to depict the gravity of the HCV pandemic, the actual trends relating to the evolution of chronic infection within given countries provide interesting insights towards predicting future problem areas. When considering age-specific prevalence data (14, 15), three broad patterns consistent with temporal patterns of HCV incidence, heavily related to iatrogenic exposure or injectable drug use, can be defined.

The first pattern describes countries where HCV is endemic and where there is very little sign of decreasing prevalence. Egypt is one such example and is characterized by very high transmission rates of which a third is still attributed to improper medical tool sterilisation and inadequate screening methods prior to blood transfusions (16). Over the 20 years leading to 2020, given its long incubation period, HCV-related mortality is predicted to increase at least 2.4 fold with more than 20 000 HCV-related predicted deaths in 2020 (17).

The second pattern describes countries such as Japan and Italy where peak HCV incidence occurred several decades ago, often through iatrogenic exposure, and where current HCV prevalence and incidence are low due to proper screening and disease control. In these countries, HCV-related mortality is likely already on the decline (18, 19).

The last of the three patterns describes countries that generally have low HCV prevalence but have an increased prevalence in middle age groups likely due to later trends of injection drug use (20). In these countries, including USA, Australia and several countries of Western Europe, trends of HCV-related advanced liver disease are expected to follow those seen in Japan except with a considerable lag given that HCV infections only peaked at the turn of the millennia (20, 21). Because the development of HCV-associated liver complications occurs a number of decades after initial infection, in the USA, annual liver-related deaths are projected to increase until 2030 (21).

Over 20 years have passed since the discovery of HCV, it is clear that HCV is of global importance, establishing itself as a widespread global health issue, and reinforces the need of proper interventions for its prevention and control.

### 1.3. Genetic Variance, Genotypic Distribution and Origins

HCV like many RNA viruses is extremely prone to mutations; human hosts are capable of producing up to $10^{12}$ virions a day (22) with a calculated $3.5 \times 10^{-5}$ mutations per replication cycle (23). While bad news for the host, high genetic diversity is generally important for the evolution of the virus as it potentially confers immune escape, vaccine evasion, drug resistance and new host adaptability mechanisms (23).

HCV is made up of seven phylogenetic clades or genotypes, each with their own subtypes. The total sequence divergence within these subcategories is approximated at ~30% and at ~20% respectively (24). Moreover, natural mutations occurring within a chronically infected individual produce a closely related but heterogeneous population of HCV isolates referred to as quasispecies. Though genotype switching is uncommon, very rarely and in some special cases it has been observed (25, 26). Though no definitive link between genotype and pathogenicity has been made (27), identifying the genotype of an infection is relevant for treatment regimen in addition to providing important insight for HCV epidemiology.

Because genotyping is based on the sequence divergence within highly conserved subgenomic regions, namely E1, core, NS5B and 5'UTR (28), determining whether the mutability of HCV alone is enough to account for the existence of different genotypes and subtypes is, to certain extent, dependent on the origins of the virus. Although the concepts of non-human primate and equine origins have been explored (29) and although multiple cross-species transmission events could explain important sequence discrepancies between genotypes, the true origins of the virus have yet to be elucidated. Though the differentiation of genotypes does not necessarily resolve the true origin of HCV, it certainly provides some insight into the circulation and the recent spread of HCV on the basis of distinctive genotypic features: affected risk groups and geographical distribution (29).

Genotype 1, 2 and 3 are relatively widespread worldwide. However, among the three, genotype 1 has the greatest geographical distribution as it affects much of the developed western world including most of North America (30), Northern and Western Europe (31). Genotypes 4, 5 and 6 have more restricted geographical distributions, but do not seem to display any less genetic diversity than genotypes 1 to 3. Genotype 4 is found predominantly in the Middle East (32). Genotype 5 is almost exclusively found in South Africa (33). Genotype 6 is common to Southeast Asia (34). Not much is known about genotype 7, but it is believed to originate from Central Africa (35).

Because transmission from mother to child or sexual contact is largely inefficient (15), the widespread use of blood transfusion and other parenterally delivered treatments, none of which were common risk factors prior to the Second World War, is perhaps not coincidently in concordance with genotype 1b, 2a and 2b being mostly prevalent in the older population of Europe and Asia (29). In parts of Europe, increases in genotype 3a (36, 37), which typically infects injection drug users (IDUs) (29), may reflect the reduced contribution of iatrogenic transmission and the increased transmission through injection drug use.

Despite similar intragenotypic diversity, discrepancies within genotype-specific distribution patterns has been suggested to be the combined result of recent epidemic spread into new risk groups overlaid on top of a much older circulation of HCV(29). Globally prevalent infections such as 1a, 1b, and 3a may fortuitously be the most successful variants to enter previously unexposed susceptible individuals through parenteral transmission.

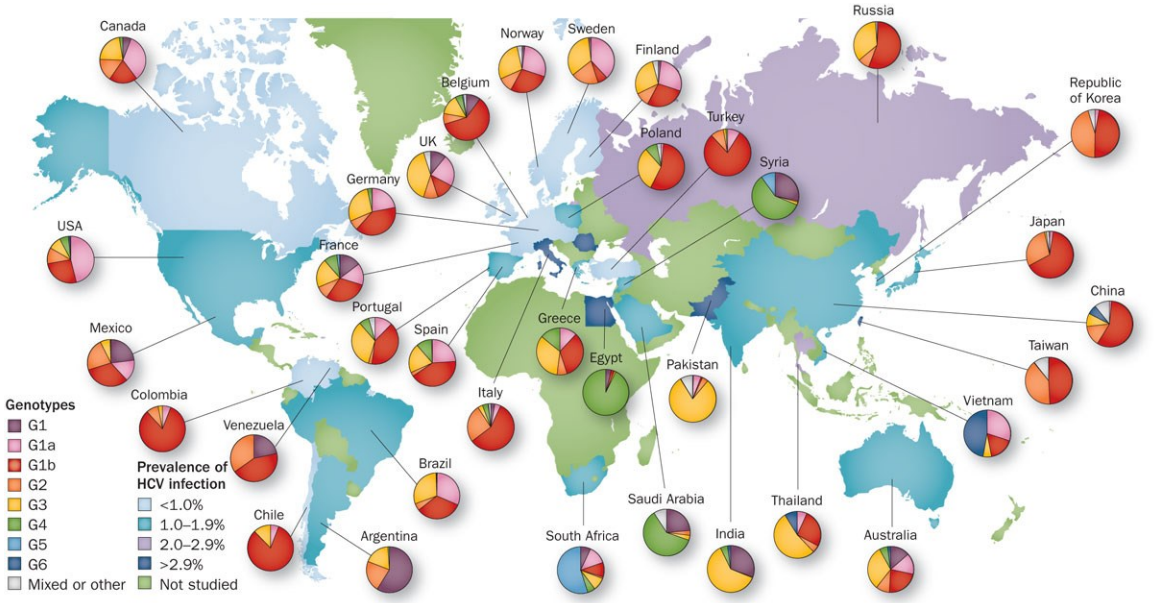

Canada
Norway
Sweden
Russia
Finland
Belgium
Republic of Korea
Turkey
Poland
UK
Syria
Germany
USA
Japan
France
China
Portugal
Greece
Spain
Taiwan
Mexico
Egypt
Pakistan
Italy
Colombia
Vietnam
Genotypes
G1
G1a
G1b
G2
G3
G4
G5
G6
Mixed or other
Venezuela
Prevalence of HCV infection
<1.0%
1.0–1.9%
2.0–2.9%
>2.9%
Not studied
Brazil
Chile
Saudi Arabia
Thailand
Argentina
South Africa
India
Australia

**Figure 1.** Worldwide estimated prevalence of HCV and genotype distribution

Visual representation of estimated region-specific prevalence of HCV infection and region-specific distribution of HCV genotypes. Countries of the middle east like Egypt have the highest prevalence of HCV infection and are predominantly characterized by genotype 4 infections. While genotype 1 has the largest worldwide distribution, genotypes 4, 5, and 6 are for the most part region specific affecting Southeast Asia, South Africa, and Egyptian middle east respectively. (permission from Hajarizadeh B, et al., *Nature Reviews Gastroenterology & Hepatology*., 2013. Copyright Nature Publishing Group 2013) (38)

# 2. HCV Lifecycle

## 2.1. Viral Entry

During primary infection, HCV particles are transported through the blood stream until they reach the basolateral surfaces of hepatocytes. There, the virus becomes concentrated by interacting with host factors with low affinity prior to interacting with subsequent essential entry factors. This initial low-affinity attachment involves interaction between host factors such as the heparan sulfate proteoglycans syndecan-1 and syndecan-4 and low-density lipoprotein receptor (LDLR) and components of the viral particles such as apolipoprotein E (apoE) and the envelope glycoproteins E1 and E2 (39-41).

The steps following this initial attachment phase are partially understood and involve a number of proposed host factors of which 4 are generally accepted: scavenger receptor B1 (SRB1) (42), tetraspanin CD81 (43), and tight junction proteins claudin-1 (CLDN1) (44) and occludin (OCLN) (45). Other factors include the cholesterol transporter Niemann-Pick C1-like 1(NPC1L1) (46), transferrin receptor 1 (TfR1) (47), and ephrin receptor A2 (EphA2) (48). Interestingly, SRB1 seems to play an intermediary role between the attachment and post-attachment phases of viral entry. Though SRB1 has demonstrated primary interaction with virion apolipoproteins, its lipid transfer activity appears to be the determining factor for productive viral entry (49, 50).

CD81, probably the most recognized among entry factors, once bound with E2 prime low pH-dependent fusion of viral endocytosis (51). Co-receptor complex formation between CD81 and CLDN1, which occurs more so at the basolateral membrane than at tight junctions, appears to be required however (52) and precedes its co-internalization with HCV particles during clathrin-dependent endocytosis. The discrepancy found within the localizations of these co-receptor complexes possibly implicates epidermal growth factor receptor (EGFR). EGFR, through downstream signaling pathways, promotes the lateral diffusion of CD81 and thus facilitates the formation of the CD81-CLDN1 complex (53). While the precise role of OCLN in the HCV life cycle has yet to be clearly defined, cell-to-cell spread has been reported as the principle mode of HCV transmission (54). Following fusion, the HCV genome is released into the cytosol where translation and replication can begin.

## 2.2. Genome Translation and Replication

Original analyses of the amino acid sequences extrapolated from the nucleotide sequences of HCV initially revealed a gene organization similar to those of flaviviruses. HCV was later assigned to a new genus, *Hepacivirus*, within the *Flaviviridae* family and is therefore a Baltimore class IV single stranded RNA virus of positive polarity (+ssRNA).

The HCV genome contains a single open reading frame (ORF) and rather than a 5'cap or a 3' poly A tail has highly structured RNA elements within its 5′ and 3′ non-translated regions (NTRs) to protect itself and assist with translation initiation (55). The 5′NTR contains an internal ribosomal entry site (IRES) to initiate translation (56) in addition to a microRNA 122 (miR-122) binding site (57). Contribution of miR-122, a liver-specific microRNA, has been shown to be indispensable to HCV replication by slowing exonuclease Xrn1 mediated degradation of HCV RNA (58) through its association with Argonaute 2 at the 5'end (59). Conversely, though no definitive consensus has been reached, the 3' untranslated region (UTR) has been shown to have stem loop structures which, in conjunction with an upstream 5BSL3.2 stem loop within the NS5B coding region, are equally as important for HCV replication and translation (60, 61).

HCV genomic RNA is translated into a single polyprotein and is subsequently processed by viral and host encoded proteases into 10 mature proteins: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (62). Host signal peptidases and signal peptide peptidases process the structural proteins of HCV by mediating the cleavage at the core/E1, E1/E2, E2/p7 and p7/NS2 junctions. Moreover, the NS2 cysteine protease, whose activity is enhanced by the N-terminus of NS3, mediates the cleavage of the NS2/NS3 junction freeing the NS3/4A serine protease. NS3/4A processes most of the non-structural (NS) proteins by mediating the cleavage of the NS4A/4B, NS4B/5A, and NS5A/5B junctions while also cleaving itself and other host factors (see chapter 2.4).

Once the polyprotein is fully processed, HCV induces massive rearrangements of intracellular membranes creating a sophisticated micro-environment called the “membranous web” to replicate its genome (55). This membranous web is particular to HCV and has been shown to be predominantly characterized by double-membrane vesicles (DMV) (63). While NS3/4A, NS4B, NS5A or NS5B alone can induce some membrane remodeling, the cooperative effect of each is required for a fully functional ultrastructure. There are, however, particular structural contributions which are differentially attributed to these NS proteins. NS4B’s oligomerization capacity has been suggested to be responsible for the scaffolding of membranous vesicles (64) while NS5A has been shown to be uniquely responsible for the induction of DMVs (65).

To follow suit, nascent genomic RNA is translated to produce either new viral polyproteins or RNA intermediates necessary for replication of genomic RNA. While RNA synthesis is the primary responsibility of the RNA-dependent RNA polymerase (RdRp) NS5B, lipid droplets (LD), triacylglycerides and cholesteryl esters deposits encapsulated by a phospholipid monolayer, are thought to play a key role in the coordination of viral RNA synthesis and the production of infectious particles (66).

## 2.3. Assembly and Release

The final stages of the HCV lifecycle require the careful spatiotemporal coordination of viral genomic RNA and structural proteins for the maturation of viral particles (67). A peculiarity of HCV morphogenesis is its complex interconnection with lipid metabolism. As a consequence, a plethora of host factors have arose in literature reporting varying degrees of contribution to HCV morphogenesis. For instance, host factors involved in very low-density lipoprotein (VLDL) synthesis pathways such as microsomal triglyceride transfer protein (MTP) (68), acyl-CoA synthetase 3 (ACSL3) (69) and hepatocyte nuclear factor 4α (HNF4α) (70) have been shown to play a role in the process.

LDs are established key players within viral assembly. While they are normally distributed throughout the cytoplasm in uninfected cells, HCV infection induces their perinuclear accumulation (71) and consequently their proximity to replication machinery. The interaction between HCV core protein, which forms the nucleocapsid, and LDs is essential for the recruitment of viral factors implicated in assembly (66) as confirmed by mutational studies in which this interaction is abrogated (72, 73). Cellular factors have also been shown to influence this core-LD association. For instance, host factors like diacylglycerol acyltransferase-1 (DGAT1) (74), an enzyme involved in lipid synthesis, and MAPK-regulated cytosolic phospholipase A2 (PLA2G4) (75), an enzyme involved in lipolysis, are reported to be involved in core trafficking to LDs. IκB kinase-α (IKK-α) has also been shown to influence this interaction through downstream lipogenic gene induction pathways (76).

While trafficking of core to LDs is vital for viral assembly, the coordination of other viral factors to assembly sites near LDs is also necessary (66). This includes HCV glycoproteins E1 and E2 that form a non-covalent heterodimer otherwise retained in the ER (77). NS2's interaction with these factors and viroporin p7 was reported to assist in this matter (78, 79).

Other NS proteins have also been shown to contribute to the assembly phase. Among them, NS5A emerges as a central player. While its interaction with LDs has been shown to be required for assembly (66), its phosphorylation at a specific serine residue by casein kinase II was shown to prompt the transition between replication and assembly (80). Similarly, NS3/4A, through its interaction with Y-box-binding protein 1 (YB-1), has been shown to influence the equilibrium between RNA replication and the production of infectious particles (81).

Following assembly, HCV particles are released through the secretory pathway (82) where the endosomal-sorting complex required for transport (ESCRT) pathway, exploited by many enveloped viruses (83) has also been proposed to play a role. It has been suggested that neutralization of acidic compartments by HCV p7 during these later stages protects newly formed particles (84).

## 2.4. Innate Response and Immune Evasion

The coevolution of hosts and viruses has granted the cell many mechanisms to recognize and defend against viral pathogens. In response to HCV infection in particular, there is a strong induction of type I IFNs and of IFN stimulated genes (ISGs). This response begins with the detection of foreign invaders through pattern recognition receptors (PRRs) (85). In the case of HCV infection, these specifically involve RIG-I and Toll-like receptors (TLR)-1, -3, and -7. Following recognition, signal transduction continues through adaptor proteins TIR-domain-containing adapter-inducing interferon-β (TRIF), myeloid Differentiation Primary Response 88 (MYD88) and mitochondrial antiviral-signaling protein (MAVS). These effectors then activate the transcription factors IRF3, IRF7 and NF-κB and consequently lead to their nuclear translocation. This allows the induction and transcription of type I and type III IFNs, whose secretion will alert neighboring cells. Although both type I and type III IFNs share a number of biological properties, they remain relatively distinct. For instance, while peroxisomal MAVS can induce type III IFNs (86), it is unable to induce type I IFNs (87).

Secreted IFNs are then detected by IFN receptor complexes (IFNAR1/IFNAR2 complex for type I IFNs and IFN-λR1/IL-10R2 complex for type III IFNs (88)) with IFN-λ receptors being more restricted to epithelial cells than the ubiquitously expressed IFN-α/β receptors (89). Signal transduction from either complex continues through the JAK/STAT pathway demonstrating differences in kinetics (90) and ultimately culminating in the induction and transcription of ISGs. The transcription of inflammatory cytokines, PRRs and effector proteins keeps cells on high alert and maintains their antiviral state (85).

In response, HCV has developed many strategies to deal with host defenses and to allow it to establish a persistent infection. Among the better characterized mechanisms is the protease activity of the NS3/4A protein responsible for the cleavage of MAVS (91) and TRIF (92). As a result, the RIG-I and TLR3 mediated early IFN response are inhibited. While NS3/4A predominantly affects the early IFN response, core has been shown to play a role in hindering the late response by inhibiting the JAK/STAT pathway through its interaction with STAT1 (93) and through the induction of suppressor cytokines SOCS1 and SOCS3 (94, 95). Additionally, HCV glycoprotein E2 (96) and NS5A (97) have been shown to inhibit IFN signalling through their interaction with ISG.protein kinase R (PKR) Beyond its interaction with PKR, NS5A has been shown to interact with another ISG 2'5' oligoadenylate synthethase (OAS) (98) achieving the same effect. Despite these evasion mechanisms, the activation of the IFN response remains detectable in patients. A rapid IFN response preceding the effect of viral evasion mechanisms and the lack of infection in all hepatocytes has been suggested to be responsible for these observations (99).

# 3. Treatment Evolution

HCV is a prime example that accurately represents the collective contribution of rigorous research to the evolution of treatment and prospective eradication of disease. From early type I IFN-based therapies riddled with side-effects which hinder treatment adherence to modern combination therapies which approach the pan-genotypic, treatment of HCV infection has a long and complex history. For HCV treatments, a therapy is considered effective when sustained virologic response (SVR) is achieved. SVR is described as the state where the virus remains undetectable following a period, typically 24 weeks, after treatment is concluded.

The Schering-Plough Corporation was the first to introduce an HCV treatment with their IFNα-2b recombinant, Intron A, being approved by the FDA in 1991 (100). Roche Pharmaceuticals would follow suit with their IFNα-2a treatment, Roferon A, being approved by the FDA in 1996. The IFN mono-therapy, however, had a poor therapeutic response with less than 20% of treated individuals achieving SVR (101). Merck, who would later acquire the Schering-Plough Corporation, brought on much needed improvements with the introduction of pegylated-IFN (PEG-IFN) and the addition of ribavirin (RBV) to treatment therapy. RBV was Originally discovered in 1972 as a guanosine analogue with broad spectrum activity against a number of RNA and DNA viruses (102), RBV was commercialized for hepatitis C treatment under the name Rebetol in 1998. On the other hand, the conjugation of IFN with polyethylene glycol allowed protection from proteolytic breakdown and thus increased its biological half-life alleviating difficulties associated with treatment adherence (103). In 2001, these milestones would establish the standard of care (SOC) for the next decade. Because genotype 1 patients were classically considered the most difficult to treat, therapeutic efficacy was still suboptimal allowing but 40% of infected individuals to attain SVR (104). In light of this, attempts continued to introduce better therapeutic regimens.

With the growing body of information surrounding the HCV life cycle and the role of individual viral proteins, the next revolution in treatment came in the form of direct-acting antivirals (DAAs). The first evidence of the potential of these novel anti-HCV agents specifically designed against essential viral enzymes was with the discovery of BILN 2061. Even though its clinical development was interrupted due to some evidence of cardiotoxicity at high doses in rhesus macaques, the efficacy of BILN 2061 in humans established the first proof-of-concept for an NS3 protease inhibitor (105). With the momentum gained from these findings, in 2011, the first generation of DAAs boceprevir (Merck) and telaprevir (Vertex) were finally approved, introduced and added to the previous PEG-IFN/RBV regimen. These new triple therapies led to higher SVR rates, but still had the adverse effects of IFN-based treatment (106). Triple therapy with boceprevir or telaprevir quickly fell out of favor with the introduction of new wave DAAs. For instance, sofosbuvir (Gilead) which was approved by the FDA at the end of 2013 in combination with PEG-IFN/RBV raised the standards by achieving SVR rates approaching 90% (107).

Competing groups gradually developed and introduced a plethora of increasingly potent DAAs using different drug design strategies (detailed in chapter **3.1**) often inspired by each other. Eventually culminating in late 2014, a new generation of all oral IFN-free combination therapies became available. Boasting excellent SVR rates, high genetic barrier to resistance as a result of the multi-pronged approach all without the undesirable IFN associated side effects, combination DAA therapies (detailed in chapter **3.3**) mark the success of years of research from discovery of a virus to near optimal treatment design.

## 3.1. Direct Acting Antiviral (DAA) – Design Strategies

In principle, every step of the HCV replication cycle is a potential target for drug design. However, early technical limitations such as a lack of a robust replication system (discussed in chapter **4.2**) and of structural information for HCV historically made designing DAAs difficult. Coincidentally, fortunate discoveries coupled with systematic research chronologically lead NS3/4A protease, NS5B polymerase, and NS5A phosphoprotein to become the primary and most successful targets for HCV-specific DAAs though other viral targets such as NS4B (108) and p7 (109) have been explored.

### 3.1.1. NS3/4A Serine Protease

The NS3/4A serine protease is a heterodimeric enzyme that belongs structurally to the trypsin superfamily and is comprised of the N-terminal domain of the NS3 as well as the NS4A protein. It is unique, however, within its classification, for its requirement of a viral cofactor and of a structural zinc atom (110).

While the N-terminal domain of NS3 is responsible for the protease function, the C-terminal two-thirds of NS3 function as an RNA helicase belonging to the DExH family (111). Though its exact function within the HCV lifecycle is unclear, based on functional homology, it may be involved in RNA folding/remodeling (112), polymerase processivity (113), and/or genome encapsidation (114). Whether the helicase activity functions as a monomer or oligomer and whether either form is more functionally fit is debatable though some evidence indicates that which form NS3 takes is dependent on relative concentrations of enzyme to substrate (115).The NS4A protease cofactor consists only of 54 residues making it a relatively small protein. The N-terminal hydrophobic region, predicted to form a transmembrane α-helix, is involved in anchoring the heterodimeric complex to the membrane while amino acids 21-34 are directly implicated in the interaction with NS3 and are absolutely required (116). Crystal structures of NS3 with and without cofactor revealed that NS4A optimizes the orientation of the catalytic triad residues and thereby stabilizes the protease domain of NS3 and increases enzymatic activity (117).

The main functional purpose of NS3/4A is the cleavage of the viral polyprotein at the junctions between NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B. The liberated viral proteins are a product of trans-cleavage events, while NS3 and NS4A result from an intramolecular cis-cleavage. These junctions possess a consensus sequence of Asp/Glu-$(Xaa)_4$-Cys/Thr ↓ Ser/Ala-$(Xaa)_2$-Leu/Trp/Tyr (118). This consensus sequence agreed with later studies that characterized the requirement of decamer peptide substrates spanning P6-P4' (Schechter & Berger nomenclature (119)) and defined preferences for an acidic residue in P6, cysteine in P1, serine or alanine in P1', and a hydrophobic residue in P4' (120-122). The NS3/4Aprotease lacks several surface loops at the substrate binding cleft found in other serine proteases (110). As a result, the solvent-exposed and relatively featureless substrate binding site posed a great challenge in the design of small molecule inhibitors and selection of non-peptidic candidates from compound collections.

#### 3.1.1.1. NS3/4A Protease Inhibitors (-previr)

Based on the enzymatic features derived mainly from X-Ray crystallography studies, three distinct strategies for developing inhibitors against the NS3/4A serine protease were contemplated. These strategies consisted of interfering with either the NS3/NS4A interaction or the binding of zinc, and preventing substrate binding to the active site. The first two strategies are still, however, considered extremely difficult (123) and drug candidates of this category are virtually nonexistent. For instance, nonpeptidic small molecule inhibitors such as certain benzimidazole-based compounds inhibit the enzyme by exploiting its interaction with zinc (124). Though noncompetitive allosteric inhibitors, emerging mainly from random screening, could have potential within combination therapies, due to the nature of the method by which they are identified, the necessity of extensive safety assessments makes their development problematic.

With the insights gained from the design of human immunodeficiency virus (HIV) protease inhibitors, and with the large body of structural data surrounding the enzyme and its substrate, active site inhibitors were the first to be successfully developed and continue to be considered the most promising and widely used approach. Though, initially, the absence of a well-defined substrate binding site had raised concerns for the design of low molecular weight inhibitors, early breakthroughs lead to the emergence of two main mechanistic classes of active site-based inhibitors, namely covalent and product analogue inhibitors(125).

#### *3.1.1.1.1. Covalent Inhibitors*

Often referred to as transition state analogues or serine-trap inhibitors, covalent NS3/4A inhibitors are product-based inhibitors conceived through the replacement of the scissile amide bond with an electrophilic warhead._When the catalytic serine (S139) of the protease attacks the electrophilic warhead, a chemical formation which mimics and locks the transition state of peptide bond cleavage is generated. Despite classic electrophile-based covalent inhibitors functioning via an irreversible mechanism and despite speculations of clinical restrictions due to the possibility of unspecific irreversible binding (126), NS3/4A protease inhibitors of this mechanistic class were the first to be approved by the FDA. Notably, of the various electrophilic warhead alternatives, the early success of both boceprevir (Merck) and telaprevir (Vertex) is owed in part to the unusual mechanism by which linear α-ketoamide derivative inhibitors allow reversible binding to the catalytic site of the viral enzyme(127).

#### *3.1.1.1.2. Product Analogues*

This mechanistic class is comprised of competitive, reversible, mostly macrocyclic, noncovalent inhibitors whose drug design efforts were initiated by early findings regarding the susceptibility of the NS3/4A protease to feedback inhibition (128, 129).

With structural studies characterizing substrate specificity, and the importance of the P1 residue (see chapter **3.1.1.**) to the potency and specificity of serine protease ligands (130), the major challenge for the development of N-terminal product-like inhibitors was designing proper chemically stable replacements for the P1 sulfhydryl group. Amino acid substitutes whether those with small hydrophobic side chains or those with larger side chains, both lead to a loss of potency either due to reduced contact surface area or steric incompatibility (128). Eventually, the continuing research efforts culminated in the successful replacement of the unstable P1 residue with aminocyclopropane carboxylic acid (ACCA) derivatives (131, 132). The addition of a macrocyclic ring that connected the side chain of the P1 and the P3 residues and the stepwise optimization which followed proved to be useful in improving affinity and specificity, while preserving bioavailability characteristics of small molecules (133). Ultimately this resulted in the discovery of the compound BILN 2061 or ciluprevir (105). Macrocyclization was truly a breakthrough in NS3/4A protease inhibitor design, as many subsequent drug candidates, though varying in C-terminal moieties for improved pharmacokinetics, retain essentially the same core P1 ACCA and accompanying structures. Simeprevir (Janssen), paritaprevir (AbbVie), and grazoprevir (Merck) are notable examples for this mechanistic class of protease inhibitors and are showcased within upcoming combination therapies.

Rather than focus on the N-terminal product, certain groups explored the C-terminal side of the scissile bond for competitive inhibition. These groups observed that P1' substitutions with proline, tetrahydroisoquinoline-3-carboxylic acid (TIC) or pipecolinic acid (PIP) generated noncleavable substrate analogues (120). Despite boasting equally high potency and selectivity, development of clinical candidates was never fully engaged due to their large molecular weights and hence their poor pharmacokinetics.

### 3.1.2. NS5B RNA-Dependent RNA Polymerase

As the RNA-dependent RNA polymerase of a positive stranded RNA virus, NS5B is required for the synthesis of negative stranded RNA intermediate and subsequently that of positive polarity RNA genomes. Early *in vitro* studies using purified NS5B had already described the enzymatic activity of NS5B as being indiscriminate, but primer-dependent (134). Further progress in regards to HCV NS5B was hindered by difficulties associated to its poor solubility, a consequence of being a component of a membrane-bound complex (135). Interestingly, while the full-length enzyme displayed rather poor catalytic activity (136) the removal of the dispensable C-terminal hydrophobic tail, consisting of 21 residues and responsible for the ER membrane targeting of NS5B (137), resulted in enhanced enzymatic activity (138) and most importantly enhanced solubility which greatly facilitated the determination of 3D structures.

The unliganded crystal structure of NS5B, reported by several groups (139-141), had revealed unique structural features which could be manipulated in later drug design. While NS5B acquired the overall classic “right hand” shape, which included fingers, palm and thumb subdomains, it differed from the majority of cellular and viral polymerases which adopted a “half-open right hand” architecture. Due to the presence of two extended loops that span finger and thumb domains near the active site, NS5B adopted a more compact shape. The presence of a unique β-hairpin in the thumb subdomain which protrudes into the active site is another structural feature particular to NS5B. This may allow greater discrimination for template binding in a model which has previously been characterized as rather unspecific.

While *in vitro* evidence supports a highly processive “copy back” mechanism, in which 3’-terminal-OH group of the template is used as a primer for polymerization (134), it is generally agreed that NS5B proceeds through de novo initiation since the alternative would lead to an eventual loss of terminal sequences that are indispensible for proper HCV replication (142).

#### 3.1.2.1. NS5B Polymerase Inhibitors (-buvir)

Due to the nature of viral replication and absence of RNA-dependent RNA polymerases in uninfected cells, targeting viral polymerases is invariably a straightforward choice in a drug design strategy and is unsurprisingly the rationale behind the majority of early approved antiviral drugs (143). For HCV, though an alternative mechanistic approach has been explored (144), polymerase inhibitors fall into two primary categories: nucleoside/nucleotide inhibitors (NI) and non-nucleoside/nucleotide inhibitors (NNI).

The NS5B polymerase has multiple binding sites that can be targeted for inhibition among which the catalytic site is the most phylogenetically conserved (145). Because NIs induce premature elongation termination by acting as substrate analogues that bind competitively to the active site of the enzyme, NIs boast a high genetic barrier with only the S282T mutation being reported to confer resistance (145, 146). Sofosbuvir (Gilead), a phosphoramidate prodrug that becomes an active uridine nucleotide analogue after triphosphorylation, is an example of a NI of current interest.

NNIs, however, can bind 4 different allosteric binding sites, located within the canonical thumb and palm domains (147, 148), and distort the precise geometry of the active site to significantly impair enzymatic function. Because these allosteric sites are not as conserved as the catalytic site, NNIs are more susceptible to resistance mutations. The thumb domain contains two binding pockets, the upper thumb (thumb I) and lower thumb (thumb II) which are characterized by distinct non overlapping resistance patterns (149). On the other hand, the palm domain contains partially overlapping palm I and II sites that also have an overlap in their resistance profiles (149). Though the design of these inhibitors is not quite as structured as that of NS3/4A protease inhibitors, palm I site inhibitors do have a recurrent structural benzothiadiazine-containing theme (149). Dasabuvir (AbbVie), an aryl dihydrouracil derivative, is an example of a palm I site directed NNI currently used in combination therapies.

### 3.1.3. NS5A Phosphoprotein

NS5A is a zinc-binding phosphoprotein spanning 447 amino acids (150) that is described to be phosphorylated at several different regions by distinct kinases and, as a consequence (151-153), exists in two forms designated p56 and p58 based on electrophoretic mobility. Though functional differences between its forms have yet to be elucidated, essential host factor and NS5A interactant lipid kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα) has been shown regulate its phosphorylation (154).

Structurally, the amino-terminus of NS5A includes an amphipathic α-helix, which is responsible for anchoring to the ER and ER-derived membranes such as lipid droplets (LDs) (155).This amphiphathic helix, comprised of the first 31 residues of NS5A, is also the only structural feature conserved in all HCV genotypes (156). NS5A can be divided into three distinct domains which are separated by linker regions (150). While domain I (D-I) residues were confirmed by biochemical assays to be involved in NS5A dimerization and RNA binding (157), domains II (D-II) and III (D-III) are thought to be responsible for NS5A's large network of host interactants by fault of their intrinsically disordered and hence flexible nature (158, 159).

NS5A's interaction with PI4KIIIα has been identified as having a central role in HCV replication as it induces the accumulation of phosphatidylinositol-4-phosphate (PI4P) within the membranous web and without which dramatic changes in ultrastructural morphology occur (160). Cyclophilin A through its contribution to de novo formation of double-membrane vesicles (DMV) (65) was also demonstrated to be a key host interactant essential for optimal HCV replication and has its own dedicated class of inhibitors (details in chapter **3.2.2.**). ADP Ribosylation Factor GTPase Activating Protein 1 (ARFGAP1) (161), a GTPase-activating protein, and vesicle-associated membrane protein-associated protein A (VAP-A) and VAP-B (162) have also been shown to interact with NS5A with varying contributions to HCV replication.

Despite intrinsically lacking in enzymatic activity (163), NS5A is indispensible to HCV replication and its promiscuous ability to interact with numerous host factors is likely the source of its relevance.

### 3.1.3.1. NS5A Inhibitors (-asvir)

Classically, it was thought that NS5A was undruggable given the lack of characterized enzymatic activity (163). However, high throughput screening (HTS) using cell-based HCV replication systems serendipitously allowed the discovery of an early monomeric candidate which after subsequent chemical optimizations eventually led to dimeric daclatasvir (Bristol-Myers Squibb) (164-166). The same study noted rapid emergence of key resistance mutations within the NS5A coding region and confirmed the inhibitor's target. A subsequent study determined that previously identified Y93H and L31V amino acid substitutions within NS5A to roughly confer a 25 fold increase in resistance to daclatasvir individually, but a 15 000 fold increase when combined (167). These findings were also related to emerging resistance mutations in clinical settings further suggesting a specific drug binding site.

Prior to the dimeric design of NS5A inhibitors, were a number of monomeric molecules which varied greatly in chemical scaffolds (166). Though emerging treatment-induced mutations were also mapped at the homodimer interface in the crystal structure of NS5A, these inhibitors were not as effective as their eventual successor becoming optimal within the nanomolar range rather than the picomolar range (166).With the encouraging clinical results from daclatasvir, NS5A inhibitor design trends shifted towards utilizing a dimeric pharmacophore, featuring a conjugated bis-biaryl core terminated by peptidic caps (166). The current trend in NS5A inhibitor designs utilizes a dimeric pharmacophore, featuring a linear conjugated bis-biaryl core terminated by peptidic caps (166). While peptidic caps are relatively conserved among various drug candidates, the chemical composition of core linkers varies widely with a tendency to remain within range of 15-18 Å (166). There is however a preference for imidazole-proline coupling for the junction between the core and caps and a preference for conjugated aryl groups to bridge the midsection of the cores (166).

The exact mechanism by which inhibition is achieved remains elusive with no definitive consensus even on whether inhibitor binding is symmetrical or asymmetrical within the NS5A homodimer (168). However, putative molecular mechanisms have been suggested to explain inhibition on HCV replication. It has proposed that this DAA class disrupts the function of new replication complexes rather than affecting preformed complexes while also causing the redistribution of NS5A to lipid droplets (LD) (169). Others have proposed mechanisms at the stage of assembly and release revolving around host factors such as PI4KIIIα and TIP47. These suggested mechanisms are unlikely exclusive and probably need to be combined to explain the biphasic clinical response to these DAAs (170).

## 3.2. Host-Directed HCV Inhibitors – Design Strategies

Ingenuity is decidedly not lacking in HCV antiviral therapies. While DAAs are largely successful at inhibiting the virus, these strategies do not compare to the diversity found in host-directed HCV inhibitors or host-targeted agents (HTAs).

### 3.2.1. RNA-Based Inhibitors

The concept of utilizing RNA molecules as therapeutic agents was initially incited from the discovery that the entire HCV genome was translated into a single polyprotein via an internal ribosome entry site (IRES) (171). From there, the potential of trans-cleaving ribozymes (172) and antisense RNAs(173) as potential inhibitors of viral translation lead to the identification of ISIS 14803, a 20-base antisense oligonucleotide inhibitor (174), which was ultimately dropped during phase 1b clinical trials after generating inconclusive data. Eventually, the discovery of the liver-specific microRNA 122 (miR-122) and its possible role in HCV replication (57) reintroduced the potential of RNA-based treatments. With host miR-122 as a target, another antisense oligonucleotide inhibitor was developed. Miravirsen (Roche), having shown moderate efficacy in treating chronically infected individuals (175), is currently undergoing phase II clinical trials as a monotherapy.

### 3.2.2. Cyclophilin A Inhibitors

Two classes of cyclophilin A inhibitors exist: classical cyclosporine and non-immunosuppressive cyclosporine derivatives. The requirement of cyclophilin A for HCV replication was only truly demonstrated when the mechanistic prerequisite was shown to be its peptidyl-prolyl isomerase activity (176, 177) and when HCV NS5A was determined to be its viral ligand (178). As the cyclophilin A-NS5A interaction is conserved across genotypes, these host-targeted agents have the benefit of being pan-genotypic.

Though its initial discovery is not merited directly to anti-HCV efforts, these discoveries definitely emphasized the potential of cyclosporine in HCV therapeutics. For instance, cyclosporine A, a cyclic peptide of 11 amino acids, was originally sought after for its immunosuppressive activity which was achieved through a ternary complex formation between cyclosporine A-bound cyclophillin A and calcineurin (179). Its immunosuppressive ability was not favorable in the context of its anti-HCV ability, and so chemical modifications lead to the development non-immunosuppressive cyclosporine derivatives like alisporivir (Debiopharm), and N-methyl-4-isoleucine cyclosporine or NIM811 (Sandoz Pharmaceuticals) (180). Unlike its parent molecule, these derivatives while retaining its binary complex formation with cyclophilin A were unable to bind calcineurin. Normally, the N-methyl leucine at position 4 of cyclosporine occupies the calcineurin binding pocket however its substitution with N-ethyl valine or N-methyl-isoleucine allows respectively alisporivir (debiorivir) and NIM811 to be non-immunosuppressive (181, 182).

Though some evidence attributed cyclosporine sensitivity to HCV NS2 (183), discrepancies in cyclosporine sensitivity between replicons lacking NS2 and full replicative systems which have NS2 were later described to be due to reduced replication competence because NS2-mediated polyprotein cleavage is the rate-limiting step in polyprotein processing (65).

### 3.2.3. Entry Inhibitors

Evidence for anti-HCV agents targeted at viral entry is varied and plentiful. The continuing development of entry inhibitors has brought forth considerable insight towards the early stages of the viral cycle (see chapter **2.1.** for details). Entry inhibitors can act at the specific stages of viral entry whether it is at initial attachment, post-binding entry or at the level of viral endocytosis/membrane fusion. Because this mostly involves some form of disruption or modification to the interaction between HCV envelope glycoproteins and host factors, as with other host-targeted agents, entry inhibitors tend to have a high genetic barrier to resistance with largely HCV genotype-independent potency, but pose a greater risk of simultaneous cellular toxicity.

Oriented towards the attachment and post-binding entry phases are: host-derived peptides like soluble low-density lipoprotein receptor (LDLR) (184), heparin-derived molecules (185), recombinant human L-ficolin (186), and claudin-1 (CLDN1)-derived molecules (187); virus-derived peptides such as p7 ion channel-derived molecules (188); glycan-binding synthetic molecules such as lectin cyanovirin-N (189), and boronic acid-modified lipid nanoparticles (190); structural mimic molecules like imidazole-based compounds (191); and natural compounds like epigallocatechin gallate (192) which is commonly found in green tea extracts. Though most candidates are in the *in vitro* stages of testing, a few have emerged in clinical trials. The savenger receptor B1 (SRB1) antagonist ITX 5061 (iTherX), an arylketoamide-based compound, is the most promising candidate of its class (193, 194) and is currently undergoing phase II of clinical trials. Because of its differing inhibitory mechanism, it also shows potential in combination with conventional DAAs (195).

Lesser characterized entry inhibitors are: those that target the acidification mechanism of virion-cell membrane fusion like vacuolar ATPase inhibitors concanamycin A and bafilomycin A (196); and those that disturb the lipid balance required for membrane fusion such as phenothiazines (197) and indole derivatives (198).

### 3.2.3. Immunomodulators

Improving tolerability, efficacy and or pharmacokinetic properties of existing treatments and characterizing other immunomodulatory candidates are venues which have also been explored by certain research groups. More specifically affecting hepatocytes and consequently reducing haematological side effects, interferon analogues such as PEG-IFN-lambda (199) and ribavirin analogues such as the taribavirin prodrug (200) have both undergone documented trials. More recent candidates which have been found to indirectly promote HCV elimination by bolstering the innate immune responses are nitazoxanide, a broad spectrum anti-parasitic that acts by improving interferon signalling, and toll-like-receptor (TLR)-7 agonists (201) which mediate endogenous interferon and cytokine release through the myd88 adaptor.

## 3.3. Current Combination Therapies

In December 2013, the approval of the polymerase inhibitor sofosbuvir (Gilead) in combination with PEG-IFN/RBV brought forth new optimism for IFN-free therapy. Boasting broad genotype coverage with high SVR rates ranging from 82% to 100% (202), the idea of dropping PEG-IFN in favor of DAA substitutes proved promising. Combined with protease inhibitor simeprevir (Janssen) or NS5A inhibitor daclatasvir (Bristol-Myers Squibb) with or without ribavirin, novel therapies which included sofosbuvir quickly demonstrated SVR rates well above previous standards covering beyond genotype 1 and proving efficacious in both naïve and previously treated patients (203, 204).

From late 2014 and onwards, proprietary therapies using a combination of pre-approved or novel re-optimized DAAs are likely to dominate the HCV therapeutic scene. Though other design strategies (see chapter **3.2.**) may continue to improve with future iterations, they are unlikely to be competitive. Outside of cost considerations, current combination therapies have the clear advantage boasting excellent SVR rates across the board and conferring more confidence in their safety when compared to host-directed inhibitors.

Currently, a number of combination therapies are available with more recent ones incorporating second-generation DAA iterations which are designed for broader genotype coverage (205). These all-oral IFN-free therapies achieve SVR rates approaching 100% within their designated genotypes, last typically between 12 to 24 weeks as opposed to the traditional 24 to 48 weeks with IFN-based treatments, and demonstrate excellent treatment adherence and good tolerability profiles (206-214). In October 2014, the combination therapy Harvoni® (Gilead) was approved by the FDA for treatment of HCV genotype 1 and subsequently approved for genotypes 4, 5, and 6. It is comprised of first-generation NS5A inhibitor ledipasvir and first-generation nucleoside inhibitor sofosbuvir. In December 2014, Viekira

Pak™ (Holkira Pak™ in Canada, AbbVie) was approved by the FDA for the treatment of HCV genotype 1 infections including individuals with compensated cirrhosis. It is comprised of first-generation NS5A inhibitor ombitasvir, second-generation protease inhibitor paritaprevir coupled to pharmacological enhancer ritonavir and non-nucleoside inhibitor dasabuvir. In July 2015, AbbVie's revised combination therapy Technivie™ was approved by the FDA for the treatment of HCV genotype 4, but is contraindicated for those with severe hepatic impairment. This package forgoes the inclusion of non-nucleoside inhibitor dasabuvir. In January 2016, Zepatier™ (Merck) was approved by the FDA for the treatment with or without ribavirin of HCV genotype 1 and 4. It is comprised of two second-generation molecules, namely protease inhibitor grazoprevir and NS5A inhibitor elbasvir. Finally, in June 2016, the most recent combination therapy Epclusa® (Gilead) was approved by the FDA for treatment of chronic HCV infection genotypes 1 through 6 including comorbidities. This all-oral IFN-free therapy is composed of second-generation NS5A inhibitor velpatasvir and first-generation nucleoside inhibitor sofosbuvir.

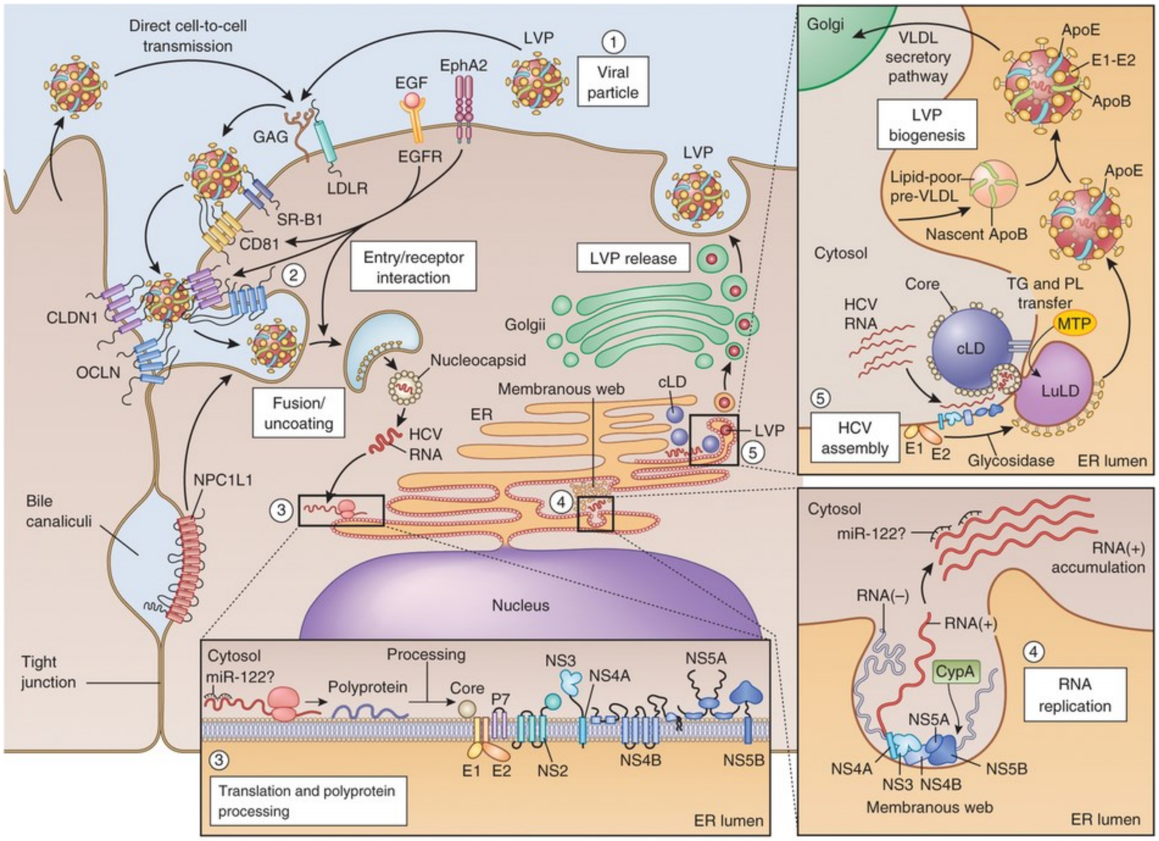

Direct cell-to-cell transmission
LVP
1
Viral particle
EGF
EphA2
GAG
EGFR
LDLR
SR-B1
CD81
LVP
Entry/receptor interaction
2
LVP release
CLDN1
OCLN
Golgii
Nucleocapsid
Fusion/ uncoating
Membranous web
cLD
ER
HCV RNA
LVP
5
NPC1L1
Bile canaliculi
3
4
Nucleus
Tight junction
Cytosol
miR-122?
Processing
Polyprotein
Core
P7
NS3
NS4A
NS5A
E1
E2
NS2
NS4B
NS5B
3
Translation and polyprotein processing
ER lumen
Golgi
VLDL secretory pathway
ApoE
E1-E2
ApoB
LVP biogenesis
Lipid-poor pre-VLDL
ApoE
Nascent ApoB
Cytosol
Core
TG and PL transfer
HCV RNA
MTP
cLD
LuLD
5
HCV assembly
E1
E2
Glycosidase
ER lumen
Cytosol
miR-122?
RNA(+) accumulation
RNA(–)
RNA(+)
4
CypA
RNA replication
NS5A
NS4A
NS5B
NS3
NS4B
Membranous web
ER lumen

**Figure 2.** HCV lifecycle – Potential points of intervention

Visual representation of the viral lifecycle of HCV. Numbered are the individual phases denoting: 1. Viral particle (where immunomodulators can be beneficial); 2. Entry/receptor interaction (detailed in chapter 2.1 and stage where entry inhibitors (chapter 3.2.3) act); 3. Translation and polyprotein processing (detailed in chapter 2.2 and stage where NS3/4A and RNA-based inhibitors (chapters 3.1.1 and 3.2.1) act); 4. RNA replication (detailed in chapter 2.2 and stage where NS5B, NS5A, Cyclophilin A inhibitors (chapter 3.1.2, 3.1.3 and 3.2.2) act); 5. Viral particle assembly (detailed in chapter 2.3 and stage where NS5A inhibitors (chapter 3.1.3) can act). (permission from Scheel T, and Rice C, *Nature Medicine*., 2013. Copyright Nature Publishing Group 2013) (215)

# 4. Models for the Study of HCV

## 4.1. Animal Models

During the earlier days of HCV research, many factors involved in HCV infection were examined in chimpanzees. These include characteristics such as transmission, genetic drift, clinical outcome of infection, and the role of the immune response during infection (216, 217). Many human vaccine trials performed or initiated for HCV along with infectivity studies of HCV clones which lead to the development of cell culture systems are owed to studies done in chimpanzees (218). Despite the universally acknowledged contribution of this non-human primate model for the advancement of HCV research, prompted by ethical concerns, chimpanzee models have either been discontinued or been on the decline since 2011.

While the first HCV infection studies in a mouse model were performed in 2001 (219), the decline of chimpanzee models in 2011 only deepened the interest in developing viable small animal models for HCV. Adaptations to this model were necessary however, given that, other than humans, persistent infection could only be established in chimpanzees (220). To this end, a number of strategies have been explored to humanize this small animal model. Because *in vivo* models are used to more closely mimic natural infection and the environment of the liver, strategies vary according to the particular host-viral protein interactions of interest. The first method attempts to gradually adapt HCV E1 and E2 to murine versions of entry factors such as CD81 and OCLN through prolonged exposure in culture. The second method uses transgenic expression of either HCV-specific or human-specific proteins in murine liver cells. FL-N/35 mice, the most well-known of transgenic mice, express the entire HCV polyprotein at close to physiological levels. They have been used to study hepatic steatosis, liver fibrosis and development of hepatocellular carcinoma. Alternatively, though its characteristics have yet to be properly defined due to the requirement of immune deficiency, expression of human CD81 and OCLN via an adenoviral vector has been attempted in mouse livers. A third method uses xenotransplantation of human hepatocytes to study HCV in mice. Immunodeficiency is required, however, to prevent rejection. This method also requires murine liver injury as a means to provide a growth advantage to transplanted primary human liver cells (220).

## 4.2. Cell Culture Based Systems

Though animal models provide a more holistic and arguably accurate picture of HCV biology, many of the major discoveries regarding the HCV lifecycle, specific viral proteins and their enzymatic properties and the derivative identification of antiviral drugs are owed to cell culture based models of HCV.

The first cell culture based system developed is the subgenomic replicon (221). The replicon system encodes viral proteins NS3 to NS5B and is therefore restricted to the study of the replicative portion of the HCV lifecycle. In this system, the IRES of HCV drives the expression of neomycin phosphotransferase which provides resistance to the G418 antibiotic and serves a selection marker. Conversely, the characteristically more efficient IRES taken from Encephalomyocarditis virus (EMCV) is used to drive the expression of the viral proteins required for replication.

Following the characterization of an HCV genotype 2a isolate derived from a Japanese patient with fulminant hepatitis designated (JFH-1), another cell culture based system was developed (222-224). Unlike before, this system was capable of emulating the full viral lifecycle. However, to increase infectivity, multiple chimeras were created, of which one is JC1. JC1 is a chimeric hybrid combining the core to NS2 coding region of another genotype 2a isolate J6 and the NS3 to NS5B coding region of JFH-1. This combination yields a viral titer 1000 times more efficient than the original JFH-1 isolate (225).

Both of these models have had undergone multiple iterations to provide adaptations for screening and microscopy purposes. This includes firefly luciferase (Fluc) reporter and green fluorescent protein (GFP) tagged alternatives. It should be noted that these culture systems typically use Huh7 derived hepatoma cell lines, which lack immune (226, 227) and metabolic (228) features of primary human hepatocytes, and consequently confer a notable disadvantage when compared to other models for the study of HCV.

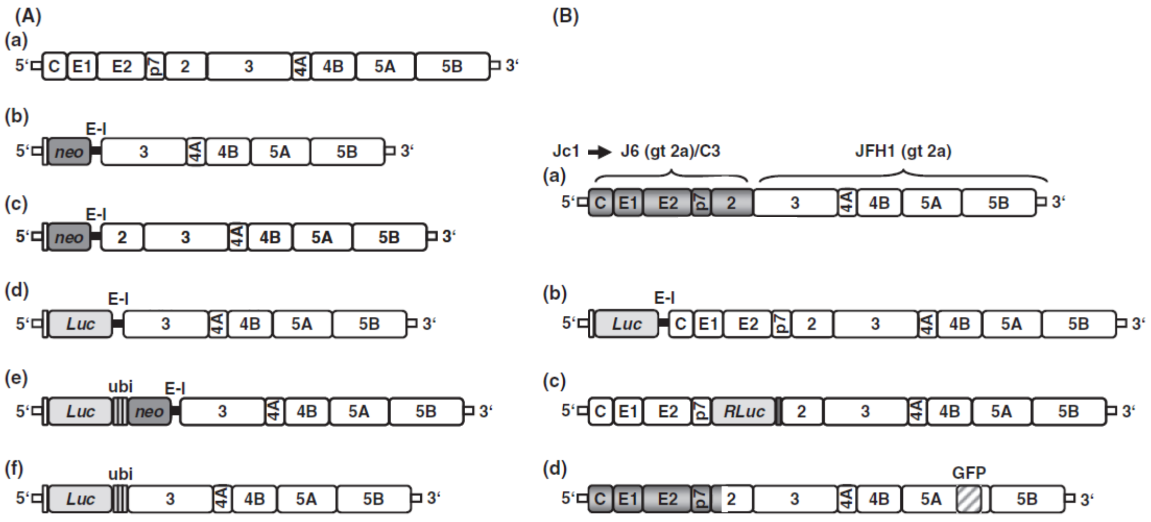


**Figure 3.** Overview of cell culture based systems for the study of HCV.

(A) Structures of HCV replicons. (a) Schematic presentation of the HCV genome. 5′ and 3′NTR are indicated by white bars. (b) and (c) Structures of bicistronic selectable subgenomic replicons. They are composed of the HCV 5′NTR directing translation of the *neo* gene, the IRES of the encephalomyocarditis virus (E-I), the HCV replicase genes NS3–NS5B (a) or NS2–NS5B (b) and the 3′NTR. (d) Structure of a subgenomic Con1 reporter replicon. (e) Structure of a selectable reporter replicon. This construct encodes a luciferase–ubiquitin–neomycin phosphotransferase fusion protein in the first cistron. (f) Structure of a monocistronic reporter replicon. The entire polyprotein is translated by the HCV IRES. (B) Structure of JFH-1 derived genomes. (a) Chimeric genomes are composed of the region encoding core to NS2 of HCV isolate J6 fused to the remainder of the JFH-1 isolate. This highly competent J6/JFH-1 chimera is called Jc1. (b) Schematic representation of a bicistronic full length JFH-1 reporter genome. The HCV 5′NTR directs translation of the firefly luciferase gene (*Luc*) whereas translation of the HCV polyprotein is under the control of the EMCV IRES (E-I). (c) Schematic diagram of a monocistronic Renilla luciferase (RLuc) reporter genome. (d) Structure of a monocistronic Jc1 reporter virus applicable for live cell imaging. The gene encoding GFP is inserted in-frame into domain 3 of NS5A. (permission from Woerz I, and Lohmann V, *J of Viral Hepatitis*., 2009. Copyright Journal compilation © 2009 Blackwell Publishing Ltd) (229)

# 5. Bioluminescence Resonance Energy Transfer (BRET)

While the yeast two-hybrid system has been widely used to identify protein-protein interactions (PPIs), resonance energy transfer methods have presented a few advantages. Unlike the classical yeast two-hybrid system that is limited to detecting interactions within the nucleus (230) and that therefore forgo protein processing and compartmentalization, these alternatives can detect interactions which depend on post-translational modifications.

BRET, first used in 1999 to study the dimerization of proteins implicated in the circadian cycle of cyanobacteria (231), is a technique that exploits the non-radioactive energy transfer between a bioluminescent energy donor protein and a fluorescent energy acceptor protein. Compatible with live cell settings, BRET can be used to determine PPIs that more easily reflect what occurs naturally within a cell and it also represents an unprecedented method to observe membrane PPIs. For instance, in a previous report where novel HCV protein interactions were identified (232), the inclusion of the interaction between NS4B and NS5A, which are located at the ER and at LDs respectively, and the exclusion of the interaction between NS3/4A and p7, which are both localized to the mitochondria, within an updated HCV interaction network attest for the specificity of BRET and its independence from protein localization.

While Renilla reniformis luciferase (Rluc) is always the energy donor different iterations of BRET such as $BRET^1$, $BRET^2$ and eBRET, utilize different combinations of substrates and energy acceptor proteins for their associated advantages (233). These include greater emission peak separation which reduce background effects and greater signal duration which facilitate time dependent experiments. These advantages are, however accompanied by inconveniences related to cell viability and the requirement of more sensitive equipment. Nevertheless, for the sake of this explanation, the case of $BRET^1$ is specifically addressed. Indeed, Rluc, following oxidation of its cell permeable substrate coelanterazine H, is able to emit a photon at a maximal wavelength of 480 nm (234). Upon excitation by photons up to a maximal wavelength of 514 nm, the yellow fluorescent protein (YFP) emits itself a photon at a maximal wavelength of 527 nm. Given the differing wavelengths of donor and acceptor protein photon emissions, the energy transfer can easily be quantified by calculating the ratio between the intensities of photons emitted by YFP and Rluc. This ratio is referred to as the BRET signal. However, because the energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor dipoles (235), energy transfer is only detectable when both YFP and Rluc are within 10–100 Å of each other and when YFP and Rluc tagged proteins are properly oriented. As a result, if YFP and Rluc tagged candidates do not interact, only bioluminescence (Rluc emission) is detected. Conversely, if both candidates interact, both bioluminescence and fluorescence (YFP emission) are detected.

# Hypothesis & Objectives

Hypothesis:

The previous optimization of live cell based assays using bioluminescence resonance energy transfer (BRET) technology had established a comprehensive hepatitis C virus (HCV) protein interaction network and had subsequently revealed an unreported NS3/4A-NS3/4A interaction (232). Given the existing proteolytic activity of NS3/4A and the novelty of the NS3/4A-NS3/4A interaction, the development of HCV inhibitors based on PPI modulation had potential. The premise of my Master's project is that inhibitors identified through BRET based assays constitute a novel class specific to HCV and function through a unique mechanism different from drugs already available on the market.

Objective:

The objective of my project is to characterize novel HCV inhibitors identified through BRET based assays that can be used in combination with currently available inhibitors. This is achieved through the evaluation of compound therapeutic potency and through the confirmation of a novel inhibitory mechanism via resistance profiling.

Aims:

- *In vitro* characterization of the resistance profile of a lead compound acting on the replication of HCV.
- Study lead compound induced modulation of previously identified cellular partners that potentially contribute to viral replication.

# Experimental Procedures

## Cell culture

The 293T, and Huh7.5 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM l-glutamine, and 1% nonessential amino acids (all from Wisent) at 37 °C in an atmosphere of 5% $CO_2$. Cells were transfected with linear (25-kDa) polyethylenimine (PEI; Polysciences) (236) or Lipofectamine 2000 (Invitrogen) as described by the manufacturer. Cell populations of HEK 293T stably harbouring the pIFNB1-LUC and of Huh7.5 stably harbouring the pEF1α-LUC were produced after selection with 200µg/ml of hygromycin B (Wisent) and were previously described (237). Huh7 cells stably expressing a reporter Con1 subgenomic replicon (Huh7-Con1-Fluc) (221) were a gift from Ralf Bartenschlager (University of Heidelberg) and were maintained in complete DMEM with 1 µg/µL G418 (Multicell).

## Expression vectors

*KPNB1* and *HSP60* cDNA were purchased from GE Dharmacon/Open Biosystems. Following PCR-amplification, PCR products were cloned into either pcDNA3.1_MCS(MB)-eYFP or pCDNA3.1_FLAG-MCS(MB) expression vectors using Pfl23II/NotI enzymes (236). 3xFLAG-NS3/4A, MYC-NS3/4A, FLAG-eYFP, NS3/4A S139A, pIFNB1-LUC and pISG56-LUC were previously described (95, 236, 238-240). All constructs were verified by the genomics platform at the Institute of Research in Immunology and Cancer (Montreal, Qc, Canada) and by subsequent Western Blot analysis when applicable.

## BRET assays

293T cells were transfected in 24-well plates with 5 ng/well of the DNA construct coding for the BRET donor (Rluc-x) and increasing amounts (0 to 200 ng/well) of the DNA construct coding for the BRET acceptor (eYFP-x), and total DNA was completed to 1 µg using salmon sperm DNA (Invitrogen). At 48 hours post-transfection, cells were harvested in Dulbecco's modified Eagle's medium and washed twice with PBS. For each sample, two aliquots of 1 ×

$10^5$ cells were distributed to wells of a 96-well microplate (Costar 3912; Corning). Total eYFP expression was measured using a Flex Station II (Molecular Devices), with an excitation wavelength at 485 nm, an emission filter at 535 nm, and a cutoff of 530 nm. The luciferase substrate coelenterazine H (Lucigen Corporation) was then added to give a final concentration of 5 μM, and emissions of luminescence and fluorescence were measured simultaneously using a Mithras LB940 (Berthold Technologies). Filters were set to 485 ± 10 nm for luciferase emission and 530 ± 12.5 nm for eYFP emission. BRET ratios were calculated as described by Angers et al (241)

## HCV replication

9-13 Huh7 cells were previously described (221) and stably express the genotype 1b Con1 subgenomic replicon. This bicistronic replicon expresses the neomycin phosphotransferase through the HCV IRES, while NS3-NS5B polyprotein production is under the control of the encephalomyocarditis virus (EMCV) IRES. The reporter Huh7 cells stably express the Con1 subgenomic replicon as well as both neomycin resistance and *Firefly* luciferase (221).

## $EC_{50}$ and $CC_{50}$ assays

For $EC_{50}$ and $CC_{50}$ assays, Huh7 stably expressing the Con1 subgenomic replicon (221) and Huh7.5 stably expressing pEF1α-LUC (236) were seeded in 96-well plates at a density of 15,000 cells in phenol-red free DMEM and incubated at 37°C in an atmosphere of 5% $CO_2$. On the second day, gradually increasing concentrations of UM42811 were added to replicate well series; DMSO was used as a negative control. On the third day, 10 µl of alamarBlue reagent (Invitrogen; diluted 1:4 in PBS) was added to each well and following a 4 hour incubation 37°C, supernatant were collected in black 96-well plates. Supernatant fluorescence at 595 nm (excitation wavelength, 531 nm) was measured with an EnVision plate reader (PerkinElmer). A control plate with medium only (no cells) or alamarBlue only was used to determine the background that was subtracted from the fluorescence value. Adherent cells were washed twice with ice-cold phosphate-buffered saline (PBS; Wisent), and subsequent cell lysis and firefly luciferase readings were perfomed in a 100 mM Tris acetate, 20 mM Mg acetate, 2 mM EGTA, 3.6 mM ATP, 1% Brij 58, 0.7% β-mercaptoethanol and 45 μg/ml luciferine pH 7.9 buffer.

## Selection of HCV replicon escape resistant variants

Huh7 stably expressing the Con1 subgenomic replicon (221) were seeded in 6 well plates at a density of 150,000 cells in DMEM containing G418 at a concentration of 1 μg/μL G418 (Multicell). On day 2, compound UM42811 dissolved in DMSO was added at indicated concentrations. On day 4, cells were transferred into 10 cm plates with fresh DMEM, compound UM42811 and G418. Media was renewed weekly, and inhibitors renewed twice a week. On day 21, cell colonies were picked and left to expand in 24 well plates containing fresh media, and both selection reagents. On day 35, RNA was extracted; 5 RT PCR reactions per clone were performed; resulting samples were put into an equimolar mixture, sonicated into 75-200 bp fragments and subsequently sent to the Illumina HiSeq platform at McGill University and Génome Québec Innovation Centre (Montreal, Qc, Canada) for deep sequencing.

## Production of mutated HCV enzymes

NS3/4A mutants K617N, I615M and L14F were individually generated via site-directed mutagenesis within the subgenomic replicon and the respective expression vectors using the Q5® kit (NEB, Ipswich, MA, USA). Substitutions were created by incorporating the desired nucleotide change in the center of the forward primer, including at least 10 complementary nucleotides on the 3´side of the mutation. The reverse primer was designed so that the 5´ ends of the two primers anneal back-to- back. PCRs were performed in a MasterCycler Gradient (Eppendorf, Hamburg, Germany) set at the following: 3min. incubation at 98°C; 25 repeating cycles of 10s at 98°C, 30s at 68°C, and 1min./1kb vector length at 72°C; and 3min. incubation at 72°C. All constructs were verified by the genomics platform at the McGill University and Génome Québec Innovation Centre (Montreal, Qc, Canada) and by subsequent Western Blot analysis when applicable.

## shRNA gene silencing

shRNAs from MISSION TRC shRNA lentiviral library (Sigma-Aldrich) were used as followed: shRNA targeting KPNB1 (TRCN0000123189), HSP60 (TRCN0000029446), or shRNA non-target (NT). shRNA were transfected in combination with a standard packaging mix (1.5 µg pMDLg/pRRE, 1.5 µg pRSV-REV and 3 µg pVSVg) as previously described (242).

## Functional firefly luciferase assays

For assays in 96-well plates, cells were seeded in white 96-well plates at a density of 5,000 HEK 293T in 100 µl of complete phenol-red free DMEM. For conditions with shRNA, polybrene was added to the phenol-red free DMEM at a concentration of 4 µg/ml. Infection with lentivirus encoding shRNA were carried out immediately after cell seeding at a MOI of 10 and incubated at 37°C in an atmosphere of 5% $CO_2$. On the second day, cells in each well were transfected with 50 ng of pIFNB1-LUC or pISG56-LUC and 200 ng of the concerned protein expression vector. BILN 2061 and UM42811 were added at the indicated concentrations immediately following transfection; DMSO was used as control. On the third day, Cells were infected with 100 HAU/ml of SeV (Cantell Strain, Charles River Labs) or stimulated with recombinant IFN-α 2A (Sigma-Aldrich) for 16 hours before cell lysis and firefly luciferase reading which were performed in a 100 mM Tris acetate, 20 mM Mg acetate, 2 mM EGTA, 3.6 mM ATP, 1% Brij 58, 0.7% β-mercaptoethanol and 45 µg/ml luciferine pH 7.9 buffer. All infections were performed in an enclosed class II cabinet.

## Western blot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS; Wisent), harvested and lysed in 10mM Tris-HCl, 100mM NaCl, 0.5% Triton X-100, pH7.6 with EDTA-free Protease Inhibitor Cocktail (Roche). Cell lysates were clarified by centrifugation at 13,000 g for 15 min at 4 °C and subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Western Blot analysis was performed using the following antibodies: ACTIN was purchased from Chemicon International (Billerica, MA MAB1501R); FLAG was purchased from Sigma (St-Louis, MO, USA, F3165); KPNB1, and NS3 were purchased from Abcam (Toronto, Ontario, Canada, ab2811, ab13830); ISG56 was purchased from Novus Biologicals (Oakville, ON, NBP1-32329); HSP60 was purchased from Cell Signaling Technology, Inc, (Danvers, MA, 2859); GAPDH was purchased from RDI (Flanders, NJ, TRK5G4-6C5); and MYC, and IRF3 were purchased from Santa Cruz Biotechnology (Dallas, TX, sc-789, fl-435). The antibody for PARP1 and Sendai Virus was a kind gift from MJ. Hébert and M. Servant, respectively. HRP-conjugated secondary antibodies were from Bio-Rad. The chemiluminescence reaction was performed using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer).

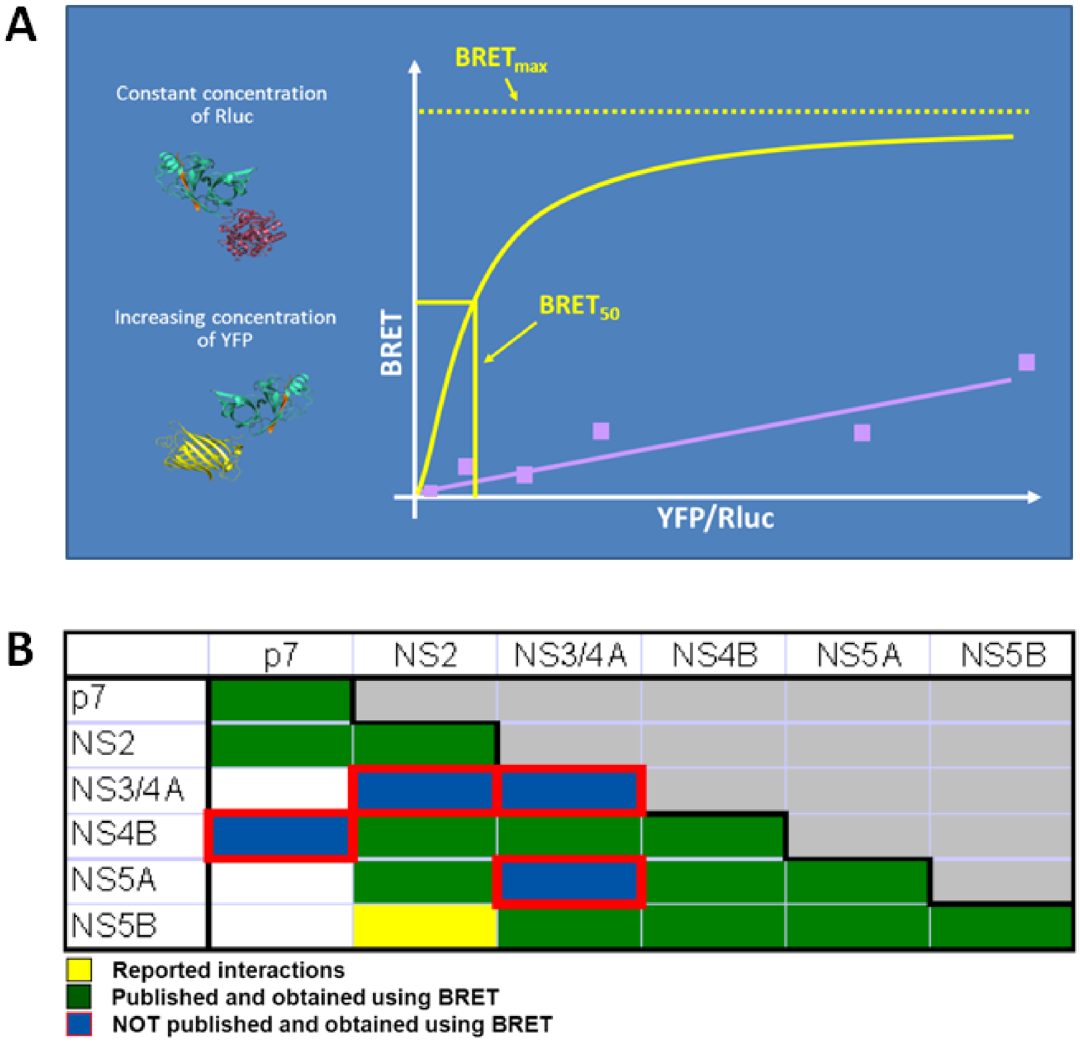
## Co-immunoprecipitation

For co-immunoprecipitation, FLAG-tagged protein expressing cells were harvested and lysed as described above. Resulting cell extracts were adjusted to 1 mg/ml and subjected to IP as follows: pre-clearing of the lysates was done by incubating lysates with 40 µl of 50:50 slurry of immunoglobulin G-Sepharose (GE Healthcare) prepared in the lysis buffer with IgG beads for 1 hour. Pre-cleared lysate were immunoprecipitated by adding 20 µl of M2 anti-FLAG affinity gel (Sigma-Aldrich) prepared in TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) overnight as described by the manufacturer. Immunoprecipitates were washed five times in lysis buffer. Elution was performed using 250 ng/µl purified FLAG peptide for 45 min at 4 °C (Sigma-Aldrich). Eluates were analyzed by Western Blotting.

# Results

## 1. Investigation of pairwise interactions between HCV proteins

In a previous work with an aim of identifying new functional HCV protein interactions (232), all possible interaction combinations between pairs of HCV proteins excluding those which are structural were tested using BRET. This included a total of 21 possible interactions between p7, NS2, NS3/4A, NS4B, NS5A, and NS5B. To identify and characterize new interactions between HCV proteins, BRET saturation curves were performed by expressing fixed level of donor fusion constructs (Rluc) with increasing amounts of acceptor fusion constructs (eYFP). In a BRET saturation curve, the y-axis represents the BRET signal which is the ratio of the light intensity emitted by YFP over that emitted by Rluc and the x-axis represents the quantitative protein ratio of YFP over Rluc (Figure 4A). Because the efficiency of BRET is dependent on the inverse sixth power of intermolecular separation and the relative orientation of the donor and acceptor transition dipole moments (235), BRET can most accurately measure molecular proximity at 10–100 Å in which case interacting dipoles are deemed sufficiently close to infer a definitive interaction. In this way, a non-linear curve is representative of the specific engagement of both YFP and Rluc fusion protein partners and is the means by which specific protein-protein interactions are discriminated from non-specific interactions. The maximum signal obtained for a saturation curve is called $\text{BRET}_{\text{max}}$ and varies according to orientation and distance between the energy donor and the acceptor within the dimer, whereas the relative affinity between the fusions proteins is reflected by the quantitative YFP/Rluc protein ratio required to obtain 50 % of the $\text{BRET}_{\text{max}}$ ($\text{BRET}_{50}$) (243). Results from the BRET pairwise interactions are summarized in a matrix table (Figure 4B). Interactions previously reported by the HCVpro database (244) and reconfirmed through the BRET assay are colored in green, whereas interactions which failed to be reconfirmed are colored in yellow. Uncharacterized interactions which were newly identified by BRET are colored in blue and outlined in red. As NS4A is considerably small relative to the size of an eYFP or a Rluc tag and as it mainly serves as a cofactor to NS3 ensuring its protease function and subcellular localization, BRET experiments were performed using constructs encoding the entire NS3/4A heterodimer rather than constructs encoding each individual protein of the protease complex. BRET analysis reproduced 13 previously reported interactions while failing to reproduce the NS2-NS5B interaction. Though the quality of the specific interactions varies (data not shown), four new pairwise specific interactions between HCV proteins were identified: the interaction between heterodimers of NS3/4A, the latter's interaction with NS2 and NS5A, along with the interaction between NS4B and p7.


A
Constant concentration of Rluc
Increasing concentration of YFP
BRET$_{max}$
BRET$_{50}$
BRET
YFP/Rluc
B

| | p7 | NS2 | NS3/4A | NS4B | NS5A | NS5B |
|---|---|---|---|---|---|---|
| p7 | 🟩 | | | | | |
| NS2 | 🟩 | 🟩 | | | | |
| NS3/4A | | 🟦 | 🟦 | | | |
| NS4B | 🟦 | 🟩 | 🟩 | 🟩 | | |
| NS5A | | 🟩 | 🟦 | 🟩 | 🟩 | |
| NS5B | | 🟨 | 🟩 | 🟩 | 🟩 | 🟩 |

🟨 Reported interactions
🟩 Published and obtained using BRET
🟦 NOT published and obtained using BRET

**Figure 4.** Bioluminescence resonance energy transfer identifies novel HCV protein-protein interactions

(A) Working principle of a BRET saturation graph. Example 3D structures of YFP and Rluc fusion proteins (left). BRET saturation graph (right): X-axis represents quantitative ratio between YFP and Rluc fusion proteins; Y-axis represents BRET signal or ratio between fluorescence and bioluminescence. (B) Reported and/or BRET identified interaction matrix between HCV proteins p7, NS2, NS3/4A, NS4B, NS5A, and NS5B.

## 2. Homodimerization of the NS3/4A heterodimer

The BRET saturation curve obtained for the pairing of the NS3/4A protease with itself increases non-linearly according to increasing protein ratios, representing the specific engagement of the Rluc-fused protein with its eYFP-fused counterpart (Figure 5A). As negative controls, TAP2, an ER-localized protein not part of the HCV replication complex which in conjunction with TAP1 forms a heterodimer implicated in the delivery of cytosolic peptides to the ER lumen, and eYFP were used. The saturation curve obtained with TAP2-Rluc and eYFP-NS3/4A leads to a linearly increasing and weak BRET signal resulting from random collisions between the two fusion proteins (orange curve in Figure 5A). This control demonstrates that the BRET technique is able to distinguish direct protein-protein interactions from interactions due to the similar subcellular localization. The NS3/4A serine protease being one of the better and earlier characterized proteins of HCV, it was interesting that the BRET system was able to identify an uncharacterized interaction of the protein with itself. Therefore to confirm this interaction, a co-IP experiment was performed (Figure 5B). 293T cells were co-transfected with Myc-NS3/4A and either FLAG-NS3/4A or TAP1-FLAG and lysates were immunoprecipitated with anti-FLAG coated beads 48 hours post-transfection. FLAG immunoprecipitates were immunobloted with an anti-Myc antibody (upper panel) and an anti-FLAG antibody (lower panel). It was observed that Myc-NS3/4A co-immunoprecipitated with FLAG-NS3/4A (lanes 3-4) but not TAP1-FLAG (lanes 1-2), therefore confirming BRET results. Together, BRET and co-IP experiments demonstrate a specific interaction between NS3/4A heterodimers.

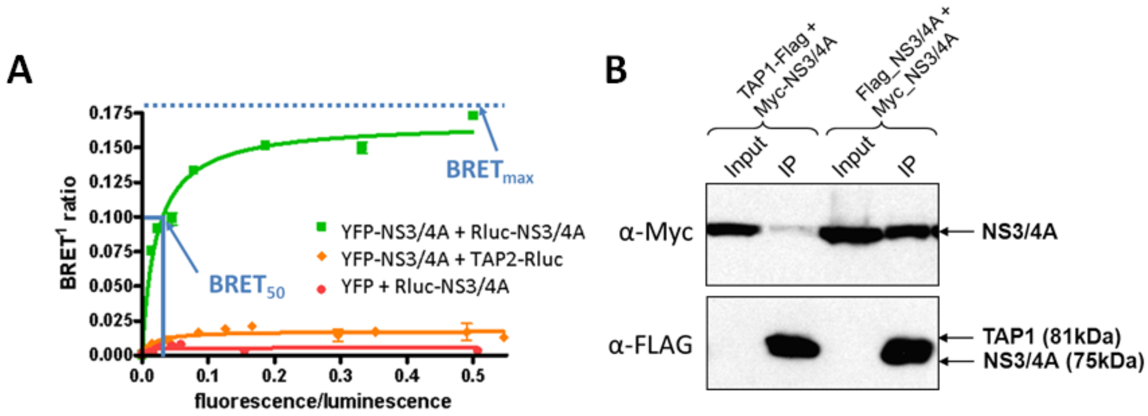
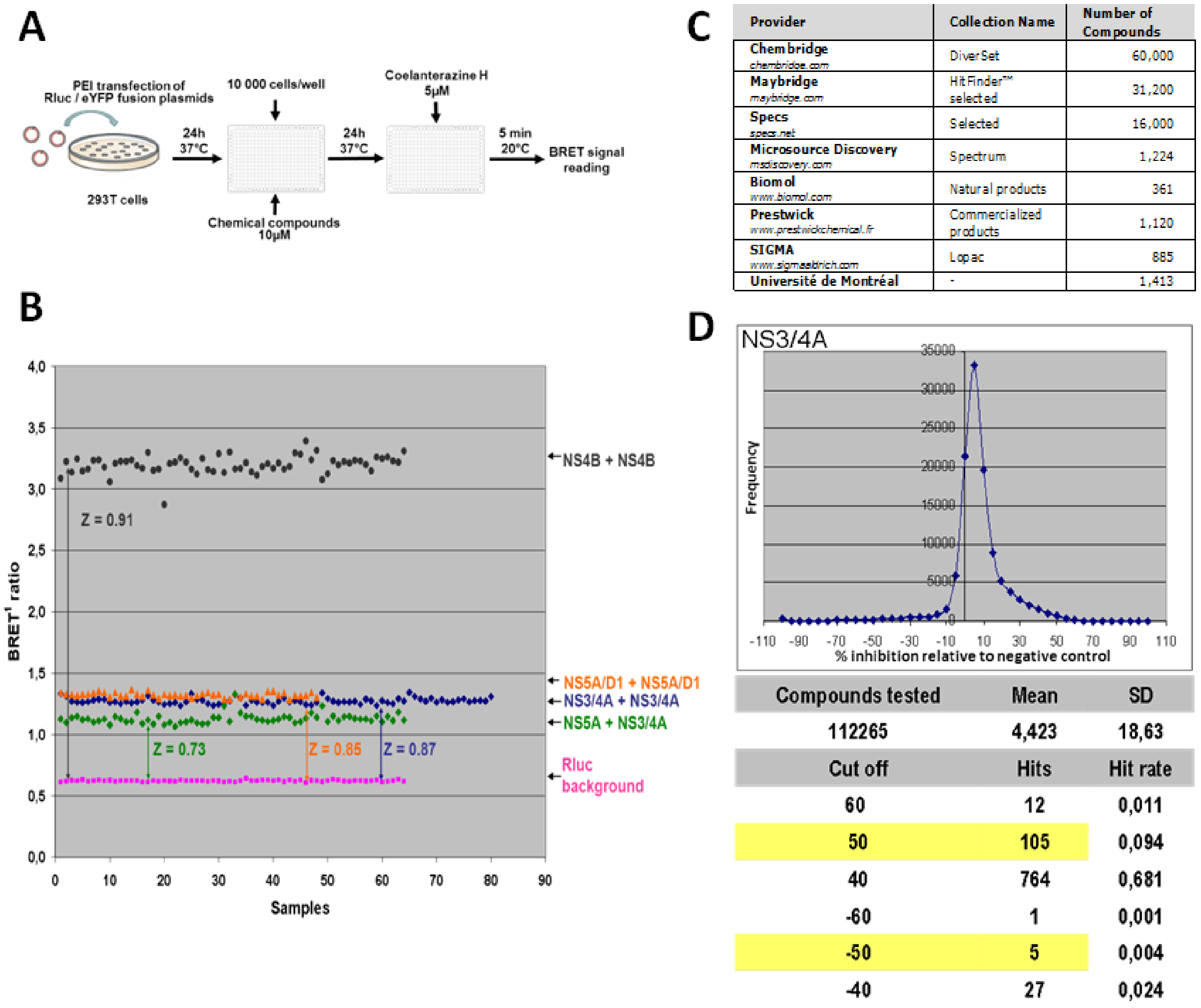


**Figure 5.** Specific interaction between NS3/4A heterodimers

(A) BRET saturation curves. In green, curve for N-terminally tagged NS3/4A fusion proteins. In red, curve with an eYFP negative control. In orange, curve with C-terminally tagged TAP2 colocalization negative control. X-axis represents quantitative ratios between YFP and Rluc fusion proteins; Y-axis represents BRET signal or ratio between fluorescence and bioluminescence. (B) HEK 293T cells are co-transfected with either TAP1-FLAG and Myc-NS3/4A or FLAG-NS3/4A and Myc-NS3/4A. 48h post-transfection, co-immunoprecipitation experiment using anti-FLAG coated beads is carried out. Both total lysate (input) and immunoprecipitated lysate (IP) are immunoblotted with anti-Myc and anti-FLAG antibodies.

# 3. Implementation of a BRET HTS assay

With the protease activity of NS3/4A being largely characterized and with potent inhibitory compound BILN2061 targeting this mechanism readily available, a BRET HTS assay using a library of compounds with potential protein-protein interaction (PPI) modulators was developed to investigate the newly identified NS3/4A homodimeric interaction. A HTS adapted version of the BRET assay mentioned in the methods was performed in 384-well plates at a cell density of 10,000 cells per well using transient transfection of both eYFP and Rluc fusion plasmids (Figure 6A). Prior to initiating HTS against the compound collection, a smaller scale BRET assay was performed on reported/identified NS3/4A-NS3/4A, NS5A/D1-NS5A/D1, NS4B-NS4B and NS3/4A-NS5A interactions to evaluate the reproducibility of BRET$^1$ ratios measured in 384 well plates (Figure 6B). Z-factors (245), denoted by a value <1 to indicate the ease with which sample values can be distinguished from control values within HTS based on standard deviations and mean value separation, were calculated for each of the interactions. Z-factors for each of the interactions were found to be well above 0.5 (Figure 6B) which is indicative of optimal suitability of a configured assay for HTS (245). Similarly, validation of a HTS assay is commonly determined using Z' factor calculations (245). Unlike the Z factor, Z' factor is a statistical parameter which accounts for the dynamic range and variation between positive and negative controls rather than those between a control and a sample condition. In this respect, Z' factor is more a reflection of the overall assay quality. Knowing that protease function is the only properly characterized function of NS3/4A, and inferring that the observed NS3/4A dimer interaction plays a role towards this end, potent protease inhibitor BILN2061 at 2 μM was taken as the positive control, while 0.5% DMSO was taken as the negative control. Z' factor for this BRET HTS assay was found to be above 0.5 (data not shown) and thus the full scale HTS was initiated. With the collaboration of the Institute of Research in Immunology and Cancer (IRIC)'s screening platform, a library of compounds comprised of approximately 110,000 compounds (details listed in Figure 6C) was tested against the NS3/4A dimer interaction and the NS5A/D1 dimer interaction as an additional screen. Because HCV antiviral drugs effective within the nanomolar range are acceptable (246), HTS using compounds at 10 μM was deemed suitable for the identification of compounds eligible for lead optimization. From the screening data, the average of all compounds tested for NS3/4A dimer in BRET signal percentage of inhibition was 4% ± 18.6% (Figure 6D). Based on these average activity values, the cut-off for the identification of hit compounds was established at ± 50% of the control (~2.5 times the standard deviation). By simultaneously screening both NS3/4A and NS5A/D1 dimer targets, hit compounds identified by their modulation of the BRET signal in primary assay were prioritized based on selectivity as determined by the lack of activity within the NS5A/D1 dimer targeted BRET HTS assay (<30% and >-30% BRET signal inhibition). Compounds were also excluded based on inherent bioluminescence quenching (>95% Rluc quenching) (Figure 6D). A total of 105 potential NS3/4A PPI inhibitors, and 5 potential NS3/4A PPI activators were identified.



| Provider | Collection Name | Number of Compounds |
|---|---|---|
| **Chembridge**<br>*chembridge.com* | DiverSet | 60,000 |
| **Maybridge**<br>*maybridge.com* | HitFinder™ selected | 31,200 |
| **Specs**<br>*specs.net* | Selected | 16,000 |
| **Microsource Discovery**<br>*msdiscovery.com* | Spectrum | 1,224 |
| **Biomol**<br>*www.biomol.com* | Natural products | 361 |
| **Prestwick**<br>*www.prestwickchemical.fr* | Commercialized products | 1,120 |
| **SIGMA**<br>*www.sigmaaldrich.com* | Lopac | 885 |
| **Université de Montréal** | - | 1,413 |

| Compounds tested | Mean | SD |
|---|---|---|
| 112265 | 4,423 | 18,63 |
| **Cut off** | **Hits** | **Hit rate** |
| 60 | 12 | 0,011 |
| 50 | 105 | 0,094 |
| 40 | 764 | 0,681 |
| -60 | 1 | 0,001 |
| -50 | 5 | 0,004 |
| -40 | 27 | 0,024 |

**Figure 6.** Identification of potential NS3/4A PPI modulators through a BRET HTS assay

(A) Schematic representation of the BRET HTS screen. HEK 293T cells were transfected with Rluc- and eYFP-fusion encoding plasmids. After 24 hours, cells were harvested and distributed in 384-well plates before the addition of chemical compounds to a final concentration of 10 µM. Twenty-four hours later, coelanterazine H was added at a final concentration of 5 µM, and emission of luminescence and fluorescence were measured simultaneously to calculate the BRET signal. (B) BRET$^1$ ratios obtained for reported/identified NS3/4A-NS3/4A, NS5A/D1-NS5A/D1, NS4B-NS4B, and NS3/4A-NS5A interactions in a preliminary 384-well plate test. Z-factors for each interaction screen are calculated. (C) Detailed breakdown of the library of compounds tested within the full-scale BRET HTS assay targeting the NS3/4A-NS3/4A interaction. (D) Distribution of the NS3/4A-NS3/4A $BRET^1$ ratio inhibition obtained in the primary screen: X-axis represents the % inhibition relative to the negative control; Y-axis represents the number of compounds found to provide a given % inhibition. Cutoffs of 50% $BRET^1$ modulation for the NS3/4A-NS3/4A interaction (with additional restrictions of bioluminescence quench < 95% and $BRET^1$ < 30% modulation for the NS5A/D1-NS5A/D1 interaction) allowed the selection of 105 specific inhibitors and 5 specific activators.

# 4. Validation and antiviral characterization of lead compound UM42811

To validate the hits from the primary screen, a replicate screen using the 110 hits was performed (data not shown). Primary screen hits that failed to meet selection criteria during the replicate screen were rejected, and the compound with the highest absolute NS3/4A PPI modulation was selected for further validation and antiviral characterization. O5-{[3-(2-chlorophenyl)-5-methylisoxazol-4-yl]carbonyl}-1,3-benzodioxole-5 carbohydroximamide which will henceforth be referred to as compound UM42811 for the sake of simplicity and readability (chemical structure shown in Figure 7A) was confirmed as a NS3/4A PPI activator in the replicate screen by demonstrating anew an inhibition less than -60%, an inhibition greater than -30% on NS5A/D1 and a quench less than 95%. A 10-point titration BRET assay (duplicate: 0.02 to 10 μM) was developed for further validation and dose-response evaluation of the lead compound. HEK 293T cells were transfected with eYFP and Rluc NS3/4A fusion expression vectors for 48 hours and incubated in the presence of BILN2061 or UM42811 for 24 hours to evaluate their effect on the BRET signal. Potent protease inhibitor BILN2061 was used as a PPI modulator control given its ability to decrease NS3/4A dimer-mediated BRET signal (left panel Figure 7B). The decrease of the BRET signal reflects a change in dimer orientation between the mature autocleaved form of NS3/4A (without bound BILN2061) and the immature uncleaved form of NS3/4A (with bound BILN2061), and demonstrates the sensitivity of the BRET readout to measure conformational changes within dimers. NS5A/D1 dimer-mediated BRET signal (orange curve) was used as a compound specificity control. From these titration curves, BRET inflection points ($BRET_{IP}$) can be determined. Significant changes were observed in the BRET signals with $BRET_{IP}$ values near 0.2 μM for BILN2061 and values within the lower micromolar range for compound UM42811. Because the autocleavage activity of NS3/4A can induce conformational changes within the homodimer and create unwanted BRET signal noise, a NS3/4A protease defective mutant constructs (NS3/4A S139A – green curve) were used alongside wild type NS3/4A constructs. UM42811 predominantly increased the BRET signal mediated by protease defective NS3/4A dimers than wild type NS3/4A dimers. To evaluate the pharmacological and antiviral properties of lead compound UM42811, biologically relevant models of HCV replication and cellular fitness were used. Huh7 cells bearing HCV subgenomic reporter Con1 replicon were used to assess antiviral activity and half-maximal response ($EC_{50}$) of UM42811 at inhibiting HCV replication using serial diluted concentration spanning 0.01 to 30 μM (left panel Figure 7C). Similarly, Huh7 stably expressing an EF-1 alpha promoter driven firefly luciferase and the respective mitochondrial reductive capacities measured by alamar blue in both cells were used to determine the cytotoxicity (alamar blue of Con1 bearing cells shown in right panel Figure 7C). Computer generated analysis of the HCV replication inhibition and cytotoxicity determined $EC_{50}$ to be 0.59μM and $CC_{50}$ to be 28.35μM. The therapeutic index, given as the $CC_{50}/EC_{50}$ ratio, was determined to be 48.05 demonstrating the pharmacological potential of compound UM42811 as a novel anti-HCV agent. The inhibition of an EF-1 alpha promoter driven luciferase in Huh7 cells was also determined to be around 28μM (data not shown). This demonstrated that the submicromolar antiviral activity of dioxole-containing compound UM42811 on HCV replicon was not due to the inhibition of firefly luciferase activity (247).

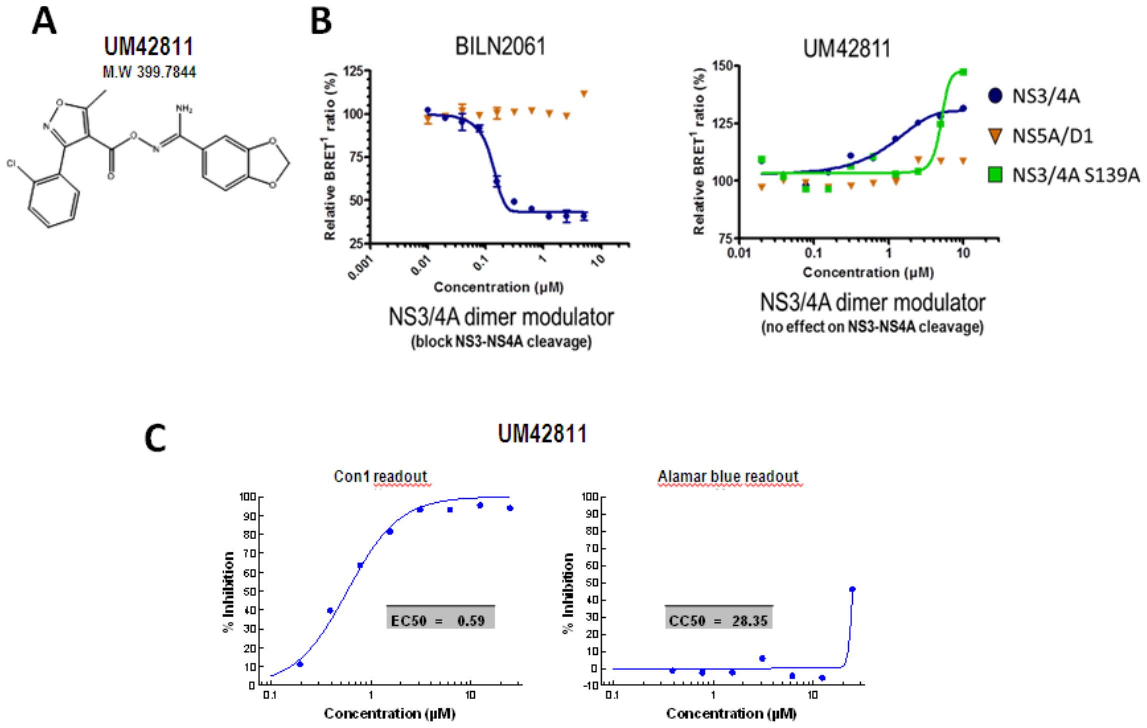


**Figure 7.** Validation and antiviral characterization of lead compound UM42811

(A) Chemical structure of lead compound UM42811 identified as a modulator of BRET NS3/4A homodimerization signal. (B) Dose-response evaluation of BILN2061 and UM42811 in cell-based BRET dimerization assays. NS3/4A S139A is a protease inactive mutant. (C) Dose-response evaluation of UM42811 in reporter Con1 subgenomic replicon denoting $EC_{50}$ (left) and in alamar blue assay denoting cellular fitness $CC_{50}$ (right).

# 5. Characterization of UM42811 resistance profile reveals mutations located at the surface of the C-terminal NS3 helicase subdomain

To ascertain UM42811 as a NS3/4A-targeted inhibitor that is mechanistically different from current protease inhibitors, selection of cells containing UM42811-resistant HCV replicons was performed. Huh7 cells bearing HCV subgenomic Con1 replicon were maintained in media containing G418 and UM42811 at either 10 μM or 20 μM for 3 to 5 weeks. Following prolonged exposure to UM42811, replicon bearing cells cured by the UM42811 were cleared in presence of 1 mg/ml G418 due to the loss of the $neo^R$ (G418 resistance) gene encoded by the HCV replicon. Cells refractory to viral inhibition, due to the selection of resistant replicon variants, were either stained for proper colony visualization or lysed. RNA was extracted from cell lysates and RT-PCR was performed using HCV specific primers. Genetic samples were then sonicated into 75 to 200 base pair fragments and subsequently sent to the Illumina HiSeq platform at McGill University and Génome Québec Innovation Centre (Montreal, Qc, Canada) for deep sequencing analysis. Crystal violet staining of emerging treatment-resistant colonies (Figure 8A) provided a preliminary quantitative measure of UM42811's ability to inhibit viral replication and to suppress emergence of resistance. 10 μM concentration of UM42811, which is much greater than the previously determined $EC_{50}$ and within range of NS3/4A homodimer BRET signal activation, was discernably able to inhibit HCV replication when compared to the DMSO control. At a 20 μM concentration, which is lower than previously determined $CC_{50}$, emergence of resistant colonies was completely suppressed and results were visually comparable to a Huh7 killing control. Deep sequencing analysis revealed a number of mutations throughout the HCV polyprotein. The 9 most common mutations and their associated frequencies are shown in Figure 8B. Though mutations found in non-structural proteins other than NS3 were relatively frequent (<1% to 6%), they were less significant than mutations occurring in NS3 with I615M and K617N alone accounting for 19% and 23% of samples respectively. Nonetheless, amino acid sequence alignment using a compiled database of full-length HCV genomes was performed to analyse variation frequency at each given position and to provide greater stringency for the identification of potential resistance mutations. Though NS3/4A I615M and K617N, NS4B P197S, NS5A E420K and E422G, and NS5B S196A were naturally uncommon and as such weighted more heavily, given the nature by which UM42811 was selected (through an NS3/4A dimerization BRET screen) and the greater frequency of associated mutations on NS3/4A, resistance studies were focused on NS3/4A associated mutations. As displayed on the NS3/4A 3D structure (Figure 8C), I615M and K617N were mapped at the surface of the C-terminal helicase domain while L14F was mapped at a region involved in membrane anchoring. Because these mutations are not associated with protease inhibitors resistance (248, 249), and given the feasibility of resistance mutations at accessible surface residues and at residues associated with potential functional alterations, these studies suggest that UM42811 specifically targets NS3/4A and differs mechanistically from protease inhibitors.

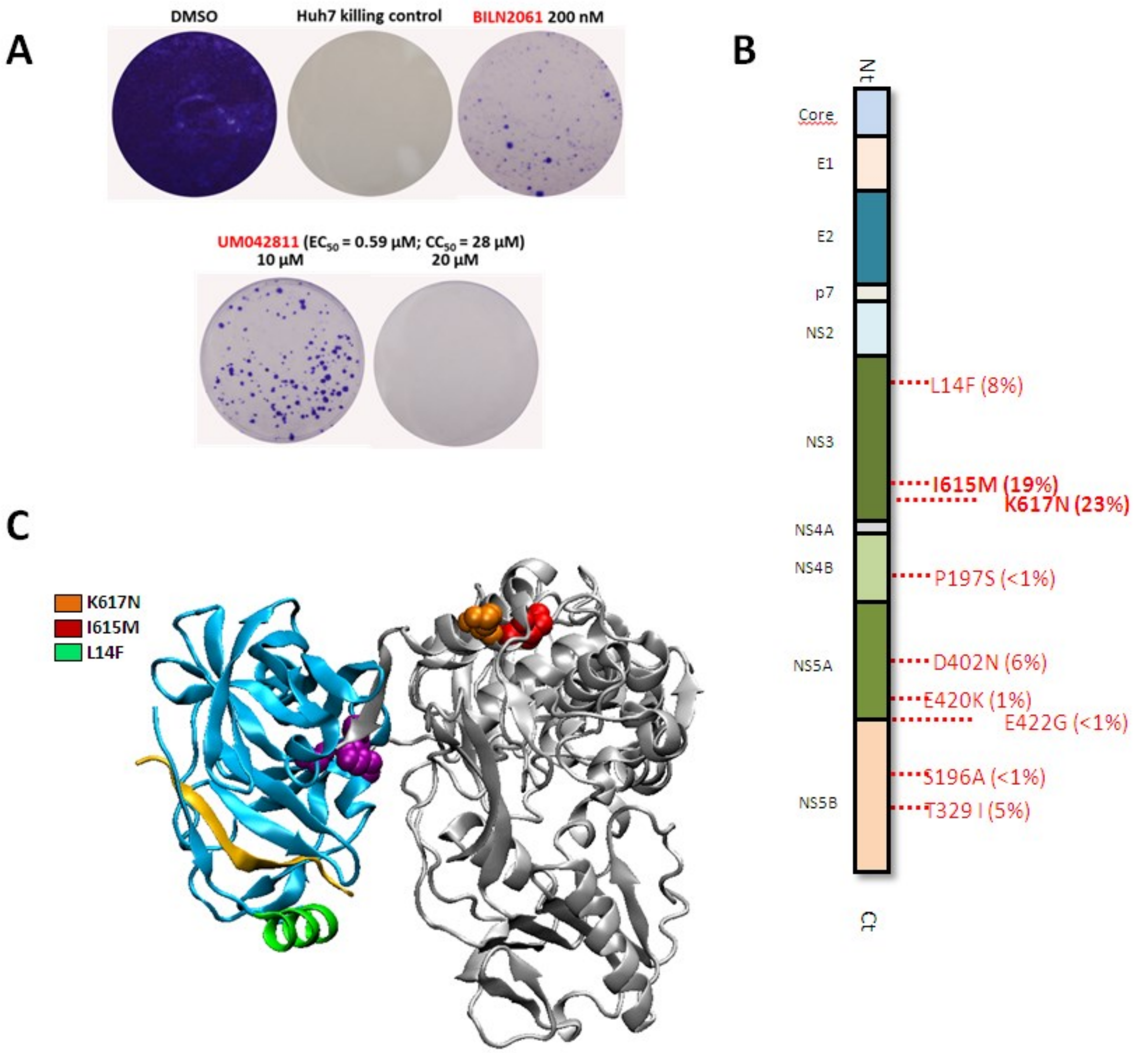

A
DMSO
Huh7 killing control
BILN2061 200 nM
UM042811 (EC$_{50}$ = 0.59 µM; CC$_{50}$ = 28 µM)
10 µM
20 µM
B
Nt
Core
E1
E2
p7
NS2
NS3
NS4A
NS4B
NS5A
NS5B
Ct
L14F (8%)
I615M (19%)
K617N (23%)
P197S (<1%)
D402N (6%)
E420K (1%)
E422G (<1%)
S196A (<1%)
T329 I (5%)
C
K617N
I615M
L14F

**Figure 8.** Characterization of UM42811 resistance profile reveals mutations located at the surface of the C-terminal NS3 helicase subdomain

(A) Crystal violet staining of BILN2061 and UM42811 emerging escape variant colonies following 3 week inhibitor exposure. (B) Schematic representation of the HCV polyprotein (top-down) punctuated by the localization of the most common mutations identified following deep-sequencing and occurring after 35 day exposure to UM42811. (C) 3D schematic of the NS3/4A heterodimer. The C-terminal helicase domain (grey), the N-terminal protease domain (blue), the NS4A cofactor (yellow), the catalytic triad (purple) and emerging resistance mutations K617N (orange), I615M (red), and L14F (green) are presented.

## 6. Generation of NS3/4A mutant fusion proteins and of mutant HCV replicon DNA precursors

For further characterization of the UM42811, emerging resistance mutations K617N, I615M and L14F were generated as fusion proteins within eYFP and FLAG expression vectors (Figure 9A) as well as within HCV replicon. To this end, different strategies were contemplated for the substitution of single nucleotides within the NS3/4A coding sequence.

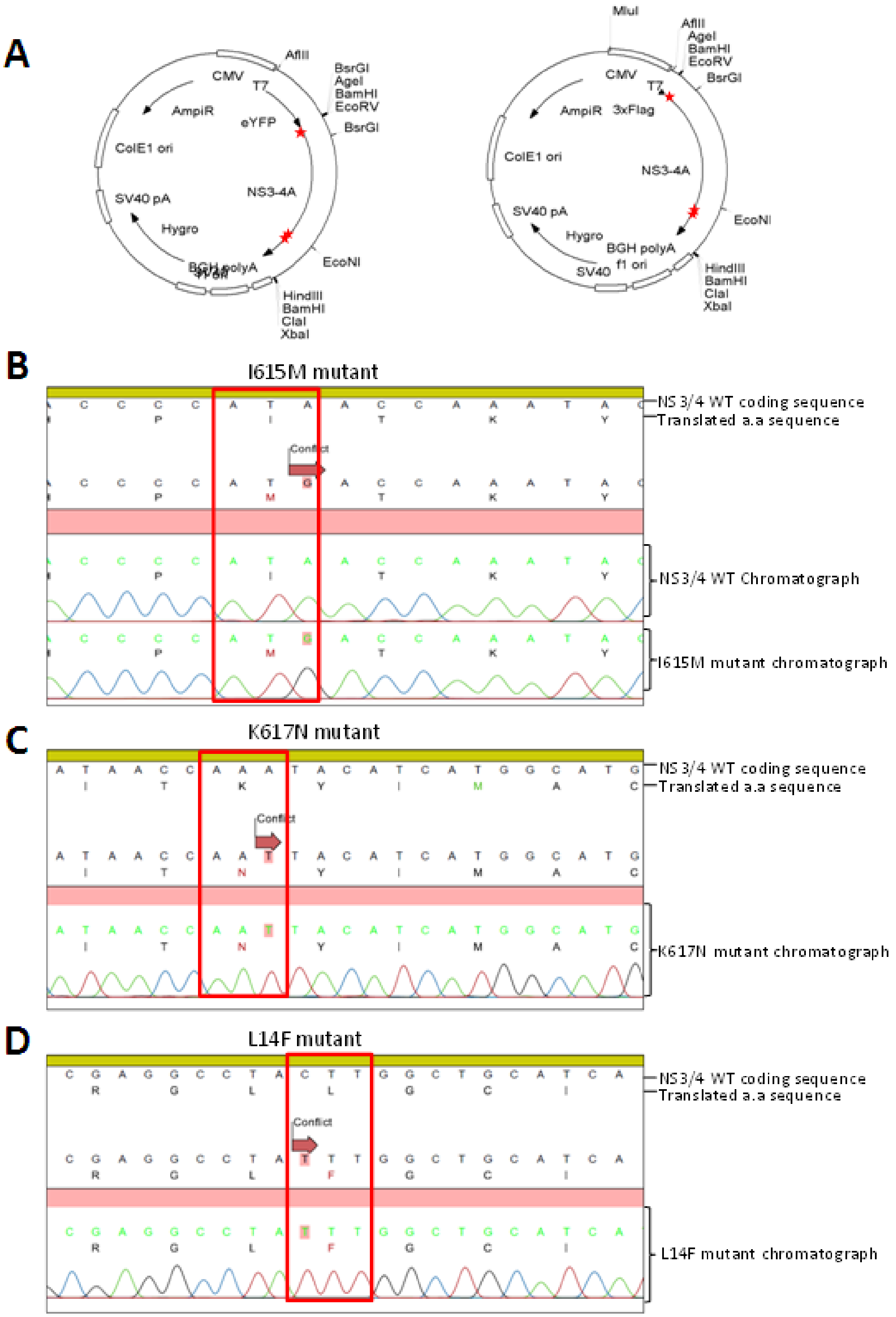
Traditional PCR site-directed mutagenesis in which sequential exponential amplification by PCR produces a fragment containing the desired mutated sequence flanked by conveniently placed restriction sites was found to be largely inefficient at introducing a point mutation within particularly large coding sequences such as that of HCV subgenomic replicon expressing DNA vectors. Whole plasmid mutagenesis which is a relatively simpler process was more successful to this end. Rather than generating fragments which needed to be inserted using recombinant molecular biology techniques, this approach to site-directed mutagenesis instead generated a nicked, circular DNA. Template DNA and mutated plasmids generated *in vitro* are discriminated based on methylation. Because only biosynthesized DNA is methylated, restriction enzymes such as *Dpn*I were used to eliminate unmutated template DNA.

After a series of optimizations of primer design, substitutions were achieved by incorporating the desired nucleotide change in the center of the forward primer, including at least 10 complementary nucleotides on the 3′side of the mutation as displayed in Table I. The reverse primer was designed so that the 5′ ends of the two primers annealed back-to-back with no overlap. PCRs were performed in a MasterCycler Gradient (Eppendorf, Hamburg, Germany) set at the following: 3min. incubation at 98°C; 25 repeating cycles of 10s at 98°C, 30s at 68°C, and 1min./1kb vector length at 72°C; and 3min. incubation at 72°C. Mutated expression vectors and replicon expressing DNA vectors were assessed for their purity and their sequences were verified at the genomics platform at McGill University and Génome Québec Innovation Centre (Montreal, Qc, Canada) (Figure 9B-C-D). While generating replicon expressing DNA vectors containing mutated enzymes was successful, introduction of *in vitro* transcribed subgenomic RNA into Huh7/7.5 cells was largely unsuccessful based on lack of luciferase activity and viral protein expression (data not shown) despite optimization attempts of classical and adapted protocols (250-252). Of the three subgenomic replicons bearing a potential punctual resistance mutation, only the one bearing an I615M was successfully reintroduced into Huh7.5 cells. Though these cells still lacked luciferase activity, because the replicon system is bicistronic and because the luciferase gene is often inexplicably lost over time, real-time PCR (qPCR) was performed directly on the HCV IRES to confirm its presence and on the luciferase gene to confirm its loss (data not shown). Though these results are fragmentary, it demonstrated that the lack of luciferase activity was not due to an incompatibility of this mutation with the replication of the subgenomic replicon.

| **Mutation** | **Primer Orientation** | **Sequence** |
|---|---|---|
| **L14F** | Forward | CCAACAGACGCGAGGCCTA*T*TTGGCTGCATCATCACTAGC |
| | Reverse | GAGTAGGCCGTAATAGGCGCCATGGTATTATCG |
| **I615M** | Forward | GGTTACTACCACACACCCCAT*G*ACCAAATACATCATGGC |
| | Reverse | TCGTTTTGAACGGCTCCCAGCCTATACAGC |
| **K617N** | Forward | CCATAACCAA*T*TACATCATGGCATGCATGTC |
| | Reverse | GGTGTGTGGTAGTAACCTCGTTTTGAACGGC |

**Table I.** Generation of single point mutations within HCV NS3

Primers used to generate potential resistance mutations to UM42811 within NS3/4A. Site directed mutagenesis was performed with Q5® kit (NEB, Ipswich, MA, USA). In red are the nucleotide substitutions which generate the desired amino acid changes.


A
MluI
AflII
AgeI
BamHI
EcoRV
BsrGI
CMV
T7
AmpiR
eYFP
3xFlag
ColE1 ori
NS3-4A
SV40 pA
Hygro
BGH polyA
f1 ori
SV40
EcoNI
HindIII
BamHI
ClaI
XbaI
B
NS 3/4 WT coding sequence
Translated a.a sequence
Conflict
A C C C C A T A A C C A A A T A C
P I T K Y
A C C C C A T G A C C A A A T A C
P M T K Y
NS3/4 WT Chromatograph
I615M mutant chromatograph
C
A T A A C C A A A T A C A T C A T G G C A T G
I T K Y I M A C
A T A A C C A A T T A C A T C A T G G C A T G
I T N Y I M A C
K617N mutant chromatograph
D
C G A G G C C T A C T T G G C T G C A T C A
R G L L G C I
C G A G G C C T A T T T G G C T G C A T C A
R G L F G C I
L14F mutant chromatograph

I615M mutant

K617N mutant

L14F mutant

**Figure 9.** Mutated NS3/4A expression vectors and mutated replicon sequences

(A) Expression vector diagrams of eYFP-NS3/4A and 3xFLAG-NS3/4A fusion proteins. Red stars indicate the approximate location of I615M, K617N, and L14F mutations. A dual Cytomegalovirus and T7 promoter drives the expression of the fusion protein. An Ampicillin resistance gene is included to allow selection upon bacterial amplification. Nucleotide sequence of I615M (B), K617N (C), and L14F (D) NS3/4A mutants as analyzed by the Sequencing Services at McGill University and Génome Québec Innovation Centre (Montreal, Qc, Canada). Viewed with the CLC Sequence Viewer (CLC bio, Aarhus, Denmark) software. Coding sequences for wild type NS3/4A are shown at the very top with the corresponding amino acid sequence displayed underneath. Chromatographs are also shown for mutant sequences (and wild type in the panel A) as indicators of sample purity. Color of chromatograph peaks correspond to specific nucleotides: cytosine (blue), guanosine (black), adenosine (green), and thymine (red). Red boxes highlight the amino acid substitution caused by the specific nucleotide substitution.

## 7. UM42811 affects the dimer/oligomer conformation of NS3/4A heterodimers

Because BRET infers PPIs based on eYFP and Rluc dipole proximity with an optimal resolution at 10–100 Å range, distances created by conformational changes and those created from PPI abolishment are somewhat ambiguous. To see whether UM42811 induced any quantitative changes in the NS3/4A heterodimeric homodimer, co-immunoprecipitation experiments were carried out using NS3/4A constructs fused with different tags. HEK 293T cells were co-transfected with NS3/4A constructs N-terminally tagged with either eYFP or FLAG and treated with increasing concentrations of UM42811 or in combination with protease inhibitor BILN2061. 48 hours post-transfection, cells were lysed and co-immunoprecipitation using anti-FLAG coated beads was performed. Western blot analysis (Figure 10) demonstrated no significant quantitative change in the interaction between FLAG-tagged NS3 (lower NS3 band) and eYFP-tagged NS3 (upper NS3 band) at any of the UM42811 concentrations when compared to a DMSO treated control. Because uninhibited NS3/4A is capable of cleaving itself which explains the presence of NS3 bands at lower molecular weight when not treated with BILN2061, NS3 immunoblot also demonstrated that UM42811 is unable to inhibit NS3/4A protease activity neither in wild type or mutated enzymes. Moreover, increasing UM42811 treatment concentrations did not result in any change in dimerization/oligomerization in wild type enzyme. Interestingly, putative resistance point mutations L14F, I615M, and K617N do not show any effect on the enzyme's autoprotease activity, but appear to result in hindered dimerization/oligomerization. This is demonstrated by the fainter eYFP-tagged NS3 immunoprecipitate bands presented in the lanes with the mutated enzyme. Though the upper NS3 bands in the mutated enzyme lanes of the lysate panel indicate slightly lower expression of eYFP-tagged NS3, it is not sufficient to explain the stark quantitative difference between eYFP-tagged NS3 immunoprecipitate bands in wild type vs mutated enzymes. Supplemental data using untreated controls would be required to ascertain and better describe the latter observation. Furthermore, the more pronounced eYFP-tagged NS3 band found in samples treated with BILN2061 when compared to a DMSO treated control showed that NS3-NS3 interactions were more significant in its heterodimeric precursor form (NS3/4A) than its mature cleaved form (NS3). A reduction in heterodimeric precursor form interaction was observed when samples were treated with UM42811. Though this observation requires a replicate experiment to confirm its robustness it may also be a reflection of the effect of UM42811 on the precursor's putative host interactions later discussed in results section 9-10.

The results from UM42811 treatment on wild type NS3/4A combined with BRET titration data suggest that the inhibitory mechanism of UM42811 involves a conformational change in NS3/4A-NS3/4A PPI. Moreover, resistance mutations conferring a handicapped dimerization/oligomerization agrees with an inhibitory mechanism which involves PPI tightening. Finally, the potency of UM42811 in inhibiting HCV replication despite its null effect on enzymatic activity suggests that the tightening conformation of these NS3/4A dimers/oligomers may hide sites for important downstream interactions.

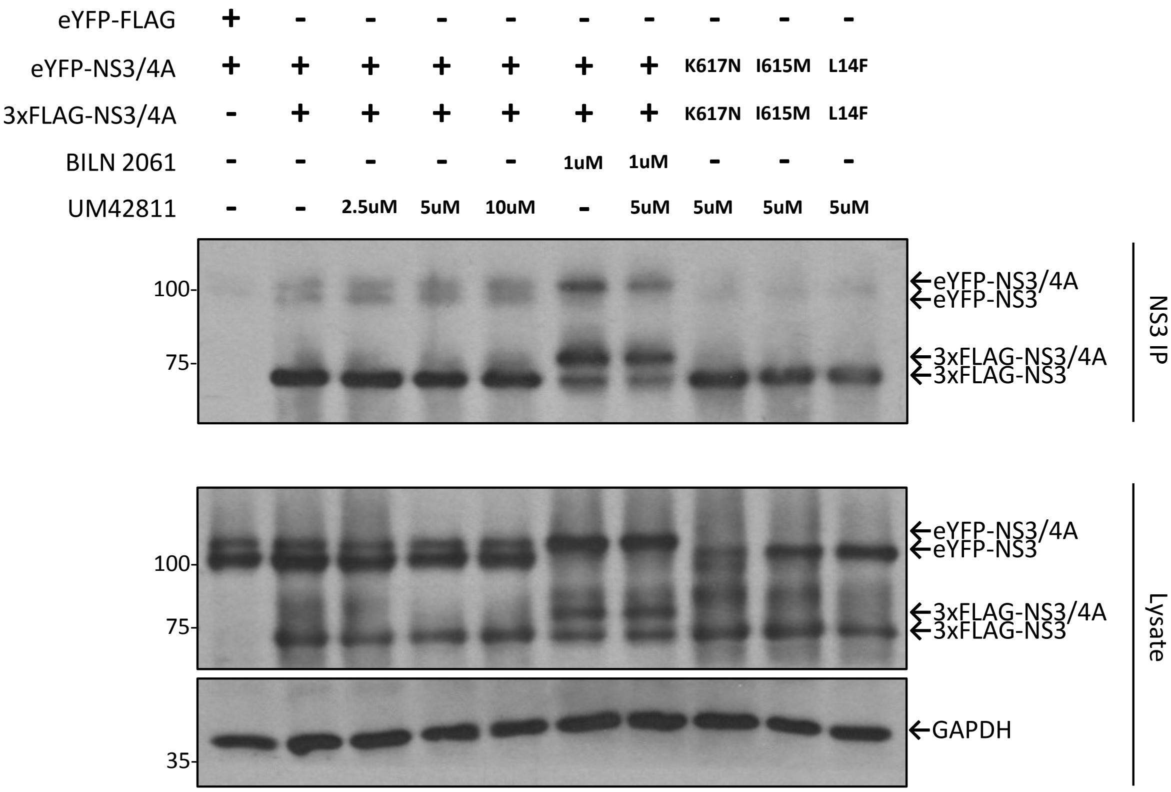

eYFP-FLAG + - - - - - - - - -
eYFP-NS3/4A + + + + + + + K617N I615M L14F
3xFLAG-NS3/4A - + + + + + + K617N I615M L14F
BILN 2061 - - - - - 1uM 1uM - - -
UM42811 - - 2.5uM 5uM 10uM - 5uM 5uM 5uM 5uM
100-
75-
eYFP-NS3/4A
eYFP-NS3
3xFLAG-NS3/4A
3xFLAG-NS3
NS3 IP
100-
75-
eYFP-NS3/4A
eYFP-NS3
3xFLAG-NS3/4A
3xFLAG-NS3
GAPDH
35-
Lysate

**Figure 10.** Point mutations at putative resistant sites of UM42811 may affect the NS3/4A heterodimeric homodimers - UM42811binding reduces precursor interaction induced by BILN2061

HEK 293T cells were transfected with the indicated expression vectors and treated with the indicated concentration of BILN2061 and/or UM42811. 48 post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between molecular weight distinguishable NS3/4A fusion constructs was resolved by immunoblot.

# 8. Functional consequences of a UM42811/BILN2061-sensitive NS3/4A-KPNB1 interaction

The contribution of NS3/4A to the viral lifecycle is not restricted to polyprotein processing alone. As demonstrated by previous results, UM42811 inhibits viral replication without affecting protease activity though resistance profiling and initial screening methods suggest NS3/4A to be the most probable target. Much like how the restoration of innate immunity through preventing the cleavage of type I IFN potentiators MAVS (91) and TRIF (92) is a contributor to the antiviral effect of protease inhibitors, contributors to the alternative inhibitory mechanism of UM42811 may be found in other previously identified unique NS3/4A host interactors (95). Among novel interactors, nucleocytoplasmic transporter karyopherin/importin subunit beta 1 (KPNB1/IMPβ1) was found to strongly associate with NS3/4A and shown, in a follow-up study on viral subversion mechanisms (253), to be implicated in antiviral signaling. Part of the data demonstrated that silencing KPNB1 in either HEK 293T or A549 cells negatively impacted ISG56 expression in favour of viral protein production following Sendai virus (SeV) infection (Figure 11A - highlighted version of original data (253)). At 8 hours post-infection, both HEK 293T and A549 cells knocked down for KPNB1 showed no expression of ISG56 while SeV proteins were expressed when compared to a non-target transduced control. At 24 hours post-infection, only A549 cells which are known to respond more rapidly than HEK 293T cells, showed restored ISG56 expression. SeV protein expression was also increased in both cell lines when compared to a non-target transduced control. Given the impact of KPNB1 silencing on antiviral priming, co-immunoprecipitation with FLAG-tagged NS3/4A was performed to validate the previously identified interaction (Figure 11B). Indeed, immunoblots confirmed the NS3/4A-KPNB1 interaction, but more interestingly, revealed the appearance of a lower secondary KPNB1 band not present in a eYFP transfected control. To further characterize the interaction between NS3/4A and KPNB1, co-immunoprecipitation experiments were performed with conditions which included BILN2061 and UM42811 treatment (Figure 11C). Surprisingly, both compounds at their given concentrations were capable of completely inhibiting the interaction though only BILN2061 treatment was associated with the disappearance of the lower secondary KPNB1 band. Taken together, NS3/4A interacts with nucleocytoplasmic transporter KPNB1 in a dynamic which can be disrupted by either protease inhibitor BILN2061 or compound UM42811.

Knowing the inherent enzymatic activity of HCV NS3/4A, validation of its interaction with KPNB1 by Western blot analysis increased the significance of the band of slightly lower molecular weight recognized by KPNB1 specific antibodies (Figure 11B). The disappearance of this band following treatment with protease inhibitor BILN2061 also contributed to its interest (Figure 11C). To explore the possibility of NS3/4A mediated cleavage, the amino acid sequence of human encoded KPNB1 was aligned with the reported consensus sequence Asp/Glu-$(Xaa)_4$-Cys/Thr ↓ Ser/Ala-$(Xaa)_2$-Leu/Trp/Tyr of NS3/4A cleaved junctions found within the HCV polyprotein (118). An amino acid sequence found at the C-terminal end of KPNB1 was found to be concordant with the aforementioned consensus sequence (Figure 12A). The predicted size of fragments generated by such a cleavage also agrees with the molecular weights of bands observed in Western analysis (97kDa for full-length KPNB1 vs 89kDa for C-terminally cleaved KPNB1). Interestingly, cleavage at this site would also occur within HEAT repeat 18 of KPNB1 which is absolutely required for its association with importin α-5 (also known as KPNA1) (254). The NS3/4A-mediated cleavage of KPNB1 could therefore have deleterious effects on nucleocytoplasmic transport (255) and more specifically on the nuclear cytoplasmic transport of its notable STAT1 cargo (256). KPNB1 silencing data (Figure 11A) and previous data which indicated that the presence of NS3/4A, but not its mature NS3 form, was responsible for the prevention of STAT1 nuclear localization upon IFN-α stimulation (95) are also consistent with a KPNB1 cleavage narrative. Given STAT1's role in the type I IFN amplification loop (257), and the previously documented effect of NS3/4A on its nuclear localization, functional assays using a luciferase reporter under the control of an ISRE promoter and IFN-α 2a stimulation were performed to confirm NS3/4A's effect on abrogating type I IFN receptor signaling (Figure 12B). Inhibition of NS3/4A's protease activity through BILN2061 treatment completely restored IFN-α mediated ISG56 induction (left panel Figure 8B). KPNB1 silencing demonstrated a greater negative effect on ISG56 induction than NS3/4A expression (middle panel Figure 12B). The lack of and additive negative effect of KPNB1 silencing and NS3/4A ectopic expression on ISG56 induction suggests that NS3/4A mediates inhibition of ISG56 induction through KPNB1. Interestingly, while UM42811 inhibits NS3/4A's interaction with KPNB1 (Figure 11C), it neither eliminates the emergence of C-terminally cleaved KPNB1 nor restores ISG56 induction (right panel Figure 12B). Altogether, NS3/4A protease activity prevents IFN-α 2a mediated ISG56 induction within a KPNB1 dependent pathway possibly by mediating KPNB1 cleavage and generating a C-terminal truncated form that leads to the prevention of STAT1 nuclear translocation.

The phenotype within the early innate response upon SeV infection of UM42811-mediated inhibition of the KPNB1-NS3/4A interaction has not yet been investigated. When bound to NS3/4A, c-terminally truncated KPNB1 is speculated to play a role in gating HCV proteins in the membranous web during replication. This could potentially explain the submicromolar antiviral activity of UM42811 in HCV replicon positive cells. On the other hand, the toxicity of UM42811 in the 10-20 uM range is believed to be due to a trans-dominant negative effect of truncated KPNB1. This cleaved form of the protein could outcompete its native uncleaved form by associating with proteins which would otherwise require transport for key cellular processes.

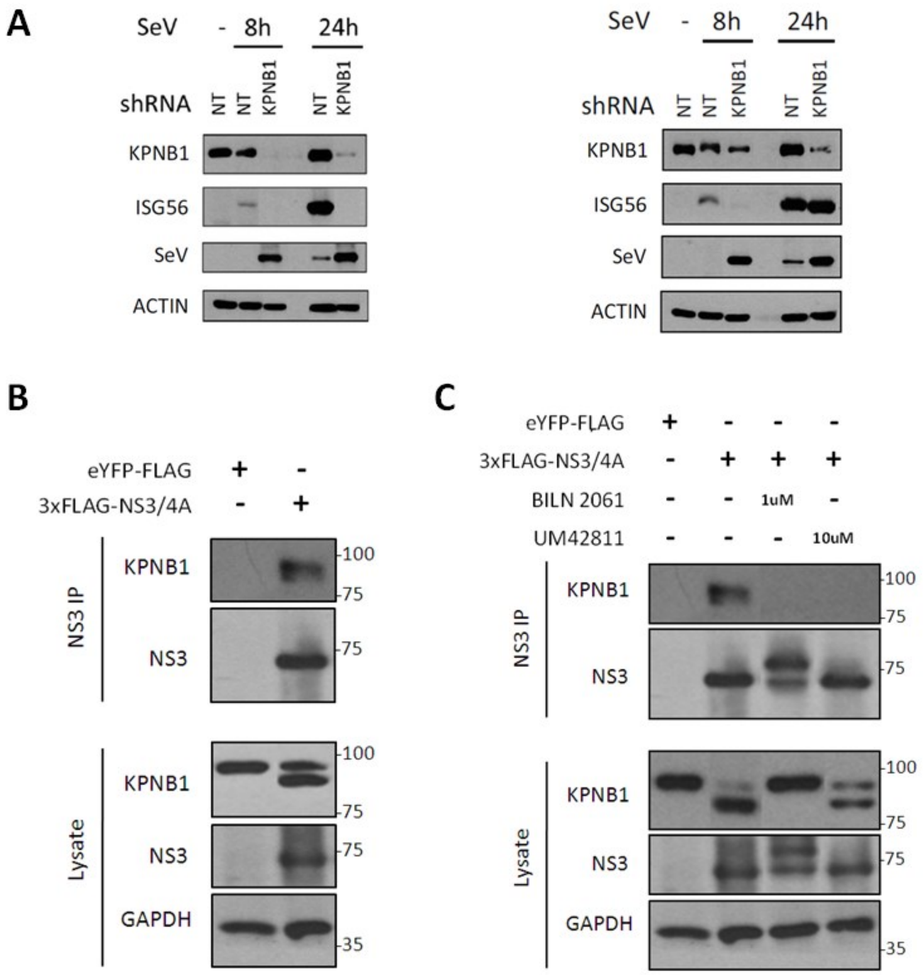

A
SeV - 8h 24h
shRNA NT NT KPNB1 NT KPNB1
KPNB1
ISG56
SeV
ACTIN
SeV - 8h 24h
shRNA NT NT KPNB1 NT KPNB1
KPNB1
ISG56
SeV
ACTIN
B
eYFP-FLAG + -
3xFLAG-NS3/4A - +
NS3 IP
KPNB1
NS3
Lysate
KPNB1
NS3
GAPDH
100
75
75
100
75
75
35
C
eYFP-FLAG + - - -
3xFLAG-NS3/4A - + + +
BILN 2061 - - 1uM -
UM42811 - - - 10uM
NS3 IP
KPNB1
NS3
Lysate
KPNB1
NS3
GAPDH
100
75
75
100
75
75
35

**Figure 11.** NS3/4A interacts with KPNB1, but is disrupted by both UM42811 and the protease inhibitor BILN2061.

(A) HEK 293T (left) and A549 (right) are transduced with shNT or shKPNB1 for 3 days and infected with SeV for 8 or 24 hours. KPNB1, ISG56, SeV protein expression are resolved by immunoblot. (B) HEK 293T cells were transfected with an 3xFLAG-NS3/4A expression vector. 48 post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between NS3/4A and KPNB1 was resolved by immunoblot. (C) HEK 293T cells were transfected with an 3xFLAG-NS3/4A expression vector and treated with the indicated concentration of BILN2061 or UM42811. 48 post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between NS3/4A and KPNB1 was resolved by immunoblot.

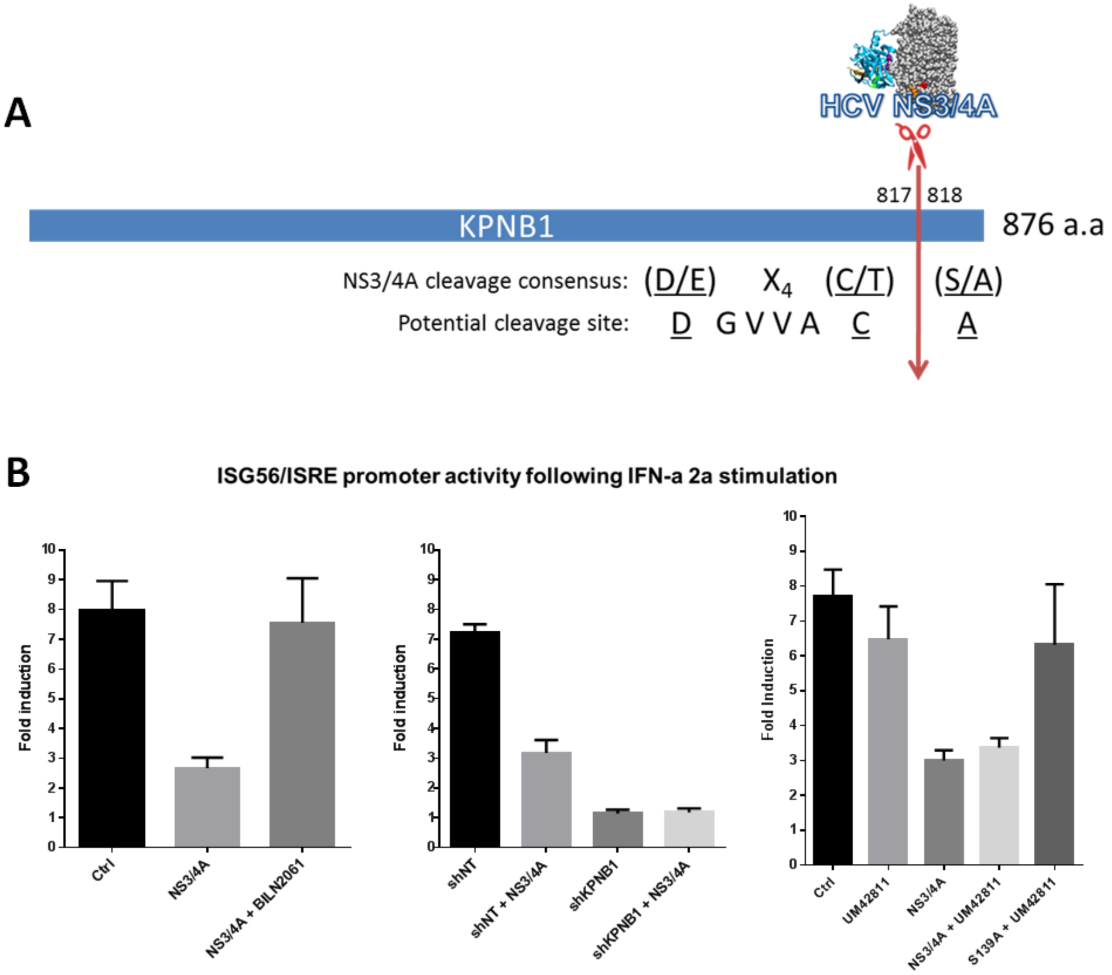


**Figure 12.** NS3/4A protease activity is required for the inhibition of KPNB1-mediated ISG56 induction following IFN-α 2A stimulation.

(A) Cartoon representation of a potential NS3/4A protease cleavage site on KPNB1. (B) HEK 293T cells were transduced with a KPNB1 shRNA for 72 hours (middle panel); transfected with an ISG56-luciferase reporter, a renilla luciferase reporter (for normalization) and indicated expression vectors; treated with either 1μM BILN2061 (left panel) or 10μM UM42811 (right panel) as indicated for 48 hours; and stimulated with IFN-α 2A for 24 hours. Luciferase fold intensity was measured and compared with control cells transduced with a non-target shRNA (middle panel) or with 0.5% DMSO treated empty vector transfected control cells (left and right panels).

## 9. Differential HSP60-IRF3-NS3/4A interaction configurations under UM42811 or BILN2061 treatment

Because Gene Ontology enrichment of specific HCV-host interactors yielded protein transport as the top hit with a large contribution from NS3/4A (95), the potential implication of chaperonins in the inhibitory mechanism of UM42811 was also explored. Chaperones constitute an important group of host cytoprotective molecules and are involved in protein folding, multimeric protein assembly, protein trafficking, and protein degradation (258). An interaction with mitochondrial heat shock protein 60 (HSP60), also known as HSPD1, is interesting not only given its possible involvement in shuttling NS3/4A to host immune factors such as MAVS, but also for its possible involvement in converting enzymes to their active state as is the case for the HBV polymerase (259). Moreover, HSP60 was previously shown to be directly implicated in apoptotic processes with important implications in tumorigenesis (260) and was shown to interact, in independent instances, with other viral factors like HCV core (261) and hepatitis B X protein (HBx) (262) in various apoptotic processes. More recently, HSP60 was shown to be implicated in type I IFN induction through its interaction with the pathway's key transcription factor IRF3(263). With these functional implications in mind, further characterization of the HSP60-NS3/4A interaction was sought to provide insights towards potential immune dampening/subversion mechanisms which could be possibly be altered by UM42811 treatment. To this end, co-immunoprecipitation experiments were performed with FLAG-tagged NS3/4A and reciprocally with FLAG-tagged HSP60 and eYFP-tagged NS3/4A in HEK 293T cells to validate the reported interactions between NS3/4A, HSP60 and IRF3 by Western blot analysis (Figure 13A-B). Indeed, immunoblots in both instances confirmed the NS3/4A-HSP60 interaction. Similar to previous experiments with KPNB1, to further characterize the interaction with HSP60, co-immunoprecipitation experiments were performed with the inclusion of BILN2061 and UM42811 treatment conditions (Figure 13C-D). Unlike the interaction with KPNB1, only UM42811 at 10 μM concentration was capable of inhibiting this interaction. Interestingly, HSP60's interaction was preferential to NS3/4A in its immature precursor form as demonstrated by the BILN2061 treated sample (Figure 13D).

Immunoblots also confirmed the previously reported interaction between HSP60 and IRF3 (Figure 13D) while demonstrating in an independent instance that NS3/4A interacted with IRF3 (Figure 13C). These interactions were, however, differentially affected by treatment with either UM42811 or protease inhibitor BILN2061 (Figure 13C-D). While the NS3/4A-IRF3 interaction was disrupted by UM42811 treatment, the HSP60-IRF3 interaction was disrupted by BILN2061 treatment. This suggests that IRF3 and HSP60 preferentially interact with precursor NS3 than with each other if the UM42811-induced tightening of the NS3/4A oligomer is assumed to preclude these interactions, and if NS3 is assumed to exist in its mature and precursor forms in similar biological proportions without BILN2061 treatment (Figure 15B). These results also indicate that the presence of specific viral factors such as NS3/4A is a prerequisite for the HSP60-IRF3 interaction. How the expression of a single viral enzyme is distinguished from its endogenously expressed counterparts is still unknown. It can be speculated that the HSP60-IRF3 interaction is a stress response triggered upon pathogenic or non-homeostatic levels of enzymatic activity.

Because HSP60’s contribution to type I IFN signalling could have further implications for HSP60’s interaction with NS3/4A, functional assays using a luciferase reporter under the control of an IFN-β promoter and SeV stimulation were performed to verify HSP60’s effect on IFN-β induction (Figure 14). Surprisingly, unlike reports by Lin et al. (263) contribution to type I IFN signaling was not reflected by IFN-β induction with quantitative changes of HSP60 expression whether silenced or overexpressed. As readout time points differ (8 vs 24 hours post-infection), the differences in IFN-β induction may reflect a temporal sensitization wherein HSP60 is not the limiting factor. When evaluated at 24 hours post-infection, any effect observed on IFN-β induction was negligible when compared to the negative impact observed with NS3/4A ectopic expression. The negative impact of NS3/4A expression on IFN-β induction following SeV infection is likely a reflection of the effect of MAVS cleavage on type I IFN production. Taken together, NS3/4A interacts with HSP60 in a dynamic which is disrupted by UM42811, but that is unlikely to have a significant bearing on IFN-β induction at 24 hours post SeV infection based on lack of effect induced by quantitative changes in HSP60 expression.

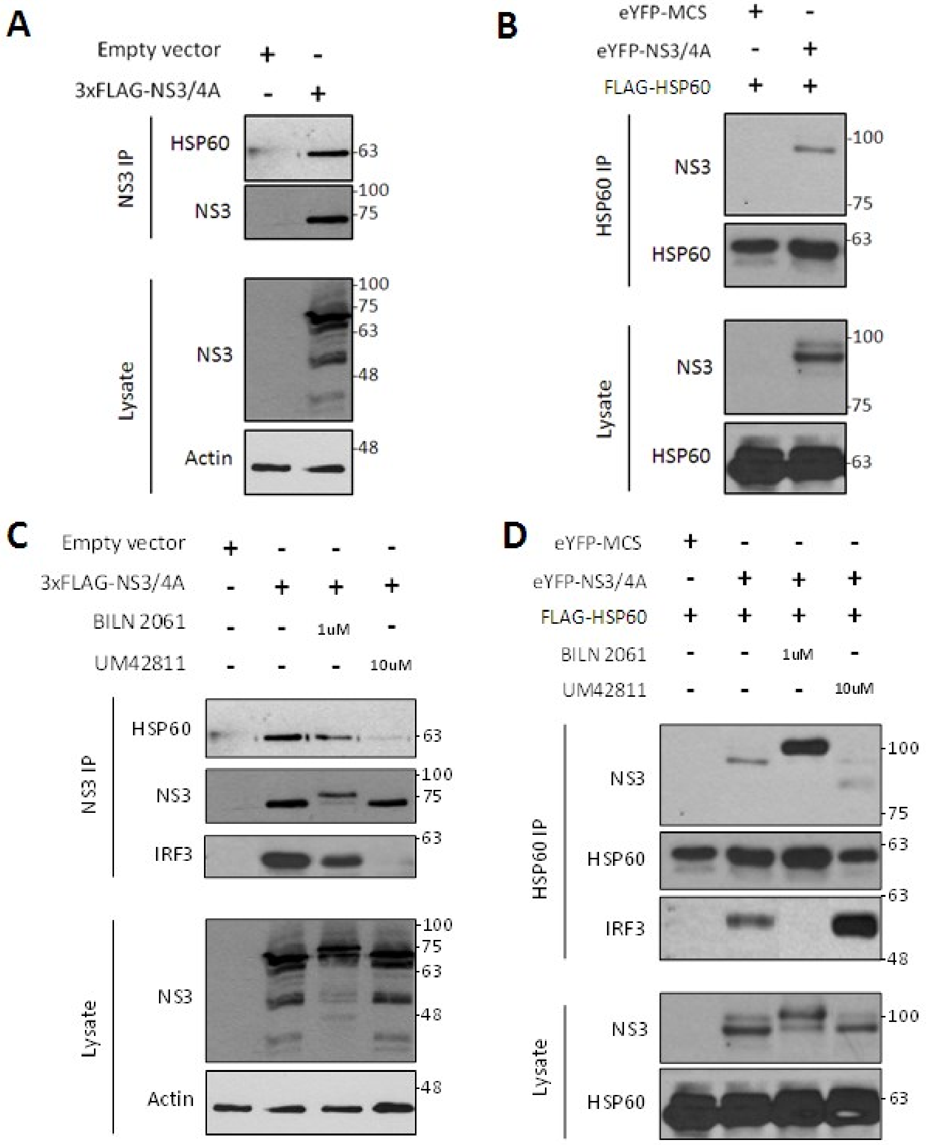

A
Empty vector + -
3xFLAG-NS3/4A - +
NS3 IP
HSP60
NS3
Lysate
NS3
Actin
63
100
75
48
B
eYFP-MCS + -
eYFP-NS3/4A - +
FLAG-HSP60 + +
HSP60 IP
NS3
HSP60
Lysate
NS3
HSP60
100
75
63
C
Empty vector + - - -
3xFLAG-NS3/4A - + + +
BILN 2061 - - 1uM -
UM42811 - - - 10uM
NS3 IP
HSP60
NS3
IRF3
Lysate
NS3
Actin
63
100
75
48
D
eYFP-MCS + - - -
eYFP-NS3/4A - + + +
FLAG-HSP60 + + + +
BILN 2061 - - 1uM -
UM42811 - - - 10uM
HSP60 IP
NS3
HSP60
IRF3
Lysate
NS3
HSP60
100
75
63
48

**Figure 13.** NS3/4A interacts with HSP60, but is disrupted by UM42811.

(A) HEK 293T cells were transfected with an 3xFLAG-NS3/4A expression vector. 48 post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between NS3/4A and HSP60 was resolved by immunoblot. (B) HEK 293T cells were co-transfected with an eYFP-NS3/4A and a FLAG-HSP60 expression vector. 48 post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between NS3/4A, HSP60 was resolved by immunoblot. (C) HEK 293T cells were transfected with an 3xFLAG-NS3/4A expression vector and treated with the indicated concentration of BILN2061 or UM42811. 48 post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between NS3/4A and HSP60 or IRF3 was resolved by immunoblot. (D) HEK 293T cells were transfected with an eYFP-NS3/4A and a FLAG-HSP60 expression vector and treated with the indicated concentration of BILN2061 or UM42811. 48 hours post-transfection, cells were harvested and co-IP using anti-FLAG coated beads was performed on cell lysates. Interaction between HSP60 and NS3/4A or IRF3 was resolved by immunoblot.

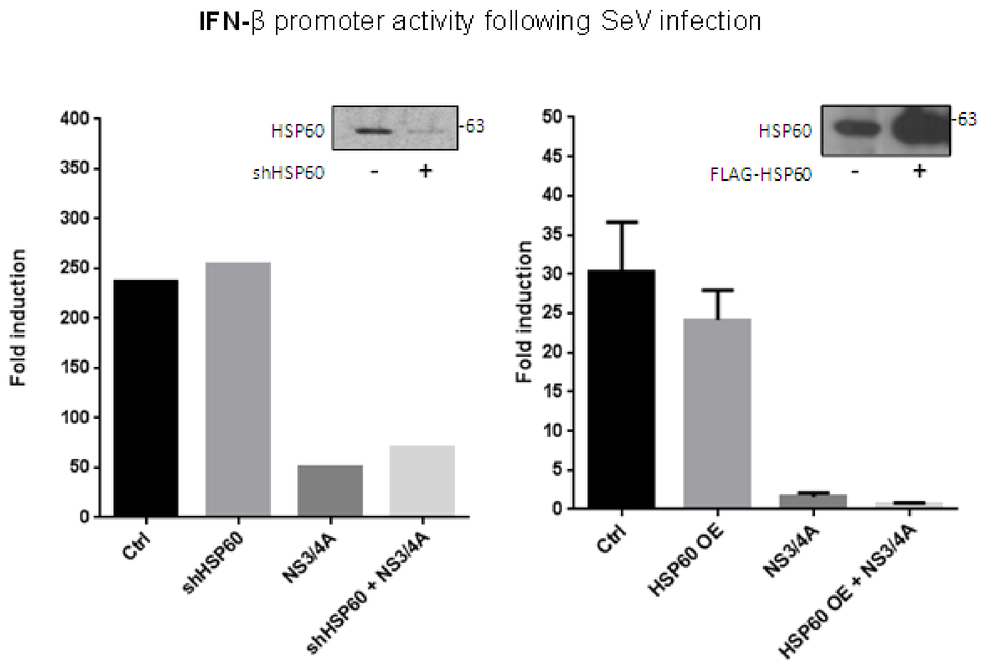


**Figure 14.** Quantitative changes in HSP60 expression have no effect on IFN-$\beta$ induction following SeV infection.

HEK 293T cells were transduced with a HSP60 shRNA for 72 hours; transfected with an IFN-β-luciferase reporter, a renilla luciferase reporter (for normalization) and NS3/4A and HSP60 expression vectors as indicated for 48 hours; and infected with SeV for 24 hours. Luciferase fold intensity was measured and compared with control cells transduced with a non-target shRNA (left) or with empty vector transfected control cells (right).

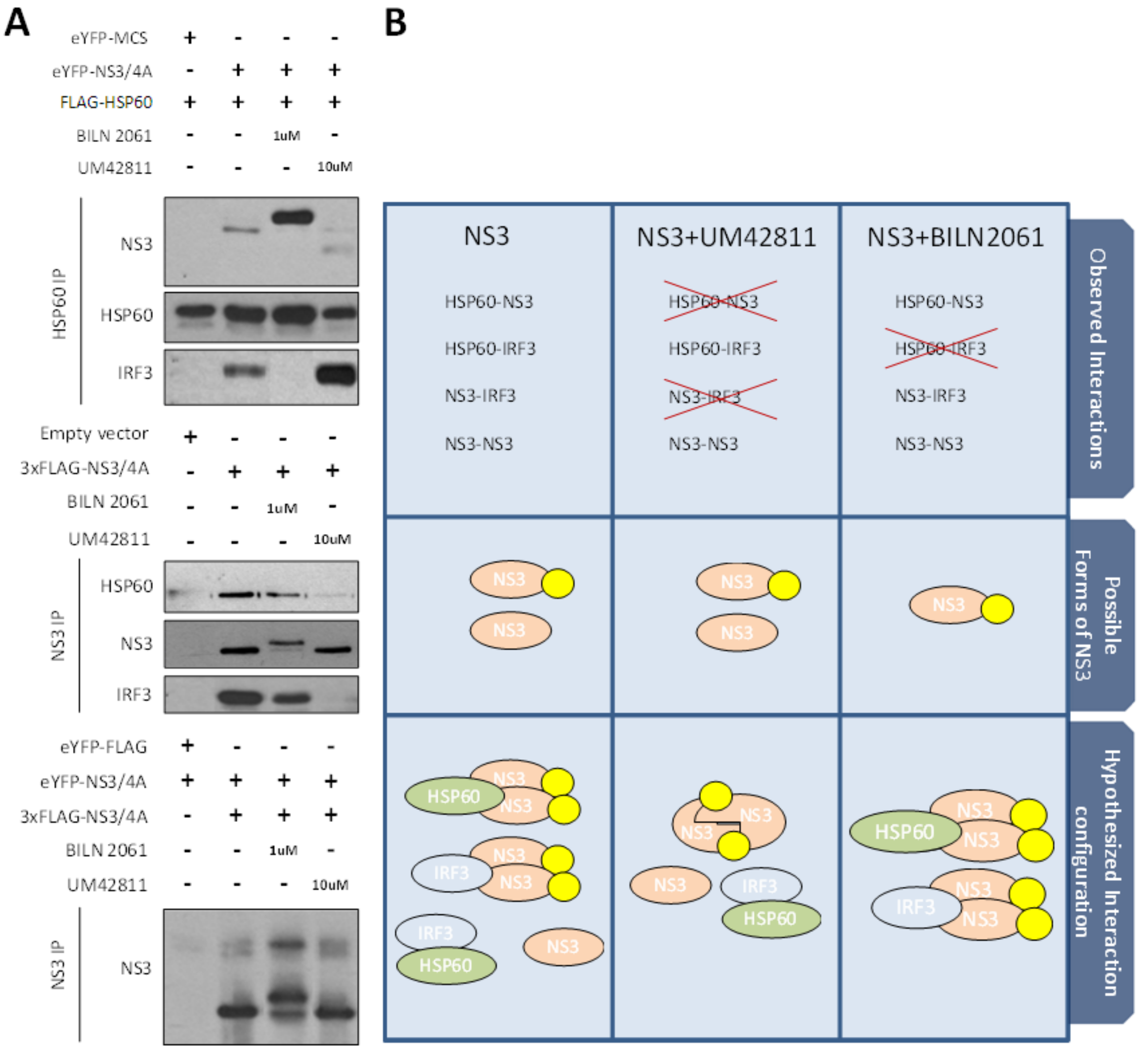

A
eYFP-MCS + - - -
eYFP-NS3/4A - + + +
FLAG-HSP60 + + + +
BILN 2061 - - 1uM -
UM42811 - - - 10uM
HSP60 IP
NS3
HSP60
IRF3
Empty vector + - - -
3xFLAG-NS3/4A - + + +
BILN 2061 - - 1uM -
UM42811 - - - 10uM
NS3 IP
HSP60
NS3
IRF3
eYFP-FLAG + - - -
eYFP-NS3/4A + + + +
3xFLAG-NS3/4A - + + +
BILN 2061 - - 1uM -
UM42811 - - - 10uM
NS3 IP
NS3
B
NS3
NS3+UM42811
NS3+BILN2061
HSP60-NS3
HSP60-IRF3
NS3-IRF3
NS3-NS3
~~HSP60-NS3~~
HSP60-IRF3
~~NS3-IRF3~~
NS3-NS3
HSP60-NS3
~~HSP60-IRF3~~
NS3-IRF3
NS3-NS3
Observed Interactions
Possible Forms of NS3
Hypothesized Interaction configuration
NS3
HSP60
IRF3

**Figure 15.** Hypothesized interaction configuration between NS3, HSP60 and IRF3 in the context of UM42811 and BILN2061 treatment

(A) Compiled co-immunoprecipitation experiments from Figures 6, 13C and 13D to facilitate the rationalization of interaction changes between NS3, HSP60 and IRF3. (B) Table denoting pairwise interactions observed in co-immunoprecipitation experiments. Cartoon visualization of the different hypothesized interaction configurations assuming protein interactions are dependent on the precursor form of NS3. Represented are NS3 (beige), NS4A (yellow), HSP60 (green), and IRF3 (blue).

# Discussion

Initiated from the same screening method designed to characterize the pairwise interaction network within the HCV proteome, the characterization of UM42811, an HCV antiviral compound identified amongst approximately 110,000 others as an NS3/4A-NS3/4A interaction modulator, is owed to an adapted high throughput BRET screening method. This approach was elected after careful consideration of its advantages and limitations. Notably, this technology is capable of providing information of specific interacting protein regions applicable in live cells and in membrane bound complexes. It does however have certain limitations associated with the use of tags and the distinction of conformational changes from PPI disengagement. The BRET signal and the intensity of $\mathrm{BRET}_{\mathrm{max}}$ are function of the distance and consequently of the orientation between dimer partners and can provide information not only about protein interactions but about specific regions mediating this interaction. For example, the $\mathrm{BRET}_{\mathrm{max}}$ of NS5A homodimerization was strong when the energy donor Rluc and the energy acceptor eYFP were N-terminally tagged whereas C-terminally tagged NS5A proteins were characterized by relatively weak $\mathrm{BRET}_{\mathrm{max}}$ (232). This observation is supported by earlier reports that NS5A proteins interact via their N-terminal domain I (264). Moreover, the sensitivity of BRET allows utility beyond resolving PPIs to be extracted from the technique. In some instances, both extremities of a protein were tagged to determine conformational changes induced by substrate binding through intramolecular BRET signals (265). BRET, as a non-radioactive energy transfer technique, also has the benefit of being compatible with live cell settings. It can therefore be used to determine PPIs with more confidence as it can more easily reflect what occurs naturally within a cell. It also represents an unprecedented method to observe membrane PPIs as more elaborate studies involving crystal structures requires soluble forms of the proteins of interest. In other words, membrane PPIs inferred from crystal structures may not be as biologically relevant. For instance, studies involving HCV NS proteins often required the removal of membrane anchoring regions despite these regions being necessary for proper formation of the iconic HCV multimeric replication complex (266). The strength of BRET is that it can be applied to any membrane bound protein interaction, and not exclusively for those involved with HCV, in a biologically relevant setting which is otherwise not possible. In the previous report where novel HCV protein interactions were identified (232), the inclusion of the interaction between NS4B and NS5A, which are located at the ER and at LDs respectively, and the exclusion of the interaction between NS3/4A and p7, which are both localized to the mitochondria, within an updated HCV interaction network attest for the specificity of BRET and its independence from protein localization. The technique is not without its pitfalls however, the requirement of a eYFP and a Rluc tag carries with it a number of assumptions. These tags are quite sizeable relative to other tags and their use assumes that they have a negligible impact on the native conformation, localization, function and dynamic of a protein. For example, a C-terminal tagging of the NS3/4A heterodimer could severely influence its mitochondrial localization as NS4A is responsible for membrane anchoring (116). These considerations are only amplified as the size of the tagged protein decreases. The use of BRET in identifying or characterizing PPIs also requires certain knowledge of the investigated proteins. As with previously mentioned examples, because of the sensitivity of BRET, an improper management of the localizations of donor and acceptor tags can yield false negatives.

Though not without contradicting evidence (267), it has previously been shown that the helicase domain of NS3 could dimerize and that dimerization was required for its enzymatic activity (268). The cysteine protease activity of NS2/3 was also shown to require dimerization (269). Because the enzymatic activity of NS2/3 precedes that of NS3/4A in polyprotein processing, this also implies an instance of polyprotein dimerization and by extension a potential key determinant for NS3/4A dimerization in generating the NS2/3 active site. There is still no real consensus on exactly how the NS3 helicase functions though it has been suggested that relative enzyme to substrate concentrations are the determinant factor (115). Nevertheless, Figure 5 demonstrates the dimerization of the NS3/4A *in cellulo.* Taken together with the UM42811-mediated BRET induced signal activation of protease inactive S139A NS3/4A mutant (Figure 7B); the greater significance of the precursor NS3/4A band particular to BILN2061 treated samples in co-immunoprecipitation experiments (Figure 10); the reduced co-immunoprecipitation of BILN 2061-mediated precursor NS3/4A-NS3/4A interaction (Figure 10 ), the homodimerization of the NS3/4A heterodimer appears to be more significant than that of NS3 in the replicative cycle of HCV. Considering many viral proteases (270) and even HCV NS2/3 protease (269) were shown to require dimerization for enzymatic activity, it would be interesting to determine whether dimerization of the NS3/4A heterodimer is also required for its protease activity. Answering this question, however, would require supplementary tools as the distinction between conformational changes and specific protein interaction disengagement is not fully addressed with BRET-based assays alone as demonstrated by BILN2061 treatment in BRET titration experiments (Figure 7B) and NS3/4A co-immunoprecipiration experiments (Figure 10).

HCV therapeutics has been quickly evolving over the last decade. Most successful DAAs currently target the NS3/4A serine protease, NS5B polymerase or the mechanistically elusive NS5A. Interestingly, the discovery of NS5A inhibitors came through fortuitous HTS with inhibitory effect achieved through the apparent influence of compounds on the NS5A dimer interaction (164, 166). Though the specific role of the homodimeric NS3/4A-NS3/4A interaction was not fully characterized within the HCV lifecycle prior to initiating BRET based compound screening, the homology found in the discovery and mechanism of NS5A inhibitors provided a level of confidence in the identification of novel anti-HCV agents.

Being positively evaluated based on statistical *Z'* and *Z* factors of each interaction configuration (Figure 6B) (245), the implementation of BRET based HTS of small molecule compounds successfully illustrated the potential of BRET not only in the assessment of membrane PPIs, but also in its adaptability for large scale drug discovery programs. To account for readout artefacts and partly as a secondary consequence of being undistinguishable from cytotoxic effect, compound selection based on bioluminescence quenching was set at a liberal <95% cutoff. For logistics purposes, all compounds were tested at 10 µM concentrations in the primary screen. As a consequence of incidental cytotoxicity below 10 µM, certain compound hits may have been false positives. These concerns were pre-emptively addressed with the implementation of a secondary screen and downstream antiviral characterization of individual hits. Out of approximately 110,000 compounds, 110 hits were identified representing a global hit rate of 0.10% for the primary BRET HTS assay, which was considered satisfactory.

Confirmation of primary screen hits in a replicate experiment, hit validation via dose-response analyses reporting antiviral potency and cytotoxicity (Figure 7C) and hit specificity confirmation via BRET titration (right panel Figure 7B) and resistance mapping (Figure 8B-C) ultimately cements BRET as a valid and attractive HTS method through the discovery and characterization of UM42811. The therapeutic index within Huh7 cells bearing subgenomic Con1 replicon determined at 48.05 was relatively favorable when compared to compounds identified in a similar anti-HCV HTS (271). In the assay designed by Yu et al., approximately 2,000 compounds were screened for their effectiveness against every stage of infection using cell culture-derived HCV at low multiplicities of infection and averaged a therapeutic index of roughly 16 within its 18 or so hits. Moreover, because amino acid sequence alignment for weighing potential resistance mutations used a compiled database of full-length HCV genomes indiscriminate of genotype, concerns of antiviral potency being skewed towards a specific genotype were lessened. It is important to recognize, however, that the use of an infectious model for HCV replication could have potentially yielded very different therapeutic indices considering NS3/4A has also been shown to affect the assembly phases of the viral lifecycle (81, 272, 273). Cytotoxicity is also susceptible to some variation depending on the cell type used to report it but cytotoxicity assays using hepatocyte cell lines are considered a good model for primary hepatocytes.

As antiviral compounds apply extreme selective pressures on virus replication, the recurrence of specific escape mutations under such pressure is undeniably highly informative in a compound's binding site. Ultimately, the mapping of resistance mutations provides strong insights towards a compound's target. In this regard, the surface localization of both I615M and K617N on the C-terminal helicase subdomain added more feasibility to their identity as potential resistance mutations; these sites were rather accessible and could very well have been involved in PPIs. Furthermore, the substitution of lysine to asparagine at position 617 of NS3 constituted a loss of a positive charge and could have seriously altered interactions at this site. Interestingly, although L14F was not weighted heavily based on naturally occurring sequence variation, its localization at the putative alpha helix of the protease domain which is involved in the membrane anchoring of the NS3/4A complex was functionally relevant. Because of the unique structural features of NS3/4A that defines its relatively featureless substrate binding cleft (110), HCV protease inhibitors are often restricted in their core designs and are consequently characterized by nearly identical resistance profiles. Mutations occurring at V36, T54, V55, Q80, R155, A156, D168, and V170 can occur naturally and have been verified in replicon studies to be associated with protease inhibitor resistance (248, 249). With these factors considered, escape variants characterized by mutations occurring within NS3/4A, but outside of the aforementioned sites would have been indicative of a novel inhibitory mechanism independent of protease inhibition.

The affect that UM42811 has on PPIs involving NS3/4A is quite striking. The disruption of the NS3/4A-KPNB1 (Figure 11C), NS3/4A-HSP60 and NS3/4A-IRF3 (Figure 13C) interactions by UM42811 in conjunction with a potential stabilization of either a mature or precursor NS3/4A-NS3/4A dimer (left panel Figure 7B) suggest that a tighter NS3/4A-NS3/4A homodimer conformation prevents host interactors from associating with the complex (Figure 15). On the other hand, the lack of any effect on self-cleavage (Figure 10) and weak effect on KPNB1 cleavage (Figure 11C) suggests that formation of a tighter dimer does not influence its protease activity. Because the reduced cleavage on KPNB1 that is derived from the consensus sequence found at NS3/4A-cleaved junctions within the HCV polyprotein (Figure 12A), it is also possible that this putative conformational tightening affects polyprotein processing and explain submicromolar antiviral activity of UM42811.

Despite resistance mutations attributing different binding locations for either compound, the inhibition of the NS3/4A-KPNB1 interaction by both UM42811 and BILN2061 coupled with the inhibition of the NS3/4A-HSP60 and the NS3/4A-IRF3 interactions only by UM42811 suggests KPNB1 interacts with NS3/4A via two distinct sites; the protease active site which contains an overlap with the binding site for UM42811, HSP60 and IRF3 and a site only inaccessible in tightly bound NS3/4A homodimers. Moreover, because both BILN2061 and UM42811 inhibit the NS3/4A-KPNB1 interaction (Figure 11C) though only BILN2061 is able to restore KPNB1 mediated ISG56 induction (Figure 12B), the NS3/4A protease activity can specifically be inferred to be responsible for ISG56 induction inhibition via the cleavage of the nucleocytoplasmic transporter KPNB1 and thereby the prevention STAT1 nuclear translocation.

Interestingly, the potential capacity of NS3/4A to cleave KPNB1 presents functional implications within innate immunity reminiscent to the characterized cleavage of MAVS (91) and TRIF (92). To expand, KPNB1 requires association with its partner KPNA1 for nucleocytoplasmic transport of its cargo (255). STAT1, which is implicated in the JAK/STAT pathway of type I IFN signalling, is a major KPNB1 cargo (256). Because the potential NS3/4A mediated cleavage site occurs within a region of KPNB1 absolutely required for its association with KPNA1 (254), the effect on innate immunity is a definite possibility.

As chaperones are involved in numerous biological functions related to protein turnover and transport (258), the NS3/4A-HSP60 interaction is interesting given its possible involvement in shuttling NS3/4A to host immune factors such as MAVS, and also for its possible involvement in converting enzymes to their active state as is the case for the HBV polymerase (259). Given HSP60's involvement in apoptotic processes (260) and type I IFN signalling (263), the NS3/4A-HSP60 interaction also provides other unclear biological implications. Interestingly, the presence of NS3/4A seemed to be required for the HSP60-IRF3 interaction described by Lin et al. (263) to occur. Why the expression of a viral enzyme is a prerequisite or rather how the expression of a single viral enzyme is distinguished from its endogenously expressed counterparts is still unknown. It can be speculated that the HSP60-IRF3 interaction is a stress response triggered upon pathogenic or non-homeostatic levels of enzymatic activity. Continuing with the HSP60-IRF3 interaction, when NS3/4A was present, this interaction was disrupted by BILN2061 treatment (Figure 13D). This suggests that IRF3 and HSP60 preferentially interact with precursor NS3 than with each other if the UM42811-induced tightening of the NS3/4A oligomer is assumed to preclude NS3/4A-HSP60 and NS3/4A-IRF3 interactions, and if NS3/4A is assumed to exist in its mature and precursor forms in similar biological proportions without BILN2061 treatment (Figure 15B).

# Perspectives

Site directed mutagenesis within the subgenomic Con1 replicon would yield variants which contain punctual mutations with the highest potential to confer UM42811 resistance. Introduction of these mutations both alone and in combination will allow the validation of these mutations as resistance bearing and provide further confirmation of UM48211's target and proposed mechanism. This would entail site directed mutagenesis within the replicon system, *in vitro* transcription reactions to generate proper RNA transcripts, electroporation or adapted transfections to introduce HCV RNA into replication-permissive Huh7.5 cells, and finally, dose-response analysis to evaluate shifts relative to wild type in $EC_{50}$ that would correspond to resistance. Given that resistance mapping and the original screening method responsible for UM42811's identification are coherent with a novel inhibitory mechanism, it is expected that atypical L14F, I615M, and K617N mutations confer resistance to UM42811 at least to varying degrees. An indirect approach based on the mutated enzymes alone could also provide further insights towards resistance. Performing BRET titration curves with mutated enzymes would show whether native NS3/4A dimer/oligomer conformation is impaired and whether UM42811 treatment no longer induces dimer/oligomer conformational tightening. The differential interaction configurations induced by UM42811 and BILN 2061 treatment could also be reevaluated in the context of individual mutated enzymes. Striking differences between these configurations with wild type versus mutated NS3/4A would be informative not only mechanistically for UM42811 but also for NS3/4A's host interaction network.

The relationship between HCV NS3/4A protease and nucleocytoplasmic transport factor KPNB1 and its potential involvement in immune evasion remains to be elucidated. Verification that KPNB1 cleavage is directly mediated by NS3/4A at biologically relevant concentrations and not a by-product of another undefined mechanism derived from the protease activity of NS3/4A is the first step. To this end, generation of KPNB1 variants, one substituting the cleavage essential P1 cysteine and one truncated from the P1 site onwards, in conjunction with silencing of endogenous KPNB1 could be used to verify NS3/4A mediated cleavage and its functional derivatives. The C-terminally truncated KPNB1 could also be used to verify, simply by its ectopic expression, whether it facilitates HCV replication or it has a dominant negative effect on full-length KPNB1 nuclear transport function. The latter could demonstrate whether the NS3/4A-KPNB1 interaction which is abrogated at 10 uM is a secondary cytotoxic effect to UM42811's HCV replication inhibition. The setup would entail PCR reactions for site directed mutagenesis or insertion of a premature stop codon, lentiviral silencing to eliminate confounding factors in downstream analysis and transfection of silencing resistant KPNB1 variant expression vectors into either replicon bearing or NS3/4A expressing cells. Assuming tags do not significantly alter its properties, C-terminal tagging of these variants could also be used to increase the resolution with which full-length and cleaved KPNB1 are distinguished. Downstream analysis would involve NS3/4A co-immunoprecipitation to verify whether truncated KPNB1 binds NS3/4A in a manner similar to N-terminal product inhibitors, western blot analysis to evaluate KPNB1 cleavage, and either IFN-α stimulation or SeV/HCV infection in immunocompetent reporter cells to evaluate the outcome of KPNB1 cleavage protection on innate immune priming. If NS3/4A truly mediates the cleavage of KPNB1, C-terminally truncated and not full-length KPNB1 would be expected to be the predominant NS3/4A interactant, P1 cysteine substituted KPNB1 would be expected to present a single unique band in Western analysis, and a functional restoration would be expected for conditions using the P1 cysteine substituted KPNB1 variant. C-terminally truncated KPNB1 could also be particularly useful, in co-immunoprecipitation experiments, to confirm whether its association with KPNA1 is truly abrogated (or if it facilitate HCV replication in KPNB1 KD cells bearing the HCV replicon). The NS3/4A-HSP60 interaction presented unclear implications in type I IFN signaling. To reconcile discrepancies with reported literature (263), a kinetic experiment with time points between 8 and 24 hours post SeV infection could be used to evaluate whether HSP60-facilitated IFN-β induction is truly a temporal sensitization. As MAVS is cleaved by NS3/4A (91) and as HSP60 is an important mitochondrial chaperone involved in protein trafficking, observing a moderate decrease in NS3/4A-dependent MAVS cleavage upon HSP60 knockdown could be used to verify whether HSP60 plays a role in shuttling NS3/4A to MAVS.

# Conclusion

By exploiting membrane protein-protein interactions, live cell assays using BRET technology have been optimized to complete a comprehensive HCV protein interaction network. Using the experience gained from this study, an HTS cell-based assay was implemented to identify novel inhibitory compounds targeting an unreported NS3/4A-NS3/4A interaction. Approximately 110,000 compounds from a small-molecule collection were screened to monitor modulation of NS3/4A homodimerization and were discriminated based on specificity and potency. UM42811 was identified as a potential activator of the NS3/4A-NS3/4A interaction and demonstrated promising antiviral activity with an excellent therapeutic window. Resistance studies and parallel interaction studies were initiated to characterize the novel inhibitory mechanism of UM42811.

In conclusion, combined deep sequencing and mutation mapping have yielded a resistance profile based on statistical and functional probability for novel small-molecule antiviral UM42811. This resistance profile coupled with the original screening method pointed towards a novel inhibitory mechanism targeting HCV NS3/4A serine protease. Previous interaction network studies, functional assays and western analysis confirmed novel NS3/4A host interactants KPNB1 and HSP60 with varying implications within cell biology. These interactions were disrupted by UM42811 treatment. NS3/4A-mediated cleavage of KPNB1 was briefly explored and had significant repercussions on the type I IFN innate immune priming. While most HCV drug campaigns have been discontinued with treatment regimens largely covered by current combination therapies, the cost of these therapies still hinder their widespread use. Chemical optimization of UM42811 could prove to yield a less costly alternative that could be used in combination with protease inhibitors. Nevertheless, the characterization of a lead compound emerging from PPI assays in live cells definitely demonstrates the potential of creative screening design in drug discovery campaigns with applications that go beyond the scope of HCV.

# Bibliographie

1. **Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV.** 1975. Transfusion-associated hepatitis not due to viral hepatitis type A or B. N Engl J Med **292:**767-770.
2. **Prince AM, Brotman B, Grady GF, Kuhns WJ, Hazzi C, Levine RW, Millian SJ.** 1974. Long-incubation post-transfusion hepatitis without serological evidence of exposure to hepatitis-B virus. Lancet **2:**241-246.
3. **Shimizu YK, Oomura M, Abe K, Uno M, Yamada E, Ono Y, Shikata T.** 1985. Production of antibody associated with non-A, non-B hepatitis in a chimpanzee lymphoblastoid cell line established by in vitro transformation with Epstein-Barr virus. Proc Natl Acad Sci U S A **82:**2138-2142.
4. **Aach RD, Szmuness W, Mosley JW, Hollinger FB, Kahn RA, Stevens CE, Edwards VM, Werch J.** 1981. Serum alanine aminotransferase of donors in relation to the risk of non-A,non-B hepatitis in recipients: the transfusion-transmitted viruses study. N Engl J Med **304:**989-994.
5. **Berman M, Alter HJ, Ishak KG, Purcell RH, Jones EA.** 1979. The chronic sequelae of non-A, non-B hepatitis. Annals of internal medicine **91:**1-6.
6. **Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359-362.
7. **Organization WH.** 1996. Fighting Disease, Fostering Development. World Health Report**:**34-35.
8. **Lavanchy D.** 2011. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect **17:**107-115.
9. **Sagnelli E, Stroffolini T, Mele A, Almasio P, Coppola N, Ferrigno L, Scolastico C, Onofrio M, Imparato M, Filippini P.** 2005. The importance of HCV on the burden of chronic liver disease in Italy: a multicenter prevalence study of 9,997 cases. Journal of medical virology **75:**522-527.
10. **Micallef JM, Kaldor JM, Dore GJ.** 2006. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. Journal of viral hepatitis **13:**34-41.
11. **Thein HH, Yi Q, Dore GJ, Krahn MD.** 2008. Estimation of stage-specific fibrosis progression rates in chronic hepatitis C virus infection: a meta-analysis and meta-regression. Hepatology **48:**418-431.
12. **Grebely J, Dore GJ.** 2011. What is killing people with hepatitis C virus infection? Seminars in liver disease **31:**331-339.
13. **Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, Alvarado M, Anderson HR, Anderson LM, Andrews KG, Atkinson C, Baddour LM, Barker-Collo S, Bartels DH, Bell ML, Benjamin EJ, Bennett D, Bhalla K, Bikbov B, Bin Abdulhak A, Birbeck G, Blyth**

**F, Bolliger I, Boufous S, Bucello C, Burch M, Burney P, Carapetis J, Chen H, Chou D, Chugh SS, Coffeng LE, Colan SD, Colquhoun S, Colson KE, Condon J, Connor MD, Cooper LT, Corriere M, Cortinovis M, de Vaccaro KC, Couser W, Cowie BC, Criqui MH, Cross M, Dabhadkar KC, Dahodwala N, De Leo D, Degenhardt L, Delossantos A, Denenberg J, Des Jarlais DC, Dharmaratne SD, Dorsey ER, Driscoll T, Duber H, Ebel B, Erwin PJ, Espindola P, Ezzati M, Feigin V, Flaxman AD, Forouzanfar MH, Fowkes FG, Franklin R, Fransen M, Freeman MK, Gabriel SE, Gakidou E, Gaspari F, Gillum RF, Gonzalez-Medina D, Halasa YA, Haring D, Harrison JE, Havmoeller R, Hay RJ, Hoen B, Hotez PJ, Hoy D, Jacobsen KH, James SL, Jasrasaria R, Jayaraman S, Johns N, Karthikeyan G, Kassebaum N, Keren A, Khoo JP, Knowlton LM, Kobusingye O, Koranteng A, Krishnamurthi R, Lipnick M, Lipshultz SE, Ohno SL, Mabweijano J, MacIntyre MF, Mallinger L, March L, Marks GB, Marks R, Matsumori A, Matzopoulos R, Mayosi BM, McAnulty JH, McDermott MM, McGrath J, Mensah GA, Merriman TR, Michaud C, Miller M, Miller TR, Mock C, Mocumbi AO, Mokdad AA, Moran A, Mulholland K, Nair MN, Naldi L, Narayan KM, Nasseri K, Norman P, O'Donnell M, Omer SB, Ortblad K, Osborne R, Ozgediz D, Pahari B, Pandian JD, Rivero AP, Padilla RP, Perez-Ruiz F, Perico N, Phillips D, Pierce K, Pope CA, 3rd, Porrini E, Pourmalek F, Raju M, Ranganathan D, Rehm JT, Rein DB, Remuzzi G, Rivara FP, Roberts T, De Leon FR, Rosenfeld LC, Rushton L, Sacco RL, Salomon JA, Sampson U, Sanman E, Schwebel DC, Segui-Gomez M, Shepard DS, Singh D, Singleton J, Sliwa K, Smith E, Steer A, Taylor JA, Thomas B, Tleyjeh IM, Towbin JA, Truelsen T, Undurraga EA, Venketasubramanian N, Vijayakumar L, Vos T, Wagner GR, Wang M, Wang W, Watt K, Weinstock MA, Weintraub R, Wilkinson JD, Woolf AD, Wulf S, Yeh PH, Yip P, Zabetian A, Zheng ZJ, Lopez AD, Murray CJ, AlMazroa MA, Memish ZA.** 2012. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet **380:**2095-2128.

14. **Alter MJ.** 2007. Epidemiology of hepatitis C virus infection. World journal of gastroenterology **13:**2436-2441.
15. **Wasley A, Alter MJ.** 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. Seminars in liver disease **20:**1-16.
16. **Paez Jimenez A, Sharaf Eldin N, Rimlinger F, El-Daly M, El-Hariri H, El-Hoseiny M, Mohsen A, Mostafa A, Delarocque-Astagneau E, Abdel-Hamid M, Fontanet A, Mohamed MK, Thiers V.** 2010. HCV iatrogenic and intrafamilial transmission in Greater Cairo, Egypt. Gut **59:**1554-1560.
17. **Deuffic-Burban S, Mohamed MK, Larouze B, Carrat F, Valleron AJ.** 2006. Expected increase in hepatitis C-related mortality in Egypt due to pre-2000 infections. Journal of hepatology **44:**455-461.
18. **Tanaka H, Imai Y, Hiramatsu N, Ito Y, Imanaka K, Oshita M, Hijioka T, Katayama K, Yabuuchi I, Yoshihara H, Inoue A, Kato M, Takehara T, Tamura S, Kasahara A, Hayashi N, Tsukuma H.** 2008. Declining incidence of hepatocellular carcinoma in Osaka, Japan, from 1990 to 2003. Annals of internal medicine **148:**820-826.

19. **Arfe A, Malvezzi M, Bertuccio P, Decarli A, La Vecchia C, Negri E.** 2011. Cancer mortality trend analysis in Italy, 1970-2007. European journal of cancer prevention : the official journal of the European Cancer Prevention Organisation **20:**364-374.
20. **Amin J, O'Connell D, Bartlett M, Tracey E, Kaldor J, Law M, Dore G.** 2007. Liver cancer and hepatitis B and C in New South Wales, 1990-2002: a linkage study. Australian and New Zealand journal of public health **31:**475-482.
21. **Davis GL, Alter MJ, El-Serag H, Poynard T, Jennings LW.** 2010. Aging of hepatitis C virus (HCV)-infected persons in the United States: a multiple cohort model of HCV prevalence and disease progression. Gastroenterology **138:**513-521, 521 e511-516.
22. **Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS.** 1998. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. Science **282:**103-107.
23. **Geller R, Estada Ú, Peris JB, Andreu I, Bou J-V, Garijo R, Cuevas JM, Sabariegos R, Mas A, Sanjuán R.** 2016. Highly heterogeneous mutation rates in the hepatitis C virus genome. Nature Microbiology **1:**16045.
24. **Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin IT, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A.** 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology **42:**962-973.
25. **Aitken C, McCaw R, Jardine D, Bowden S, Higgs P, Nguyen O, Crofts N, Hellard M.** 2004. Change in hepatitis C virus genotype in injecting drug users. Journal of medical virology **74:**543-545.
26. **Eyster ME, Sherman KE, Goedert JJ, Katsoulidou A, Hatzakis A.** 1999. Prevalence and changes in hepatitis C virus genotypes among multitransfused persons with hemophilia. The Multicenter Hemophilia Cohort Study. The Journal of infectious diseases **179:**1062-1069.
27. **Zein NN.** 2000. Clinical significance of hepatitis C virus genotypes. Clinical microbiology reviews **13:**223-235.
28. **Ohno T, Lau JY.** 1996. The "gold-standard," accuracy, and the current concepts: hepatitis C virus genotype and viremia. Hepatology **24:**1312-1315.
29. **Simmonds P.** 2013. The origin of hepatitis C virus. Current topics in microbiology and immunology **369:**1-15.
30. **Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS.** 1999. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med **341:**556-562.
31. **Cornberg M, Razavi HA, Alberti A, Bernasconi E, Buti M, Cooper C, Dalgard O, Dillion JF, Flisiak R, Forns X, Frankova S, Goldis A, Goulis I, Halota W, Hunyady B, Lagging M, Largen A, Makara M, Manolakopoulos S, Marcellin P, Marinho RT, Pol S, Poynard T, Puoti M, Sagalova O, Sibbel S, Simon K, Wallace C, Young K, Yurdaydin C, Zuckerman E, Negro F, Zeuzem S.** 2011. A systematic review of hepatitis C virus epidemiology in Europe, Canada and Israel. Liver Int **31 Suppl 2:**30-60.
32. **Kamal SM, Nasser IA.** 2008. Hepatitis C genotype 4: What we know and what we don't yet know. Hepatology **47:**1371-1383.

33. **Murphy DG, Willems B, Vincelette J, Bernier L, Cote J, Delage G.** 1996. Biological and clinicopathological features associated with hepatitis C virus type 5 infections. Journal of hepatology **24:**109-113.

34. **Pham VH, Nguyen HD, Ho PT, Banh DV, Pham HL, Pham PH, Lu L, Abe K.** 2011. Very high prevalence of hepatitis C virus genotype 6 variants in southern Vietnam: large-scale survey based on sequence determination. Jpn J Infect Dis **64:**537-539.

35. **Murphy DG, Sablon E, Chamberland J, Fournier E, Dandavino R, Tremblay CL.** 2015. Hepatitis C virus genotype 7, a new genotype originating from central Africa. Journal of clinical microbiology **53:**967-972.

36. **Chlabicz S, Flisiak R, Kowalczuk O, Grzeszczuk A, Pytel-Krolczuk B, Prokopowicz D, Chyczewski L.** 2008. Changing HCV genotypes distribution in Poland--relation to source and time of infection. J Clin Virol **42:**156-159.

37. **Tallo T, Norder H, Tefanova V, Krispin T, Schmidt J, Ilmoja M, Orgulas K, Pruunsild K, Priimagi L, Magnius LO.** 2007. Genetic characterization of hepatitis C virus strains in Estonia: fluctuations in the predominating subtype with time. Journal of medical virology **79:**374-382.

38. **Hajarizadeh B, Grebely J, Dore GJ.** 2013. Epidemiology and natural history of HCV infection. Nature reviews. Gastroenterology & hepatology **10:**553-562.

39. **Shi Q, Jiang J, Luo G.** 2013. Syndecan-1 serves as the major receptor for attachment of hepatitis C virus to the surfaces of hepatocytes. J Virol **87:**6866-6875.

40. **Lefevre M, Felmlee DJ, Parnot M, Baumert TF, Schuster C.** 2014. Syndecan 4 is involved in mediating HCV entry through interaction with lipoviral particle-associated apolipoprotein E. PloS one **9:**e95550.

41. **Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, Roitelman J, Barbaras R, Graber P, Ghersa P, Smolarsky M, Funaro A, Malavasi F, Larrey D, Coste J, Fabre JM, Sa-Cunha A, Maurel P.** 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. Journal of hepatology **46:**411-419.

42. **Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J **21:**5017-5025.

43. **Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S.** 1998. Binding of hepatitis C virus to CD81. Science **282:**938-941.

44. **Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature **446:**801-805.

45. **Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM.** 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature **457:**882-886.

46. **Sainz B, Jr., Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S, Marsh KA, Yu X, Chayama K, Alrefai WA, Uprichard SL.** 2012. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. Nature medicine **18:**281-285.

47. **Martin DN, Uprichard SL.** 2013. Identification of transferrin receptor 1 as a hepatitis C virus entry factor. Proc Natl Acad Sci U S A **110:**10777-10782.
48. **Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, Royer C, Fischer B, Zahid MN, Lavillette D, Fresquet J, Cosset FL, Rothenberg SM, Pietschmann T, Patel AH, Pessaux P, Doffoel M, Raffelsberger W, Poch O, McKeating JA, Brino L, Baumert TF.** 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nature medicine **17:**589-595.
49. **Dao Thi VL, Granier C, Zeisel MB, Guerin M, Mancip J, Granio O, Penin F, Lavillette D, Bartenschlager R, Baumert TF, Cosset FL, Dreux M.** 2012. Characterization of hepatitis C virus particle subpopulations reveals multiple usage of the scavenger receptor BI for entry steps. J Biol Chem **287:**31242-31257.
50. **Zahid MN, Turek M, Xiao F, Thi VL, Guerin M, Fofana I, Bachellier P, Thompson J, Delang L, Neyts J, Bankwitz D, Pietschmann T, Dreux M, Cosset FL, Grunert F, Baumert TF, Zeisel MB.** 2013. The postbinding activity of scavenger receptor class B type I mediates initiation of hepatitis C virus infection and viral dissemination. Hepatology **57:**492-504.
51. **Sharma NR, Mateu G, Dreux M, Grakoui A, Cosset FL, Melikyan GB.** 2011. Hepatitis C virus is primed by CD81 protein for low pH-dependent fusion. J Biol Chem **286:**30361-30376.
52. **Harris HJ, Davis C, Mullins JG, Hu K, Goodall M, Farquhar MJ, Mee CJ, McCaffrey K, Young S, Drummer H, Balfe P, McKeating JA.** 2010. Claudin association with CD81 defines hepatitis C virus entry. J Biol Chem **285:**21092-21102.
53. **Zona L, Lupberger J, Sidahmed-Adrar N, Thumann C, Harris HJ, Barnes A, Florentin J, Tawar RG, Xiao F, Turek M, Durand SC, Duong FH, Heim MH, Cosset FL, Hirsch I, Samuel D, Brino L, Zeisel MB, Le Naour F, McKeating JA, Baumert TF.** 2013. HRas signal transduction promotes hepatitis C virus cell entry by triggering assembly of the host tetraspanin receptor complex. Cell Host Microbe **13:**302-313.
54. **Wieland S, Makowska Z, Campana B, Calabrese D, Dill MT, Chung J, Chisari FV, Heim MH.** 2014. Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. Hepatology **59:**2121-2130.
55. **Lohmann V.** 2013. Hepatitis C virus RNA replication. Current topics in microbiology and immunology **369:**167-198.
56. **Friebe P, Lohmann V, Krieger N, Bartenschlager R.** 2001. Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. J Virol **75:**12047-12057.
57. **Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P.** 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science **309:**1577-1581.
58. **Li Y, Masaki T, Yamane D, McGivern DR, Lemon SM.** 2013. Competing and noncompeting activities of miR-122 and the 5' exonuclease Xrn1 in regulation of hepatitis C virus replication. Proc Natl Acad Sci U S A **110:**1881-1886.
59. **Shimakami T, Yamane D, Jangra RK, Kempf BJ, Spaniel C, Barton DJ, Lemon SM.** 2012. Stabilization of hepatitis C virus RNA by an Ago2-miR-122 complex. Proc Natl Acad Sci U S A **109:**941-946.

60. **You S, Rice CM.** 2008. 3' RNA elements in hepatitis C virus replication: kissing partners and long poly(U). J Virol **82:**184-195.
61. **Cantero-Camacho A, Gallego J.** 2015. The conserved 3'X terminal domain of hepatitis C virus genomic RNA forms a two-stem structure that promotes viral RNA dimerization. Nucleic acids research **43:**8529-8539.
62. **Moradpour D, Penin F.** 2013. Hepatitis C virus proteins: from structure to function. Current topics in microbiology and immunology **369:**113-142.
63. **Ferraris P, Blanchard E, Roingeard P.** 2010. Ultrastructural and biochemical analyses of hepatitis C virus-associated host cell membranes. J Gen Virol **91:**2230-2237.
64. **Gouttenoire J, Roingeard P, Penin F, Moradpour D.** 2010. Amphipathic alpha-helix AH2 is a major determinant for the oligomerization of hepatitis C virus nonstructural protein 4B. J Virol **84:**12529-12537.
65. **Madan V, Paul D, Lohmann V, Bartenschlager R.** 2014. Inhibition of HCV replication by cyclophilin antagonists is linked to replication fitness and occurs by inhibition of membranous web formation. Gastroenterology **146:**1361-1372 e1361-1369.
66. **Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K.** 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nature cell biology **9:**1089-1097.
67. **Lindenbach BD.** 2013. Virion assembly and release. Current topics in microbiology and immunology **369:**199-218.
68. **Huang H, Sun F, Owen DM, Li W, Chen Y, Gale M, Jr., Ye J.** 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc Natl Acad Sci U S A **104:**5848-5853.
69. **Yao H, Ye J.** 2008. Long chain acyl-CoA synthetase 3-mediated phosphatidylcholine synthesis is required for assembly of very low density lipoproteins in human hepatoma Huh7 cells. J Biol Chem **283:**849-854.
70. **Li X, Jiang H, Qu L, Yao W, Cai H, Chen L, Peng T.** 2014. Hepatocyte nuclear factor 4alpha and downstream secreted phospholipase A2 GXIIB regulate production of infectious hepatitis C virus. J Virol **88:**612-627.
71. **Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Brechot C.** 1997. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci U S A **94:**1200-1205.
72. **Boulant S, Targett-Adams P, McLauchlan J.** 2007. Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus. J Gen Virol **88:**2204-2213.
73. **Shavinskaya A, Boulant S, Penin F, McLauchlan J, Bartenschlager R.** 2007. The lipid droplet binding domain of hepatitis C virus core protein is a major determinant for efficient virus assembly. J Biol Chem **282:**37158-37169.
74. **Herker E, Harris C, Hernandez C, Carpentier A, Kaehlcke K, Rosenberg AR, Farese RV, Jr., Ott M.** 2010. Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. Nature medicine **16:**1295-1298.

75. **Menzel N, Fischl W, Hueging K, Bankwitz D, Frentzen A, Haid S, Gentzsch J, Kaderali L, Bartenschlager R, Pietschmann T.** 2012. MAP-kinase regulated cytosolic phospholipase A2 activity is essential for production of infectious hepatitis C virus particles. PLoS pathogens **8:**e1002829.
76. **Li Q, Pene V, Krishnamurthy S, Cha H, Liang TJ.** 2013. Hepatitis C virus infection activates an innate pathway involving IKK-alpha in lipogenesis and viral assembly. Nature medicine **19:**722-729.
77. **Dubuisson J, Hsu HH, Cheung RC, Greenberg HB, Russell DG, Rice CM.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J Virol **68:**6147-6160.
78. **Ma Y, Anantpadma M, Timpe JM, Shanmugam S, Singh SM, Lemon SM, Yi M.** 2011. Hepatitis C virus NS2 protein serves as a scaffold for virus assembly by interacting with both structural and nonstructural proteins. J Virol **85:**86-97.
79. **Popescu CI, Callens N, Trinel D, Roingeard P, Moradpour D, Descamps V, Duverlie G, Penin F, Heliot L, Rouille Y, Dubuisson J.** 2011. NS2 protein of hepatitis C virus interacts with structural and non-structural proteins towards virus assembly. PLoS pathogens **7:**e1001278.
80. **Tellinghuisen TL, Foss KL, Treadaway J.** 2008. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. PLoS pathogens **4:**e1000032.
81. **Chatel-Chaix L, Melancon P, Racine ME, Baril M, Lamarre D.** 2011. Y-box-binding protein 1 interacts with hepatitis C virus NS3/4A and influences the equilibrium between viral RNA replication and infectious particle production. J Virol **85:**11022-11037.
82. **Coller KE, Heaton NS, Berger KL, Cooper JD, Saunders JL, Randall G.** 2012. Molecular determinants and dynamics of hepatitis C virus secretion. PLoS pathogens **8:**e1002466.
83. **Welsch S, Muller B, Krausslich HG.** 2007. More than one door - Budding of enveloped viruses through cellular membranes. FEBS Lett **581:**2089-2097.
84. **Wozniak AL, Griffin S, Rowlands D, Harris M, Yi M, Lemon SM, Weinman SA.** 2010. Intracellular proton conductance of the hepatitis C virus p7 protein and its contribution to infectious virus production. PLoS pathogens **6:**e1001087.
85. **Gale M, Jr., Foy EM.** 2005. Evasion of intracellular host defence by hepatitis C virus. Nature **436:**939-945.
86. **Odendall C, Dixit E, Stavru F, Bierne H, Franz KM, Durbin AF, Boulant S, Gehrke L, Cossart P, Kagan JC.** 2014. Diverse intracellular pathogens activate type III interferon expression from peroxisomes. Nat Immunol **15:**717-726.
87. **Dixit E, Boulant S, Zhang Y, Lee AS, Odendall C, Shum B, Hacohen N, Chen ZJ, Whelan SP, Fransen M, Nibert ML, Superti-Furga G, Kagan JC.** 2010. Peroxisomes are signaling platforms for antiviral innate immunity. Cell **141:**668-681.
88. **Wack A, Terczynska-Dyla E, Hartmann R.** 2015. Guarding the frontiers: the biology of type III interferons. Nat Immunol **16:**802-809.
89. **Sommereyns C, Paul S, Staeheli P, Michiels T.** 2008. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS pathogens **4:**e1000017.

90. **Kohli A, Zhang X, Yang J, Russell RS, Donnelly RP, Sheikh F, Sherman A, Young H, Imamichi T, Lempicki RA, Masur H, Kottilil S.** 2012. Distinct and overlapping genomic profiles and antiviral effects of Interferon-lambda and -alpha on HCV-infected and noninfected hepatoma cells. Journal of viral hepatitis **19:**843-853.
91. **Li XD, Sun L, Seth RB, Pineda G, Chen ZJ.** 2005. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. Proc Natl Acad Sci U S A **102:**17717-17722.
92. **Li K, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M, Jr., Lemon SM.** 2005. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. Proc Natl Acad Sci U S A **102:**2992-2997.
93. **Lin W, Kim SS, Yeung E, Kamegaya Y, Blackard JT, Kim KA, Holtzman MJ, Chung RT.** 2006. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. J Virol **80:**9226-9235.
94. **Yao ZQ, Waggoner SN, Cruise MW, Hall C, Xie X, Oldach DW, Hahn YS.** 2005. SOCS1 and SOCS3 are targeted by hepatitis C virus core/gC1qR ligation to inhibit T-cell function. J Virol **79:**15417-15429.
95. **Germain MA, Chatel-Chaix L, Gagne B, Bonneil E, Thibault P, Pradezynski F, de Chassey B, Meyniel-Schicklin L, Lotteau V, Baril M, Lamarre D.** 2014. Elucidating novel hepatitis C virus-host interactions using combined mass spectrometry and functional genomics approaches. Mol Cell Proteomics **13:**184-203.
96. **Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM.** 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science **285:**107-110.
97. **Pawlotsky JM, Germanidis G.** 1999. The non-structural 5A protein of hepatitis C virus. Journal of viral hepatitis **6:**343-356.
98. **Taguchi T, Nagano-Fujii M, Akutsu M, Kadoya H, Ohgimoto S, Ishido S, Hotta H.** 2004. Hepatitis C virus NS5A protein interacts with 2',5'-oligoadenylate synthetase and inhibits antiviral activity of IFN in an IFN sensitivity-determining region-independent manner. J Gen Virol **85:**959-969.
99. **Loo YM, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM, Gale M, Jr.** 2006. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc Natl Acad Sci U S A **103:**6001-6006.
100. **Swartz ML.** 1991. Intron A (interferon-alpha-2b recombinant) for injection/Schering-Plough Corporation. Gastroenterol Nurs **14:**40-43.
101. **Tong MJ, Reddy KR, Lee WM, Pockros PJ, Hoefs JC, Keeffe EB, Hollinger FB, Hathcote EJ, White H, Foust RT, Jensen DM, Krawitt EL, Fromm H, Black M, Blatt LM, Klein M, Lubina J.** 1997. Treatment of chronic hepatitis C with consensus interferon: a multicenter, randomized, controlled trial. Consensus Interferon Study Group. Hepatology **26:**747-754.
102. **Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT, Robins RK.** 1972. Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. Science **177:**705-706.
103. **Bailon P, Palleroni A, Schaffer CA, Spence CL, Fung WJ, Porter JE, Ehrlich GK, Pan W, Xu ZX, Modi MW, Farid A, Berthold W, Graves M.** 2001. Rational design

of a potent, long-lasting form of interferon: a 40 kDa branched polyethylene glycol-conjugated interferon alpha-2a for the treatment of hepatitis C. Bioconjug Chem **12:**195-202.

104. **Lee SS, Bain VG, Peltekian K, Krajden M, Yoshida EM, Deschenes M, Heathcote J, Bailey RJ, Simonyi S, Sherman M, Canadian Pegasys Study G.** 2006. Treating chronic hepatitis C with pegylated interferon alfa-2a (40 KD) and ribavirin in clinical practice. Aliment Pharmacol Ther **23:**397-408.
105. **Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, Bos M, Cameron DR, Cartier M, Cordingley MG, Faucher AM, Goudreau N, Kawai SH, Kukolj G, Lagace L, LaPlante SR, Narjes H, Poupart MA, Rancourt J, Sentjens RE, St George R, Simoneau B, Steinmann G, Thibeault D, Tsantrizos YS, Weldon SM, Yong CL, Llinas-Brunet M.** 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature **426:**186-189.
106. **Shiffman ML, Esteban R.** 2012. Triple therapy for HCV genotype 1 infection: telaprevir or boceprevir? Liver Int **32 Suppl 1:**54-60.
107. **Lawitz E, Mangia A, Wyles D, Rodriguez-Torres M, Hassanein T, Gordon SC, Schultz M, Davis MN, Kayali Z, Reddy KR, Jacobson IM, Kowdley KV, Nyberg L, Subramanian GM, Hyland RH, Arterburn S, Jiang D, McNally J, Brainard D, Symonds WT, McHutchison JG, Sheikh AM, Younossi Z, Gane EJ.** 2013. Sofosbuvir for previously untreated chronic hepatitis C infection. N Engl J Med **368:**1878-1887.
108. **Cho NJ, Dvory-Sobol H, Lee C, Cho SJ, Bryson P, Masek M, Elazar M, Frank CW, Glenn JS.** 2010. Identification of a class of HCV inhibitors directed against the nonstructural protein NS4B. Sci Transl Med **2:**15ra16.
109. **Chatel-Chaix L, Germain MA, Gotte M, Lamarre D.** 2012. Direct-acting and host-targeting HCV inhibitors: current and future directions. Curr Opin Virol **2:**588-598.
110. **De Francesco R, Steinkuhler C.** 2000. Structure and function of the hepatitis C virus NS3-NS4A serine proteinase. Current topics in microbiology and immunology **242:**149-169.
111. **Suzich JA, Tamura JK, Palmer-Hill F, Warrener P, Grakoui A, Rice CM, Feinstone SM, Collett MS.** 1993. Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. J Virol **67:**6152-6158.
112. **Rajkowitsch L, Chen D, Stampfl S, Semrad K, Waldsich C, Mayer O, Jantsch MF, Konrat R, Blasi U, Schroeder R.** 2007. RNA chaperones, RNA annealers and RNA helicases. RNA Biol **4:**118-130.
113. **Jarvis TC, Newport JW, von Hippel PH.** 1991. Stimulation of the processivity of the DNA polymerase of bacteriophage T4 by the polymerase accessory proteins. The role of ATP hydrolysis. J Biol Chem **266:**1830-1840.
114. **Sun S, Rao VB, Rossmann MG.** 2010. Genome packaging in viruses. Curr Opin Struct Biol **20:**114-120.
115. **Jennings TA, Mackintosh SG, Harrison MK, Sikora D, Sikora B, Dave B, Tackett AJ, Cameron CE, Raney KD.** 2009. NS3 helicase from the hepatitis C virus can function as a monomer or oligomer depending on enzyme and substrate concentrations. J Biol Chem **284:**4806-4814.

116. **Lin C, Thomson JA, Rice CM.** 1995. A central region in the hepatitis C virus NS4A protein allows formation of an active NS3-NS4A serine proteinase complex in vivo and in vitro. J Virol **69:**4373-4380.
117. **Kim JL, Morgenstern KA, Lin C, Fox T, Dwyer MD, Landro JA, Chambers SP, Markland W, Lepre CA, O'Malley ET, Harbeson SL, Rice CM, Murcko MA, Caron PR, Thomson JA.** 1996. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. Cell **87:**343-355.
118. **Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J Virol **67:**2832-2843.
119. **Schechter I, Berger A.** 1967. On the size of the active site in proteases. I. Papain. Biochem Biophys Res Commun **27:**157-162.
120. **Landro JA, Raybuck SA, Luong YP, O'Malley ET, Harbeson SL, Morgenstern KA, Rao G, Livingston DJ.** 1997. Mechanistic role of an NS4A peptide cofactor with the truncated NS3 protease of hepatitis C virus: elucidation of the NS4A stimulatory effect via kinetic analysis and inhibitor mapping. Biochemistry **36:**9340-9348.
121. **Urbani A, Bianchi E, Narjes F, Tramontano A, De Francesco R, Steinkuhler C, Pessi A.** 1997. Substrate specificity of the hepatitis C virus serine protease NS3. J Biol Chem **272:**9204-9209.
122. **Zhang R, Durkin J, Windsor WT, McNemar C, Ramanathan L, Le HV.** 1997. Probing the substrate specificity of hepatitis C virus NS3 serine protease by using synthetic peptides. J Virol **71:**6208-6213.
123. **De Francesco R, Pessi A, Steinkuhler C.** 1999. Mechanisms of hepatitis C virus NS3 proteinase inhibitors. Journal of viral hepatitis **6 Suppl 1:**23-30.
124. **Sperandio D, Gangloff AR, Litvak J, Goldsmith R, Hataye JM, Wang VR, Shelton EJ, Elrod K, Janc JW, Clark JM, Rice K, Weinheimer S, Yeung KS, Meanwell NA, Hernandez D, Staab AJ, Venables BL, Spencer JR.** 2002. Highly potent non-peptidic inhibitors of the HCV NS3/NS4A serine protease. Bioorg Med Chem Lett **12:**3129-3133.
125. **De Francesco R, Tomei L, Altamura S, Summa V, Migliaccio G.** 2003. Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase. Antiviral Res **58:**1-16.
126. **Sanderson PE.** 1999. Small, noncovalent serine protease inhibitors. Med Res Rev **19:**179-197.
127. **Lin C, Kwong AD, Perni RB.** 2006. Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. Infect Disord Drug Targets **6:**3-16.
128. **Llinas-Brunet M, Bailey M, Fazal G, Goulet S, Halmos T, Laplante S, Maurice R, Poirier M, Poupart MA, Thibeault D, Wernic D, Lamarre D.** 1998. Peptide-based inhibitors of the hepatitis C virus serine protease. Bioorg Med Chem Lett **8:**1713-1718.
129. **Steinkuhler C, Biasiol G, Brunetti M, Urbani A, Koch U, Cortese R, Pessi A, De Francesco R.** 1998. Product inhibition of the hepatitis C virus NS3 protease. Biochemistry **37:**8899-8905.
130. **Lu W, Apostol I, Qasim MA, Warne N, Wynn R, Zhang WL, Anderson S, Chiang YW, Ogin E, Rothberg I, Ryan K, Laskowski M, Jr.** 1997. Binding of amino acid side-chains to S1 cavities of serine proteinases. J Mol Biol **266:**441-461.

131. **Llinas-Brunet M, Bailey M, Fazal G, Ghiro E, Gorys V, Goulet S, Halmos T, Maurice R, Poirier M, Poupart MA, Rancourt J, Thibeault D, Wernic D, Lamarre D.** 2000. Highly potent and selective peptide-based inhibitors of the hepatitis C virus serine protease: towards smaller inhibitors. Bioorg Med Chem Lett **10:**2267-2270.
132. **Rancourt J, Cameron DR, Gorys V, Lamarre D, Poirier M, Thibeault D, Llinas-Brunet M.** 2004. Peptide-based inhibitors of the hepatitis C virus NS3 protease: structure-activity relationship at the C-terminal position. J Med Chem **47:**2511-2522.
133. **Tsantrizos YS, Bolger G, Bonneau P, Cameron DR, Goudreau N, Kukolj G, LaPlante SR, Llinas-Brunet M, Nar H, Lamarre D.** 2003. Macrocyclic inhibitors of the NS3 protease as potential therapeutic agents of hepatitis C virus infection. Angew Chem Int Ed Engl **42:**1356-1360.
134. **Behrens SE, Tomei L, De Francesco R.** 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J **15:**12-22.
135. **Ivashkina N, Wolk B, Lohmann V, Bartenschlager R, Blum HE, Penin F, Moradpour D.** 2002. The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. J Virol **76:**13088-13093.
136. **Tomei L, Vitale RL, Incitti I, Serafini S, Altamura S, Vitelli A, De Francesco R.** 2000. Biochemical characterization of a hepatitis C virus RNA-dependent RNA polymerase mutant lacking the C-terminal hydrophobic sequence. J Gen Virol **81:**759-767.
137. **Schmidt-Mende J, Bieck E, Hugle T, Penin F, Rice CM, Blum HE, Moradpour D.** 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J Biol Chem **276:**44052-44063.
138. **Ferrari E, Wright-Minogue J, Fang JW, Baroudy BM, Lau JY, Hong Z.** 1999. Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in Escherichia coli. J Virol **73:**1649-1654.
139. **Ago H, Adachi T, Yoshida A, Yamamoto M, Habuka N, Yatsunami K, Miyano M.** 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure **7:**1417-1426.
140. **Bressanelli S, Tomei L, Roussel A, Incitti I, Vitale RL, Mathieu M, De Francesco R, Rey FA.** 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc Natl Acad Sci U S A **96:**13034-13039.
141. **Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC.** 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nature structural biology **6:**937-943.
142. **Luo G, Hamatake RK, Mathis DM, Racela J, Rigat KL, Lemm J, Colonno RJ.** 2000. De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J Virol **74:**851-863.
143. **De Clercq E.** 2001. Antiviral drugs: current state of the art. J Clin Virol **22:**73-89.
144. **Summa V, Petrocchi A, Pace P, Matassa VG, De Francesco R, Altamura S, Tomei L, Koch U, Neuner P.** 2004. Discovery of alpha,gamma-diketo acids as potent selective and reversible inhibitors of hepatitis C virus NS5b RNA-dependent RNA polymerase. J Med Chem **47:**14-17.
145. **Bhatia HK, Singh H, Grewal N, Natt NK.** 2014. Sofosbuvir: A novel treatment option for chronic hepatitis C infection. J Pharmacol Pharmacother **5:**278-284.

146. **Le Pogam S, Jiang WR, Leveque V, Rajyaguru S, Ma H, Kang H, Jiang S, Singer M, Ali S, Klumpp K, Smith D, Symons J, Cammack N, Najera I.** 2006. In vitro selected Con1 subgenomic replicons resistant to 2'-C-methyl-cytidine or to R1479 show lack of cross resistance. Virology **351:**349-359.

147. **Le Pogam S, Kang H, Harris SF, Leveque V, Giannetti AM, Ali S, Jiang WR, Rajyaguru S, Tavares G, Oshiro C, Hendricks T, Klumpp K, Symons J, Browner MF, Cammack N, Najera I.** 2006. Selection and characterization of replicon variants dually resistant to thumb- and palm-binding nonnucleoside polymerase inhibitors of the hepatitis C virus. J Virol **80:**6146-6154.

148. **Tomei L, Altamura S, Bartholomew L, Biroccio A, Ceccacci A, Pacini L, Narjes F, Gennari N, Bisbocci M, Incitti I, Orsatti L, Harper S, Stansfield I, Rowley M, De Francesco R, Migliaccio G.** 2003. Mechanism of action and antiviral activity of benzimidazole-based allosteric inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J Virol **77:**13225-13231.

149. **Kati W, Koev G, Irvin M, Beyer J, Liu Y, Krishnan P, Reisch T, Mondal R, Wagner R, Molla A, Maring C, Collins C.** 2015. In vitro activity and resistance profile of dasabuvir, a nonnucleoside hepatitis C virus polymerase inhibitor. Antimicrob Agents Chemother **59:**1505-1511.

150. **Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM.** 2004. The NS5A protein of hepatitis C virus is a zinc metalloprotein. J Biol Chem **279:**48576-48587.

151. **Kim J, Lee D, Choe J.** 1999. Hepatitis C virus NS5A protein is phosphorylated by casein kinase II. Biochem Biophys Res Commun **257:**777-781.

152. **Chen YC, Su WC, Huang JY, Chao TC, Jeng KS, Machida K, Lai MM.** 2010. Polo-like kinase 1 is involved in hepatitis C virus replication by hyperphosphorylating NS5A. J Virol **84:**7983-7993.

153. **Katze MG, Kwieciszewski B, Goodlett DR, Blakely CM, Neddermann P, Tan SL, Aebersold R.** 2000. Ser(2194) is a highly conserved major phosphorylation site of the hepatitis C virus nonstructural protein NS5A. Virology **278:**501-513.

154. **Reiss S, Harak C, Romero-Brey I, Radujkovic D, Klein R, Ruggieri A, Rebhan I, Bartenschlager R, Lohmann V.** 2013. The lipid kinase phosphatidylinositol-4 kinase III alpha regulates the phosphorylation status of hepatitis C virus NS5A. PLoS pathogens **9:**e1003359.

155. **Shi ST, Polyak SJ, Tu H, Taylor DR, Gretch DR, Lai MM.** 2002. Hepatitis C virus NS5A colocalizes with the core protein on lipid droplets and interacts with apolipoproteins. Virology **292:**198-210.

156. **Penin F, Brass V, Appel N, Ramboarina S, Montserret R, Ficheux D, Blum HE, Bartenschlager R, Moradpour D.** 2004. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J Biol Chem **279:**40835-40843.

157. **Lim PJ, Chatterji U, Cordek D, Sharma SD, Garcia-Rivera JA, Cameron CE, Lin K, Targett-Adams P, Gallay PA.** 2012. Correlation between NS5A dimerization and hepatitis C virus replication. J Biol Chem **287:**30861-30873.

158. **Gupta G, Qin H, Song J.** 2012. Intrinsically unstructured domain 3 of hepatitis C Virus NS5A forms a "fuzzy complex" with VAPB-MSP domain which carries ALS-causing mutations. PloS one **7:**e39261.

159. **Liang Y, Ye H, Kang CB, Yoon HS.** 2007. Domain 2 of nonstructural protein 5A (NS5A) of hepatitis C virus is natively unfolded. Biochemistry **46:**11550-11558.

160. **Reiss S, Rebhan I, Backes P, Romero-Brey I, Erfle H, Matula P, Kaderali L, Poenisch M, Blankenburg H, Hiet MS, Longerich T, Diehl S, Ramirez F, Balla T, Rohr K, Kaul A, Buhler S, Pepperkok R, Lengauer T, Albrecht M, Eils R, Schirmacher P, Lohmann V, Bartenschlager R.** 2011. Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. Cell Host Microbe **9:**32-45.

161. **Li H, Yang X, Yang G, Hong Z, Zhou L, Yin P, Xiao Y, Chen L, Chung RT, Zhang L.** 2014. Hepatitis C virus NS5A hijacks ARFGAP1 to maintain a phosphatidylinositol 4-phosphate-enriched microenvironment. J Virol **88:**5956-5966.

162. **Paul D, Hoppe S, Saher G, Krijnse-Locker J, Bartenschlager R.** 2013. Morphological and biochemical characterization of the membranous hepatitis C virus replication compartment. J Virol **87:**10612-10627.

163. **Macdonald A, Harris M.** 2004. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol **85:**2485-2502.

164. **Lemm JA, O'Boyle D, 2nd, Liu M, Nower PT, Colonno R, Deshpande MS, Snyder LB, Martin SW, St Laurent DR, Serrano-Wu MH, Romine JL, Meanwell NA, Gao M.** 2010. Identification of hepatitis C virus NS5A inhibitors. J Virol **84:**482-491.

165. **Romine JL, St Laurent DR, Leet JE, Martin SW, Serrano-Wu MH, Yang F, Gao M, O'Boyle DR, 2nd, Lemm JA, Sun JH, Nower PT, Huang XS, Deshpande MS, Meanwell NA, Snyder LB.** 2011. Inhibitors of HCV NS5A: From Iminothiazolidinones to Symmetrical Stilbenes. ACS Med Chem Lett **2:**224-229.

166. **Cordek DG, Bechtel JT, Maynard AT, Kazmierski WM, Cameron CE.** 2011. Targeting the Ns5a Protein of Hcv: An Emerging Option. Drugs Future **36:**691-711.

167. **Fridell RA, Wang C, Sun JH, O'Boyle DR, 2nd, Nower P, Valera L, Qiu D, Roberts S, Huang X, Kienzle B, Bifano M, Nettles RE, Gao M.** 2011. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. Hepatology **54:**1924-1935.

168. **Nettles JH, Stanton RA, Broyde J, Amblard F, Zhang H, Zhou L, Shi J, McBrayer TR, Whitaker T, Coats SJ, Kohler JJ, Schinazi RF.** 2014. Asymmetric binding to NS5A by daclatasvir (BMS-790052) and analogs suggests two novel modes of HCV inhibition. J Med Chem **57:**10031-10043.

169. **Targett-Adams P, Graham EJ, Middleton J, Palmer A, Shaw SM, Lavender H, Brain P, Tran TD, Jones LH, Wakenhut F, Stammen B, Pryde D, Pickford C, Westby M.** 2011. Small molecules targeting hepatitis C virus-encoded NS5A cause subcellular redistribution of their target: insights into compound modes of action. J Virol **85:**6353-6368.

170. **Guedj J, Dahari H, Rong L, Sansone ND, Nettles RE, Cotler SJ, Layden TJ, Uprichard SL, Perelson AS.** 2013. Modeling shows that the NS5A inhibitor daclatasvir has two modes of action and yields a shorter estimate of the hepatitis C virus half-life. Proc Natl Acad Sci U S A **110:**3991-3996.

171. **Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A.** 1992. Internal ribosome entry site within hepatitis C virus RNA. J Virol **66:**1476-1483.

172. **Welch PJ, Tritz R, Yei S, Leavitt M, Yu M, Barber J.** 1996. A potential therapeutic application of hairpin ribozymes: in vitro and in vivo studies of gene therapy for hepatitis C virus infection. Gene Ther **3:**994-1001.
173. **Wu CH, Wu GY.** 1998. Targeted inhibition of hepatitis C virus-directed gene expression in human hepatoma cell lines. Gastroenterology **114:**1304-1312.
174. **Soler M, McHutchison JG, Kwoh TJ, Dorr FA, Pawlotsky JM.** 2004. Virological effects of ISIS 14803, an antisense oligonucleotide inhibitor of hepatitis C virus (HCV) internal ribosome entry site (IRES), on HCV IRES in chronic hepatitis C patients and examination of the potential role of primary and secondary HCV resistance in the outcome of treatment. Antivir Ther **9:**953-968.
175. **van der Ree MH, van der Meer AJ, de Bruijne J, Maan R, van Vliet A, Welzel TM, Zeuzem S, Lawitz EJ, Rodriguez-Torres M, Kupcova V, Wiercinska-Drapalo A, Hodges MR, Janssen HL, Reesink HW.** 2014. Long-term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients. Antiviral Res **111:**53-59.
176. **Liu Z, Yang F, Robotham JM, Tang H.** 2009. Critical role of cyclophilin A and its prolyl-peptidyl isomerase activity in the structure and function of the hepatitis C virus replication complex. J Virol **83:**6554-6565.
177. **Chatterji U, Lim P, Bobardt MD, Wieland S, Cordek DG, Vuagniaux G, Chisari F, Cameron CE, Targett-Adams P, Parkinson T, Gallay PA.** 2010. HCV resistance to cyclosporin A does not correlate with a resistance of the NS5A-cyclophilin A interaction to cyclophilin inhibitors. Journal of hepatology **53:**50-56.
178. **Coelmont L, Hanoulle X, Chatterji U, Berger C, Snoeck J, Bobardt M, Lim P, Vliegen I, Paeshuyse J, Vuagniaux G, Vandamme AM, Bartenschlager R, Gallay P, Lippens G, Neyts J.** 2010. DEB025 (Alisporivir) inhibits hepatitis C virus replication by preventing a cyclophilin A induced cis-trans isomerisation in domain II of NS5A. PloS one **5:**e13687.
179. **Liu J, Farmer JD, Jr., Lane WS, Friedman J, Weissman I, Schreiber SL.** 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66:**807-815.
180. **Naoumov NV.** 2014. Cyclophilin inhibition as potential therapy for liver diseases. Journal of hepatology **61:**1166-1174.
181. **Landrieu I, Hanoulle X, Bonachera F, Hamel A, Sibille N, Yin Y, Wieruszeski JM, Horvath D, Wei Q, Vuagniaux G, Lippens G.** 2010. Structural basis for the non-immunosuppressive character of the cyclosporin A analogue Debio 025. Biochemistry **49:**4679-4686.
182. **Billich A, Hammerschmid F, Peichl P, Wenger R, Zenke G, Quesniaux V, Rosenwirth B.** 1995. Mode of action of SDZ NIM 811, a nonimmunosuppressive cyclosporin A analog with activity against human immunodeficiency virus (HIV) type 1: interference with HIV protein-cyclophilin A interactions. J Virol **69:**2451-2461.
183. **Ciesek S, Steinmann E, Wedemeyer H, Manns MP, Neyts J, Tautz N, Madan V, Bartenschlager R, von Hahn T, Pietschmann T.** 2009. Cyclosporine A inhibits hepatitis C virus nonstructural protein 2 through cyclophilin A. Hepatology **50:**1638-1645.

184. **Albecka A, Belouzard S, Op de Beeck A, Descamps V, Goueslain L, Bertrand-Michel J, Terce F, Duverlie G, Rouille Y, Dubuisson J.** 2012. Role of low-density lipoprotein receptor in the hepatitis C virus life cycle. Hepatology **55:**998-1007.

185. **Barth H, Schnober EK, Zhang F, Linhardt RJ, Depla E, Boson B, Cosset FL, Patel AH, Blum HE, Baumert TF.** 2006. Viral and cellular determinants of the hepatitis C virus envelope-heparan sulfate interaction. J Virol **80:**10579-10590.

186. **Hamed MR, Brown RJ, Zothner C, Urbanowicz RA, Mason CP, Krarup A, McClure CP, Irving WL, Ball JK, Harris M, Hickling TP, Tarr AW.** 2014. Recombinant human L-ficolin directly neutralizes hepatitis C virus entry. Journal of innate immunity **6:**676-684.

187. **Si Y, Liu S, Liu X, Jacobs JL, Cheng M, Niu Y, Jin Q, Wang T, Yang W.** 2012. A human claudin-1-derived peptide inhibits hepatitis C virus entry. Hepatology **56:**507-515.

188. **Hong W, Lang Y, Li T, Zeng Z, Song Y, Wu Y, Li W, Cao Z.** 2015. A p7 Ion Channel-derived Peptide Inhibits Hepatitis C Virus Infection in Vitro. J Biol Chem **290:**23254-23263.

189. **Helle F, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J.** 2006. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. J Biol Chem **281:**25177-25183.

190. **Khanal M, Barras A, Vausselin T, Feneant L, Boukherroub R, Siriwardena A, Dubuisson J, Szunerits S.** 2015. Boronic acid-modified lipid nanocapsules: a novel platform for the highly efficient inhibition of hepatitis C viral entry. Nanoscale **7:**1392-1402.

191. **VanCompernolle SE, Wiznycia AV, Rush JR, Dhanasekaran M, Baures PW, Todd SC.** 2003. Small molecule inhibition of hepatitis C virus E2 binding to CD81. Virology **314:**371-380.

192. **Ciesek S, von Hahn T, Colpitts CC, Schang LM, Friesland M, Steinmann J, Manns MP, Ott M, Wedemeyer H, Meuleman P, Pietschmann T, Steinmann E.** 2011. The green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. Hepatology **54:**1947-1955.

193. **Masson D, Koseki M, Ishibashi M, Larson CJ, Miller SG, King BD, Tall AR.** 2009. Increased HDL cholesterol and apoA-I in humans and mice treated with a novel SR-BI inhibitor. Arterioscler Thromb Vasc Biol **29:**2054-2060.

194. **Syder AJ, Lee H, Zeisel MB, Grove J, Soulier E, Macdonald J, Chow S, Chang J, Baumert TF, McKeating JA, McKelvy J, Wong-Staal F.** 2011. Small molecule scavenger receptor BI antagonists are potent HCV entry inhibitors. Journal of hepatology **54:**48-55.

195. **Zhu H, Wong-Staal F, Lee H, Syder A, McKelvy J, Schooley RT, Wyles DL.** 2012. Evaluation of ITX 5061, a scavenger receptor B1 antagonist: resistance selection and activity in combination with other hepatitis C virus antivirals. The Journal of infectious diseases **205:**656-662.

196. **Meertens L, Bertaux C, Dragic T.** 2006. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. J Virol **80:**11571-11578.

197. **Chamoun-Emanuelli AM, Pecheur EI, Simeon RL, Huang D, Cremer PS, Chen Z.** 2013. Phenothiazines inhibit hepatitis C virus entry, likely by increasing the fluidity of cholesterol-rich membranes. Antimicrob Agents Chemother **57:**2571-2581.
198. **Boriskin YS, Leneva IA, Pecheur EI, Polyak SJ.** 2008. Arbidol: a broad-spectrum antiviral compound that blocks viral fusion. Curr Med Chem **15:**997-1005.
199. **Muir AJ, Arora S, Everson G, Flisiak R, George J, Ghalib R, Gordon SC, Gray T, Greenbloom S, Hassanein T, Hillson J, Horga MA, Jacobson IM, Jeffers L, Kowdley KV, Lawitz E, Lueth S, Rodriguez-Torres M, Rustgi V, Shemanski L, Shiffman ML, Srinivasan S, Vargas HE, Vierling JM, Xu D, Lopez-Talavera JC, Zeuzem S, group Es.** 2014. A randomized phase 2b study of peginterferon lambda-1a for the treatment of chronic HCV infection. Journal of hepatology **61:**1238-1246.
200. **Marcellin P, Gish RG, Gitlin N, Heise J, Halliman DG, Chun E, Rodriguez-Torres M.** 2010. Safety and efficacy of viramidine versus ribavirin in ViSER2: randomized, double-blind study in therapy-naive hepatitis C patients. Journal of hepatology **52:**32-38.
201. **Bergmann JF, de Bruijne J, Hotho DM, de Knegt RJ, Boonstra A, Weegink CJ, van Vliet AA, van de Wetering J, Fletcher SP, Bauman LA, Rahimy M, Appleman JR, Freddo JL, Janssen HL, Reesink HW.** 2011. Randomised clinical trial: anti-viral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV. Aliment Pharmacol Ther **34:**443-453.
202. **Kowdley KV, Lawitz E, Crespo I, Hassanein T, Davis MN, DeMicco M, Bernstein DE, Afdhal N, Vierling JM, Gordon SC, Anderson JK, Hyland RH, Dvory-Sobol H, An D, Hindes RG, Albanis E, Symonds WT, Berrey MM, Nelson DR, Jacobson IM.** 2013. Sofosbuvir with pegylated interferon alfa-2a and ribavirin for treatment-naive patients with hepatitis C genotype-1 infection (ATOMIC): an open-label, randomised, multicentre phase 2 trial. Lancet **381:**2100-2107.
203. **Kwo P, Gitlin N, Nahass R, Bernstein D, Etzkorn K, Rojter S, Schiff E, Davis M, Ruane P, Younes Z, Kalmeijer R, Sinha R, Peeters M, Lenz O, Fevery B, De La Rosa G, Scott J, Witek J.** 2016. Simeprevir plus sofosbuvir (12 and 8 weeks) in hepatitis C virus genotype 1-infected patients without cirrhosis: OPTIMIST-1, a phase 3, randomized study. Hepatology **64:**370-380.
204. **Sulkowski MS, Gardiner DF, Rodriguez-Torres M, Reddy KR, Hassanein T, Jacobson I, Lawitz E, Lok AS, Hinestrosa F, Thuluvath PJ, Schwartz H, Nelson DR, Everson GT, Eley T, Wind-Rotolo M, Huang SP, Gao M, Hernandez D, McPhee F, Sherman D, Hindes R, Symonds W, Pasquinelli C, Grasela DM, Group AIS.** 2014. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. N Engl J Med **370:**211-221.
205. **Clark VC, Peter JA, Nelson DR.** 2013. New therapeutic strategies in HCV: second-generation protease inhibitors. Liver Int **33 Suppl 1:**80-84.
206. **Mizokami M, Yokosuka O, Takehara T, Sakamoto N, Korenaga M, Mochizuki H, Nakane K, Enomoto H, Ikeda F, Yanase M, Toyoda H, Genda T, Umemura T, Yatsuhashi H, Ide T, Toda N, Nirei K, Ueno Y, Nishigaki Y, Betular J, Gao B, Ishizaki A, Omote M, Mo H, Garrison K, Pang PS, Knox SJ, Symonds WT, McHutchison JG, Izumi N, Omata M.** 2015. Ledipasvir and sofosbuvir fixed-dose combination with and without ribavirin for 12 weeks in treatment-naive and previously

treated Japanese patients with genotype 1 hepatitis C: an open-label, randomised, phase 3 trial. Lancet Infect Dis **15:**645-653.

207. **Ferenci P, Bernstein D, Lalezari J, Cohen D, Luo Y, Cooper C, Tam E, Marinho RT, Tsai N, Nyberg A, Box TD, Younes Z, Enayati P, Green S, Baruch Y, Bhandari BR, Caruntu FA, Sepe T, Chulanov V, Janczewska E, Rizzardini G, Gervain J, Planas R, Moreno C, Hassanein T, Xie W, King M, Podsadecki T, Reddy KR, Study P-I, Study P-I.** 2014. ABT-450/r-ombitasvir and dasabuvir with or without ribavirin for HCV. N Engl J Med **370:**1983-1992.
208. **Poordad F, Hezode C, Trinh R, Kowdley KV, Zeuzem S, Agarwal K, Shiffman ML, Wedemeyer H, Berg T, Yoshida EM, Forns X, Lovell SS, Da Silva-Tillmann B, Collins CA, Campbell AL, Podsadecki T, Bernstein B.** 2014. ABT-450/r-ombitasvir and dasabuvir with ribavirin for hepatitis C with cirrhosis. N Engl J Med **370:**1973-1982.
209. **Keating GM.** 2016. Ombitasvir/Paritaprevir/Ritonavir: A Review in Chronic HCV Genotype 4 Infection. Drugs **76:**1203-1211.
210. **Lawitz E, Gane E, Pearlman B, Tam E, Ghesquiere W, Guyader D, Alric L, Bronowicki JP, Lester L, Sievert W, Ghalib R, Balart L, Sund F, Lagging M, Dutko F, Shaughnessy M, Hwang P, Howe AY, Wahl J, Robertson M, Barr E, Haber B.** 2015. Efficacy and safety of 12 weeks versus 18 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin for hepatitis C virus genotype 1 infection in previously untreated patients with cirrhosis and patients with previous null response with or without cirrhosis (C-WORTHY): a randomised, open-label phase 2 trial. Lancet **385:**1075-1086.
211. **Sulkowski M, Hezode C, Gerstoft J, Vierling JM, Mallolas J, Pol S, Kugelmas M, Murillo A, Weis N, Nahass R, Shibolet O, Serfaty L, Bourliere M, DeJesus E, Zuckerman E, Dutko F, Shaughnessy M, Hwang P, Howe AY, Wahl J, Robertson M, Barr E, Haber B.** 2015. Efficacy and safety of 8 weeks versus 12 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin in patients with hepatitis C virus genotype 1 mono-infection and HIV/hepatitis C virus co-infection (C-WORTHY): a randomised, open-label phase 2 trial. Lancet **385:**1087-1097.
212. **Zeuzem S, Ghalib R, Reddy KR, Pockros PJ, Ben Ari Z, Zhao Y, Brown DD, Wan S, DiNubile MJ, Nguyen BY, Robertson MN, Wahl J, Barr E, Butterton JR.** 2015. Grazoprevir-Elbasvir Combination Therapy for Treatment-Naive Cirrhotic and Noncirrhotic Patients With Chronic Hepatitis C Virus Genotype 1, 4, or 6 Infection: A Randomized Trial. Annals of internal medicine **163:**1-13.
213. **Younossi ZM, Stepanova M, Sulkowski M, Foster GR, Reau N, Mangia A, Patel K, Brau N, Roberts SK, Afdhal N, Nader F, Henry L, Hunt S.** 2016. Ribavirin-Free Regimen With Sofosbuvir and Velpatasvir is Associated With High Efficacy and Improvement of Patient-Reported Outcomes in Patients With Genotypes 2 and 3 Chronic Hepatitis C: Results From Astral-2 and 3 Clinical Trials. Clin Infect Dis.
214. **Feld JJ, Jacobson IM, Hezode C, Asselah T, Ruane PJ, Gruener N, Abergel A, Mangia A, Lai CL, Chan HL, Mazzotta F, Moreno C, Yoshida E, Shafran SD, Towner WJ, Tran TT, McNally J, Osinusi A, Svarovskaia E, Zhu Y, Brainard DM, McHutchison JG, Agarwal K, Zeuzem S, Investigators A-.** 2015. Sofosbuvir

and Velpatasvir for HCV Genotype 1, 2, 4, 5, and 6 Infection. N Engl J Med **373:**2599-2607.

215. **Scheel TK, Rice CM.** 2013. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nature medicine **19:**837-849.
216. **Prince AM, Brotman B.** 1994. The biology of hepatitis C virus infection. Lessons learned from chimpanzees. Current studies in hematology and blood transfusion**:**195-207.
217. **Walker CM.** 1997. Comparative features of hepatitis C virus infection in humans and chimpanzees. Springer seminars in immunopathology **19:**85-98.
218. **Bukh J.** 2012. Animal models for the study of hepatitis C virus infection and related liver disease. Gastroenterology **142:**1279-1287 e1273.
219. **Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP, Churchill TA, Lakey JR, Tyrrell DL, Kneteman NM.** 2001. Hepatitis C virus replication in mice with chimeric human livers. Nature medicine **7:**927-933.
220. **Vercauteren K, de Jong YP, Meuleman P.** 2014. HCV animal models and liver disease. Journal of hepatology **61:**S26-33.
221. **Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110-113.
222. **Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nature medicine **11:**791-796.
223. **Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623-626.
224. **Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV.** 2005. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A **102:**9294-9299.
225. **Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R.** 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A **103:**7408-7413.
226. **Blight KJ, McKeating JA, Rice CM.** 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol **76:**13001-13014.
227. **Sumpter R, Jr., Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M, Jr.** 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol **79:**2689-2699.
228. **Meex SJ, Andreo U, Sparks JD, Fisher EA.** 2011. Huh-7 or HepG2 cells: which is the better model for studying human apolipoprotein-B100 assembly and secretion? Journal of lipid research **52:**152-158.
229. **Woerz I, Lohmann V, Bartenschlager R.** 2009. Hepatitis C virus replicons: dinosaurs still in business? Journal of viral hepatitis **16:**1-9.
230. **Fields S, Song O.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245-246.

231. **Xu Y, Piston DW, Johnson CH.** 1999. A bioluminescence resonance energy transfer (BRET) system: application to interacting circadian clock proteins. Proc Natl Acad Sci U S A **96:**151-156.
232. **Racine ME.** 2008. Étude du réseau d'interactions entre les protéines du Virus de l'Hépatite C. Université de Montréal.
233. **Pfleger KD, Seeber RM, Eidne KA.** 2006. Bioluminescence resonance energy transfer (BRET) for the real-time detection of protein-protein interactions. Nature protocols **1:**337-345.
234. **Lorenz WW, McCann RO, Longiaru M, Cormier MJ.** 1991. Isolation and expression of a cDNA encoding Renilla reniformis luciferase. Proc Natl Acad Sci U S A **88:**4438-4442.
235. **Masters BR.** 2014. Paths to Förster's resonance energy transfer (FRET) theory. The European Physical Journal H **39:**87-139.
236. **Baril M, Racine ME, Penin F, Lamarre D.** 2009. MAVS dimer is a crucial signaling component of innate immunity and the target of hepatitis C virus NS3/4A protease. J Virol **83:**1299-1311.
237. **Baril M, Es-Saad S, Chatel-Chaix L, Fink K, Pham T, Raymond VA, Audette K, Guenier AS, Duchaine J, Servant M, Bilodeau M, Cohen E, Grandvaux N, Lamarre D.** 2013. Genome-wide RNAi screen reveals a new role of a WNT/CTNNB1 signaling pathway as negative regulator of virus-induced innate immune responses. PLoS pathogens **9:**e1003416.
238. **Berube P, Barbeau B, Cantin R, Sekaly RP, Tremblay M.** 1996. Repression of human immunodeficiency virus type 1 long terminal repeat-driven gene expression by binding of the virus to its primary cellular receptor, the CD4 molecule. J Virol **70:**4009-4016.
239. **Lin R, Mamane Y, Hiscott J.** 2000. Multiple regulatory domains control IRF-7 activity in response to virus infection. J Biol Chem **275:**34320-34327.
240. **Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J.** 2003. Triggering the interferon antiviral response through an IKK-related pathway. Science **300:**1148-1151.
241. **Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M.** 2000. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci U S A **97:**3684-3689.
242. **Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L.** 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol **72:**8463-8471.
243. **Mercier JF, Salahpour A, Angers S, Breit A, Bouvier M.** 2002. Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. J Biol Chem **277:**44925-44931.
244. **Kwofie SK, Schaefer U, Sundararajan VS, Bajic VB, Christoffels A.** 2011. HCVpro: hepatitis C virus protein interaction database. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases **11:**1971-1977.

245. **Zhang JH, Chung TD, Oldenburg KR.** 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen **4:**67-73.
246. **Rehman S, Ashfaq UA, Javed T.** 2011. Antiviral drugs against hepatitis C virus. Genet Vaccines Ther **9:**11.
247. **Thorne N, Shen M, Lea WA, Simeonov A, Lovell S, Auld DS, Inglese J.** 2012. Firefly luciferase in chemical biology: a compendium of inhibitors, mechanistic evaluation of chemotypes, and suggested use as a reporter. Chem Biol **19:**1060-1072.
248. **Romano KP, Ali A, Royer WE, Schiffer CA.** 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc Natl Acad Sci U S A **107:**20986-20991.
249. **Wu S, Kanda T, Nakamoto S, Imazeki F, Yokosuka O.** 2013. Hepatitis C virus protease inhibitor-resistance mutations: our experience and review. World journal of gastroenterology **19:**8940-8948.
250. **van den Hoff MJ, Christoffels VM, Labruyere WT, Moorman AF, Lamers WH.** 1995. Electrotransfection with "intracellular" buffer. Methods Mol Biol **48:**185-197.
251. **Yi M.** 2010. Hepatitis C virus: propagation, quantification, and storage. Curr Protoc Microbiol **Chapter 15:**Unit 15D 11.
252. **Schmitt M, Scrima N, Radujkovic D, Caillet-Saguy C, Simister PC, Friebe P, Wicht O, Klein R, Bartenschlager R, Lohmann V, Bressanelli S.** 2011. A comprehensive structure-function comparison of hepatitis C virus strain JFH1 and J6 polymerases reveals a key residue stimulating replication in cell culture across genotypes. J Virol **85:**2565-2581.
253. **Gagné B.** 2015. The nuclear pore complex and its transporters : from virus-host interactors to subverting the innate antiviral immunity. Université de Montréal.
254. **Kutay U, Izaurralde E, Bischoff FR, Mattaj IW, Gorlich D.** 1997. Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. EMBO J **16:**1153-1163.
255. **Cingolani G, Petosa C, Weis K, Muller CW.** 1999. Structure of importin-beta bound to the IBB domain of importin-alpha. Nature **399:**221-229.
256. **Sekimoto T, Imamoto N, Nakajima K, Hirano T, Yoneda Y.** 1997. Extracellular signal-dependent nuclear import of Stat1 is mediated by nuclear pore-targeting complex formation with NPI-1, but not Rch1. EMBO J **16:**7067-7077.
257. **Taniguchi T, Takaoka A.** 2002. The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Current opinion in immunology **14:**111-116.
258. **Sherman MY, Goldberg AL.** 1996. Involvement of molecular chaperones in intracellular protein breakdown. Exs **77:**57-78.
259. **Park SG, Jung G.** 2001. Human hepatitis B virus polymerase interacts with the molecular chaperonin Hsp60. J Virol **75:**6962-6968.
260. **Ghosh JC, Dohi T, Kang BH, Altieri DC.** 2008. Hsp60 regulation of tumor cell apoptosis. J Biol Chem **283:**5188-5194.
261. **Kang SM, Kim SJ, Kim JH, Lee W, Kim GW, Lee KH, Choi KY, Oh JW.** 2009. Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-alpha-mediated apoptosis. Cancer letters **279:**230-237.

262. **Tanaka Y, Kanai F, Kawakami T, Tateishi K, Ijichi H, Kawabe T, Arakawa Y, Kawakami T, Nishimura T, Shirakata Y, Koike K, Omata M.** 2004. Interaction of the hepatitis B virus X protein (HBx) with heat shock protein 60 enhances HBx-mediated apoptosis. Biochem Biophys Res Commun **318:**461-469.
263. **Lin L, Pan S, Zhao J, Liu C, Wang P, Fu L, Xu X, Jin M, Zhang A.** 2014. HSPD1 interacts with IRF3 to facilitate interferon-beta induction. PloS one **9:**e114874.
264. **Tellinghuisen TL, Marcotrigiano J, Rice CM.** 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature **435:**374-379.
265. **Salahpour A, Espinoza S, Masri B, Lam V, Barak LS, Gainetdinov RR.** 2012. BRET biosensors to study GPCR biology, pharmacology, and signal transduction. Frontiers in endocrinology **3:**105.
266. **Gosert R, Jendrsczok W, Berke JM, Brass V, Blum HE, Moradpour D.** 2005. Characterization of nonstructural protein membrane anchor deletion mutants expressed in the context of the hepatitis C virus polyprotein. J Virol **79:**7911-7917.
267. **Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C, Caron PR.** 1998. Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. Structure **6:**89-100.
268. **Khu YL, Koh E, Lim SP, Tan YH, Brenner S, Lim SG, Hong WJ, Goh PY.** 2001. Mutations that affect dimer formation and helicase activity of the hepatitis C virus helicase. J Virol **75:**205-214.
269. **Lorenz IC, Marcotrigiano J, Dentzer TG, Rice CM.** 2006. Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. Nature **442:**831-835.
270. **Tong L.** 2002. Viral proteases. Chemical reviews **102:**4609-4626.
271. **Yu X, Sainz B, Jr., Petukhov PA, Uprichard SL.** 2012. Identification of hepatitis C virus inhibitors targeting different aspects of infection using a cell-based assay. Antimicrob Agents Chemother **56:**6109-6120.
272. **Mousseau G, Kota S, Takahashi V, Frick DN, Strosberg AD.** 2011. Dimerization-driven interaction of hepatitis C virus core protein with NS3 helicase. J Gen Virol **92:**101-111.
273. **Jones DM, Atoom AM, Zhang X, Kottilil S, Russell RS.** 2011. A genetic interaction between the core and NS3 proteins of hepatitis C virus is essential for production of infectious virus. J Virol **85:**12351-12361.

# Annex 1: Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response.

This annex is to state that I contributed to the paper "**Spliceosome SNRNP200 Promotes Viral RNA Sensing and IRF3 Activation of Antiviral Response."** by Tremblay et al., published in the peer-reviewed open-access journal "PLOS Pathogens" in July 2016. As secondary co-author, I performed experiments to address issues in previous revisions (unpublished), contributed to the overall flow and clarity of explanations and had meaningful input in the design of figures, particularly Figure 3 and Figure 10.

# Annex 2: HCV NS3/4A Protease Inhibitors and the Road to Effective Direct-Acting Antiviral Therapies.

This annex is to state that I contributed to the review chapter "**HCV NS3/4A Protease Inhibitors and the Road to Effective Direct-Acting Antiviral Therapies."** by Tremblay et al., that will be published in the Springer Japan book "Hepatitis C Virus II: Infection and Disease" by Miyamura et al. expected later in 2016. As secondary co-author, I contributed to the overall flow and clarity of the text as well as verified coherence with updated patent literature.