Sophoraflavenone G is the major active compound of sophora flavecens against HCV replication

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Abstract

Plant-derived compounds can provide an alternative approach to new medical therapies with features including effectiveness, lower cost of production and milder side effects in clinical experiments compared with conventional treatments. SF is an important ingredient in a number of Chinese medications used for the treatment of chronic HCV infection and represents an important and largely untapped source of potential antiviral compounds. Results of preliminary screening indicated that SF is the most effective and specific medicine against HCV replication among the 45 test Chinese medicines in HCV-JFH-1 infectious assay. Based on anti-viral guided extraction and fractionation, we discovered the most active anti-HCV compound of SF and MS, ^{1}H NMR, ^{13}C NMR, HMBC and NOESY assays confirm it to be SFG. SFG significantly suppressed HCV replication by almost 90% at a concentration of 10μm. In the HCV replicon system, we demonstrated that SFG inhibited HCV RNA replication. No significant cytotoxicity was observed with higher concentrations of SFG. We further showed that SFG induced lipid storage and increases in LDs size. Enlarged LDs are able to induce changes in the environment around LDs and thus interfere with HCV replication complex formation. Additionally, SFG significantly induced both HO-1 production and antiviral interferon responses that are able to affect different aspects of the HCV life cycle. These findings suggested that SFG could be a new potential drug for HCV treatment in the future.

Résumé

Les composés dérivés de plantes peuvent fournir une approche alternative aux nouvelles thérapies médicales, de par leur efficacité, leur coût de production faible et leurs effets secondaires bénins pendant les expériences cliniques comparé aux traitements conventionnels. *SF* est un ingrédient important dans de nombreux médicaments chinois utilisés pour le traitement des infections chroniques au virus de le HCV, et représente une source importante et largement inexploitée de composés potentiellement antiviraux. Suite au criblage de composés isolés de SF contre le HCV, dans des expériences d'infectivité du HCV-JFH-1, nous avons découvert le composé le plus actif, et les tests MS, ^{1}H NMR, ^{13}C NMR, HMBC et NOESY ont permis de l'identifier comme étant le SFG. SFG inhibe significativement la réplication du HVC (presque 90%) à une concentration de 10μM. Dans le système de réplicon du HCV, nous avons démontré que le SFG inhibe la réplication de l'ARN du HCV. Aucune cytotoxicité significative n'a été observée avec des concentrations plus élevées de SFG. Nous avons par la suite montrée que le SFG induit le stockage des lipides et l'augmentation de la taille des corps lipidiques. Des LDs plus grands peuvent induire des changements de l'environnement autour des LDs et donc d'interférer avec la formation du complexe de réplication du HCV. De plus, le SFG augmente de façon significative la production de la HO-1 et induit des réponses antivirales de type interféron, qui sont capables d'affecter différents aspects du cycle de vie du HCV. Ces résultats suggèrent que le SFG pourrait potentiellement devenir une nouvelle drogue pour le traitement du HCV dans le futur.

Publications

1. Olagnier D, Sze A, Bel Hadj S, Chiang C, Steel C, **Han X**, Routy JP, Lin R, Hiscott J, van Grevenynghe J. HTLV-1 tax-mediated inhibition of FOXO3a activity is critical for the persistence of terminally differentiated CD4+ T cells. PLoS Pathog. 2014 Dec 18; 10(12):e1004575. doi: 10.1371/journal.ppat.1004575. eCollection 2014 Dec.

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Contribution

Xiaoying Han

Generation of HCV virus stock

Inhibitory activity of SFG on HCV replication

Mechanisms of SFG anti-HCV activity

Qingwen Shi in Dr. Jianhui Wu's lab

Extraction and identification of SFG

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Abbreviations

^{1}H NMR	^{1}H nuclear magnetic resonance
3'X-tail	3' terminal nucleotides
3D	3-dimensional
^{13}C NMR	^{13}C nuclear magnetic resonance
aa	amino acid
ADP	adenosine diphosphate
AH	amphipathic helices
ALT	alanine transaminase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
AP-1	activator protein-1
ApoB	apolipoprotein B
ApoE	apolipoprotein E
ATP	adenosine triphosphate
CD81	cluster of differentiation 81
CK	casein kinase
CLDN	claudin
CM	Chinese medicine
CO	carbon monoxide
COX	cyclooxygenases
CREs	Cis-acting RNA elements
CypA	cyclophilin A
Da	dalton
DAAs	direct-acting antivirals
DALYs	disability-adjusted life years
DGAT	diacylglycerol acyltransferase
DMV	double-membrane vesicle

EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EtOAC	ethyl ethanoate
EVR	early virologic response
FAH	fumaryl acetoacetate hydrolase
FASN	fatty acid synthase
FDA	Food and Drug Administration
GAG	glycosaminoglycan
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
H	helice
HMBC	heteronuclear multible bond correlation spectroscopy
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	cell culture-derived HCV
HCVpp	HCV pseudoparticles
HDL	high-density lipoprotein
HHV-6	human herpes virus type 6
HO	heme oxygenase
HRP	horseradish peroxidase
HS	heparin sulfate
HT22	hippocampal neuronal cells
HTAs	host-targeted antivirals
Huh	human hepatoma cell line
HVR	hypervariable regions
IgVR	intergenotypic variable region
IRES	internal ribosome entry site
ISGs	IFN-stimulated genes
IVDU	intravenous drug users

JAK	janus kinase
JFH-1	Japanese patient suffering from fulminant hepatitis
JNKs	c-Jun N-terminal kinase
LCS	low complexity sequences
LDL	low-density lipoprotein
LDLR	LDL receptor
LDs	lipid droplets
LEL	large extracellular loop
LOX	lipoxygenases
LVP	lipoviral particles
MAPK	mitogen-activated protein kinases
MBD	membrane binding domain
MDA5	melanoma differentiation-associated gene 5
miRNAs	microRNA
MLV	murine leukemia virus
MP	metalloporphyrin
MRSA	methicillin-resistant Staphylococcus aureus
MS	mass spectrometry
MTP	microsomal triglyceride transfer protein
MV	measles virus
MW	membranous web
NASH	non-alcoholic steatohepatitis
NF-kB	nuclear transcription factor kappa-B
NNIs	non-nucleoside analog inhibitors
NOESY	nuclear overhauser effect spectroscopy
NP	nucleoprotein
Nrf2	NF-E2-related factor 2
NS	nonstructural
NT	nucleotides

NTBC	2- (2- nitro- 4- trifluoro- methylbenzoyl)- 1, 3- cyclohexanedione
NTP	nucleoside triphosphate
NTPase	nucleoside triphosphatase
NTRs	non-translated regions
NIs	nucleoside analog inhibitors
OR6	Huh-7 derived genome-length HCV-RNA replicon cells
ORF	open reading frame
P70S6K	p70S6 kinase
PBS	phosphate-buffered saline
PEG-IFN	pegylated interferon
PG	prostaglandins
PGE2	prostaglandins E2
PI3K	phosphatidylinositol 3-kinase
PI4KIIIa	phosphotidylinositol 4-kinase III alpha
PIP2	phopshotidylinositol 4-5-bisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PlK I	polo-like kinase I
PPARα	peroxisome-proliferator-activated receptor α
PP-IX	iron-protoporphyrin IX
PR8	influenza A virus PR8 strain
RBV	ribavirin
RdRp	RNA dependent RNA polymerase
RIG-I	retinoic acid-inducible gene I
RNA	ribonucleic acid
RVR	rapid virologic response
SCID	severe combined immunodeficiency

SE	sterol esters
SEL	small extracellular loop
SeV	sendai virus
SF	sophora flavescens
SFG	sophoraflavenone G
shRNA	short hairpin RNA
siRNA	small interfering RNA
SL	stem-loop structures
SnPP	tin protoporphyrin
SP	signal peptidase
SPP	signal peptide peptidase
SR-BI	scavenger receptor class B type I
SREBP	sterol regulatory element binding protein
STAT	signal transducers and activators of transcription
SVR	sustained virological response
TCID50	tissue culture infectious dose 50
TLR	toll-like receptor
TLC	thin layer chromatography
TMD	trans-membrane domain
uPA	urokinase-type plasminogen activator
VLDL	very-low-density lipoprotein
WHO	World Health Organization

Chapter 1- Rationale and objectives

Due to the limitations of presently used anti-HCV drugs and the advantages of herbal medicine, we wanted to discover the herbal medicines with anti-HCV activity and its precise active compound. During our investigation, we found that SF was one of the most effective herbal medicines that inhibited HCV infection. Additionally, we isolated and identified the most active compound of SF and studied its mechanisms.

This project was conducted in multiple steps. Firstly, the anti-HCV activity of different herbal medicines was detected by western blotting and SF was found to be the most active one. Secondly, pure compounds of SF were isolated and fractionated by column chromatography and TCL; **KS-211**, the most active anti-HCV compound isolated from SF was identified as SFG by MS, ^{1}H NMR, ^{13}C NMR, HMBC and NOESY analysis. Thirdly, enough JFH-1 viruses had to be generated from transfected Huh7.5 cells and HCV sub-genomic replicon system has to be constructed and selected. Fourthly, the relationship between LDs and SFG was studied. Finally, many host factors regulated by SFG or involved in HCV RNA replication were investigated by qPCR to determine which one is associated with the anti-HCV activity of SFG.

Chapter 2- General introduction

Since the identification of the hepatitis C virus (HCV) genome in 1989 by Choo *et al.,* it has been recognized that HCV is a major cause of chronic liver disease [1]. Patients with chronic HCV infection can generate approximately 10^{12} viral particles each day, representing a challenge for pharmacological treatment [2]. Over the past decades, many approaches have been made to study the characteristics of HCV in order to develop new and better treatments to combat the virus. This chapter presents a brief review of HCV discussing the epidemiology, genome organization, life cycle, the importance of lipid metabolism in HCV life cycle, model systems and treatments option for HCV. This chapter will also describe the mechanism of SFG, which is the most active compound against HCV purified from SF that is an important ingredient used in many traditional Chinese medical formulas for HCV treatment.

2.1 Epidemiology

2.1.1 Prevalence of HCV worldwide

HCV infection has become a major global health burden. About 54,000 deaths and 955,000 disability-adjusted life years (DALYs) are associated with acute HCV infection worldwide. The most recent estimates indicated that more than 185 million people have persistent HCV infection worldwide, which translates to 3% of the world's total population. There are approximately 4 million people newly diagnosed with HCV infection each year [3]. Chronic HCV infection is the major cause of fibrosing hepatitis that subsequently leads to cirrhosis and hepatocellular carcinoma (HCC), which is now the third leading cause of cancer related mortality worldwide. Data indicates that around 2% to 5% of HCC in patients is associated with HCV infection [4].

2.1.2 Genotypes and distribution of HCV

The distribution of HCV infection varies geographically. For example, the prevalence rate in the developed countries such as Germany (0.6%) [5], France (1.1%) [6] and Australia (1.1%) [7] are lower than those found in developing countries such as Pakistan (range between 2·4% and 6.5%) [8-10] and Egypt (22%) [11]. In Canada, 251,990 individuals (uncertainty interval 177,890 to 314,800) were presumed to live with HCV infection in 2013. Fortunately, this number is estimated to decline to 188,190 (uncertainty interval 124,330 to 247,200) by 2035, and the number of HCC patients due to HCV infection is also expected to decline by the same period [12].

A current study divides HCV into 7 genotypes (1 through 7) and further subdivided into 67 subtypes (a, b, c, etc.) [13]. Among these, genotype 1 is most commonly detected worldwide. Genotype 1b is also prevalent in Europe but more frequent in Japan (more than 70% of HCV infected patients in Japan). Genotype 2 is detected in clusters in the Mediterranean region. Genotype 3 is most common in the Indian subcontinent and among European intravenous drug users (IVDU) [14]. Genotype 4 is common in the Middle East and in Africa. In Egypt, almost 90% of HCV infection is caused by genotype 4; the proportion of infection due to this genotype has also risen with in the IVDU in European [15]. Genotype 5 is rarely found, and genotype 6 is mainly observed in south China and Vietnam [16, 17]. As of today, there is only one genotype 7 infection report and the virus was isolated in Canada from a Central African immigrant [18].

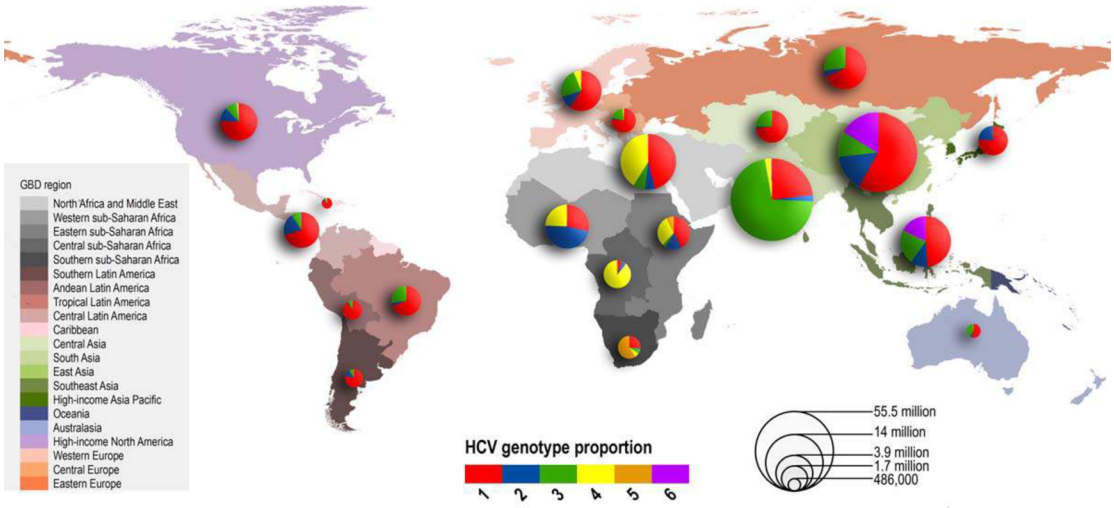

Figure 1: Global prevalence of HCV genotypes. Size of pie charts is proportional to the number of seroprevalence of HCV antibody. Figure is adapted from Jane P. Messina *et al.*, 2015 [19].

2.2 Virology

2.2.1 HCV viral genome and proteins

HCV, a member of the genus hepacivirus within the family flaviviridae, is an enveloped, positive-sense, single-stranded RNA virus of about 9600 nucleotides (NT) in length [20]. The viral genome encodes a single polyprotein of about 3,000 amino acids (aa) that is cleaved by cellular and viral proteases into structural proteins which include the core protein, the envelope glycoproteins (E) E1, E2, and non-structural (NS) proteins: p7 viroprin; NS2, NS3, NS4A, NS4B, NS5A and NS5B [21]. Among these proteins, the structural proteins (core, E1 and E2) form the viral particle; non-structural proteins NS3 to NS5B are involved in the replication of the viral genome; P7 and NS2, are dispensable for RNA replication but important for viral production.

2.2.1.1 Genome

HCV viral genome contains a single long open reading frame (ORF) flanked by highly structured 5' and 3' non-translated regions (NTRs) [22]. Both the 5’ NTR and 3’NTR play important roles in many different biological functions, such as viral genome reorganization, replication, translation initiation, and viral assembly. The 5’ NTR of HCV is ~341 NT in length and its secondary structural models reveal four distinct domains: domain I, II III and IV [23]. More over, the 5’ NTR harbors an internal ribosome entry site (IRES) that is composed of domain II, III, IV and the following 15 NT of 5’ NTR to initialize the cap-independent translation of the polyprotein [24]. The IRES also functions as a promoter for the initiation of RNA synthesis [25].

The 3’ UTR of HCV is composed of three distinct domains: an upstream genotype-specific variable region of about 40 nucleotides, a tract of long solely poly (U/UC) and 98 highly conserved 3’ terminal nucleotides (3’X-tail) sequence that contain three stem-loop (SL) structures [26, 27]. Studies indicated that the variable region is dispensable but it could reduce HCV replication significantly. Deletion of either the poly (U/UC) or the individual SL structures in 3’ X tail was not viable, indicating that the poly (U/UC) tract and the X tail is very important for HCV replication [28]. Cis-acting RNA elements (CREs) are required for viral RNA replication. It has been identified that CREs are located in the coding sequences of HCV genome, as well as in the NTR regions.

2.2.1.2 Structural proteins

2.2.1.2.1 Core protein

HCV viral nucleocapsid is composed of multiple copies of the core protein, which is the first protein encoded by the HCV ORF. After its translation, the polyprotein is targeted to the endoplasmic reticulum (ER) membrane by a signal peptide located between the core and the E1 and cleaved between residues 191 and 192 via host signal peptidase (SP), in order to release a 191 aa immature type of core protein (23kDa). To form the mature and stable form of core protein (21kDa of 177aa) that is found in cell culture and in viral particles isolated from virus-infected patients [29], the immature core protein is retained in the ER through a C-terminal hydrophobic region, getting further processed by the signal peptide peptidase (SPP) [30, 31].

2.2.1.2.2 Envelope glycoproteins

The other two main components of the HCV virion are the envelope glycoproteins E1 (aa 192 to 383) and E2 (aa 384 to 750), which play crucial roles in HCV entry into host cells. E1 and E2 are type I trans-membrane proteins with a highly glycosylated N-terminal ectodomain and a short C-terminal trans-membrane domain (TMD) that is essential for the subcellular localization and heterodimer formation of HCV. The non-covalent bonds between E1 and E2 were believed to be the major interaction in the past [32-34]. However, recent studies have shown that disulfide bridges exist between E1 and E2, suggesting that the E1-E2 heterodimer may also rely on covalent bonds [35]. Several hypervariable regions (HVR) including HVR-1, HVR-2 and intergenotypic variable region (IgVR) have been identified within the E2 envelope glycoprotein, differing by up to 80 % among different HCV genotypes. HVR-1 is an immunodominant region that elicits the neutralizing

antibodies response in natural infection [36]. Although humoral immune response does not target HVR2 and IgVR, their deletion would abolish E1-E2 heterodimer formation [37].

2.2.1.3 Nonstructural proteins

2.2.1.3.1 P7

P7 (aa 751 to 783) is a membrane-associated polypeptide. It has characteristics similar to those of viroporins, which oligomerize to form ion channels and play an important role in virus assembly and release [38-40]. The p7 monomer consists of two trans-membrane segments connected by a hydrophilic loop. Currently, the oligomerization of p7 monomers into hexamers [41, 42] or heptamers [43] is still debated. Data indicates that the ion channels formed by P7 are cation-selective channels, with a higher affinity for Ca^{2+} than K^{+} and Na^{+} [38, 39]. Besides that, p7-mediated transfer of protons across intracellular membranes was also reported. Interestingly, virus production was strongly reduced by replacing P7 with variants from other isolates, indicating that some of its functions are genotype specific [44, 45]. Although evidence has suggested P7 is essential for the assembly and release of HCV [45], its precise function is still unknown.

2.2.1.3.2 NS2

NS2 (aa 814 to 1029) is a long cysteine-protease, which contains a hydrophobic N-terminal membrane binding domain (MBD), and a C-terminal globular and cytosolic protease subdomain [46]. NS2 is not required for RNA replication, but it encodes a cysteine protease, which along with NS3, is responsible for the cleavage between NS2 and NS3 to liberate a fully

functional NS3 protein that is required for HCV replication [47, 48]. Recently, it was shown that in addition to its protease activity, NS2 also plays an important role in HCV assembly, and may be involved in a complex network of interactions with other viral proteins [49, 50].

2.2.1.3.3 NS3-4A complex

NS3 (aa 1030 to 1661) is a multifunctional protein, consisting of a serine protease located in its N-terminus and a RNA-stimulated nucleoside triphosphatase (NTPase)/RNA helicase in its C-terminus [51]. NS3 helicase is a member of the helicases superfamily 2 DExH/ D-box, and utilizes the energy from nucleoside triphosphate (NTP) to separate the double-stranded RNA into single strands, from a 3′ to 5′ direction, during the replication of viral genomic RNA [52]. The individual NS3 catalytic domain is normally inactive and requires the NS4A co-factor, *in vitro* and *in vivo,* [53] to form a non-covalent heterodimer complex, which is responsible for the cleavage of the viral polyprotein precursor at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junction regions [54-56]. NS4A (aa 1662 to 1715) is a 54-residue amphipathic peptide, containing a hydrophobic N-terminus and a hydrophilic C-terminus. The N-terminus of NS4A is involved in the integral membrane association of the NS3-4A complex [57], while the C-terminus has been shown to interact with other replicase components, contributing to HCV RNA replication and virus particle assembly [58, 59]. When the NS4A interacts with NS3 to form the NS3-4A complex, the N-terminal region of NS3 is rearranged, leading to optimized localization of residues His-57, Asp-81, and Ser-139 of the catalytic triad [60, 61]. X-ray crystal structures of the NS3-4A complex have shown that the protease adopts a chymotrypsin-like structure, stabilized by a Zn^{2+} ion by coordinating with Cys-97, Cys-99, Cys-145, and His-149 [62].

2.2.1.3.4 NS4B

NS4B (aa 1716 to 1976) is an integral membrane protein with a highly hydrophobic character. The structure is composed of an N-terminal portion, a central part harboring four putative trans-membrane passages, and a C-terminal part [63]. There are two amphipathic helices (AH) located at the N-terminal region (AH1 and AH2), four TMDs at the central portion and two helices (H1 and H2) at the C-terminal portion [64]. NS4B is able to induce the alteration of ER membrane to form the membranous web (MW), which consists of membranous vesicles and serves as a scaffold for the HCV replication complex [65, 66]. It has been reported that NS4B is able to bind viral RNA and is involved in viral assembly [67, 68]. In addition, NS4B is indicated to harbor NTPase activity, so it can hydrolyze guanosine triphosphate **(**GTP) and catalyze the synthesis of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) from two adenosine diphosphate (ADP) molecules [69].

2.2.1.3.5 NS5A

NS5A (aa 1977 to 2443) is a proline-rich, hydrophilic phosphoprotein that plays multiple roles in mediating HCV RNA replication and particle formation. NS5A is comprised of an N-terminal membrane anchor that contains an unconventional zinc-binding motif, followed by three domains (1, 2 and 3) separated by two low complexity sequences (LCS) [70]. There are predominantly two types of NS5A: the basally phosphorylated form (56 kDa) and the hyperphosphorylated form (58 kDa), which is conserved among HCV genotypes [71-73]. Phosphorylation of NS5A protein primarily occurs on a serine residue of the central and C-terminal regions [72]. It has been reported that many cellular kinases are responsible for the phosphorylation of NS5A either *in vitro* or *in vivo*, including the α isoform of casein kinase I (CKI α) [74],

CKII [75], polo-like kinase 1 (Plk1) [76], cAMP-dependent protein kinase A (PKA) [77], glycogen synthase kinase 3 (GSK3), protein kinase B (PKB), p70S6 kinase (p70S6K) as well as mitogen-activated protein kinases (MAPK) [78].

2.2.1.3.6 NS5B

NS5B (aa 2443 to 3033), the RNA dependent RNA polymerase (RdRp) of HCV, is a key enzyme that catalyzes both the generation of a complementary RNA (negative-strand) using the genome as a template and the subsequent synthesis of genomic a positive-strand RNA from this negative-strand RNA template [79, 80]. NS5B is composed of an N-terminus, a 40-aa linker and a C-terminal trans-membrane tail that occludes the active site. The N-terminus is the catalytic domain, and it is homologous to other viral RdRp enzymes that consist fingers, palm and thumb subdomains [81].

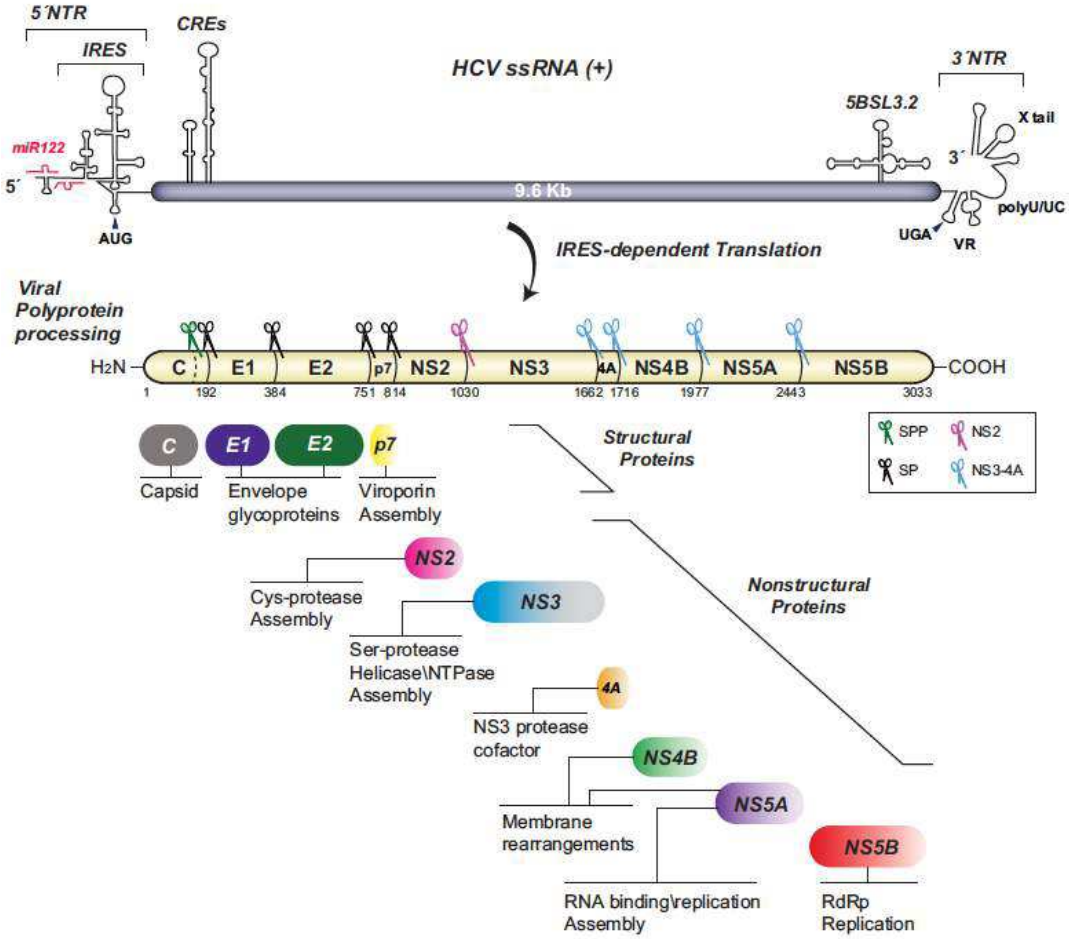

Figure 1: HCV viral genome and polyproteins. The structure of viral genome containing the ORF flanked by 5' and 3' NTRs is shown on the top. IRES and CREs and miR-122 Interaction sites are indicated. The polyprotein precursor and processing products are shown below. After its synthesis, HCV polyprotein is cleaved by viral and host encoded proteases that are indicated by scissors with different color. Figure is adapted from David Paul *et al.* [82].

2.2.2 HCV life cycle

2.2.2.1 HCV entry

Although multiple barriers exist on the cell surface to prevent virus entry, enveloped viruses still remarkably exploit fundamental cellular processes to gain access to their cellular hosts and release their genome. The interactions between HCV virus particles and host cells are largely defined by the E1 and E2. The development of HCV pseudoparticles (HCVpp) and cell culture-derived HCV (HCVcc) has been successfully used to study virus entry. Currently, the precise function of E1 in HCV entry is not completely understood, but it seems to be involved in the fusion process [83]. E2 seems to be the major protein held responsible for viral entry, because it binds well to two HCV entry factors: scavenger receptor class B, type I (SR-BI) and cluster of differentiation 81 (CD81) [84, 85]. The tetraspanin CD81 is a membrane protein that is composed of four trans-membrane domains: intracellular N-and C-terminus, two small (SEL) and one large (LEL) extracellular loop that is the soluble E2 binding component of CD81 [84]. The CD81 binding region of E2 is a conformational binding site that is composed of several non-contiguous sites further downstream in E2, and requires a correctly folded E2 [86]. SR-BI is a cell surface glycoprotein with two trans-membrane domains, two small cytoplasmic tails (N- and C-terminus) and a LEL and is expressed in many different mammalian cells but most abundantly in the liver and steroidogenic tissues [87]. The SR-BI binding site is located in the HVR-1of the N-terminus of E2 [85]. Several studies have indicated that cell entry of HCV needs the cooperation of SR-BI and CD81 [88]. Antibodies against HVR-1 and non-contiguous CD81 [89] binding sites of E2 block the cell entry of HCVpp [90]. However, It has been noted that the sole expression of the HCV receptors SR-BI and CD81 was often insufficient to allow HCVpp entry [91]. Claudin-1 (CLDN-1), a tight junction component has been recently identified as essential

for HCV entry [92]. The other members of CLDN, including CLDN-6 and CLDN-9 have also been found to contribute in HCV entry [93]. Glycosaminoglycans (GAGs) are linear polysaccharides that serve as low affinity first-attachment sites, and are able to interact with a number of different viruses including HCV [94]. Among these different types of GAGs, the highly sulfated GAGs such as heparin sulfate (HS), are thought to initiate the binding of HCV to the cell surface [95]. Because of the association of HCV virions in serum with low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL), the LDL receptor (LDLR) is suggested to be an attractive candidate receptor [96]. Together with GAGs and other cell surface proteins, LDLR functions as a primary collector of HCV particles for further targeting to CD81 and additional receptor components [97]. Indeed, binding of HCVpp to the target cells was strictly related to the expression levels of LDL-R even under the high expression of CD81 and SR-BI on the surface of cells [98].

After the attachment of virus to several receptors at the cell surface, HCV is taken up by clathrin-dependent endocytosis that transports incoming viruses into endosomes [99-101]. It is reported that the acidic pH (optimum at about pH 5.5) in endosomes, which play a key role in triggering penetration and uncoating of endosomes, is required to deliver the HCV genome into the host cell cytosol. Ergo, substances that inhibit acidification of early endosomes could block the entry of HCVpp and HCVcc [102]. Roohvand, F *et al.* have shown that microtubule affecting drugs, which inhibit microtubule formation or depolymerize or stabilization, block HCV infection. HCV core protein is able to bind to tublin to enhance microtubule plymerzation *in vitro*, suggesting that an intact microtubule network also plays an essential role in HCV cell entry and post-entry steps of the virus cycle [103].

2.2.2.2 HCV RNA translation and replication

2.2.2.2.1 HCV RNA translation

After being released into the cytoplasm, HCV RNA genome is directly translated into a polyprotein at the rough ER with the positive-strand HCV RNA as the template. HCV translation initiation needs the help of many cellular factors. It starts with the binding of HCV IRES to the small ribosomal 40S subunit [104], followed by the formation of 48S initiation complexes that require the recruitment of eukaryotic translation initiation factor 3 (eIF3) and eIF2 to associate with the initiator tRNA and GTP to form the ternary complex (eIF2-GTP-Met-tRNAi) [105]. Subsequently, eIF5 promotes the ternary complex by recognizing the AUG initiation codon and acting as a GTPase-activator protein for eIF2. Finally, after the GTP hydrolysis and eIF2 release, the large ribosomal 60S subunit joins to assemble the translation competent 80S ribosomes [103].

After the initiation of HCV RNA translation, the ORF of HCV is translated into a polyprotein that is processed by cellular and viral proteases into the mature structural and non-structural proteins. Host SP cleaves the structural proteins, whereas the non-structural proteins are cleaved by HCV proteases, NS2-3 and the NS3-4A.

2.2.2.2.2 HCV RNA replication

HCV RNA replication is indicated to occur predominantly on the specific MW. This could be induced by NS4B alone in Huh-7 cells harboring sub-genomic HCV replicons [106]; similar structure have been observed in the livers of HCV-infected chimpanzees [66]. The mechanisms of MW formation are still poorly understood. It is believed that the MW is derived from ER membranes

[66]. Analyses with electron microscopy and three-dimensional (3D) reconstructions of the MW have indicated that this structure is predominantly composed of double-membrane vesicles (DMVs), which are 150nm in average diameter and embedded in a membrane matrix and accumulate in parallel to the peak of RNA replication [106]. Formation of MW facilitates HCV RNA replication in several ways, including the organization of the HCV RNA replication complex that contains all of the viral nonstructural proteins, replicating RNA and other host factors; the protection of viral RNA from recognition by innate sensors (e.g. RIG-I, retinoic acid-inducible gene I, and MDA5, melanoma differentiation-associated gene 5) or RNA interference [66, 107].

The 3′ NTR of HCV genome is imperative for the initiation and regulation of negative-strand RNA synthesis and contributes to viral RNA replication [28, 108]. NS5B is the key enzyme responsible for HCV RNA synthesis through a primer-dependent mechanism or de novo. [80, 109]. Although it has not been demonstrated, the de novo RNA synthesis method seems to be the physiological mode of initiation of RNA synthesis in HCV infected cells [110]. Because of the high error rate of NS5B, HCV replication generates a heterogeneous population of viral variants. Even within a single patient, HCV exists not as a single genotype but a collection of microvariants, centering on a master sequence [111].

2.2.2.2.2.1 Host factors

Many host factors have been identified as important components required by the replication of HCV genome. For example, the liver specific microRNA-122 (miR-122) that comprising >50% of mature miRNAs in human hepatocytes regulates the expression of numerous hepatic genes and plays a critical role in

HCV replication [112]. Unless other miRNAs that bind to the 3'NTR of mRNA to promote mRNA destabilization or repress its translation [113], miR-122 interacts with the 5'NTR of HCV genomic RNA and recruits argonaute 2 (ago2) to it, thereby protecting the HCV RNA genome from degradation by RNase or induction of innate immune responses [114]. In addition, miR-122 is also reported to bind to the 5'NTR of the positive-strand HCV RNA genome to stimulate the translation of HCV genome [115].

2.2.2.3 HCV assembly and release

There is still a lot of work to do in the study of HCV morphogenesis. It has been indicated that aside from the components of HCV particle (RNA genome, core protein, E1, E2), non-structural proteins including P7, NS2 and NS5A are also essential elements in HCV assembly process [116].

Lipid droplets (LDs) are dynamic and cytoplasmic organelles, consisting of a neutral lipid core, mainly of triglycerides and sterol esters that are surrounded by a phospholipid monolayer and many associated proteins [117]. LDs were mainly perceived as lipid storage for energy homeostasis in cells. However, more studies demonstrated that LDs are indispensable for the production of infectious HCV particles. The core-LD association plays a very important role in the recruitment of other viral proteins and virus production. Following maturation by SPP, HCV core protein accumulates at the surface of LDs by its domain II [31, 118]. The basic clusters of the domain I of core is mainly involved in viral RNA binding [119]. After loading on the LD surface, core protein further recruits NS5A that serves as the scaffold for the recruitment of replication complexes to this compartment [120, 121]. HCV NS5A is detected on LDs in NS5A-expressing cells by binding to diacylglycerol acyltransferase 1 (DGAT1) [122]. To produce infectious HCV, the core-NS5A interaction around

LDs is required [120, 123]. The phosphorylation of some conserved serines at the C-terminal end, domain III of NS5A, is essential for their interaction. Alanine replacement of the C-terminal serine cluster of NS5A impairs its phosphorylation, thereby disrupting the co-localization of core protein and NS5A around LDs, and abrogating HCV particle production without affecting the efficiency of the RNA replication [121]. Interestingly, mutations in NS5A that stabilize replication events impair virion production, suggesting there is a close relationship between these processes [124-126]. In fact, and phosphorylation of serine residues in domain III of NS5A has been proposed as a regulator of these processes [75, 120, 126]. In addition to the enzymatic activities of NS3, multiple subdomains located near the N terminus of helicase/NTPase domain are also involved in the early step of Infectious HCV particle assembly [127]. Since NS2 is not required for HCV RNA replication or incorporated into viral particle, it has been doubted to contribute to the assembly process of the HCV particle. Several studies have demonstrated the specific involvement of NS2 during the assembly of infectious HCV particles by bringing together the glycoproteins and nascent particles [49, 128]. It has been reported that the protease domain of NS2 is essential for infectious virus assembly and production [48, 49]. Furthermore, Kenneth A. *et al.* indicated that NS2 physically interacts with viral E1, E2 and NS3-4A complex instead of core or NS5A, and that p7 was essential for NS2 complex formation [50].

Although HCV belongs to flaviviridae family, its secretion and maturation seem to be fundamentally different from flaviviruses. Currently, it is accepted that maturation and release of HCV virions hijack the triglyceride-rich VLDL secretion pathway, and virus particle release depends on VLDL production and secretion [129]. Hepatic assembly and secretion of VLDLs plays an essential role in maintaining intrahepatic and plasma lipid homeostasis by exporting cholesterol and triglycerides from hepatocytes. The main protein component of VLDL is the secretory glycoprotein, apolipoprotein B 100 (apoB

100) a fully translated protein capable of binding lipids because of its amphipathic nature. To assembly VLDL, microsomal triglyceride transfer protein (MTP) transfers apoB during its translation to form a lipid-poor precursor particles, followed by fusion with triglyceride droplets to form mature VLDL with the help of apolipoprotein E (apoE) [130-132]. Data indicates that NS5A specifically interacts with apoE to facilitate the transport of lipoproteins to LDs; silenced apoE expression leaded to the impairment of virus assembly and release without affecting its entry and replication [133]. Moreover, both the MTP inhibitor and small interfering RNA (siRNA) targeting apoB inhibited secretion of VLDL and virus particles [134].

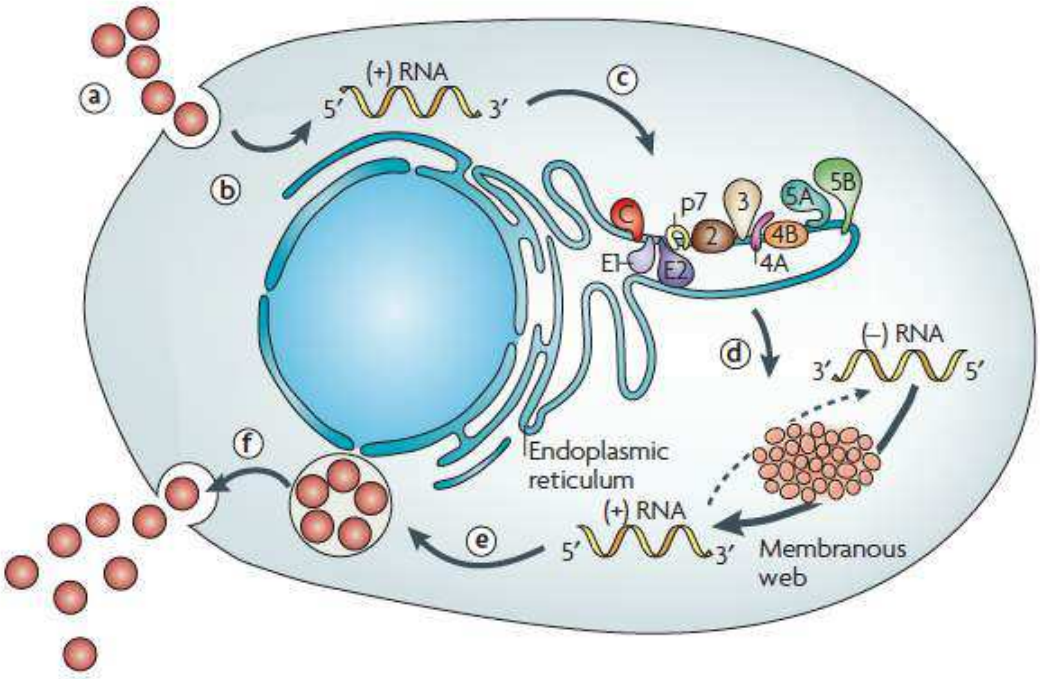

Figure 3: HCV viral life cycle. (a) Virus entry stage; (b) core release from endosomes and its uncoating; (c) IRES-mediated translation of virus RNA and polyprotein processing into structural and non-structural proteins; (d) HCV RNA replication on MW; (e) HCV genome packaging and virion assembly; (f) maturation and release of HCV virions. Figure is adapted from Darius Moradpour *et al.*[135].

2.3 Lipid droplets

There is a close connection between each step of HCV life cycle and host cell lipid metabolism. HCV modulates cellular lipid metabolism to create an environment rich in lipids and favorable to its infection. Genomic analyses of the host response to HCV infection in chimpanzees have suggested that genes involved in the biosynthesis, degradation and transport of intracellular lipids are altered during the early stage of HCV infection [136]. HCV circulating in the blood of infected patients as lipoprotein complexes that are rich in cholesteryl esters and contain apolipoproteins such as apoE and apoB have been termed as lipoviral particles (LVPs). LVPs display a varying range of densities, between 1.03 g/ml and 1.25 g/ml and bind to LDLR followed by the interaction with SR-BI and CD81 [97, 137, 138]. Interestingly, HCV found in lower density fractions, similar to serum LDL and VLDL, from plasma of infected chimpanzees displays the highest infectivity, whereas HCV found in higher density fractions is poorly infectious [139, 140].

Hepatocytes are primary target cells for HCV infection. Liver steatosis, a common histological feature in patients, is the accumulation of LDs and occurs in 73% of genotype 3 infected patients and 50% other genotypes infected patients [141]. Core-transgenic mice develop steatosis and some of them progress to HCC [142-144]. When the core proteins were expressed in various LD-expressing cells from different species, they are detected on the ER membrane and at the surface of LDs [31, 118, 145]. It has also been reported that, after HCV infection, the core proteins are detected initially at a single punctate site on each LD by 12h, and then they progressively accumulate in the entire organelle [146]. Perlemuter, G et al. indicated that core proteins induce lipid accumulation in mice by inhibiting the activity of MTP [147]. Clinical evidence has indicated that steatosis is associated with an increased

rate of fibrosis and the development of HCC, finally serving as a specific marker for HCV reinfection following liver transplantation [148-150].

As previously mentioned, LDL-R that delivers the cholesterol-rich LDL from the extracellular medium into cells is involved in the cellular entry of HCV. HCV binding is competitively inhibited by serum LDL and VLDL. LDL-R antibodies, as well as LDL-R depletion, reduced HCV infection of hepatoma cells [94, 151]. Another receptor, SR-BI, plays a critical role in the metabolism of lipoproteins, and is also a high-density lipoprotein (HDL) receptor regulating the supply of cholesterol to the cells [152]. Interestingly, virion density plays an important role in HCV/SR-BI interaction. Viet Loan Dao Thi *et al.* indicated that intermediate density virions utilize SR-BI to attach to hepatocytes through apoE, whereas lower density particles do so through E2. However, SR-BI-mediated entry of HCV particles was not associated with HCV buoyant density, relying only on its lipid transfer function [153]. Although there is no evidence suggesting direct interaction between HCV and HDLs, the latter were found to enhance HCV infectivity while anti-SR-BI antibodies abrogated this enhancement [154, 155].

The site of HCV replication, MW, is highly enriched in proteins required for VLDL assembly, including apoB, apoE, MTP as well as phopshotidylinositol 4,5-bisphosphate (PIP2), the product of a lipid kinase, phosphotidylinositol 4-kinase III alpha (PI4KIIIa) [134]. Silencing of PI4KIIIa by siRNAs reduced the accumulation of altered membrane structures, therefore inhibiting HCV RNA replication in infected cells [156]. HCV RNA replication dramatically depends on intracellular levels and content of fatty acids, including cholesterols. It has been suggested that HCV deregulate lipid homeostasis by activating transcription factors and sterol regulatory element binding proteins (SREBPs), to increase lipogenesis and reduce fatty acid oxidation and lipid export [141]. The expression of SREBPs that control both fatty acid and sterol biosynthesis

through transcription of related genes is stimulated both by HCV infection and expression of individual viral proteins [157]. Data indicated that 25-hydroxycholesterol, which suppresses the synthesis of fatty through inhibiting the function of SREBPs, blocked HCV replication [136]. Up-regulation of fatty acid synthase (FASN) and other genes related to its synthesis and transport were observed in HCV infected cells and replicon cells [158]. Moreover, inhibition of FASN activity suppressed HCV RNA replication and influenced formation of infectious virus particles [159]. Additionally, HCV infection inactivates AMP-activated protein kinase (AMPK) that serves as a sensor of energy status regulating the lipid homeostasis to reducing oxidation of fatty acids therefore increasing lipid accumulation in cells [160]. Currently, studies suggest the important roles of cholesterol in HCV infection. Cholesterol can be either synthesized in the ER from acetyl-CoA via the mevalonate pathway, or acquired by internalization of LDL associated cholesterol through receptor-mediated endocytosis [161]. However, the steps in which the HCV replication cycle requires cholesterol are still incompletely understood.

2.4 Model

2.4.1 HCV replicon systems

According to studies, subgenomic RNA molecules of many positive strand RNA viruses are able to replicate in transfected cells [162, 163]. The subgenomic replicons of HCV that autonomously amplify in cultured human hepatoma cells was established by trimming the HCV genome to those structural components required for RNA replication. The prototype replicon was a bicistronic RNA of genotype 1b (Con1 isolate) in which the region encoding for the core, E1, E2, NS2 and p7 was replaced by the neomycin phosphotransferase and the IRES from picornavirus. Upon its transfection into the human hepatoma cell line Huh7 and a subsequent G418 treatment, few

G418-resistant colonies that supported the high levels of autonomous replication of HCV RNA (1000- 5000 positive-strand RNA molecules per cell) were selected [164]. The HCV replicon system has been widely applied to facilitate the study of mechanisms of HCV RNA replication since it was invented. During the following years, many replicon constructs with different reporter genes, such as luciferase and fluorescent proteins, were also developed.

2.4.2 Retroviral pseudoparticles

The establishment of infectious HCVpp systems has been another important achievement used to investigate virus entry into cells independently from the other parts of the viral life cycle such as HCV RNA replication. HCVpp is composed of retroviral nucleocapsids surrounded by an envelope lipid bilayer that contains HCV E1-E2 glycoprotein complexes. HCVpp was produced by co-transfecting human 293T cells with expression vectors encoding HCV E1 and E2, the murine leukemia virus (MLV) or human immunodeficiency virus (HIV) gag-pol proteins and a packable reporter gene (e.g. luciferase) [165]. These pseudoparticles exhibit a preferential tropism for hepatic cells. After their entry into the hepatic cells, they deliver their retroviral capsid into the cytoplasm, followed by the reverse transcription and integration of the viral genome into the genome of the host cells. The reporter gene is expressed and the productive entry events are easily detected by measuring reporter gene activity. HCVpp entry is strictly E1/E2-dependent and can be neutralized with antibodies targeting the viral glycoproteins E1and E2 [166].

2.4.3 Cell culture-derived HCV

Although there has been great progress with HCV replicons and HCVpp, a

full-cycle culture system is still needed to study HCV life cycle. In 2001, Kato and his colleagues constructed a genotype 2a consensus genome termed JFH1 from a Japanese patient suffering from fulminant hepatitis. Interestingly, these replicons did not require adaptive mutations and replicated with very high efficiency [167]. Most importantly, researchers indicated that transfection of the complete wild-type JFH1 genome into Huh-7 cells supports the production of virus particles that are infectious in cell culture and animal models [168, 169]. Researchers routinely use these particles, now designated HCVcc. Recently, many efforts have been made to improve the system in order to acquire higher infectivity titres (about 10^4 tissue culture infectious dose 50 (TCID50) per mL obtained with JFH-1) and overcome the limitation of HCV genotype. A comprehensive panel of chimeric genomes was constructed; it consists of the JFH1 replicase genes NS3-NS5B fused to the core to NS2 region of alternative HCV genomes. One of these constructs is the J6CF/JFH1chimera, composed of the structural region of a genotype 2a isolate (J6CF) fused to the JFH-1 replicase [170]. Pietschmann and colleagues found that the most efficient virus production was generated when the genome fragments were fused via a more efficient fusion site located after the first TMD of NS2; they created the most efficient construct, the GT2a/2a chimera that is made of J6CF- and JFH1-derived sequences connected at that junction (generate 10^6 TCID50 per mL) [171].

2.4.4 Animal models

Relevant animal models are important for basic and clinical studies of HCV infection and development of new drugs and effective vaccines. However, there is no suitable small animal model, which allows us to understand the entire life cycle of HCV infection. The only true model for study of HCV is the chimpanzee that can be infected with isolates of the six major genotypes,

triggering innate and adaptive immune responses similar to those observed in infected humans [172, 173].

In 2001, Mercer and his colleagues reported that the severe combined immunodeficiency (SCID) mice, which overexpress a urokinase-type plasminogen activator (uPA) that lead to degeneration of their hepatocytes, could be transplanted intrasplenically with fresh or cryopreserved primary hepatocytes within the first 2 weeks of life and subsequently robustly infected with HCV. However, this model has an impaired immune system for studies of adaptive immunity and high mortality rates [174]. Bissig *et al.* developed a regulatable system from immunodeficient mice (specially the $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mouse) with genetic alterations. In this model, the hepatocyte degeneration caused by fumaryl acetoacetate hydrolase (FAH) is prevented by oral administration of 2- (2- nitro- 4- trifluoro- methylbenzoyl)- 1, 3- cyclohexanedione (NTBC), which blocks hydroxyphenylpyruvate dioxygenase activity upstream of FAH [175]. Obviously, the disadvantage of these two models is that they cannot be utilized for the studies of adaptive immune response to HCV infection.

Chimpanzees are historically linked to the discovery of HCV and greatly contribute to our studies of innate and adaptive immune responses in the process of HCV infection [176, 177]. Because of their limited availability and high cost, chimpanzees are only permitted to use in the study of important biological questions of HCV that cannot be studied by any other means, such as generation of vaccines and drugs [178]. In most studies, there are only two to four chimpanzees involved. Therefore, the observed correlations might represent biological variation among the animals and conclusions must be based on statistical significance. However, the chimpanzee is an irreplaceable model for HCV research [179].

2.5 Treatments

In most countries, the standard treatment for patients with chronic HCV is still the combination therapy consisting of pegylated interferon (PEG-IFN) α and ribavirin (RBV). This therapy could achieve a SVR in patients with HCV genotype 2 and 3 (80%). However, it could only result in a SVR of 50% in patients infected with HCV genotype 1, which represents the most common type of HCV [180]. Currently, novel anti-HCV drugs can be broadly divided into two categories: direct-acting antivirals (DAAs) and host-targeted antivirals (HTAs). DAAs are designed to target specific nonstructural proteins of the virus to disrupt viral replication and infection, and have significantly improved treatment outcomes in chronic HCV infection. HTAs are drugs that target host factors involved in the viral life cycle.

2.5.1 DAAs

2.5.1.1 NS3/4A protease inhibitors

HCV NS3-4A protease is essential for viral replication and therefore has been one of the most attractive targets for drug development. its Inhibitors could also prevent the activity of several other nonstructural viral proteins. The first two HCV NS3/4A protease inhibitors, telaprevir and boceprevir, were approved by the U.S.A Food and Drug Administration (FDA) for clinical use in HCV genotype 1-infected patients in May of 2011. The addition of both agents to standard therapy has been indicated to significantly improve rates of SVR among treatment-naïve patients and previous non-responders and relapsers [181-184]. Although they have excellent efficacy against HCV infection, there are still additional side effects and patients require taking medicine every 8 hours. The most common side effects with telaprevir include anemia, rash, and

anorectal discomfort [185] and the most common side effects of boceprevir are anemia, neutropenia, and dysgeusia [186]. Both of these drugs have a low barrier to resistance [187]. Moreover, telaprevir and boceprevir were designed to block protease activity of genotype 1 HCV; consequently their efficacy varies among different genotypes. Analysis of data from a series of small clinical trials indicated that telaprevir is effective against patients infected with genotype 2, but not those with genotype 3 and uncertain for genotype 4 [188]. In vitro experimental data has demonstrated that both telaprevir and boceprevir exhibit antiviral activity against genotypes 2, 5, and 6, but not 3 [189]. Recently, enormous efforts have been made to develop new drugs to overcome these shortcomings. Numerous so-called second-wave protease inhibitors with pan-genotypic effect, now in phase II and III trials, have improved pharmacokinetic profiles and generated fewer adverse effects and higher barriers to viral resistance. For example, macrocyclic NS3/4A protease inhibitor simeprevir/TMC435 (which blocks the catalytic site through a reversible, noncovalent mechanism, whereas linear protease inhibitors including telaprevir and boceprevir via a reversible, covalent mechanism) is one of the second-wave protease exhibiting potent antiviral activity against genotypes 1, 2, 4, 5 and 6, but limited efficacy against genotype 3 of HCV [190]. In 2013, simeprevir was approved in Japan, Canada and the U.S.A for the treatment of genotype 1 chronic HCV. Because of its upgraded pharmacokinetic properties with a 40-hour half-life, it allows once-daily administration as a single capsule of 150-mg. Analysis of a Phase II trial indicated that simeprevir treatment induces fewer resistant variants compared to the llinear protease inhibitor [191]. Another highly effective macrocyclic protease inhibitor is danoprevir. However, increased levels of aminotransferases were observed in patients with high-dosed danoprevir [192]. The studies exploring the efficacy and safety of danoprevir in patients are still under research. Data indicated that co-administration of danoprevir and ritonavir, which inhibit the cytochrome P450 3A4 enzyme, allows a lowers

therapy doses and limits hepatotoxicity of danoprevir. In addition, together with ritonavir, PEG-IFN and RBV, danoprevir was indicated to profoundly and robustly reduce serum HCV RNA in patients without safety-related discontinuations [193].

2.5.1.2 NS5A inhibitors

The most advanced NS5A inhibitor is daclatasvir, the first known inhibitor of NS5A and currently in Phase III. It was identified in a screen that aimed to find compounds functionally distinct from NS3/4A and NS5B inhibitors by using a cell-based HCV replicon assay [194]. Although its exact mode of action is still unclear, daclatasvir seems to bind to domain I of NS5A [195]. The *in vivo* mechanism of action of daclatasvir has been studied for years， but still remains unclear. Choongho Lee *et al.* indicated that daclatasvir could alter the subcellular localization of HCV NS5A, therefore disrupting the formation of HCV replication complexes [196]. Jeremie Guedj and his colleagues reported that daclatasvir inhibits intracellular HCV RNA synthesis, virus assembly and secretion [197]. Daclatasvir seems to be effective in patients with HCV genotype 1 infection but relatively weak in other genotypes [198]. It is safe at low concentrations and pharmacokinetic analyses support once-daily dosing. Combined with PEG-IFN and RBV, daclatasvir led to a high SVR of 83%- 92% compared to 25% in patients who received only PEG-IFN and RBV at week 12 post-treatment in treatment-naïve patients with HCV genotype-1 infection [199]. Daclatasvir has a low barrier to resistance and genotype 1a, compared to genotype 1b, was more prone to develop drug resistance variants. Fortunately, PEG-IFN and RBV and other HCV protease and non-nucleoside polymerase inhibitors can prevent daclatasvir-resistant variants. Analysis of a phase IIa, open-label study indicated that the combination of daclatasvir and asunaprevir, a selective inhibitor of the HCV NS3, achieve a SVR of 90% in patients with

HCV genotype 1b infection who previously had no response to PEG-IFN and RBV and also fail to respond to the treatment of PEG-IFN, RBV plus telaprevir or boceprevier [200].

2.5.1.3 NS5B RdRp inhibitors

HCV RdRp inhibitors include nucleoside analog inhibitors (NIs) and non-nucleoside analog inhibitors (NNIs). NIs target the active or catalytic sites of NS5B [201, 202]. They mimic the natural nucleoside substrates of polymerase and are incorporated into the growing viral genome at the active site of NS5B, thus resulting in the premature chain termination. The active site of NS5B is a highly conserved domain among all HCV genotypes. Consequently, NIs have a potentially effectiveness against the various genotypes with a high barrier to resistance [202-204]. Several NIs, such as purine and pyrimidine analogues, are undergoing clinical development. They are effective against all HCV genotypes and subtypes. Nowadays, sofosbuvir, a prodrug of uridine, is the most advanced NI in clinical development. Sofosbuvir has a high genetic barrier for resistance and several amino acid substitutions have been identified (including L159F, S282T, and V321A) in patients who received it [205]. However, these changes in the NS5B gene do not reduce SVR rates. Therefore, the combination of sofosbuvir with other classes of inhibitors might be an effective antiviral regimen in patients with emergence of NS3/4A or NS5A variants [206]. Clinical data demonstrated that the combination of sofosbuvir with PEG-IFN and RBV resultied an early virologic response (EVR) of 95% in patients with HCV genotype 1 infection and a SVR of 96% in patients with genotype 2 and 3 infection after 12 weeks of therapy [207]. One study of the optimal duration of PEG-IFN in HCV treatment indicated that the only combination of sofosbuvir and RBV has a rate of SVR 100% at 12 weeks post therapy in genotype 2 or 3 patients, indicating that

sofosbuvir could be used in IFN-free regimens and is the only IFN-free regimen for genotypes 2/3 that has been submitted for approval to the U.S.A FDA [208].

NNIs bind to the different allosteric site of NS5B, resulting in conformation changes before the initiation or elongation of the RNA strand. There are several allosteric sites on the RdRp that are targeted by NNIs and catalog them [209]. The aa sequences at allosteric sites are not conserved among all HCV genotypes; therefore, NNIs have a low barrier to resistance and most of them have been developed to target genotype 1 [210]. Currently, multiple NNIs are in clinical development, including filibuvir and tegobuvir. Although there are increases in rates of rapid virologic response (RVR), clinical data reported that it could only achieve a SVR of about 50% in HCV genotype 1 patients treated with the combination of filibuvir or tegobuvir with PEG-IFN and RBV. Additionally, in phase IIb clinical trials, filibuvir treatment results in a high rate of relapse [211, 212].

2.5.2 HTAs

Each step of HCV life cycle requires a set of host factors [213]. Thus, numerous HTAs have been developed and tested to against HCV. Some of them are broad target HTAs that trigger innate immune response including IFNα, IFNγ or agonists targeting Toll-like receptor (TLR). Many host enzymes or cellular factors, which are involved in the HCV lifecycle including cyclophilin A (CypA), FASN and miRNA-122, are potential targets of HTAs. There are several advantages provided by HTA. Firstly, under the viral or chemotoxic stress, the genetic variability within the host are much more lower than virus.

Therefore, HTAs impose a higher barrier to drug resistance and minimize the potential of viral breakthrough [214, 215]. Secondly, HTAs exhibit a broad pan-genotypic activity. It has been indicated that HTAs are able to inhibit infection by HCV of all major genotypes [216]. Finally, many different viruses could utilize similar cellular machines. Thus, HTAs are able to interfere with new and emerging infectious diseases.

2.5.2.1 IFNα

IFNα is one of the best-studied and tested HTAs. For the past 25 years, recombinant IFNα has been used for the treatment against HCV infection [217]. There are, at least, 14 human IFNα non-allelic functional genes found on the short arm of chromosome 9 which encode for 13 structurally related human IFNα proteins: IFNα1, IFNα2, IFNα4, IFNα5, IFNα6, IFNα7, IFNα8, IFNα10, IFNα13, IFNα14, IFNα16, IFNα17 and IFNα21. The circulating IFNα within a patient binds to their receptor and subsequently activate signaling cascades that induce transcription of hundreds of interferon-stimulated genes (ISGs) [218]. These ISGs play key roles in host immune defense against HCV infections through various processes, such as suppression of viral protein synthesis and inhibition of negative strand RNA of HCV (replicative intermediate) generation [219-221].

2.5.2.2 HO-1

Heme or iron-protoporphyrin IX (PP-IX) is a common metalloporphyrin (MP) in prokaryotes and eukaryotes cells. Heme has long been known to be important in a wide array of functions, such as respiration, oxygen metabolism, electron transfer and signal transduction. Heme functions as a regulatory molecule that

controls DNA transcription, RNA translation, protein stability and targeting, and cell differentiation [222, 223]. Heme oxygenase (HO) is an enzyme that catalyzes the oxidative degradation of heme into bilirubin, ferric ion, and carbon monoxide (CO). Among the different isoforms of HO, only HO-1 is usually induced in response to inflammation, infection and other forms of cellular stress [224]. Data indicated that HO-1 induction is associated with protection from oxidative stress and cellular [225, 226]. Recently, it was shown that overexpression or induction of HO-1 displays a wide range of antiviral activity against HIV, HBV and HCV. HO-1 is an attractive target for HTAs [227-229]

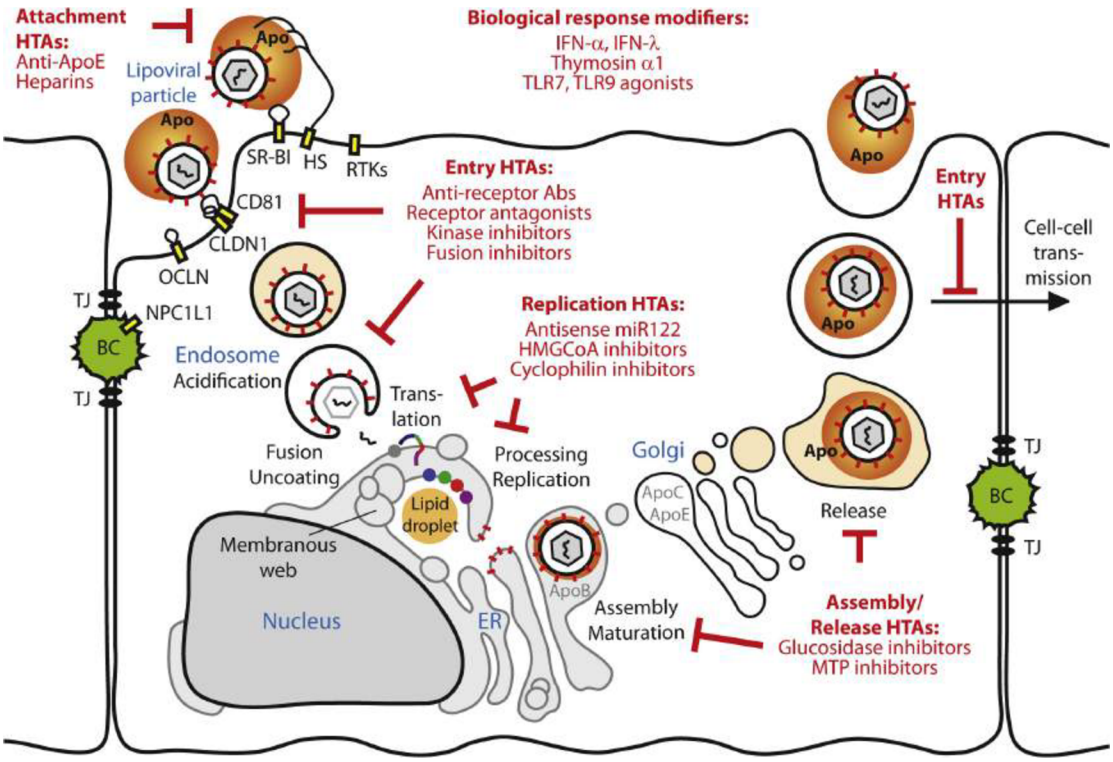

Figure 4: Host factors involved in the hepatitis C virus life cycle are potential targets of HTA. Different HTAs that interference with the various steps of HCV life cycle are indicated. Figure adapted from Mirjam B. Zeisel *et al.* [230].

2.6 Herbal medicines

Herbal medicines are a major part of complementary and alternative therapies that are used by 70-80% of the world population to treat diseases according to World Health Organization (WHO). They have gained increasing popularity and widespread use because of their effectiveness, limited side effects in clinical experiments and relatively low cost. Notably, a significant number of important modern drugs have been developed from molecules originally isolated from natural sources based on their use in traditional medicine. Many efforts have been made to identify the precise active ingredients in these herbal medicines. Numbers of herbal formulations from China and Japan show promising activity in the treatment of liver diseases including HCV infection. Over the last decades, there have been an increasing number of reports on the identification of active natural products from plants harboring anti-HCV activities. For example, silymarin, an extract from the seeds of milk thistle plant, has been reported to possess protective effects on the liver and used to treat chronic liver disease for decades [231]. Silibinin, the major active constituent of silymarin composed of two diastereoisomers, silybin A and silybin B, is thought to be responsible for the hepatoprotective effects of silymarin and has also been reported to have anti-HCV activity [232]. It was reported that silymarin reduced the expression of core genes and proteins in a dose dependent manner in cells transiently transfected with HCV 3a core plasmid [233]; silibinin may also inhibit HCV replication by blocking the interaction between NS5A and HCV RNA [234].

2.6.1 Sophora flavescens

For thousands of years, sophora flavescens (SF) has been used broadly in traditional Chinese medication to treat viral hepatitis, cancer, viral myocarditis,

gastrointestinal hemorrhage, and skin diseases [235-238]. It is thus administered in formulas for the treatment of many diseases including virus infection. SF is a main ingredient in numerous Chinese medicines that have been used to treat HCV infection. One of these most popular formulas is Bing Gan Tang. Data from clinical analyses indicated that the combination therapy of herbal mixture Bing Gan Tang and IFNα resulted in better effects on clearance of HCV RNA and the normalization of serum alanine transaminase (ALT) level than IFNα alone in patients with chronic HCV infections [239]. Recently, alkaloids, pterocarpans, and a variety of flavonoid compounds including flavanone, flavanol, valvanonol, chalcone, isoflavone, isoflavanononе and pterocarpans have been reported in this plant，such as matrine, oxymatrine, kurarinone, and several sophoraflavanones [240-242]. More studies are focusing on the precise function of these compounds.

2.6.1.1 Sophoraflavanone G

Sophoraflavanone G (SFG, 5,7,D, 20,40 -tetrahydroxy-8-lavandulylflavanone) is a lavandulyl flavanone isolated from SF and found to have multiple functions. For instance, SFG exerts antimicrobial activities against methicillin-resistant Staphylococcus aureus (MRSA) [243] and antibacterial action by reducing membrane fluidity [244]. Dong Wook Kim *et al.* reported that SFG significantly inhibited the activity of cyclooxygenases (COX) and lipoxygenases (LOX) that catalyze the generation of proinflammatory mediators such as prostaglandins (PG), therefore exhibiting anti-inflammatory activity in LPS-stimulated macrophages [245]. Another group further indicated that SFG inhibited the levels of nitric oxide and prostaglandins E2 (PGE2) and the production of pro-inflammatory cytokines and mediators through the interruption of the nuclear transcription factor kappa-B (NF-κB) and MAPK signaling pathways [246]. Recently, Byung-Hak Kim and his colleagues identified novel biological

activities of SFG, such as the capability of inhibiting tyrosine phosphorylation of signal transducers and activators of transcription 1 (STAT1), STAT3 and STAT5 through regulating their upstream signaling pathways including Janus kinases

(JAKs), Src family kinases, phosphoinositide 3-kinase (PI3K)/Akt, and extracellular signal-regulated kinases (ERK)1/2 and NF-kB signaling in the tested cancer cells. Additionally, they indicated that SFG could inhibit cancer cell proliferation and induce apoptosis by regulating STAT3 mediated expression of apoptotic and anti-apoptotic proteins [247].

Chapter 3-Materials and methods

3.1 Cell culture

Human hepatoma cell line (Huh7.5) and OR6 cells, as well as HCV sub-genomic replicon cell line (SGRmJFH1BlaRL) were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen, CA) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin, 1% streptomycin and 1% nonessential amino acids. Medium for OR6 contained G418 (500g/ml; Geneticin, Invitrogen) and for SGRmJFH1BlaRL contained blasticidin (300g/ml; Invitrogen). Cells were digested with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and split twice a week.

3.2 Western blotting

Cells were lysed in lysis buffer composed of 50mMTrisHCL (pH8.0), 150mM NaCl, 1% Triton X-100, with 100ug/ml PMSF added before use. Protein concentration was quantitated by the Bradford assay (Bio-Rad). 20µg of proteins were electrophoresed on a 10% SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane (Amersham Biosciences). The blot was blocked in 5% non-fat dry milk in PBS with 0.01% Tween-20 for 1h at room temperature, then incubated with primary antibodies over night at 4 ^{0}C, and then incubated with horseradish peroxidase (HRP) labeled secondary antibodies for 1h at room temperature. Proteins were visualized via enhanced chemiluminescence (Thermo).

The primary antibodies used for western blotting are: mouse monoclonal anti-NS3 (Abcam) (1:1000); mouse monoclonal anti-NP (Senta Cruze) (1:1000); mouse monoclonal anti-NS1 (Senta Cruze) (1:1000); mouse anti-SeV (gift from Ganes Sen, Cleveland Clinic) (1:1,000); rabbit monoclonal anti-HO-1 (Abcam) (1:1000) and mouse monoclonal anti-actin ((Santa Cruz Biotechnology) (1:5000). The secondary antibodies used for western blotting are: horseradish peroxidase-linked rabbit anti-mouse IgG (Amersham Biosciences, Inc, NJ, USA) (1:5000) and horseradish peroxidase-linked sheep anti-rabbit IgG (Amersham Biosciences, Inc, NJ, USA) (1:5000).

3.3 Quantification of HCV RNA and cellular mRNA

Huh-7.5 cells were infected with HCVcc and treated with SFG for 48h. After that, cells were harvested, and total RNA was extracted for assay with RNeasy Mini Kit (Qiagen). cDNA was synthesis was done with iScript cDNA Synthesis Kit (Bio-Rad). 2µg of total RNA was used for a 20µl reverse transcription reaction. Real-time quantitative PCR (qPCR) was performed using the Applied Biosystems 7500 Fast Real-Time PCR System with 2xSYBRGreen Master Mix (Diagenode) in a 10µL reaction. The applied cycling conditions were 50 ^{o}C for 2min and 95 ^{o}C for 10min,followed by 40 cycles at 95 ^{o}C for15s, 60 ^{o}C for 30s, and 72 ^{o}C for 30s each as well as a final dissociation stage of 95 ^{o}C for 15s and 60 ^{o}C for 1min. The primers used are listed in Table 1.

Table 1. Oligonucleotide used for reverse transcriptase PCR (RT-PCR) and qRT-PCR.

Oligonucleotide name	Sense	Anti-sense
HCV	AGCCATGGCGTTAGTATGAGTGTC	ACAAGGCCTTTCGCAACCCAA
HO-1	GAGATTGAGCGCAACAAGGAG	CTGACTGCGGGAGTCATCTC
IFNa2	GCAAGTCAAGCTGCTCTGTG	GATGGTTTCAGCCTTTTGGA
IFNa17	AGGAGTTTGATGGCAACCAG	CATCAGGGGAGTCTCTTCCA
OAS 1	CAAGCTCAAGAGCCTCATCC	TGGGCTGTGTTGAAATGTGT
PKR	ATGATGGAAAGCGAACAAGG	GAGATGATGCCATCCCGTAG
GAPDH	GCCATCAATGACCCCTTCATT	TTGACGGTGCCATGGAATTT

3.4 Cellular toxicity

Cell viability was determined by SRB assay with CytoScan*TM* SRB Cytotoxicity Assay (Biosciences). Briefly, Huh7.5 cells were digested with Trypsin/EDTA, and cell numbers were obtained with a cytometer. Then, 100µL of a culture with $1X10^5$ cells/mL was plated into 96-well plates. Fresh culture medium containing SFG at various concentrations was added next day. After 48h, remove the medium in the 96-well plate and gently layer 50µL Fixation Reagent onto each well. Incubate the plate for 1 hour at 4^oC and then wash the wells 3 times with water. Dry the plate by incubating in a 45^oC incubator for 30 minutes to remove excess wash. Add 100µL SRB Dye Solution to cover the culture surface of the well for 30min at room temperature in the dark. Wash the wells using 1X Dye Wash Solution. Dry the plate and then add 200µL SRB Solubilization Buffer to each well. Mix by pipetting up and down to dissolve the dye completely. Finally, we measure the absorbance at 570nm with a microplate reader.

3.5 HCV viral stock

The production of infectious HCV in hepatocytes was done as described. The plasmid pFL-J6/JFH1 containing the full length chimeric HCV complementary DNA was restricted with XbaI and treated with mung bean nuclease (New England Biolabs) to generate the according HCV cDNA with T7 promoter. The cDNA were purified and use as templates for RNA synthesis. HCV RNA was synthesized in vitro using a MEGAscript T7 kit (Ambion) and purified with RNeasy Mini Kit (QIAGEN). The synthesized HCV RNA was used to electroporate into naïve Huh7.5 cells. Cells were plate every 3 days and after 15 days, the culture medium was collected and cleaned by centrifugation at 3000 rpm for 10 minutes. The supernatants were stored at -80^{o}C as HCV viral stock. All infectious experiments were performed in Huh7.5 cells infected with HCV at a multiplicity of infection (MOI) of 0.1.

3.6 HCV sub-genomic replicon cell line

The pSGRmJFH1BlaRL replicon was kindly provided by Dr. Kui Li (university of Tennessee Health Science Center) and used to measure the inhibition activity of SFG on HCV RNA replication. In this construct, the HCV structural proteins (core-E1-E2-p7) and NS2 were replaced by cDNA sequences encoding the blasticidin resistance gene product that directly fused to the N-terminus with renilla luciferase. Plasmid DNA templates was linearized with XbaI and purified. Replicon RNAs was synthesized in vitro using a MEGAscript T7 kit (Ambion) and subsequently transfected into Huh7.5 [248]. Stable transfected cells were selected with blasticidin.

3.7 Luciferase assay

For the luciferase assay 1.0-1.5 x 10^4 OR6 or Huh-7.5 harboring HCV sub-genomic replicon (SGRmJFH1BlaRL) cells were plated onto 24-well plates in triplicate and precultured for 24 h. the cells were treated with SFG for 48h. After that, cells were collected, washed in ice-cold phosphate-buffered saline (PBS), and assayed for reporter gene activities according to the manufacturer's protocol (Promega) and normalized to the protein content in the individual samples.

3.8 BODIPY (493/503)

To prepare a stock solution, BODIPY (493/503) was dissolved in ethanol at a concentration of 1mg/ml and diluted 1:500 in PBS immediately before use. Cells were fixed with 4% paraformaldehyde for 30 min and after that, samples were washed 3 times in PBS. Following fixation, incubate samples with diluted BODIPY (493/503) for 30 min in the dark and then rinse with PBS three times. Samples were mounted with mounting medium (Thermo) on glass slides and store specimens in a lightproof box overnight to dry the mounting medium. We example samples under a fluorescence microscope with a filter set for Alexa488.

Chapter 4-Results

4.1 Preliminary screening identified Chinese medicines (CMs) with anti-HCV activity.

Due to the advantages of herbal medicine, our aim was to discover herbal medicines with effective anti-HCV activity and identify their active compounds. In this study, the HCVcc system was used to perform screen to explore which CM elicits any anti-HCV activity. Huh 7.5 cells were seeded on day 1 infected with HCV, and then treated with different crude extracts of test CMs (CM1 to CM45) at a concentration of 8 μg/ml on day 2. After 48h, cell lysates were subjected to western blotting with an anti-HCV NS3 antibody. Results of preliminary screening indicated that all the 45 herb crude extracts had not affect in directly eradicating HCV, but CM 17, CM3 and CM15 could significantly inhibit HCV replication. As shown in Fig. 5A, CM17 (lane 3), CM3 (lane 4) and CM15 (lane 5) significantly inhibited HCV NS3 protein expression compared with the mock control (0.1% DMSO) (lane 2). To further confirm the antiviral activity of CM17, CM3 and CM15, RT-PCR was performed with primers corresponding to HCV genome. As shown in Fig. 5B, a clear decrease in HCV RNA levels occurred upon treatment with CM17, CM3 and CM15 (especially CM3, lane 4) compared to the cells upon treatment with DMSO (lane 2). Data confirmed that CM17, CM3 and CM15 contain bioactive compounds against HCV infection.

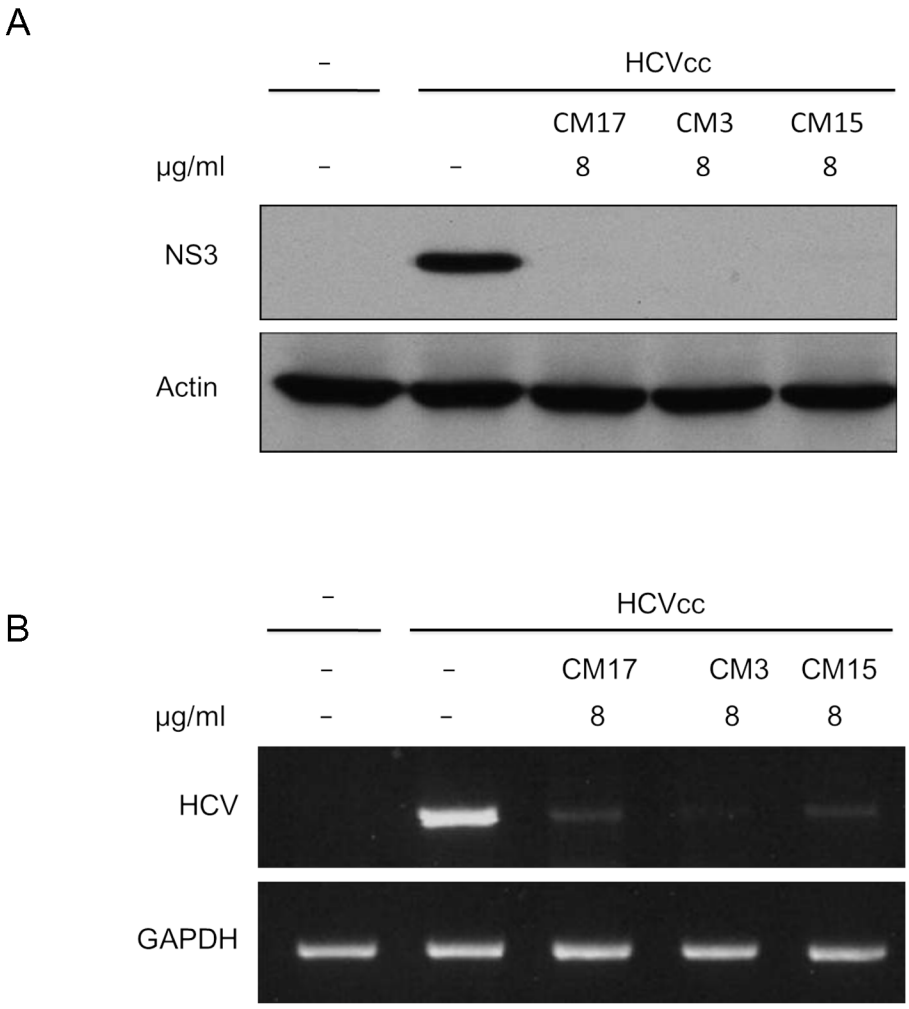

Figure 5: CM 17, CM3 and CM15 exhibited effective Inhibitory effect on HCV infection. (A) Inhibition of HCV NS3 protein expression in HCV infected Huh7.5 cells with CM17, CM3 and CM15 treatment respectively. Huh7.5 cells were infected with HCV and treated with CM17，CM3 or CM15 for 48h. 0.1% DMSO served as the control. Western blotting was performed using anti-NS3 and anti-actin (a loading control) antibodies. (B) CM17, CM3 and CM15 significantly reduced HCV RNA replication. After 48h of treatment with CM17, CM3 or CM15, HCV RNA levels were analyzed by RT-PCR by using HCV-specific primers and the GAPDH gene served as the loading control.

4.2 CM17, CM3 and CM15 affected different steps in HCV life cycle.

To determine which steps in the HCV life cycle were affected by CM17, CM3 and CM15 treatment: entry or post entry, we tested their effect on the HCV when they were provided at different times. Huh7.5 cells were infected with HCV and treated with CM17, CM3 or CM15 either during infection or after infection. HCV infection was analyzed by western blotting at 48 h post-infection. As we showed before, all the three medicines could inhibit HCV NS3 protein expression when they were present throughout the infection (Fig. 6A). However only CM15 was able to significantly inhibit HCV NS3 protein expression when HCV and crude extracts were removed 2h post infection (Fig. 6B), indicating that CM15 could inhibit the entry step of HCV life cycle, but CM17 and CM3 did not. Inhibition of NS3 protein synthesis by CM17 and CM3 was observed when each was added 2h (enough time for HCV entry) post infection. Since CM17 and CM3 still inhibited NS3 protein expression after viral entry, we consider that these two medicines interfered with HCV post-entry.

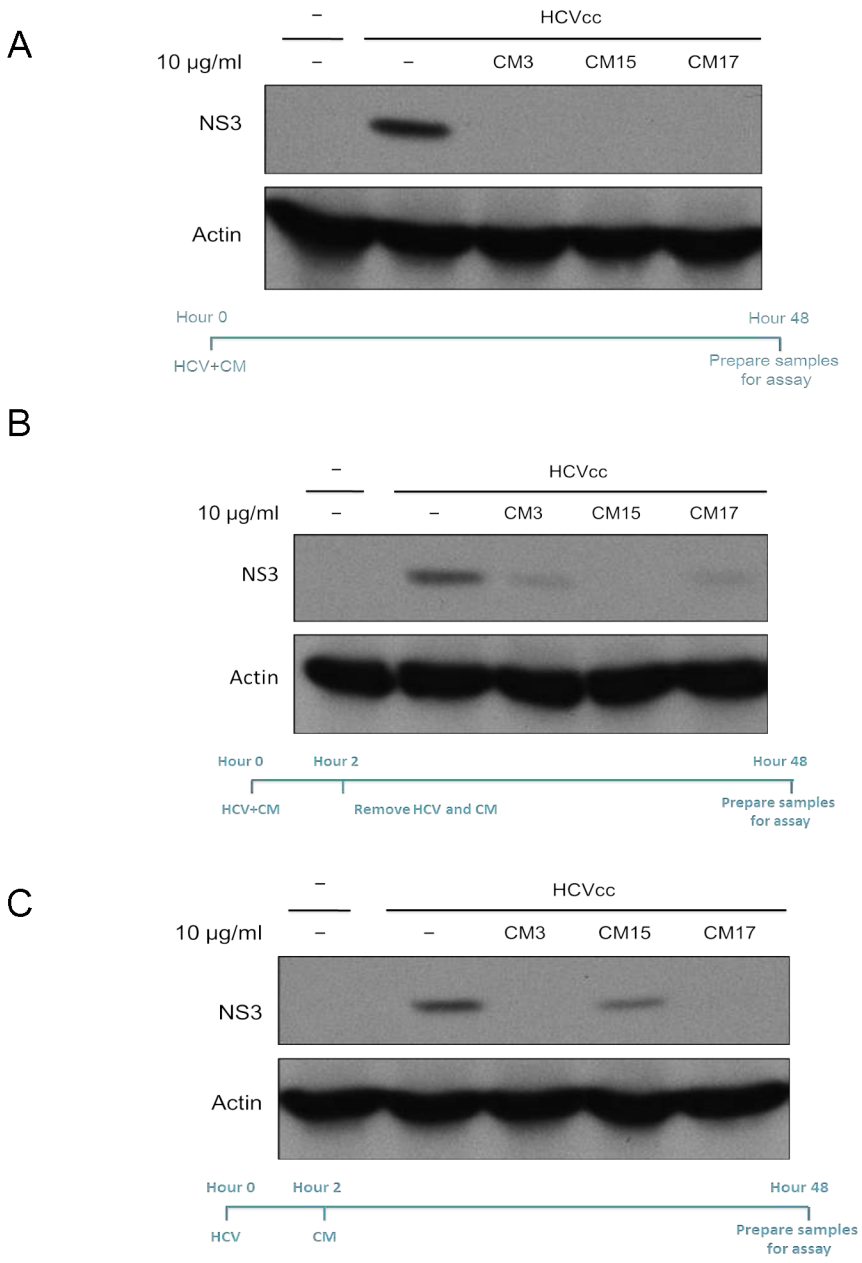

Figure 6: Effects of CM17, CM3 and CM15 on the entry and post-entry steps of the HCV life cycle. Huh7.5 cells were infected with HCV and treated with CM17, CM3 or CM15 for 48h (A), just for 2h (B), or CMs were added 2h post infection (C). The cells were washed with PBS after 2 h of inoculation after the removing of HCV and medicines.

4.3 Specific inhibition of HCV replication by CM3.

Next we wanted to study whether CM17, CM3 or CM15 could also block the replication of other viruses, such as measles virus (MV), influenza A/Puerto Rico/8/34 (PR8) virus and sendai virus (SeV). Huh7.5 cells were treated with CM17, CM3 or CM15 at a concentration of 5μg/ml and simultaneously infected with MV, RP8 or SeV at a MOI of 0.1. After 24h, cell lysates were subjected to western blotting with anti-NP (MV), anti-NS1 (PR8) or anti-SeV antibody respectively. As shown in Fig. 7A, CM17 was also able to strongly inhibit nucleoprotein (NP) protein expression of MV (lane 2) and slightly interfere with NS1 protein expression of RP8 (Fig. 7B, lane 2) and SeV infection (Fig. 7C, lane 2). CM15 could marginally suppressed MV and SeV infection (Fig. 7A lane 4 and 7C lane 4). However, the protein expression of those viruses was not affected by CM3, indicating that CM3 had no effect on their replication and specifically inhibited HCV replication.

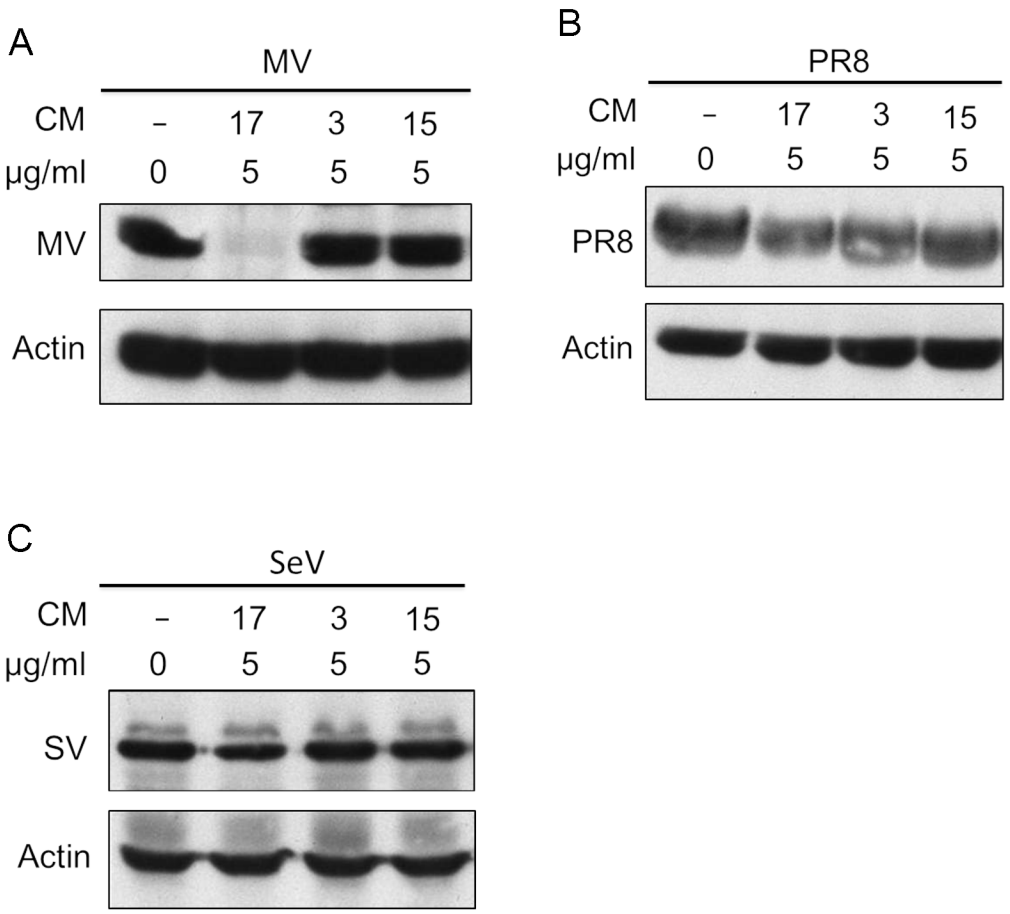

Figure 7: CM3 is a specific inhibitor of HCV replication. (A) MV infection was significantly inhibited by CM17 and marginally by CM15. PR8 infection and SeV infection was slight suppressed by CM17 (B) and CM15 (C) respectively. However, CM3 did not interfere with the replication of either of these viruses (MV, PR8 or SeV). Western blotting was performed using anti-NP (MV), anti-NS1 (PR8), anti-SeV and anti-actin (a loading control) antibodies.

4.4 Antiviral-guided extraction and fractionation of the active extracts from SF.

The CM3 (crude extract) was generated from SF, an important ingredient in many Chinese formulas used to treat HCV infection, such as Bing Gan Tang. Due to the effective and specific inhibitory activity of CM3 against HCV infection, we wanted to isolate the pure compound of CM3 and study its mechanisms. Aired-dried roots of SF (2.5Kg) were chipped and ground to powder and then extracted under reflux with ethanol. The powder was filtered and extracted again with fresh solvent for two times (each time with 1.5 L solvent for 500 g material, totaling 7.5 L). Concentration of the solvent afforded an ethanol extract, which was suspended in salted water (4 L) and extracted with hexane, CH_2Cl_2, ethyl ethanoate (EtOAC) and *n*-BuOH, successively. The extracts were dried with anhydrous sodium sulfate, filtrated and evaporated, yielding four dark brown extracts in order: the hexane soluble extract **KSH**, CH_2Cl_2 soluble extract **KSD**, EtOAC soluble extract **KSA** and *n*-BuOH soluble extract **KSE**. As shown in Fig. 8A, the *n*-BuOH (KSE), EtOAC (KSA) and CH_2Cl_2 (KSD) extract from SF showed anti-HCV activity in a dose-dependent manner. In particular, the EtOAC extract, KSA, exerted the strongest inhibitory effect on HCV NS3 protein expression, with inhibition of greater than 70% at 5μg/ml and more than 90% at 10μg/ml.

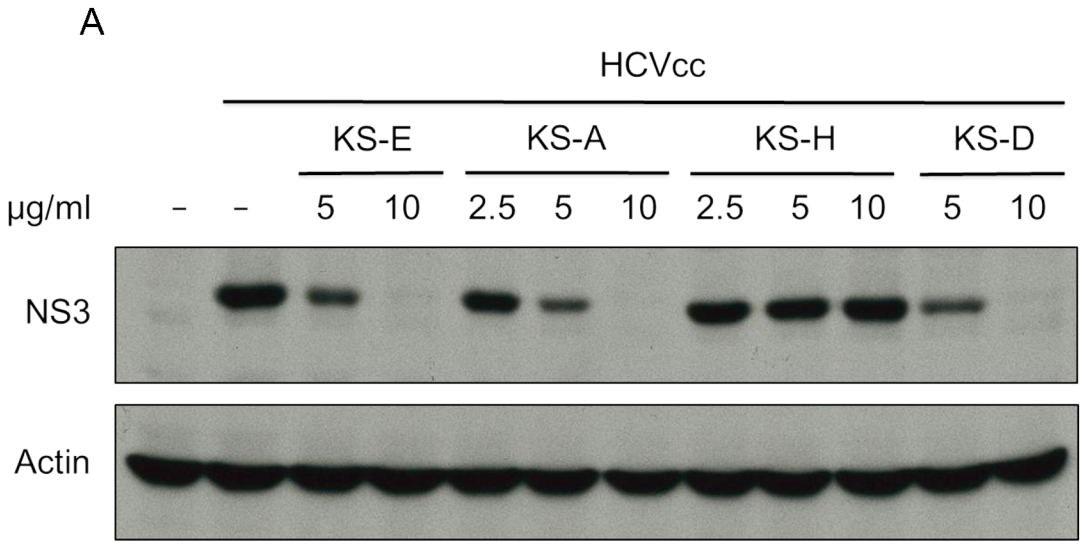

Figure 8: KSE, KSA and KSD showed anti-HCV activity in a dose-dependent manner. (A) The EtOAC soluble extract KSA exerted the strongest inhibitory effect on HCV NS3 protein expression. Huh7.5 cells were infected with HCV and treated with four different extracts respectively. After 48h, cell lysates were subjected to western blotting with an anti-HCV NS3 antibody.

4.5 KS-211 is the major active compound of SF against HCV infection.

Then, the most active antiviral fraction **KSA** (30g) was absorbed onto 27 g of silica gel and subjected to column chromatography (silica gel 230-400 mesh). Successive elution with hexane-acetone (5:4) yielded 9 fractions: **KS-21** to **KS-29,** which were individually assayed for inhibition of HCV NS3 protein expression, respectively (data not shown). **KS-21** (powder, 0.9 g), the most active fraction was applied to preparative thin-layer chromatography (TLC) developed with Hexane-EtOAC (1:1) and yielded **KS-211**, **KS-212** and **KS-213**.

Subsequently, Huh7.5 cells were treated with **KS-211**, **KS-212** and **KS-213** 0.5h prior to HCV infection. These pure compounds exhibited anti-HCV activity and **KS-211** was shown to be the most active compound (Fig. 9A). Next, Huh7.5 cells were infected with HCV firstly and after 4h were treated with different compounds. As we expected, Inhibition of NS3 protein synthesis was observed (Fig. 9B), suggesting that these compounds interfered with HCV post-entry step. Because of the high activity of **KS-211** and time constraint, only **KS-211** was studied in-depth.

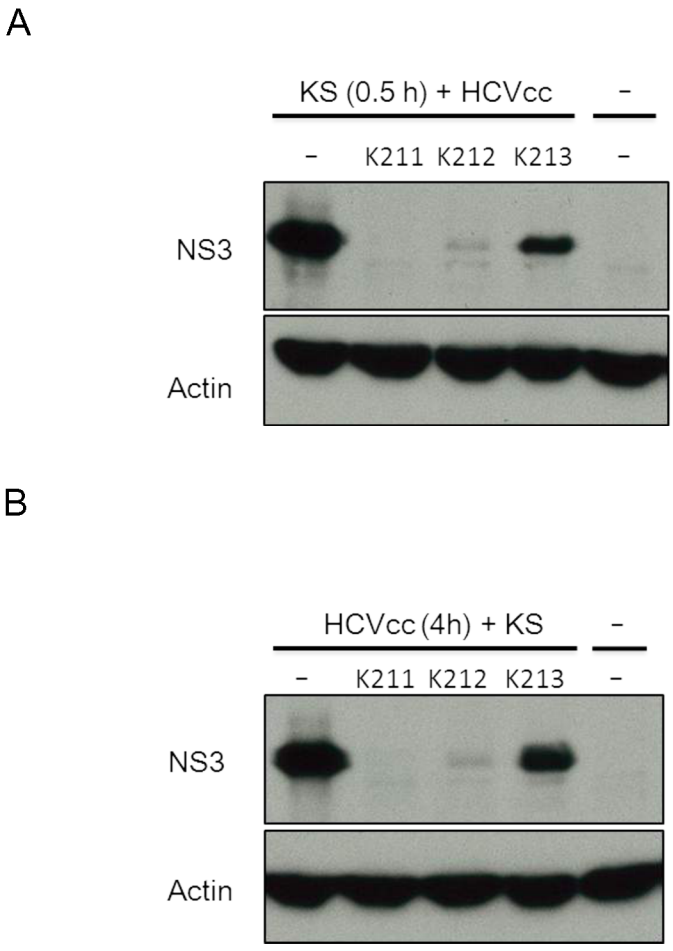

Figure 9: KS-211 is the most active anti-HCV compound isolated from SF. (A) **KS-211**, **KS-212** and **KS-213** inhibited HCV infection in a dose dependent manner. Huh7.5 cells were treated with **KS-211, KS-212** and **KS-213** 0.5 h prior to HCV infection. (B) **KS-211**, **KS-212** and **KS-213** interfered with HCV post-entry step. Huh7.5 cells were infected with HCV 4h prior to **KS-211, KS-212** and **KS-213** treatment. After 48h, cell lysates were subjected to western blotting with an anti-HCV NS3 antibody.

4.6 Identification of KS-211.

The structure of **KS-211** was identified mainly by mass spectrometry (MS), ^{1}H nuclear magnetic resonance (^{1}H NMR), ^{13}C NMR, heteronuclear multible bond correlation spectroscopy (HMBC) and nuclear overhauser effect spectroscopy (NOESY) analyses. The negative ESI-MS of compound **KS-211** showed molecular ion peaks at *m*/*z* 423.1803 ($[M-H]^{-}$), corresponding to the molecular formula $C_{25}H_{28}O_6$. As showed in table 2, the ^{1}H NMR spectrum of peaks at δ 5.67 (1H, *dd*, 13.3, 2.7Hz, H-2), δ 3.07 (1H, *ddd*, 17.0, 13.3, 1.6, H-3a) and δ 2.76 (1H, *ddd*, 17.1, 2.8, 0.8, H-3e) represent the protons of ring C. The proton signals at δ 4.59 (1H, *m*, H-19a), δ 4.55 (1H, *m*, H-19b), δ 4.99 (1H, *m*, H-14), δ 2.63 (2H, ABx, H-11), δ 2.53 (1H, *m*, H-12), δ 2.07 (1H, *m*, H-13a), δ 2.02 (1H, *m*, H-13b), 1.64 (3H, *s*, H-20), 1.56 (3 H, *d*, 0.9Hz, H-17) and δ 1.49 (3 H, *s*, H-16) were assigned as lavanduyl functional group. These 1H and 13C NMR chemical shifts of compound **KS-211** were consistent with those of SFG (Fig. 10) [249] and were further demonstrated by 2D NMR correlations (NOSY and HMBC, Table 2).

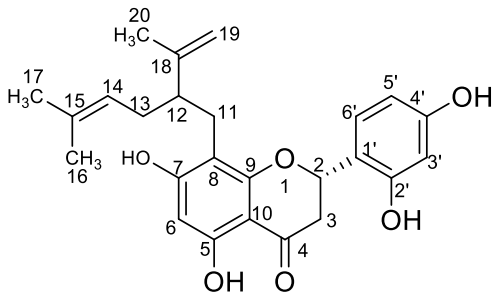

Figure 10: Chemical structure of KS-211.

Table 2: ^{1}H NMR, ^{13}C NMR, HMBC and NOESY spectral data of KS-211.

Position	δ H – mult	*J* (Hz)	δ C	HMBC	NOESY
1 O	--		--		
2	5.67 (dd)	13.3, 2.7	75.46	4, 1', 2', 6'	3e, 6'
3a 3e	3.07 (ddd) 2.76 (ddd)	17.0, 13.3, 1.6 17.1, 2.8, 0.8	42.96 43.01	2, 4, 1' 4, 10	3e, 6' 2, 3a
4 C=O	--		198.31 198.24		
5 =C-OH	12.18 (s)		163.13 162.84	5, 6, 10, (weak 7)	6
6	6.01 (2xs)		96.29 96.22	5, 7, 8, 10	H2O (2.9), OH5
7	--		165.34 165.28		
8	--		107.96		
9	--		162.23 162.21		
10	--		103.32 103.26		
11 CH2	2.63 (ABx)		27.87	7, 8, 9, 12, 13, 18	13, 14, 19b, 20 13, 14, 19b, 20
12 CH	2.53 (m)		47.91	13, 17, 18, 19	
13 a CH2 13 b	2.07 (o.m) 2.02 (m)		32.03	12, 14, 15, 18	
14 =CH	4.99 (t.m)	6.8, 1.3	127.57	12, 13, 16	11, 12, 13, 17(1.56)
15 C=			131.72		
17 CH3	1.56 (d)	0.9	25.91	14, 15, 17	14
16 CH3	1.49 (s)		17.95	14, 15, 16	13
18 C=			149.24		
19a =CH2 19b	4.59 (small J) 4.55 (small J)		111.28	12, 20	Me 1.64 12
20 CH3	1.64 (s)		19.25	12, 18, 19	11, 12, 13, 14, 19a
1	--		117.94		

4.7 SFG significantly attenuated HCV production in a dose-dependent manner.

To assess the potential of SFG in inhibiting HCV replication, Huh7.5 cells were infected with HCV and treated with SFG at concentration levels of 2.5 μM, 5 μM, and 10 μM respectively for 48h. Subsequently, cell lysates were subjected to western blotting with an anti-HCV NS3 antibody. The results indicated that SFG significantly inhibits HCV NS3 protein synthesis compared with the mock control (0.1% DMSO) in a dose-dependent manner (Fig. 11A). The inhibitory effect of SFG on HCV RNA replication was further examined by qRT-PCR with primers corresponding to HCV RNA. As expected, a clear decrease in HCV RNA level occurred upon treatment with SFG for 48h compared to the treatment of mock control cells (Fig. 11B). SFG significantly suppressed HCV replication by almost 90% at a concentration of 10μm. These data supports the conclusion that SFG is a promising anti-HCV reagent of SF and warrants further study. The cytotoxic index of the SFG was evaluated by SRB assay and no toxicity was observed at higher concentrations (Fig. 11C).

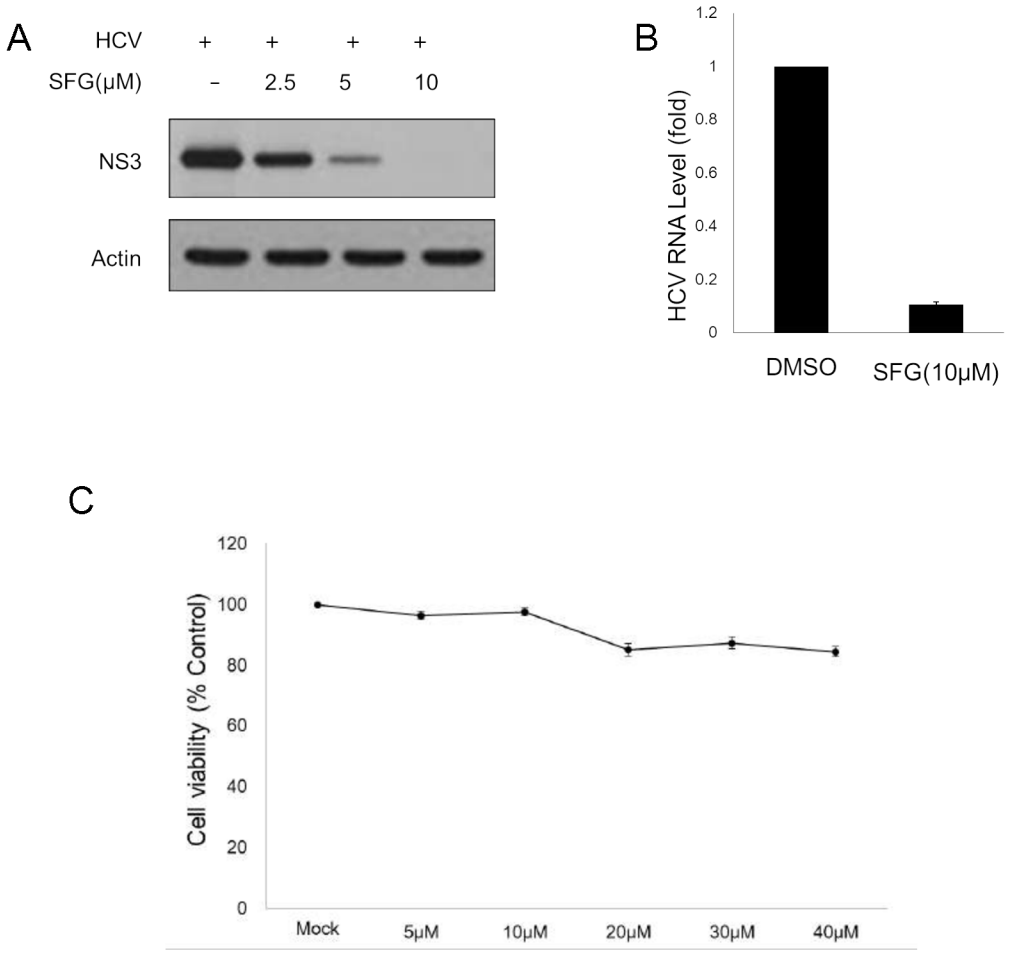

Figure 11: SFG inhibited HCV replication. (A) Dose-dependent inhibition of HCV NS3 protein expression in HCV infected Huh7.5 cells by SFG. Huh7.5 cells were exposed to HCV at different concentrations (2.5μm, 5μm and10μm) of SFG for 48h. Western blotting was performed with anti-HCV NS3 and anti-actin antibodies. (B) Reduction in HCV RNA replication by SFG. qRT-PCR was performed to quantify the total RNA extracted from HCV infected and SFG (10μm) treated Huh7.5 cells. The mRNA level in non-SFG treated cells was defined as 1. (C) Cellular toxicity was evaluated by the SRB assay.

4.8 SFG inhibited HCV RNA replication.

OR6 is a Huh-7 derived genome-length HCV-RNA replicon cell line (O strain of genotype 1b) with renilla luciferase as a reporter, and useful for the discovery of anti-HCV reagents [250]. To study the mechanisms of SFG action in more detail and whether SFG inhibits HCV viral RNA replication, the OR6 cell line was used to study the effect of SFG on HCV RNA replication. We seeded the OR6 cells in 24-well plates 24h prior to the treatment with SFG at concentration levels of 2.5µm, 5µm, 10µm and 20 µm for 48h. The inhibitory effect of SFG on HCV RNA replication was then examined by a luciferase assay. The results indicated that SFG markedly decreased the luciferase activity in a dose-dependent manner (Fig. 12A). In addition, we also used Huh-7.5 cells harboring the HCV sub-genomic replicon (SGRmJFH1BlaRL) to confirm the inhibitory effect of SFG on viral RNA replication. Similarly, cells were treated with SFG at increasing concentrations for 48h. As we expected, dose-dependent reduction of luciferase signal by SFG treatment was also detectable (Fig. 12B).

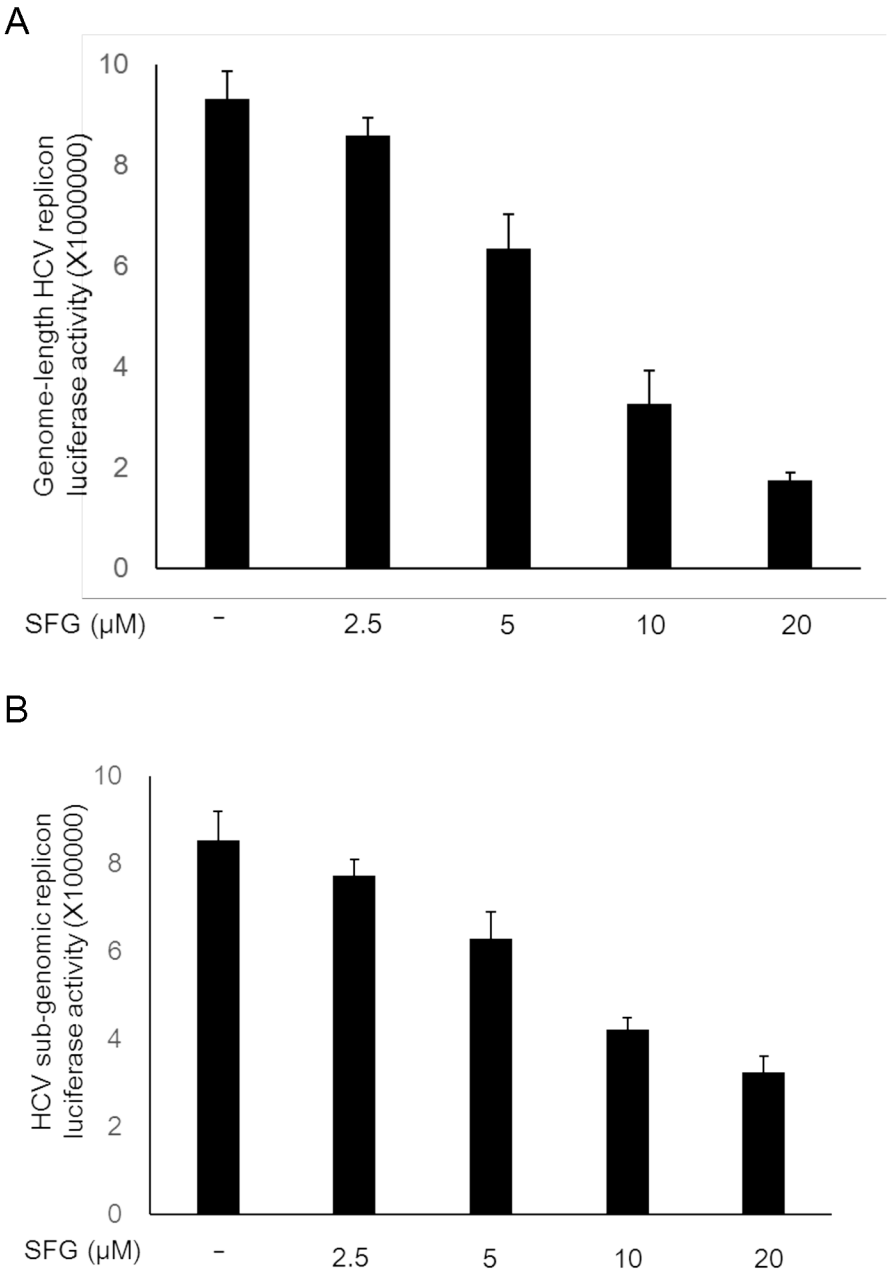

Figure 12: SFG inhibited HCV RNA replication in a dose-dependent manner. (A) OR6 cells or (B) Huh7.5 cells harboring HCV sub-genomic replicon (SGRmJFH1BlaRL) were incubated in the presence of SFG at 2.5μm, 5μm, 10μm and 20μm for 48h. HCV RNA replication was measured by a luciferase reporter assay, normalized to the protein content of the individual sample.

4.9 SFG resulted hyperlipidemia in Huh7.5 cells.

Because of the important role of lipid metabolism in the HCV life cycle, we analyzed the phenotype of lipid droplet in Huh7.5 cells upon the treatment with SFG. Huh7.5 cells were treated with SFG using concentrations of 2.5µM, 5µM, and 10µM for 48 h. After that, cells were fixed and stained with a fluorescent dye, BODIPY (493/503), for observation under a fluorescence microscope. A lipid phenotype observed after treatment showed dramatic hyperlipidemia induced by SFG at 5µM, a concentration shown to significantly reduce HCV replication in cell culture. The increases in LDs have already been observed upon the treatment with SFG at the concentration of 2.5µm. Significant lipid accumulation was mainly localized around the per-nuclear regions of cell appearing as larger LD aggregates (Fig.13). In the mock treatment (0.1% DMSO), a low abundance of small LDs was mainly distributed throughout the cell.

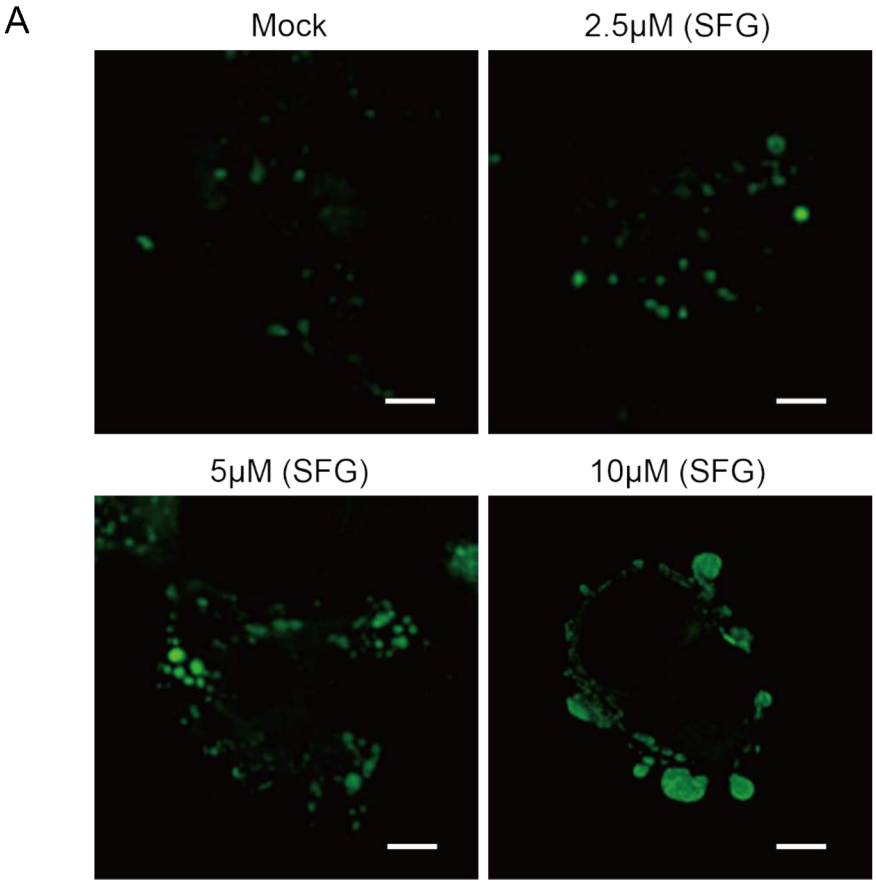

Figure 13: Huh7.5 cells treated with SFG displayed larger LD aggregates. (A) SFG induced LD aggregates in Huh7.5 cells. Huh 7.5 cells were treated with SFG at concentration levels of 2.5μM, 5μM, and 10μM. 0.1% DMSO served as the mock control. After 48h, cells were fixed and stained with BODIPY (493/503) for 30min. A fluorescence microscope was used to observe the cells. Scale bar: 10μm.

4.10 SFG increased HO-1 expression in HCV replicon cells.

It has been demonstrated that SFG effectively induced HO-1 expression in mouse hippocampal neuronal cells (HT22). Besides that, induction or overexpression of HO-1 could inhibit HCV replication [229, 251]. Next, we sought to detect whether SFG could regulate HO-1 expression in HCV replicon cells. OR6 cells were seeded 24h before treatment with SFG at different concentration levels: 2.5μM, 5μM, 10μM and 20μM. After 48h, cell lysates were subjected to western blotting with anti-HO-1 and anti-HCV NS3 antibody; the level of actin served as the loading control. As shown in Fig. 14A, SFG increased HO-1 protein expression in a dose dependent manner. qRT-PCR analysis clearly revealed that SFG increased HO-1 RNA levels dose-dependently (Fig. 14B).

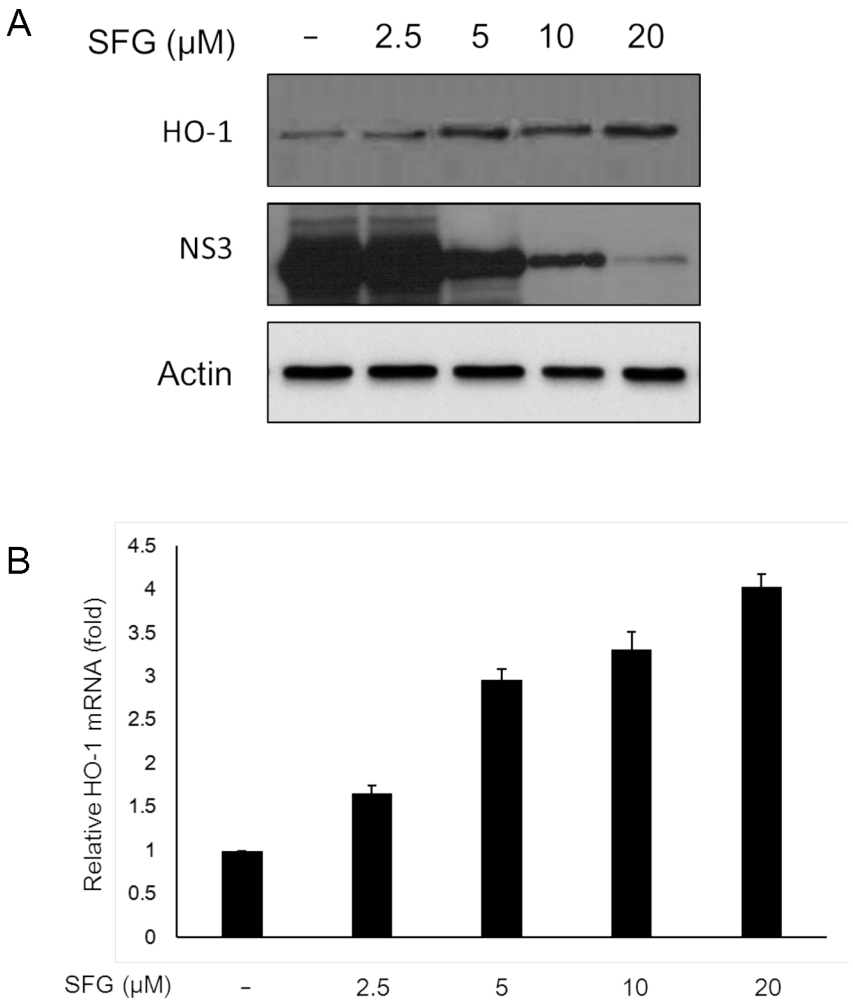

Figure 14: SFG up-regulated HO-1 expression in OR6 cells. (A) SFG induced HO-1 expression and inhibited HCV NS3 protein synthesis in a dose-dependent manner. OR6 cells were treated with SFG at concentration levels of 2.5μM, 5μM, 10μM and 20μM for 48h. Western blotting was performed using anti HO-1, anti-NS3 and anti-actin (a loading control) antibodies. (B) SFG induced HO-1 gene expression in a dose-dependent manner. Total RNA was extracted to quantify the mRNA by qRT-PCR analysis under the same assay conditions. The relative mRNA levels were normalized by cellular GAPDH mRNA expression. The mRNA level in non-SFG treated cells was defined as 1.

4.11 SFG increased antiviral IFN responses.

Recent data indicated that HO-1 inhibits HCV replication by increasing the antiviral IFN responses through its product. To study whether SFG could trigger the antiviral IFN responses because of the increased expression of HO-1 protein, we measured the gene expression of IFNα2 and IFNα17 in SFG treated OR6 cells by qRT-PCR analysis. Cells were treated with 1% DMSO (mock group) or SFG at concentration levels of 2.5μM or10μM for 48 hrs. As shown in Fig. 15A and B, SFG significantly induced the mRNA levels of both IFNα 2 and IFNα17 compared to the mock group. Then, we detected the mRNA levels of essential antiviral genes regulated by IFN, such as protein kinase R (PKR) and 2'-5'- oligoadenylate synthetase 1 (OAS1) under the same experimental conditions. As expected, SFG significantly induced the gene expression of both PKR and OAS1 compared to their expression in non-SFG treated cells (Fig. 15C and D).

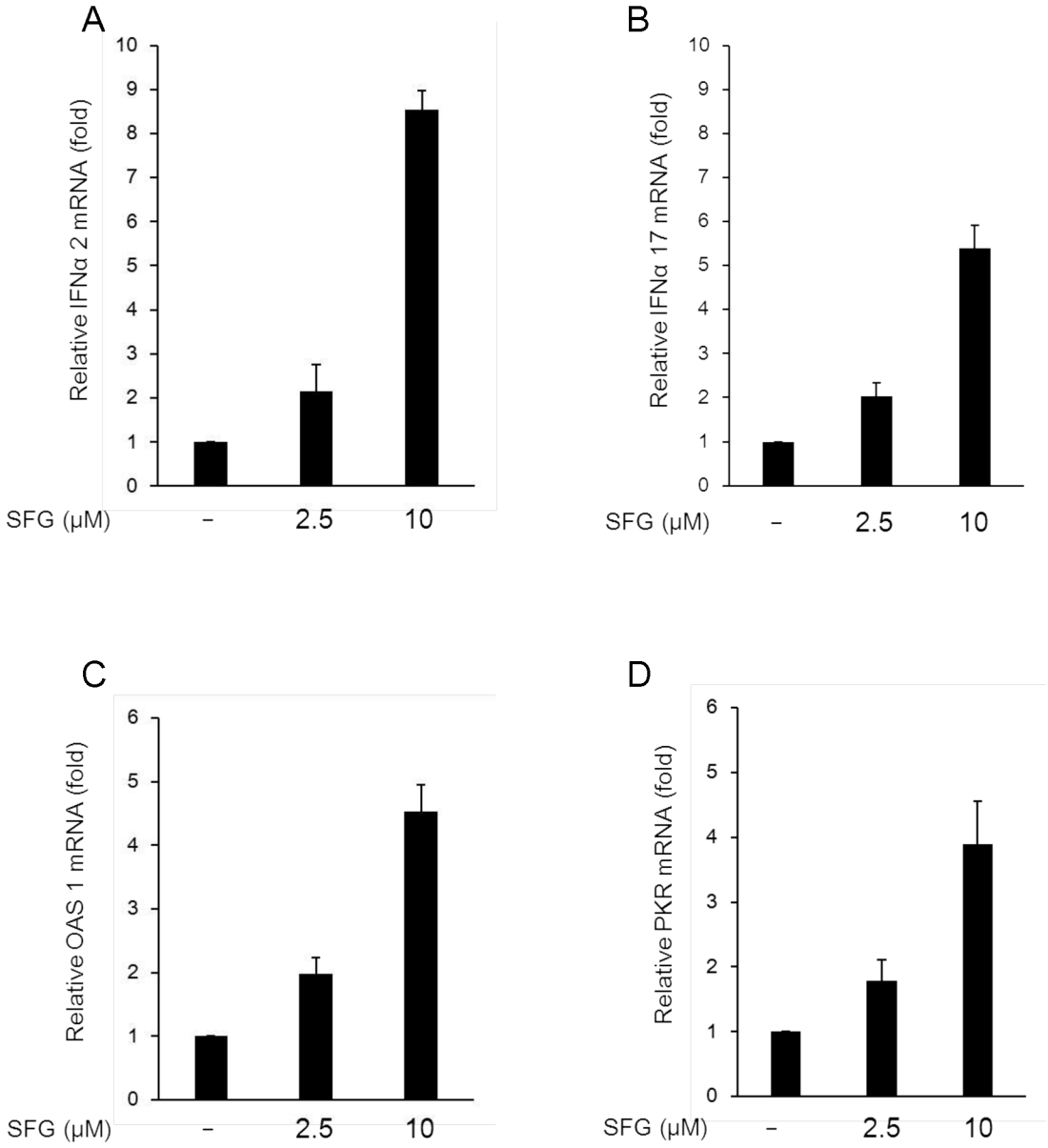

Figure 15: SFG triggered antiviral IFN responses in OR6 cells. SFG induces gene expression of IFNα2 (A), IFNα17 (B), OAS1 (C) and PKR (D). OR6 cells were treated with 1% DMSO or SFG at concentration levels of 2.5μM or 10μM for 48h. Total RNA was extracted to quantify the mRNA levels of IFNα2, IFNα17, OAS1 and PKR by qRT-PCR analysis under the same assay conditions. The relative mRNA levels were normalized by cellular gapdh mRNA expression. The mRNA level in non-SFG treated cells was defined as 1.

Chapter 5-Discussion

In the present study, we identified the most active and specific anti-HCV herbal medicine (SF) among the 45 different CMs tested. Most herbal medicines had little or no anti-HCV activity (data not shown) or did not specifically inhibit HCV replication (Fig. 7). SF is an essential ingredient in many Chinese herbal formulas used for the treatment of chronic HCV infection. After isolation and purification by column chromatography and TLC, we finally obtained three pure active compounds from SF and **KS-211** is the most active anti-HCV compound (Fig. 9A). By using MS, ^{1}H NMR, ^{13}C NMR, HMBC and NOESY assays, KS-211 was identified to be the flavonoid compound, SFG (Fig. 10 and table 2). Subsequently, we indicated that SFG displayed an efficient inhibition of the NS3 protein in a dose-dependent manner (Fig. 11A) and of HCV RNA syntheses in HCVcc infected Huh7.5 cells (Fig. 11B). Almost 90% of HCV protein and RNA expression were decreased by SFG treatment at a concentration of 10μM (Fig. 11A and B). By providing SF or its pure active compound, SFG, at different time: either during infection or after, we identified that SF and SFG affected the post entry step of the HCV life cycle (Fig. 6, 9A and B). In more detail, by using HCV replicon cells we demonstrated that SFG inhibits HCV RNA replication (Fig. 12). Interestingly, the inhibition activity of SFG was found to be more prominent in the genome-length HCV replicon cells (OR6) (Fig. 12A) compared with the HCV sub-genomic replicon cells (SGRmJFH1BlaRL) (Fig. 12B), suggesting that SFG may be able to interact with structural proteins or other non-structural proteins such as NS2, which are important for HCV RNA replication.

Previous studies have demonstrated that host cell lipid metabolism is essential for HCV propagation and LDs primarily as storage organelles for energy are

necessary for the replication complex formation [123]. Based on the important role of lipids in the HCV life cycle, we detected the phenotype of LDs by staining the cells with the fluorescent dye BODIPY (493/503), upon the treatment with SFG. Interestingly, we found that Huh7.5 cells treated with SFG displayed LDs with increased size and number. Additionally, these enlarged LDs predominantly localized at the perinuclear region where the replication complex is known to reside, whereas in the mock-treated cells, LDs were evenly distributed throughout the cell (Fig. 13). Rodney K. Lyn and his colleagues have also found similar results; in their studies, they discovered that inhibitors of peroxisome proliferator-activated receptor α (PPARα) (benzamide) and the mevalonate pathway (lovastatin) resulted in rapid hyperlipidemia and simultaneously inhibited HCV replication in Huh7.5 cells. Additionally, they observed the dispersion of HCV RNA throughout the cytoplasm upon treatment with those inhibitors. Moreover, changes in lipid phenotype and RNA diffusion happened at the same time. Because HCV core proteins are well known to induce rapid increases in LDs size, K. Lyn and his colleagues overexpressed core proteins in cells to determine whether the increased LDs induced by core proteins could disrupt the formation of HCV replication complex. However, no dispersion of HCV RNA was observed. They hypothesized that core protein induced hyperlipidemia is not high enough to provoke changes in replication complexes [252]. These observations suggested that the environment surrounding HCV replication is very important. It has already been reported that replication complexes are located at the interstitial space between the LDs and the ER membrane [123, 253]. When hyperlipidemia is induced, it is possible that the enlarged LDs change the local environment around LDs and MW, therefore resulting in inhibition of HCV propagation.

Further, we observed that SFG elevated the expression of cellular HO-1 protein (Fig. 14A) and RNA (Fig. 14B) in OR6 cells in a dose-dependent

manner, which may also be responsible for the anti-HCV activity of SFG. Gil-Saeng Jeong and his colleagues reported that SFG was able to efficiently induce the expression of HO-1 and increase its activity in dose- and time-dependent mannersin HT22 cells. The increased HO-1 was able to protect HT22 cells against glutamate-mediated neurotoxicity [251]. Data has demonstrated that HO-1 has antiviral effects on many viruses, although their replication machinery is quite different. For example, Ulrike Protzer *et al.* demonstrated that the induction of HO-1 inhibited HBV replication in hepatocytes by reducing stability of HBV core protein, and consequently blocked the refill of nuclear HBV covalently closed circular DNA [227, 228]. In the case of HCV, Weihong Hou *et al.* indicated that miRNA-196 significantly repressed the bach1 protein, a transcriptional repressor of HO-1, resulting in the up-regulation of HO-1 and the subsequent suppression of HCV replication [254]. Zhu, Z. *et al.* reported that HO-1 overexpression decreased HCV RNA replication through its protection activity to hepatocytes from oxidative damage that interferes with IFNα induced antiviral gene expression [229, 255]. Consequently, HO-1 represents a potential therapeutic target against HCV infection. Indeed, targeting host factors required for the viral life cycle has been widely recognized as a very attractive strategy to overcome viral mutations that occur more frequently compared with host gene mutations.

We also observed that SFG induces the expression of endogenous IFNα2 (Fig. 15A), IFNα17 (Fig. 15B) and IFN-dependent antiviral genes including OAS 1 (Fig. 15C) and PKR (Fig. 15D). These results are supported by recent findings of Lehmann et al. demonstrating that HO-1 inhibited viral replication by targeting the IFN signaling pathway. The IFN response was predominantly induced by a metabolic product of HO-1, biliverdin [256]. The IFN system provides the first line of defense against viral infection in mammals and IFNα in combination with RBV is still the standard treatment used for HCV infection. Induced IFNα could act on the producing and neighboring cells to induce

transcriptional activation of hundreds of antiviral ISGs that establish an antiviral state rapidly targeting viruses at various steps of their life cycle [257, 258]. In addition, biliverdin has been reported to interfere with cellular processes to inhibit replication of several viruses, such as human HIV and human herpes virus type 6 (HHV-6) [259, 260].

The present study is the first to identify SFG as a protective reagent against HCV replication and the most active compound isolated from SF for HCV treatment. In our experiment, we further demonstrated that two possible pathways might lead to the inhibition of HCV infection upon the treatment of SFG. First, SFG induced hyperlipidemia in cells that changed the environment around LDs and MW. These changes might disrupt HCV replication complex formation and consequently inhibit HCV RNA replication. Second, SFG induced expression of HO-1, which triggered IFN pathway through its product and thus interfered with HCV propagation.

There are still many tasks left to accomplish to discover the precise mechanism. Tin protoporphyrin (SnPP) is a competitive inhibitor of HO-1. Our ongoing investigations would firstly include studies on whether inhibition of HO-1 expression by SnPP or short hairpin RNA (shRNA) could reduce the anti-HCV activity of SFG. Secondly, whether SnPP could reverse the hyperlipidemia induced by SFG. Thirdly, Lucia Malaguarnera *et al.* demonstrated that non-alcoholic steatohepatitis (NASH) patients exhibited increased expression of HO-1, which was related with severity of the disease and might be associated with the appearance of the initial lesions of fatty liver disease and the development of the disease in fibrosis [261]. In addition, HO-1 has also been reported in association with many other liver diseases [262, 263]. Therefore, we will study the modulation of lipid metabolism by HO-1 in the future. Fourthly, the HO-1 product Biliverdin has been indicated to suppress HCV NS3/4A protease activity; consequently, we will study whether

SFG is a novel HCV NS3/4A protease inhibitor. Finally, many intracellular signaling molecules are involved in HO-1 gene expression including NF-E2-related factor 2 (Nrf2), NF-κB, activator protein-1 (AP-1), MAPK, ERK, c-Jun N-terminal kinase (JNKs), phosphatidylinositol 3-kinase (PI3K) and protein kinase C (PKC) [264, 265]. SFG was previously reported to regulate NF-κB and ERK signaling. Therefore, we will further study the molecules or signaling pathways involved in induction of HO-1 by SFG.

Chapter 6-Conclusions

In conclusion, SF is the most effective and promising anti-HCV medicine among all the 45 test CMs. After isolation and identification, we discovered that the major active compound of SF is SFG, which can significantly inhibit HCV NS3 protein expression in a dose-dependent manner in HCVcc system and suppress sub-genomic HCV RNA replication. An investigation of the mechanisms disclosed that SFG interfered with the metabolism of host cell lipids that are of such critical importance for the infectivity of HCV. Moreover, we have shown that SFG trigged interferon response that may be because of the increased protein expression of HO-1. All the data indicated that SFG is an efficient prodrug of the potent anti-HCV agent and warrants further study.

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