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Development of a Targeted Drug Delivery System for the Treatment of Hepatitis C Virus Infection

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Abstract

Background: Hepatitis C virus infection affects more than 170 million people worldwide and is frequently associated with chronic liver disease and hepatocellular carcinoma. No protective vaccine is yet available and the current standard of care, consisting of pegylated interferon alpha and ribavirin, has limited efficacy. Ribavirin is a key component of any effective anti-HCV regimen. However, accumulation of ribavirin in the red cell compartment not only reduces drug efficacy as a result of diversion to extra-hepatic sites but also produces haemolytic anaemia which can lead to dose reduction or discontinuation of treatment. Lipid or polymer based nanoparticles can be used to deliver therapeutic agents, such as drugs or small interfering RNAs (siRNAs) directly to their site of action. We therefore elected to develop new antiviral strategies based on the targeted delivery of ribavirin to hepatocytes, coupled with the identification of new therapeutic targets. In order to inform the rational use of direct intracellular delivery of ribavirin, we enquired whether variation in expression of the ribavirin transporter may determine drug uptake and permit the identification of individuals who would benefit from these alternative approaches to treatment.

Aims: The aims of this study were to:

- identify host proteins involved in virus replication
- demonstrate reduction of viral replication by modulation of host gene expression
- develop and test a nanoparticle based system for the delivery of therapeutic molecules, including siRNAs either alone or in combination with ribavirin.
- assess the relationship between ribavirin uptake by primary human hepatocytes and expression of ribavirin receptors

Methods: A subgenomic HCV replicon system was established to study the virus-host relationship and identify host proteins supporting viral replication by using stealth siRNA. Viral RNAs were *in vitro* transcribed and transfected into Huh7 cells and expression assessed using engineered GFP as a reporter gene.

siRNAs were co-transfected with viral RNAs using a nucleofector. Modulation of host gene expression was measured by both quantitative RT-PCR and protein blotting. Liposomal nanoparticles containing ApoB-100 duplexes were supplied by Lipoxen. Primary human hepatocytes were isolated by a modified two step collagenase perfusion method and cultured on collagen coated plates. HPLC and real time PCR conditions were used to measure and correlate drug uptake and receptor expression respectively. Equilibrative nucleoside transporter (ENT1) gene was analysed by direct sequencing.

Results: A JFH1 (HCV genotype 2a) virus based subgenomic replicon system was successfully established. Using this model system, host proteins VAP-A and STAT3 were shown to positively regulate virus replication while ACTN1 had no effect. Liposomes failed to deliver either siRNA targeted at apoB-100 or ribavirin and this was found to be due to structural instability of the delivery vehicle. In contrast, fluorescently labelled liposomes were stable and could be taken up by human hepatocyte cell lines under optimised conditions. A protocol capable of efficient isolation and culture of hepatocytes from human donor was validated. Data from primary human hepatocytes show that ENT1 expression was highly variable in different sets of primary livers and correlated strongly with ribavirin uptake. Strikingly, Huh7 cells did not take up ribavirin despite expressing wild type ENT1. It was also found that interferon alpha does not modulate ENT1 expression and therefore ribavirin uptake, suggesting it to be a highly unlikely mode of synergism between the two drugs.

Conclusion: Modulation of host proteins VAP-A and STAT3 inhibited viral replication, confirming that host genes can be used as a potential target to inhibit viral replication. Liposomes used in this study were, however, found to be ineffective vehicles for the delivery of ribavirin or siRNA, as the majority of drug leaked before cellular uptake. Polymer based nanoparticles are currently being assessed for antiviral drug delivery. Variation in ENT1 expression may account for differences in response rate in patients receiving anti-HCV therapy. Results in the Huh 7 cell line suggest that, while ENT1 is necessary, other factors are also required to mediate ribavirin uptake.

Abstracts and scientific communications

Baby Kanwal Baloch *et al*: Equilibrative nucleoside transporter 1 (ENT1) expression is highly variable in primary human hepatocytes and determines the uptake of Ribavirin. Hepatology (Vol 541, Number 4 Supplement October 2011). Presented at the 62nd Annual meeting of the American Association for the study of Liver Diseases, San Francisco, California, 4-8 November, 2011.

Baby Kanwal Baloch *et al*; Ribavirin uptake by Primary human Hepatocytes corresponds to Equilibrative Nucleoside Transporter 1 expression. Gut (September 2011 Volume 60 Supplement 2). Presented at British Association for the Study of the Liver, Annual Meeting, London, United Kingdom, 7-9 September 2011.

Baby Kanwal Baloch *et al*; The use of nanotechnology to deliver intracellular therapies for the treatment of Hepatitis C virus infection. Presented at 110th Annual meeting of American Society of General Microbiology, San Diego, California, 23-27 May 2010.

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Lastly, and most importantly, I am thankful to my son, Ahmed for making this journey joyful and a memorable one.

Abbreviations

2D	two-dimensional
3D	three-dimensional
Apo	Apolipoprotein
Ara-C	Cytarabine
C-terminus	carboxy terminus
cDNA	complementary deoxyribonucleic acid
CF	carboxyfluorescein
CLDN1	Claudin-1
bp	base pair
DMSO	dimethy sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
EMCV	encephalomyocarditis virus
ENT1	Equilibrative nucleoside transporter
ER	endoplasmic reticulum
EVR	Early virological response
GFP	green fluorescent protein
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HPA	Health Protection Agency
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HVR	Hypervariable region
IC	internal control
IDU	intravenous drug use
IFN	Interferon
IMPDH	inosine monophopshate dehydrogenase
IRES	internal ribosome entry site
IRF-3	interferon regulatory factor 3
ISG	Interferon stimulated gene
JFH1	Japanese fulminant hepatitis clone 1
kb	kilobase

kDa	kilo dalton
L	litre
LDL	low density lipoprotein
Luc	luciferase
ml	millilitre
mRNA	messenger ribonucleic acid
miRNA	microRNA
µl	microlitre
µg	microgram
µM	micromolar
NF-κB	nuclear factor κB
NP	nanoparticle
N-terminus	amino terminus
nm	nanometre
nM	nanomolar
NS	non-structural
ORF	open reading framework
PBS	phosphate buffer saline
PC	phosphatidyl choline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
rpm	revolutions per minutes
RBI	Rhodamine B isothiocyanate
RdRp	RNA dependent RNA polymerase
RIG1	retinoid-inducible gene 1
RNA	ribonucleic acid
RSD	relative standard deviation
RT	reverse trascriptase
RV	ribavirin
RVR	Rapid virological response
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis

SEAP	Secreted Alkaline Phosphatase
SGR	subgenomic replicon
siRNA	small interfering RNA
SVR	Sustained virological response
TLR-3	toll-like receptor 3
UK	United Kingdom
UTR	untranslated region
UV	ultraviolet
VLDL	Very low density lipoprotein
WHO	World Health Organisation

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Chapter One

1 General introduction

1.1 Hepatitis C virus infection

1.1.1 Natural history of hepatitis C virus infection

Hepatitis C virus (HCV) was first identified in 1989 as a major cause of parenterally transmitted non-A, non-B hepatitis (Choo et al., 1989). Since then, it has gained considerable attention because of the high morbidity and significant mortality associated with chronic infection. HCV infection frequently leads to chronic liver disease, cirrhosis and hepato-cellular carcinoma and is now an important cause for liver transplantation worldwide (Forman et al., 2002, Hoofnagle, 2002).

1.1.1.1 Epidemiology of hepatitis C virus infection

Estimates show that approximately 170 million people (equalling 3% of world's population) are infected with HCV (Hutin et al., 2004, Shepard et al., 2005, Lavanchy, 2009). The figures vary among different regions (Figure 1.1), with highest prevalence found in the African and the Eastern Mediterranean regions (Lavanchy, 2009). Recently, a systemic review assessing the prevalence and risk factors for HCV in different regions of Asia, Australia and Egypt (an area containing 40% of the world population) estimated that 49.3 to 64.0 million adults are HCV positive (Sievert et al., 2011). Egypt had the highest prevalence rate (14%), followed by Pakistan (4.7%) and Taiwan (4.4%) whereas it was lowest, yet still significant at 1-1.9 % for countries such as China, Japan, India, Saudi Arabia and Syria. In the United States, HCV prevalence is estimated to be about 2%, affecting approximately 5.2 million

people (Chak et al., 2011). These figures reflect the magnitude of the health care burden associated with HCV infection and great need to have effective measures to reduce the pool of infected individuals and minimise HCV related disease.

In England, no population based survey of HCV infection has been conducted and the prevalence is estimated at around 0.76% of the population (Sweeting et al., 2007). A recent report published by Health Protection Agency (Hpa) shows a progressive rise in reported HCV cases between 1992 and 2010 and an estimated 0.2 million people chronically infected with HCV in the UK (Figure 1.2). More than two thirds of these patients (69%) were males and almost half (49%) were aged between 25 and 39 years. Of all the reported risk factors, intravenous drug use was by far the most common and accounted for the acquisition of infection in 89% of all known cases (Hpa, 2011).

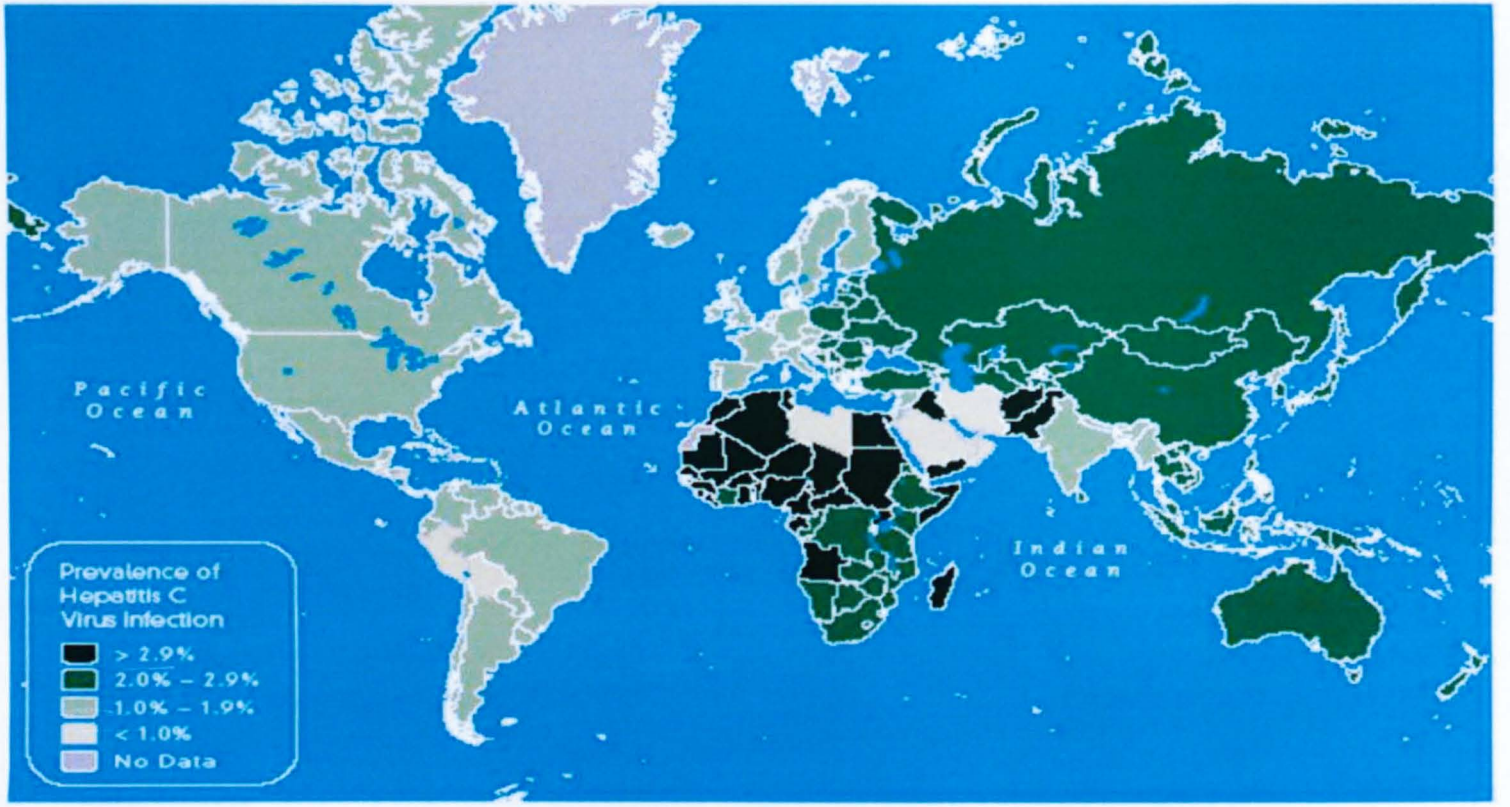


Figure 1.1 Global prevalence of HCV infection

(Source: Centre of Disease Control)

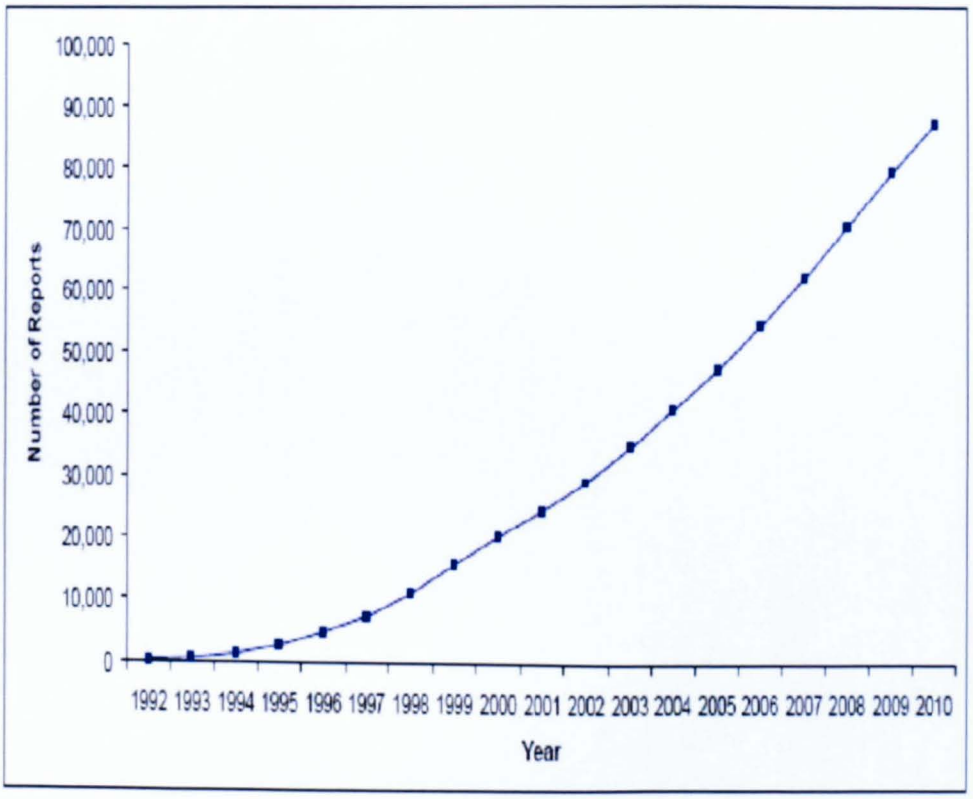


Figure 1.2 Laboratory reports of HCV infection from England from 1992 to 2010

The graph shows consistent rise in newly reported cases of HCV infection in England over a period of ~20 years. Source: the Health Protection agency (Hpa, 2011).

1.1.1.2 Course of hepatitis C virus induced liver disease

Chronic HCV infection is a major cause of liver disease worldwide and a potentially huge healthcare burden. No protective vaccine is available as yet. Individuals who acquire an acute infection usually remain asymptomatic but a majority (50-80%) progresses to chronicity (Marcellin, 1999). The mechanism of spontaneous resolution in one fifth of the infected individuals remains unknown but is now recognised to have strong host genetic basis. Among those with persistent infections, disease severity varies from mild to severe but generally progresses over time. A significant proportion will develop fibrotic liver disease (cirrhosis) over a period of 20 years with consequent decompensation and the development of end stage liver disease (Figure 1.3). About one fourth will die as a result of the complications of cirrhosis or hepatocellular carcinoma (Pawlotsky, 2004, Qureshi, 2007). Globally, HCV infection is responsible for 27% and 25% of cases of cirrhosis and hepato-cellular carcinoma respectively (Perz et al., 2006).

Several factors have been implicated in determining disease outcomes. Female sex, perhaps due to protective effect of oestrogen hormone, has been associated with higher rate of spontaneous viral clearance i.e. 40% in female subjects vs. 19% in male subjects (Micallef et al., 2006). Similarly, a very young age at the time of acute infection (Zhang et al., 2006) and HBV co-infection have also been associated with higher rate of spontaneous resolution. The latter may be due either to reciprocal inhibition of viral replication in patients infected with these two viruses or a more vigorous immune response (Zhang et al., 2006, Shores et al., 2008). In contrast to HBV, co-infection with human immuno-

deficiency virus (HIV) reduces rates of spontaneous clearance and accelerates the development of cirrhosis and its complication (Sulkowski et al., 2000). This is likely to be the result of the underlying immune imbalance seen in HIV infected individuals altering response to HCV antigens (Sulkowski, 2003). Interestingly, among HIV infected patients, those contracting HCV via sexual transmission have greater chance of clearing infection than those who acquire it via the intravenous route (21.9 % vs. 11.6% respectively) (Shores et al., 2008).

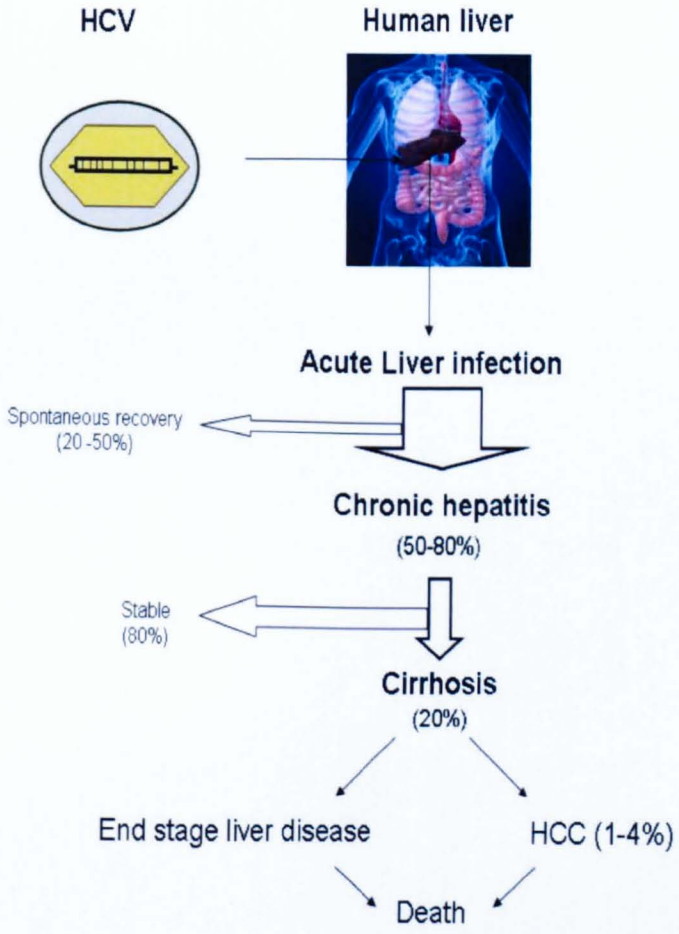


Figure 1.3 Natural course of acute HCV infection

HCV infects liver cells (hepatocytes) and persists in more than half of those acutely infected. With the development of chronic hepatitis, virus induced changes lead to fibrotic disease (cirrhosis) in at least one fifth of these livers over a period of 20-30 years. A significant number of these patients eventually die due to liver cancer or complications of end stage liver disease (HCV = hepatitis C virus, HCC = hepatocellular carcinoma)

1.1.1.3 Virus transmission to host and target cell

The infectious viral particles circulate in the blood stream of infected individuals so transmission is mainly through contact with blood or products contaminated with it such as blood transfusion, IV drug abuse or use of non-sterile equipment during medical and surgical procedures (Alter, 1997, 2002). Transfusion mediated transmission has been dramatically reduced by implementation of blood screening programmes (Donahue et al., 1992) but use of non-sterile medical equipment and unscreened blood remain an important risk factor in many parts of the world. In contrast, intravenous drug abuse has emerged as the major risk factor in industrialised countries (Sievert et al., 2011, Sweeting et al., 2007). Transmission to babies from infected mothers (vertical transmission), transplant recipients from infected donors and sexual transmission are some other but less frequent modes of virus transmission (Albeldawi et al., 2010).

Within the infected host, the virus circulates in various forms. It can be found in association with plasma lipoproteins like low density lipoproteins (LDL) and very low density lipoproteins (VLDL), immunoglobulin or as free virion. The first two forms are considered to represent the infectious fraction (Andre et al., 2002) (Andre et al., 2005) and the majority of infectious viral particles are found in association with apolipoproteins B and E (Owen et al., 2009, Sheridan et al., 2009).

For HCV, humans are the only natural hosts and liver is the principal organ involved in infection. Although the virus is primarily hepatotropic, evidence exists for presence of virus or its negative strand intermediates (as occurs

during viral replication) in peripheral blood mononuclear cells (Okuda et al., 1999), including B lymphocytes (Baré et al., 2005), T lymphocytes (Macparland et al., 2006), monocytes (Laskus et al., 2000) and dendritic cells (Goutagny et al., 2003). In vivo studies have suggested a biological relationship between HCV infection and cognitive impairments seen in patients with HCV infection (Forton et al., 2005). This has been also shown in vitro where the ability of the virus to infect and propagate in certain neuronal cell lines has indicated neurotropic involvement of HCV which may account for neuropschiatric features like fatigue, weakness, inability to concentrate etc seen in a proportion of patients infected with HCV (Fletcher et al., 2010).

1.1.2 Molecular biology of HCV

1.1.2.1 Classification of virus

HCV belongs to the Flaviviridae family which comprises three genera namely Flavivirus, Pestivirus and Hepacivirus (Robertson et al., 1998). Yellow fever virus, dengue fever virus etc are members of the genus Flavivirus while bovine viral diarrhea virus, classical swine fever virus etc belong to the Pestivirus genus (Pawlotsky, 2006). HCV is a prototype member of genus Hepacivirus as a consequence of its tropism for the liver and is structurally closer to the Flavivirus than the Pestiviruses genus (Choo et al., 1991). Flaviruses infect a broad range of vertebrates with humans being dead end host (i.e. does not participate in virus transmission) while no known Pestivirus infects humans. HCV naturally infects only humans but virus can be propagated in a Chimpanzee model.

HCV comprises at least six major genotypes (1, 2, 3, 4, 5, and 6) and several subtypes (a, b, c and d....) differing from each other by approximately 30 and 15-20 percent in their nucleotide sequence respectively (Simmonds et al., 2005). Greatest sequence variation is seen in the region designated the hypervariable region (HVR-1). The high viral replication rate resulting in production of around 10^{12} virions per day, coupled with the inability of viral RdRp to proof read its transcript, accounts for the genetic variability seen among HCV isolates and drives formation of quasi-species within an infected individual (Holland et al., 1992). Figure 1.4 illustrates a phylogenetic analysis of various HCV genotypes and their epidemiological association (Simmonds et al., 2005).

HCV genotypes vary in their worldwide distribution and therapeutic responsiveness, with some evidence for differences in transmission and disease outcome (Gottwein et al., 2009). With respect to differences in viral distribution between different regions, America, Europe and Japan harbour genotype 1a, 1b and 3b followed by 2a and 2b whereas genotype 4 and 5 are mainly found in the Middle East and Africa and genotype 6 is the predominant type in Southeast Asia (Gottwein et al., 2009). In Egypt, genotype 4 accounts for 90% of all HCV cases as a likely consequence of mass parenteral anti-schistosomal therapy programmes (Antaki et al., 2010). In contrast, genotype 1, 2 and 3 in Europe are uniformly distributed and are thought to have been transmitted mainly by intravenous drug abuse. In terms of disease outcome, liver steatosis is common and virus specific in genotype 3 infection (Rubbia-Brandt et al., 2000). Response rates to treatment with pegylated interferon and

ribavirin is much higher (~70%) for genotype 2 and 3 than for genotype 1 (40%) and intermediate for types 4-6 (Manns et al., 2006).

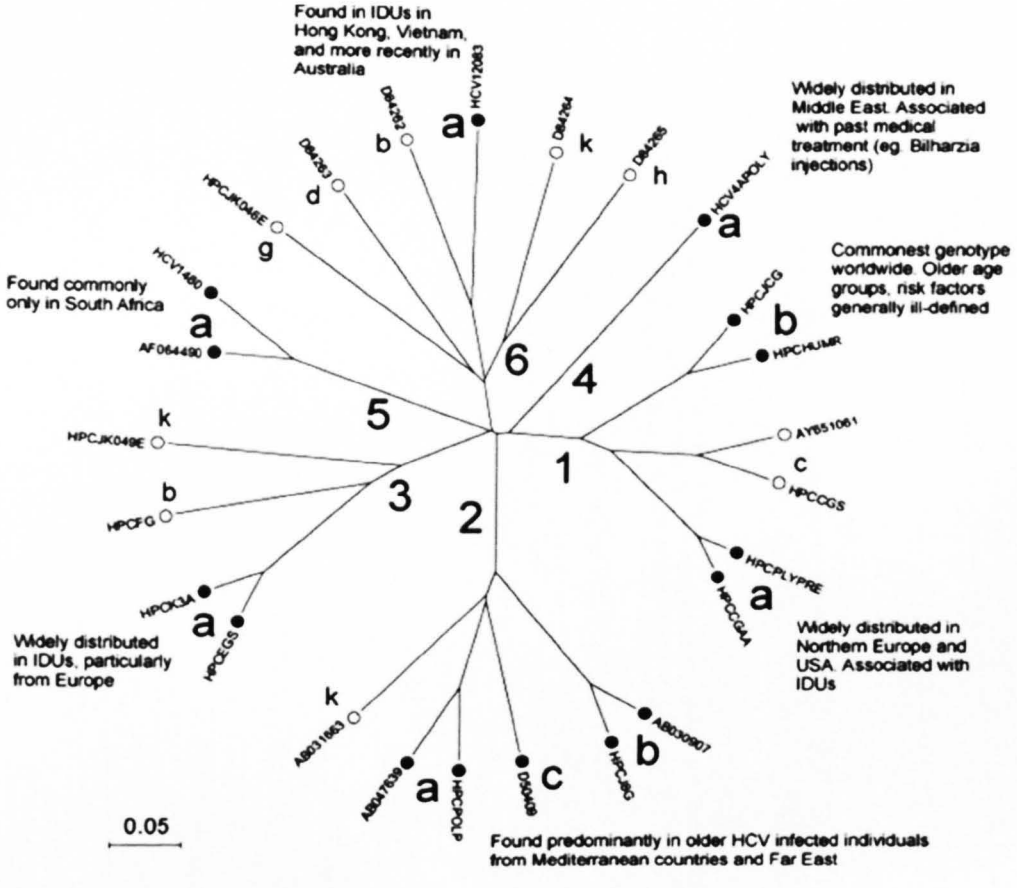


Figure 1.4 Evolutionary tree illustrating major HCV genotypes

Major HCV genotypes and their geographical association are shown. Also given are the available complete open-reading frame sequences for each HCV genotype (Simmonds et al., 2005)

1.1.2.2 HCV genome

The HCV genome is a 9.6 kilobases (kb) long positive-sense single-stranded RNA molecule which serves as template for both replication and translation. It consists of a single open reading frame (ORF) flanked on either side by a 341 and 230 nucleotides long 5' and 3'-UTR respectively (Penin et al., 2004). The ORF encodes for synthesis of ~ 3000 amino acid long single polyprotein which gives rise to HCV structural proteins from N-terminus and non- structural (NS) proteins from its C-terminus (Figure 1.5).

Both UTRs play an important role in protein translation and virus replication. The 5'UTR is composed of four highly structured domains (I-IV) of which I and II are essential for viral replication (Friebe et al., 2001). Domains II, III and IV together with the first 12 to 30 nucleotides of the ORF constitute an internal ribosome entry site (Madeira et al.) that plays a role in cap-independent polyprotein translation, a key step in translation initiation (Pawlotsky, 2006). The 3' UTR consists of a 40 nucleotide long variable region, a poly U/UC tract of 80 nucleotides and a 98 nucleotide long X-tail which is highly conserved (Kolykhalov et al., 1996). There are a number of studies showing that the X tail and part of the poly U/UC tract of at least 25 nucleotides are mandatory for viral replication in *in vitro* culture models and productive infection *in vivo*. You et al identified a cis-acting replication element (CRE) in the 3' end of NS5B designated as 5BSL3.2 (You et al., 2004). Interaction of the middle stem loop (SL2) of the X tail and 5BSL3.2 seems to be indispensable for HCV RNA replication (Friebe et al., 2005).

The IRES facilitates cap-independent translation of viral RNA through binding to the 40S ribosome. This complex recruits eukaryotic initiation factor (eIF)-3 and results in formation of a 48S complex. eIF-3 is later released during joining with the 60S ribosomal subunit resulting in production of a translationally competent 80S complex which then proceeds with viral protein translation (Qureshi, 2007). Translation results into production of a 3000 amino acids polyprotein which is then modified by both viral and host enzymes to yield structural proteins including core protein (C) and envelope glycoproteins E1 and E2, the p7 ion channel and non- structural (NS) proteins (NS2, NS3, NS4A and 4B, NS5A and 5B). Host signal peptidases mediate processing of the structural proteins and at the p7/NS2 junction, while two virally encoded proteases (NS2 and NS3/4A) are essential for maturation of non-structural proteins (Penin et al., 2004).

1.1.2.3 Structure and function of HCV proteins

Post translational processing of the HCV polyprotein gives rise to structural proteins including core, E1 and E2 as well as non-structural proteins including NS2, NS3, NS4A, NS4B, NS5A and NS5B as illustrated in Figure 1.5.

CORE PROTEIN: Along with the genomic RNA, the 191 amino acids long or 21kDa core protein forms the viral nucleocapsid (Yasui et al., 1998) and has a key role in both viral replication and pathogenesis. It has a highly basic N-terminal hydrophilic domain (D1) which is thought to be involved in RNA binding and nuclear localisation (Suzuki et al., 2005). The C-terminal D2 domain is hydrophobic and its association with lipid droplets may lead to the

development of steatotic liver disease seen in HCV infection (Asselah et al., 2006). Wang *et al* showed that the core protein is an inhibitor of RNA interference suggesting that this could be a possible mechanism of viral escape (Wang et al., 2006). The core protein has also been implicated in modulating cellular processes involved in the development of hepatocellular carcinoma (Moriya et al., 1998). Using an HCV cell culture system, a recent study by Alsaleh *et al* identified four basic amino acids at the N-terminal of the core protein required for production of infectious viral particles (Alsaleh et al., 2010). The F protein or alternate reading frame protein (ARFP) is produced as a result of a ribosomal frame shift in the core protein N-terminus. Its role in the viral lifecycle is unclear but is thought to facilitate persistence of HCV and associated induced liver pathogenesis (Baril and Brakier-Gingras, 2005, Fiorucci et al., 2007).

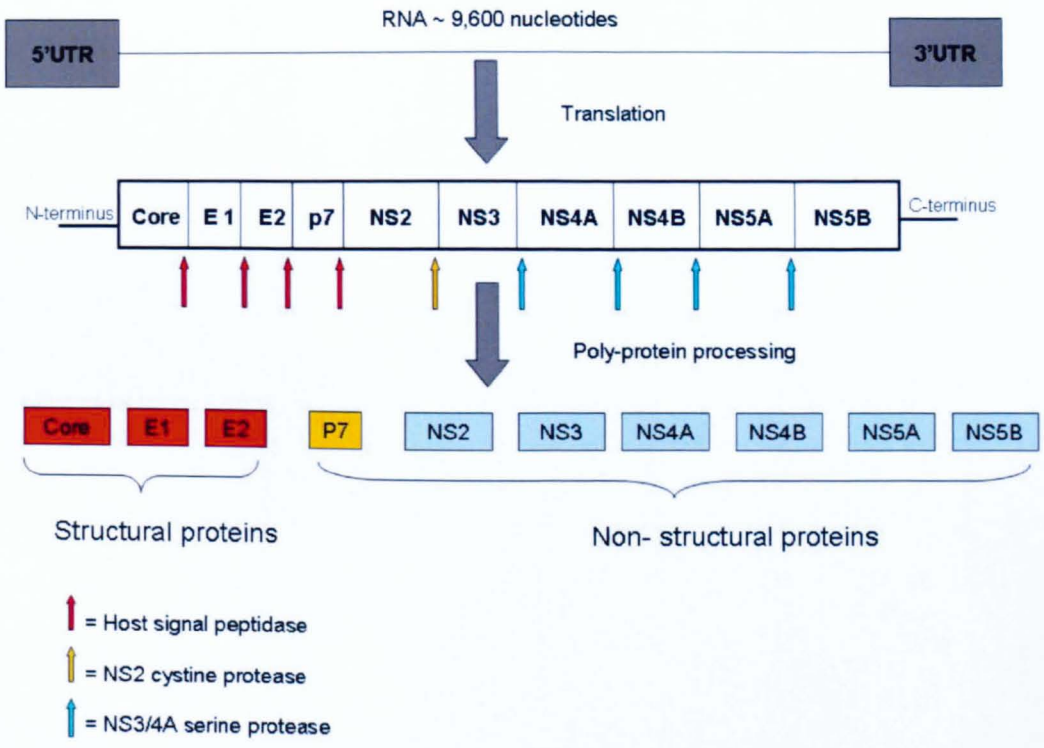


Figure 1.5 Structure of HCV genome and viral polyprotein

The HCV genome is a single stranded RNA molecule having an ORF flanked on either side by 3'- and 5'-UTR. The ORF encodes for viral structural proteins (core, E1 and E2) as well as non-structural proteins (p7, NS2-5B). After being translated, the HCV polyprotein is cleaved by both host and virus encoded enzymes into mature structural and non-structural proteins which perform their specific role in virus replication and pathogenesis inside host cells. (ORF = open reading frame work, UTR = untranslated region, NS = non-structural, E1 and E2 = envelope proteins).

ENVELOPE PROTEINS (E1 & E2): The two glycoproteins E1 and E2 are the key components of the viral envelope and also play a key role in viral attachment and entry into the host cell (Dubuisson et al., 2002). In general, E1 is fusogenic while E2 serves as a receptor binding subunit. E2 protein has a binding site for CD81, a tetraspanin expressed on hepatocytes and B-lymphocytes and is essential for virus entry (Pileri et al., 1998). A study by Wakita *et al* demonstrating efficiency of JFH1 replication and production of infectious viral particles *in vitro* showed that envelope proteins are required for production of infectious viral particles (Wakita et al., 2005). An in frame deletion of envelope proteins coding sequence in HCV clones impaired production of infectious viral particles (Wakita et al., 2005).

The hypervariable region 1 (HVR-1) of HCV is near the amino terminus of E2 (Kato, 2001) and contains amino acid sequences which can differ up to 80% between HCV genotypes and subtypes. This variability may function to promote viral evasion of immune response and support persistence (Boulestin et al., 2002). In spite of sequence variability, the physico-chemical properties of the residues at each position and overall conformation of the HVR-1 region are highly conserved among HCV genotypes, underlining the importance of the HVR-1 region in the viral life cycle (Penin et al., 2001). This is further supported in a chimpanzee model in which Farci and colleagues showed that neutralising antibodies can be developed against the first 27 amino acids of HVR-1 (Farci et al., 1996).

P7: This is a small, 63 amino acid long polypeptide located between E2 and NS2. It belongs to the viroporin family and has putative cation channel activity (Griffin et al., 2003, Pavlović et al., 2003). Intrahepatic transfection studies in Chimpanzees have highlighted its role in viral maturation and release (Sakai et al., 2003). This was confirmed by a later study using a HCV infectious system which showed that p7 is required for efficient assembly and release of infectious viral particles (Steinmann et al., 2007).

NON STRUCTURAL PROTEINS (NS): Although NS proteins are not involved in virus particle assembly, they are required for polyprotein processing and virus replication. NS proteins are cleaved by two viral enzymes, a serine and a cysteine protease (Qureshi, 2007, Sharma, 2010). NS2 encodes a cysteine protease, also known as auto protease and participates in cleavage at the NS2/3 junction (Hijikata et al., 1993). NS3 is a multifunctional protein. At its N terminal $1/3^{rd}$, the sequence encodes for a viral serine protease which along with its co-factor NS4A, mediates cleavage of the NS3/4A, NS4A/B, NS4B/5A and NS5A/B junctions (Figure 1.5). Due to its critical role in viral protein processing, the NS3 protease is a prime target for development of antivirals (Lin, 2010). The remaining C-terminal $2/3^{rd}$ part of NS3 has Helicase-NTPase activity thought to be involved in RNA binding, unwinding of RNA, reduction of RNA secondary structures and NTP hydrolysis (Kolykhalov et al., 2000, Tai et al., 1996, Tomei et al., 1993). The RNA unwinding activity of NS3 is modulated through its interaction with NS5B. Through inhibition of RIG-1 signalling pathways required for induction of the

interferon pathway (Meylan et al., 2005), NS3-4A also plays a role in antagonising the host immune response to viral infection (Li et al., 2005).

NS4A acts as a co-factor to the NS3 serine protease (Tomei et al., 1993) and is responsible for membrane association and stabilisation of the NS3/4A complex (Wolk et al., 2000). By interacting with other HCV proteins like NS4B/5A, NS4A facilitates formation of the viral replication complex. It also modulates viral replication by altering the phosphorylation status of NS5A (Asabe et al., 1997, Kaneko et al., 1994). NS4A has also been shown to localise in mitochondria and mediate apoptotic cell damage, thus contributing to viral cyto-pathogenesis (Nomura-Takigawa et al., 2006).

NS4B has an essential role in viral replication by promoting the assembly of membranous structures, which then along with other non-structural proteins, serves as sites for RNA replication (Egger et al., 2002). It has also been implicated in HCV induced carcinogenesis (Park et al., 2000b) and modulation of cellular processes involved in virus pathogenesis (Yi Zheng, 2005).

NS5A belongs to a group of phosphoproteins having a phosphorylated (56kDa) and hyper- (58kDa) phosphorylated form and an RNA binding activity (Huang et al., 2005). Its phosphorylation status has been shown to inversely affect its function (Appel et al., 2006, Neddermann et al., 2004). Subgenomic replicons have adaptive mutations (predominantly in NS5A) which enable high replication efficiency in the Huh7 cell line (Blight et al., 2003). Studies using an HCV genotype 1b clone subgenomic replicon (Con1) have shown that

reduction of the NS5A hyperphosphorylation state by specific kinases stimulates RNA replication, producing a non-adapted replicon that replicates efficiently in the culture system (Neddermann et al., 2004). Similarly, the hyperphosphorylated form of NS5A also inhibits its interaction with human VAP-A (a host protein supporting virus replication, discussed in detail in chapter 3), consequently inhibiting viral replication (Evans et al., 2004). Use of specific NS5A inhibitors like BMS-790052 (Fridell et al., 2011) has been shown to be an effective anti-HCV agent in phase 1 clinical trials (Gao et al., 2010). In addition to modulating replication of HCV RNA, variation in amino acid sequence between 237-276, the so called interferon sensitivity determining region (ISDR), is shown to modulate the responsiveness of an individual to interferon (IFN) treatment in some populations (Pawlotsky and Germanidis, 1999, Katze et al., 2002, Ishii et al., 2011). A study using transgenic mice showed that NS5A impairs both innate and adaptive immune responses and therefore favours chronic hepatitis (Kriegs et al., 2009). All these factors make NS5A an attractive target for antiviral intervention.

NS5B is an RNA dependent RNA polymerase (RdRp) having a three amino acid GDD (Glycine-Aspartic acid-Aspartic acid) motif in its active site (Lesburg et al., 1999). The GDD is a highly conserved motif in the RdRp in all HCV genotypes and is indispensable for polymerase activity (Yamashita et al., 1998). NS5B catalyzes the replication of HCV and is obligatory for its survival (Behrens et al., 1996). Like all other RdRp, it cannot proof read its transcript with a consequent mis-incorporation rate of 10^{-3} per nucleotide per generation. Its activity is modulated by viral factors like NS3 and NS5A as well as host

factors like Cyclophilin B (Watashi et al., 2005, Heck et al., 2009) and human VAP-A (Gao et al., 2004) and VAP-B (Hamamoto et al., 2005). NS5B is therefore, together with NS3, the premier target for antiviral drug development (Appel et al., 2006). During the course of years, several nucleoside (e.g. R-7128) and non-nucleoside polymerase inhibitors like benzothiadiazine and related analogs have been developed and have shown efficacy in *in vitro* studies and in clinical trials (Das et al., 2011).

1.1.3 Lifecycle of HCV

1.1.3.1 HCV receptors

HCV infection begins by viral attachment to hepatocytes by interaction of specific cell surface receptors with viral envelope glycoproteins (Von Hahn and Rice, 2008). Pseudo-particles (HCVpp) based on envelope glycoproteins E1 and E2 on a retroviral or a lentiviral core are a useful system to understand processes of virus attachment and entry into host cells (Bartosch et al., 2003). Using this and other systems (section 1.1.3), several receptor molecules for HCV have been identified including CD81, scavenger receptor class B type I (SR-BI), claudin 1 (CLDN1), occludin and the low density lipoprotein (LDL) receptor.

The most widely studied of the HCV receptors is the tetraspanin CD81, which binds to E2 (Pileri et al., 1998). This multi-functional protein is expressed on the surface of various cells and has diverse functions including cell adhesion and activation (Levy et al., 1998). Various studies have confirmed the essential role of CD81 in HCV based model systems like HCVpp (Bartosch et al., 2003,

Hsu et al., 2003, Mckeating et al., 2004, Zhang et al., 2004) and HCV cell culture systems (Lindenbach et al., 2005, Wakita et al., 2005). Similarly, a hepatoma cell line such as HepG2 which is not normally permissive to HCV becomes infectable when engineered to express CD81 (Zhang et al., 2004). However, other reports have made clear that, while necessary, CD81 alone is not sufficient for viral entry as not all CD81 positive cell lines can be infected (Cormier et al., 2004). This suggested that other factors are also required for HCV binding and entry into hepatocytes.

SR-BI has been identified as a mediator of HCV entry and, like CD81, has E2 binding properties (Scarselli et al., 2002). An SR-BI ligand, high density lipoprotein (HDL), has been shown to enhance infectivity in the HCVpp system (Voisset et al., 2005) suggesting the importance of this molecule in viral entry. A tight junction protein CLDN1 has also been shown to mediate a late step in viral entry (Evans et al., 2007) and its expression was found to be higher in infected livers than that of normal tissue (Reynolds et al., 2008). Similarly, CLDN1 expression was found to restore HCV infectivity in an otherwise resistant cell line (positive for CD81 and SR-BI) while its down regulation blocked viral entry in a HCV susceptible hepatoma cell line (Evans et al., 2007).

Low density lipoprotein receptor (LDL) is another molecule implicated in virus entry into hepatocytes (Agnello et al., 1999). The association of virus particles with lipoproteins like LDL and VLDL and discovery that infectivity can be reduced by blocking apolipoprotein B and E secretion from infected liver

further supports the role of the LDL receptor in viral entry (Andre et al., 2002, Huang et al., 2007, Owen et al., 2009) but additional studies are needed to confirm its role more precisely.

The co-location of CD81, SR-BI and CLDNI on the sinusoidal surface of hepatocytes, which is the site of the first encounter with HCV, is consonant with their role as the principal receptor molecules (Reynolds et al., 2008). In addition to these receptors other molecules like occludin, glysosaminoglycans (GAG's), mannose binding lectins like DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) and the related L-SIGN (liver-specific intercellular adhesion molecule 3-grabbing nonintegrin) have also been identified as putative entry molecules (Von Hahn and Rice, 2008). Despite all these findings, some cell lines remain non-permissive for HCV suggesting some other factors also affect virus entry. Recently, EW1-2wint (a CD 81 associated protein) was shown to inhibit HCV entry by blocking its interaction with the envelope proteins (Rocha-Perugini et al., 2008).

1.1.3.2 Virus entry, replication and release

After entering into the target cell by receptor mediated endocytosis, uncoating and release of the nucleocapsid into the cytoplasm takes place (Figure 1.6) (Lavillette et al., 2006). The positive sense viral RNA is directly translated into HCV proteins in association with ribosomes. With the help of host and mature viral proteins, HCV initiates the replicative cycle in which a complex is formed consisting of viral non-structural proteins and replicating RNA in association with a membranous web alteration derived from endoplasmic reticulum

(Bartenschlager et al., 2004, Salonen et al., 2004). This is known as the HCV replication complex (HRC).

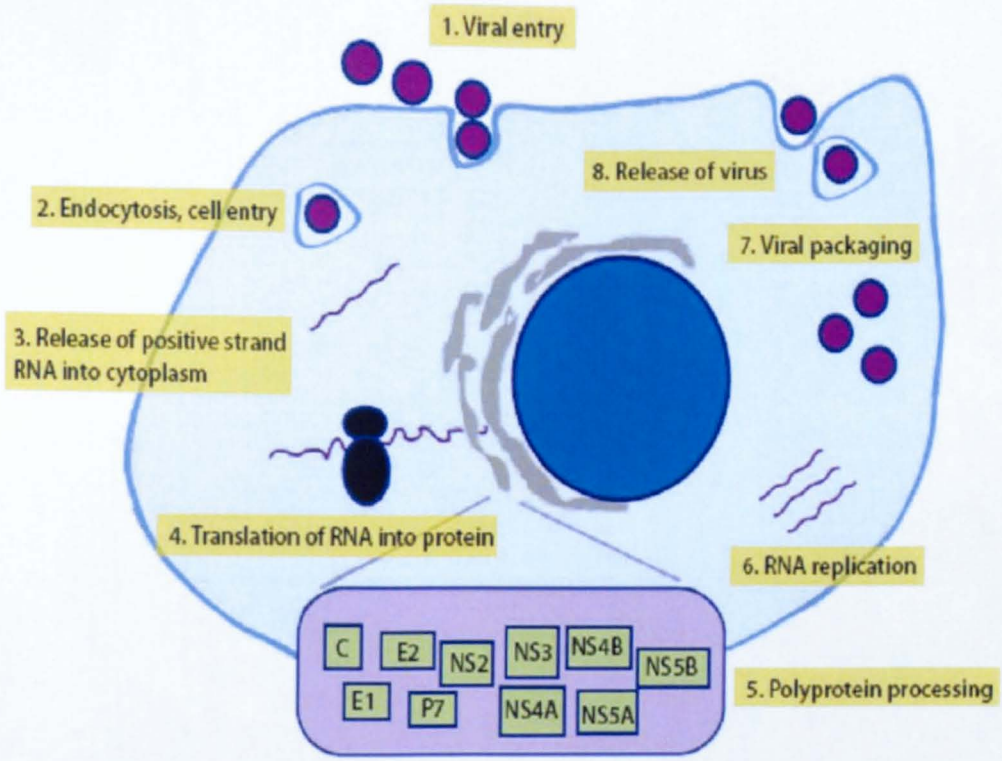


Figure 1.6 Life cycle of HCV

Schematic representation of the HCV life cycle starting from (1) virus attachment and (2) entry into host cell, (3) uncoating and release of viral RNA, (4) translation and (5) processing of viral proteins followed by (6) replication, (7) packaging and (8) release of infectious virus particles (Jazwinski, 2011).

The viral NS4B protein facilitates formation of the membranous web (Egger et al., 2002), which not only provides the physical support and lipid components required for replication but also protects double stranded RNA intermediates from degradation by the host antiviral defences (Schwartz et al., 2002). Saturated fats promote HCV replication while poly-unsaturated fats are inhibitory, demonstrating the influence of fatty acids and cholesterol in viral replication (Kapadia and Chisari, 2005). In light of reports suggesting that saturated fatty acids are required for the formation of the membranous web (Sakamoto et al., 2005), it has been postulated that altering lipid metabolism by drugs may have an effect on down-regulating HCV replication (Torres and Harrison, 2008).

Both untranslated regions (UTRs) and non-structural proteins harbour essential elements for RNA replication. NS5B catalyses the formation of a full length negative strand which then serves as a template for newly synthesised positive stranded HCV genomes which can be either directed to polyprotein translation, participate as replication intermediate (negative strand) or packaged as virus particle. The presence of negative strand serves as an indication of active virus replication (Moradpour et al., 2007). The positive strand RNA is enveloped by budding into the ER lumen and transported to the Golgi apparatus where newly synthesized particles are further matured before being released into the pericellular space by exocytosis as virions (Serafino et al., 2003) as illustrated in Figure 1.7.

1.1.3.3 HCV particle assembly and secretion

The envelope and core proteins are essential for virus particle assembly, while the non-structural proteins (NS3-5B) constitute the minimal viral components required for efficient replication (Blight et al., 2000, Lohmann et al., 1999a). Regardless of their specific roles, the structural proteins, the p7 ion channel and the non-structural proteins are essential for the production of infectious viral particles.

Production of virus particles has been linked to lipid droplets and a number of studies have shown that infectious virus particle assembly is dependent on components of the VLDL biosynthetic machinery (Huang et al., 2007, Gastaminza et al., 2008, Nahmias et al., 2008). Using a cell culture system supporting full length viral particles secretion, Gastaminza et al showed that intracellular HCV particles have higher density than their secreted counterparts suggesting that virus may bind low density particles just before its release (Gastaminza et al., 2008). This was further supported by Huang et al, who identified the presence of proteins involved in the lipoprotein synthetic pathway, such as microsomal triglyceride protein (MTP) and apoB and E within the replication complex, indicating a role for lipoproteins in virus release (Huang et al., 2007). Another study suggested that silencing of apoB-100 by siRNA leads to 70 percent reduction in virus particle production (Nahmias et al., 2008). Electron microscopic studies of cell culture produced HCV particles suggest that HCV virions have spherical shape and a mean diameter of approximately 55 nm (Yu et al., 2007) (Gastaminza et al., 2010).

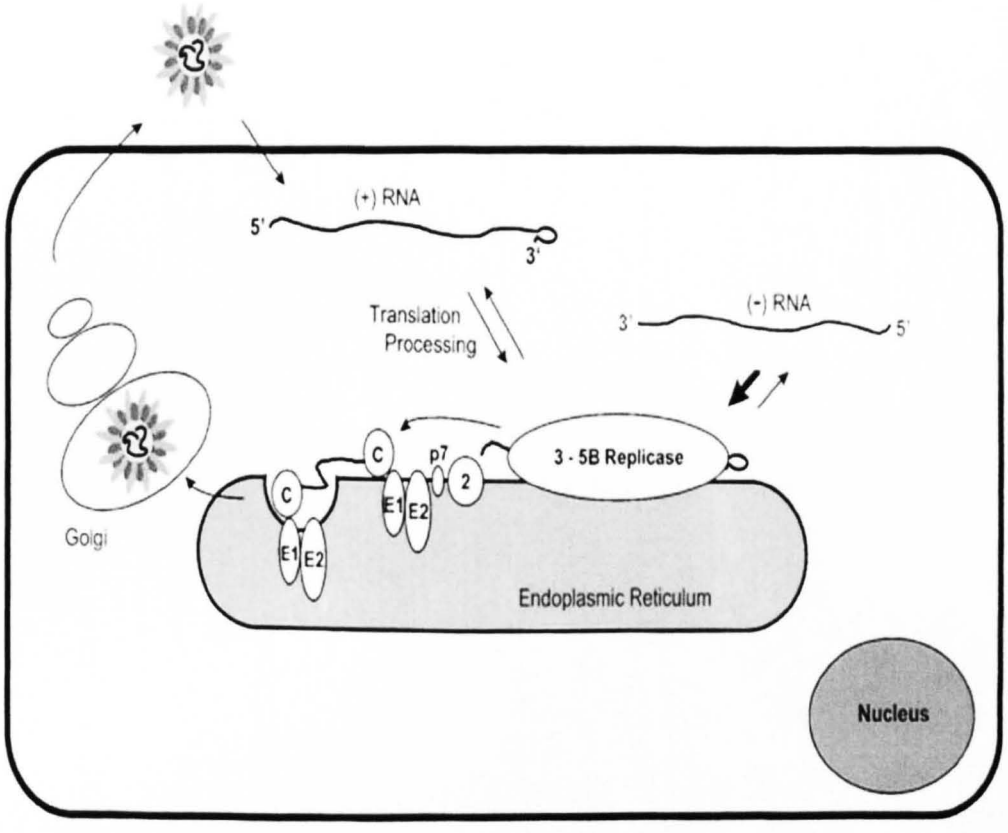


Figure 1.7 HCV replication cycle

Infectious viral particles enter into cells and release the single stranded viral genome into the host cytoplasm which serves as a template for RNA translation and replication. This process takes place in a membranous web derived from the ER. Newly synthesised RNA is then enveloped and assembled in the ER and matured within the Golgi apparatus before being released as infectious virions. (Bartenschlager and Lohmann, 2000)

1.1.3.4 Model systems to study HCV

After the HCV discovery in 1989 (Choo et al., 1989), efforts focused on developing infectious systems which would permit the study of the viral lifecycle that could facilitate the development of effective anti-viral therapy. Such systems, however, proved very difficult to develop and HCV research was slowed for many years due to the lack of a reliable and productive *in vitro* model. Humans are the only natural hosts. Chimpanzees can be infected but their use is restricted due to ethical issues, limited availability, and high cost. In order to create a deeper insight into virus attachment and entry, the replication process and host virus interaction, numerous *in vitro* culture systems have now been developed.

Initial attempts to infect primary human foetal hepatocytes using HCV containing patient sera resulted in an encouraging albeit lower replication potency and infection was maintained for at least one month (Iacovacci et al., 1997). Another study by Lazaro et al demonstrated the infectability of untransformed human foetal hepatocytes with genotype 1a HCV RNA (Lázaro et al., 2007). Considering the short passage life, contamination issues and lack of regular availability of primary cells, immortalised/transformed human hepatoma cell lines were developed and have been extensively used to study cellular processes involved in virus replication.

Liver cell lines, primarily the human hepatoma Huh7 cell line and its derived clones have been used extensively to study HCV pathogenesis. The Huh7 cell line was derived in 1982 from a well differentiated hepatocellular carcinoma and proliferates efficiently in culture conditions (Nakabayashi et al., 1982). It is

a fairly well differentiated cell line retaining some hepatocyte specific function and production of plasma proteins like glucose-6-phosphatase (G6Pase) and Fructose 1,6-diphosphatse (FDPase) (Nakabayashi et al., 1982). However, Huh7 cells do not completely reflect primary liver cell characteristics as they lack cytochrome P450 function and unlike hepatocytes, has high proliferation rate. Huh7.5 is a subclone of Huh7 and supports higher levels of viral replication owing to the loss of retinoid-inducible gene 1 (RIG1) which helps in viral RNA recognition and production of interferon (Blight et al., 2002).

Lack of suitable culture systems that efficiently allow viral replication was a major hurdle in understanding viral-host relationship, but an important breakthrough occurred in 1999 when Lohmann et al developed a bi-cistronic subgenomic replicon system based on genotype 1b (Lohmann et al., 1999a). This subgenomic replicon was created by replacing the portion of genome encoding core to P7 by the neomycin resistance gene and the IRES of the encephalomyocarditis virus (EMCV). Translation of the first cistron was mediated by the HCV IRES while translation of the second cistron was mediated by the IRES of the EMCV (NS2-5B). These replicons were capable of high levels of autonomous replication when transfected into the human hepatoma cell line Huh7. With this system, it became possible for the first time to study HCV replication in long term cultures based on Huh7 cells *in vitro*. It also enabled researchers to understand the formation of the replication complex, host virus interactions, drug targeting and resistance. Some cell culture adaptive mutations were found in the replicon system that increased the efficiency of virus production by many folds but reduced or almost completely

abolished its *in vivo* infectivity (Bukh et al., 2002). More recently, replicons of genotype 1a and 2a have also been developed. Genomic replicons consisting of full length HCV genome including structural proteins were constructed but their replication capacity was lower than that of subgenomic replicons, and there was no virus production (Blight et al., 2003, Ikeda et al., 2002, Pietschmann et al., 2002).

The development of HCV cell culture (HCVcc) was a great step forward. A specific clone of genotype 2a was isolated from a Japanese patient with fulminant hepatitis, known as JFH-1 (Kato et al., 2003). A replicon system based on JFH-1 was constructed and was used to transfect Huh7 cells without the requirement of adaptive mutations (Kato et al., 2003). Wakita et al demonstrated that transfection of full length JFH-1 genomes into Huh7 cells resulted in production of a virus that can be infectious for naïve Huh7 cells and human liver (Wakita et al., 2005). This system allowed the study of unexplored steps in the virus life cycle like the entry process, the replication mechanism, host defence and production of virions. Later on Zhong et al established another JFH1 based culture in Huh7.5, a subclone of Huh7 (Blight et al., 2002) which allowed the virus to replicate at a higher rate in naïve and serially passaged cells (Zhong et al., 2005). A full length chimeras of JFH1 with components of other viral genotypes, including J6, have now been developed and shown to efficiently replicate and produce infectious viral particles in Huh7.5 cells (Lindenbach et al., 2005).

Other model systems include HCV like particles and HCV pseudo particles. HCV like particles (HCVlp) were first produced in insect cells and include

HCV structural proteins E1 and E2. Based on this model system, it was shown that HCV binding to the host cell might induce some changes in gene expression facilitating its infection (Fang et al., 2006b). Another study found that humoral and cellular immune responses induced by HCVlp were able to downregulate viral infection (Elmowalid et al., 2007). To understand the virus entry process, HCV pseudoparticles (HCVpp) were developed by replacing the natural envelope of other viruses, like vesicular stomatitis virus, with the HCV envelope proteins E1 and E2. Both of these proteins assemble to form a noncovalent heterodimer and the correct folding and orientation of this complex is essential for ER retention of virus and interaction with host molecules mediating entry (Dubuisson et al., 2002).

1.1.4 Host cell response to viral infection

1.1.4.1 Pathophysiology of chronic HCV infection

Lack of an obvious relationship between viral load and severity of liver damage seen in chronic infection has suggested that the virus itself is not cytopathic. The only exception to this is steatosis seen in association of genotype 3 infections (Poynard et al., 2003). Instead, the local immune response mediated by T cells appears to be largely responsible for the tissue destruction. The lesions characterizing the chronic infection include portal lymphoid infiltration, focal and bridging necrosis and degenerative lesions. The presence of large numbers of activated $CD4^+$ T cells (producing IFN-gamma) and $CD8^+$ T cells in these lesions further validates their role in progression of the hepatic inflammatory lesions seen in chronic infection (Bertoletti et al., 1997, Bertoletti and Ferrari, 2003, Fiore et al., 1997).

The main complication of chronic HCV infection is liver fibrosis which determines the outcome of disease. Fibrosis in HCV disease shares a complex set of mechanisms with fibrosis of other aetiologies like alcoholism, HBV infection, metabolic disorders, autoimmune diseases etc (Henderson and Iredale, 2007). Liver injury triggers recruitment of various inflammatory cells, production of wide range of cytokines like TGF-β, Platelet derived growth factor (PDGF) etc, and results in changes in populations of sinusoidal and periportal cells into a myofibroblast phenotype. Hepatic stellate cells (HSC) are the most abundant of all the hepatic non-parenchymal cells and chief source of myofibroblasts. Once activated, they play a key role in mediating fibrotic changes in liver which ultimately progresses to cirrhosis and its attendant complications of end stage liver disease and HCC (Reeves and Friedman, 2002). Activated HSCs not only produce fibrillar collagen but also express tissue inhibitors of metalloproteinases (TIMPs) with resultant inhibition of matrix degradation. These events favour scar formation and development of cirrhosis. In HCV related fibrosis, the virus directly contributes to the progression of disease by producing pro-fibrogenic stimuli and activation of HSCs (Schulze-Krebs et al., 2005). Moreover, a direct interaction between HCV core protein and non-structural protein may also play a role in stellate cell activation (Bataller et al., 2004).

Several host related factors like chronic alcoholism, immunocompromised states and co-infection with HIV also contribute in this process (Peters and Terrault, 2002, Sulkowski et al., 2000). In the case of HIV, reduced $CD4^+/CD8^+$ ratio has been held responsible as $CD8^+$ cells act to amplify the

fibrogenic response, but direct infection of HSC may also contribute to this process (Scott L, 2008).

1.1.4.2 Immune response to HCV infection

Once inside the body of its host, the virus acutely triggers an immune reaction consisting of both an innate (non-specific) and adaptive (specific) immune response. The innate or non-specific immune response is the first line of defence mechanism. It is mediated by complement, interferon secretion and natural killer (NK) cells activation. NK cell cause enzymatic lysis of infected cells and produce type II interferon γ and tumour necrosis factor alpha (TNF-α). Presence of double stranded viral RNA intermediates activate host cell interferon genes via toll like receptors (TLR3) (Alexopoulou et al., 2001), which leads to production of type I IFN α and β, that inhibit virus replication (Goodbourn et al., 2000, Randall and Goodbourn, 2008).

The innate immune response provides an immediate host defence to inhibit virus replication (Randall and Goodbourn, 2008) and facilitates the initiation of a more specific and effective adaptive immune responses (Fearon and Locksley, 1996). Studies have shown that this response fails to control the acute infection as, in most of the cases, the virus evades these defence mechanisms by various means like reducing responsiveness of inflammatory cells to IFN (Jinushi et al., 2003) and inhibition of NK cells (Tseng and Klimpel, 2002). Despite the importance of the innate immune response in controlling the acute infection, the effectiveness of the adaptive immune response largely decides its progression to chronic infection (Thimme et al., 2001).

Adaptive immune responses are of two types. A humoral response executed by production of neutralizing antibodies by B lymphocytes, and a cell mediated response elicited by activation of T lymphocytes (both $CD4^+$ or helper T cells and $CD8^+$ or cytotoxic T cells). The antibodies are formed within 7 to 31 weeks after exposure to the virus and are dominantly directed against HVR-1, a 27 amino acid sequence located in the N terminus of the E2 glycoprotein. The role of these antibodies in combating the virus infection is questionable as the variability in this region continuously generates new HCV variants capable of evading this response and naturally acquired antibodies fail to neutralise viral infection.

The more specific and effective cell mediated immune response is mediated by both the $CD4^+$ (helper T) and $CD8^+$ (cytotoxic T) cells (Thimme et al., 2001). $CD4^+$ T cells are stimulated by MHC class II molecules expressed on the surface of antigen presenting cells (APC) and produce cytokines that play a role in macrophage (IFN-γ), B cell and $CD8^+$ T cell activation. On the other hand, they also produce IL-4 and IL-10 that help in limiting inflammatory reaction in order to prevent excessive tissue destruction (Moser and Murphy, 2000). $CD8^+$ T cells recognise antigens in association with MHC class I molecules, and mediate killing of infected cells and secretion of cytokines like IFN-γ and TNF- α which in turn also inhibit viral replication in bystander cells (Kägi and Hengartner, 1996). It has been observed that patients who have spontaneous viral clearance have a powerful, sustained and specific $CD4^+$ and $CD8^+$ T cell response (Thimme et al., 2001). By contrast, a weak, transient and

non-specific T cell response is associated with progressive infection (Bertoletti and Ferrari, 2003).

1.1.4.3 Factors responsible for viral evasion or persistence in host cell

HCV induces a host response which involves numerous signalling pathways and gene products that create a hostile environment for the virus. The presence of viral replication products like dsRNA is sensed both in the extra-cellular environment during viraemia and intra-cellularly by TLR-3 and RIG-1 respectively (Qureshi, 2007). These two pathways converge together and ultimately lead to activation of interferon response genes via interferon regulatory factor (IRF)-3, resulting in secretion of interferon which then exerts its antiviral role (detailed in section 1.2.2.1). However, in the majority of cases, the virus makes use of various host evasion strategies favouring its survival. This is a complex set of functions, including signalling interference, effectors modulation and generation of a population of progeny viral variants at a very high rate (Gale and Foy, 2005).

Viral proteins play a major role in its escape from host immune response. NS3/4A by blocking phosphorylation and activation of IRF-3 (Foy et al., 2003), inhibits interferon signalling via attenuation of RIG -1 and TLR-3 signalling (Foy et al., 2005, Meylan et al., 2005). Similarly, the core protein induces expression of suppressor of cytokine signalling-3 (SOCS-3) which inhibits the JAK-STAT pathway downstream of interferon signalling (Bode et al., 2003). HCV E2 inhibits protein kinase R (PKR) and natural killer cell activation

(Crotta et al., 2002). NS5A also contributes to blunting the host interferon response by inhibiting PKR (Gale et al., 1998) and producing IL-8, a chemokine which inhibits IFN's antiviral actions (Polyak et al., 2001). Other possible mechanisms of HCV evasion include high mutational frequency due to the low fidelity of RNA polymerase (Weiner et al., 1992) leading to mutational inactivation of B- and T- cell epitopes (Mondelli et al., 2001) (Bowen and Walker, 2005) and functionally incompetent $CD8^+$ T cells with poor cytotoxity, proliferative ability and cytokine secretion (Spangenberg et al., 2005). Similarly, HCV attachment and entry into hepatocytes induces a number of host genes that may facilitate viral invasion, entry, replication or persistence inside the host cell (Fang et al., 2006b).

1.2 Clinical aspects of HCV infection

1.2.1 Clinical features, diagnosis and screening of HCV infection

Acute HCV infection is often sub-clinical (80-90%) and in the majority of cases, it progresses to chronicity. People with chronic hepatitis may present with non-specific symptoms of fatigue, abdominal pain, nausea, vomiting, malaise, arthlagia, myalgia, or features of end stage liver disease (cirrhosis) such as jaundice, ascites, palmar erythema, portal hypertension, upper GI bleeding etc (Modi and Liang, 2008).

Laboratory diagnosis of suspected HCV infection is usually made by serological tests, detecting anti-HCV antibodies or molecular techniques for the presence of viral RNA (Patel et al., 2006). Anti-HCV antibody detection

(directed against core or non-structural proteins) is mainly performed by enzyme-linked immunosorbent assay (Lanford et al.)ELISA) or enzyme immune assay (EIA) having 99% specificity. These assays allow for screening of large numbers of samples but can produce false negative results due to a narrow spectrum, in the event of acute infection (before development or antibodies) or immunocompromised states (inadequate immune response). A positive immunoassay result does not mean current infection so needs further confirmation by other tests like HCV RNA quantification by PCR. These molecular based techniques have 98-99% specificity and detection limit of 50-100 copies/ml (viral load usually in range of 0.5-5 million IU/ml), which will not only establish current or active infection but can also be used to monitor response to therapy.

HCV genotyping helps in predicting treatment response and deciding treatment duration. Liver biopsy determines grade and stage of fibrosis which indicates the degree of disease progression and is a key prognostic indicator. HCV screening by means of serological tests is usually recommended for patients at high risk of acquiring infection including intravenous drug users, recipients of blood or blood components before 1992, health care exposure, haemodialysis recipients, HIV infected, children or partners of HCV infected individuals (Ferguson, 2010). The resultant early diagnosis helps in better control of infection and provides the opportunity to prevent or delay disease progression to chronicity. In order to quickly screen individuals aged 15 years or more at high HCV risk, the U.S. Food and Drug Administration (FDA) in June 2010

approved the first rapid blood test for HCV (OraQuick HCV Rapid Antibody Test; OraSure Technologies, Bethlehem, PA).

1.2.2 Current treatment regimens

The goal of therapy is to eradicate the replicating virus and avert progression of chronic liver disease and liver related death. Symptomatic acute HCV infection has greater chance of clearing the virus and approximately 50% of these people undergo spontaneous viral clearance within 12 weeks of onset of symptoms (Gerlach et al., 2003). Those with persistent HCV infection or asymptomatic infected individuals who have an insignificant chance of self resolution, should be treated with either standard or pegylated interferon alpha (PEG-IFN α) monotherapy in order to prevent progression to chronic infection. In the event of chronic infection, combination therapy with PEG-IFN α and ribavirin is given and its duration depends on viral genotype. Current EASL guidelines applied for the management of chronic HCV are summarised in Figure 1.8.

Long term response or virologic cure is best measured by sustained virological response (SVR), which is defined as un-detectable HCV RNA as measured by a sensitive PCR assay 24 weeks after cessation of therapy. SVR is currently regarded as indicative of viral eradication and is clearly associated with reduction in liver related morbidity and improved outcomes. The best predictor of SVR is rapid virological response (RVR) defined as absence of HCV RNA 4 weeks after therapy. Early virological response (Ducat et al.) defined as $\geq$ 2 log reduction in HCV RNA levels than baseline (partial EVR) or total disappearance of viral RNA (complete EVR) within 12 weeks of therapy is

used as a forecaster of response to ongoing treatment. Failure to achieve EVR suggests non-response and is an indication for stopping therapy.

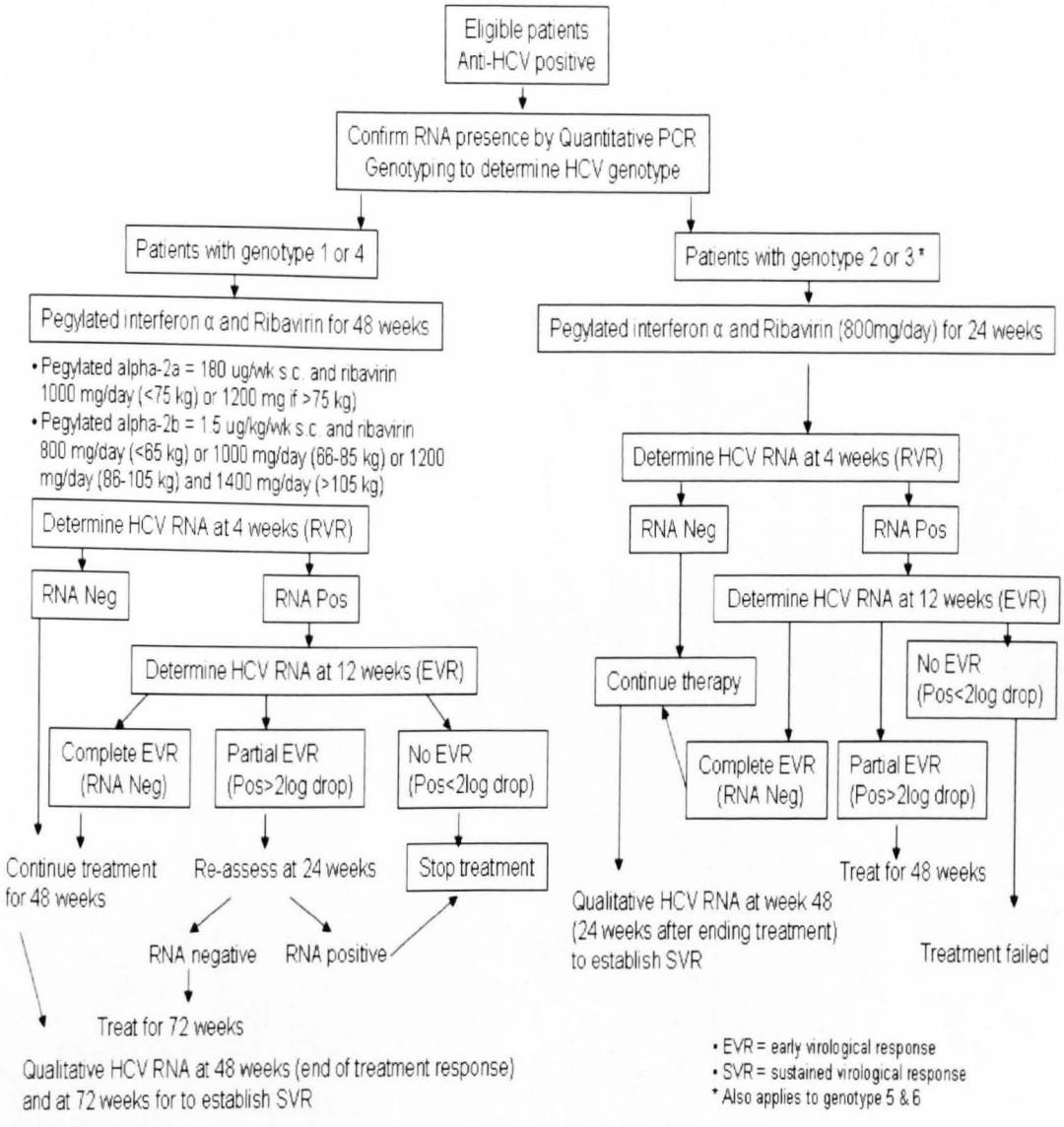


Figure 1.8 Treatment guidelines for chronic HCV infection

European Association for the Study of the Liver Guidelines (Easl, 2011)

1.2.2.1 Pegylated interferon

Interferons (IFNs) are a group of naturally occurring cytokines which have both antiviral and immuno-regulatory properties. Type I IFNs, including interferon alpha (IFN-α) and beta (IFN-β), are produced by many cell types such as fibroblasts, hepatocytes and epithelial cells, whereas type II IFNs (IFN-γ) are only produced by certain types of immune cells like natural killer and T lymphocytes. The presence of viral replication products, especially double stranded (ds) RNA intermediates, acts as a stimulus for the production of various factors required for interferon synthesis like IFN regulatory factors (IRFs) (Barnes et al., 2002).

Upon binding to its cell surface receptor, IFNs triggers a complex signalling pathway leading to activation of gene transcription (Katze et al., 2002). Hundreds of these interferon stimulated genes (ISGs) have been identified (De Veer et al., 2001). The majority induce an antiviral state within the infected cells. A well known type I IFN ISG is protein kinase R, PKR (Meurs et al., 1990, Clemens, 1997). PKR inhibits eukaryotic initiation factor 2 (eIF2) which in turn down regulates viral protein synthesis (Guo et al., 2004). Since response to interferon varies between HCV genotypes, several viral and host factors have been implicated. An interferon sensitivity determining region (ISDR) has been identified in viral NS5A and has been suggested to determine response to interferon based regimens as patients with treatment response carried mutations in this region (Miyamura, 1996). Inhibition of PKR either by viral NS5A (Gale et al., 1998) or direct phosphorylation (Garaigorta and Chisari, 2009) may also account for interferon resistance.

IFN α was initially approved as a monotherapy for HCV but was successful only in a small proportion of individuals (Di Bisceglie and Hoofnagle, 2002). Standard or conventional IFN is administered subcutaneously three times a week and available as Interferon-alpha-2a (Roferon-A, Roche) and Interferon-alpha-2b (Intron-A, Merck). The limitations of conventional interferon, such as short half life and variable peak-trough concentrations requiring frequent administration were overcome by surface modification with polyethylene glycol (pegylated or PEG) (Lindsay et al., 2001). Addition of PEG coating creates a shield around interferon molecules and delays its breakdown and clearance from the body. This allows dosing once a week and since the drug molecule stays longer in the body, there is even greater suppression of HCV. The two currently FDA approved PEG-IFN are Pegasys (pegylated INF-alpha-2a) from Roche and Peg-Intron (pegylated IFN-alpha-2b) from Merck. Albinterferon (Zalibin) is a newer type of interferon genetically fused with plasma protein albumin, producing a long acting molecule and thus requiring dosing once every two weeks. It was successful in phase III clinical trials and is now awaiting FDA approval (http://www.nelm.nhs.uk). Further addition of ribavirin to interferon resulted in a significant improvement in response rate, especially for genotypes 2 and 3 (Mchutchison, 1999) and transformed the treatment paradigm.

1.2.2.2 Ribavirin

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) is a purine nucleoside analogue and was used initially for the treatment of respiratory syncytial virus (RSV) infection. A later trial used ribavirin alone for HCV infection but was found to have negligible effect on HCV RNA levels (Di Bisceglie et al., 1995). Another trial comparing efficacy of ribavirin monotherapy vs. placebo also showed that it had no effect on HCV RNA levels despite improved serum aminotransferases (Dusheiko et al., 1996). Ribavirin was, however, found to have dramatic effects on viral clearance and reduction of relapse rate when used in combination with interferons. In a study by Fried et al, only 29% of patients receiving IFN monotherapy achieved a SVR whereas ~56% of those on combination regimen did so, highlighting the importance of ribavirin on viral clearance. Ribavirin was subsequently approved for use in combination therapy for HCV infection (Mchutchison, 1999). The exact mechanism by which ribavirin exerts its antiviral action is currently unknown. A number of possible modes have been proposed:

(i) After penetrating the cells, ribavirin is phosphorylated to form ribavirin mono- (MTP), di- (DTP) and tri-phosphate (RTP) (Wu et al., 2005). Maag et al showed that RTP is misincorporated into newly produced RNA leading to early chain termination and blockage of viral replication (Maag et al., 2001). Although this effect can be produced for all HCV genotypes, it requires a high ribavirin concentration. In addition, since ribavirin monotherapy fails to exert a direct anti-viral action, this is an unlikely mode of action.

(ii) Ribavirin monophosphate (RMP) acts as an inhibitor of inosine monophopshate dehydrogenase (IMPDH) and leads to depletion of cellular

GTP levels required for protein synthesis, hence it down regulates viral replication (Feld and Hoofnagle, 2005). However, use of specific IMDPH inhibitors failed to produce such inhibition as is seen in the case of ribavirin suggesting that other mechanisms are also required (Zhou et al., 2003).

(iii) It has been proposed that ribavirin also acts as an immuno-modulator by augmenting the CD4+ response and down regulating the CD8+ response (Lau et al., 2002). In support of this, patients receiving combination therapy with interferon α and ribavirin possess a strong helper T cell response than those receiving interferon monotherapy (Cramp et al., 2000).

(iv) HCV has a RNA polymerase that lacks proof reading ability and this accounts for the presence of a large number of quasipsecies in infected individuals. Using a Poliovirus model, Crotty et al showed that presence of ribavirin in the form of RTP leads to mis-incorporation of cytidine and uridine bases into RNA, which exert a mutagenic effect leading to replication error catastrophe (Crotty et al., 2000, Cameron, 2001). Some *in vitro* studies have highlighted the importance of this mechanism in HCV infection (Contreras et al., 2002) but others have provided evidence to the contrary (Chevaliez et al., 2007).

A limitation of the majority of the above mentioned mechanisms of ribavirin was requirement of a very high concentration of ribavirin. A recent study using clinically relevant concentrations of ribavirin showed that inhibition of IMPDH may be the main mechanism of ribavirin's anti-HCV action (Mori et al., 2011). Clinically available formulations of ribavirin include Rebetol (Merck), Copegus (Roche) and Ribasphere (Three Rivers Pharmaceutical, LLC).

Ribavirin is administered orally twice a day and dosage is based on body weight depending on viral genotype (800-1400 mg/day for genotype 1 and 800 mg for 2 and 3). Further details are given in Figure 1.8. Taribavirin (formerly known as Viramidine), a 3-carboxamidine derivative of ribavirin, is a pro-drug which is activated and converted to ribavirin by adenosine deaminase (Wu et al., 2003). Due to favourable properties like its ability to be converted and concentrated in liver, and a positive charge with fewer propensities to be entrapped by RBCs, Taribravin was in clinical trials for the treatment of chronic HCV (Ferguson, 2010). However recent data suggest that at higher dosage, anaemia did not differ greatly from ribavirin.

1.2.2.3 Side effects of current therapy

Treatment related adverse effects are a common consequence of interferon and ribavirin based regimens and often result in dosage reduction or treatment discontinuation. Common side effects affecting 20-40% of patients are flu like symptoms (fatigue, headache, and fever), gastrointestinal upsets (nausea, anorexia and diarrhea), and psychiatric disturbance (depression, insomnia or irritability). Interferon based products have the potential to aggravate existing neuropsychiatric, autoimmune, ischemic and infectious disorders and are contraindicated in patients with hepatic decompensation.

Ribavirin adds to both the efficacy and side effects of IFN monotherapy. The most common adverse reaction is accumulation of ribavirin in red blood cells (RBCs) resulting in often severe haemolytic anaemia (Bodenheimer et al., 1997). As ribavirin concentration increases, more and more ribavirin enters into RBCs and is phosphorylated to its active forms i.e. mono-, di-, and tri

phosphates (RMP, RDP and RTP). Once phosphorylated, ribavirin can neither be metabolised nor transported out of erythrocytes thus resulting in >100 fold higher intracellular concentration than in the plasma (Krishnan and Dixit, 2011). Presence of high amount of phosphorylated ribavirin causes ATP depletion with resultant oxidative stress and cell damage leading to red cell death (De Franceschi et al., 2000). Interferon mediated bone marrow suppression also contributes to ribavirin induced haemolytic anaemia through impaired cell renewal (Peck–Radosavljevic et al., 2002). Symptomatic anaemia occurs in a substantial number of patients and requires either treatment with erythropoietin or blood transfusion or discontinuation of therapy in extreme cases (De Franceschi et al., 2000, Sulkowski, 2003, Sulkowski et al., 2004). A study investigating the effect of ribavirin and IFN combination on hemoglobin levels showed that more than 50% of patients suffered a reduction of $\geq$3g/dl, and reducing ribavirin dose improved it by ~1g/dl (Sulkowski et al., 2004). However, a reduction in ribavirin dose often compromises its therapeutic response (Manns et al., 2001, Sulkowski, 2003, Reddy et al., 2007). A report by Reddy et al assessing effects of ribavirin dose reduction on SVR in patients infected with genotype 1 virus suggested that patients receiving less than 60% of planned ribavirin dose had significant (p value = 0.0006) reduction in SVR and increase in relapse rate at the end of treatment (Reddy et al., 2007).

Ribavirin has the potential to induce birth defects or foetal death and is contraindicated in pregnant women or people hypersensitive to it making it practically not available to these patients. Despite these limitations, current research suggests that ribavirin will remain the cornerstone of any future anti-

HCV regimen and further approaches are needed to overcome these undesirable effects and improve therapeutic responsiveness.

In addition to the factors like viral genotype, ethnic origin, presence or absence of cirrhosis or HIV infection, an important recent advance has been the identification of single nucleotide polymorphisms (SNPs) in the interleukin-28B gene or IL28B which is located on chromosome 19 (rs12979860), as a predictor of spontaneous viral clearance and treatment response (Ge et al., 2009). Several studies have confirmed the importance of IL28B polymorphisms and response to therapy. In a recent report by Thompson et al, CC type, when compared with CT or TT types, was associated with improved early viral kinetics and RVR (28% vs 5% and 5%), EVR (87% vs 38% and 28%) and SVR (69% vs 33% and 27%) respectively confirming its role as a pre-treatment response predictor (Thompson et al., 2010). Another report by Arends et al underlined the association of IL28B and early first phase viral decline (Arends et al., 2011). It is likely that measuring IL28B polymorphisms will become an essential part of the pre-treatment assessment of HCV infected individuals.

1.2.3 Anti-HCV drugs in clinical development

The limited efficacy of current anti-HCV therapy has lead to a search for new therapeutic molecules. Distinct stages of the virus lifecycle which present an opportunity to target numerous drugs are being investigated. These drugs either inhibit viral factors essential for virus entry, RNA replication/protein processing or host factors supporting its survival. Unlike HIV, HCV does not integrate into the host genome so it can potentially be eradicated with a

sustained effect (Monto et al., 2010). Direct-acting antiviral (DAA) agents or Specifically Targeted Antiviral Therapy for hepatitis C (STAT-C) are a class of molecules specifically targeting enzymes involved in virus replication. Several of these compounds are in different stages of clinical trials (Table 1.1) and are of great promise when combined with interferon-α and ribavirin.

1.2.3.1 Inhibitors of viral entry

HCV structural proteins (E1 and E2) interact with host cell receptors (CD81, SRB-1, Claudin, occludin etc) to mediate intracellular entry. Interference with entry can be accomplished by using molecules which either prevent E1 and E2 mediated attachment or fusion to host cell membranes. To achieve this monoclonal and polyclonal antibodies are being developed and evaluated in early phase clinical trials (Mir et al., 2009, Zeisel et al., 2011). The most significant of monoclonal antibodies are HCV AB68, HCV AB6865 and Bavituximab (Peregrine Pharmaceuticals), together with polyclonal antibodies such as HCIg or Civacir (Biotest Pharmaceuticals).

1.2.3.2 Inhibitors of viral translation

The IRES located in the 5'UTR controls viral translation by initiating its binding to ribosomes and its efficiency is affected by HCV proteins like core, NS4A and NS5B. Drug groups which can inhibit IRES can potentially block its translation and hinder its persistence and propagation. These include antisense oligonucleotides (DNA or RNA strands capable of binding to an inhibiting complementary mRNA and thus inhibiting translation), ribozymes (RNA molecules capable of sequence mediated recognition and degradation of target RNA) and small molecule inhibitors. An example of each of these categories

is given in Table 1.1. AVI-4065 is a synthetic phosphorodiamideate morpholino oligonucleotide targeting viral 5'UTR. It has shown efficacy in phase I trials but was stopped in phase II trial due to limited efficacy in reducing viral load (http://clinicaltrials.gov/ct2/show/NCT00229749). Similarly, Heptazyme (a chemically modified ribozyme) was also halted in phase II due to evidence of animal toxicity (Pawlotsky et al., 2007).

MicroRNAs (miRNAs) are a class of small molecules involved in gene regulation. miRNA122 is specifically and abundantly expressed in liver cells and has been shown to positively regulate HCV replication and translation (Jopling et al., 2005). This may be used as a potential target to inhibit HCV replication and drugs based on anti-sense oligonucleotides like SPC3469 (Santaris Pharma) are in phase I trials (Lares et al., 2010).

1.2.3.3 Inhibitors of post-translational polyprotein processing

Once the viral protein is translated, it undergoes processing mainly by the NS3/4A serine protease. Numerous studies have shown the efficacy of targeting the viral protease. The most advanced protease inhibitors are two linear ketonamide compounds called Telaprevir and Boceprevir which have been shown in phase III clinical trials to have clear efficacy in both treatment naïve and treatment experienced individuals and are now approved for clinical use. Phase II trials (PROVE I and II) have shown that combination of Telaprevir with PEG-IFN α and ribavirin improved SVR by 20% but also caused more adverse effects like skin rash, gastrointestinal events and anaemia, than in patients who were on the standard regimen (Hezode et al., 2009, Mchutchison et al., 2009). Another phase II trial (PROVE III) demonstrated

that non-responders to PEG-IFN α and ribavirin showed significant improvement in response rate to triple therapy (51-52%) than the standard regimen (14%) (Mchutchison et al., 2010). Despite these benefits, addition of Telaprevir resulted in higher incidence of adverse effects like anaemia and skin rash resulting in the treatment discontinuation in a few patients. Boceprevir has also shown similar potency. In a phase II trial (SPRINT-1) in genotype 1 treatment naïve patients, Boceprevir based triple therapy resulted in 67-75% SVR compared to only 38% achieved with PEG-IFN α and ribavirin (Kwo et al., 2010). However, the Boceprevir treated group had a higher incidence of anaemia (52-56%) than the control group (35%). One common observation in all of these studies was that ribavirin was of integral importance in the combination regimen as excluding it not only significantly reduced response rate but also increased relapse rate.

1.2.3.4 Inhibitors of replication machinery

HCV's positive stranded RNA genome acts as a template for replication and with the help of viral and host factors, a replication complex is formed consisting of host derived membranous alterations, a negative stranded RNA replicative intermediate and viral proteins. Among the viral factors, the NS5B RNA polymerase is central to this process and has been an attractive target for drug development (Pawlotsky et al., 2007). Although the exact mechanism of NS5A is not known, it is also involved in replication so inhibitors targeting this viral protein are also under development (Table 1.1).

The NS5B inhibitors can be nucleoside inhibitors (NI) or non-nucleoside based inhibitors (NNI) (Jazwinski, 2011). The NI are a natural substrate for the

polymerase and therefore become incorporated in growing RNA chains resulting in early chain termination and viral inhibition. On the other hand NNI directly bind to the viral polymerase and inhibit it. Table 1.1 shows examples of some of these compounds in clinical development.

1.2.3.5 Other inhibitors

In addition to the above mentioned drugs which are still under development, other potential therapeutic targets including RNA interference mediated inhibition of the HCV genome (e.g ALN-VSP), cyclophilin inhibitors (Debio 025), immuno-modulators (Oglufanide disodium), Silibinin (a major component of Silymarin with known anti-oxidant activity) etc are also being tested in phase II clinical trials. Cyclophilins are host proteins involved in protein folding and also act as regulators of the NS5B polymerase. Targeting host factors like cyclophilin not only complements anti-viral effects but will also provide a greater genetic barrier for viral escape mutants (Gaither et al., 2010, Heck et al., 2009, Watashi et al., 2005). In addition, several other host based targets have also been identified using siRNA based screening methodology (Ashfaq et al., 2011).

Table 1.1 Summary of anti-HCV drugs in clinical development

Drug Category	Drug name	General remarks	Current status
Interferon related	Albinterferon	An albumin conjugated longer acting interferon requiring once/2-4 weeks dosing.	Awaiting approval
Ribavirin Related	Taribavirin (Viramidine)	A ribavirin pro-drug which can be concentrated in liver and can avoid ribavirin induced haemolytic anaemia. However, at higher dose, anaemia rate increased and did not differ greatly from ribavirin.	Phase III
Host based			
Cyclophilin inhibitors	DEB025 (Alisporivir)	A synthetic non-immunosuppressive analogue of cyclosporine A which inhibits effects of cyclophilin on HCV replication. Efficacious against genotype 1-4.	Phase II
Virus entry			
Neutralising antibody	Bavituximab	A monoclonal neutralising antibody that binds to phosphatidyl-serine exposed to surface of virally infected cells, thus inhibiting virus entry	Phase II
IRES function			
Antisence-oligonucloetide	AVI-4065	A synthetic phosphorodiamidate morpholino oligomer targeting 5'UTR. Shown efficacy in phase I trials but phase II trial was halted due to limited reduction in viral load.	Phase II
Ribozyme	Heptazyme	Chemically modified ribozyme with improved stability. Shown efficacy in early phase clinical studies but halted because of animal toxicity.	Phase II
Small molecule inhibitors	VGX-410C	Orally active small molecule anti-IRES inhibitor which blocks its association with eIF3 and therefore blocks translation initiation.	Phase II
DAA/STAT-C			
NS3A/4A Protease inhibitors	BMS-850032	Interferes with viral poly-protein processing and activation into mature form after being translated. These are most extensively studied and successful DAA therapies. Two of these, Bocepravir (SCH-503034) and Telaprevir (VX-950) have successfully completed phase III trial and are awaiting approval for clinical use. These compounds have been found to be most effective for genotype 1 than other HCV genotypes.	Phase I
	Vaniprevir, Danoprevir		Phase II
	Telaprevir, Boceprevir		Approved
NS5A inhibitor	PPI-461	Inhibits NS5A of all HCV genotypes and has shown to improve efficacy of current drugs.	Phase I
NS5B NI	R7128	An oral cytidine nucleoside analog polymerase inhibitor. In phase I trial, ~60% achieved RVR when combined with standard regimen and appear to be safe and well tolerated in early trial.	Phase II
NS5B NNI	VX-222	An oral non-nucleoside analog shown potency in early clinical trial and is being evaluated as a combination with Telaprevir as an adjuvant to standard regimen.	Phase II

(NI = Nucleoside inhibitors, NNI = Non-nucleoside inhibitors)

1.3 Current status of anti-HCV therapies and strategies proposed to improve response rate

Current anti-viral regimens based on pegylated IFN alpha and ribavirin, are not only toxic but have lower efficacy for certain genotypes (Manns et al., 2001). Sustained virological response (SVR), although is achievable in 76–82% of those with genotype 2/3 infection, is very low i.e. 42–52% in patients infected with the most abundant genotype 1 (Manns et al., 2001, Fried et al., 2002, Hadziyannis et al., 2004, Zeuzem et al., 2004). Despite the fact that DAAs have shown great promise in clinical trials, the majority of them still require ribavirin. These are also commonly associated with development of clinical resistance due to appearance of mutants resulting in viral rebound. One of the most advanced of DAAs is Telaprevir. In phase II clinical trials with genotype 1 virus, the Telaprevir receiving group achieved SVR of 67-69% (Prove 1/2) and 24-53% (Prove 3) when compared to 41-46% (Prove 1/2) and 14% (Prove 3) in patients on the standard regimen. Removing ribavirin not only lowered anti-viral efficacy but also caused higher relapse rate indicating that ribavirin remains important for achieving SVR. Also Telaprevir appeared to be less efficacious for genotytpe 2 and minimally effective for genotype 3 and 4.

Compliance with therapy and the dose of RV appear to be vital determinants of therapeutic efficacy (Feld and Hoofnagle, 2005). In a comparative study assessing the efficacy of lower dose of ribavirin (800mg daily) vs. standard dose (1000 or 1200mg daily), clearance rates were higher (52%) in patients receiving standard dose, suggesting that lower dose ribavirin will compromise

efficacy (Hadziyannis et al., 2004). Conversely, Lindahl *et al* showed that using a higher daily dose of ribavirin, sufficient to achieve plasma concentration of 15uM/L (mean dose 2540mg/day, range 1600-3600), achieved a response rate of 90% even in genotype 1 infection. The majority of these patients developed severe haemolytic anaemia and required treatment with either erythropoietin or blood transfusion, implying that the improved response rates associated with higher doses of ribavirin are only achievable at the expense of greater side effects (Lindahl et al., 2005) The sequestration of ribavirin into the red cell compartment also reduces its availability in liver, and is a crucial factor in limiting the efficacy of ribavirin for the treatment of HCV infection (Takaki et al., 2004). All of these features point towards the need for an effective delivery method for the currently available drugs, which will not only reduce associated side effects but will direct high concentrations of the drug to hepatocytes. The principle of targeted therapy for ribavirin is also applicable to novel therapeutic agents that may be used in combination with ribavirin based regimens (Jazwinski, 2011). This is particularly relevant to small molecule inhibitors like Telapevir and Boceprevir, which have now been approved for clinical use but still require ribavirin and also cause additional side effects (Hezode et al., 2009).

Although next generation anti-viral agents have improved in efficacy in comparison to standard regimens, there remains a need to identify new therapeutic drugs for HCV with higher efficacy and fewer side effects. Many such agents will be used in combination with other drugs, so minimal side effects and lacks of cross resistance are important considerations. A specific

delivery vehicle for transport of multiple drugs to their site of action in hepatocytes could therefore enhance the efficacy of next generation anti-viral therapy. Small interfering RNAs are therapeutic molecules which can be utilised to silence viral or host gene expression and are ideal candidates for development as novel anti-viral agents. The process of viral replication presents a number of highly specific targets for siRNA action. A number of such siRNA based targets have been identified but are associated with development of resistance to siRNAs due to the intrinsic error prone nature of HCV replication (Ashfaq et al., 2011). Host derived genes, which are involved in virus replication provide relatively invariant targets which may have therapeutic potential. Once validated, these can be combined with current therapy to improve response rate of existing drugs. Two of these host proteins (VAP-A and STAT-3) have been tested and validated in this study (chapter 3). Such small molecules will, however, require a suitable delivery vehicle.

Taken together, current evidence indicates that ribavirin and interferon will remain the cornerstone of HCV therapy for the foreseeable future. New therapeutic targets based on either the viral genome or siRNA targeting of host proteins are an attractive therapeutic option. A key requirement for realising the value of this approach is the development of an efficient, targeted drug delivery system. Such a system may improve viral clearance and minimise the systemic toxicity of anti-viral drugs. Additionally, it will also enable delivery of more the one therapeutic molecule as is the case of DAAs, either alone or in combination with current drugs.

1.4 Research objective

In light of the inadequacies in the current therapeutic options available at the time of planning this thesis, we aimed to improve therapy for HCV through identification of new targets and improving efficacy of current treatments. Thus, the aims of this thesis were:

A) Identification of new targets: In this part of the project we aimed to test whether specific host molecules could be targeted to inhibit viral replication. This firstly required the establishment of a subgenomic replicon system in which subgenomic viral replication could be quantified. This was then used to assess the effect of inhibition of a number of cellular proteins (by siRNA) on viral replication.

B) Improved delivery of therapies: Ribavirin is an effective therapy but it can become sequestered in red blood cells resulting in haemolysis. siRNAs can also be degraded by RNAses present in the blood. We hypothesised that it would be useful to exploit the potential of nanoparticles (NPs), either liposomal or polymer based for drug delivery. These molecules can potentially deliver anti-viral drugs to the liver in a manner which may have many advantages over conventional systematically administered drugs. Since the majority of a drug reaches its target site, this potentially powerful approach will increase drug responsiveness at a lower dosage. We aimed to test whether ribavirin and siRNAs to ApoB-100 could be targeted accurately to cells using liposomal nanoparticles.

C) Improved targeting in patients: Ribavirin is a key component of anti-HCV therapy and being a nucleoside analogue requires a nucleoside transporter to gain intracellular entry. Addition of ribavirin substantially improves response rates and reduces relapse rate when added to interferon alpha. It is therefore also important to recognize the factors responsible for variation in response rate in different individuals exposed to ribavirin based regimen. Earlier studies have highlighted the importance of intracellular ribavirin concentration and response to therapy but the primary reasons remain obscure. In this part of the project, we aimed to understand the mechanism of ribavirin entry in primary human hepatocytes. It was hypothesised that the nucleoside transporter mediated ribavirin uptake in human hepatocytes is responsible for variation in response rate in treated individuals. This also involved validation of an effective method to isolate and culture primary human hepatocytes.

Figure 1.10 summarises the outline of this study and the specific aims to address these research questions.

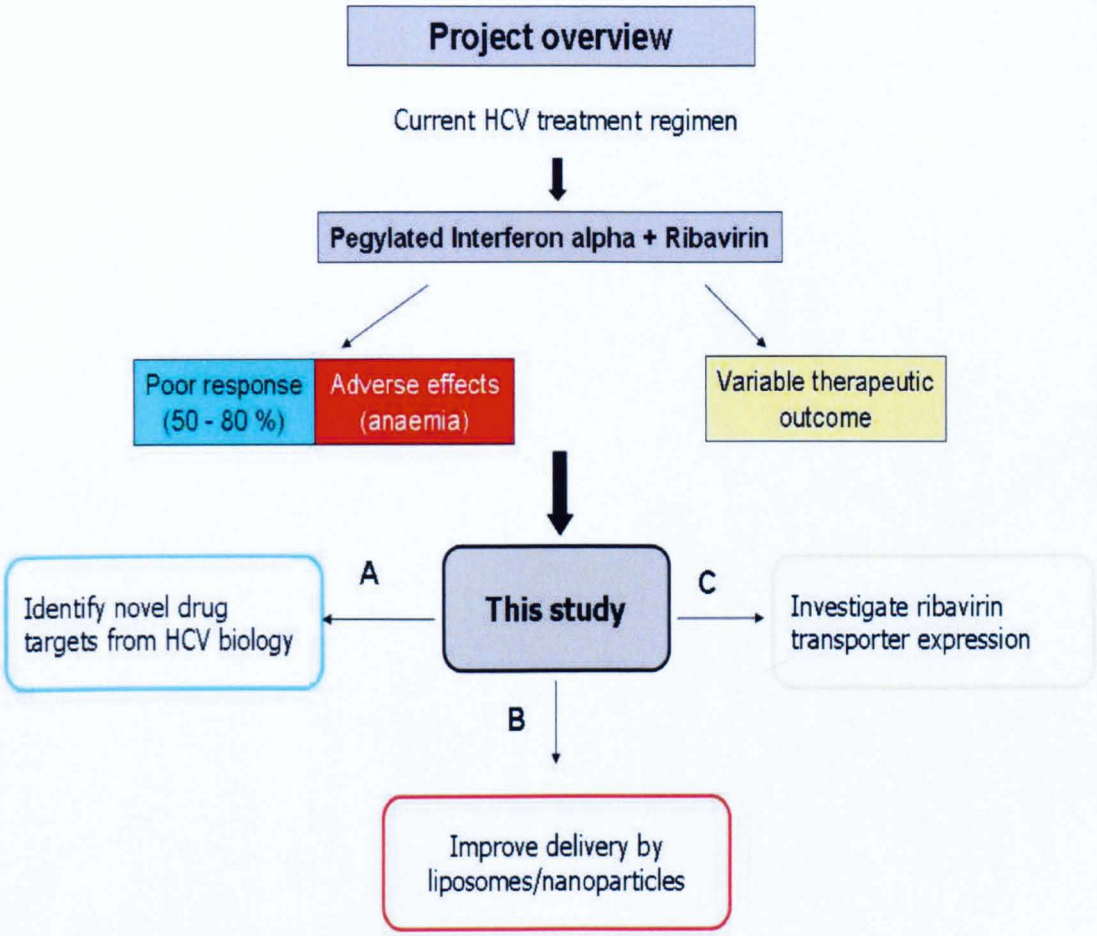


Figure 1.9 Project overview

A. Chapter 3

B. Chapter 4

C. Chapter 5

Chapter Two

2 Materials and Methods

2.1 Cell culture

Tissue culture treated flasks (75 cm^2) and 6 well tissue culture plates were obtained from Corning, UK. Collagen coated 6 well plates were purchased from BD Biosciences, UK. All the buffers and solutions used for culture were prepared fresh under sterile conditions and details of all the chemicals used are given in Appendix 8.1.

Human hepatoma cell lines Huh7 and Huh7.5 were maintained in culture medium made up of Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL, UK) supplemented with 10 % Foetal calf serum (FCS, Sigma, UK), 2mM L-glutamine (Gibco, UK) and antibiotic/antimycotic solution (Hyclone Thermoscientific). Cell culture was maintained at 37°C in a humidified atmosphere containing 5% CO_2 and passaged when ~80-90% confluent. Huh7.5 was also grown in the same conditions except that FCS used was from Biosera.

2.2 Preparation of JFH1 subgenomic replicon:

A subgenomic replicon plasmid (Figure 2.1) based on the HCV genotype 2a, JFH1 (pSGR -LUC-GFP-JFH1) and GND (pSGR -LUC-GFP-JFH1-GND) was a kind gift from John McLauchlan's lab (MRC, Glasgow). The Plasmid was linearised by restriction digestion with XbaI (Roche) following the manufacturer's instructions. Any overhangs generated in the linearised DNA were removed with the help of Mung bean treatment (New England BioLabs) at a concentration of 1unit/μg DNA, and the resulting template was used for *in*

vitro transcription using the Ambion T7 Megascript kit following its manual. Briefly, 5 µg of DNA was prepared in 20 µl of reaction containing nitrogenous bases (ATP, CTP, UTP, GTP) and enzyme mix and incubated at 37°C for 2 hours. DNAase was added to remove input DNA and incubated for another 15 minutes at room temperature. Replicon RNA was further purified using the Qiagen RNAesy kit and quantified using the Nanodrop (ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK). Samples having an A260/A280 ratio between (1.8-2.00) were considered suitable for subsequent steps. 10µg aliquots of replicon RNA were store at -80 °C until use. RNA quality was assessed after every prep using agarose gel electrophoresis.

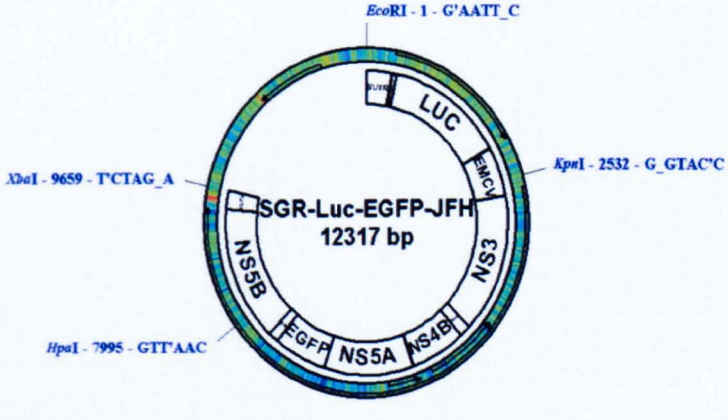


Figure 2.1 Plasmid map for subgenomic replicon

2.3 Transfection of siRNA, plasmid DNA and viral RNA:

Pre-designed stealth siRNAs targeting the gene of interest were obtained from Invitrogen (Block IT) and their sequence is given in Table 2.1. For transfection using Lipofectamine2000, the manufacturer's instructions were followed. In brief, Huh7 cells were plated overnight for initial attachment at a density of 2.5 x 10^5cells/well. The required volume of duplexes was prepared in 500 µl of Optimem medium (GIBCO, UK) and incubated with 5 µl of lipofectamine for 20 minutes at room temperature. The mixture was added to cells cultured in antibiotic free medium and incubated for 6 hours at 37°C before replacing with fresh Huh7 medium (with antibiotics). Cultures were harvested at 48 hours for RNA and protein extraction. For transfection of DNA expression plasmid encoding GFP using Lipofectamine2000, the same protocol was followed except that the cells were harvested at 48 hours for flow cytometry to analyse for GFP expression (Section 2.7).

For nucleofection, siRNA (at a final concentration of 33-500nM) either alone or with viral RNA (5-20µg) were nucleofected by using the Amaxa nucleofector device in conjunction with the nucleofector kit T (Lonza) and T-020 programme. A number of nucleofection controls were used like pmaxGFP, mock-transfected cells, and siRNA for GFP, JFH1 and GND alone. Cells were resuspended in RPMI medium (GIBCO, UK) and cultured for 48 or 72 hours before analysis by flow cytometry and Western blotting. The scrambled control has the same bases but arranged randomly and has been recognised to have no homology to any known RNA sequence.

Table 2.1 Stealth siRNA sequences used in the present study

Target gene	Sense strand (5' –> 3') Anti-sense strand (5'-> 3')
STAT3	UGGCCCAAUGGAAUCAGCUACAGCA UGCUGUAGCUGAUUCCAUUGGGCCA
VAP-A	GGGAAUGCUCCGACUGUCACUUCAA UUGAAGUGACAGUCGGAGCAUUCCC
ACTN1	GGCCCUGGAUUUCAUAGCCAGCAAA UUUGCUGGCUAUGAAAUCCAGGGCC
APOB-100	GUCAUCACACUGAAUACCAAU AUUGGUAUUCAGUGUGAUGACAC

2.4 Total RNA (ribonucleic acid) extraction

RNA was extracted by RNeasy mini kit (Qiagen) following manufacturers instruction. In brief, culture medium was aspirated and 600 µl of buffer RLT (lysis buffer) was added to the cells. The cell suspension was pipetted up and down several times to ensure complete lysis and filtered through shredder columns (Qiagen) for homogenisation. An equal volume of 70% ethanol was added to the resulting suspension and applied to the RNeasy spin column for centrifugation at 13,000 rpm for 15 seconds. The flow through was discarded and the spin column was washed twice with buffer RW1 by centrifugation at 13,000 rpm. DNA free treatment was done by preparing 10µl of reconstituted DNase in 70µl DNase buffer (supplied with the kit) and applying it to the spin column. After incubation for 15 minutes at room temperature, the column was

washed with buffer RPE by spinning at 13,000 rpm for 15 seconds (wash 1) and 2 minutes (wash 2). Finally RNA was eluted in 30-50 µl of RNase free water and quantified by a NanoDrop ND-1000 UV-Vis Spectrophotometer (LabTech International Ltd, Ringmer, UK). The samples having an A260/A280 ratio between (1.8-2.00), indicating sufficiently pure RNA were considered suitable for subsequent steps. The eluted RNA was stored at -80°C until used.

2.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

Complementary deoxyribonucleic acid (cDNA) was synthesised by reverse transcription of RNA. Briefly, 1µg of RNA was prepared in 20 µl of water and 1µl of random hexamers (pD(N)6) and incubated at 70°C for 10 minutes as initial denaturation step. Samples were placed on ice for 5 minutes and a master mix was prepared by adding 1 µl (200 units) of Moloney Murine Leukemia Virus Reverse Transcriptase enzyme [M-MLV RT (Invitrogen, UK)], 50mM of dithiothreitol [DTT (Invitrogen, UK)] and 1.5 µl of deoxyribonucleotide triphosphate (dNTP) mix. In RT negative (RT -) samples, water was added to replace the enzyme. The master mix was added to each sample up to a final volume of 50 µl and incubated at 37°C for 1 hour followed by 10 minutes incubation at 95°C.

2.6 Quantitative real time PCR (Q-PCR)

Primers for real time PCR were designed by the Primer 3 (web version 0.040) programme and targeted at exon-exon junctions. PCR amplification was done using a SYBR green II (reporter dye) based assay (Stratagene). The reaction mixture consisted of 12.5 µl of 1X SYBR Green Master Mix (Stratagene, UK),

1 µl of each forward and reverse primer (final concentration of 250nM), 0.38 µl of ROX (reference) dye and 5µl of DNA template (10ng/5µl). Cycling conditions were 10 minutes denaturation at 95°C followed by 40 cycles of: 30 seconds denaturation at 95°C; 30 seconds annealing at a temperature according to the primer used (see table 2 below); 30 seconds extension at 72°C and a final melt for 60 seconds. The reaction was conducted using a thermal cycler (MX3005P Stratagene, UK) and data analysed by Mxpro-QPCR software version 3.20. A standard curve was generated using serial dilution of neat cDNA. No template control (NTC) without any cDNA and no RT (RT-) control without reverse transcriptase enzyme were used with every reaction.

Table 2.2 Primers used for real time PCR

Gene	Forward primer (FP) (5'-3') Reverse primer (RP) (5'-3')	Annealing Temp (°C)	Amplicon size (bp)
SLC29A1/ ENT1	AGCCAGGGAAAACCGAGA ACCCAGCATGAAGAAGATAAGC	55	95
HPRT	AAATTCTTTGCTGACCTGCTG TCCCCTGTTGACTGGTCATT	60	122
STAT-3	AGTTTCTGGCCCCTTGGATT AAGCGGCTATACTGCTGGTC	58	118
VAP-A	CAACACCTGCCAGTTATCACAC GGCATAGGTCCATCTTGCTT	58	133
ACTN-1	AAATCGTGGATGGGAATGTG CATTTTTGTAAGGGGCTGTCTT	52	150
ApoB-100	GGGCATGGATATGGATGAAG CGGACCCTCAACTCAGTTTT	60	111

2.7 Flow cytometry and GFP expression:

Single cell suspension for flow cytometry was prepared by trypsinising cells using low EDTA (Lonza). The cell pellet was washed twice and resuspended into 1x PBS. Half of the samples were analysed by Beckman Altra to assess GFP expression whereas the rest was used for protein extraction. At least 10,000 events were collected per sample (usually 50,000 cells) and flow cytometric analyses were performed using an EPICS Altra Flow Cytometer (Beckman Coulter, Buckinghamshire, UK). Data were analysed using WinMDI version 2.9 (Joseph Trotter, Scripps Institute, La Jolla, CA, USA) and expressed as mean fluorescence intensity (MFI). Dead cells were excluded from analysis according to their forward and side scatter characteristics.

2.8 Protein extraction and quantification:

Culture medium was aspirated and cells were washed with cold PBS (1X). Lysis solution consisting of RIPA buffer (Thermo scientific) and 1% protease and phosphatase inhibitor cocktail (Thermo scientific) was added to cells and incubated for 30 minutes on ice. The cell suspension was then transferred to ice cold ependorf tubes and spun in a pre-cooled centrifuge at 13,000 rpm for 30 minutes. The supernatant was transferred to fresh tubes and aliquots stored at -20°C until use. Protein quantification was performed using the Pierce® BCA Protein Assay kit (Thermo scientific) following the enclosed manual (microplate procedure).

2.9 SDS-PAGE gel and Western blotting:

10-30 µg of protein lysate was treated with 4X SDS loading dye [100mM Tris-HCl (pH 6.8), 200mM DTT, 4% SDS, 0.2% glycerol and 0.2% bromophenol

blue] supplemented with 5% β-mercaptoethanol. Samples were boiled for 5 minutes at 95°C in a heat block, and then electrophoresed using 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) at 30mA/gel. Gel composition and preparation method is given in Appendix 8.2. Proteins were transferred to a PVDF membrane (Amersham Hybond-P PVDF membrane, GE Healthcare, UK) using a semi-dry transfer method at 60mA for 2 hours. The membrane was incubated in blocking buffer consisting of 5% dried milk dissolved in Tris-buffer saline (TBS) with 0.1 % Tween-20 (Sigma) for 1 hour at room temperature. Immunoblotting was performed by incubating the membrane with mouse monoclonal antibodies for ApoB-100 (1:200, Santa-Cruz Biotechnology), STAT-3(1:50, Abcam), VAP-A (1:500, Abnova) and ACTN1 (1:100, Abcam) overnight with rolling at room temperature. The membrane was rinsed with wash buffer (TBS with 0.1% Tween-20) three times for 5 minutes each and was exposed to horse-raddish peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature. Bands were visualized using an Enhanced Chemiluminescence detection kit (Supersignal West Pico Chemiluminescent Substrate, Thermoscientific, UK) and exposed to X-ray film (Kodak, UK).

2.10 Isolation and culture of primary human hepatocytes

Composition and preparation of all the solutions and culture media used in this procedure are given in Appendix 8.1. Primary human hepatocytes were isolated by a modified two step Collagenase perfusion method described previously (Gottschalg et al., 2006). Briefly, equipment was set up in a class II safety hood and pump speed was set up at 50rmp. Perfusion channels were sterilised by recirculating with 70% ethanol (Sigma) followed by 1L of sterile water.

Hepatocyte isolation medium and solutions were pre-warmed at 43°C and perfusion buffers were also aerated with carbogen (95% Oxygen plus 5% Carbon-dioxide). Liver tissue was flushed well with diluted Soltran solution in order to remove any blood clots. About 500ml of buffer 1 was flushed at a speed of 45rpm to set up flow. Once the perfusion was established (indicated by change in temperature and colour of liver tissue), the liver was perfused with 1L of buffer 1 (contains EGTA to break cell-cell interaction). Buffer 2 (No EGTA) was then flushed to remove any EGTA before shifting to buffer 3 which was recirculated for approximately 20 minutes. Buffer 3 contains collagenase which destroys cell-matrix interactions. Once the liver tissue was softened, cells were released by teasing digested tissue with blunt forceps placed in a petri dish containing isolation medium. The resulting cell suspension was filtered through a nylon membrane and centrifuged at 50g for 5 minutes. Hepatocytes were pelleted and supernatant was removed. The cell pellet was resuspended in isolation medium containing 90% Percoll solution (3:1 ratio) and centrifuged at 100g for 10 minutes. The supernatant containing dead cells was discarded and cells were dissolved in cold hepatocyte plating medium. Cell number and viability were assessed by Trypan blue (TB) exclusion. An equal volume of cell suspension and TB were mixed and placed on a 0.0025mm^2 haemocytometer (Sigma, UK) under a glass cover slip. Dead cells (blue) were subtracted from the total cells (yellow) and the percentage calculated as viability. A viability of ~85% was considered necessary to proceed. Cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Donor information for human liver used is given in Table 2.3.

Table 2.3 Human liver donor information and cell viability

Liver #	Sample ID	Age (years)	Sex	Cell viability %	Additional information
Liver 1	L89/10/N	67	M	89	right Hepatectomy
Liver 2	L90/10/N	82	M	84	-
Liver 3	L93/10/N	70	M	85	Liver resection VII/VIII
Liver 4	L96/10/N	74	F	87	-
Liver 5	L110/10/N	66	M	86	Left Hepatectomy
Liver 6	L111/10/N	64	F	85	Left Hepatectomy

2.11 Ribavirin and interferon alpha treatment

Ribavirin solution was prepared by dissolving 10mg of powder (Sigma, UK) in 10 ml of water to obtain a concentration of 1mg/ml. The required volume of drug was added to the cell culture medium at a final concentration of 12μM/well as found in patients receiving therapy (Tsubota et al., 2002) and cells were incubated for 24hours. Recombinant human interferon alpha-2a (Roferon - A, Roche) was obtained as a solution and was added either alone or in combination with ribavirin at a final concentration of 5000pg/ml (Lopez-Cortes et al., 2008). Parallel samples were taken for HPLC, RNA and cell viability after 4, 8 and 24 hours.

2.12 High performance liquid chromatography (HPLC)

2.12.1 Sample preparation (Cell lysis and enzyme diges tion)

Culture medium was aspirated and cells were detached using trypsin-EDTA solution (Lonza). The resulting cell suspension was centrifuged at 250g for 5 minutes to pellet. Cells were lysed by adding 2% Triton X-100 (Sigma) in PBS and vortexing the tubes for 5 minutes until no cell debris were visible. After centrifugation at 100rpm for 5 minutes, the supernatant was transferred to fresh tubes and stored at -20°C. About 200µl of stored cell suspension was treated with 300µl of 30 µM Tris-HCL buffer (pH 7), 25 µl of 1M sodium acetate (Sigma) pH 4.0 and 2.5 µl of Acid phosphatase type IV from sweet potato (500 Units, Sigma) after incubation at 37°C for 1 hour. The best incubation time and concentration of enzyme was determined by monitoring the ribavirin peak at a time course of 30, 60, 90 and 120 minutes with a plateau achieved at 60 minutes. The reaction was stopped by adding 2.5 µl of KOH (10M) and spinning the tubes to collect the supernatant which was then used for column extraction.

2.12.2 PBA column extraction

Phenylboronic acid cartridges (PBA Bond Elute, Varian) were used to remove any impurities from HPLC samples. Cartridges were positioned on a 10 port vacuum elution manifold under reduced pressure and washed with 1ml of methanol containing 0.5% v/v H_3PO4 (pH=2) and 2ml of Ammonium phosphate buffer [$(NH_4)H_2PO_4$ (250mM, pH8.5)]. Samples treated with Ammonium phosphate buffer [$(NH_4)H_2PO_4$ (250mM, pH8.5)] and 2.5 µl

(1mg/ml) of internal standard (3-methylcytidine methosulfate) were applied to the column and washed with 3 ml of Ammonium phosphate buffer [$(NH_4)H_2PO_4$ (250mM, pH8.5)] and 2ml of methanol. Finally, ribavirin and the internal standard were eluted into glass tubes with 2 ml of methanol containing 2.5% formic acid. The effluents were dried under nitrogen gas and reconstituted in 200 µl of water. Twenty microliter aliquots of reconstituted samples were injected onto the HPLC column. Preparation and composition of all the solvents and buffers is given in Appendix 8.3.

2.12.3 Chromatographic conditions:

The HPLC system used was HP1050 with a four channel pump, an autosampler and UV detector operating at a wavelength of 207nM. Drug separation was done at room temperature using an Atlantics dC18 column (3µm, 150 x 4.6mm, Waters), coupled to a Guard column: Atlantics dC18 3µm (20mm × 4.6mm, Waters). The mobile phase consisted of 10mM Ammonium phosphate buffer after adjusting the pH to 6.5 using 10M NaOH. The flow rate was maintained at 1ml/min. The HPLC assay is further described in Chapter 5 (section 5.1.1.3) and Figure 5.2 shows a schematic representation of the HPLC set up.

2.13 Analysis of Huh7 ENT1 gene sequence

2.13.1 PCR amplification of ENT1 coding sequence

The Huh7 cell line was cultured in DMEM based culture medium (appendix 8.1) followed by RNA extraction and cDNA synthesis as described before. The amplification primers (MWG-BiotecH AG) for the ENT1 coding sequence were designed manually and had the following sequence:

Forward primer: 5'-ATGACAACCAGTCACC- 3'

Reverse primer: 5'-TCACACAATTGCCCGGAACAGG-3'

$1/10^{th}$ of the cDNA reaction mixture was amplified using Phusion (pfu) high-fidelity DNA polymerase (Finzyme, NEB, UK) to produce a blunt ended PCR product following the manufacturer's instructions. The PCR reaction contained primers at a final concentration of 500nM and was performed in a thermal cycler (Perkin Elmer GeneAmp PCR system 2400) under the following conditions: Initial denaturation at 98°C for 30 sec followed by: 40 cycles of denaturation 98°C for 10 sec, Annealing 60±10°C (gradient) for 15 sec and extension 72°C for 90 sec, followed by a final extension at 72°C. At an optimal annealing temperature of 62.1°C, there was a single product of the right size (1.3kb). The PCR product was purified by column filtration using the QIAquick PCR Purification Kit (Qiagen) following the manual's instructions. DNA was visualised by agarose gel electrophoresis and quantified by NanoDrop.

2.13.2 Agarose gel electrophoresis

A 2% agarose gel was prepared by dissolving 2g of Agarose powder (Gibco-BR Life technologies, USA) in 100ml of 1X Tris Acetate-EDTA (Oh and Park)

buffer (Sigma Aldrich) and heating for 3 minutes in a microwave. The gel was allowed to cool at room temperature (RT), and after addition of Ethidium bromide (EB) at a concentration of 0.5μg/ml, it was poured into a casting apparatus to set. The running gel buffer consisted of 1xTAE and contained EB as a visualising dye. Samples were prepared by adding 2μl of loading dye to 5μl of PCR reaction and loaded on to the gel in parallel with a 10kb DNA marker (GeneRuler DNA Ladder mix, Fermentas). Electrophoresis was performed at 100v for 45 minutes and bands were visualised under an ultraviolet light transilluminator.

2.13.3 Cloning of ENT1 into pCR® 2.1-TOPO® TA cloning vector

The plasmid vector pCR® 2.1-TOPO® has single 3'deoxythymidine (T) residues which permits ligation with deoxyadenosine (A) at the 3' ends of the PCR product. As Phusion polymerase generates blunt ended PCR products, Taq DNA polymerase (Fermentas) was used to add single adenosines to the 3' end of the PCR products. About 10 ng of the resulting template was used to set up a ligation reaction (final volume 6 μl) containing TOPO vector as given in the manual and incubated at RT for 30 minutes.

Transformation was performed by adding 2μl of the cloning reaction to a vial of TOP10 chemically competent E. coli (Invitrogen) and incubated on ice for 30 minutes. Cells were then heat shocked for 45 seconds at 42°C in a heat block and immediately transferred back to ice for another 5 minutes. About 250 μl of SOC medium (Invitrogen) was added to the tubes and incubated at

37°C for 1 hour with horizontal shaking at 200rpm. Lysogeny broth (LB) agar plates were prepared by dissolving 1.5 g of LB agar (Sigma) into 100ml water and heated in a microwave. After addition of 100μg/ml of Ampicillin, the mixture was poured into sterile petri dishes and left to set. The transformed bacteria were spread onto the agar plates containing X-gal (70ug/ml) and bacterial colonies containing plasmid allowed to grow overnight at 37°C. White colonies indicate the presence of both vector and insert, whereas blue colour colonies lack insert.

2.13.4 Analysis of transformants

Five white colonies were picked and allowed to grow overnight in LB culture medium (Sigma, UK) and ampicillin by gentle shaking at 37°C. Bacterial cells were collected by centrifugation and plasmid DNA was isolated by QIAprep Miniprep kit (Qiagen) following the steps given in the manual. DNA concentration and purity was checked by NanoDrop. Samples were submitted for sequencing using M13 forward and reverse primers supplied with the kit (Invitrogen).

Chapter Three

3 Effect of host gene silencing on replication of HCV subgenomic replicon

3.1 Introduction

3.1.1 Gene silencing by small interfering RNAs (siRNAs)

3.1.1.1 Mechanism of RNA interference (RNAi)

RNA interference (RNAi) is a naturally occurring regulatory mechanism in which short double stranded RNA (dsRNA) mediates sequence dependent inhibition of a target gene by either degradation or blockage of the corresponding mRNA. The concept of RNAi was initially described in a nematode worm (Fire et al., 1998). Two years later, the first evidence for the ability of siRNA to block expression of target genes in mammalian cells for therapeutic purposes, offered a novel tool to study gene function *in vivo* and develop gene specific therapeutics (Elbashir et al., 2001).

The principal players of the RNAi pathway are small interfering RNAs (siRNA). These are short double stranded duplexes derived either from the processing of long dsRNA or exogenously introduced synthetic siRNAs. Figure 3.1 describes the siRNA pathway and its processing. Synthesis of siRNAs begins by entry of long dsRNA in the cytoplasm, followed by their cleavage by the endoribonuclease enzyme Dicer to give rise to approximately 21-23 nucleotide long duplexes (Bernstein et al., 2001). These siRNAs are loaded onto a ribonucleoprotein complex known as RISC. The catalytic action of Argonaute 2 (Ago-2), an endonuclease present in RISC, releases the

passenger strand (sense strand) from the duplexes resulting in activation of RISC. The guide strand (antisense strand) then binds to the target mRNA by perfect base matching resulting in its cleavage and gene silencing (Matranga et al., 2005).

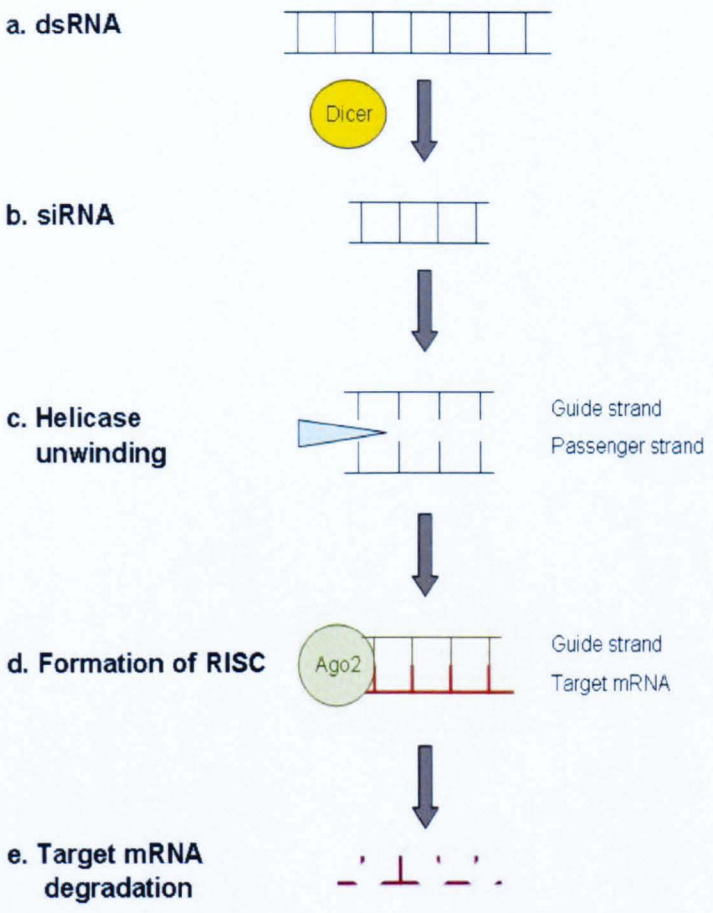


Figure 3.1 Mechanism of RNA interference by siRNAs

Double stranded RNA or dsRNA (a) is cleaved by Dicer to produce short duplexes or siRNA (b) which are then unwound by a Helicase (c) into single strands (passenger or guide). The release of the passenger strand and binding of the guide strand to RISC (d) leads to its activation by Argonaute2 (Ago2) and degradation of target mRNA (e). Synthetic siRNAs skip the initial processing step (a) by Dicer.

3.1.1.2 Advantages and limitations of siRNA based gene silencing

There has been an immense progress in the field of RNAi due to its potential therapeutic application and ability to act as a tool to understand protein function in biological systems. At present, a number of synthetic siRNA are under development to treat various human diseases like cancers and viral infections (Guo et al., 2010, Lares et al., 2010). However, various challenges need consideration before it can be applied in humans, such as off target effects, immune recognition, plasma stability and most importantly, efficient delivery.

siRNA mediated gene silencing can be induced by means of viral or plasmid based vectors which, after being transcribed in the nucleus, are exported to the cytoplasm in the form of short hairpin RNA (shRNA) (Lares et al., 2010). These are then processed by Dicer to produce siRNA duplexes similar to synthetic siRNA. The clear advantage of using this approach is that cells can be stably transfected to produce siRNA but the disadvantage is that viral based vector can cause immuno-toxicity and mutagenesis (Guo et al., 2010).

Another rather simple and convenient way is to directly transfect short sequences which by-passes Dicer's processing and can be repeated to achieve long term gene silencing (Jackson and Linsley, 2010). These siRNAs can potentially cause off target effects which could be either sequence dependent or due to immune responses. The former is due to base pair matching of nucleotides, which requires as few as eight nucleotides at the 3' end of the cellular mRNA to be complementary to the 5'end of the siRNA's guide strand

(Jackson et al., 2003). The latter effect is due to the presence of exogenous dsRNA molecules which are prone to recognition by immune cells; mainly via Toll like receptors (TLRs) resulting in production of inflammatory cytokines and up regulation of various IFN stimulated genes (Robbins et al., 2009). These can be overcome by improving siRNA design by various means like shorter length (<30nucleotides), chemical modifications such as addition of a 2'O-methyl group in the siRNA sense strand, avoiding certain GU rich motifs etc (Hornung et al., 2005, Judge and Maclachlan, 2008, Robbins et al., 2009). Similarly, additions of 3' overhangs, as is the case with Dicer processed siRNA not only enhances gene silencing ability but also helps in evading the immune response (Marques et al., 2006).

3.1.1.3 Delivery systems for siRNA

Despite having some practical challenges in the use of siRNA, their therapeutic potential is very promising. In order to exert its action, however, synthetic siRNA needs to be transported to their target cells. An important prerequisite to achieve this is to devise a suitable delivery vehicle by means of which siRNAs can efficiently and specifically achieve mRNA knockdown. Carrier molecules for siRNA can be either viral based or non-viral, but the former are least preferred because of associated toxicity. Non-viral delivery of siRNA could be achieved by siRNA bound to a positively charged vector (e.g cationic cell penetrating peptides), siRNA conjugated with small molecules (e.g lipids), polymers, antibodies or siRNA entrapped in a nanoparticle formulation (Wang et al., 2010)

3.1.2 HCV replicons

Several *in vitro* model systems have been developed to study HCV infection *in vitro*. One extensively used system is subgenomic replicons (SGRs) in which viral structural proteins (Core, E1 and E2) are replaced by the neomycin phosphotransferase (neo) gene and are translated under the control of the HCV IRES. The non-structural proteins (NS3-NS5B) which are responsible for virus processing and packaging are under the influence of an IRES derived from the encephalomyocarditis virus (EMCV) (Blight et al., 2003).

SGRs derived from JFH1 (Kato et al., 2003), are found to be superior to other replicons from genotype 1a (Blight et al., 2000) or 1b (Lohmann et al., 1999b) in terms of both colony formation under G418 selection or transient replicon assay without selection (Kato et al., 2003, Targett-Adams and Mclauchlan, 2005). Also, these replicons replicate efficiently in Huh7 cells without the requirement of cell culture adaptive mutations as is the case with other genotype based replicons. This system is useful in analysis of HCV replication and protein function, but production of infectious virus particles is lacking. Wakita *et al* demonstrated that *in vitro* transcribed full length RNA from the JFH1 genome cannot only replicate efficiently when transfected into Huh7 cells but also be incorporated into virus particles which can infect naïve Huh7 cells (Wakita et al., 2005).

A report by Target-Adams and John McLauchlan indicated development of such transient replicons based on the JFH1 genome containing a Luciferase reporter instead of the neo gene (Targett-Adams and Mclauchlan, 2005). The

same group subsequently established a JFH1 based SGR that includes a GFP tagged into the C-terminal of the NS5A protein (Jones et al., 2007). A clear advantage of using this system was its simplicity and ability to act as a direct measure of viral RNA replication. In addition to its use in evaluating molecular mechanisms of HCV replication, subgenomic replicons can provide a useful *in vitro* system to test the efficacy of antiviral drugs (Randall and Rice, 2001). Figure 3.2 gives a schematic representation of subgenomic replicons derived from the full length JFH1 HCV genotype 2a genome.

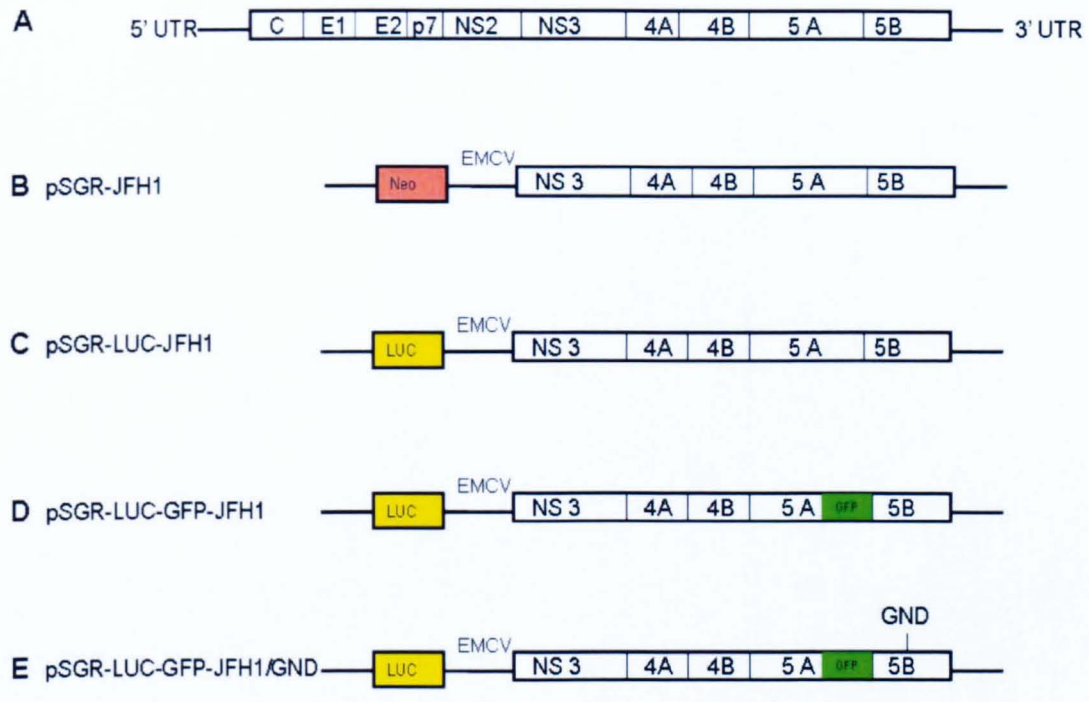


Figure 3.2 Schematic diagram of the structure of JFH1 (genotype 2a) HCV subgenomic replicons

(A) Full length or wild type virus genome structure, (B) subgenomic (SG) di-cistronic replicon carrying resistance gene (neo) for selection and EMCV IRES for translation of non-structural (NS) proteins, (C) SG replicon containing reporter protein (luciferase) replacing neo, (D) SG replicon carrying the green fluorescent protein (GFP) gene within the viral NS5A for detection of translation in infected cells, (E) Same as D but with a point mutation in the GDD motif to GND in NS5B which abolishes the replicative ability of the viral RNA polymerase (NS5B).

3.1.3 Nucleofector technology

Intracellular entry of large exogenous nucleic acids requires physical methods of transfection, which is a process through which genetic material enters the target cell, enabling the expression of non-native proteins. Electroporation is an established transfection method based on use of an electric wave to induce changes in membrane potential causing pores. These temporary pores allow naked nucleic acids present in the buffer solution to enter the cells. The ionic composition of the electrophoretic solution affects the passage of current and its effect on the cell membrane. The nucleofector technology is a specialised electroporation system combining specific buffer solutions and an electroporation device (Nucleoporator® Amaxa®, AG, Germany) that delivers specifically optimised electrical parameters. It has the dual advantage of being able to produce efficient transfection (even for difficult to treat cell lines like primary cells) without causing massive cell damage. As this technique is independent of the type of nucleic acid being transfected, the same protocols can be used for delivery of any type of nucleic acid (DNA, RNA or siRNA).

3.1.4 RNAi based therapies for human diseases

The potential benefit of siRNA to act as a therapeutic tool instigated a drive to identify possible disease targets. Since its first demonstration as an inhibitor of vascular endothelial growth factor in patients with acute macular degeneration (Bevasiranib), it became apparent that any human disease can be targeted (Mousa and Mousa, 2010). Some of its noteworthy applications entering clinical trials belong to eye disorders, cancers, inflammatory diseases and viral infections (Lares et al., 2010). Human immunodeficiency virus (HIV),

respiratory syncytial virus (RSV) and HCV are some of the viruses where utility of siRNA as an antiviral has been demonstrated (Ali Ashfaq et al., 2011, Liu et al., 2009, Zhang et al., 2005).

Use of single siRNA to target viral infection is associated with development of resistant mutants and necessitates combination of multiple siRNAs (Liu et al., 2009). Targeting cellular genes involved in virus replication is therefore an attractive alternative to circumvent resistance and has been demonstrated to work well for difficult targets like HIV (Zhang et al., 2007). A number of cellular targets have also been identified for HCV, and a few are making their way to the clinic. Cyclophilins involved in protein folding and trafficking are one of these, and are thought to play a role in virus replication by direct interaction with NS5A and altering viral protein folding and trafficking to the site of replication (Gaither et al., 2010). Specific cyclophilin inhibitors, such as NIM811, have shown potency against HCV when used in combination with pegylated IFN (Lawitz et al., 2011).

3.1.5 siRNA based therapies for HCV

Hepatitis C virus infection poses a huge disease burden due to its chronic nature and a treatment regimen which is ineffective in half of the patients receiving it (Manns et al., 2001). The exact underlying mechanism for the limited success of pegylated IFN and ribavirin remains obscure but various viral and host related factors have been implicated (Feld and Hoofnagle, 2005). There is a major need to develop new antiviral targets which can be combined with current therapy to improve its success (Mchutchison et al., 2006). Drug

targets aiming at the virus itself have gained considerable popularity and some of them have already entered clinical trials (Jazwinski, 2011).

Since the first demonstration of ability of the siRNA to knock down gene expression in mammalian cell cultures, virologists have been striving to show its efficacy as an anti-HCV agent (Elbashir et al., 2001). Co-localisation of viral replication and siRNA duplexes into the host cell cytoplasm further enhances the utility of this system to knock down viral replication (Randall et al., 2003). Similarly, siRNA targeting each step of the viral replication and various sites within the viral genome including the 5' UTR, Core, NS3, NS4B and NS5B have been tested for their efficacy (Ashfaq et al., 2011, Jazwinski, 2011). However, a limitation of virally targeted drug could be emergence of drug resistant HCV variants in patients receiving this treatment arising as a result of the error prone nature of the HCV polymerase (Lin, 2010). Similarly, the majority of these siRNA based anti-HCV drug targets are less effective for variants other than those to which they are specifically targeted. To get around these hurdles, host genes involved in the viral replication cycle could be identified and targeted to knock down viral replication (Lin, 2010 #320).

3.1.6 Role of host protein in HCV replication

In HCV infection, hepatocytes are the principal site of viral replication and host cell factors are critical in every step of the virus life cycle. The full length HCV genome encodes for a large polyprotein (~3,000 amino acids long) which is processed by viral and host enzymes to produce structural (Core, E1 and E2) and non-structural proteins (NS2, NS3, NS4A, NSAB, NS5A and NS5B). The viral replication complex consisting of NS proteins and host proteins co-

localise on cytoplasmic membranes derived from the endoplasmic reticulum (ER), called a membranous web or lipid raft associated membranous complex (Shi et al., 2003). The precise role of various components of this complex in modulating viral replication is poorly understood.

A number of host genes have been implicated in supporting virus replication within the infected cells (Ng et al., 2007). Expression profiling studies of cells containing replicating HCV genomes have identified various pathways belonging to oxidative stress, lipid metabolism and vesicular trafficking to be significantly differentially regulated (Blackham et al., 2010). Identification of these molecular pathways interacting with HCV will improve our understanding of the HCV replication cycle which will in turn aid in devising new anti-HCV drugs. Targetting siRNAs towards these cellular genes may also circumvent genotype resistance due to the high fidelity of host enzymes and reduce genotype specificity in response. Once validated, these can be combined with current treatment regimen and would help in improving response rates.

A report by Blackham *et al.* highlighted involvement of proteins related to the cytoskeleton (e.g. ABLIM), regulation of secretory vesicles (e.g. RAB40B) and mediators of oxidative stress (e.g. TXNIP) indicating that knock down of these genes result in significant reduction in virus replication (Blackham et al., 2010) Using a wide RNAi screen, Randall and co-workers identified a number of host gene interactions with HCV non-structural proteins including vesicle associated membrane protein (VAMP)-associated protein A (VAP-A), signal transducer and activator of transcription 3 (STAT-3) and alpha actinin 1

(ACTN1) (Randall et al., 2007). The significance of these genes was tested by analysing the effects of gene knock down on viral replication and was shown to have a more than three fold reduction in production of infectious virus.

3.1.6.1 Vesicle-associated membrane protein - associated protein A (VAP-A)

Vesicle associated membrane protein (VAMP)-associated proteins or VAP proteins are ubiquitously expressed integral ER membrane proteins involved in diverse cellular functions like membrane trafficking, neurotransmitter release and lipid transport and metabolism (Lev et al., 2008). In humans, VAP-A and VAP-B are the two main subtypes encoded by two genes where VAP-B has 63% sequence homology to VAP-A and gives rise to an alternatively spliced variant i.e. VAP-C, composed of its N-terminal one third only (Nishimura et al., 1999).

Studies have suggested that both VAP-A and B interact with NS5A and NS5B and facilitate viral replication in association with lipid rafts. Gao *et al.* reported that human VAP-A due to its intrinsic NS5A and NS5B ability helps the association of the viral replication complex to lipid raft membranes and knocking down VAP-A with siRNA inhibits HCV RNA replication (Gao et al., 2004). Another study suggested that hyperphosphorylation of NS5A prevents its interaction with VAP-A which negatively regulates virus replication (Evans et al., 2004). A similar role has been reported for VAP-B in a report by Hamamoto *et al.* which suggested that VAP-B forms a complex with VAP-A, as well as binding to NS5A and NS5B, enhancing viral replication (Hamamoto et al., 2005). A contrasting role has been reported for VAP-C. Using a co-

immunoprecipitation assay, Kukihara *et al.* revealed that human VAP-C was capable of binding to NS5B but not NS5A, due to the absence of the C-terminal part of the protein or the NS5B binding site, and negatively regulated HCV replication through interfering with VAP-A and B binding to NS5B (Kukihara et al., 2009). They also showed that VAP-C was not expressed in liver which may suggest that its absence may partly account for the tissue tropism of HCV.

3.1.6.2 Signal transducer and activator of transcription 3 (STAT-3)

STAT-3 is one of the seven members of the STAT family involved in the JAK/STAT signalling pathway activated in response to various stimuli like interferon, interleukins and growth hormones (Schindler et al., 2007, Zhang et al., 2011). STAT-1 and -2 are downstream of interferon signalling pathways activated in response to viral infection like HCV and induce expression of various interferon stimulated genes culminating in an anti-viral state within hepatocytes (Chevaliez and Pawlotsky, 2007, Durbin et al., 1996, Park et al., 2000a).

STAT-1 and STAT-3 have distinct target genes and, depending on duration of activation or the cell type involved, display contrasting roles in related biological processes like inflammation, tumorigenesis and survival/growth (Regis et al., 2008). As opposed to STAT-1, STAT-3 is an oncogene considered to favour cell survival and inhibits apoptosis. Its constitutive activation leads to cellular transformation (Azare et al., 2007). However, the

specific function that this molecule exerts appears to be dependent on the specific pathological or physiological environment as it is found to be pro-apoptotic in normal tissues like mammary gland and bone marrow (Chapman et al., 1999, Lee et al., 2002). Also, in contrast to STAT-1, STAT3 mainly acts as an anti-inflammatory factors and it is likely that the relative abundance of these molecules will determine the fate of many cells. The role of STAT3 in the case of viral infection is currently unclear. Interaction of HCV core protein with STAT3 has been implicated in its activation and cellular transformation (Yoshida et al., 2002). Similarly HCV induced oxidative stress has been associated with activation of cellular kinases and activation of transcription factors like STAT3 and NF-κB (Gong et al., 2001).

3.1.6.3 Alpha actinin 1 (ACTN1)

Alpha actinin is an actin binding cytoskeletal protein ubiquitously expressed in both muscle and non-muscle tissues. There are four main isoforms classified as muscle alpha actinins (3 and 4) and non-muscle alpha actinin (1 and 2) (Dixson et al., 2003). The former group mainly function as cytoskeletal proteins providing shape and stability, while the latter group additionally provides a link to various transmembrane proteins and receptors (Otey and Carpen, 2004).

ACTN1 and ACTN4 share 80% nucleotide homology but a different subcellular localisation. ACTN4 mainly exists in the cytoplasm and nucleus while ACTN1 is plasma membrane associated (Honda et al., 1998). The different location within intracellular compartments may suggest a distinct function for these two isoforms. The exact function and localisation of these isoforms in human liver is currently unclear. Some reports have indicated their

presence on parenchymal and ductal cells of liver (Inada et al., 2008) and interaction with NS5B (Lan et al., 2003).

To explore whether these potential HCV co-factors can act as functional anti-HCV targets, this study focuses on evaluating effects of silencing these molecules on virus replication using a subgenomic replicon model and siRNAs. Use of pre-designed stealth siRNAs (Invitrogen) combines the advantage of efficient gene silencing and low toxicity with high specificity and no off-target side effects. Once validated, these anti-viral drug targets can be either used alone or as an adjuvant therapy for patients not responding to conventional regimens.

3.2 Aims:

The specific aims of this part of study were to assess the potential utility of modulating host gene expression as a therapeutic tool in HCV infection. The majority of the host proteins targeted interact with hepatitis C virus non-structural proteins (Randall et al., 2007), so a subgenomic replicon was used to evaluate changes in viral replication after knocking down these targets by:

- Preparation of high quality subgenomic replicon RNA derived from JFH1
- Assessment of optimal nucleofection conditions for JFH1
- Testing gene silencing ability of selected duplexes
- Optimising nucleofection condition for siRNAs
- Co-transfection of duplexes with viral RNA and validation of gene silencing
- Monitoring the effects on viral replication by measuring GFP expression.

3.3 Results:

3.3.1 Preparation of replicon RNA

In this study, a subgenomic replicon based on JFH1 genotype 2a virus was used as a model system to study virus replication *in vitro* and analyse effects of host gene silencing. Plasmid pSGR-LUC-GFP-JFH1 (JFH1) and a replication incompetent control plasmid pSGR-LUC-GFP-JFH1-GND (GND) were used as a template to generate replicon RNA by *in vitro* transcription (IVT).

Plasmid linearization was done by restriction digestion using the enzyme XbaI as shown in Figure 3.3 (A). Linearised plasmid was then subjected to mung bean enzyme digestion to clean overhangs followed by column purification to yield neat DNA template (Figure 3.3, B). Replicon RNA was *in vitro* transcribed and after a number of optimisations, high quality RNA was obtained as determined by agarose gel analysis. Figure 3.3 (C) gives a comparison between intact vs. degraded RNA. Agarose gel electrophoresis was repeated every time to ensure RNA quality was not compromised (Figure 3.3, D) and only high quality RNA was used in all the experiments.

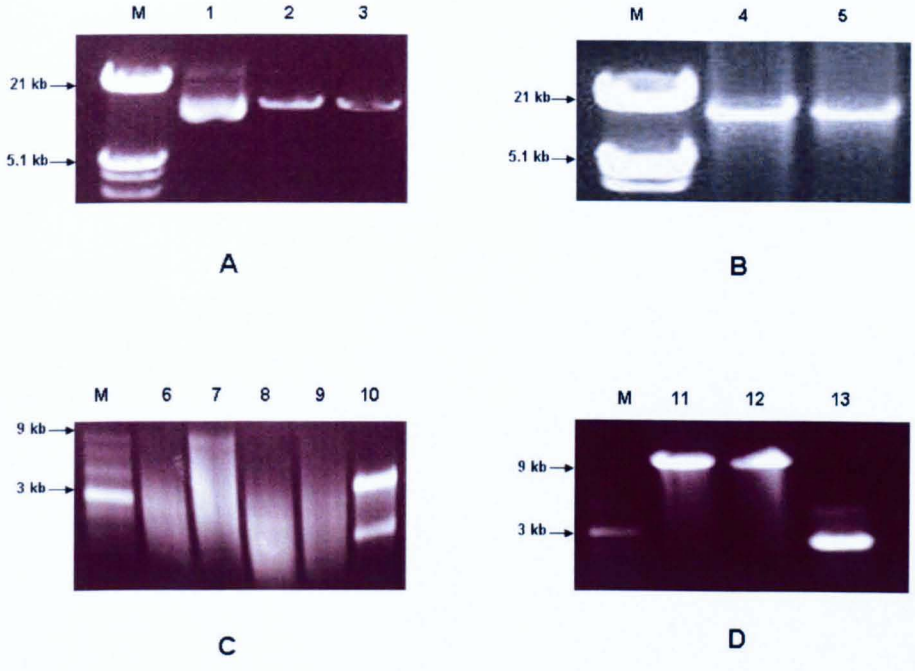


Figure 3.3 Example images of JFH1 plasmid DNA or RNA as analysed by ethidium bromide gel electrophoresis.

Nucleic acids (DNA or RNA) were visualised on agarose gels after every step to ensure high quality (A) undigested or circular plasmid DNA (lane 1) vs. XbaI digested JFH1 (lane 2) and GND (lane 3). Note that undigested plasmid has a close circular structure so it migrates a little faster than its linearised counterpart. (B) Mung bean treated and phenol chloroform extracted JFH1 (lane 4) and GND (lane 5) DNA, (C) example of degraded RNA (lane 6-9) illustrated by smearing produced by multiple randomly sized small RNA fragments as a result of RNase activity. Intact Huh7 cell line total RNA (lane 10) is shown for comparison having two distinct 28S and 18S ribosomal bands. (D) High quality *in vitro* transcribed replicon RNA, JFH1 (lane 11,), GND (lane 12) and IVT positive control (13).

3.3.2 Nucleofection of replicon RNA

In this study, nucleofection was used as a mode of transfecting both viral RNA and siRNAs. A number of optimisations steps were required to achieve transfection conditions which would produce optimal JFH1 expression in the Huh7 cell line with minimal cytotoxicity. Once validated, the same protocol was used for co-transfection.

Nucleofection was initially attempted by using a control RNA derived from the phMGFP expression plasmid (Promega) following the same steps as for JFH1 replicon RNA synthesis. Huh7 cells were nucleofected with control RNA using different programmes from the nucleofector device (there are a number of pre-recorded programmes with different electrical parameters generated by the nucleofector device). Cells were then subjected to flow cytometric analysis for GFP expression after 24 hours. Figure 3.4 outlines work stages for a typical transfection experiment performed in this study.

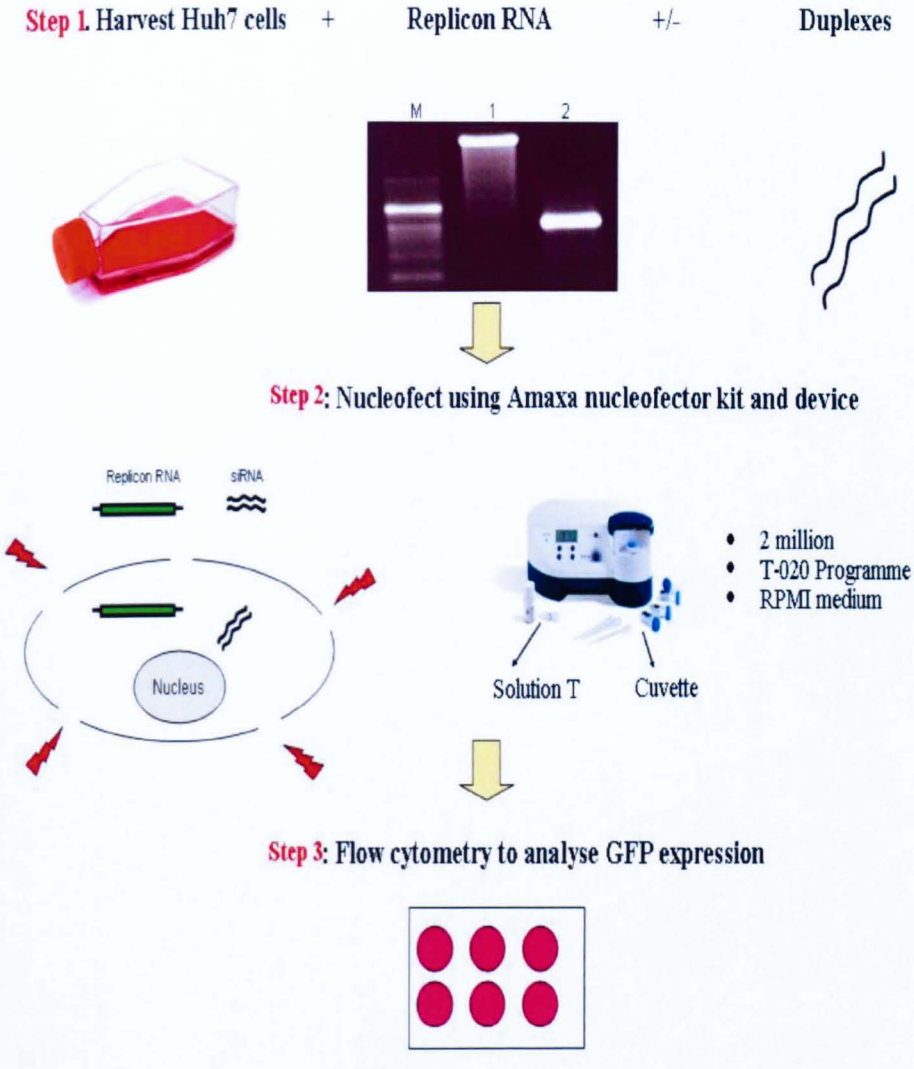


Figure 3.4 Work stages for a typical transfection procedure

Step 1: About 2 million Huh7 cells and 10 µg of replicon RNA were prepared in 100 ul of buffer solution with or without gene specific duplexes (M = marker, lane 1= JFH1 RNA, lane 3 = IVT positive control). Step 2: Cells were permeabilised by an electric current generated by the nucleofector device using an appropriate programme and specialised buffer solution. Step 3: Cells were transferred to plates and GFP expression is analysed after selected time points (e.g. 72 hour post electroporation).

Transfection efficiency was expressed as a percentage of positive cells above baseline fluorescence (mock transfected cells). Cell viability was determined by trypan-blue exclusion test and subtracting dead cells (floating in culture medium) from total cells (attached to plates) /number of plated cells (~2 million) x 100 after 24 hours. As given in the table in Figure 3.5 (A), all tested programmes were associated with cell viability of more than 90% but T-020, T-030 and T-014 were chosen as they also had superior transfection efficiency.

Nucleofection was then performed by means of these three programmes using 10 μg of replicon RNA (JFH1) and GFP expression was measured at 72 hours. As illustrated in Figure 3.5 (B), the T-020 and T-030 programmes were found to have comparable efficiency of ~ 35 % but T-020 was chosen as it also had higher cell viability. A time course and dose response experiment was done as illustrated in Figure 3.5 (C and D). In terms of quantity, 10μg of input RNA was clearly better than 5 μg as it doubled the % of positive cells (from 10% to 22%) but, increasing it further to 20 μg did not improve it significantly. Time course experiments proved that JFH1 replication efficiency is greatest at 72 hours post nucleofection. These optimal conditions were used next for co-transfections.

A

Programme	Cell viability (%)	Transfection efficiency for phMGFP (%)	Transfection efficiency for JFH1(%)
T-020	98.5	17.26	35.19
T-030	97	16.08	36.75
T-014	96	10.24	31.20
A-020	98	0.73	-
X-005	92	4.23	-
X-001	98	4.67	-
No programme control	99	-	-

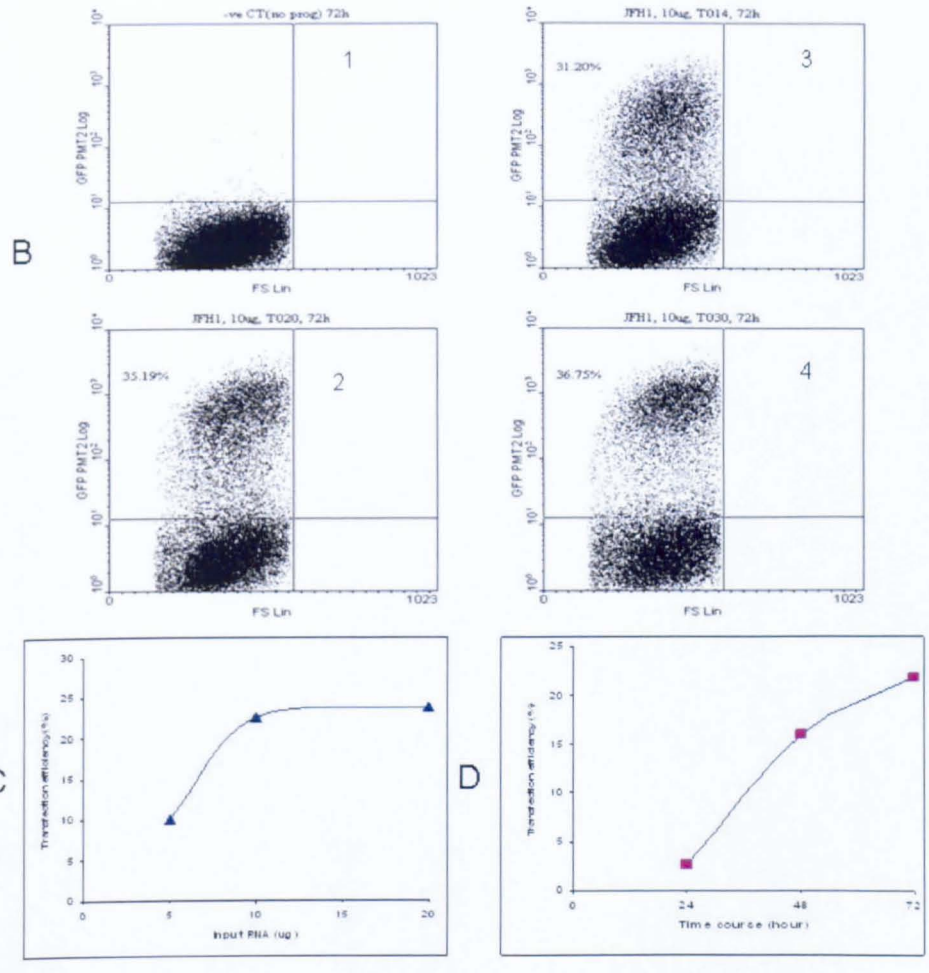


Figure 3.5 Optimisation of nucleofection procedure

Optimal transfection conditions for replicon RNA were determined by testing different programmes as well as varying amount of input RNA; (A) Table illustrating cell viability and transfection efficiency for different nucleofector programmes, both for control RNA (phMGFP) as well as replicon RNA (JFH1); (B) Dot plot demonstrating percentage of Huh7 cells expressing GFP (y-axis) in un-transfected (1) or nucleofected cells by different programmes, (2) T-020, (3) T-014 and (4) T-030; (C) Graph showing percentage efficiency of JFH1 replication (y-axis) as a result of different amounts of input RNA in μg (x-axis); (D) Graph showing percentage efficiency of JFH1 replication (y-axis) plotted against various time points post-electroporation (x-axis).

3.3.3 Validation of siRNA duplexes by Lipofectamine2000

Small interfering RNAs (siRNA) designed to target selected host genes were tested to verify their ability to silence target genes in this cellular environment. Pre-designed stealth siRNAs were obtained from Invitrogen (BLOCK-iT™) and used at a final concentration of 100nM. Huh7 cells were transfected with duplexes by means of a commercial reagent Lipofectamine 2000 (invitrogen) following the manufacturers' instructions (siRNA sequence is given in materials and methods). At 48 hours post-transfection, Q-PCR and western blot were performed to analyse knock down at the mRNA and protein level respectively.

For real time PCR, RNA extraction and cDNA synthesis was performed as detailed in materials and methods. PCR primers were designed with the help of the Primer3 programme (version 0.4.0) targeting exon-exon junctions and specificity was checked with primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Optimal primer annealing conditions were achieved by doing a gradient PCR with a temperature range of $60\pm10^{o}C$ and the temperature which gave maximum amplification subsequently used (primer sequence and optimal annealing temperature are given in materials and methods). A standard curve was prepared by serially diluting neat cDNA in order to ensure best PCR efficiency (range = $100\pm15\%$).

As depicted in Figure 3.6, the standard curve shows exponential amplification with efficiency of 85%, 99%, 106% and 98% for STAT-3, VAP-A, ACTN1 and HPRT primers respectively. The melting curve confirmed specificity of all

primer pairs by producing a single peak for the desired amplicon and no additional peaks were seen which may appear due to the presence of contaminating DNA and primer dimers. Non-template control (NTC) and no RT (RT negative) prepared without cDNA template and enzyme in the cDNA synthesis reaction respectively were used with every run. The melt curve also demonstrated that there was no amplification in NTC and RT negative control (baseline).

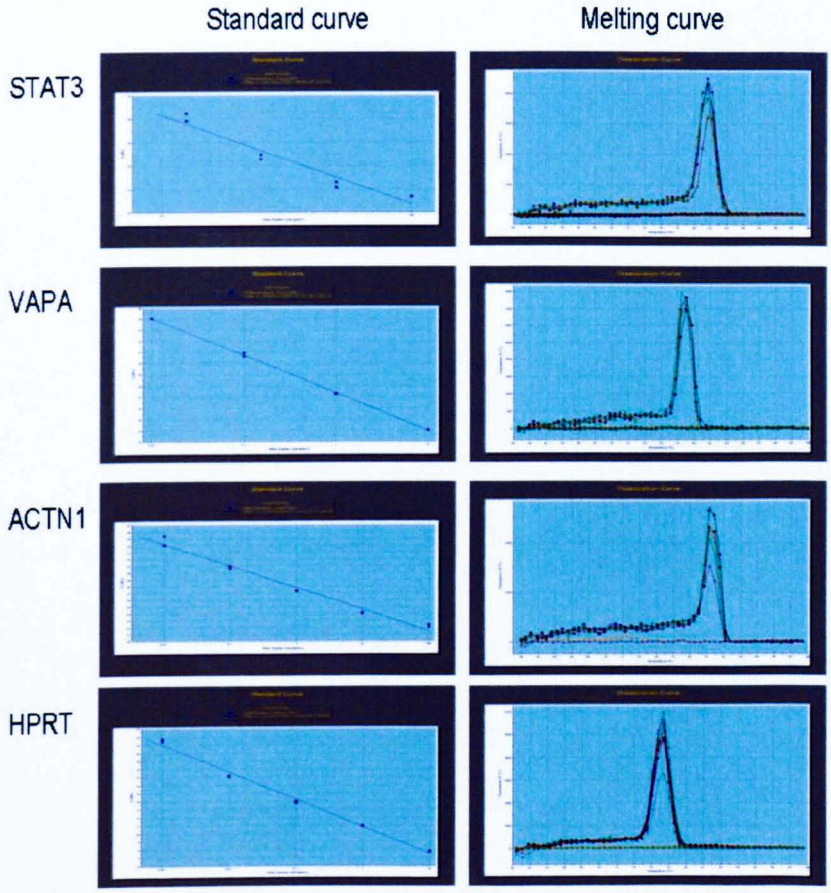


Figure 3.6 Real time PCR plots for STAT3, VAP-A, ACTN1 and HPRT primer pairs.

The Standard curve shows change in cycle threshold (Ct on y axis) of 1 with every cycle in a 2 fold dilution series (x-axis) and an R^2 value close to 1. Melting curve demonstrates that every primer pair generated a single product and absence of contaminating products which may give rise to additional peaks separate from the desired amplicon. No amplification in the NTC and RT negative controls is observed (baseline in the melt curve).

To ascertain knock down efficiency of these duplexes by real time PCR, gene expression was quantified in unknown samples treated with either gene specific siRNA or a scrambled control and levels were normalised to the house keeping gene HPRT. As shown in Figure 3.7, when compared to scrambled control, gene specific duplexes reduced the mRNA level of their respective genes, albeit to different degrees. The silencing efficiency was ~99% for STAT-3, 90% for VAP-A and 85% for ACTN1 mRNA as compared to that in the scrambled control.

Parallel samples were taken at 48 hours for Western blotting to determine knock down at the protein level. Protein extraction followed by quantification was performed as detailed in materials and methods. About 10-30 μg of protein were separated on a 10% SDS-PAGE gel and immuno-blotted with specific monoclonal antibodies. Blots were analysed visually and as shown in Figure 3.7, when compared to control samples; there was almost 90% and 50% reduction in STAT-3 and VAP-A protein expression while no significant difference was observed for ACTN1. It was hypothesised that ACTN1 has a long protein half life masking any knock down by Western blot. β-actin was used as a loading control and showed equivalent protein levels in parallel samples.

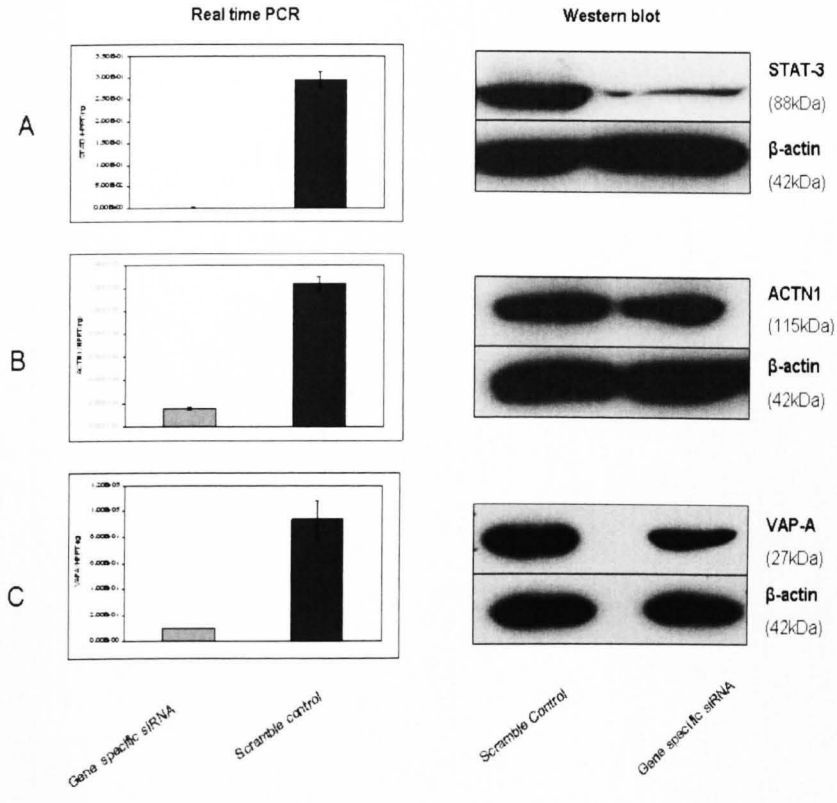


Figure 3.7 Validation of gene knock down by siRNA (48 hour)

Gene knock down was performed by 100 nM of gene specific siRNA using Lipofectamine2000 and analysed at 48 hours. For real time PCR, mRNA quantification was done using Maxpro software (stratagene) and STAT-3 (A), ACTN1 (B) and VAP-A (C) mRNA levels were normalised to HPRT (house keeping gene). The ratio of specific gene vs. HPRT quantity in nanograms is plotted on the y axis. Bars represent mean and error bar depicts standard deviation. For Western blots, total proteins harvested from parallel samples were separated on a 10% SDS-PAGE gel followed by transfer to nitrocellulose membrane. Specific monoclonal antibodies were used to detect each of the target proteins and β-actin which was used as a loading control.

3.3.4 Optimal nucleofection conditions for duplexes

In order to assess the importance of host genes in virus replication, nucleofection was used for co-transfection of siRNA and viral RNA. To ensure that the duplexes can be successfully transfected by this technique and to ascertain best concentration of siRNAs, elecroporation conditions were optimised. Conditions which produced efficient replication of the subgenomic replicon were used to carry out a dose response experiment using a range of 30 to 500nM siRNA to find the optimal siRNA concentration for use in co-transfection experiments. Following nucleofection, cells were plated and incubated for 72 hours before protein extraction to analyse gene silencing by Western blot.

Figure 3.8, shows that STAT-3 and ACTN1 were successfully inhibited at 200nM (final concentration of siRNA per well) while for VAP-A, there was no significant reduction in protein expression at all doses tested. Previous analysis at 48 hours demonstrated protein knockdown. It was hypothesised that higher protein turn over could replenish any reduction in protein levels if it is left for 72 hours so the knock down experiment was repeated at 48 hr and showed significant reduction in VAP-A protein expression (see section 3.3.5.2 and Figure 3.11). β-actin expression was consistent in all samples tested in parallel.

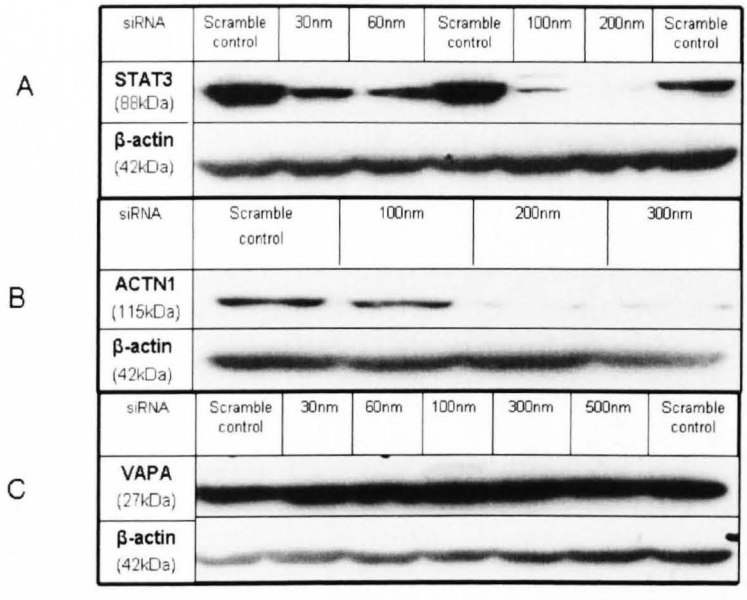


Figure 3.8 Dose response for gene silencing by nucleofection (72 hours)

Gene specific siRNA as shown and respective scramble control siRNA were transfected at different doses (final concentration per well). Appropriate nucleofection conditions were used to deliver siRNA into Huh7 cells and samples were collected at 72 hours to assess knock down efficiency by western blot. 10-30μg of protein was separated on a 10% SDS-PAGE gel and immuno-blotted using specific antibodies. β-actin was used as a control to exclude loading artefacts.

3.3.5 Co-transfection of duplexes with the JFH1 replicon

3.3.5.1 Analysis of GFP expression by flow cytometry

The subgenomic replicon plasmid used in this study has a green fluorescent protein (GFP) fused to the NS5A. Therefore, green fluorescence was used as an indirect measure of viral replication and translation. Optimised nucleofection conditions (10 μg RNA and the T-020 programme) and GFP expression was measured at 72 hour post-electroporation.

A number of experimental controls were used to ensure optimal efficiency of the procedure and make sure that conclusions drawn from the RNAi experiment were valid. These were: (1) Mock transfected cells treated with identical transfection conditions but without any viral RNA or siRNA duplexes; (2) GFP (DNA) positive control cells treated with a DNA plasmid (pmaxGFP™) encoding GFP protein; (3) Positive siRNA control cells treated with positive control plus siRNA targeting GFP (4) Negative siRNA control: cells treated with positive control plus scrambled control siRNA (GFP) (5) JFH1 alone cells treated with subgenomic replicon RNA JFH1 fused with GFP and (6) GND alone cells treated with a replication incompetent GND mutant fused with GFP.

Cells were subjected to flow cytometric analysis and gated on the basis of forward and side scatter signals to restrict the analysis to viable cells. Mean fluorescence intensity (MFI) was measured in the cells as a measure of GFP expression. As shown in Figure 3.9 (A), mock treated cells had GFP

expression close to baseline (0.5%) while the majority of cells treated with the DNA positive control expressed GFP at high levels (96%). Knocking down GFP expression by specific siRNA not only reduced the percentage of positive cells to 88% but more importantly, expression levels (indicated by MFI) also showed a three fold reduction. Transfection with scrambled control had minimal effect on GFP expression (95% positive). JFH1 expression (GFP tagged) was positive in 36% of cells whereas in GND (GFP tagged) treated cells, only 1% of cells were positive.

Figure 3.9 (B) depicts shift in fluorescence in the different populations tested. As expected, cells transfected with siRNA specific for GFP show a significant left shift as compared to the scrambled control. GFP fused replicon treated cells (JFH1 alone) show two distinct peaks corresponding to the negative (grey area) and positive control populations, whereas the GFP fused GND mutant treated cells overlapped the negative control. Transfection efficiency for GFP tagged JFH1 replicon was 26.7% $\pm$ 6.7 (n=7) and 52.1% $\pm$ 11.5 (n=7) for Huh7 and Huh7.5 respectively. Due to the higher transfection efficiency, the Huh7.5 cell line was used for co-transfection experiments in which a number of host genes were selected and targeted using siRNAs. All the graphs and data analysis was performed using GraphPad Prism version 4 and unpaired student's t test was used to compare the two groups.

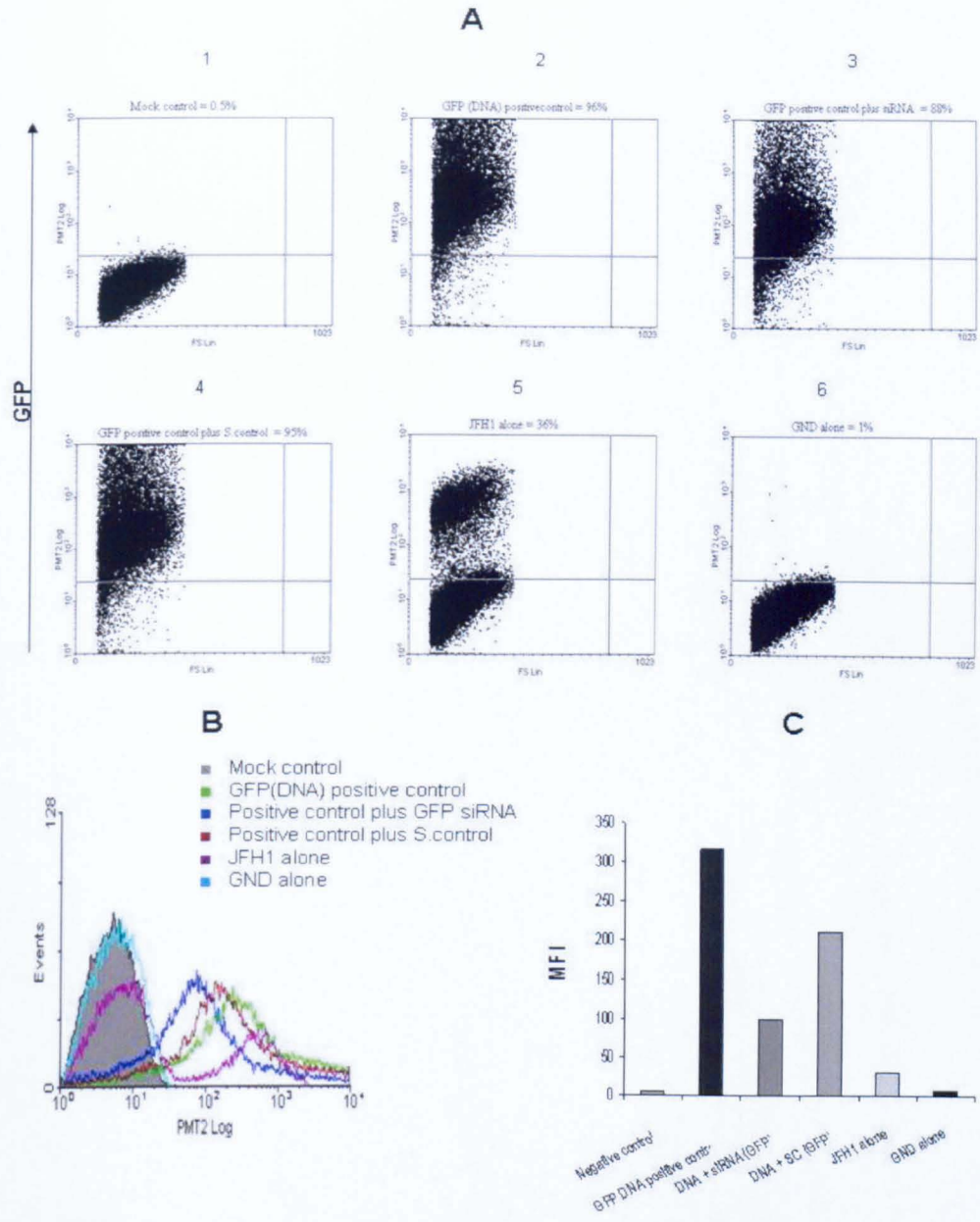


Figure 3.9 Experimental controls for RNAi by nucleofection

A) Different types of experimental controls were used to validate our transfection protocol including (1) a mock transfected/negative control, (2) a GFP (DNA) positive control, (3) a positive siRNA control, (4) a negative siRNA control. JFH1 (GFP tagged) alone (5) and GND (GFP tagged) alone (6) are also shown for comparison. Cells were gated on the basis of forward and side scatter to include live cell populations and baseline fluorescence (y-axis) based on negative control was selected as a cut off for positive populations. B) Histogram demonstrating change in fluorescence intensity (x-axis) and shift in different populations as compared to negative cells (grey filled area). (C) Mean fluorescence intensity (MFI) measured in the different populations (1-6 in A) and plotted on y-axis.

3.3.5.2 Effect of VAP-A knock down on viral replication

The HCV subgenomic replicon derived from JFH1 was used in this study to observe the effect of VAP-A knock down on virus replication. Huh7.5 cells were co-transfected with GFP tagged JFH1 replicon RNA and siRNA targeting human VAP-A and GFP expression was calculated by flow cytometry. Mean fluorescence intensity (MFI) was measured at 72 hours and compared in the knock down sample versus control. As shown in Figure 3.10 (A), VAP-A knock down resulted in ~30% reduction in viral replication as indicated by the difference in MFI between the two populations (p value = 0.03).

Western blot was performed to confirm protein knock down but as shown in Figure 3.10 (B), there was no reduction in VAP-A protein expression. The reduction in virus replication did not correlate with the level of protein knock down so it was hypothesized that rate of VAP-A protein turn over is high and the half life of protein is short so that its levels come back up again if left longer. To prove this, the experiment was repeated with a shorter analysis time of 48 hours. As shown in Figure 3.11, there was more than 80% reduction in VAP-A protein expression at 48 hours as indicated by the Western blot which translated into a more robust or statistically significant inhibitory effect on viral replication ($p = 0.007$).

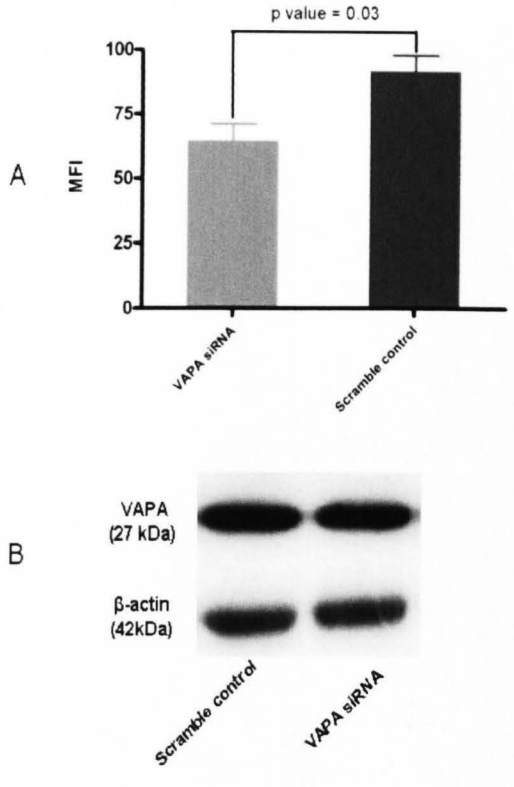


Figure 3.10 Effect of VAP-A knock down on JFH1 replication at 72 hour

Huh7.5 cells were co-transfected with JFH1 replicon RNA and either VAP-A specific siRNA or scramble control by nucleofection and effects on viral replication were measured at 72 hours. A) Graph illustrating MFI plotted on y-axis and significant difference between control and knock down group. Bars indicate mean and error bars denote standard deviation B) Western blot image showing VAP-A protein (27kDa) in both groups and β-actin as a loading control. This diagram is a representative of three repeat experiments with each sample performed in triplicate.

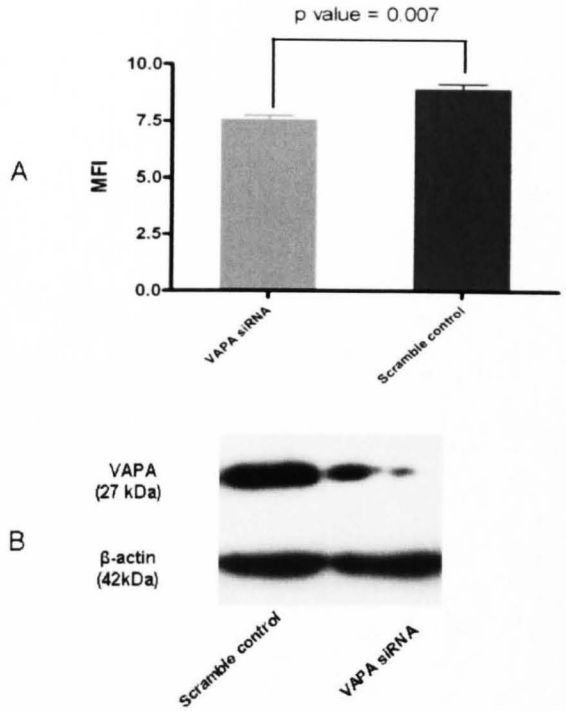


Figure 3.11 Effect of VAP-A knock down on JFH1 replication at 48 hour

Huh7.5 cells were co-transfected with JFH1 replicon RNA and either VAP-A specific siRNA or scrambled control and its effect on viral replication was measured at 48 hours. A) Graph illustrating MFI plotted on y-axis and significant difference between control and knock down group. Bars indicate mean and error bars denote standard deviation. B) Western blot image showing VAP-A protein (27kDa) in both knock down and control groups and β-actin as a loading control. This diagram is a representative of three repeat experiments with each sample performed in triplicate.

3.3.5.3 Effect of STAT-3 knock down on viral replication

STAT-3 was next chosen as a target for gene knock down and the effect on viral replication assessed by co-transfection with replicon RNA. Huh7.5 cells were co-nucleofected with either STAT-3 specific siRNA or respective scrambled control together with GFP tagged JFH1 viral RNA. Flow cytometric analysis of GFP expression was carried out at 72 hours by measuring mean fluorescence intensity (MFI) in the knock down sample and comparing it to the scramble control.

As shown in Figure 3.12 (A), silencing STAT-3 had an inhibitory effect on virus replication indicated by reduction in fluorescence signal and this difference was statistically significant (p value = 0.0004). Western blot was performed to confirm protein knock down (Figure 3.12, B) which showed that there was almost 100% reduction in STAT-3 protein expression in knockout samples in contrast to control treated samples. β-actin expression was equal in the samples tested indicating equal loading.

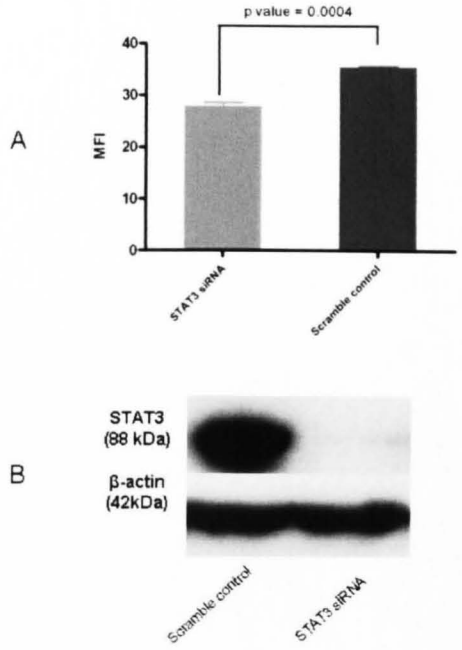


Figure 3.12 Effect of STAT-3 knock down on JFH1 replication

Huh7.5 cells were co-electroporated with JFH1 replicon RNA and either STAT3 specific siRNAor scrambled control by nucleofection and its effect on viral replication was measured at 72 hours. A) MFI plotted on y-axis to compare its difference between control and the knock down group. Bars indicate mean and error bars denote standard deviation; B) Western blot image showing STAT-3 protein (88kDa) expression only in the control group while it is absent in siRNA treated samples. β-actin expression shows equivalent loading in the two samples. This diagram is a representative of three repeat experiments with each sample performed in triplicate.

3.3.5.4 Effect of ACTN1 knock down on viral replication

To analyse the effects of ACTN1 knock down, cells were transfected with ACTN1 specific siRNA along with GFP tagged JFH1 viral RNA. Flow cytometric analysis of GFP expression was carried out at 72 hours by measuring mean fluorescence intensity (MFI) in the knock down sample as compared to the scrambled control. As depicted in Figure 3.13 (A), ACTN1 knock down did not suppress virus replication and the signal was comparable in both populations (p value = 0.95). Western blot was performed to analyse protein knock down and as shown in Figure 3.13 (B), there was almost 100% reduction in ACTN1 protein expression while β-actin expression remained unaltered indicative of equivalent protein quantity in the parallel wells under comparison.

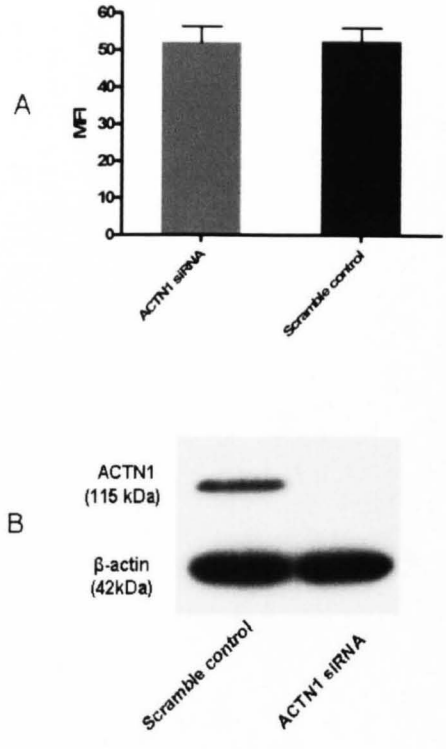


Figure 3.13 Effects of ACTN1 knock down on viral replication

Huh7.5 cells were co-electroporated with JFH1 replicon RNA and either ACTN1 specific siRNA or scrambled control by nucleofection and its effect on viral replication was measured at 72 hours. A) Graph illustrating MFI plotted on the y-axis and showing no significant difference between the control and knock down group. Bars indicate mean and error bars denote standard deviation; B) Western blot image showing ACTN1 protein (115kDa) expression only in the control group while it is absent in siRNA treated samples. β-actin expression shows equivalent loading in the two samples. This diagram is a representative of three repeat experiments with each sample performed in triplicate.

3.4 Discussion:

Limited success in developing an effective form of therapy and lack of a protective vaccine has allowed HCV infection to emerge as a significant public health problem. Current therapeutic options not only have a low success rate (depending on virus genotype) but are also expensive and produce often intolerable adverse effects (Ferguson, 2010). All of these features point towards the need to develop better treatment regimens. Use of directly acting anti-viral agents targeting viral proteins is an important option but remains toxic, expensive and may be limited by pre-existing or acquired resistance due to the error prone nature of the virus RdRp. A complementary approach would be targeting host factors required for virus replication which will provide greater barrier to resistance. Designing siRNAs acting on host genes will also have the advantage of being effective for different HCV genotypes. A number of such siRNAs targeting three host genes were used to analyse effects on virus replication. Gene silencing potential of these duplexes was initially validated by both real time PCR and western blotting. For real time PCR relative quantification using a standard curve method was used. Maxpro real time PCR software was used to automatically quantify gene expression in ng and normalised to house keeping genes to compare between knock down and control. This is a simpler method than doing absolute quantification in which exact concentration of DNA needs to be measured by an independent method for example by cloning into a plasmid.

Intracellular delivery of molecules into biological systems has been used as a useful tool to study gene function or design therapeutic agents. A variety of

techniques are in use for this purpose including chemical, liposomal or viral based methods. Nucleofection is a type of transfection technique which allows entry of nucleic acids (DNA, RNA or siRNA) into mammalian cells with the help of a nucleofector device (electroporator) and special solutions. High efficiency and use of identical transfection protocols enables its use for simultaneous delivery of both siRNA and viral RNA. Nucleofection is also suitable for delivering larger size products like the HCV subgenomic replicon (~10 kB) than other lipid based transfection procedures.

A subgenomic replicon based on HCV non-structural proteins was used in this study. The advantages of this system are that it does not require Category III facilities and has a GFP reporter protein fused to the viral NS5A, which not only makes analysis simple but also enables measurement of HCV replication/translation in transient assays without the need for time consuming and laborious stable transfection.

In the current study, strict analysis of subgenomic RNA quality was carried out to exclude experimental artefacts arising from poor quality RNA by using fresh RNA, preventing repeated freeze thawing by producing aliquots, monitoring quality by agarose gel electrophoresis after every step during its production etc. Further, we used a number of experimental controls to exclude any sequence independent effects, toxicity associated with transfection procedure or hypersentivity to introduction of double stranded RNA. These included, i) mock transfected or negative controls indicating that the procedure does not give rise to any non-specific effects and is not associated with cytotoxicity; ii)

a DNA positive control (pmaxGFP™) to verify that the nucleofector solution and programme are suitable for the Huh7.5 cell line which then means that similar conditions can be used for RNA or siRNA; iii) a positive siRNA control which successfully knocked down GFP expression showing that siRNAs can efficiently enter and knock down the target gene in this cellular environment; iv) a negative control siRNA or scrambled control which had the same sequence as of the gene specific siRNA but arranged randomly. Of all the three siRNAs tested, the scrambled control that had no effect on protein expression indicating that the results obtained are real and are due to sequence-specific silencing of the target genes rather than sequence-independent effects associated with the delivery of siRNA into the cells.

Optimisation of replicon transfection was needed to determine the nucleofection conditions which can simultaneously provide high GFP expression and moderate cell number. To achieve this, a number of programmes were tested to select the one with the highest transfection efficiency without causing massive cell death. T-020 appeared to be the best, so it was used for transfection of viral RNA. In addition, the highest replication efficiency was achieved by using 10μg of replicon RNA and measuring GFP expression at 72 hours. In agreement with Blight *et al.*, when comparing the two cell lines, we found that transfection efficiency was higher for Huh7.5 as compared to Huh7 cells (Blight et al., 2002). Huh7.5 is a sub clone of Huh7 cells containing a deletion of the retinoid-inducible gene 1(RIG1). The higher permissiveness of the Huh7.5 cells is related to loss of the RIG1 protein, which

plays a role in recognition of viral RNA and triggers production of Type 1 IFN, thereby suppressing virus replication.

The field of RNAi for gene silencing has been revolutionised in the past decade due to its potential to act as a potential therapeutic and screening tool (Lares et al., 2010). Many studies have tested the utility of siRNAs for inhibiting HCV replication and some of those specifically which targeted viral proteins are in the later stages of clinical trials (Lin, 2010). Although promising, the high replication rate and error prone nature of the viral polymerase can be a hurdle. A report illustrating the efficacy of RNAi for inhibiting HCV replication also showed the development of escape mutants which could limit its utility (Randall et al., 2003). Similarly, some studies have already reported the development of mutant HCV replicons resistant to protease or polymerase inhibitors (Mo et al., 2005) or other therapies which target specific binding sites in HCV like microRNA122 (Li et al., 2011). The same effect has been observed for other RNA viruses, such as polio viruses where a study by Gitlin *et al.* provided evidence for rapid development of mutant viruses (Gitlin et al., 2002).

A report by Randall *et al.* identified a number of cellular proteins involved in virus replication (Randall et al., 2007). In the present study, a number of host genes were selected and their potential as an anti-viral target was assessed. Prior to their use in nucleofection, the intended siRNAs were validated for their gene silencing ability. Lipofectamine2000 was used for delivery of duplexes and showed that these siRNAs can successfully knock down target genes. In all

cases, the transcripts were consistently knocked by 85 to 99% of the levels observed in control samples (section 3.3.3). Reduction in protein levels was also highly significant for STAT-3 (90%) followed by VAP-A (~50%), showing reduction in protein expression in knock down sample. However for ACTN1, protein levels remained more or less unaltered and may reflect a variation in the dynamics of protein turn over (Figure 3.7). In this study, prior to co-transfection of siRNAs with viral RNA, the utility of nucleofector technology for delivering siRNA was tested. SiRNAs were successfully transfected and silenced target genes in a dose dependant manner. The minimum concentration having greatest knock down efficiency was chosen for co-transfection experiments in order to minimise toxicity or off target effects.

HCV NS3 protease inhibitors are the most advanced directly acting anti-viral (DAA) as they have now been approved for use as a combination with pegylated-interferon and ribavirin. However, a key concern with these viral based drug targets is emergence of resistance due to the high replication rate producing 10^{10-12} virions per day and the lack of proof reading ability of the RNA polymerase leading to high mutation rate when used as monotherapy. Also since virally targeted inhibitors bind to a specific site within the protein, even a single nucleotide difference can make it ineffective. Using host based therapies has the potential to circumvent these issues and also expand the list of available targets. Once validated, these can be used either alone or in combination to effectively suppress virus replication. This principle has been tested for HCV where siRNA targeting HCV receptors like CD81 and SRBI or other cellular proteins like phosphatidylinositol 4-kinase III alpha can inhibit

HCV replication (Berger et al., 2009). In this study, a number of cellular proteins involved in viral replication have been tested to evaluate their efficacy in suppressing virus replication.

VAP-A has been previously identified as a NS5A and NS5B binding protein (Tu et al., 1999) and is thought to aid in the formation of HCV replication complexes while enhancing viral replication (Gao et al., 2004). Our findings validate these results indicating that knock down of VAP-A results in a significant reduction in virus replication. HCV has been shown to replicate in a complexes consisting of lipid rafts and viral proteins (Shi et al., 2003), and VAP-A is known to be involved in lipid biosynthesis and trafficking, so we can speculate that VAP-A knock down interferes with the formation of replication complex and therefore suppresses viral replication. This is supported by a recent report demonstrating that knock down of seven lipid raft associated proteins including VAP-A inhibited HCV replication (Chan et al., 2011). We also found that VAP-A protein has a short half life as protein levels recover after 48hours and inhibitory effects on viral replication also diminish.

STAT-3 belongs to the STAT (signal transducer and activator of transcription) family but has distinct biological functions. It is mainly activated in response to cytokines like interleukin-6 (IL-6) and growth factors like epidermal growth factor (EGF) and has been implicated in oncogenesis (Lim and Cao, 2006). It has also been reported that in addition to cytokines, disturbance of intracellular calcium (Ca^{+}) by viral NS5A results in production of reactive oxygen species (ROS) which in turn activate STAT3 (Gong et al., 2001). A similar

phenomenon of STAT-3 activation by ROS has also been observed in lymphocytes (Carballo et al., 1999). A later study by Waris *et al.* also indicated involvement of ROS and Ca^{+} signalling in mediating HCV induced activation of STAT-3 (Waris et al., 2005). They also showed that blocking STAT-3 by inhibitors of cellular kinases like tyrosine or MAP kinases which are believed to activate STAT3 or using a dominant negative mutant for STAT-3 suppressed virus replication (Waris et al., 2005). The results presented in this study also validate these findings. We found that in both Huh7 and Huh7.5 cells, co-transfection and knock down of STAT-3 inhibited replication of the subgenomic replicon. Previous studies have suggested that HCV gene expression also influences expression of anti-oxidant pathways (Blackham et al., 2010). By causing oxidative stress and production of ROS, STAT3 may favour virus induced damage to cells and an inflammatory state, therefore favouring viral survival and persistence (Tardif et al., 2005).

Contrasting results have been reported by Zhu *et al.* showing that STAT-3 activation in response to IL-6 exerts an anti-viral mechanism in HCV infection (Zhu et al., 2004). Similarly, the role of ROS in modulating HCV replication is not clear yet and different reports provide diverse results (Choi et al., 2004) (Mccartney et al., 2008). Expression profile studies have also highlighted the importance of host genes involved in oxidative stress in virus replication (Blackham et al., 2010). Another possibility is that inhibition of STAT-3 may result in reciprocal increase in STAT-1 signalling leading to a pronounced antiviral response within the cells (Regis et al., 2008). Differences in results, however, may be due to different experimental conditions, including

differences in siRNA sequences, transfection techniques, HCV genotype or replication system. In our system, we found unequivocal evidence that STAT-3 acts to support virus replication.

ACTN1 is a cytoskeletal protein with an as yet undefined role in liver disease. Lan *et al.* used yeast two hybrid screening and co-immunoprecipition to demonstrate that alpha actinin interacts with viral NS5B and its silencing leads to reduction in viral replication (Lan et al., 2003). In our study, ACTN1 specific siRNA was co-transfected with viral RNA to assess changes in virus replication but its silencing did not suppress virus replication. We hypothesize that this difference may be isoform specific as the previous study did not analyse the presence of isoforms in liver. Contrasting function has been reported earlier for ACTN1 and ACTN4 in controlling survival and motility in astrocytoma (Quick and Skalli, 2010). Using proteome analysis, Fang *et al.* compared the expression profile of proteins in the Huh7 cell line and Huh7 cells containing replicating subgenomic replicon and found alpha actinin to be upregulated suggesting a role for this protein in HCV replication (Fang et al., 2006a). This may suggest ACTN1 is involved in establishment of infection in cells containing replicating virus. Our study, however, found no evidence that expression of ACTN1 was required to support viral replication as assessed in the sub-genomic replicon system.

In conclusion our study has validated the feasibility and potential of siRNA based therapies targeting cellular co-factors in down regulating HCV replication and this can be used either alone or in combination with existing or

forthcoming therapies to provide additive anti-HCV effect. In this study, VAP-A and STAT-3 have been shown to positively regulate HCV replication. Other cellular targets need to be identified and screened for their anti-HCV efficacy. Using drug delivery properties of liposomes/nanoparticles, more than one therapeutic agent can be targeted to liver either alone or in combination with the current treatment regime to further enhance its efficacy and this suggests that our approach could become an important adjunct to new virus specific therapies. It will now be important to confirm our findings in a system which accommodates viral replication and the production of infectious progeny virus.

Chapter Four

4 Liposome for delivery of therapeutic molecules

4.1 Introduction

4.1.1 Principle of targeted delivery for anti-HCV therapy

Current anti-HCV therapy has a variable outcome and is limited by a low response rate so that even the most favourable genotypes (2 and 3) do not have 100% chance of eradicating the virus. Compliance with therapy and the dose of ribavirin appear to be vital determinants of therapeutic efficacy (Feld and Hoofnagle, 2005). Lowering the dose has shown to diminish (Hadziyannis et al., 2004) while increasing it further improves outcome but only at the expense of higher adverse effects (Lindahl et al., 2005). This further lessens both patient compliance and quality of life. There is thus a need to devise effective and specific delivery system to directly deliver anti-viral drugs to infected hepatocytes and thereby minimise adverse effects arising from accumulation of drug in non-target tissue. Once successful, the same approach can be employed for delivering other small molecule inhibitors. These are a class of anti-HCV therapeutics aimed directly at viral enzymes like the protease or the viral RNA polymerase. The most successful and advanced are NS3A/4B protease inhibitors such as Telaprevir and Boceprevir which have now been licensed for clinical use. These agents do, however, require to be used together with both interferon and ribavirin (Hezode et al., 2009) and have additional drug specific toxicities.

This part of the study aimed to investigate whether nanoparticles (NP) could be used to specifically and efficiently deliver antiviral agents to hepatocytes. There are many advantages of using such an approach; Firstly, NP can be readily modified to enhance their physical properties (smaller size, low charge, surface conjugation with liver targeting molecules etc). This will improve their uptake by hepatocytes so the majority of drug will be concentrated to its site of action i.e. virally infected cells. Secondly, the enhanced uptake of drug molecules to a particular tissue will not only improve its therapeutic efficacy but may shorten total duration of treatment and achieve higher cure rates. Thirdly, tissue specific sequestration of drug molecule will reduce side effects arising from accumulation of drugs in off target sites, such as red blood cells in the case of ribavirin. Fourthly, more than one therapeutic molecule can be conjugated in a single formulation that will further enhance therapeutic efficacy. All these properties make them a suitable carrier molecule to deliver anti-HCV or other therapeutic molecule requiring targeted delivery.

4.1.2 Liposomes as vehicles for targeted drug delivery

An ideal drug delivery vehicle should be efficient, non-cytotoxic, non-immunogenic, biodegradable and specific in delivering a therapeutic molecule to its site of action. In case of siRNA, the delivery vehicle should also be able to protect duplexes from nucleases during its transit in the circulation and prevent rapid clearance by reticulo-endothelial cells. A number of nanoparticles are available for drug delivery (Moghimi et al., 2001). Liposomes, micelles and polymeric nanoparticles are used widely for delivery of drugs, genes, imaging agents and vaccines (Fahmy et al., 2005, Schwendener et al., 2010, Torchilin, 2007). Lipid based drug carriers represent

vehicles made up of physiological lipids such as phospholipids, cholesterol and triglycerides and fulfil the above criteria for a delivery vehicle suitable for human use. The only recognized side effect of carrier mediated delivery is a hypersensitivity reaction caused by complement activation, but this can be prevented by altering the dosage administration method, and exhibits tachyphylaxis (Szebeni et al., 2000).

Liposomes are lipid based nanoparticles. They are spherical vesicles having an aqueous core and an outer layer made up of phospholipid (Figure 4.1). Hydrophilic molecules like drugs and nucleic acids (e.g. siRNA or plasmid DNA) can be enclosed in the water soluble centre while lipid soluble compounds can be entrapped in the hydrophobic outer coat (lipid soluble drugs e.g. AmBisome). Liposomes mainly enter the cells by endocytosis (Düzgüneş and Nir, 1999, Pollock et al., 2010) and are characterised in terms of lipid content, size (50-1000 nm in diameter), surface charge or zeta potential (positive, negative or neutral) and number of lipid layers (uni or multi lamellar liposomes). Smaller size (<200nm) and neutral surface charge protects them from removal by the reticuloendothelial system (Vonarbourg et al., 2006).

Production of 'stealth' liposomes is achieved by attaching a hydrophilic polymer such as polyethylene glycol (PEG) outside the lipid bilayer. This prolongs circulation half life due to invisibility by the immune system as a result of less interaction with plasma proteins, reduced uptake by reticulo-endothelial cells (REC) in the liver or spleen, and reduced renal clearance (Owens Iii and Peppas, 2006, Rawat et al., 2008). Pegylated interferon alpha-

2b (PEG-Intron) is a classic example of using the pegylation technique to significantly improve response rates when compared to the unmodified drug (Glue et al., 2000).

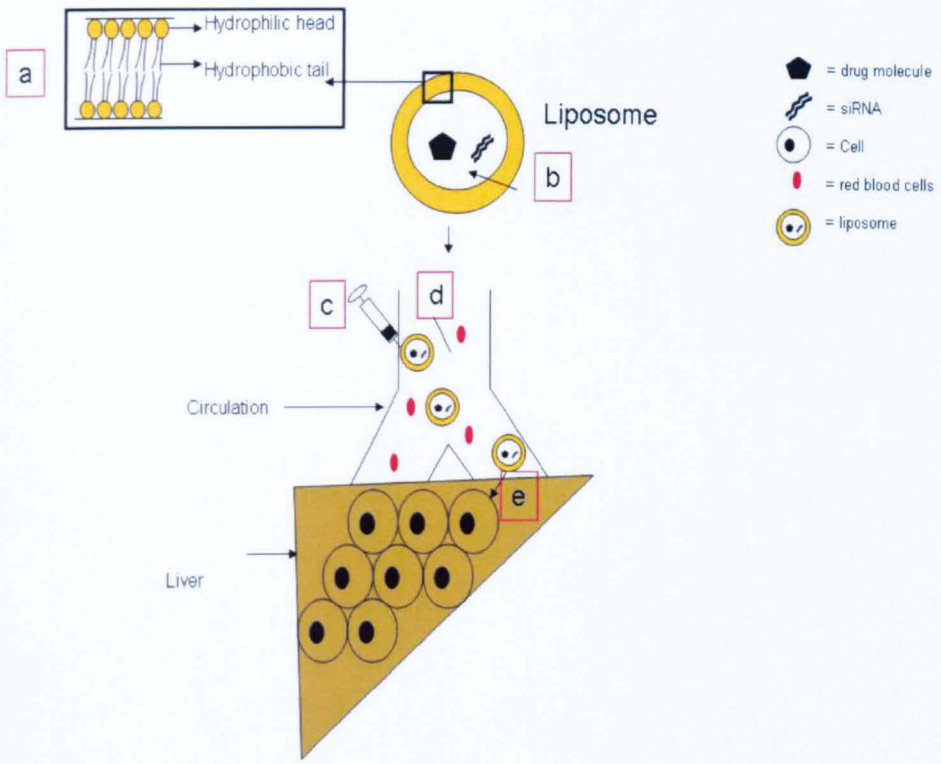


Figure 4.1 Liposomes for drug delivery

Liposomes are made of a phospholipid bilayer (a) having an inner hydrophilic core which can be loaded with drug or siRNA molecules (b). These can be used for systemic administration (c) and protect drug molecules from degradation or uptake by non-target cells (d). Further specificity can be improved by surface conjugation with ligands like antibodies or antibody fragments to improve uptake in specific locations, like the liver (e).

Target specificity of a liposomal delivery agent can be further increased by anchoring a ligand molecule on its surface which can identify and bind to a biological antigen or receptor enabling by the target organ. This is called active or ligand mediated drug targeting. One of the most frequently used methods of active targeting is immuno-liposomes, in which liposomes are coated with antibody or its antigen-binding or Fab fragment as a surface moiety to mediate uptake in tumour sites or other specific locations (Mastrobattista et al., 1999). Using haemoglobin conjugated ribavirin-HRC 203 (Levy et al., 2006) and lactosylated liposomes-CL-LA5 (Watanabe et al., 2007) are methods to mediate targeted delivery of therapeutic molecules to the liver. Apolipoprotein A-I (apo A-I) is a protein component of high density lipoprotein (HDL). Cationic liposomes artificially associated with apo A-I have been targeted to the liver through its cellular receptor. This vehicle has been shown to be able to transport siRNA to the liver thus mediating effective and specific delivery (Kim et al., 2007). In a later study based on an HCV mouse model (expressing all the structural proteins in liver), Kim *et al* also showed that the apo A-I targeting delivery vehicle can transport siRNA targeting HCV and cause 65-75% inhibition of viral replication (Kim et al., 2009).

Several liposome or polymer based nanoparticle drugs are already in clinical use for the treatment of human diseases such as cancer and fungal infections (Allen and Cullis, 2004, Schwendener, 2007). This approach is particularly advantageous in cancer treatment, where accumulation of a drug at the tumour site can improve its effectiveness and minimise non-specific adverse effects associated with conventional chemotherapeutics. Additionally tumours usually

have less efficient lymphatic drainage which further reduces drug clearance from its site of action and hence improves its efficacy. Doxorubicin (Doxil/Caelyx) indicated for use in the treatment of Kaposi's sarcoma and refractory ovarian and breast cancer, was one of the first pegylated liposomal formulations approved for human use (Allen and Cullis, 2004). In comparison to free drug, liposomal doxorubicin considerably improved the therapeutic efficacy due to higher bioavailability and reduced cardiotoxicity and myelosuppression (Martín et al., 2011, Soloman R, 2008, Uziely et al., 1995). Other examples of liposomal drugs approved for clinical use include liposomal amphotericin B (AmBisome) (Allen et al., 1994) and cytosine arabinoside (DepoCyt) indicated for lymphomatous meningitis (Murry and Blaney, 2000).

4.1.3 Role of Apolipoprotein B-100 in HCV replication

Plasma lipoproteins are particles made of phospholipids, cholesterol and apoproteins and function mainly to transport lipid and lipid soluble materials to and from the liver. Within an infected host, HCV particles circulate in association with lipoproteins such as very low density lipoprotein (VLDL) and low density lipoprotein (LDL). These are referred to as lipo-viral particles (LVP) (Andre et al., 2002). Apolipoprotein B-100 (apoB-100) forms the protein component of lipoproteins and is required for the assembly and release of LVP from the liver (Blasiole et al., 2007). The microsomal triglyceride transfer protein (MTP) present in the ER lumen regulates VLDL assembly by enhancing apoB-100 lipidation and preventing its degradation. Huang *et al* used an immuno-isolation approach to purify membrane vesicles containing HCV replication complexes. Proteome analysis of HCV containing membranes revealed that these vesicles are rich in apoB-100, apoE and MTP. They also

reported that blocking VLDL secretion by the liver through inhibiting apoB-100 expression by siRNAs and MTP inhibitor (BMS-2101038) decreases the release of infected viral particles in the circulation (Huang et al., 2007).

In this study, we planned to test the capability of liposomes to deliver two different types of therapeutic molecules i.e. siRNAs (targeting apoB-100), and drug (ribavirin). ApoB-100 was chosen as a siRNA target as it's related to HCV and is specifically expressed in the liver. For this, apoB-100 expression was silenced using siRNAs and knock down efficacy was compared following delivery by either using liposomes (supplied by Lipoxen) or a commercially available reagent like Lipofectamine 2000 (Invitrogen). Similarly, we planned to measure cellular uptake of free and liposomal ribavirin by Huh7 cells, a human hepatoma cell line used for propagation of infectious clones of HCV. It was hypothesised that once a suitable delivery vehicle has been identified, the same formulation could be used to deliver current drugs (ribavirin) alone or in combination with other directly acting or siRNA-based antiviral therapeutics. The ultimate aim of the project was to generate preclinical data resulting in the declaration of a candidate product suitable for phase-I clinical trials.

4.2 Aims:

The aims of this part of the study were to test the utility of liposomes for delivering siRNA (ApoB-100) and drug (ribavirin) by:

- Validating gene knock down of naked duplexes and comparing gene knock down efficiency of naked duplexes (delivered by lipofectamine2000) and those enclosed in liposomes.
- Analysing cellular uptake of a GFP encoded plasmid DNA when using liposomes or Lipofectamine2000 as a delivery vehicle.
- Analysing intra-cellular uptake of liposomes by using fluorescent labelled liposome.
- Modifying liposomal formulation or cell culture conditions to improve cellular uptake.
- Validating efficacy of liposomes as a delivery vehicle using apoB-100 siRNA and ribavirin as test molecules.

4.3 Results:

4.3.1 Comparisons of ApoB-100 knock down by using naked siRNA vs. liposomally entrapped siRNAs

4.3.1.1 Validation of gene silencing ability of anti-ApoB100 siRNA

Before analysing siRNA delivery by liposomes, the capacity of naked siRNA to silence ApoB-100 was assessed by using lipofectamine2000 (Invitrogen). ApoB-100 targeting siRNA used in this study was supplied by our commercial collaborator Lipoxen. The sequence of siRNA and details of transfection method is given in materials and methods. Briefly, Lipofectamine2000 manual instructions were followed to transfect 2.5 x 10^5 Huh7 cells/well in six well plates. Cells were pre-plated by overnight incubation and once 50-70% confluent, duplexes in transfection medium were added to the wells at a final concentration of 100nM. RNA extraction and cDNA synthesis was performed after 48 hours.

PCR primers for quantification of ApoB-100 and HPRT expression were designed using Primer3 programme (version 0.4.0) targeting exon-exon junction. Specificity was checked with primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Optimal primer annealing conditions were achieved by gradient PCR with a temperature range of $60\pm10^{o}C$ (primer sequence and optimal annealing temperature are given in materials and methods). A standard curve was prepared by serially diluting neat cDNA in order to ensure best PCR efficiency (range = $100\pm15\%$). As

shown in Figure 4.2 (A and B), the efficiency for both primer pairs was close to 100% as depicted by linear standard curve. Melting or dissociation curve confirmed that the primer pairs generated a single product indicated by the presence of a single peak. Similarly, the RT negative control and non-template control did not amplify, indicating that there is no genomic DNA or exogenous contamination (Figure 4.2, C and D).

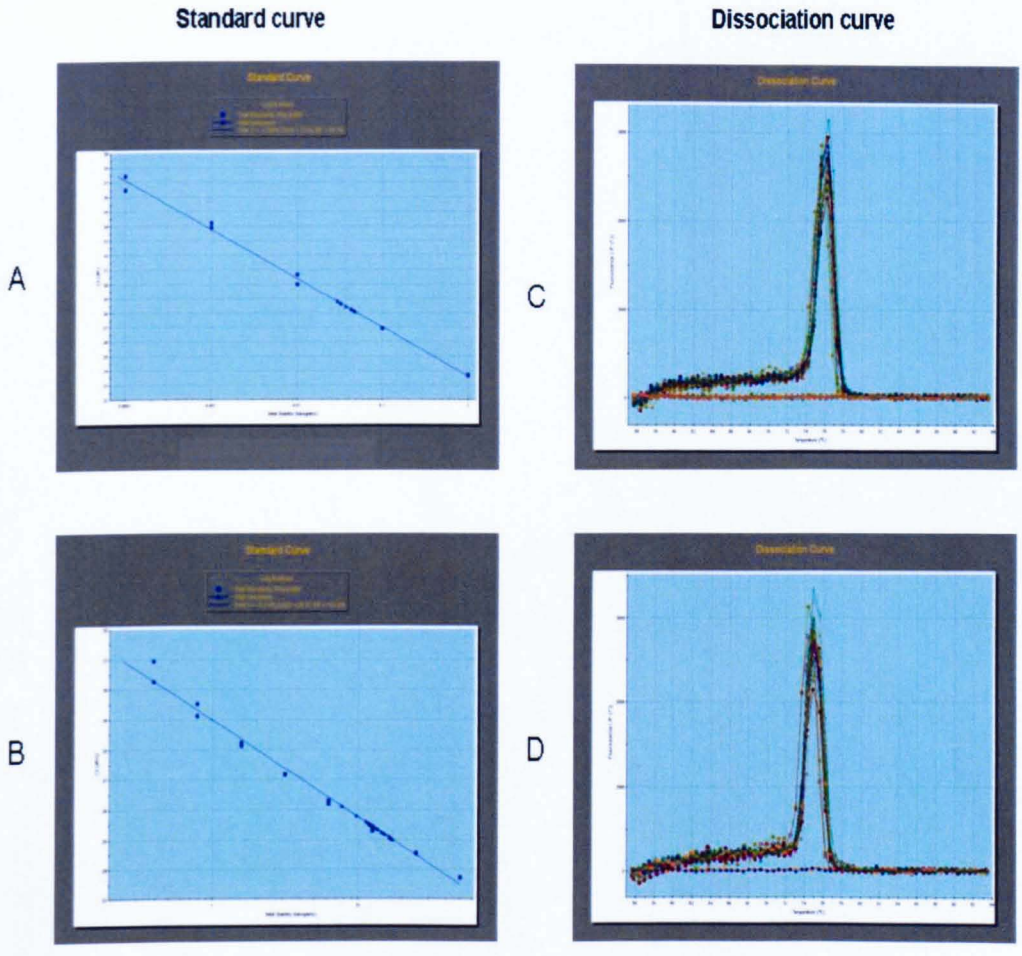


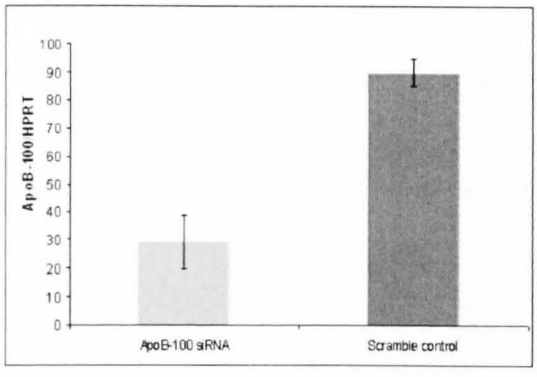
Figure 4.2 Real time PCR plots for ApoB-100 and HPRT primer

Standard curve for apoB-100 (A) and HPRT (B) was prepared by serial dilution of neat cDNA (x-axis) and change in cycle threshold (Ct, y axis) was measured. PCR efficiency was 98% for ApoB-100 and 104% for HPRT whereas R^2 value close to 1. Melting curves for ApoB-100 (C) and HPRT (D) demonstrated that both primer pairs generated a single product and absence of contaminating products, which may give rise to additional peak separate from the desired amplicon. No amplification of NTC and RT negative controls is observed (baseline in the melt curve).

MaxPro real time PCR software was used to quantify ApoB-100 expression in samples treated with either gene specific siRNA or a scrambled control and levels were normalised to the house keeping gene HPRT. As given in Figure 4.3 (A), cells treated with siRNAs had a 3 fold reduction in the expression of apoB-100 in comparison to cells treated with scrambled control. We then sought evidence that knock down at the message level was correlated with reduction in ApoB-100 protein expression. Figure 4.3 (B) shows results of a dot Blot which confirms that ApoB-100 specific siRNAs but not scramble control caused a significant reduction in ApoB-100 protein expression. In view of very faint detection of β-actin in these dot blots, implying inadequate protein detection, we elected to confirm specific protein knock down using Western blot.

ApoB-100 encodes for a large protein with a molecular weight of ~510kDa. Such a large molecule required optimisation of the western blot protocols. A number of methods, including agarose gel electrophoresis were tried unsuccessfully. Finally, protein expression was successfully quantified using a 4% SDS-PAGE resolving gel. β-actin was used as a loading control. The Western blot shown in Figure 4.4 (A) demonstrated that there is 100% reduction in expression of apoB-100 protein in siRNA treated samples as compared to the control siRNA. β-actin expression remained consistent in the parallel samples, indicating equal loading. The specificity of the Western was confirmed by using two other apoB-100 negative cell lines and secondary antibody alone (Figure 4.4, B). As expected bands were only detected in Huh7 cells exposed to both primary and secondary antibody.

A



B

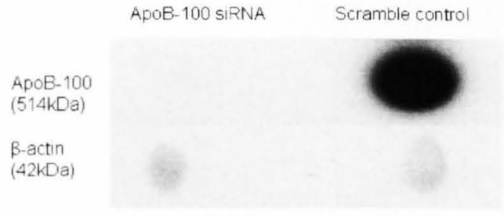


Figure 4.3 Analyses of apoB-100 knock down by siRNA delivered by Lipofectamine2000.

(A) Real time PCR: ApoB-100 expression (ng) was normalised to HPRT and the ratio plotted on the y-axis to compare levels in knock down samples vs. control. Bars indicate mean and error bars denote standard deviation.
(B) Dot Blot indicating absence of ApoB-100 protein in knock down samples as opposed to cells treated with the scrambled siRNA control. β-actin protein, although faintly detected, shows equal expression.

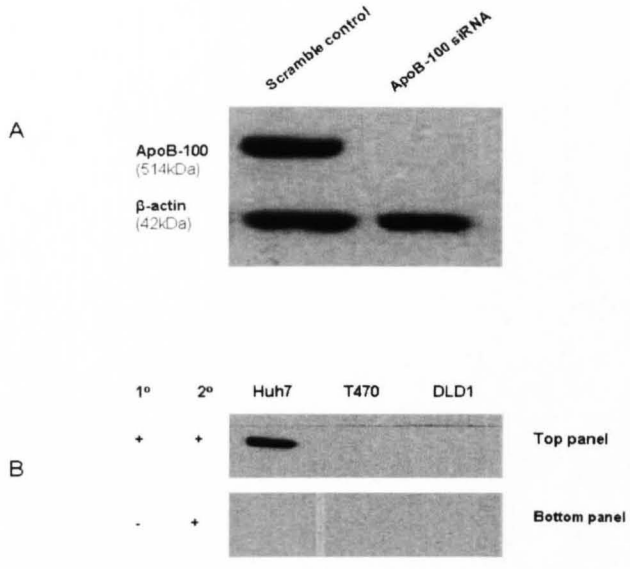


Figure 4.4 Western blot for ApoB-100 knock down by naked duplexes

(A) ApoB-100 expression by western blot confirmed complete loss of protein expression in knock down samples while β-actin expression was equivalent, demonstrating equal loading.
(B) The specificity of ApoB-100 antibody was demonstrated by using an apoB-100 positive (Huh7) and two negative (DLD1 and T470) cell lines. As expected, a 514 kDa band representative of ApoB-100 was only detected in Huh7 cells (top panel). Specificity of the secondary antibody was also confirmed by exposing the membrane to secondary antibody only. No signal was present in membrane incubated with secondary antibody alone (lower panel).
(1^{o} = ApoB-100 primary antibody, 2^{o} = Anti-mouse secondary antibody, Huh7= Human hepatoma cell line, T470= Breast cancer cell line, DLD1= Colorectal cancer cell line).

4.3.1.2 ApoB-100 knock down by liposomally entrapped siRNAs

After validating the gene silencing ability of duplexes, the efficacy of liposomally entrapped siRNAs was investigated. Huh7 cells were plated overnight for initial attachment as before. Liposomes made up of egg phosphatidylcholine were supplied by Lipoxen (Lipoxen Technologies Ltd) in a freezed/dried form and were resuspended in nuclease free water by vortexing to form siRNA containing vesicles. The volume of liposome suspension required to give a final concentration of 100nM siRNA was added to the wells and incubated for 48 hours prior to RNA extraction. siRNA delivered by Lipofectamine2000 was used as a positive control.

In order to compare efficiency of the two delivery methods, real time PCR was performed to quantify inhibition of apoB-100 mRNA expression. It was found that siRNA delivered by Lipofectamine2000 caused a significant reduction in transcript levels in knock down samples vs. control. In contrast, siRNA delivered by liposomes appeared to have no significant effect on apoB-100 mRNA levels (Figure 4.5). Dose response was assessed by increasing the amount of duplexes (range 30 – 300nM) but did not improve knock down with either of the delivery systems. Similarly, longer incubation of cells with liposomal siRNA (24-72 hours) also failed to cause any reduction in gene expression.

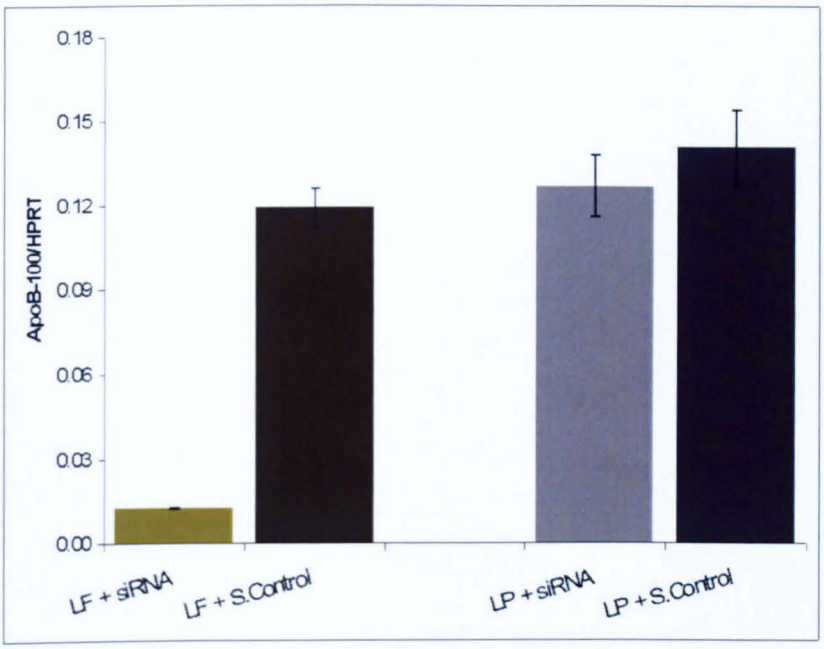


Figure 4.5 Comparison of apoB-100 knock down by naked vs. liposomally entrapped siRNA

Huh7 cells were exposed to either naked siRNA using Lipofectamine2000 or duplexes enclosed in liposome at a final concentration of 100nM/well and harvested after 48 hours. Real time PCR was done to quantify gene expression and normalised to HPRT. Using Lipofectamine2000 (LF) as a delivery vehicle, apoB-100 specific duplexes produced a significant reduction in transcript levels (less than 10% of control treated cells). In contrast, liposomal (LP) siRNAs failed to cause any significant reduction in mRNA levels. (LF = lipofectamine2000, LP = liposomes, S.control = scrambled control). Bars denote mean of ApoB-100 vs. HPRT ratio and error bars represent standard deviation.

4.3.2 Cellular uptake of liposomes (F1 and F2) containing a GFP expression vector

Liposomal siRNAs failed to silence target gene expression. This could be either due to liposomal failure to deliver siRNA, siRNA degradation or something unique to Huh7 cells. The ability of liposomes to deliver a GFP expression vector was next assessed in two cell lines i.e. Huh7 cells and the colorectal cancer cell line HCT116, to exclude that results are siRNA or cell line specific. Plasmid pGFP was entrapped in liposomes made up of egg phosphatidylcholine lipid either without cholesterol (F1) or with cholesterol (F2) and were supplied as a suspension by Lipoxen (Table 4.1). Huh7 and HCT116 cells were treated with a volume of liposome suspension required to give a final concentration of 2.8 μg of plasmid DNA/well (this dose has been previously validated by Lipoxen). The positive control consisted of plasmid at the same concentration delivered by means of Lipofectamine2000.

Liposomal uptake was expressed as percentage of cells positive for GFP above baseline fluorescence (negative control). As demonstrated in Figure 4.6 (top panel), a substantial population of Huh7 cells in the positive control population expressed GFP. On the other hand, Huh7 cells exposed to plasmid entrapped in liposomal formulations had GFP expression indistinguishable from the negative control. Similar results were found in HCT116 cells where the majority of cells in the positive control expressed GFP above baseline whereas expression was negligible in liposomally treated lines (Figure 4.6, bottom panel).

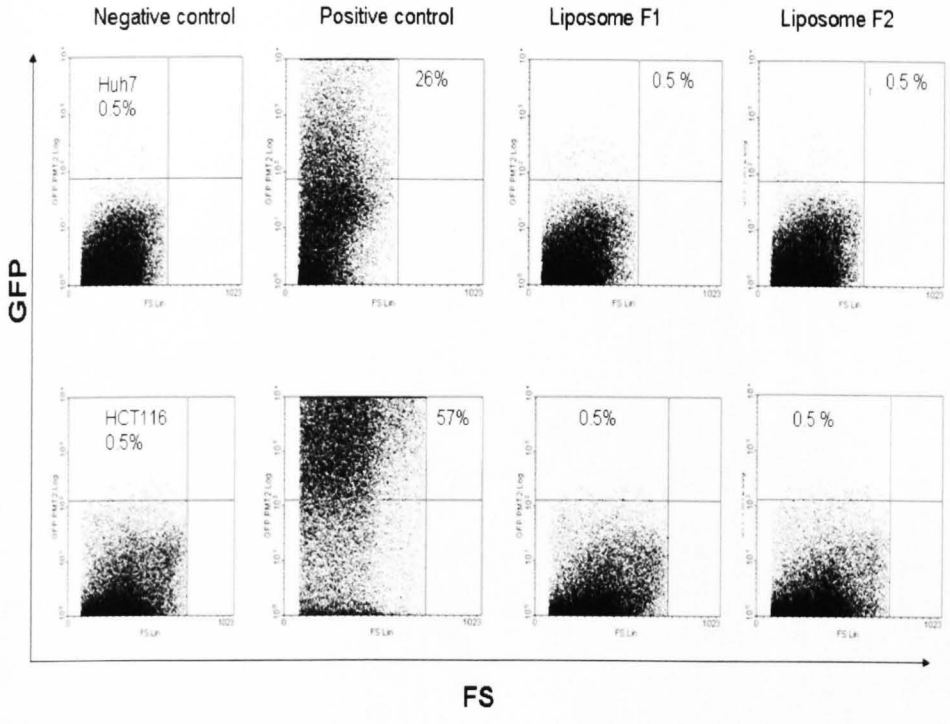


Figure 4.6 Transfection efficiency of GFP labelled liposome

Huh7 (top panel) and HCT116 cells (bottom panel) were either untreated (negative control), delivered with 2.8μg/ml of plasmid DNA encoding GFP protein using either lipofectamine2000 (positive control) or liposome formulations (F1 and F2). After 48 hours, the cell suspension was subjected to flow cytometric analysis for GFP expression. Cells were gated on the basis of forward and side scatter to exclude apoptotic or dead cells. Any cells expressing GFP above the levels in the negative control (untreated) were measured as percentage (%) of GFP positive cells in the total population. GFP expression is shown on the y-axis while forward scatter (FS) in presented on the x-axis.

4.3.3 Analysis of liposomal uptake by labelled vehicles

In order to further understand the failure of liposomally entrapped siRNA to effectively silence apoB-100, we next planned to evaluate liposome uptake inside Huh7 cells using fluorescent labelled liposomes. Information regarding various fluorescent liposomal preparations used in this study is given in Table 4.1.

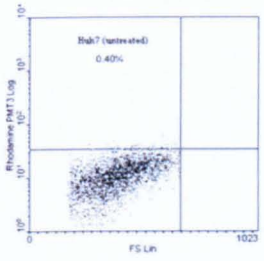
4.3.3.1 Cellular uptake of positive control nanoparticles and carboxy-fluorescence labelled liposomes

To demonstrate intra-cellular uptake, liposomes made up of either DPPC (F3) or HSPC (F4) lipid and conjugated with carboxy fluorescent (CF) dye were prepared by Lipoxen (Table 4.1). A positive control consisting of nanoparticles made up of polyglyceroadipate (PGA) and labelled with Rhodamine B isothiocyanate (RBI), known to be avidly taken up by cells, was prepared and supplied by Weina Meng (School of Pharmacy, University of Nottingham). Huh7 cells were exposed to 500μg/ml of nanoparticles for 2 hours and analysed by FACS. We found that Huh7 cells treated with nanoparticles were strongly positive for RBI with an uptake efficiency of 100% when compared to the negative control (Figure 4.7, A-C).

We next performed a series of dose response experiments using different concentration of liposomes F3 and F4. Cells were incubated with different concentrations (0-500μg) of liposome suspension for 2 hours and fluorescence expression was analysed by flow cytometry. In contrast to the positive control, cells treated with liposome formulations F3 and F4 at all doses tested, had

minimal fluorescence expression (Figure 4.7, D). The same experiment was repeated by prolonging incubation duration to 24 hours but failed to enhance uptake significantly. Analysis in HCT116 cell line produced identical findings and ruled out the possibility that results are Huh7 cell specific.

A



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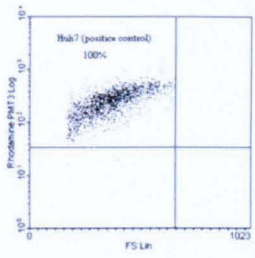

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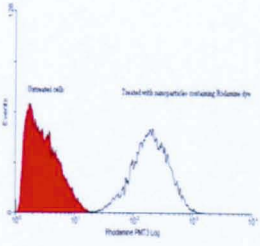


D

Formulation ID	Liposome dose (µg)	Mean fluorescence intensity (MFI)		Liposomal uptake (%)	
		2 hours	24 hours	2 hours	24 hours
Positive control	500		-	100	-
F3	0	7.5	30	<1	<1
	50	7.1	-	0.28	-
	100	6.3	29.1	0.11	0.32
	200	5.6	-	0.14	-
	500	6.0	39	0.20	1.15
F4	0	17.9	30	<1	<1
	50	15.0	-	0.09	-
	100	13.3	25.5	0.12	0.34
	200	12.1	-	0.10	-
	500	12.0	25.9	0.09	0.25

Figure 4.7 Cellular uptake of carboxy-fluorescence labelled liposomes

Huh7 cells were exposed to either RBI labelled nanoparticles or various doses of liposome formulation F3 and F4 composed of DPPC and HSPC lipid respectively. Cellular uptake was expressed as number of cells containing fluorescent dye (RBI or CF) above the baseline in untreated cells. Dot plot demonstrated 100% of cells positive for nanoparticles (B) when compared to the untreated population (A). Histogram depicts shift in fluorescence signal in the two populations on the x-axis (C). Red filled area = negative control and no filled area = nanoparticle treated Huh7. Table shows MFI and uptake of CF labelled liposomes by Huh7 cells (D). MFI = mean fluorescence intensity.

4.3.3.2 Altering physical characteristics of liposomal formulations to improve cellular uptake

Fluorescent labelled liposomal data showed that there is no fluorescence inside the cells. This suggested that either the liposomes are not being taken up by the cells or the fluorescent marker is leaking out of the liposomes. The leakage of fluorescence was ruled out by using liposomes incorporating a fluorescent label embedded within the lipid bilayer (F5-F12). These liposomes were supplied as a suspension by Lipoxen and varied with respect to lipid composition, molar ratio causing variation in surface charge and sucrose content. Details of the fluorescent liposomal preparations (F5-F12) are given in Table 4.1. Huh7 cells were exposed to various liposome formulations at different concentrations and subjected to flow cytometric analysis.

Figure 4.8 illustrates the cellular uptake of various liposomal preparations. Dose response analyses indicated that using 500 μg/ml of liposomes achieved a higher uptake than using a lower dosage. Among the first four liposomal formulations (F5-F8), F5 demonstrated the highest uptake level of around 10%. Notably among these preparations, F5 had the least surface charge or zeta potential, so it was hypothesised that this property may have accounted for the higher liposomal uptake. To prove this, further formulations with lower zeta potential (F9-12) were prepared and supplied by Lipoxen. In addition, F12 liposomes also had lowest sucrose content. Data in Figure 4.8 shows that the F12 formulation demonstrated a substantial uptake of around 63% which was superior to any of the preparations tested so far and was even five times higher than F5.

Table 4.1 Physical properties of liposomal formulations used in the current study.

A number of liposomal formulation labelled with the below mentioned fluorescent marker were used to track intra-cellular entry of delivery vehicles. (PC = egg Phosphatidylcholines, DPPC = dipalmitoylphosphatidylcholine, HSPC = hydrogenated soy phosphatidylcholine, CHL = cholesterol, CF = carboxyfluorescein, DPE = N-dansyl phosphatidyl ethanolamine, NBD = 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine, 7-nitrobenzofurazan-labelled lipid). Different colours indicate various batches.

Formulation ID	Lipid composition	Fluorescent material	Particle size (nm)	Zeta potential (mV)
F1	PC	GFP	-	-
F2	PC/CHL	GFP	-	-
F3	DPPC	CF	227.3±42.6	-
F4	HSPC	CF	153.1±30.3	-
F5	DPPC	DPE	92.7	-26.7
F6	DPPC/CHL	DPE	102.7	-41.9
F7	DPPC	DPE	94.77	-40.7
F8	DPPC/Gal-Lipid	DPE	70.52	-37.4
F9	DPPC	DPE	92.3	-16.4
F10	DPPC	DPE	79.52	-14.1
F11	DPPC	DPE	95.76	-7.01
F12	DPPC	NBD	109.5	-6.05

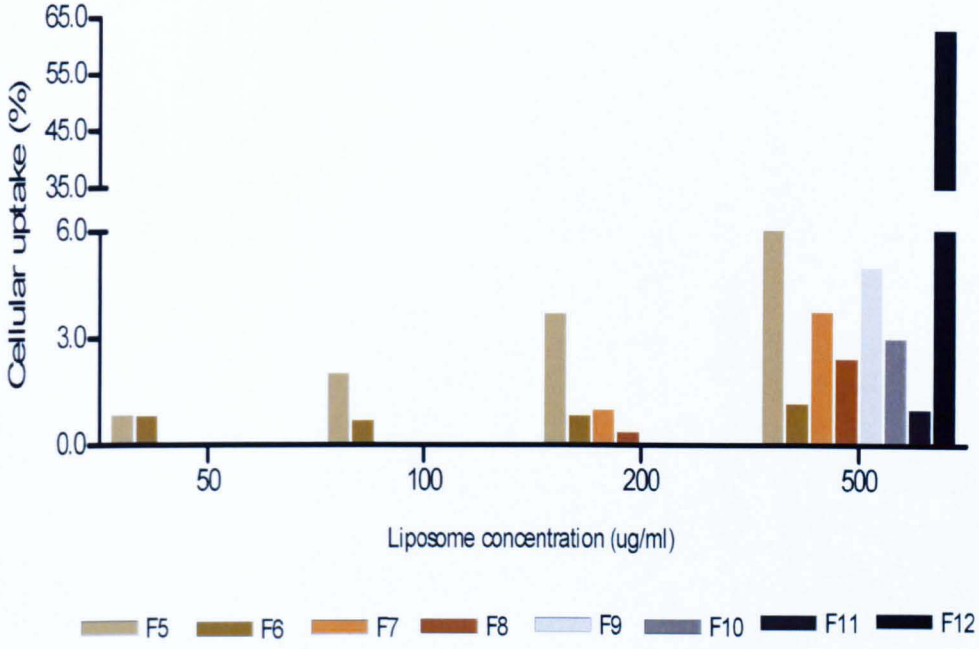


Figure 4.8 Cellular uptake of DPE and NBD labelled liposomes with variable physical characteristics

Huh7 cells were exposed to different liposomal formulation (F5-F12) and uptake was analysed by flow cytomtery. A dose response for F5-F8 was performed at the indicated concentrations (x-axis) to evaluate liposomal uptake after 2 hours (y-axis). Huh7 cells were also treated with liposomes with variable surface charge (F9-F12) and lower sucrose content (F12) in order to analyse uptake efficiency. Liposomal uptake was expressed as percentage of positive cells expressing fluorescent marker above the baseline fluorescence (negative control).

4.3.3.3 Altering serum conditions to enhance liposomal uptake

Liposomal formulations with a lower surface charge generally had higher uptake efficiency. In order to further enhance cellular entry, the effect of serum on liposomal uptake was evaluated. Huh7 cells were delivered with liposomes with the lowest surface charge (F5, F9, F10, F11 and F12). For this, cells were plated overnight and incubated with 500μg/ml of liposomes in Huh7 culture medium with or without serum for 4 hours.

It was found that depriving cells of serum improved liposomal uptake by at least 2 fold (Figure 4.9-A). The Highest uptake levels were achieved by formulation F12 in which 80% of cells expressed the fluorescent marker above the baseline. In contrast, the same delivery vehicle in the presence of serum produced an uptake level of 60% as illustrated in Figure 4.9-B. In light of these findings, F12 was considered to be suitable for subsequent analysis.

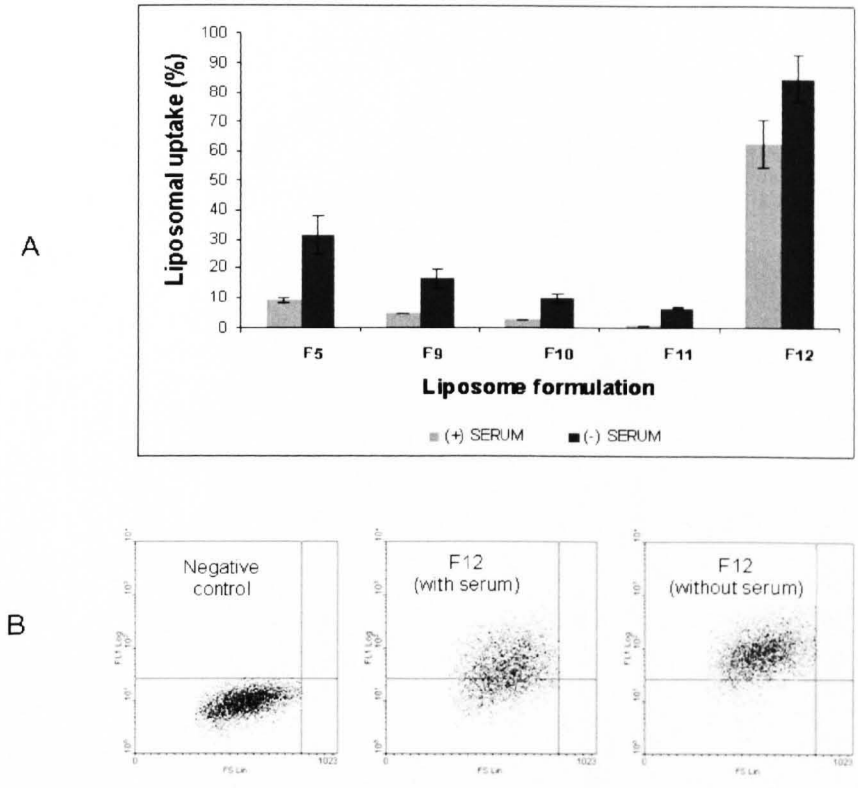


Figure 4.9 Influence of serum conditions on liposomal uptake by Huh7 cells

(A) Huh7 cells were exposed to different liposomal formulations (F5, F9-12) in the presence or absence of serum and cellular uptake was analysed by flow cytomtery. Bars indicate average and error bars indicate standard deviation.
(B) Dot plot indicating liposomal uptake in Huh7 cells treated with 500μg/ml of F12 in the presence or absence of serum. Y-axis = fluorescence, x-axis= forward scatter.

4.3.4 Efficiency of liposome as a drug delivery vehicle

Analysis of fluorescent liposomes (F12) revealed that lowering the surface charge and sucrose content of liposomes significantly enhanced intracellular uptake achieving optimal uptake *in vitro*. These liposomes were then tested for their efficacy to deliver exogenous molecules, either siRNA or ribavirin.

4.3.4.1 ApoB-100 gene silencing by liposomal siRNAs

A new liposomal formulation enclosing ApoB-100 specific siRNA was supplied by Lipoxen. These liposomes had physical properties similar to the F12 formulation which was found to be efficiently taken up by Huh7 cells. Liposomes were packaged with siRNA and supplied fresh as a ready to use suspension by Lipoxen. The siRNA sequence is the same as mentioned earlier. Huh7 cells were delivered with 100nM of either naked siRNAs or liposomally entrapped siRNAs. Lipofectamine2000 was used for delivery of naked duplexes. For liposome delivery, Huh7 cells were plated overnight and the required volume of liposomes was added to the culture medium. Cells were harvested for RNA and protein extraction at 48 hours. Real time PCR and Western blot analysis was done to evaluate gene knock-down.

ApoB-100 mRNA expression was compared between knock down and control samples, and transcript levels normalised to HPRT. As shown in Figure 4.10, naked siRNA delivered by Lipofectamine2000 caused a drop of more than 80% in mRNA levels (A) which also translated into approximately 90% reduction in apoB-100 protein expression (B). In contrast, the same duplexes when delivered by liposomes failed to produce such an effect where apoB-100

mRNA dropped less than 10% in the control vs. siRNA treated samples and protein levels also remained unaltered between the two sets of samples.

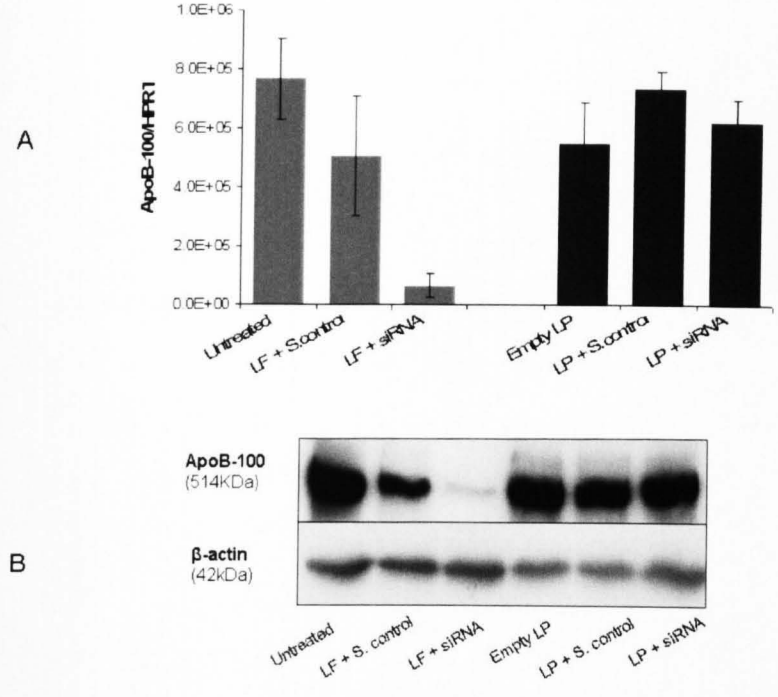


Figure 4.10 Analysis of siRNA delivery ability of low sucrose liposomes

Huh7 cells were treated with gene specific and control siRNA at a final concentration of 100nM. Effects of gene silencing were analysed at 48 hours by real time PCR and Western blot. (A) ApoB-100 mRNA was quantified in knockdown and control samples and after normalisation, the ratio of ApoB-100 and HPRT was plotted on the y-axis to examine differences in expression levels among different samples. (B) Protein samples extracted from parallel samples were run on a 4% SDS-PAGE gel and immuno-blotted with mouse monoclonal antibody. β-actin was used as a loading control. (LP = liposome, LF = lipofectamine2000, S.control = scrambled control).

4.3.4.2 Comparison of free and liposomal ribavirin uptake

The capacity of liposomes to deliver drug was next analysed by comparing efficacy of free ribavirin uptake to that packaged inside liposomes. These were supplied by Lipoxen as a ready to use suspension and had physical properties similar to F12 previously shown to have the highest cellular uptake.. Huh7 cells were pre-plated overnight and exposed to various doses of both free and liposomal ribavirin. Samples were extracted at 1, 2, 4 and 24 hours to measure total ribavirin uptake by Huh7 cells. The drug uptake experiments were done by Weina Meng (School of Pharmacy) using high performance liquid chromatography (HPLC). Uptake of liposomal ribavirin by Huh7 cells was very limited. Almost all of the drug was recovered from the cell culture medium and less than 1% of the total drug from the cellular compartment. Surprisingly, it was found that Huh7 cells did not take up free ribavirin either. This finding has subsequently been confirmed in repeat experiments during this study (given in Chapter 5). In order to further analyse the failure of liposomes to transport ribavirin, the stability of these liposomes was assessed. Liposomes were resuspended in Huh7 culture medium in a dialysis bag and incubated at 37° C in a shaker bath at a speed of 90 cycles per min. Samples were taken out at pre-determined time points and replaced with fresh culture medium. It was found that almost 50 % of the ribavirin was released after 4 hours. This figure rose to ~70 % after 24 hours. It is therefore likely that for incubation times longer than four hours, Huh7 cells were exposed to higher concentration of free ribavirin than liposomally entrapped drug. There was, however, no evidence of early uptake suggestive of liposomally mediated entry of ribavirin into cells.

4.4 Discussion

Drug delivery systems (DDS) have been recognised as an important tool for specific delivery of large number of therapeutic molecules. Since their discovery in 1961 by Alec D. Bangham, liposomes have been used as a versatile tool in various fields of science and medicine including DDS, for both lipophilic and hydrophilic molecules. Physical properties such as the ability of targeted delivery and a non-toxic, non-immunogenic, bio-degradable, flexible structure makes them an ideal carrier molecule for delivering drugs, imaging molecules or vaccination peptides. This could be particularly valuable for treating diseases like cancers in which entrapment of drug molecules in non-target sites not only compromises the efficacy but also produces intolerable adverse effects. Hepatitis C virus is another example in which delivery of anti-virals to hepatocytes might play a vital role in a patient's response to the treatment. Ribavirin is a vital component of current HCV therapy but causes severe haemolytic anaemia due to its accumulation in the red cell compartment, reducing its efficacy as well. Similarly, the cure rate can be boosted by using a higher dose but occurs at the expense of more side effects. Both of these factors can be surmounted by using a specific and targeted drug delivery vehicle.

This study was aimed at evaluating efficacy of liposomal formulations (supplied by Lipoxen) as a carrier to transport therapeutic molecules. To do so, siRNAs targeting apoB-100 and ribavirin were used as test molecules. It was anticipated that liposomal preparations which could successfully deliver these test molecules may then be used to deliver other siRNA or small molecule

based drug targets. Moreover, more than one agent can in theory be conjugated in a single formulation to produce a synergistic effect. This concept is in line with other studies showing feasibility of use of liposomes for delivery of more than one agent, particularly chemotherapeutic drugs which usually require separate administration and more complex dosing regimens (Mendonça et al., 2010, Wong and Chiu, 2011). We set out to test a number of liposomal formulations which varied in physical properties like particle size, surface charge, and lipid content.

ApoB-100 plays an integral role in assembly and release of VLDL and LDL required for the transport and metabolism of cholesterol (Blasiole et al., 2007, Brown and Goldstein, 1986). Drug targets for apoB-100 based on antisense oligonulceotides (Crooke et al., 2005), MTP inhibitors (Magnin et al., 2003) or siRNA (Zimmermann et al., 2006) are being evaluated for the treatment of hypercholesterolemia. Previous studies have found that apoB-100 plays a role in the release of infectious HCV particles from the liver (Huang et al., 2007). We therefore planned to use apoB-100 as a target for inhibiting HCV infection. The gene silencing ability of siRNAs incorporated in liposomes was compared to that of naked duplexes delivered by Lipofectamine2000 as a control. The results presented earlier demonstrated no inhibition of apoB-100 expression by liposomally delivered siRNA while it was significantly reduced in control cells. Similarly, GFP containing liposomes also failed to produce any evidence of liposome mediated uptake of plasmid DNA and hence GFP expression and confirmed that the findings are not unique to siRNA or Huh7 cell line. We rationalised that one or more factors may account for the failure of liposomally

entrapped siRNA to modulate target gene expression in two distinct cell lines. These factors are described below in Figure 4.11.

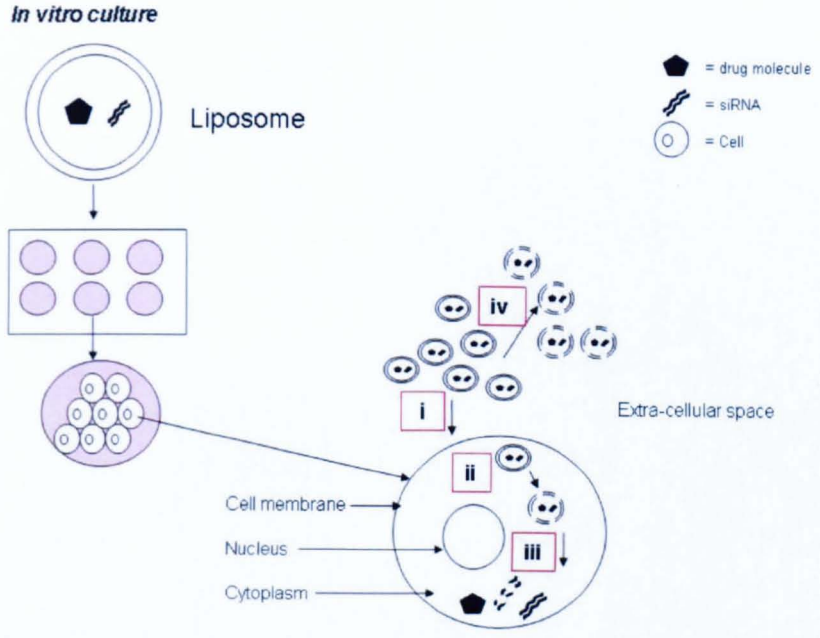


Figure 4.11 Possible mechanism of liposomal failure

Failure of liposomes to silence apoB-100 gene expression and deliver ribavirin may be due to one or more of the following factors:

i. Liposomes are unable to enter the cells or liposomes are taken up, but at low levels delivering insufficient siRNAs to mediate gene silencing or GFP expression by plasmid

ii. Liposomes are taken up by the cells but intracellular processing is inhibiting release of siRNA, plasmid DNA or drug

iii. Intracellular inhibition of molecules by degradation

iv. Disintegration of liposomes due to intrinsic instability is resulting in degradation of particles prior to intracellular uptake.

The first logical measure was to assess whether liposomes are being transported inside Huh7 as the first three questions are completely (i) or partly dependent on it (ii and iii) as given in Figure 4.11. We used fluorescent labelled liposomes for characterising liposomal uptake as it is the most common approach to monitor particle entry under physiological conditions (Madeira et al., 2011). We also used RBI labelled PGA NPs shown to be avidly taken up by primary brain cells and brain tumour cells in previous studies by our colleagues in Pharmacy (Meng et al., 2006). Fluorescent analysis carried out by flow cytometry revealed that almost all the cells expressed the fluorescent marker in the positive control but it was minimal in the liposomally (F3-F4) treated group.

Failure of liposomal uptake suggested that either the formulation themselves (a) or the *in vitro* culture conditions (b) were not suitable for uptake. To address (a), factors like the vehicle's physical properties (size, charge, lipid and sugar content etc) were altered whereas the osmotic environment and serum conditions of culture were modified to overcome (b) and further improve uptake. Additionally, use of another cell line (HCT116) and a positive control NP ensured that the culture conditions were appropriate.

The physical characteristics of a lipid carrier are an important determinant of its cellular uptake. Important variables are vesicle size and surface charge, and these can be altered by modifying the preparation method and lipid content (molar ratio). We initially tested F5-F8 formulations and of these, F5 had the highest uptake. Especially, this preparation had the lowest negative charge so

further carrier molecules with lower zeta potentials were supplied by Lipoxen for testing. Our data showed that formulation F12 having the lowest or near neutral charge showed a significant uptake of almost 60 percent. This may be due to the cell membrane's hydrophobic nature that causes electrically neutral and small molecules to pass through the membrane easier than charged or large ones. So a neutral or near neutral surface charge facilitates liposomal penetration through the plasma membrane (Lee et al., 2011). In the case of siRNAs incorporated in liposomes, this further helps by forming stable complexes between negatively charged siRNA molecules and neutral or positively charged liposome. Our data also validates these earlier reports as lowering particle size and surface charge seemed to improve intracellular uptake.

Since the ultimate aim of this study was to test a formulation suitable for *in vivo* testing, factors favouring physiological stability of particles were also considered. After entering into the circulation, the fate of a carrier molecule also depends on its size, range between 70 and 200nM. Smaller particles are likely to be lost via renal excretion or by crossing the endothelial barrier, and larger particles can be removed by the bone marrow, heart, kidney and stomach (Gaumet et al., 2008, Litzinger et al., 1994, Vonarbourg et al., 2006). In a report assessing role of liposome characteristics, Rhomberg et al found that liposomes with a mean size of 120 nm were removed from the bloodstream at a lower rate than liposomes of 230 and 360 nm, respectively suggesting that particle size of less than 150nM had long circulating life than larger size

vesicles (Romberg et al., 2007). All the liposomal preparations used in this study had sizes within this range.

Another observation was that most of the formulations used in this study were prepared with high sucrose content except F12 formulation. Sugar molecules are known to interact directly with the polar head group of phospholipids by forming a hydrogen bond (Crowe et al., 1994). Addition of carbohydrates like sucrose and trehalose to the liposomal preparation helps in stabilising the vesicle membrane and preventing liposome flocculation and fusion (Anchordoguy et al., 1987, Womersley et al., 1985). Our results suggest that the high sugar content of the majority of vehicles may have increased osmotic forces in the cell culture environment disfiguring particle structure and thus contributing to poor uptake levels.

It has been previously suggested that serum free medium can alter efficiency of liposomal uptake by the cells (Düzgüneş and Nir, 1999). In order to enhance delivery properties of vehicles, influence of serum on liposomal uptake compared by incubating cells in the presence or absence of serum. We found that, starving cells with serum improved liposomal uptake least 2 fold. This might suggest that either the serum components alter cell:liposome interactions or cells in the presence of serum remain in a comparatively quiescent state, while depriving them of serum which contains essential lipids for cell proliferation induces an activated phagocytic state to uptake more extra cellular molecules. This is consistent with the observation that liposomes are mainly taken up by endocytosis as in most of cases the cell's outer exoskeleton prevents direct attachment and fusion (Düzgüneş and Nir, 1999). The same

effect can be achieved *in vivo* by using PEG coating that prevents interaction with serum protein and hence improves their stability and uptake. Such an example is use of stable nucleic acid liposomes (SNALP).

Among all those tested, F12 was identified as a formulation suitable for delivery of therapeutic molecules like siRNA or drugs so its ability to transport these molecules was assessed. Huh7 cells were treated with apoB-100 siRNAs entrapped in the F12 preparation but failed to cause gene silencing. Similarly, no significant difference was achieved by using free or liposomal ribavirin. In order to further understand the basis of this failure, the stability of liposomes was assessed at 37°C. An *In vitro* release assay revealed that these formulations were very unstable in serum and the majority of the drug leaked out within 24 hours, thus accounting for poor uptake in the cells.

Stability of liposome *in vivo* has been questioned by many investigators and has been rationalised to liposome-plasma protein interactions. These studies generally suggested that these interfaces result in destabilisation and break down of vesicles or opsonisation thus reducing their efficacy *in vivo* (Semple et al., 1998). Liposomal charge and lipid composition seemed influence adsorption of proteins on the liposomal surface. A report by Hernandazcaselles *et al* showed that liposomal instability indicated by release of the entrapped fluorescent probe positively correlated with the amount of protein adsorbed on its surface. They also demonstrate that liposomes with neutral or positive charge bound the least protein, while those of negative charge were dependant on lipid composition, as the vesicles with phosphatidylcholine bound less

protein than the ones devoid of it (Hernandezcaselles et al., 1993). Thus there is need to improve these formulation by either changing lipid content and include helper lipids like cholesterol, which will increase liposomal stability. Similarly use of stealth liposomes can be advantageous (Bege et al., 2011). Surface coating of liposomes with hydrophilic molecules like PEG not only prolongs circulation time but also increases their stability. This has been found due to blockage of direct attachment of serum proteins with the liposomal membrane (Hioki et al., 2010). In the present report, positive control PGA nanoparticles were shown to be avidly taken up by cells and have been identified as highly potent delivery vehicle in previous studies (Meng et al., 2006) so these can be used for intracellular delivery of siRNAs or anti-HCV medicine instead of liposomes.

Surprisingly, uptake levels of free ribavirin by Huh7 cells were very poor. This was a much unexpected finding as a number of studies have shown efficacy of ribavirin in inhibiting HCV replication in Huh7 cells *in vitro* (Kato et al., 2005). However, this is in line with the data produced by our collaborator in Glasgow who found that free ribavirin failed to inhibit JFH-1 replication in a SEAP assay by Huh7 (Arvind et al, personal communication). SEAP is a cell based reporter assay which serves as a direct measure of viral translation and replication. In this system Enhanced Green Fluorescent Protein (EGFP) is fused in frame with Secreted Alkaline phosphatase (SEAP) via a viral N3/4A serine protease recognition sequence and is secreted in the culture medium when cleaved by HCV NS3/4A protease (Iro et al., 2009). We next wish to examine the presence of the ribavirin transporter in primary human hepatocytes

and compare it to the Huh7 cell line. We will then be able to evaluate if ribavirin uptake correlates with the expression of major ribavirin transporters such as the equilibrative nucleoside transporter.

Chapter Five

5 Correlation of ribavirin uptake and ENT1 expression by primary human hepatocytes

5.1 Introduction

5.1.1 Treatment for chronic hepatitis C virus infection (HCV)

5.1.1.1 Combination therapy with ribavirin and interferon

The current standard of care for patients with chronic HCV infection consists of pegylated interferon alpha (IFN-α) and ribavirin. Ribavirin is a purine nucleoside analogue used as an adjuvant with interferon based therapy. When used as monotherapy, its effects on viral replication are negligible but improve dramatically when combined with IFN-α (Mchutchison, 1999). The exact mechanism of ribavirin's anti-viral action is currently not known but a number of mechanisms have been proposed. These are summarised in Figure 6.1 and discussed in greater detail in Chapter 1 (General introduction).

Of the proposed mechanisms for ribavirin action, most require ribavirin to be transported into the cells. Levy *et al* found that targeting ribavirin to hepatocytes improves its antiviral potency and survival rate in mice with hepatitis (Levy et al., 2006). Further, the response rate to ribavirin improves with increasing dose of ribavirin (even for the less responsive genotype 1) but at the expense of more side effects (Lindahl et al., 2005). Conversely, a recent prospective study comparing efficacy of ribavirin with a reduced initial dose showed that despite having higher haemoglobin levels, SVR cannot be

achieved with a lower dosage (Konishi I, 2010). It is therefore evidently clinically important to achieve as high levels of ribavirin as possible, but the capacity to do so is limited by increasing side effects. In these circumstances, it is legitimate to interrogate the factors which regulate ribavirin entry into hepatocytes, particularly those which may provide a mechanistic basis for variations in the outcome of therapy.

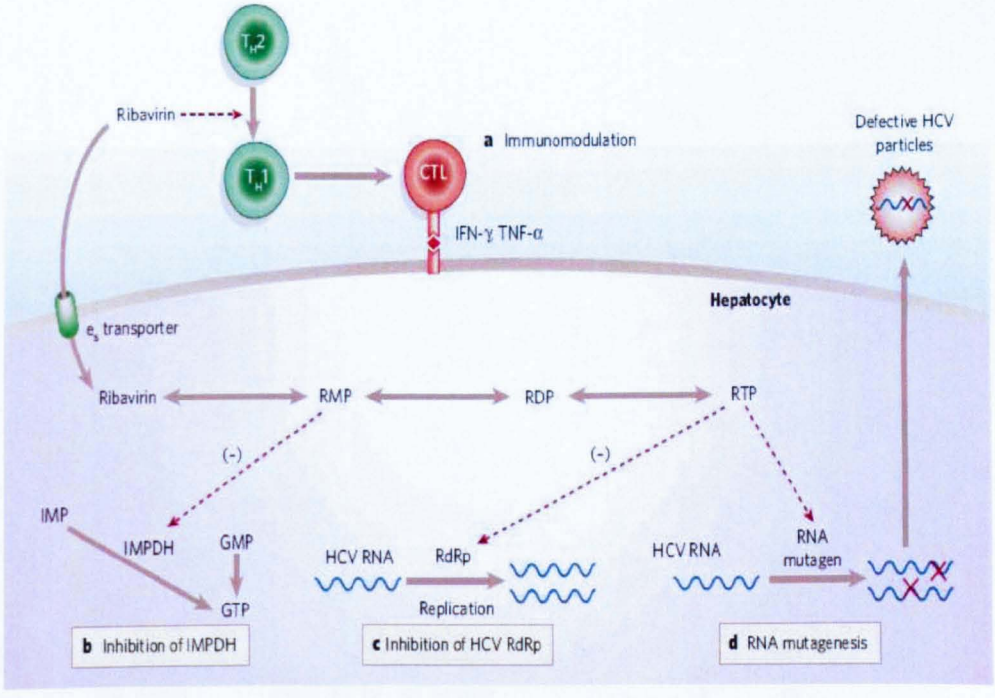


Figure 5.1 Possible mode of ribavirin anti-HCV actions

A number of mechanisms have been proposed for ribavirin effects in combination therapy for hepatitis C virus. **a**) Immunomodulation (by eliciting a strong helper T cell response), **b**) Inhibition of IMPDH (by reducing GTP levels) **c**) Inhibition of viral RNA polymerase, RdRp (by RTP) and **d**) RNA mutagenesis (by incorporation of RTP into replicating RNA chains and inducing mutagenesis resulting into production of defective HCV particles) (Feld and Hoofnagle, 2005)

5.1.1.2 Limitations of current regimen

Established treatment guidelines for chronic HCV comprise either a 24- or 48-week course of pegylated interferon and ribavirin, depending on the viral genotype (Excellence, 2004). Using these regimens, an SVR has been reported in 42–52% of patients infected with HCV genotype 1 and in 76–82% of those with genotype 2/3 infection (Manns et al., 2001, Fried et al., 2002, Hadziyannis et al., 2004, Zeuzem et al., 2004). Combination therapy, however, has a significant and often serious side effect profile, including depression, insomnia and flu like symptoms attributed to interferon and haemolytic anaemia due to accumulation of ribavirin in the red blood cell compartment.

Owing to great disease burden (having approximately 180 million people infected worldwide), there has been tremendous effort to improve treatment response rate by identifying new drugs (Lin, 2010 #320) or revising the current regimen. This includes a better understanding of the mechanisms of action of interferon and ribavirin. One study explored the pharmacokinetic interactions of interferon and ribavirin and found that interferon does not affect ribavirin metabolism or prolong its availability or vice versa, and the safety profile of these drugs in combination is similar to that of either drug used alone (Khakoo et al., 1998). Feld et al provided evidence that ribavirin acts by improving interferon signalling via upregulating cytokine production (Feld et al., 2010). It is, however, still unclear how these two drugs act to provide synergistic effects and dramatically improve response rate.

5.1.1.3 Assay for ribavirin quantification

A number of assays have been developed for ribavirin quantification. High performance liquid chromatography (HPLC) is one of the widely used techniques due to the simplicity of the method and lack of the radioactivity associated with some other methods (Granich et al., 1989). It is a type of liquid chromatography which separates compounds based on differences in their polarity to solid phase as detected by ultraviolet light.

Figure 5.2 shows the HPLC flow scheme. A pump provides constant pressure to propel the solvent (mobile phase) through the column at a fixed rate. An unknown sample is injected into the column which is pre-packed with a solid material (solid phase). The speed at which any specific substance moves through the column depends on its relative polarity with the solid phase. Compounds with least polarity will elute first and those with high polarity will come out later. The UV lamp detects any eluted metabolite and a signal is produced in the form of a specific peak proportion to the amount of substance eluted.

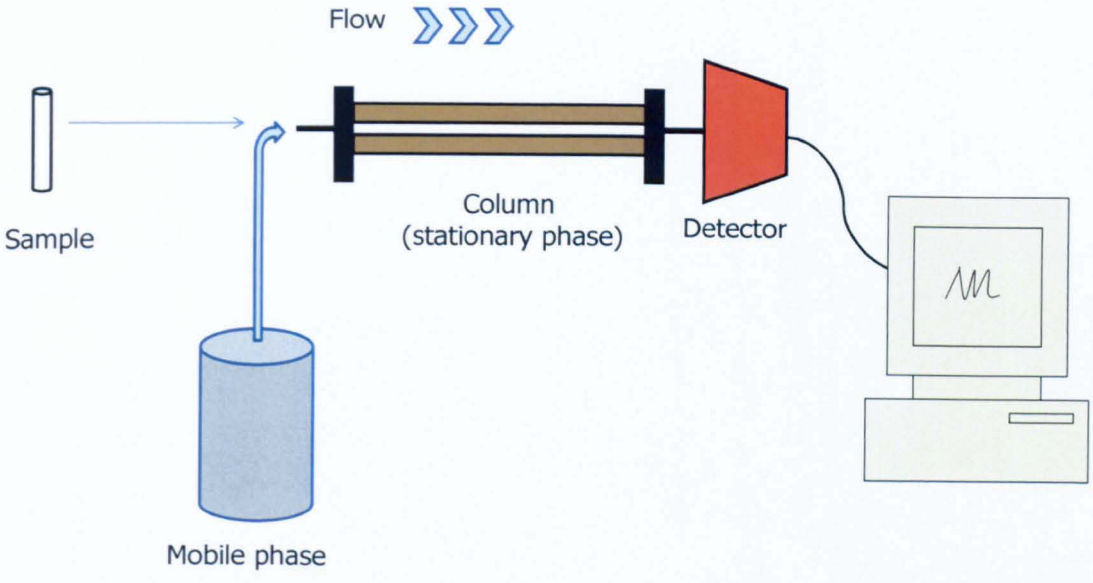


Figure 5.2 Flow scheme for HPLC

Mobile phase (buffer) is propelled into the column with the help of constant pressure generated by a pump at a flow rate of 1ml/min. the sample is injected into the column and is separated as it passes through the column. The detector has a UV source which detects the substance at a specific wavelength. The signal is then transmitted to a computer and is displayed in the form of chromatogram (peak).

5.1.2 Nucleoside transporters

5.1.2.1 Introduction and classification of nucleoside transporters

Nucleosides are the building blocks of nucleic acid synthesis and play a vital role in growth and metabolism. Several nucleoside analogues are being used as antiviral and anti-cancer molecules like ribavirin, acyclovir, fluorouracil, cytarabine (Ara-C), gemcitabine etc. Due to their hydrophilic nature, nucleoside analogues require a transport system to gain entry inside the cells and exert their action. Expression levels and function of these transporters are therefore probably an important determinant of the efficacy and toxicity of various antiviral and anticancer drugs (Clarke et al., 2006).

There are two main types of nucleoside transporters; the Equilibrative nucleoside transporter (ENT), encoded by SLC29A, is a facilitative type of transporter in which the substrate concentration is the driving force. The other type, the concentrative nucleoside transporter (CNT, SLC28A), is a Na^+ dependent secondary active transporter (Kong et al., 2004). Each of these transporters is further classified into subtypes. ENTs have two major classes depending on their inhibitor sensitivity to a compound called Nitrobenzylthioinosine (NBMPR). The ENT1 gene is located on chromosome 6 and is inhibited by nanomolar concentrations of NBMPR whereas ENT2 requires micromolar concentrations of NBMPR to be inhibited. ENT3 and ENT4 are two less well characterised types of nucleoside transporters. ENT3

transports purines and pyrimidines and ENT4 transports adenosine only (Baldwin et al., 2004).

CNTs are also classified into subtypes (1-3) based on substrate selectivity. CNT1 and 2 transport pyrimidines and purines respectively whereas CNT3 transports both (Marzena Podgorska, 2005).

5.1.2.2 Functional importance and regulation of human equilibrative nucleoside transporters 1 (hENT1)

Human ENT1 is a 456- residue glycoprotein made up of 11 transmembrane domains (TMDs) with an intracellular N-terminus and extracellular C-terminus (Sundaram et al., 2001). It is widely distributed in mammalian tissue with high levels of expression found in erythrocytes, placenta, brain, heart, liver, lung colon etc. In contrast, CNTs have specific expression sites like the liver, kidney and intestine (Kong et al., 2004). hENT1 are mainly localised to the plasma membrane where they play a key role in movement of nucleotide and nucleic acids in and out of cells. For cells that lack intrinsic biosynthesis pathways like erythrocytes, these are supplied by the liver (Griffith and Jarvis, 1996). By modulating concentration of nucleoside substrates like adenosine (a purine nucleoside) these transporters play an important physiological role in tissues like the heart, brain and placenta (Baldwin et al., 2004).

Inflammatory cytokines have been shown to alter hepatic gene expression and alter transporter function (Petrovic et al., 2007). It has been shown that nitric oxide alters SLC29A1 promoter activity and reduction in ENT1 expression leading to reduced adenosine uptake by the cell and vascular abnormalities

(Farlas et al., 2010). Stress induced c-Jun N-terminal kinases (JNK), a type of mitogen-activated protein (MAP) kinase has been implicated in down regulating ENT1 function, mRNA expression and promoter activity (Leisewitz et al., 2011). It has been shown that TMDs 3-6 are implicated in nucleoside binding and transport (Sundaram et al., 1998). A later study by Sengupta et al identified a highly conserved glycine residue at position 179 of TMD 5 of human and rat ENT1(Sengupta et al., 2002). By using a yeast based transporter model, they showed that this residue is essential for transporter function and sensitivity to NBMPR regardless of its localisation in the plasma membrane. The requirement of nucleoside transporters in cellular uptake of many nucleoside analogues used as anticancer or antiviral agents necessitates studying the mechanisms regulating their expression and function. This is clearly also true for ribavirin (purine nucleoside analogue) used for HCV infection.

5.1.2.3 Ribavirin uptake is mainly mediated by equilibrative nucleoside transporters 1 (ENT1)

Hepatocytes are the principal site of viral replication in HCV infection. To exert its antiviral actions, ribavirin must be transported into hepatocytes via a nucleoside transporter. Jarvis et al demonstrated that ribavirin transport into human erythrocytes is mainly mediated through an NBMPR sensitive equilibrative nucleoside transporter (Jarvis et al., 1998). Studies done by Endres et al also suggest that ENT1 plays an important role in erythrocyte uptake of ribavirin, both *in vitro* and *in vivo* (Endres et al., 2009). Using in situ hybridization, Govindarajan et al showed that higher levels of mRNA were

present for ENT1, CNT1 and ENT2 than for CNT2 in human hepatocytes (Govindarajan et al., 2007). A later study showed that ENT1 and CNT1 as well as ENT2 and CNT have comparable expression and the majority of ribavirin transport into hepatocytes is mediated through ENT1 (89%) (Govindarajan et al., 2008). A study using cryopreserved hepatocytes also suggested that ENT1 may be the major transporter involved in ribavirin uptake (Fukuchi et al., 2010)

5.1.3 In vitro model based on primary human hepatocytes

5.1.3.1 Indication for use of primary human hepatocytes

Although there are well defined limitations to growth and maintenance of hepatocyte specific functions *in vitro,* primary hepatocyte culture remains the most suitable system to study hepatocyte function *in vitro*. Maintenance of metabolism and transporter systems makes them a good model to undertake drug based studies (Runge et al., 2000). Also, as humans are the only natural host for hepatitis C, primary human hepatocytes provide a useful tool to study response to anti-virals and host virus interaction (Runge et al., 2000). Although rodent hepatocytes are a useful alternative to human cells for certain purposes, species differences and earlier loss of liver specific function are limiting factors (Battle and Stacey, 2001, Morel et al., 1990, Runge et al., 2000). Another advantage of human cells is that expression of sinusoidal transport proteins remains relatively constant and mimics closely that found *in vivo* when compared to rat hepatocytes (Jigorel et al., 2005).

5.1.3.2 Culture systems

Hepatocytes *in vivo* are arranged in a three dimensional (3D) configuration surrounded by non-parenchymal cells and extra cellular matrix components (Figure 5.3). This environment is required for cells to proliferate, maintain phenotype and perform specific functions. Advances in tissue culture techniques, such as the addition of extracellular matrix component (e.g. collagen), co-culture with other non-parenchymal cells (e.g. hepatic stellate cells) and serum free conditions have allowed development of healthy longer living cultures (Battle and Stacey, 2001, Chen et al., 1998, Katsura et al., 2002, Thomas et al., 2005) . Growing cells on extracellular matrix also helps in maintenance of liver specific function and receptor expression (Kataropoulou et al., 2005). Further, cells grown on extracellular matrix components grow in a spatially organised manner, rather as a flat monolayers when cultured on tissue culture plastic, and form well characterised structures known as spheroids. Spheroid formation promotes hepatocyte polarisation, cell-cell and cell-matrix interaction and therefore creates a micro-environment in which cellular transporters such as ENT1 may be physiologically localised.

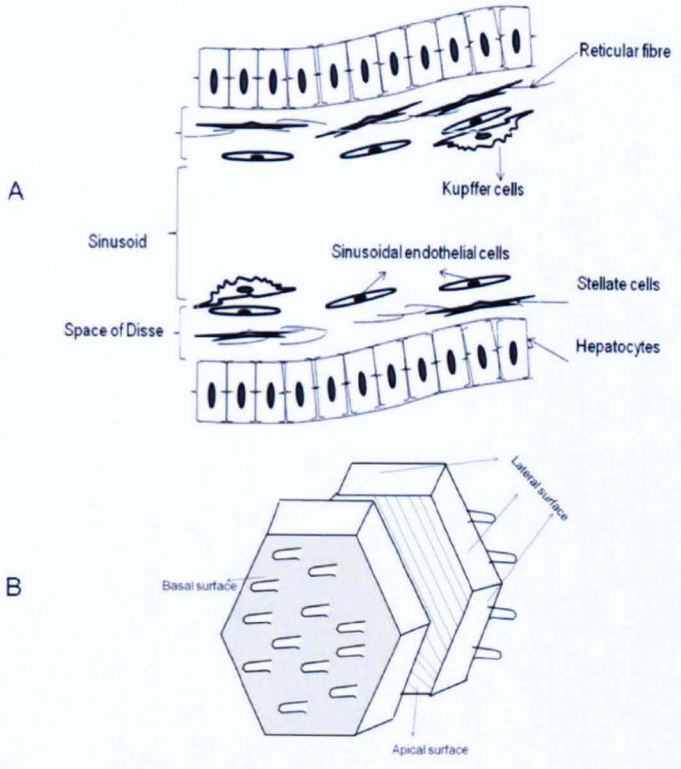


Figure 5.3 Schematic illustration of various liver cells arranged *in vivo*

A) Relationship between hepatocytes to other non-parenchymal liver cell types such as sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells.

B) Three dimensional structure of a hepatocyte having a basal (facing sinusoid and space of Disse), apical (facing bile canaliculous) and lateral side (facing adjacent hepatocytes).

5.2 Aims:

New agents are currently being evaluated for a better efficacy against HCV. Although a number of these agents are likely to be used for human therapy in future, current data suggest that ribavirin will remain a cornerstone of any future therapy for HCV infection. It is therefore important to study mechanisms involved in ribavirin action including transporter systems responsible for uptake into hepatocytes. In order to do so, primary human hepatocytes provide a most suitable model to study hepatocyte function *in vitro*. The specific aims of this study were:

- To isolate and culture primary human hepatocytes *in vitro*.
- To quantify ribavirin uptake into human hepatocytes by HPLC.
- To quantitatively evaluate expression of the major ribavirin transporter (ENT1) in human hepatocytes.
- To correlate drug uptake with receptor expression.
- To assess whether interferon alpha treatment modulates ENT1 expression and ribavirin uptake.

5.3 Results

5.3.1 Experimental design and data analysis

Human hepatocytes were isolated and cultured according to a procedure described in material and methods (section 2.10). After the addition of ribavirin, samples were taken at 4, 8 and 24 hour for liver 1-4 and 24 hour for liver 5-6, and the Huh7 cell line. The 24 hour time point was chosen to compare drug uptake and receptor expression as ribavirin uptake was maximum at this point. Additionally, at this time point cell number remains the same or comparable between the different liver preps (Tissue Engineering group, University of Nottingham). Although a similar seeding density was used for all livers, total protein concentration measured in the cell suspension used for HPLC was used to normalise for variation caused by cell number which may arise due to errors during cell count. Non-parametric Spearman's Correlation test (r_s) was used to correlate ribavirin uptake with total ENT1 expression. All the graphs and data analysis was done in GraphPad Prism version 4. Bar graph shows means ± standard deviation from three repeat experiments.

5.3.2 Human hepatocytes culture

Liver tissue was obtained from human donors undergoing liver resection. Full ethical approval and patient consent was obtained for the use of human tissue in this study. This work was done in collaboration with the FRAME group (Biomedical Sciences, University of Nottingham). Donor information is given in Table 2.3 and liver from each of these donors is referred to as Liver 1-6. Primary human hepatocytes were isolated by a modified two step collagenase

perfusion and after being resuspended in plating medium, cells were seeded on 6 well collagen coated plates at a density of 1.68 million/well (cell density previously optimised by Tissue Engineering Group, University of Nottingham). The use of optimal seeding density and collagen as a culture surface has been found to create in our hands a healthy *in vitro* environment for hepatocyte growth. For hepatocytes grown on standard tissue culture surface, identical conditions were used except that the cells were cultured on tissue culture plastic instead of collagen. After overnight attachment, medium was changed to hepatocyte culture medium.

Human hepatocytes were successfully isolated with consistently good cell viability of ~ 85% and purity (Figure 5.4). Cells grown on collagen coated plates maintained typical cuboidal shape characteristic of hepatocytes, with prominent nuclei and cell boundaries throughout the culture period (yellow arrows in Figure 5.4-A). Hepatocytes also continued to proliferate in the form of three dimensional spheroids (white arrows in figure 5.4-A). In contrast, hepatocytes grown on tissue culture plastic lost their cuboidal shape and appeared circular because of failure to properly adhere to the culture surface (Figure 5.4-B). Despite having used the same initial density, hepatocytes were less confluent on tissue culture surface, indicative of lower proliferation rate.

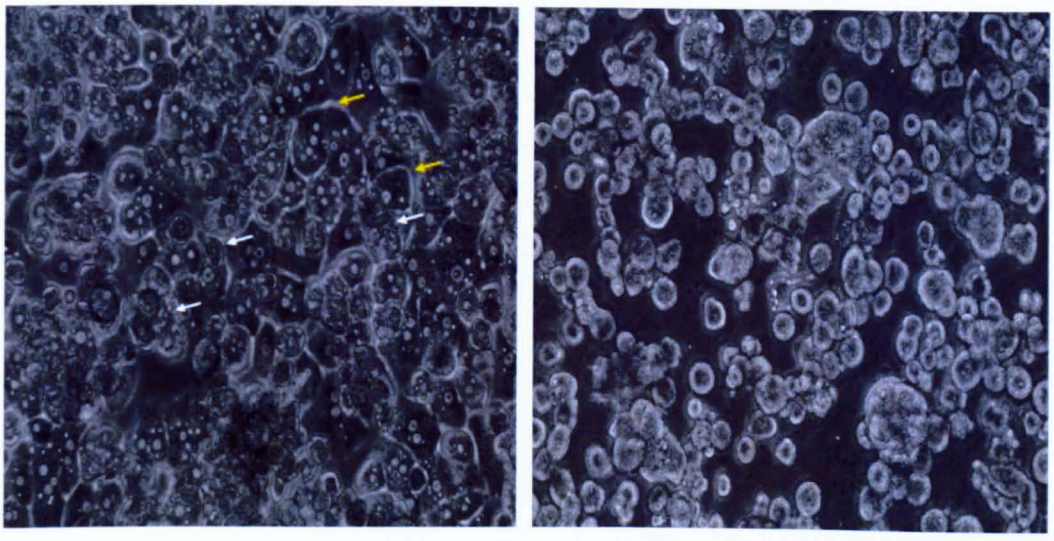


Figure 5.4 Human hepatocytes on day 3 of *in vitro* culture

A) Human hepatocytes cultured on collagen coated plates yellow arrows show hepatocytes attached to culture plate maintaining liver cell morphology whereas white arrows show areas of spheroid formation (cells growing in a three dimensional pattern). B) Human hepatocytes cultured on tissue culture plastic: cells grow in a two dimensional mono-layer rather than forming spheroids and appear less proliferative than A.

5.3.3 Optimal chromatographic conditions for ribavirin

HPLC was used to quantify ribavirin uptake by the cells. For this, cells were treated with ribavirin and the sample extracted at given time points. Samples for HPLC were prepared by cell lysis and enzyme digestion followed by PBA column extraction (to purify the sample for the subsequent step). Ribavirin levels in the cell fraction were quantified by a method described previously (Loregian et al., 2007). The HPLC conditions for measuring ribavirin in the cell fraction had been previously validated by Weina Meng (Pharmacy) and are described in section 2.12.

HPLC conditions were re-validated to exclude operator related variation and ensure reproducibility. For this, samples with known ribavirin quantity were either prepared in water (to identify specific peak for ribavirin) or in cell suspension (to exclude interference from any metabolites in the cell suspension). Initially, there were some difficulties in obtaining optimal peaks. Peaks either become very broad or split, leading to double peaks and unreliable data. These difficulties were entirely resolved by revalidating and using a new column.

Inter-assay precision (one reading taken on three separate days) and intra-assay precision (three separate readings taken on the same day) tests were performed to indicate both long term and short term reproducibility and accuracy for ribavirin quantification. Table 5.1 shows that at a given ribavirin dose, the variation in observed levels was low as indicated by standard deviation and relative standard deviation (RSD). The assay linearity was determined by

means of a calibration curve in which a known ribavirin concentration was plotted against the ratio of the area for ribavirin (RV) vs. the area for the internal standard (IS). As shown in Figure 5.5, the data points form a straight line and the value of correlation co-efficient (R^2) is 0.999 indicating that there is a good linear relationship. This calibration curve was repeated along with any unknown samples in order to quantify drug uptake and ensure assay conditions were identical. Drug sensitivity was also measured by using a number of serial dilutions and it was found that the method is sensitive enough to measure drug concentration down to 0.04 μg/ml. Figure 5.6 shows specificity of ribavirin and internal standard peaks, prepared either in water (top panel) or cell fraction (bottom panel). It can be seen that there is a single sharp peak for both ribavirin and internal standard showing no interference from any non-specific metabolites.

Table 5.1 HPLC assay reproducibility and precision

Samples were prepared by adding known ribavirin quantities (2, 4 and 8 ug/ml) to cell suspension. These samples were injected through the column and repeated on the same day (to measure intra-assay variation) or on a different day (to measure inter-assay). Mean indicates the average of three experiments and variation is denoted by standard deviation (Baldwin et al.) and relative standard deviation (RSD).

Added concentration (ug/ml)	Observed concentration (ug/ml)					
	Intra-assay (n=3)			Inter-assay (n=3)		
	Mean	SD(±)	RSD (%)	Mean	SD(±)	RSD (%)
8	7.9	0.03	0.42	7.8	0.30	3.9
4	4.1	0.05	1.30	4.0	0.12	3.0
2	2.0	0.01	0.60	1.94	0.13	6.7

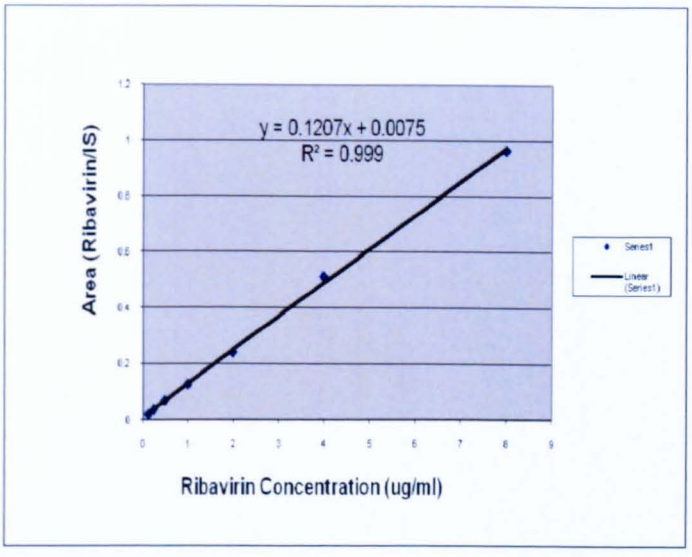


Figure 5.5 Calibration curve for ribavirin in cell fraction

A series of samples were prepared by adding known ribavirin quantity and then doing a double dilution to produce a calibration curve. Area under the curve was automatically determined by the machine for both ribavirin and internal standard. Data points were obtained by plotting the ratio of the area of ribavirin vs. the area of internal standard on the y-axis against known ribavirin concentrations on the x-axis. Equation (y=0.1207x-0.0075) was derived from these data points and used to work out the ribavirin concentration in unknown samples. Co-relation co-efficient (R^2) close to 1 indicates good linearity.

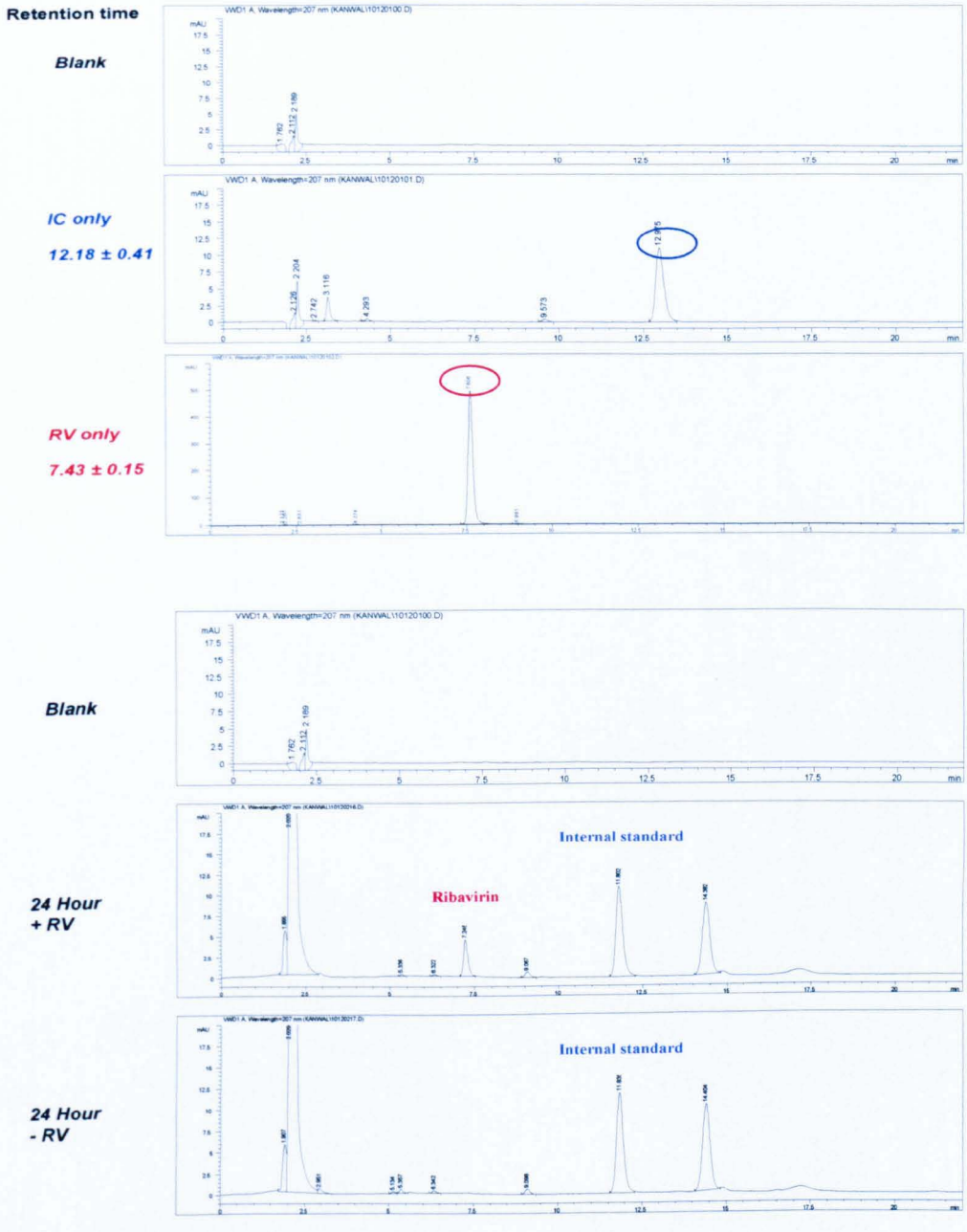


Figure 5.6 Typical chromatogram for ribavirin

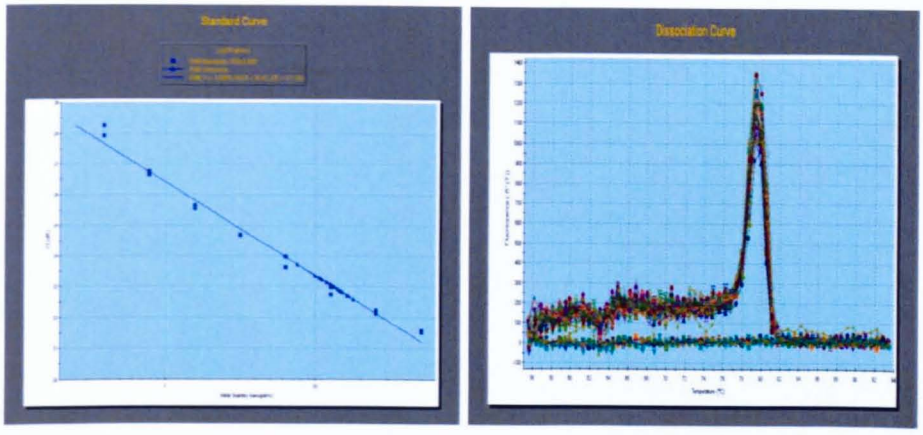
Top: Samples prepared by adding ribavirin (RV) and internal control (IC) in water to identify specific peaks for each of them. Blue circle indicates IC peak and red circle indicates RV peak. Corresponding retention times are given on the left. **Bottom:** Samples from a cell fraction with or without ribavirin were added to the culture medium to show that there is no interference caused by any metabolites in cell suspension.

5.3.4 Time course for ribavirin uptake and ENT1 expression by primary human hepatocytes (liver 1-4)

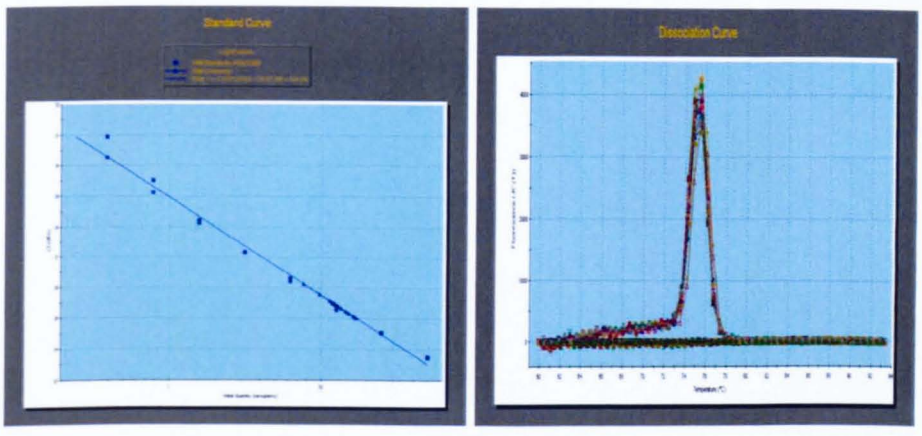
Human hepatocytes from livers 1-4 were cultured on 6 well collagen coated plates and exposed to ribavirin diluted in culture medium at a concentration of 3μg/ml for 24 hours. Quantification of ribavirin uptake by cells was based on the equation derived from the calibration curve (Figure 5.5). Value of x (unknown) was calculated by taking a ratio of the area of ribavirin with that of the internal standard (y) and using the equation derived from the calibration curve. The resulting figure gives ribavirin concentration (μg/ml) which is then divided by the total protein content (mg/ml) in each sample to normalise for cell number. The protein levels were used as a control for discrepancies arising as a result of variable cell count and were found to be equivalent between triplicate wells. As shown in Figure 5.9(A), liver 1, 2 and 4 showed a progressive increase in drug uptake over a period of 24 hours whereas this was less evident in liver 3. At 24 hours, Liver 4 had the highest uptake of ribavirin whereas liver 3 had the least.

ENT1 expression was estimated by real time PCR. RNA extraction and reverse transcriptase PCR was done as described in sections 2.4 and 2.5. Gene specific primers were designed with the help of Primer 3 programme and blasted against the known sequences present in the database to check for specificity. Primer sequences are given in Table 2.2 while method for real time PCR is described in section 2.6. Gene expression levels were measured by Maxpro software (Stratagene) by deriving a standard curve and normalised to a house keeping gene (HPRT). Figure 5.7 shows representative real time PCR plots for

both ENT1 and HPRT. The standard curve shows almost 100% efficiency of the PCR reaction, indicating exponential multiplication of PCR products with every cycle. Further, the dissociation curve shows a single peak for each primer pair, confirming the absence of non-specific products or primer dimers. PCR products were also run on a 2% Agarose gel and gave single bands of the expected size as shown in Figure 5.8. Quantification of total ENT1 expression by real time PCR is shown in Figure 5.9B. Consistent with trends in ribavirin uptake shown in Figure 5.9 A, livers 1, 2, and 4 had comparable expression of ENT-1 but levels were much lower in liver 3.



ENT1



HPRT

Figure 5.7 Real time PCR plots for ENT1 and HPRT primer pairs

A typical standard curve for ENT1 (top panel) and HPRT (bottom panel). Each data point indicates amount of gene (nanograms) on the x axis plotted against cycle threshold value on y axis. Also shown are amplification plots (top right) and dissociation peaks (bottom right). All samples were done in triplicate.

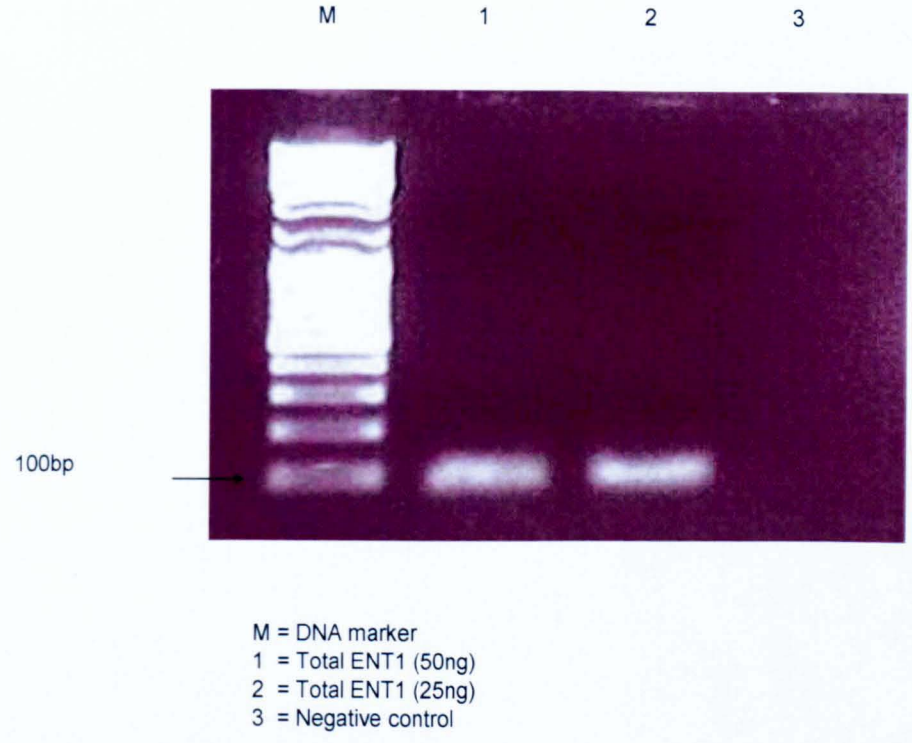


Figure 5.8 Analysis of real time PCR amplified ENT1 by agarose gel electrophoresis

50ng (lane 1) and 25 ng (lane 2) of human liver cDNA was amplified by real time PCR using primers for total ENT1. The resulting PCR reaction was analysed for the presence of a single band in order to validate specificity of the primers used in this study.

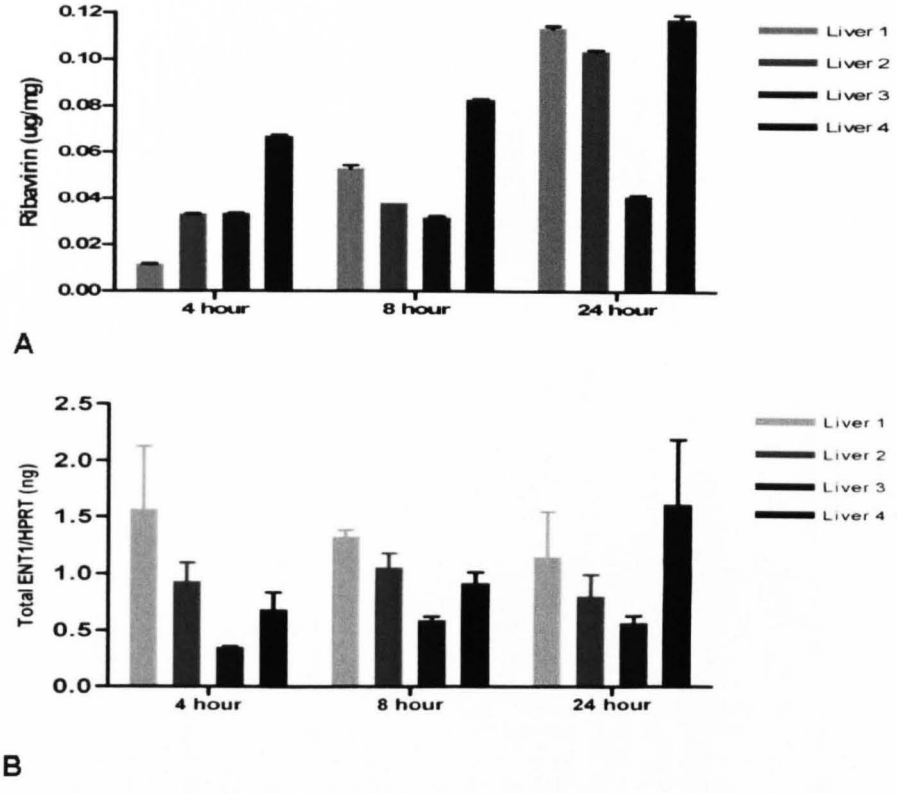


Figure 5.9 Time course for ribavirin uptake and ENT1 expression

Human hepatocytes from livers 1-4 were cultured on collagen coated plates and exposed to Ribavirin diluted in culture medium. After 4, 8 and 24 hours, cells were harvested and analysed for ribavirin uptake (A) and ENT1 expression (B). Bars indicate mean and the standard deviation is represented by error bars.

5.3.5 Correlation of ribavirin uptake and ENT1 expression in human hepatocytes (Livers 1-6) and Huh7 at 24 hours

Hepatocytes were treated with ribavirin at a final concentration of 12μM and samples were harvested at 24 hours. A concentration of 12μM ribavirin lies within the therapeutic range achieved in patients on anti-viral therapy (Kato et al., 2005, Khakoo et al., 1998). Ribavirin levels in primary hepatocytes from 6 human livers and the Huh7 cell line were analysed and correlated with total ENT1 expression. As illustrated in Figure 5.10, there was a greater than threefold variation in the levels of ENT1 expression in the six human livers. Further, this variation was associated with a similar order of difference in ribavirin uptake. Thus, liver 4 had the highest total ENT1 expression and the highest drug uptake, whereas livers 3 and 6 had lower levels of receptor expression and proportionately lower levels of ribavirin uptake. Liver 5 is an apparent outlier as, despite having higher uptake of drug than liver 2, liver 5 had 1/3rd less receptor expression. Extremely interestingly, Huh7 cells did not detectably take up ribavirin despite expressing levels of ENT1 higher than all but one of the primary hepatocytes tested. A non-parametric Spearman correlation test (r_s) was done to assess any relationship between the two variables (A value of $r_s = 1$ indicates perfect correlation and $r_s = 0$ indicates that two variables do not vary together. p value less than 0.05 was considered to be significant). The correlation test gave a value of r_s equal to 0.94 and a p value of 0.01 showing that there is a strong positive correlation between ribavirin uptake and ENT1 receptor expression.

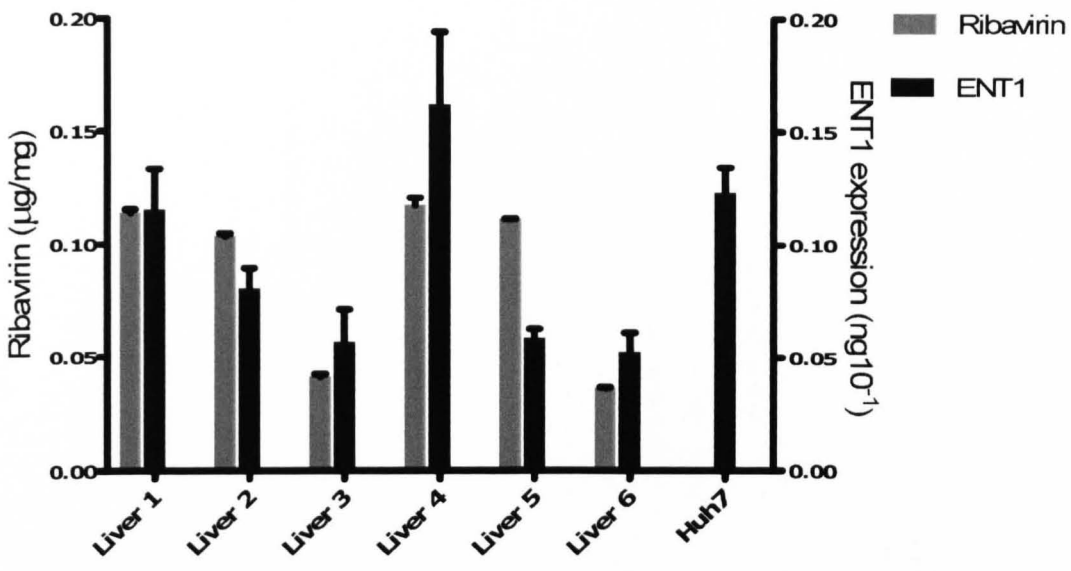


Figure 5.10 Correlation of ribavirin uptake and ENT1 expression

Human hepatocytes from livers 1-6 and Huh7 cells were exposed to ribavirin and drug uptake and ENT1 expression levels were quantified at 24 hour. Ribavirin concentration plotted on the left y-axis indicates µg of drug over mg of total proteins in the sample. Total ENT1 plotted on the right y-axis is the quantity in ng and is normalised to the house keeping gene HPRT. Bar graphs indicate the mean of three repeat experiments whereas error bars denote standard deviation.

5.3.6 Analysis of Huh7 ENT1 (SLC29A1) gene sequence

The lack of ribavirin uptake by the Huh7 cell line despite relatively high levels of ENT1 expression suggests either that the receptor itself is non-functional, or that other factors required for ribavirin uptake are not present in this cell line. In order to screen for the presence of any mutation, the Huh7 ENT1 coding sequence was analysed by cloning into pCR® 2.1-TOPO® TA cloning vector/kit (Invitrogen) followed by direct sequencing. For this purpose, gene specific primers were designed and blast analysis of the primer pair was done to confirm specificity. Gradient PCR followed by agarose gel electrophoresis was performed to check efficiency and optimal annealing temperature. As shown in Figure 5.11 (A), there was a single product of the right size (1.3kb) without any non-specific bands. The resulting PCR product was filtered by column purification and used for cloning with the TOPO TA cloning kit (invitrogen) as elaborated in section 2.13. Plasmid analysis by direct sequencing revealed the SLC29A1 gene sequence in Huh7 cells which was then blasted against the wild type sequence (Genebank). It was found that the ENT1 gene expressed by the Huh7 cell line has the same coding sequence as the wild type. A representative chromatogram is shown in Figure 5.11 (C).

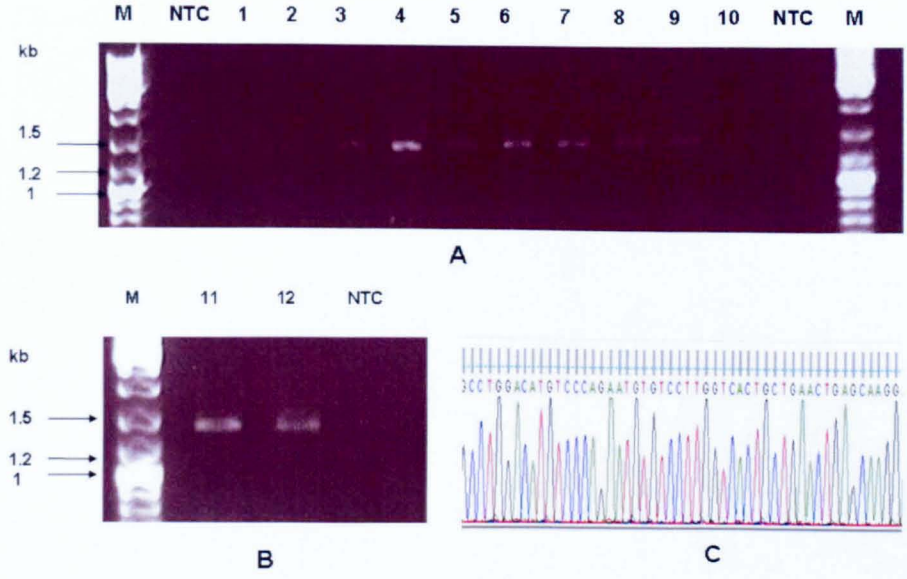


Figure 5.11 Agarose gel analysis of PCR product

The ENT1 coding sequence was amplified using specific primers and analysed by agarose gel electrophoresis. A) Gradient PCR to select optimal annealing temperature (lane 1-10 and temperature range 60±10°C). B) The temperature with the highest amplification was used for PCR amplification and the resulting product was purified for cloning (lane 11 & 12) followed by sequencing (C). M = DNA marker, NTC = non-template control

5.3.7 Effect of interferon-alpha on ribavirin uptake and ENT1 Expression

In clinical practice, ribavirin is used in conjunction with interferon alpha 2a for the treatment of HCV infection. This combination significantly improves the treatment response rate from either of these agents administered alone. We therefore aimed to examine whether interferon modulates ENT1 expression and ribavirin uptake in two sets of primary human hepatocytes. We found that in the case of liver 5, addition of interferon did result in a modest up-regulation of ENT1 expression but this was not associated with increased ribavirin uptake (Figure 5.12). In the case of liver 6, the addition of interferon did not modulate either ribavirin uptake or ENT1 expression.

5.3.8 Effect of culture conditions on ribavirin uptake and ENT1 expression

It is conceivable that the expression of ENT-1 may be modulated by the polarisation status of the hepatocytes, and that this may vary with culture conditions, particularly whether or not cells are in spheroids or 2D cultures. It follows that the variable degree of spheroid formation alone may simply be responsible for the variation of ribavirin uptake seen in different livers. In order to exclude this, we next wanted to see if using different culture systems could potentiate or knock down receptor expression and drug uptake. In our system, we found that culturing cells either on tissue culture plastic or collagen coated plates did not affect ribavirin uptake as seen in Figure 5.13.

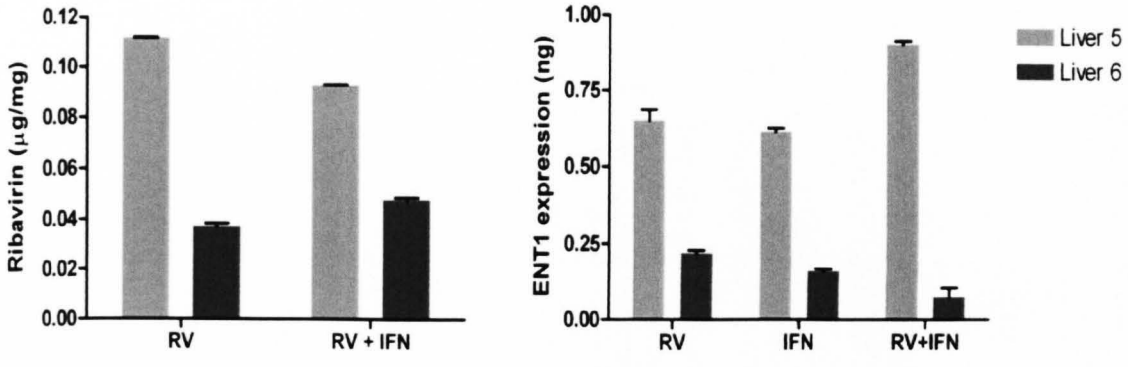


Figure 5.12 Effect of interferon alpha on ribavirin uptake and ENT1 expression

Hepatocytes from human livers 5 and 6 were cultured on collagen coated plates. Ribavirin and interferon alpha 2 either alone or in combination were added to the culture medium and cells harvested at 24 hours. Ribavirin quantity (A) and ENT1 expression levels (B) were compared in the presence and absence of interferon. (RV = ribavirin, IFN = interferon). Bar graphs indicate an average of three repeat experiments whereas error bars denote standard deviation.

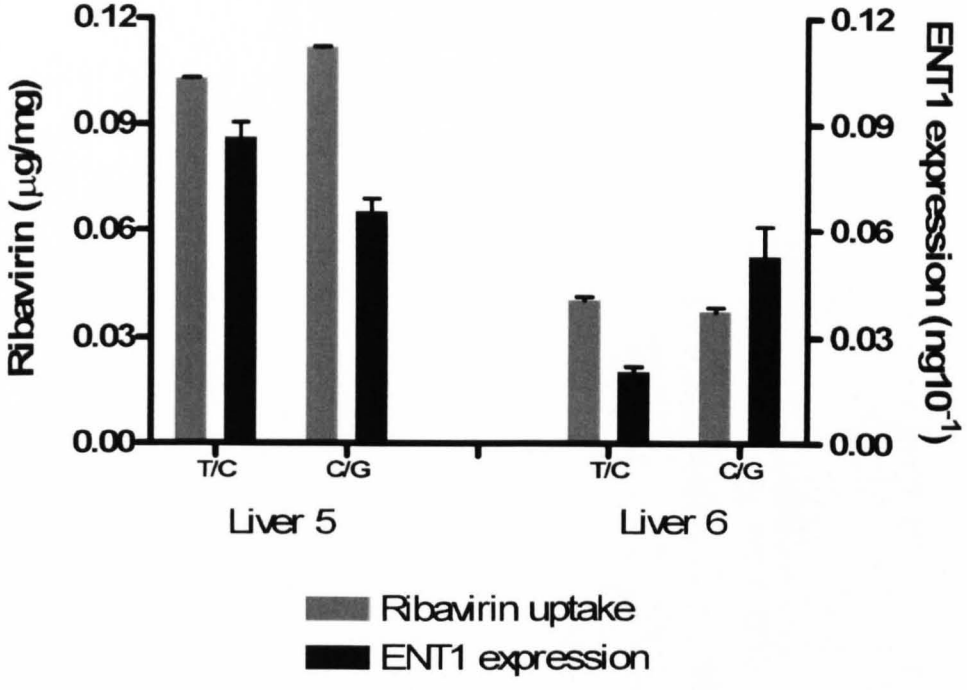


Figure 5.13 Effect of culture condition on transporter expression

Hepatocytes from human livers 5 and 6 were cultured either on tissue culture plastic or on collagen coated plates. Ribavirin quantity and ENT1 expression were measured after 24 hours and compared between cells grown on the two culture surfaces (T/C = tissue culture surface, C/G = collagen coated surface). Bar graphs indicate an average of three repeat experiments whereas error bars denote standard deviation.

5.4 Discussion

Studies have shown that the anti-HCV effects of ribavirin are dose related and response to treatment can be improved by increasing ribavirin delivery to hepatocytes (Levy et al., 2006). Of the main nucleoside transporters, ENT1 has been identified as a major ribavirin transporter (Govindarajan et al., 2008, Jarvis et al., 1998). Using a model system based on polio virus, Ibarra and Pfeiffer demonstrated that in all the cell lines tested, ribavirin uptake was mainly mediated by ENT1 and resistance to ribavirin can be overcome by over expression of ENT1 (Ibarra and Pfeiffer, 2009). A recent study using cryopreserved human hepatocytes suggested that ENT1 may be the major transporter involved in ribavirin uptake and out of the three hepatocyte lines tested, one having the highest ENT1 expression had the greatest drug uptake (Fukuchi et al., 2010). However, none of the previous studies have explored expression of nucleoside transporters by sets of primary human hepatocytes isolated from human donors and cultured *in vitro* and their correlation with ribavirin uptake.

Human hepatocytes are the most suitable tool to study function of cells inside the human body (Jigorel et al., 2005). In this study, we have successfully isolated and cultured human hepatocytes validating a method described previously (Gottschalg et al., 2006). Good cell viability was achieved and cultured cells maintained hepatocyte-like phenotype during the course of therapy. As shown in Figure 5.4(A), we isolated a highly homogenous population of healthy hepatocytes with no evidence of significant numbers of contaminating cells. We can therefore be confident that the differences in

ribavirin uptake among livers 1-6 are hepatocyte specific and not due to contamination and uptake by non-parenchymal cell types. Time course experiments demonstrated that hepatocytes continued to take up ribavirin, which itself is evidence that transport systems are intact in this *in vitro* culture model. Our principal finding is that ENT1 expression varies by up to threefold in hepatocytes obtained from different donors and that there is a highly significant correlation between ribavirin uptake and the level of ENT1. We anticipate that this relationship may be very important *in vivo* where even a moderate increase in drug dosage correlates to better treatment outcome. Thus levels of ENT1 expression may be an important predictor of treatment response in patients receiving combination therapy for HCV infection. In support of this concept, a recent study by Fujita et al implied that low ENT1 expression levels is a predictor of poor response to Gemcitabine (a pyrimidine nucleoside analogue) in patients with pancreatic ductal carcinoma (Fujita et al., 2010).

In order to further explore mechanisms for the dramatic improvement in outcomes of combination therapy for HCV infection, we wished to see if the addition of interferon enhanced ribavirin uptake by an effect on ENT1 expression. Based on data from two livers, we did not find any evidence that interferon modulated either ENT1 expression or ribavirin uptake, suggesting that it is an unlikely mode of synergism between the two drugs.

Within the liver, hepatocytes are arranged in a three dimensional framework in complex relationship with non-parenchymal cells, sinusoidal spaces and extra cellular matrix components (Figure 5.3). This arrangement maintains

hepatocytes in a polarised state which is lost in most culture models and this could be a factor determining transporter expression and function. In contrast to growth on tissue culture plastic, hepatocytes cultured on collagen coated surfaces form spheroids (Figure 5.3) which maintain the polarised state (Thomas et al., 2006). To investigate whether differences in ENT1 expression and ribavirin uptake could simply be a consequence of differences in the proportion of hepatocytes within spheroids, we compared ENT1 expression and ribavirin uptake in hepatocytes from the same donor cultured on either tissue culture plastic or on collagen coated plates. No differences were observed in either transporter expression or drug uptake by primary hepatocytes in these different models. This indicated that differences in uptake levels are unlikely to be a direct consequence of the polarisation status of hepatocytes used in this study, but due to intrinsic variation in the levels of transporter expression.

A fascinating observation in this study was that the Huh7 cell line, despite having ENT1 expression levels which were at least as high as the majority of the livers tested, failed to take up any ribavirin. This interesting observation opens up new avenues to understand whether ENT1 expression is sufficient for ribavirin uptake. Gene mutations could lead to defects in transporter function resulting in poor uptake of nucleosides and their analogues. Cytarabine (Ara-C) is a pyrimidine nucleoside analogue and is used for the treatment of acute myeloid leukaemia. A study by Zimmerman et al suggested that mutation in Glycine 24 in TMD 1 of human ENT, a highly conserved amino acid among different ENT isoforms including ENT1 and ENT2, abolished transporter

function without affecting gene expression and its plasma membrane localisation (Zimmerman et al., 2009). This could explain resistance to Ara-C therapy in leukaemia patients. It was therefore important to explore the ENT1 gene for the presence of any mutations which may provide an explanation for defect in ribavirin uptake by Huh7. Sequencing results, however, indicated that the ENT1 gene had the wild type sequence in Huh7 cells and there was no mutation in the coding sequence. This might suggest that ENT1 is necessary but may not be sufficient to mediate ribavirin uptake alone. On the other hand, factors affecting mRNA stability, promoter function and post-translational modification may play a role which needs further investigation.

Alternative mRNA processing leads to formation of various splice variants which may or may not code for the same protein. A number of splice variants have been reported for the ENT1 gene. The study by Furihata et al also explored ENT1 promotor region and showed that four different promoters give rise to at least 12 different ENT1 isoforms (Fukuchi et al., 2010). They also showed that expression levels of d1-d4 isoforms were higher in hepatocytes having higher drug uptake. However the primers used in the Furihata study were in fact non-specific and picked up ENT1 variants other than d1/d3 which they claimed were responsible for higher drug uptake. In view of the complexity of the splice variants of ENT1, we elected to exploit the relationship between ENT1 and ribavirin uptake using a primer pair which captures all variants. It may be interesting in the future to explore presence of ENT1 splice variants in primary human hepatocytes and Huh7 cells.

In summary, this study has successfully achieved:

1. Validation of the method for human hepatocytes isolation

2. Culture of human hepatocytes *in vitro*

3. Development of robust HPLC methodology for the measurement of ribavirin

4. Development of quantitative methodology for measurement of ENT1 gene expression

5. Demonstration of a highly significant correlation between levels of ENT1 expression and ribavirin uptake

6. Demonstration that interferon does not modulate ENT1 expression levels

7. TheHuh7 cell line does not avidly take up ribavirin despite expressing wild type ENT1.

Overall, our observations suggest that intrinsic differences in ENT1 expression may determine uptake of ribavirin and have important consequences for the outcomes of current antiviral regimens for hepatitis C infection.

Chapter Six

6 Final discussion

6.1 Project summary

HCV infection is highly prevalent world wide and leads to life threatening liver diseases including cirrhosis and hepatocellular carcinoma. HCV is already the most common cause of liver transplantation in the USA and is a major and growing health burden in the UK. Despite its importance, present anti-viral drugs have inadequate and unpredictable therapeutic potency. Additionally, current therapies are associated with a wide range of side effects, and anaemia as a consequence of extra hepatic uptake of ribavirin is an important barrier to completion of therapy and viral eradication. It is therefore extremely important to study and devise measures to overcome these limitations. This could be achieved by both improving efficacy and reducing side effects of currently available drugs and identifying novel treatment targets. Furthermore, the highly variable response rate to current HCV drugs warrants independent investigation. The principal aims and outcomes of this study, set within the current framework of understanding of HCV, are illustrated below.

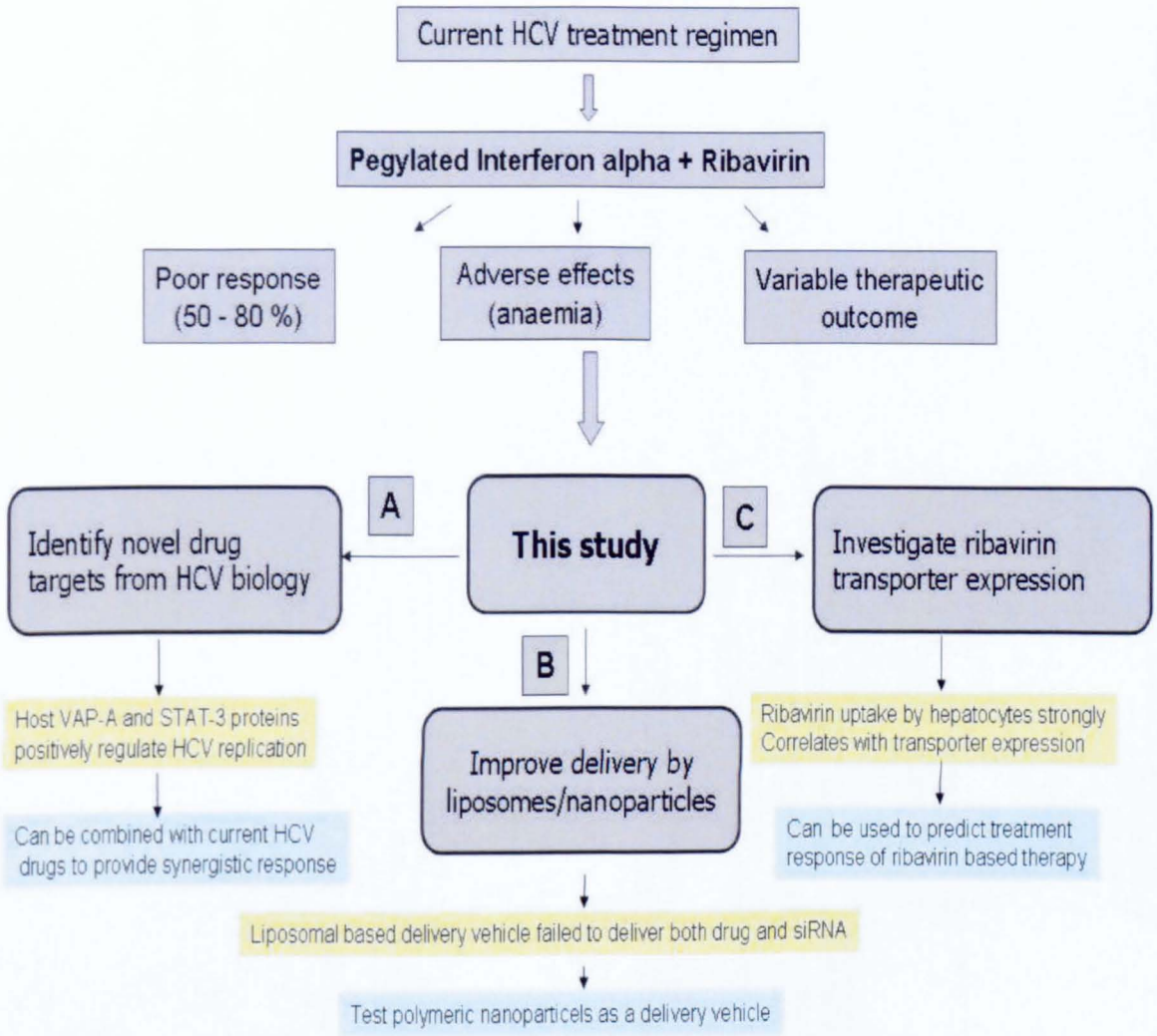


Figure 6.1 Project overview with principal findings

Yellow filled areas specify important findings and blue filled areas indicate future implication.

6.2 Cardinal findings and future implications of the present study:

6.2.1 Role of host protein in viral replication

Previous studies have highlighted the importance of host genes involved in HCV replication (discussed in chapter 3). Using siRNA to dissect the virus-host relationship is independently informative and provides a potential tool for inhibiting viral replication. The first challenge was to develop a subgenomic replicon model system to study the effects of our interventions on viral replication. Although this system is not new to people working in this field, it required considerable optimisations before an efficient replication system was established in our hands. We found that quality of *in vitro* transcribed JFH1 RNA was the key factor in determining outcome.

Several transfection methods are in use for *in vitro* delivery of nucleic acids. Most of these lipid based transfection reagents cause significant cell toxicity and have an unpredictable outcome. Additionally these methods are not suitable for delivering large molecules such as viral RNA. To overcome these issues, we used nucleofection as a transfection method with the aim of obtaining consistent and reproducible results for the co-transfection of both siRNA and viral RNA. We successfully developed a protocol for co-transfection of replicon RNA and gene specific duplexes. In this system, VAP-A and STAT-3 proteins were found to positively regulate viral replication as specifically knocking down these genes resulted in significant reduction in viral replication. It was noted that the reduction in viral replication achieved

after knock down of both proteins, although statistically significant, was less than 1 log reduction. An effective therapeutic intervention for HCV would normally be expected to result in a reduction in replication of several logs. This factor may be less significant for the host genes we targeted as these interventions are likely to be used as an adjuvant to current therapy to enhance their therapeutic potency. Moreover, long term therapy may produce a more robust and sustained inhibition of viral replication. We were unable to validate our findings using a full length infectious virus as we did not have access to a category III facilities during the course of this study. However, the recent establishment of this resource, allowing us to use an infectious replicon system producing viral particles will be invaluable and will provide a more meaningful interrogation of the effects of these proteins on the full viral life cycle and infectability. This will not only help to validate currently identified targets in this or previous studies but will also enable screening for other proteins that will give us a deeper insight into the mechanism of virus replication and help in identification of novel anti-viral targets. An important practical requisite for using duplexes as therapeutic tools is the development of an efficient delivery vehicle. We therefore next wanted to assess feasibility of liposomal nanoparticles as carrier molecules.

6.2.2 Liposomal nanoparticles as delivery vehicles

Advances in nanotechnology have improved delivery of various therapeutic and imaging agents (Torchilin, 2007). This is particularly relevant for diseases like HCV affecting liver (Harivardhan Reddy and Couvreur, 2011), where targeting of therapeutic molecule to hepatic tissue is an important requisite. Testing liposomal formulations suitable for human use was the principal goal

of this project. It was done as part of collaboration with our industry partner (Lipoxen PLC ltd) to develop projects in translational research which could potentially have a great influence in the field of medicine. We note that, in addition to hepatitis C, other liver diseases like hepatic fibrosis and carcinoma will be optimally treated by a methodology which enhances the targeted accumulation of drug at the desired location with a consequent reduction in side effects.

The results presented and discussed in chapter 4, however, demonstrate that the liposomal formulations containing siRNA used in this study were not effective. Initial testing suggested that siRNA containing liposomes were intrinsically unstable and unable to silence target gene expression irrespective of the liposomal formulation. In contrast, liposomal uptake and delivery of fluorescent label was subsequently boosted, both by changing the formulation's physical properties as well as modifying culture conditions. The difference between the two delivery vehicles is that fluorescent label is embedded within the lipid bilayer while siRNAs duplexes are enclosed in the hydrophilic centre. Further testing with more liposomal formulations is clearly required to develop an efficient and targeted drug delivery vehicle. This could be achieved by devising pH-sensitive liposomes which release their contents only when exposed to the appropriate pH. Previous reports have shown the feasibility and efficacy of pH-sensitive liposome for intra-cellular delivery of drugs to a specific cellular compartment like the endoplasmic reticulum (Ducat et al., 2011, Düzgüneş and Nir, 1999). The practicability of such an approach for delivery of anti-virals needs to be further assessed. In contrast, our in-house

prepared polymeric nanoparticles were taken up effectively when exposed to cells. Their physical properties, such as charge and size were comparable to the liposomal preparations and such particles have potential as delivery vehicles for therapeutic interventions. Unlike liposomes, however, polymer based nanoparticles are untested in human trials and are therefore at a very early stage in development as clinical tools.

6.2.3 Equilibrative nucleoside transporter 1 expression regulates ribavirin uptake by primary human hepatocytes

Ribavirin addition to interferon alpha based anti-HCV regimens was an important breakthrough as it greatly improved the otherwise poor response rate associated with interferon monotherapy. Despite this advance, a significant proportion of treated individuals fail to respond. It is therefore important to identify the factors responsible for the limited cure rate in certain individuals. Being a nucleoside analogue, ribavirin requires a specific portal system as the hydrophilic nature of the drug prevents free penetration through the cell membrane. Of all the transporters, ENT1 has been identified as the likely candidate ribavirin transporter. We therefore aimed to assess the relationship between ENT1 expression and ribavirin uptake using an *in vitro* culture system based on primary human hepatocytes. The results obtained in this study suggest that ENT1 expression varies up to threefold in primary hepatocytes derived from different sets of human livers and these inherent differences may determine ribavirin uptake. Our data strongly suggest that ENT1 is the primary

ribavirin transporter but this requires further interrogation *in vivo*. It may also be useful to explore various ENT1 isoforms in our patient populations and identify their contribution to ribavirin uptake. On the basis of these observations, we next planned to interrogate the correlation of ENT1 single nucleotide polymorphisms and SVR rates in our patient populations. Further, it would be interesting to examine ENT1 expression on PBMCs as a surrogate marker of hepatocyte expression.

Another interesting finding of our study was that Huh7 cells, despite expressing ENT1 to levels comparable to most of the liver samples, failed to take up any detectable ribavirin. Sequencing analysis of the ENT1 coding sequence in Huh7 cells failed to show any mutation, suggesting a role for other factors in accounting for this discrepancy. It is known that hENT1 protein is mainly localised to the cell membrane with some evidence of its presence on the mitochondrial membrane (Lai et al., 2004). However, regulatory signals underlying processing, membrane targeting or activity of transporter protein are not understood completely. For a membrane protein like ENT1, correct folding as well as trafficking to plasma membrane is the key for transporter function and specific motifs in the N- and/or C- terminal of the protein have been shown to play a regulatory role in this process (Loo et al., 2005, Williamson et al., 2008). A study by Nivillac *et al* identified essential motifs in both the N- and C- terminals of human ENT1 and showed that loss of these sequences prevents the plasma membrane localisation of the transporter and ER retention respectively (Nivillac et al., 2009). This suggests that factors

responsible for proper protein folding and membrane localisation may be deficient in Huh7 cells and needs further investigation.

6.3 Future work

The outlook for anti-HCV therapy has been entirely transformed during the last three years since the start of this study. Directly acting anti-viral agents, such as protease inhibitors (Telaprevir and Boceprevir) have now entered clinical practice and are standard of care for the treatment of HCV genotype 1 infection (Chapter 1). Additionally, other drugs like NS5A and polymerase inhibitors have also shown very high efficacy in recent clinical trials. PSI-7977, for example, is a highly potent uridine nucleotide analogue polymerase inhibitor. It has been shown to be associated with 100% SVR in genotype 1 infection, when used as combination therapy with pegylated interferon alpha 2a and ribavirin for 12 weeks. Recent data presented in AASLD (November, 2011) assessed optimal duration of interferon addition when PSI-7977 was used with ribavirin for 12 weeks (Edward J. Gane et al., 2011). Remarkably, 12 weeks of combination therapy with PSI-7977 was associated with 100% SVR across viral genotypes 1-3, as assessed in robust Phase 2B trials. With the advent of these and other DAA based novel drug targets, the possibility of orally administered, interferon free, treatment regimens with 100% efficacy appears realistic and will transform the treatment algorithms for HCV infection. Nonetheless, these projected therapies all require a ribavirin backbone and are associated with an even more profound haemolytic anaemia than standard interferon and ribavirin. Moreover, the new therapies themselves also cause side effects, so the concept of using an efficient carrier molecule to specifically deliver drugs to hepatocytes remains valid. Further, the capacity to understand

factors responsible for variable ribavirin uptake could contribute to personalisation of drug regimens, and markers of ENT1 expression may inform cost effective strategies for the administration of ribavirin.

6.4 Conclusion

In summary, the important observations/achievements presented in this thesis are:

- ✓ Establishment of an efficient subgenomic replicon system supporting HCV replication. It will be useful for screening other host genes required for viral replication and, once a suitable delivery vehicle is identified, testing anti-viral efficacy of these agents.
- ✓ Confirmation of VAP-A and STAT-3 as positive regulators of viral replication. Using specific drug delivery vehicle, effects of combination of ribavirin with these targets need to be assessed.
- ✓ That liposomes used in this study are not suitable delivery vehicles. Further testing with either new liposomal preparation or polymer based nanoparticles is required.
- ✓ Validation of an efficient method for isolation and culture of primary human hepatocytes. It can be used for any future studies requiring primary human cells, either hepatocytes or non-parenchymal cells like hepatic stellate cells.
- ✓ Ribavirin uptake in primary human hepatocytes strongly correlates with ENT1 expression. Relationship between SNP in ENT1 gene in patients need to be assessed with responsiveness of ribavirin based regimens. Additionally various ENT1 isoforms need to be explored and correlated with ribavirin uptake.

Chapter Seven

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Chapter Eight

8 Appendix

8.1 Cell culture solutions

A. Solution required for isolation of primary human hepatocytes

1. 10x Hanks-HEPES buffer

NaCl (, Fisher Scientific)	1.37 M
KCl (Sigma)	54 mM
KH_2PO_4 (Sigma)	4.4 mM
$Na_2HPO_4.12H_2O$ (Fluka)	3.6 mM
HEPES Sigma)	200 mM
NaOH (Sigma)	100 mM
Sterile distilled water	1000 ml

- Syringe filtered and stored at 4°C
- Diluted with autoclaved/sterile distilled water to make up 1x

2. EGTA solution (25mM)

EGTA (Sigma, UK)	0.48 g
Sterile 1x Hanks HEPES	25 ml
(no bicarbonate/glucose)	
NaOH 1M (Sigma, UK)	2.5 ml

- Made up to 50ml with sterile distilled water
- Syringe filtered and aliquots stored at 4°C.

3) Calcium choloride (CaCl2) solution (250mM)

$CaCl_2$ (Fluka)	1.84 g
Sterile distilled water	50 ml

- Syringe filtered and stored at 4°C.

4) Bicarbonate/glucose solution

NaHCO3 (Sigma)	0.74 M
D-(+)-Glucose (Sigma)	0.28 M
DL-Methionine (Sigma)	0.1 M
Sterile distilled water	50 ml

- Syringe filtered and stored in aliquots at -20°C.

5) 90% Percoll

Percoll (Sigma)	90 ml
HBSS (10×) (GIBCO)	10 ml

- Syringe filtered and stored at 4°C

6) Diluted Soltran solution for transport and flushing of tissue

Soltran (Baxter)	500 ml
Sterile distilled water	300 ml

B. Isolation and growth media

7) Primary human hepatocytes isolation medium

Ingredient	Volume	Final concentration
Dulbecco's modified Eagles Medium (DMEM) (GibcoBRL, UK)	500 ml	N/A
L-glutamine (GIBCO)	5 ml	2 mM
Antibiotic (Hyclone Thermo Scientific)	5 ml	100 U penicillin 100 ug streptomycin
Foetal calf serum (Sigma, UK)	50 ml	10 %

8) Primary human hepatocytes plating medium

Ingredient	Volume	Final concentration
Williams medium E (GibcoBRL, UK)	500 ml	N/A
Antibiotic (Hyclone Thermo Scientific)	5 ml	100 U penicillin 100 µg streptomycin
Insulin solution (Sigma, UK)	2 µl	0.1 µM
Dexamethasone (Sigma, UK)	50 µl	0.1 µM
L-glutamine (GIBCO)	5 ml	2 mM

9) Primary human hepatocytes culture medium

Ingredient	Volume	Final concentration
Dulbecco's modified Eagles Medium (DMEM) (GibcoBRL, UK)	500 ml	N/A
Antibiotics (Hyclone Thermo Scientific)	5 ml	100 U penicillin 100 µg streptomycin
Insulin solution (Sigma, UK)	2 µl	0.1 µM
Dexamethasone (Sigma, UK)	500 µl	1 µM
Foetal calf serum (Sigma, UK)	25 ml	5 %

10) Human hepatoma cell line (Huh7) culture medium

Ingredient	Volume	Final concentration
Dulbecco's modified Eagles Medium (DMEM) (GibcoBRL, UK)	500 ml	N/A
L-glutamine (200 mM) (GibcoBRL, UK)	5 ml	2 mM
Antibiotic/antimycotic (GibcoBRL, UK)	5 ml	100 U penicillin 100 µg streptomycin 250 ng amphotericin B
Foetal calf serum (Sigma, UK)	50 ml	10 %

C. Preparation of buffers for human liver perfusion

1) Buffer 1 (EGTA/Chelating buffer)

1X Hanks Hepes buffer	500ml/1L
Bicarbonate/glucose solution	10ml/20ml
EGTA solution	10ml/20ml

2) Buffer 2 (No-EGTA buffer)

1X Hanks Hepes buffer	500ml
Bicarbonate/glucose solution	10ml

3) Buffer 3 (Collagenase buffer)

1X Hanks Hepes buffer	500ml
Bicarbonate/glucose solution	10ml
*Collagenase enzyme	65U
*Trypsin inhibitor(250mg, NB 4G proved Grade from Clostridium histolyticum, 0.263 U/mg)	80mg

- *Added only prior to use.

8.2 SDS Polyacrylamide gel electrophoresis

A) Solutions:

- Filtered all solutions and used the free base forms of Tris and glycine when making up solutions. HCL was used to adjust pH of Tris solutions.
- 4x Gel Running Buffer (1.5M Tris-Cl, pH 8.8)

9.0g of Tris in 50ml of dH_2O (pH 8.8). Stored up to 3 months at 4°C in dark.

- 4x Gel Stacking Buffer (0.5M Tris-Cl, pH 6.8)

3.0g of Tris in 50ml of dH_2O (pH 6.8). Stored up to 3 months at 4°C in dark.

- 10% SDS

1g of SDS to 10ml of dH_2O. Stored up to 6 months at room temperature.

- 2x Sample Buffer (100mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol (BME), 15% glycerol, Bromophenol Blue to colour)

Stored in a tightly sealed container to prevent the BME from going off.

- Tris-glycine electrophoresis buffer (25mM Tris base, 192mM glycine, 0.1% SDS, pH 8.3)

A 5x stock solution was prepared in 500ml by adding: 7.56g Tris, 36g glycine, 2.5g SDS and dH_2O to 500ml. Its pH is around 8.2-8.4 and is Stored at room temperature.

- Transfer buffer: Prepare 1x solution of 10X TGS bugger with 20% Methanol in dH_20.

▪ 10X TGS buffer	50ml
▪ 20% ethanol	100ml
▪ water	350 ml

B) Preparation of the SDS polyacrylamide gel:

- Plates were cleaned with mild detergent and soft cloth and loaded onto mini gel apparatus (Biorad). Monomer solution for the running gel was prepared as per table below and was poured in the glass plate frame. Gel was allowed to polymerise at room temperature.

Final gel concentration (1 0ml, 2 each, 0.75mm thick SE250 gels)

	10%
Monomer solution (gel)	*3.3ml*
4x Running Gel Buffer (1.5M Tris-HCL PH 8.8)	*2.5ml*
10% SDS	*0.1ml*
dH2O	*4 ml*
*APS**	*50 µl*
TEMED	*3.3µl*

* 10% APS should be prepared first

- While the gel is polymerising, prepare the stacking gel as per table below.

	0.75mm
Monomer solution	*0.44ml*
4x Stacking Gel Buffer(0.5M Tris-HCl Ph 6.5)	*0.83ml*
10% SDS	*33µl*
dH2O	*2.03ml*
APS	*16.7µl*
TEMED	*1.7µl*

- Insert the gel into electrophoresis unit with the larger thicker plate to the outside. If not running two gels insert the clear plastic plate against the gasket and close the tabs. Mark the location of the well by a permanent marker. Add tris-glycine electrophoresis buffer (*10% Tris-glycine buffer*). Remove the comb carefully. Make 4X loading buffer by adding *95µl of loading buffer + 5µl β- mercaptoethanol.*

- Prepare the protein samples by adding *5µl of loading buffer + 15µl sample*, then heat at 90°C for 5 minutes, and then keep in ice for 5 minutes.
- Load the samples onto the gel, and run the gel at *30mA per gel* for 60-90 minutes. Electrophoresis is complete when the dye reaches the bottom of the gel. Remaining procedure of western blotting is given in materials and methods.

8.3 Solution and buffers used for HPLC:

1) Ammonium Phosphate (NH4)H2PO4) Buffer, 250mM

Ammonium phosphate (Sigma)	7.2g
Distilled water	250ml

- pH adjusted to 8.5 using 10M sodium hydroxide and filtered through 0.2 µm nylon filter membrane before use.

2) Mobile phase (Ammonium Phosphate (NH4)H2PO4) Buffer, 10mM

Ammonium phosphate (Sigma)	1.15g
Distilled water	1L

- pH adjusted to 6.5 using 10M sodium hydroxide and filtered through 0.2 µm nylon filter membrane before use.

3) 2.5% v/v formic acid in methanol

Formic acid (Sigma)	2.5ml
Methanol (Sigma)	97.5ml

- Stored at room temperature.