COMBATING HCV RECURRENCE:

THE PAST, PRESENT AND FUTURE OF ANTI-VIRAL WARFARE

SCOT HENRY

INVITATION
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OF THE PHD THESIS

COMBATING HCV RECURRENCE:
THE PAST, PRESENT AND FUTURE
OF
ANTI-VIRAL WARFARE

OF
SCOT HENRY

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COLLEGEZAAL 7 OF THE
ERASMUS MC
'S-GRAVENDIJKWAL 230
IN ROTTERDAM

PARANIMFEN:
TERRY HENRY
LEONIE BOVEN
LA.BOVEN@MEANDERMC.NL

RECEPTION TO FOLLOW

SCOT HENRY
UNGERPLEIN 8B
ROTTERDAM
0643203006
S.HENRY@ERASMUSMC.NL

1. Steroids have no direct effect on the replication of HCV *in vitro*. (This thesis)

2. Mycophenolic Acid reduces the ability of HCV to replicate independent of guanosine depletion *in vitro.*(This thesis)

3. Lentiviral delivered simultaneous combinatorial shRNA reduces HCV replication and its entry receptors *in vitro*. (This thesis)

4. Delivery of lentiviral vectors to hepatocytes is enhanced by the hydroxyethyl starch in UW solution and facilitates hypothermic transduction during cold perfusion of a liver graft. (This thesis)

5. RNAi and Interferon therapy can be combined in the treatment of HCV without cross interference. (This thesis)

6. HCV **re**infection is a misnomer in that, post liver transplantation, HCV infects, *de novo,* the newly placed naïve liver graft, while the patient has retained the infection throughout the course of disease.

7. Mycophenolic acid's anti-HCV effect is masked by its immunosuppressive capacity *in vivo*.

8. Most anti-viral monotherapies fail due to the pre-treatment existence of a significant quasispecies population of viral mutants that confer resistance.

9. It has been said that the primary function of schools is to impart enough facts to make children stop asking questions. Some, with whom the schools do not succeed, become scientists. - Knut Schmidt-Nielson.

10. Almost always, the men who achieve these fundamental inventions of a new paradigm have been either very young or very new to the field whose paradigm they change. – Thomas S. Kuhn.

11. Any man who can drive while kissing a pretty girl is simply not giving the kiss the attention it deserves. – Albert Einstein.

These propositions are considered opposable and defendable and as such have been approved by the supervisor Prof.dr. H.W. Tilanus.
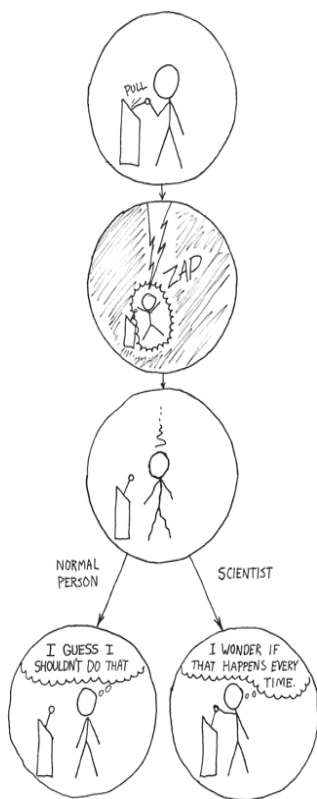
Combating HCV Recurrence:

The Past, Present and Future of Anti-Viral Warfare

Scot Henry

s.henry@erasmusmc.nl

Commission

Promoters:

Prof.dr. H.W. Tilanus

Prof.dr. H.J. Metselaar

Inner Committee:

Prof.dr. G. Wagemaker

Prof.dr. H.L.A. Janssen

Prof.dr. B. Berkhout

Copromoter:

Dr. L.J. van der Laan

I dedicate this thesis to
Jeanne and Gary Henry
my mom and dad
who always told me to
"don't be smart"

Combating HCV Recurrence:

The Past, Present and Future of Anti-Viral Warfare

De strijd tegen HCV-reïnfectie:

Verleden, heden en toekomst van antivirale oorlogsvoering

Thesis

*to obtain the degree of Doctor
from the Erasmus University Rotterdam
by command of the rector magnificus
Prof. dr. S.W.J. Lamberts
and accordance to the decision of the
Doctorate Board*

*The public defense shall be held on
Wednesday 18 of June 2008 at 9:45 hrs
by*

*Scot Dowler Henry
born in Ottawa, Ontario, Canada*

Erasmus

ERASMUS UNIVERSITEIT ROTTERDAM
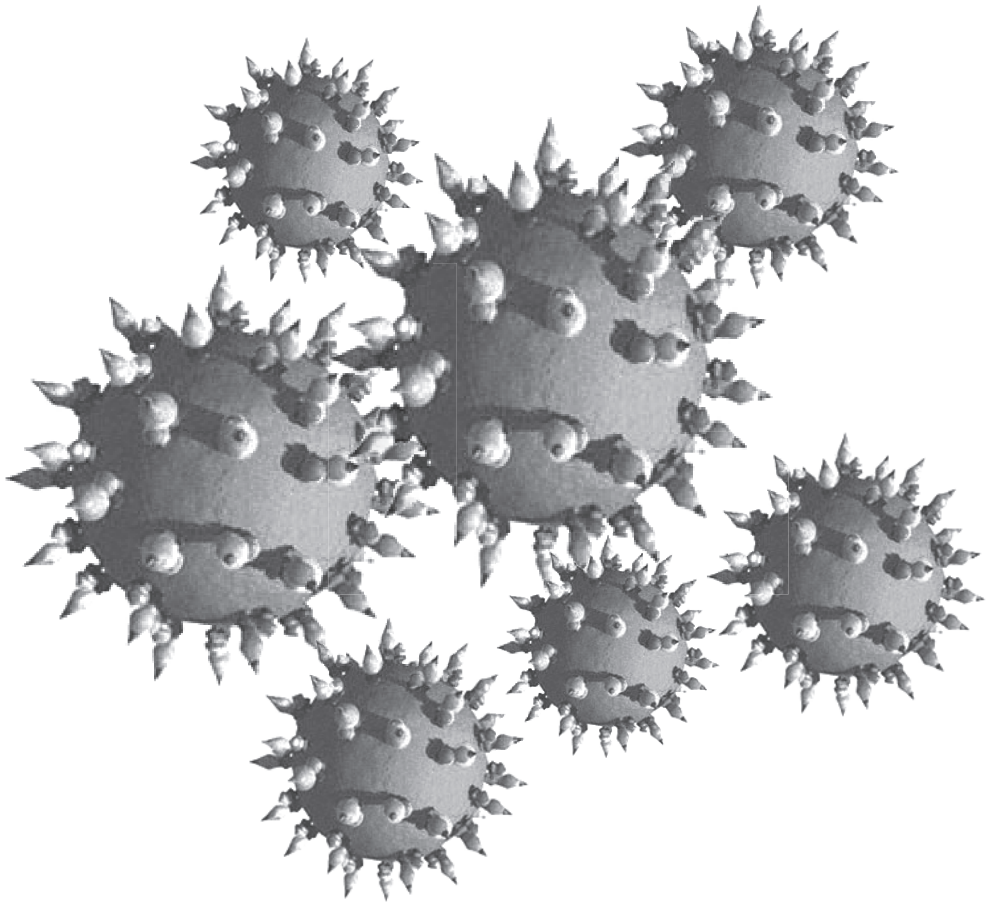
CHAPTER 1

General Introduction

Scot Henry

Contents

$\mathcal{T}$he face of medicine is changing. Advancements in every aspect of medicine have begun to accelerate. Throughout history doctors have utilized science to some degree or another but never more than in the last 50 years. Today, doctors are turning to science more and more to answer their unanswerable questions. This both increases the ability to treat and manage patients as well as provides the necessary funding in order to propel medical sciences at unprecedented speeds.

Newly discovered viruses and the complications of their infections present doctors and scientists with some of the most complex problems with regard to treatment options. In order to establish a viable therapeutic option to eradicate the virus a thorough understanding of a given virus's biology and epidemiology must be achieved. Once accomplished, focus shifts to the production of a suitable vaccine. While this helps prevent the spread of new infections, established or undetected viral infections still present a large health care puzzle that needs to be solved. Viral infections often go unnoticed until symptoms appear, long after the initial infection has occurred.

Drug development produces the first line, rapid response therapy option to new infections and disease but predominately focuses on retroactively treating the symptoms of infection, not often the source. Established infections can only be treated with lifelong drug application that are often not very efficacious in viral eradication but rather just attempt to control viral replication to slow the progression of viral related diseases. These drug therapy options often come at a heavy price, in monetary sums as well as in the patients well being and quality of life.

The Hepatitis C virus (HCV) is one of those infections, which can remain undetected for many years and is often untreatable or with low efficacy. This thesis aims to explore the development of these therapies, and to provide new information and insight about classical, current and future options to HCV therapy.

1.1 HCV

During the early 1970's Harvey J. Alter, the then Chief of Infectious Disease Section in the Department of Transfusion Medicine at the National Institutes of Health (NIH) demonstrated that another agent, a non-A non-B hepatitis virus was responsible for post transfusion hepatitis. First identified as a unique virus and published to the world in 1989 in two separate articles in Science[1, 2], the hepatitis C virus affects more than 200 million people worldwide[3] and is recognized as a major cause of chronic liver disease. Together with the hepatitis B virus (HBV), HCV now accounts for 75% of all cases of liver disease around the world.

The HCV is a small, enveloped, positive-strand RNA virus classified as the sole member of a distinct genus called hepacivirus in the family *Flaviviridae*[4], which also includes such viruses as dengue and yellow fever viruses as well as the pestviruses, such as bovine diarrhea virus[5].

The genome of HCV encompasses a single ~9600 nucleotide (nt) RNA molecule carrying one large open reading frame (ORF) that is flanked by non-translated regions (NTRs)[1, 6]. The 3000 amino acid residue precursor polyprotein created from this ORF is cleaved co- and post-translationally by cellular and viral proteinases into at least ten different products, with structural proteins; Core, E1, E2 and p7 located in the amino-terminal one-third and the non-structural replicative proteins; NS2, NS3, NS4A, NS4B, NS5A, NS5B, located in the remainder[7,8] (Figure 1).

Figure 1
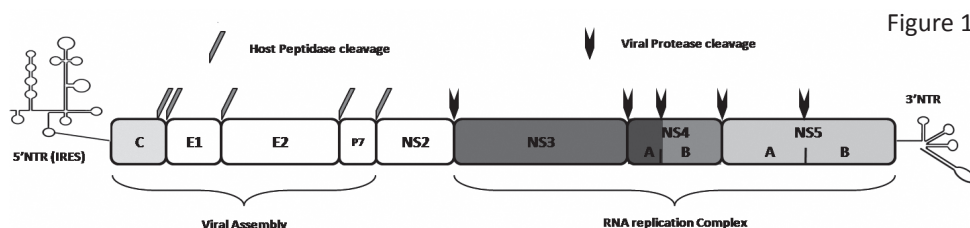
Each protein in the HCV genome is required for assembly of new infectious viral particles, though only the non-structural proteins are needed for replication of new positive strand HCV RNA. The Core protein, derived from the N-terminus of the polyprotein, likely forms the central nucleocapsid, though is probably multifunctional, being involved in not only assembly but also regulatory functions within the cell[9]. The E1 and E2 proteins are components of the viral envelope and give HCV its tropism to hepatocytes. The P7 protein, though largely unknown for most of HCV's discovery lifetime, has recently been shown to be involved in the production of infectious virions *in vivo*[10] and may belong to a small family of viroporins, which enhance membrane permeability. The non-structural proteins are involved in the formation and function of the HCV replication complex. The NS2, a transmembrane protease, is not essential for replication *in vitro*, as its removal does not hinder replication in cell cultures[11], though has been shown to be essential for completion of the viral replication cycle *in vivo*[12]. Proteins NS3 and

NS4A form a complex bifunctional molecule with both helicase and NTPase activity used for polyprotein processing and RNA replication[9]. The NS4b protein, a transmembrane protein, is believed to form the anchor of the HCV replication complex, allowing for the formation of a specialized membrane compartment where viral RNA replication takes place[13]. The remaining two proteins are thought to be essential to viral replication, though the NS5a function is still largely unknown. The NS5b is an RNA dependent RNA polymerase (RdRp), responsible for the creation of new positive strand HCV RNA from the negative strand intermediate RNA (Fig .2).

Figure 2

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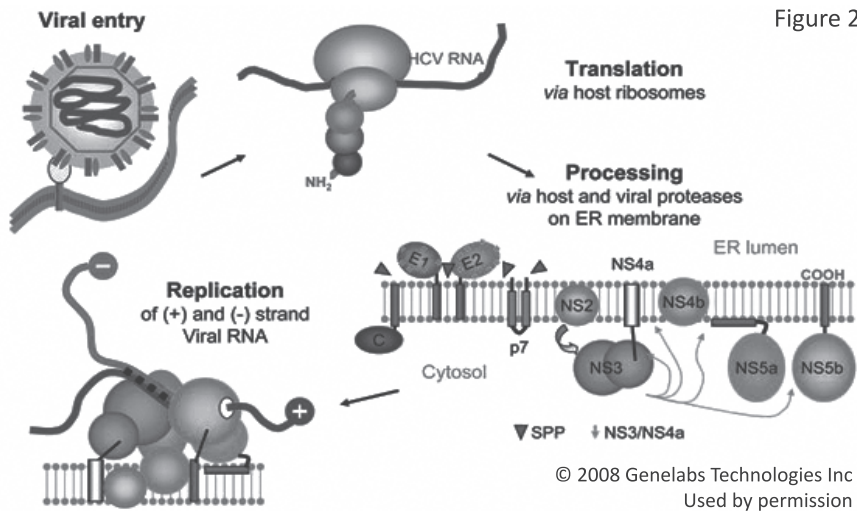
As with most RNA viruses, HCV is highly mutational[14]. The rates of mutation differ throughout the genome, with the NTRs being the most conservative and the envelope proteins E1 and E2 being the most variable. These mutations are due to the high error rate of the HCV RdRP and has lead to an evolution of viral strains to now include six principle genotypes, 1 to 6, each with their own subclasses that often circulate in infected individuals as closely related quasispecies[15]. In chronically infected patients, the presence of quasispecies relates to viral persistence, often with sub populations possessing genotypic mutants that can confer drug resistance and as such has made successful long term treatment of HCV difficult[14].

Though the initial study of hepatitis C was hindered by slow development of an effective method to culture the virus *in vitro* as well as the lack of a small animal model, recent developments have accelerated the process of discovery. These have included the development of alternative models focused towards viral binding and entry through the use of recombinant HCV envelope proteins, including virus-like particles produced by baculovirus[16], retrovirus pseudotypes (HCVpp)[17]. Hepatitis C virus predominantly infects hepatocytes, though can infect other cell types, due in part, to the recognition of envelope protein E2 by several cell surface proteins that have recently been implicated in viral entry. The first to be identified, CD81, is a widely expressed cell surface tetraspanin that was capable of binding soluble E2[19]. The

human scavenger receptor class B type 1 (SR-B1) was the second to be identified as a putative receptor for HCV[20]. Though several cells lines co-expressing both CD81 and SR-B1 were found to be nonpermisible to HCVpp[21], suggesting that others cell surface molecules were required for entry. A few other proteins have been implicated, such as the LDL receptor[22], L-SIGN[23], DC-SIGN[24], and the asialoglycoprotein receptor[25] though each still have controversial elements in their direct role in HCV entry. Claudin-1, a tight junction protein, was recently shown to be critical for HCV entry into hepatocytes and is thought to be a co-receptor acting during the late stages of viral entry[26]. The current model of HCV entry to hepatocytes suggests that HCV first associates to lipoproteins in the blood stream, which then bind to cell surface low-density lipoprotein receptors. The virus then interacts with SR-B1 and/or CD81, with Claudin-1 coming into play in the late stages of entry, before internalization in a clatherin pit[27] (Fig. 3).

HCV
LDL
Figure 3
LDLR/GAG
SR-B1
CD81
Claudin-1, 6 or 9
Clatherin Coated Pit
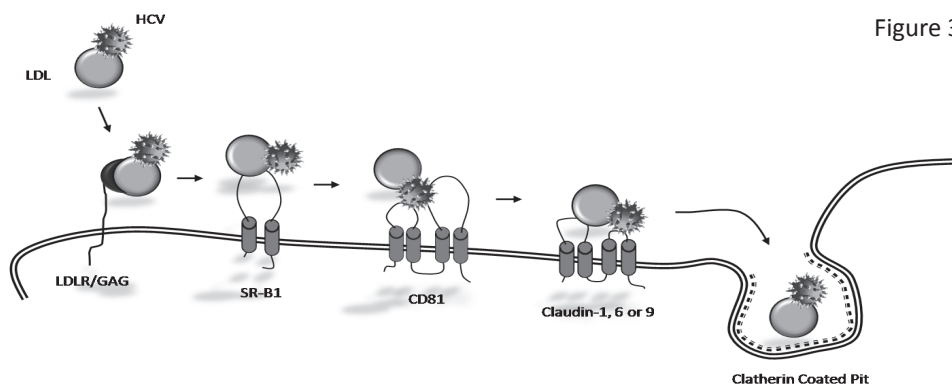
Advances in the understanding of the replication of HCV have been facilitated by the development of *in vitro* RNA replicon based model[11]. This replicon system has aided in the development of new HCV therapies, improved the development speed of antiviral drugs as well as facilitated a new level of understanding of HCV and its relationship to the host. This replication system was the experimental standard for many groups worldwide and soon spawned other groups looking to improve or create newer, more robust replicon systems. In 2005, Takaji Wakita produce a full length, infectious HCV molecule based on a clone extracted from an individual with fulminant hepatitis[18]. For the first time, the world had an infectious HCV particle that was suitable for long term *in vitro* culture or experimentation and the speed of discovery increased again.

Persons with acute HCV infection typically are either asymptomatic or have a mild clinical illness; most have no discernible symptoms; some develop jaundice; and some have nonspecific symptoms seemingly unrelated to the viral infection[28, 29]. The course of acute hepatitis C is variable, though fluctuating elevations in serum alanine aminotransferase (ALT) levels are its most characteristic feature. After acute infection, 15-25% of persons appear to resolve their infection without detectable HCV RNA in serum and a normalization of ALT levels[30]. After a period of fluctuation, normalization of ALT levels may occur and suggests full recovery, but

this is frequently followed by progressively elevating ALT levels that indicate progression to chronic disease[31]. Chronic HCV infection develops in most infected individuals[31], with persistent or fluctuating ALT elevations indicating actively developing liver damage. The development of chronic liver disease is subtle, progressing at a slow rate without symptoms or physical signs in the majority of patients during the first two or more decades after infection. Chronic hepatitis C is often not recognized until asymptomatic persons are identified as HCV-positive during blood-donor screening, or elevated ALT levels are detected during routine physical examinations. Hepatitis related liver disease progresses from mild fibrosis to cirrhosis in 10-20% of chronically infected patients over a period of 20-30 years with 1-5% of those developing hepatocellular carcinomas[32-34]. The current treatment options for HCV infections are limited to viral replication inhibitors and Interferon alpha (INF-α), given alone or in combination. The worldwide standard for treatment of HCV follows the flowchart in Figure 4. Patients were initially treated with INF-α

Figure 4

* If dose and duration were inadequate

** If dose and duration were adequate
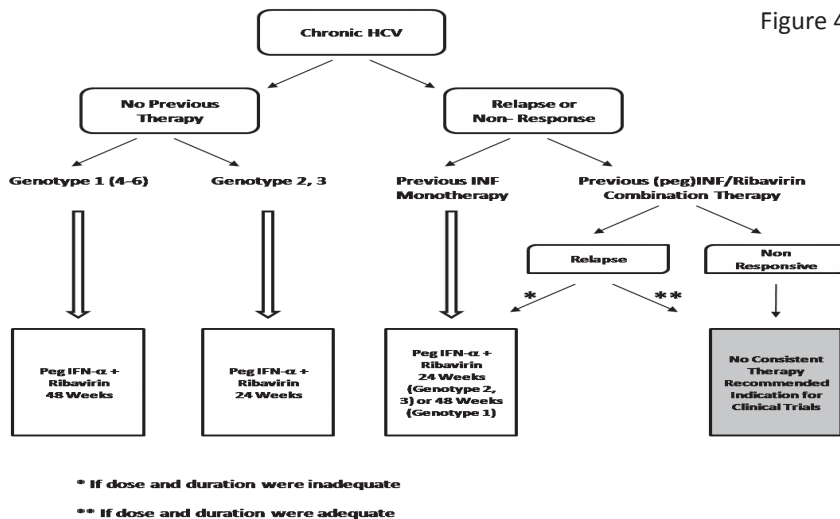
monotherapy, though sustained virological response (SVR), defined as no detectable HCV RNA after a 24 week treatment period followed by follow-up period, rates were far too low; between 2-9% for patients with genotype 1 and between 16-23% for patients with genotypes 2 and 3[35, 36]. When the treatment regime was altered to include a 48 week period as well as the addition of the replication inhibitor ribavirin (RBV), SVR rates improved to 28-31% for genotype 1 and 65% for genotypes 2 and 3[35, 37]. A few years later IFN-α was altered to include a pegylated molecule (pegIFN-α) that slowed its catabolism and allowed it to remain in the body much longer. When pegIFN-α was combined with RBV, SVR's rose to 80% in patients with genotypes 2 and 3[38]. Though genotype 1 is the most common infection it remains the most difficult to treat, even when treated with pegIFN-α and RBV, SVR's remain slightly lower than 50%[39]. In the remaining 50%, new treatment options are needed. Currently, the only option for HCV related end stage liver disease is liver transplantation.

1.2 Liver Transplantation

Liver transplantation is the surgical procedure used to remove a damaged or diseased liver and replace it with a healthy liver allograft. The first human liver transplant was performed by a surgical team lead by Dr. Thomas Starzl in 1963 though it wasn't until 1967 that successful short term transplantation[40] was achieved. The process of liver transplantation remained largely experimental during most the 1970s with low year survival rates. Rates began to improve with the introduction of cyclosporine[41], a powerful immunosuppressive drug, to transplantation by Sir Roy Calne in 1978[42]. During the 1980's liver transplantation became recognized as a standard clinical treatment for both adult and paediatric patients with end stage liver diseases. During the past decades liver transplantation has become an effective treatment for end-stage liver disease, often with high success rates over the first years.

The transplant operation occurs in three phases: the hepatectomy (liver removal) phase, the anhepatic (no liver) phase, and the post implantation phase. The hepatectomy involves removal of the liver from all ligamentous attachments, including the common bile duct, hepatic artery, and portal vein. Usually, the retrohepatic portion of the inferior vena cava is removed along with the liver, although an alternative technique preserves the recipient's vena cava, known as a piggyback technique can be employed.

The donor graft undergoes multiple perfusions in order to clear the liver of blood, preserve and prepare it for transplant. Hypothermic liver perfusion is most commonly performed with University of Wisconsin (UW) or Histadine Thymadine Ketogluterate (HTK) preservation solutions[43]. After the liver is recovered from the donor within the minimum warm ischemic time (<30min), a cold perfusion is performed to clear the vasculature and cool the liver for storage. The graft is then placed on ice, ready for up to 12-18 potential hours for allocation to the receiving hospital. Generally, a second perfusion is performed with cold human albumin at the receiving hospital, in part to remove transportation outflow and also to dilute the high potassium levels in some perfusion solutions. Once in place and all connections established, the new graft is reperfused with blood and the patient's bile duct anastomosis is constructed to either the existing bile duct or to the small intestine. Post transplantation, patient's received a cocktail of drugs including immunosuppressive agents, antibiotics as well as steroids to facilitate healing and prevent rejection.

1.3 Immunosuppression

Corticosteroids have been used since the early years of organ transplantation. Prednisolone (Pred), first introduced to the world as an anti-inflammatory immunosuppressant agent in the early 1950's, saw use in liver transplantation in 1968 with some of the first "successful" liver transplantations[40]. By modulating cellular and inflammatory responses via stimulation or inhibition of gene transcription, Pred and its close analogue Dexamethasone (Dex) are potent suppressors of the immune system. As such they are used as therapeutic treatments in a broad range of autoimmune and inflammatory diseases. Within liver transplantation, steroids are given during surgery as an induction protocol. Low doses of steroids, in combination with other drugs, are used as maintenance immune suppression post transplantation. In the case of an acute cellular rejection episode patients receive several boluses of methylprednisolone, often effective to reverse the rejection. Since the start of transplantation for end-stage liver failure due to chronic HCV, the use of corticosteroids has become a point of debate with groups suggesting that steroids affect HCV levels post transplantation, in turn affecting the success of the surgery.

Major improvements in the outcomes of liver transplantation can be attributed to the introduction of new immunosuppressive agents like calcineurin inhibitors, IL-2 receptor blocking antibodies and mycophenolic acid. Calcineurin inhibitors cyclosporine A (CsA) and FK506 (tacrolimus) have proven efficacy as immunosuppressive agents. In the last decade, FK506 was introduced and replaced much of CsA use[44] due to nephrotoxic complications associated with CsA. This replacement has possibly contributed to the acceleration of HCV recurrence[45]. *In vitro*, CsA has a potent anti-viral side-effect on several viruses including HIV[46], HBV[47], and HCV[48, 49]. Recent publications suggest that the anti-viral activity of CsA acts via cyclophilins, particularly cyclophilin B[50, 51], a functional regulator of HCV RNA polymerase (NS5B). As FK502 inhibits calcineurin via FK binding proteins and not cyclophilins, it does not exhibit anti-viral activity[48, 49] in this pathway.

The activated form of Mycophenolate mofetil (MMF), Mycophenolic acid (MPA), is a highly effective immunosuppressant which lacks the nephrotoxicity associated with calcineurin inhibitors that is often used to prevent rejection post organ transplantation. MPA inhibits inosine monophosphate dehydrogenase (IMPDH), the enzyme responsible for the conversion of inosine 5' monophosphate (IMP) into xanthosine 5' monophosphate (XMP) at the beginning of the guanosine triphosphate (GTP) and deoxy-guanosine triphosphate (dGTP) *de novo* synthesis pathways[52]. Inhibition of IMPDH decreases levels of intracellular guanosine nucleotide pools resulting in inadequate quantities for nominal DNA duplication. As such, MPA has been shown to have anti-proliferative[53], immunosuppressive[54], and anti-viral effects[55]. Its anti-viral effects have been shown *in vitro* for dengue virus[56], HBV[57], avian reovirus[58], yellow fever[59] and West Nile virus[60] with new evidence suggesting an effect on the replication of HCV[61].

1.4 HCV Recurrence

Liver transplantation for end stage HCV related liver disease is often the only option, though success of this treatment is often complicated further by a rapid reinfection (though reinfection is a misnomer in that a new infection of a newly placed organ is occurring, while the patient has retained the infection through the course of disease) of the new graft from extrahepatic reservoirs of HCV. These reservoir location remain controversial as they have not been fully characterized. Early studies have reported the detection of negative strand HCV-RNA, a viral replication intermediate, in whole PBMC's[62] indicating that PBMC's were a possible source reinfection. It was shown later that the assay itself was not as specifically detecting negative strand HCV RNA[63] and as such was insufficient to prove replication and indeed when similar samples were tested with a more accurate assay, the PBMCs were found to be negative[64]. Lanford's group could detect positive strand HCV RNA in liver, spleen, pancreas, muscle, and lymph node though no trace of the negative strand (proof of HCV replication) were found only in liver and not in any of the other tissues assayed. More recent data has re-inspected the possible sources of reinfection and indicated yet again, the blood itself, with the initial reinfection occurring during reperfusion of the graft[65]. Though it may not be actively replicating (at least not at detectable levels) in extrahepatic reservoirs or the blood, it is clear that infectious HCV particles can remain extrahepatic for long periods of time[66].

Recurrent HCV is universal and often very rapid due in part to the necessity for immunosuppression post liver transplantation. Many factors, viral load, host, donor age, modalities of treatment, have been implicated, though no single marker or combination of variables can be used to predict progression[67, 68]. Reinfection of the graft is not only rapid, but accelerated, leading to advanced disease stages within a few years. Development of HCV-related disease occurs in at least 50% of patients after 1 year[69]. Within 5 years, 30% of transplant patients and, within 10 years, as many as 50%[67, 70] will have developed cirrhosis. If cirrhosis occurs, there is a high risk of decompensation within the years following, associated with a 60% risk of death within one year after the first episode of decompensation[71]. When a chronic reinfection occurs post liver transplantation treatment with INF-α or pegINF-α and RBV only achieves SVR's between 25-35%[72-74]. As well, tolerance is poor, due mainly to the development of cytopenias[38] from pegIFN-α and or anemias due to slower clearance of RBV during immunosuppressive induced renal dysfunction[75], as such the rate of discontinuance of anti-viral therapy is higher in these patients than in non-transplant patients[76]. At least 10% of patients who undergo liver transplantation for HCV related disease progression will require retransplantation for recurrent HCV related liver disease[77]. Clearly new alternative treatments are needed to treat not only the hard to treat genotype 1 patients, but the rate and incidence of HCV recurrence post liver transplantation. Gene therapy provides an attractive option to this end.

1.5 Gene Therapy

Genetic engineering was once a catch phrase of science fiction and future fantasy; today it is revolutionizing the world around us. Genetic modification is possible in all forms of life, from single cell alteration to the building of modified organisms, as witnessed when Dolly the sheep was born. Though genetic modification has vast potentials for many fields, the majority of advancements have been made in the field of medicine. The capacity to correct a genetic defect or to alter protein expression that gives rise to disease has always been a very attractive proposition to doctors and scientists alike. Gene therapy is, at its most basic, the modification or introduction of genes or non-native RNA or DNA into existing cells to prevent or to cure a specific aliment. Since its introduction to the world as a viable therapy option in 1989, some 1309 clinical trials have been run to varying degree of success and setbacks.

Midway into 1990, Rosenberg et al used a retrovirus vector to achieve a relatively simple gene transfer to patients tumor-infiltrating lymphocytes (TIL) and was able to detect the non-native DNA in circulation for as long as two months[78], with no detectable side effects of the transfer. Though the transfer did not include a therapeutic agent, it showed the very real potential of viral vector based gene delivery and therapy. Early after this proof of concept another group successfully treated two X-linked severe combined immunodeficiency (XSCID) children[79]. This disease presents itself due to a mutation in the adenosine aminotransferase (ADA) gene, an enzyme which breaks down purines. The lack of a viable ADA gene results in a negative cascade that ends in a lack of proliferating lymphocytes (T-Cells) and thus a compromised immune system. Using an integrating adenoviral vector, a functional ADA gene was delivered to the patients T-Cells in an attempt to compensate for the genetic defect. The persistence of the ADA gene was shown to increase the patients T-Cell counts as well as improving cellular and humoral responses. Following the success of this initial trial, other groups and countries (England[80], Italy[81], Japan[82], USA[83]) soon initiated their own trials, with similar successes; until two of eleven patients in a France based XSCID trial developed leukaemia[84-86]. Its onset was initially thought to be related to the retroviral delivered integration of the ADA gene into or near the proto-oncogene, LOM2, which subsequently activated it. It was later found that the combination of the specific insertional gene and its proximity to the LOM2 was reponsible[86-88] and was not specific to the vector used. While XSCID therapy trials were suspended in the USA following the report of leukaemia, the reported news quickly lost site that in that same trial nine of eleven patients were treated successfully, with no adverse side effects.

Today, the scientist and clinician have a variety of tools at their disposal to place genes within cells. Viral vectors have been the delivery vehicle of choice due to their natural abilities to efficiently deliver a gene or set of genes to a host cell. Viruses have evolved over millions of years and are often particular in their permissible cells due to selection pressures. Viruses that infect cells that are incapable of supporting viral replication are soon overpopulated by ones that infect cells that can support their replication. Scientist have utilized these properties and

forced viral vector evolution to their own ends. Vectors are now derived from many different sources including human pathogenic viruses, to equine or even bacterial viruses. Each has been modified from its wild type to eliminate pathogenesis, alter their ability to self-replicate by removing replicative proteins, and designed to target cells never intended for its wild type by altering its envelope proteins. Of the 1309 clinical gene therapy trials begun since 1989, 305 have used retroviruses[90, 91], a class of virus that includes the human immunodeficiency virus (HIV), termed lentiviruses[92, 93]; these viruses usually contain an RNA copy of their genome and are capable of making a double stranded DNA version that can integrate into the host cells genome. The ability to integrate is of distinct importance to gene therapists in that the transferred gene can be passed on to daughter cells through mitosis, thereby prolonging the effectiveness of the new gene. Non-integrating versions of vectors have been used extensively as well to produce a short term therapy option. These vectors come from a variety of different sources including adenoviruses[94], the virus that causes the common cold. Modified adenoviruses have been used extensively in cancer gene therapy trials to provide anti-tumor agents such as p53[95], particularly due to the transient nature of the gene transfer. These vectors produce a transient therapy so additional treatments are needed to maintain the benefit, but lack the associated problems with integration as seen in the France trials.

Regardless of the vector used and the intended target, the sole purpose of gene therapy is to correct an existing condition. Two methods are employed to facilitate this effectively, *in vivo*, or *ex vivo*. *In vivo* implies that the therapy be transferred directly into a patient, whereas the *ex vivo* method involves removal of the intended target tissue or cells and genetically altering them before returning them to the patient.

Viral vectors are emerging as a new treatment option for use in infectious diseases. Clinical trial aimed to reduce the destructiveness of the HIV virus by introducing genetically modified immune cells to reconstitute the damaged immune system typical with a chronic HIV infection are being attempted in various countries worldwide including, but not exclusively, in Australia[96], Switzerland[97], Italy[98] and the USA[99, 100]. Though HIV has seen the majority of initiation into clinical trials, other viruses such as HBV and HCV are gaining momentum towards trials.

1.6 RNA Interference

Along with the variety of vectors available to scientists, so are the myriad of techniques to remove, replace, correct or mutate specific proteins of interest. RNA interference (RNAi) is one of the most powerful tools, with the ability to sequence-specifically, post-transcriptionaly silence gene expression. It was first observed after the introduction of an extra copy of an endogenous gene into plants was silenced by an unknown mechanism[101-104]. During the 1990s, a number of these gene-silencing phenomena were observed in plants, fungi, animals and ciliates, which introduced the concept of post-transcriptional gene silencing (PTGS) or RNA silencing[105, 106]. RNA silencing is triggered by the occurrence of double-stranded RNA (dsRNA)[107, 108] in plants and animals. dsRNA can elicit a host immune response in mammalian cells as many viruses produce a transitory dsRNA intermediate during replication, as such was once thought to be a vestigial anti-viral defence mechanism, though new developments have shown RNAi to be an active process, regulating protein expression[109, 110] as well as viral replication[111, 112].

Figure 5

http://www.microbiologybytes.com/virology/3035pics/rnai.jpg Used by permission
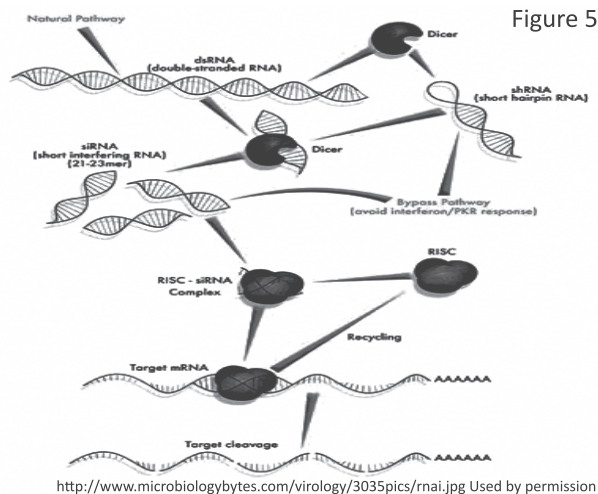
When the RNAi pathway is activated by dsRNA, an enzyme termed Dicer seeks out and cleaves the dsRNA into 21 nucleotide pieces, ensuring that further copying of the viral intermediate is halted. These small pieces of dsRNA are then termed small interfering RNA (siRNA)[113]. One strand of the siRNA associates with RNA-induced silencing complex (RISC)[113, 114] which guides the siRNA to its homologous mRNA target resulting in cleavage of the target sequence[107, 114] (Fig. 5). To date RNAi has been used effectively to reduce infection in many *in vitro* viral systems including human immunodeficiency virus (HIV)[115, 116], poliovirus[117], influenza virus[118], hepatitis B[119] with recent studies showing that RNAi can be effective in blocking HCV replication[120-122]. In addition to viral RNAs, RNAi can be an effective tool to downregulate host cell genes involved in viral infection such as CD4 and CXCR4 in HIV[116, 123] and CD81 in HCV infections[124]. Though still in its infancy, RNAi shows real potential to scientists and doctors alike, providing the potential to control and regulate defective genes, reduce the ability of viruses to replicate as well as reverse the effects of disease.

CHAPTER 2

Research Aims & Thesis Outline

Research Aims

The Hepatitis C virus is a global problem, with some 170 million people infected worldwide, and an estimated 3 to 4 million new infections per year. Its treatment options are limited. Among those patients treated for the infection, almost 50% do not respond to current therapy. Its infection leads to a chronic disease state that will eventually cause severe liver dysfunction requiring a liver transplantation to correct. After liver transplantation HCV infects the new liver from non-hepatic sources of the virus. Once infection recurs, the virus proliferates and damages the new graft at an accelerated rate, partially due to the immunosuppressive regimes needed post transplantation to prevent rejection. This can lead to both a loss of graft as well as lose of the patient's life.

Treatment options for HCV and HCV recurrence has evolved over time, though success rates and the development of a vaccine have been less than perfect. The need for a more complete understanding on the ways these various treatments affect the life cycle of the virus, and by proxy, the outcome of infection, is needed. This thesis aims to explore the past, present and future of these therapies with an attempt to shed new insight into the understanding of antiviral mechanisms as well as the biology of the HCV virus itself.

Thesis Outline

Part I: The Past

This section includes a study of the use of corticosteroids and their direct effects on the replication of hepatitis C. The use of corticosteroids during and post liver transplantation in HCV infected recipients has been a point of debate since their inclusion in standard of care protocols. Never shown in an HCV *in vitro* setting before, evidence is presented that determines that corticosteroids have little direct effects on HCV replication.

Part II: The Present

This section explores the use of immunosuppression in HCV patients. Using an *in vitro* model of HCV replication, various immunosuppressants currently used in liver transplant patients were tested to determine if the choice of agent affects the severity of HCV recurrence. Cyclosporine A and Mycophenolic Acid showed direct effects on the ability of HCV to replicate. As the mechanism for Cyclosporine A has been elucidated, a second study sought to determine the mechanism of action that was responsible for Mycophenolic Acid's ability to reduce HCV replication.

Part III: The Future

This section sought to explore not only the newest options of therapy but also to pave a pathway to the near future by developing a gene therapy system directed specifically against HCV recurrence. Novel lentiviral based shRNA delivery systems were built to target multiple HCV proteins and host cell receptors simultaneously. These vectors were applied to the *in vitro* HCV replication model and showed high potency against HCV replication. As this therapy was to be delivered to an *ex vivo* liver graft and not systemically to the patient, a second study explored the conditions in the liver transplant setting and determined whether these were suitable for gene delivery with minimal interruption in existing transplant procedures. It is not only important to have a patient treated but a long follow up is required to ensure the therapy has been successful. A final study in this section explores the interaction of the existing anti-viral therapy, Interferon-α with the use of RNA interference. Cells were given both treatments and their interactions were elucidated to determine if RNA interference strategies would be affected during existing treatments and vice versa.

Part I: The Past

Study the past if you would divine the future

~ Confucius ~
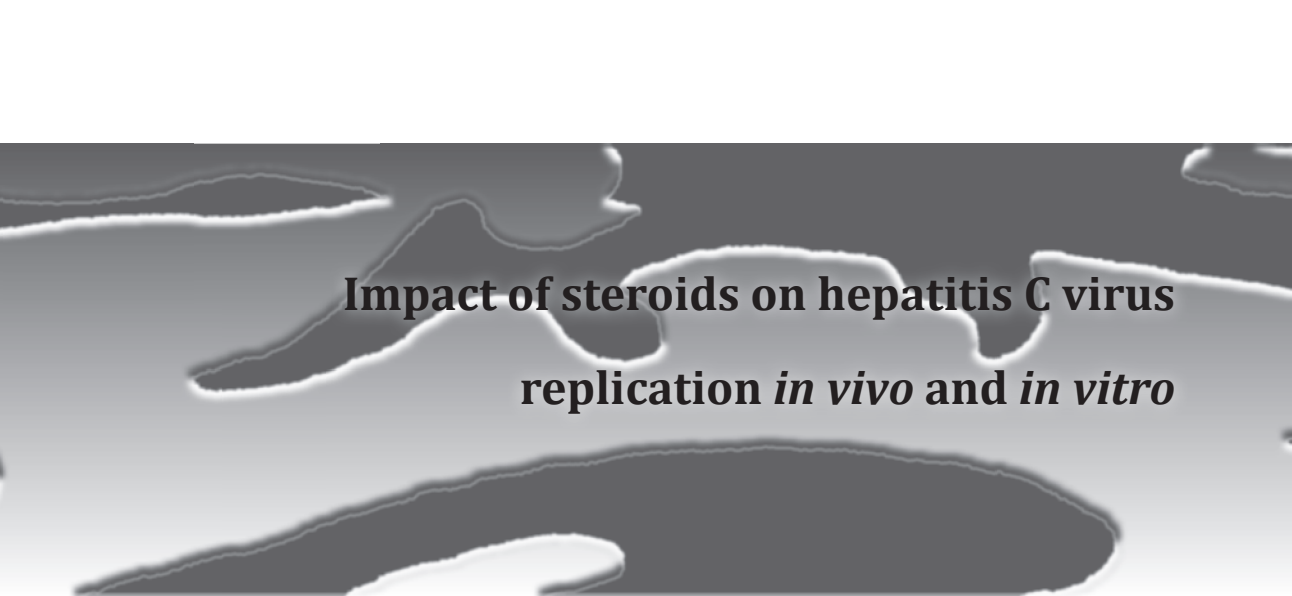
CHAPTER 3

Impact of steroids on hepatitis C virus replication *in vivo* and *in vitro*

Scot D. Henry[1]

Herold J. Metselaar[2]

Jeroen van Dijck[1]

Hugo W. Tilanus[1]

Luc J.W. van der Laan[1]

Departments of [1]Surgery and [2]Gastroenterology & Hepatology,

Erasmus MC - University Medical Center, Rotterdam, Netherlands.

Abstract

Chronic hepatitis C virus (HCV) infection is the leading indication for liver transplantation. Transplantation outcome is often compromised by a rapid re-infection of the graft. Several factors have been implicated in the increased severity of recurrence, including steroid-based immunosuppression. Evidence suggests that steroids boluses used to treat acute rejection are associated with an increase in HCV viral load and the severity of recurrence. Two possible mechanisms for a steroid-mediated effect on HCV viral loads can be postulated; the first being a direct effect of steroids on the virus, by enhancing its replication. The second, an indirect effect due to the suppression of the HCV immune response, allowing HCV unrestricted replication.

To investigate the direct effect of the steroids on HCV replication Dexamethasone (Dex) and Prednisolone (Pred) were tested in an *in vitro* replicon model. HCV replication was assessed based on luciferase reporter expression (luminescence) and HCV RNA (RT-PCR). At clinically relevant concentrations (1-10 nM), treatment with both Dex and Pred did not enhance but resulted in a slight reduction of relative luciferase activity (HCV replication) which was independent of increased cellular protein content and reduced cell proliferation. This minor reduction of HCV replication was confirmed by RT-PCR showing over 41% reduction in HCV RNA levels.

In conclusion, despite clinical evidence that the use of steroids aggravates recurrence of HCV, our *in vitro* study suggests that there is no direct stimulatory effect of steroids on the replication of HCV. As such the increased viral loads after high-dose steroid treatment are more likely due to a down-regulation of the immune response. In such patients, a dampened immune response allows viruses, like HCV, to replicate free of immune mediated killing of their host cells. When a change occurs, such as a tapering or an alteration of immunosuppressant drugs, the immune system reinitiates and vigorously attempts to control the virus, resulting in acceleration of liver damage. Therefore, either steroid avoidance or maintaining low levels, coupled with a slow tapering of corticosteroids may be beneficial to HCV-infected transplantation recipients.

Introduction

Clinical evidence of the effect of steroids on HCV viral load and recurrence of HCV

Since the past decades liver transplantation has become an effective treatment for end-stage liver disease. Major improvements in the outcomes of liver transplantation can be attributed to the introduction of new immunosuppressive agents like calcineurin inhibitors (cyclosporine A and tacrolimus), IL-2 receptor blocking antibodies and mycophenolic acid. These newer immunosuppressants are often used in combination with corticosteroids that have been used since the early years of organ transplantation. Prednisolone (Pred), first introduced to the world as an anti-inflammatory immunosuppressant agent in the early 1950's, saw use in liver transplantation in 1968 with some of the first "successful" liver transplantations[1]. By modulating cellular and inflammatory responses via stimulation or inhibition of gene transcription, Pred and its close analogue Dexamethasone (Dex) are potent suppressors of the immune system. As such they are used as therapeutic treatments in a broad range of autoimmune and inflammatory diseases. Within liver transplantation, steroids are given during surgery as an induction protocol. In addition, low doses of steroids are often used in combination with other drugs as maintenance immune suppression. In case of an acute cellular rejection episode patients receive several boluses of methylprednisolone, which is often effective to reverse the rejection. Since the start of transplantation for end-stage liver failure due to chronic hepatitis C, the use of corticosteroids has become a point of debate.

Most hepatitis C virus (HCV)-positive patients receiving transplantation experience a reduction in HCV RNA levels within the anhepatic phase followed by a rapid increase in viral load due to re-infection of the graft. This re-infection results in an accelerated recurrence of liver fibrosis that compromises patient and graft survival. Several host and viral factors are associated with a more aggravated course of HCV recurrence including genotype and donor age with many studies showing a strong correlation between severe recurrence of HCV and treatment of rejection[2].

Steroid boluses and their effect on HCV viral load

The most compelling evidence that steroids affect HCV replication comes from the non-transplant setting. In a study of nine patients, during a seven-week course of Prednisolone, the mean serum HCV RNA levels increased by at least one log as detected by two separate quantitations. This was a transient effect with HCV RNA levels decreasing to pretreatment values after the discontinuation of Prednisolone[3,4]. Similar results were reported by Magrin et al[5]. The best evidence from the transplant setting comes from studies on the use of corticosteroid boluses to treat acute rejection episodes. It was first reported by Gane and colleagues, that steroid boluses resulted in an increased serum viral load[6,7]. They detailed a 4 to 100-fold increase in HCV RNA seven days after treatment, with the observed raise in HCV RNA also being transient and declined with decay in methylprednisolone concentrations (Fig. 1A).
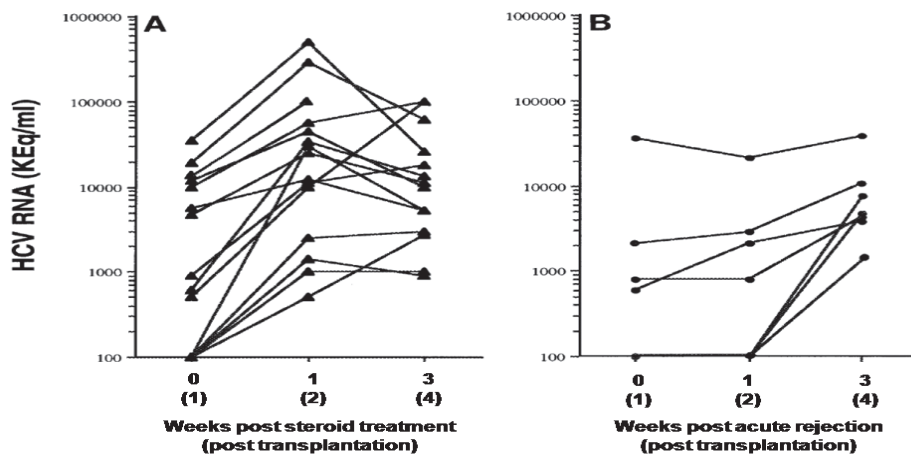
Fig 1. Effect of high dose steroids for acute rejection on viremia levels. A) methyl prednisolone give. B) methyl prednisolone not given. Reprinted from publication Gane EJ, Naoumov NV, Qian KP, Mondelli MU, Maertens G, Portmann BC, Lau JY, Williams R. A longitudinal analysis of hepatitis C virus replication following liver transplantation. Gastroenterology 1996; 110:167-77, with permission from American Gastroenterological Association.

No such increase was seen at seven days in a similar group of patients that experienced acute rejection but were not treated with steroids (Fig. 1B). Soon after Gane's study, a study published in Hepatology did not confirm this effect, though only included a limited number of patients[8]. Neither study explored the specific effect of steroids on HCV replication in the liver. Two possible mechanisms for a steroid-mediated effect on HCV viral loads can be postulated; the first being a direct effect of steroids on the virus, by enhancing its replication. The second, an indirect effect due to the suppression of plasmacytoid dendritic cells[9] or Tcells[10], allowing HCV unrestricted replication, thus rapidly giving rise to higher viral RNA concentrations.

Controversial clinical evidence on the impact of steroid maintenance therapy of HCV recurrence

Though the data on increases of HCV viral loads due to steroid boluses are convincing, the effects of steroid maintenance therapies are still controversial. Studies have shown that complete steroid avoidance could be beneficial for patients undergoing liver transplantation. A study by Papatheodoridis reports that HCV RNA levels were increased with the duration of steroid treatment as well showed that more severe fibrosis was seen in patients with triple or double rather than single agent immunosuppression[11,12]. Though a randomized trial found that there was no significant difference between steroid maintenance (n=51) and steroid free patients (n=48) in liver fibrosis and viral loads at twelve months after liver transplantation, though serum HCV RNA was significantly higher in the steroid group at one month[13].A more recent report also found no differences in HCV viral load, at three or six months, in HCV positive liver transplant patients with (n=45) or without (n=43) Pred treatments[14].In the transplant setting Pred is always used in combination with other immunosuppressive compounds, making

it difficult to interpret the individual effect of steroids. As well, when treatments involve steroid withdrawal or steroid-free regiments, they are often used in combination with T-cell depleting or IL-2-receptor blocking antibody treatments, again confounding analysis[15].

Rapid reduction of steroid dose is associated with more aggravated HCV recurrence

With the suggestion that steroid use is detrimental to patients undergoing liver transplantation due to hepatitis C complications, studies have attempted to wean patients off steroids early. Evidence about the speed of the tapering of steroids has been published, with most suggesting that a slow withdrawal of steroids is more beneficial than a rapid weaning in HCV-infected liver transplant patients[16,17]. It has been postulated that rapid weaning of immunosuppression can unleash an anti-HCV immune response that is detrimental to the graft and results in accelerated recurrence of liver injury and fibrosis[18,19]. Consistent with this, Fong et al show that during Pred treatment, in chronic HCV patients, transaminase levels decline significantly, suggesting a direct effect of steroids on immune-mediated liver damage[4]. These studies support the idea that it is the rapid change in immunosuppressive levels that are deleterious, possibly due to the increase in viral replication and a change in the anti-viral response. Any changes in regime that includes rapid withdrawal or alteration in the level or type of suppression can allow a vigorous immune response and killing of HCV-infected hepatocytes, which lowers the overall HCV content in the liver, but creates extensive fibrosis, rapidly degrading the efficiency of the new graft and putting it at risk for accelerated cirrhosis.

Effects of steroids on HCV replication and anti-HCV immune response

Considering the body of clinical evidence on changes in HCV viral load due to steroid treatment, a distinct lack of information regarding the exact underlying mechanism of steroids on the course viral infection exists. To address whether steroids directly enhance viral replication, we have examined the use of Pred and Dex, in an *in vitro* system of HCV replication.

The direct effects of steroids on the replication of HCV *in vitro*

The replication of HCV was studied using a luciferase based replicon cell line model for HCV replication (Huh-7 ET cells), as described before[20-22]. Treatments with corticosteroids Pred and Dex were applied at clinically relevant concentrations (1-10nM) to replicon cell monolayers and incubated for a period of 18 hours. The medium was replaced to remove the steroids and the global protein content, replicon cell proliferation, luciferase expression (synonymous with HCV replication in this model); and HCV RNA were assessed.

Global protein production was determined with a colorimetric protein stain. Giemsa was applied to fixed cells and total protein quantified via spectrophotometry and normalized against non-treated cells. The protein content within the cells increased slightly, though significantly, across the doses of steroids (average 9.0%±0.6 SEM), indicating that there was a direct effect on protein production, though at the same time cell proliferation was significantly reduced

(average 18%±1 SEM compared to untreated cells). As the luciferase reporter construct is directly linked to the replication of the HCV replicon, any change in luciferase expression levels can be interpreted as a direct change in viral replication. After treatment with steroids, cells were treated with luciferin salt, allowed a rest time for uptake and light emission was measured with an illuminometer. HCV replication was observed to slightly increase with all treatments of either Pred or Dex though lacked significance (average 5.1%±1.4 SEM increase). When the luciferase activity was normalized for the general increase of the protein content there was no specific increase in HCV replication after steroid treatment (Fig. 2A). Indeed, observed this way, steroids had a slight decrease, rather than increase, on the replication of HCV.

Though the Huh-7 ET replicon model has clear advantages and has allowed many advances in the HCV field, it falls short of perfect (as any model will, given time). In order to compensate for the shortfall of the luciferase assay in this experimental setup, we applied the same steroid treatments to an interferon hypo-responsive, cell-cycle independent replicon model which lacks the luciferase reporter protein, the Huh-6 Con1[23]. Interestingly, when looking at non-normalized

expression of replicon RNA with real-time PCR for IRES specific primers, relative to the reference gene GAPDH, treatment for 18hr with either Dex or Pred did not increase HCV RNA but rather reduced levels by more than 41% when compared to untreated cells (Fig. 2B). This strengthens the plausibility of the slight reduction seen in the normalized HCV replication data. In this set of experiments, our data suggests that there is no direct evidence of an increase in HCV replication due to the presence of steroids. Indeed if there is an affect, we can conclude that, *in vitro*, steroids reduce cell cycling and proliferation in hepatocytes, increase the cellular protein content and independent of this has a slight effect on the ability of HCV to effectively replicate. Though no mechanism has been established thus far, these *in vitro* data give new insight into the observed effects of steroids in the clinical setting.
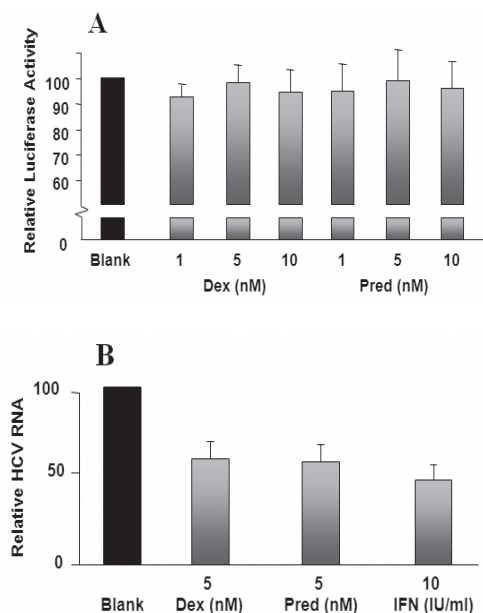
Fig 2. Steroids reduce HCV replication. Replicon cells (Huh-7 ET) were treated with various doses of Dex or Pred. A) Luciferase expression was measured and normalized against total protein content of the replicon cells. (n=6). B) Replicon cells (huh-6 Con1) were treated for 18hr with Dex, Pred or peg-Interferon-α (IFN). HCV RNA was extracted and RT-PCR was performed using IRES and GAPDH specific primers. Quantitation of HCV RNA, relative to GAPDH RNA was determined by the ΔΔCT method. (n=4) (* p<0.02, using Wilcoxin signed rank test (paired T-Test)).

Direct effects of steroids on the anti-HCV immune response

The question remains, why do clinical studies see an increase in HCV viral loads due to steroids? Clearly, our experiments show it is not due to a direct effect of steroids on viral replication, but rather more likely the second postulate that was explained earlier as an indirect effect due to their immunosuppressive properties. In the last 10 years, many aspects of cellular function have been found to be affected by steroids, including a dowregulation of inflammatory mediating cytokines and chemokines in various cell types[24-26]. Steroids are thought inhibit NF-kB signaling by either activating the glucocorticoid receptor binding to p65 thus interfering with the binding of NF-kB to DNA[27] or via altering cofactor recruitment[28-31]. It is known that HCV-specific T-cell responses are detectable in liver transplant patients and play a role in the pathogenesis of HCV recurrence. Patients with minimal histological recurrence can show a multi-specific response to HCV. In contrast, PBMCs from patients with severe HCV recurrence, despite being able to proliferate in response to non-HCV antigens, fail to respond to the HCV antigens[10]. To date, it has not been fully investigated whether steroids directly affect the immune response against HCV. Though, two studies have shown a reduction in transaminase levels in chronic HCV patients[4,5], suggesting the inhibition of the anti-HCV immune response by steroids.

Conclusions

Though steroid treatments help to reverse the acute rejection episodes within a liver transplant patient, they may be doing more harm than good in patients transplanted for hepatitis C related disease. Our *in vitro* study suggests that there is no direct stimulatory effect of steroids on the replication of HCV, so the increased viral loads after high-dose steroid treatment are more likely due to a down-regulation of the immune response. In these patients, a dampened immune response allows viruses, like HCV, to replicate free of immune-mediated elimination of infected hepatocytes. When a change occurs, such as a tapering or an alteration of immunosuppressant drugs, the immune system reinitiates and vigorously attempts to control the virus, resulting in acceleration of fibrosis. Therefore, either steroid avoidance or maintaining low levels, coupled with a slow tapering of corticosteroids may be beneficial to HCV-infected transplantation recipients.

Part II: The Present

The future influences the present just as much

as the past
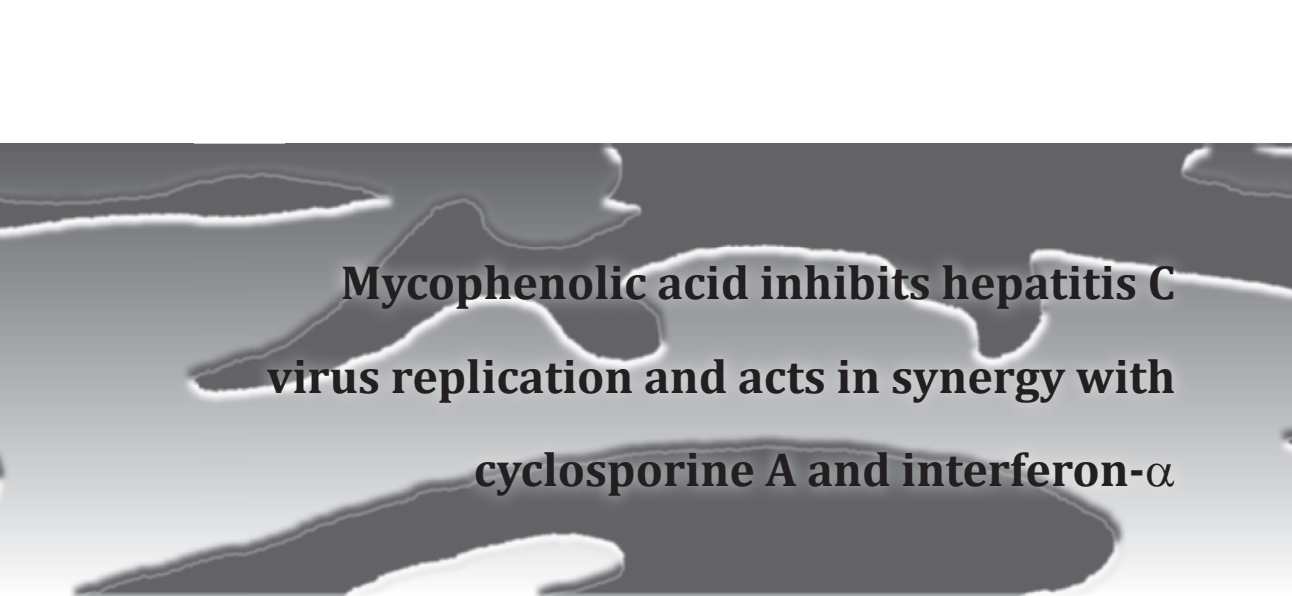
~ Friedrich Nietzsche ~
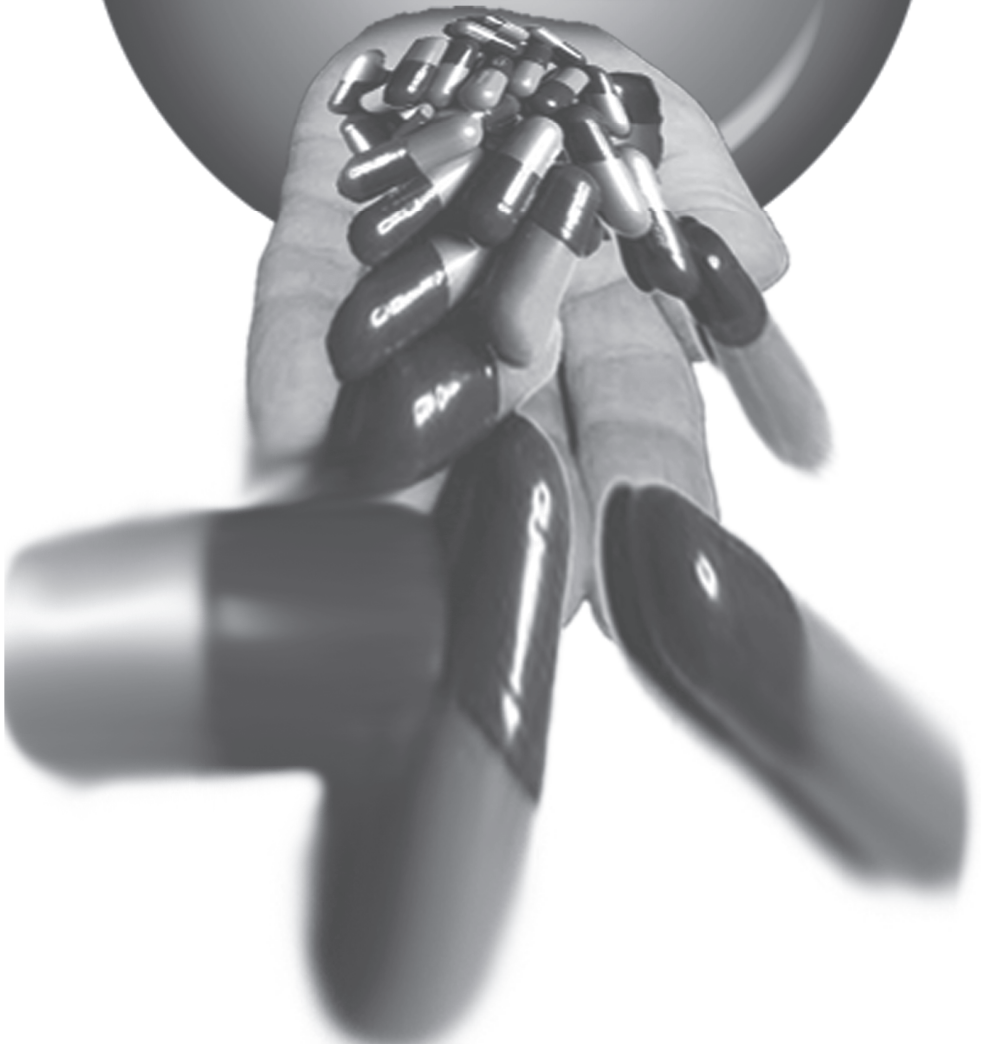
CHAPTER 4

Mycophenolic acid inhibits hepatitis C virus replication and acts in synergy with cyclosporine A and interferon-α

Scot D. Henry[1]

Herold J. Metselaar[2]

Richard C. B. Lonsdale[3]

Alice Kok[1]

Bart L. Haagmans[3]

Hugo W. Tilanus[1]

Luc J. W. van der Laan[1*]

Departments of [1]Surgery , [2]Gastroenterology and Hepatology and [3]Virology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands.

Abstract

Chronic hepatitis C virus (HCV) infection is the leading indication for liver transplantation. Clinical evidence suggests that particular immunosuppressive agents can have an influence on HCV recurrence. Cyclosporine A (CsA) specifically inhibits HCV replication through blocking the viral RNA polymerase enzyme, NS5b. In this study we investigated the effect of mycophenolic acid (MPA) and other immunosuppressants on HCV replication.

   MPA and other compounds were tested *in vitro* using an HCV-replication model containing a luciferase reporter gene. At clinically relevant concentrations (1.0-6.0 μg/ml), MPA inhibited HCV replication to approximately 75%. CsA and interferon (IFN-α) also showed inhibition in a dose dependent manner. In these short-term (18 hr) experiments, MPA did not inhibit replicon cell proliferation or induce cell death, which could have accounted for the anti-viral effect. In contrast to the anti-viral activity of MPA against West Nile virus, the effect of MPA on HCV replication was guanosine-independent. When combined, MPA and CsA showed significant synergistic inhibition of replication, reaching maximum inhibition ~90% at highest doses. Synergistic effects were observed with suboptimal concentrations of IFN-α with MPA or CsA. The kinetics of HCV inhibition by MPA, CsA and IFN-α were clearly distinct, with earliest effects seen with IFN-α. No specific inhibitory effects were observed with tacrolimus, or rapamycin.

   The immunosuppressive drug MPA is as potent as CsA as an inhibitor of HCV replication. MPA was shown to have a distinct anti-HCV mechanism of action, independent of cell proliferation and guanosine depletion.

Introduction

Chronic hepatitis C virus (HCV) infection is the leading indication for liver transplantation worldwide. The success of transplantation, however, is compromised by re-infection of the liver graft by persisting HCV. Re-infection results in an accelerated recurrence of liver cirrhosis that compromises patient and graft survival. Several studies have shown that the problem of HCV recurrence has worsened in recent times[1]. The reasons for this deterioration are unknown, though changes in immunosuppressive therapy within the last decade could provide a possible explanation[2].

Calcineurin inhibitors cyclosporine A (CsA) and FK506 (tacrolimus) have proven efficacy as immunosuppressive agents. In the last decade, FK506 was introduced and replaced much of the CsA use[3], possibly contributing to the acceleration of HCV recurrence[4]. *In vitro*, CsA has a potent anti-viral side-effect on several viruses including HIV[5], HBV[6], and HCV[7, 8]. Recent publications suggest that the anti-viral activity of CsA acts via cyclophilins, particularly cyclophilin B[9, 10], a functional regulator of HCV RNA polymerase (NS5B). As FK502 inhibits calcineurin via FK binding proteins and not cyclophilins, it does not exhibit anti-viral activity[7, 8].

The activated form of Mycophenolate mofetil (MMF), Mycophenolic acid (MPA), is a highly effective immunosuppressant which lacks the nephrotoxicity associated with calcineurin inhibitors that is often used to prevent rejection post organ transplantation. MPA inhibits inosine monophosphate dehydrogenase (IMPDH), an enzyme responsible for the conversion of inosine 5' monophosphate (IMP) into xanthosine 5' monophosphate (XMP) at the beginning of the GTP and dGTP de novo synthesis pathways[11]. Inhibition of IMPDH decreases levels of intracellular guanosine nucleotide pools resulting in inadequate quantities for nominal DNA duplication. As such, MPA has been shown to have anti-proliferative[12], immunosuppressive[13], and anti-viral effects[14]. Its anti-viral effects have been shown *in vitro* for dengue virus[15], HBV[16], avian reovirus[17], yellow fever[18] and West Nile virus[19] but no clear effect has been reported for HCV. Inhibition of West Nile virus and yellow fever virus by MPA could be overcome by supplementation of exogenous guanosine[18, 19]. This suggests that the anti-viral effect of MPA for these viruses acts solely via IMPDH blockade and the prevention of de novo guanosine nucleotide production, though other mechanisms have been recently postulated[20].

There is still controversy as to whether MPA has anti-HCV effects in the clinical setting as compared to other immunosuppressants. Several studies demonstrate that the use of MPA is associated with a reduction of serum HCV-viral load and/or HCV-related fibrosis after liver transplantation[21-24], whereas others report no change or a slight increase in HCV viral load[25-27]. Here we present the individual and combined effects of MPA and CsA in an *in vitro* HCV replicon model and show a specific, distinct anti-viral effect on HCV by MPA, not due to the depletion of intracellular guanosine pools. When combined, MPA was shown to have potent synergistic effects with both CsA and IFN-α. Immunosuppressive therapy based on a combination of MPA and CsA could be beneficial to reduce HCV recurrence after transplantation and might act as an adjuvant to IFN-α anti-viral therapy.

Materials And Methods

Reagents

MPA (dissolved in DMSO), CsA (dissolved in a 1:1 mixture of EtOH with 10% Tween-20 and water) and rapamycin-derivative SDZ RAD (dissolved in EtOH) were provided by Novartis Pharma AG, Basel, Switzerland. Tacrolimus (as intravenous fluid) was provided by Fujisawa Holland (Houten, The Netherlands). Pegylated-interferon (IFN)-α2a (dissolved in water) was provided by Roche Ltd (Basel, Switzerland). All agents were stored in 15 ml aliquots and frozen at $-20°$C, excluding CsA which was stored at $4°$C. Guanosine (Sigma, Zwijndrecht, The Netherlands) was diluted to 1 mM in PBS and stored at -20°C. Hygromycin (diluted in PBS) was purchased from Sigma and stored in 100x concentrated aliquots; Geneticin (G418-Sulfate dissolved in PBS) was purchased from Invitrogen-Gibco (Breda, The Netherlands) and stored in 100x concentrated aliquots.

Cell Cultures & Treatments

Cell monolayers of the human hepatoma cell line Huh-7[28], were maintained in Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% v/v fetal calf serum (PerBio Science), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-Glutamine (Invitrogen-Gibco) (cDMEM). Huh-7 cells containing subgenomic HCV monocistronic replicon (I_{389}/NS3-3'/HygUbi/5.1) or bicistronic replicon (I_{389}/NS3-3'/LucUbiNeo-ET)[29] were maintained with 25 mg/ml Hygromycin (Sigma) or 250 mg/ml G418 (Sigma) respectively. Vero-E6 cells were maintained in cDMEM, supplemented with 0.02 M HEPES buffer and 0.15% Sodium Bicarbonate.

Replicon cells were plated in cDMEM at 70-80% confluence and treated with various concentrations and combinations of drugs, exogenous guanosine or vehicle controls and incubated at 37°C for 18 hrs (or 48 hrs for immunocytochemistry). Concentration-range tested for MPA, 0.1-10 μg/ml; CsA, 0.1-10 μg/ml; Tacrolimus, 0.05-0.5 μg/ml; SDZ RAD, 0.1-3.0 μg/ml; Pegylated IFN-α, 0.1-10 IU/ml; and guanosine, 25-100 μM. Vehicle control treatments (DMSO or EtOH) were equivalent to the highest concentrations in the dose range experiments for each of the drugs. Cell were retrieved from monolayers by Trypsin/EDTA (Invitrogen/Gibco) digestion or directly lysed in the wells.

Luciferase Assay

Cells for endpoint assays were lysed and luciferin substrate added using Steady Glo Luciferase System (Promega) according to manufacturer protocols. Kinetic and live cells experiments were performed as described above (excluding lysis step) with the addition of 100 mM luciferin potassium salt (Sigma). Luciferase activity was quantified using a Top Count scintillation/luminescence counter (Packard BioSciences Top Count-NXT, Milan, Italy).

West Nile Virus Replication

Vero-E6 cells were seeded at 1.5×10^5 cells per well, in 24-well tissue culture plates and incubated overnight to ~90% confluency. Monolayers were washed with PBS and infected with West Nile virus, NY99 strain, at a multiplicity of infection of approx. 0.3 in medium containing 2% v/v fetal calf serum. Virus was allowed to absorb for 2 hrs before washing and refreshing the cells and followed by 18hr treatment with CsA or MPA at 2.5 or 5 µg/ml and supplemented with or without 50 µM guanosine. Supernatants were harvested and 50% tissue culture infectious doses ($TCID_{50}$/ml) determined by titration on Vero-E6 for 6 days, scoring for cytopathic effect and using Reed-Muench method[30].

Viability, Proliferation And Cell Quantification

Cell viability was determined by the addition of 7AAD to resuspended replicon cells followed by FACS analysis. 7AAD negative cells were considered viable, whereas apoptotic and necrotic cells were positive for 7AAD [31]. Proliferation profiling was achieved with Carboxy-fluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands) staining. In brief, 150,000 Huh-7 replicon cells were treated with 20 mM CFSE and incubated according to manufactures protocol and seeded in 12-well plates. After 18 hr or 5 days, cells were treated with 7AAD (BD Pharmigen, San Diego, USA) at a 1:300 dilution and proliferation staining was measured via FACS (BD FACS Calibur). Generational analysis was performed with ModFit LT v3.0 (BD Pharmigen) software and was gated to exclude 7AAD positive cells. Parent generation was assessed at highest CFSE concentration. Cell quantification was assessed using Giemsa (Merck, Darmstadt, Germany) staining[32]. Adherent replicon cells were fixed with methanol followed by 20 min staining with 40% Giemsa solution. After five washes with PBS, the cell bound Giemsa was resolved in methanol and quantified using a spectrophotometer at 655 nm with a Model 680 Microplate Reader (BioRad, Hercules, USA). The cell count per well is directly related to the Giemsa staining. Expansion of cultures was measured by comparing ODs of treated cells to cells fixed at time zero (addition of drugs).

Real-time RT-PCR

Confluent monolayers of replicon cells were lysed by Trizol (Invitrogen-Gibco) and RNA precipitated with 75% EtOH and captured with a Micro RNAeasy silica column (Qiagen). RNA was quantitated using a Nanodrop ND-1000 (Wilmington, Delware, USA) and adjusted to total cell numbers for each sample. cDNA was prepared using Promega's AMV reverse transcriptase following standard protocols. Real-time PCR (MJ Research Opticon, Hercules California, USA) was performed using primers NS3 (F-5'-GGT TCT GTG CGA GTG CTA TG-3', R-5'-TCT CCT GCC TGC TTA GTC TG-3'), and GAPDH (F-5'-CCA TGG AGA CTG GGG-3', R-5'-CAA AGT TGT CAT GGA TGA CC-3'). Semi-quantitation of sample amplicons was performed with SybrGreen (Sigma) using Taq DNA polymerase (Invitrogen-Gibco) as previously described[33].

Immunocytochemistry

Monocistronic replicon cells were grown in 8-well chamber slides. After 48 hr treatment, cells were fixed in 2% paraformaldehyde and labeled with an antibody conjugated to HCV-non-structural protein 3 (NS3, Novo Castra, Newcastle, UK). Secondary antibody binding and amplification of signal was accomplished with EnVision™ horse radish peroxidase then visualized with 3'-Amino-ethyl-carbozole (Sigma). Images were created on a Zeiss Axioskop microscope (Sliedrecht, The Netherlands) fitted with Nikon's (Badhoevedorp, The Netherlands) Digital Sight DS-U1 imaging system. Images were captured and formatted using Eclipse Net digital software. Quantitation of NS3 staining was performed by blinded scoring of 4 optical fields by two independent observers.

Synergy Isobologram

Additive effects were calculated with a modified Bliss Independence calculation[34] in which the expected effect for a combination of independently acting drugs can be calculated from the single dose effects. Additive effects curves were created by fitting tested concentrations on a Boltzsman sigmoidal dose response curve and interpolating effects through the range of doses using Graph Pad Prism 4.0 software (Graph Pad Software, San Diego, USA). These points were plotted and EC50 determined and compared to the EC50 of observed combined doses. Synergy was defined as when the observed effects were at lower concentrations than the expected additive values.

Statistical Analysis

Statistical analysis of the expected and observed results was performed using either matched pair non-parametric test (Wilcoxon signed rank test) for the cell viability data, or the non paired, non-parametric test (Mann Whitney test) using GraphPad Prism 4.0 software.

Results

Specific inhibition of HCV replication by MPA and CsA

In order to determine the effect of immunosuppressants on HCV replication, sub-confluent Huh-7 replicon cells were treated with various doses of these drugs and cultured for 18 hrs. Total luciferase activity was measured and compared to the activity of untreated (Con) cells. As shown in figure 1, both MPA and CsA reduced HCV replicon levels in a dose dependent manner with a maximum reduction of 75% with the highest dose tested (10 µg/ml).
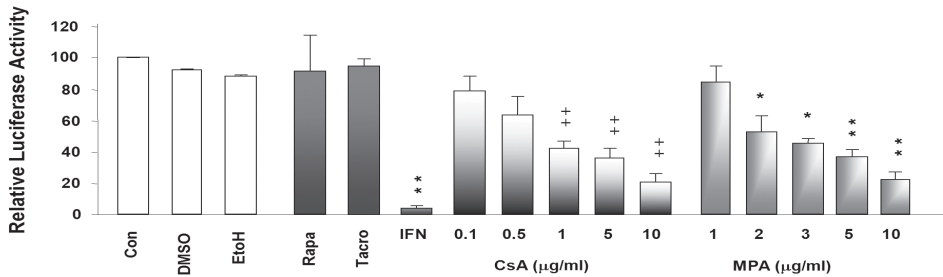

Fig 1. Dose-dependent inhibition of HCV replication by mycophenolic acid (MPA) and cyclosporine A (CsA). Huh-7 replicon cells were cultured for 18 hrs in the presence of various immunosuppressants and their vehicle controls. Luciferase activity, as a measure of HCV replication, was quantitated by luciferase-derived luminescence and represented as a percentage of the activity from untreated (Con) cells. No inhibition of HCV replication was seen with 500 ng/ml Tacrolimus (Tacro) or 3 µg/ml Rapamycin (Rapa). Almost complete inhibition was seen with 10 IU/ml of pegylated-IFN-α 2a (p<0.01). MPA showed a significant (* p<0.05, ** p<0.01) dose-dependent decrease in HCV replication up to approximately 75% as compared to DMSO control. CsA also showed a significant (+ p<0.05, ++ p<0.01) dose-dependent decrease in HCV replication up to 75% as compared to EtOH control. Shown is the mean ± SEM from 4 to 13 independent experiments.

Long-term incubation with MPA or CsA maintained inhibition of HCV replicons up to seven days (data not shown). HCV replicon RNA, as detected by RT-PCR for IRES and NS3 sequences showed a significant decrease by both MPA and CsA (Fig. 2A). Quantification of the NS3 RNA levels relative to the house-keeping gene GAPDH by Real-Time RT-PCR confirmed this reduction
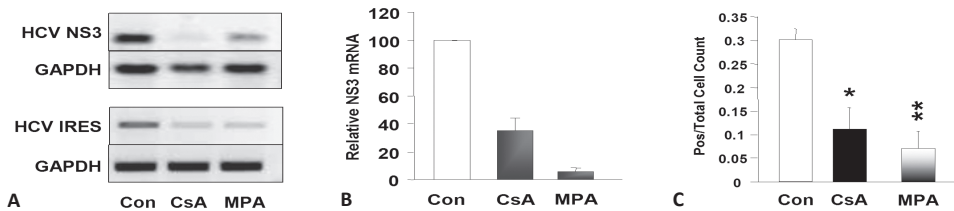
(Fig 2B). Treatment with MPA or CsA also reduced the NS3 protein expression in replicon cells as detected by immunocytochemistry (Fig. 2C). Quantification of NS3 staining as shown in figure 2D, confirmed the reduction in viral protein expression. Treatment with MPA or CsA did not result in detectable levels of IFN-α production (data not shown). Tacrolimus and rapamycin-derivative SDZ RAD did not show specific inhibition of HCV replication (Fig. 1).
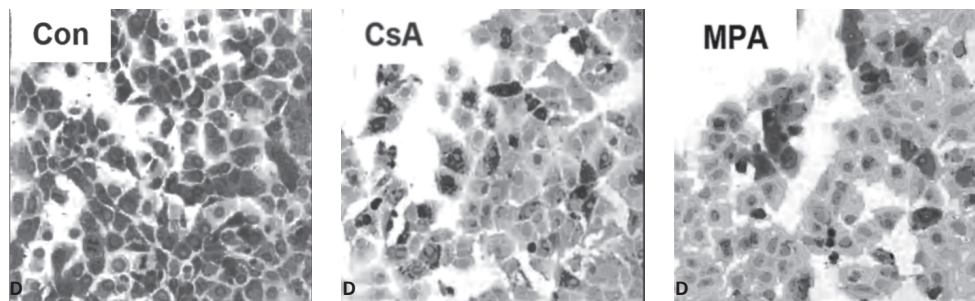
Fig 2. MPA and CsA reduce HCV RNA and NS3 protein levels. Replicon cells were cultured for 18hr in the presence of MPA (5 μg/ml) or CsA (5 μg/ml). To determine RNA levels of HCV (NS3 or IRES) or GAPDH, total RNA from replicon cells was extracted normalized to total cell numbers and RT-PCR was performed. A) Representative agrose gel analysis of RT-PCR product for NS3, IRES and GAPDH are shown. B) Real-time RT-PCR of NS3 normalized to GAPDH levels using ΔΔCT calculation of two independent experiments is shown. Both MPA and CsA show a marked reduction in HCV RNA. C) Immunocytochemistry detection of NS3 protein. MPA and CsA treatment significantly (* p<0.05, ** p<0.01) reduced the levels of intracellular NS3 protein levels, as scored by two independent blinded observers (D).
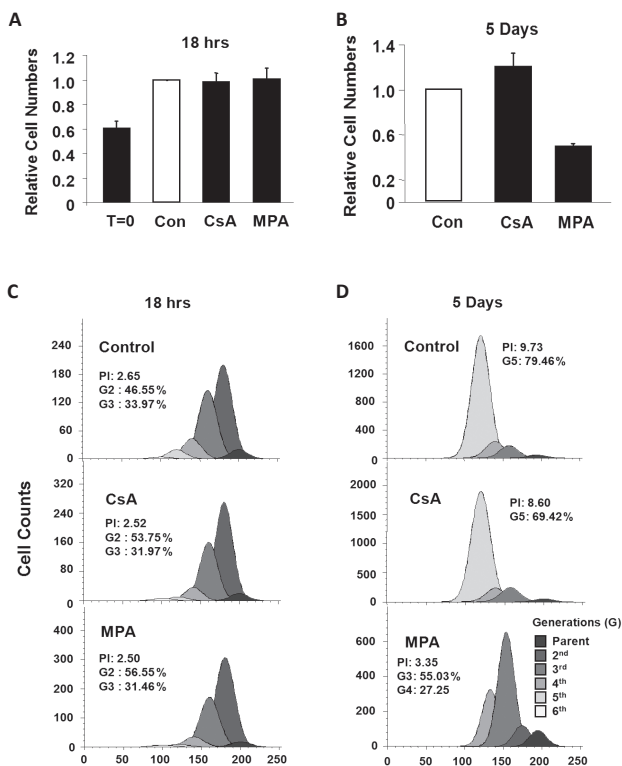
Inhibition of HCV replication was independent of cell proliferation and cytotoxicity

MPA is a potent inhibitor of cell proliferation. To exclude that the anti-viral effect of MPA was due to a reduction of proliferation of the replicon cells in these experiments, cell-numbers, -proliferation and -viability were determined. As shown in figure 3A, control cells showed approximately 55% increase in numbers after 18 hrs of culture as compared to start of experiment (t=0). Cells treated with either MPA or CsA showed an increase in numbers as much as 65% as

Fig 3. Anti-HCV effect of MPA is unrelated to replicon cell proliferation. Huh-7 replicon cells were cultured for 18 hrs (A, C) or 5 days (B, D) in the presence or absence of CsA (5 μg/ml) or MPA (5 μg/ml). Cell numbers were assessed by Giemsa staining of adherent cells. Cell numbers increased over 18 hrs cultures (A) and were comparable for all treatments as compared to control. At 5 days (B), MPA showed a marked reduction in cell numbers when compared to controls. C) CFSE labeled cells showed similar proliferation index (PI) for all conditions at 18 hrs, but at 5 days (D), MPA shows a distinct arrest in proliferation generations (G) 3 and 4. At that time, untreated controls and CsA or IFN-α (not shown) treated cells continued proliferation to G5. These findings suggest that the anti-HCV effect of MPA after 18 hrs treatment is unrelated to inhibition of cell proliferation.

compared to t=0, suggesting these compounds have no effect on cell proliferation at 18 hrs. In contrast, MPA but not CsA reduced cell expansion in 5 day cultures (Fig. 3B). CFSE profiling confirmed no difference in proliferation due to MPA treatments within 18 hrs (Fig. 3C), whereas arrested proliferation was observed at 5 days (Fig. 3D). Furthermore, no significant increase in cell death was detected after 18 hrs or 5 day treatments with MPA or CsA as compared to controls (89% vital cells in control and 87% and 88% vital cells for CsA and MPA, respectively). In summary, loss of cell-proliferation, -numbers or -toxicity could not have accounted for the inhibition of HCV replication by MPA.

Inhibition of HCV replication by MPA is independent of guanosine-depletion

MPA is a potent inhibitor of the IMPDH enzyme and effectively reduces the cellular guanosine nucleotide pool. It has been shown for several viruses that supplementation with exogenous guanosine can overcome the inhibition by MPA and restore replication. To determine whether inhibition of HCV replication by MPA is due to a blockade of guanosine production, the effect of guanosine supplementation was tested. As shown in figure 4A, the addition of exogenous guanosine slightly (approx. 17%) inhibited HCV replication alone, but did not reduce cell proliferation or induce cell death (data not shown). Addition of 50 μM guanosine slightly reversed MPA-induced inhibition of HCV replication (observed inhibition of 55% compared to the expected 62% inhibition), though this did not reach statistical significance. As expected, inhibition by CsA or IFN-α was not affected by the addition of guanosine (Fig. 4A). Inhibition of West Nile virus replication by MPA could be completely overcome with the addition of exogenous guanosine (Fig. 4B). These findings suggest that MPA has a largely IMPDH-independent anti-HCV mechanism, whereas inhibition by MPA of other viruses (like West Nile) seems entirely dependent on guanosine depletion.
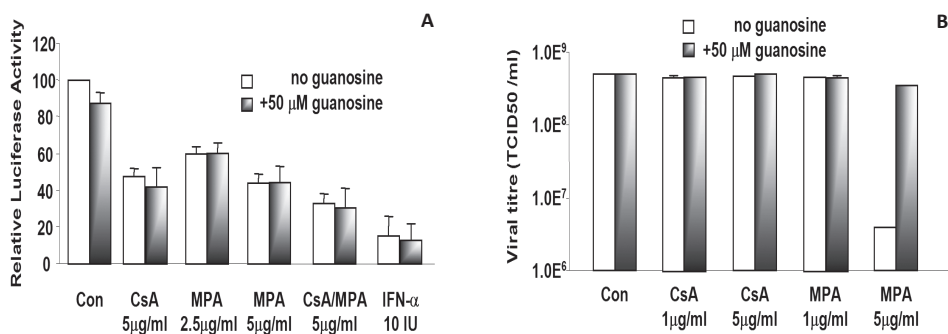
Fig 4. Inhibition of HCV replication is not dependent on guanosine, whereas inhibition of West Nile Virus is guanosine-dependent. A) Huh-7 replicon cells were incubated for 18 hrs with CsA (5 μg/ml), MPA (2.5 or 5 μg/ml), MPA/CsA combined (both 5 μg/ml) or peg-IFN-a 2a (10 IU/ml) with or without 50 μM guanosine. The relative luciferase activity, as a measure of HCV replication, was calculated as a percentage of untreated cells (Con). When used in combination, exogenous guanosine did not reversed the MPA-induced inhibition had no effect on inhibition of HCV replication by CsA or IFN-α (n=8). B) Guanosine supplementation of West Nile virus infected Vero-E6 cells completely overcomes the inhibition by MPA. Guanosine and CsA alone had no effect on West Nile virus replication. Shown are the mean of triplicates of one representative experiment.

Chapter 4
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MPA acts synergistically with CsA and IFN-α

MPA and CsA have distinct molecular targets. To determine if these compounds could potentially act synergistically against HCV, different combination doses were tested. As shown in figure 5, when optimal concentrations of MPA and CsA were combined, increased inhibition of HCV replication was observed, reaching maximum inhibition of 90% (± 2 SEM). Increased inhibition was also seen with a combination of MPA and a suboptimal dose (0.1 µg/ml) of CsA (Fig. 5A). MPA and CsA inhibition of HCV replication is not merely additive, but synergistic, based on Bliss Independence Zero Interaction calculations. A significant shift (p<0.001) from expected (additive) to the observed synergistic line was seen (Fig. 5B). The maximal concentration shift occurred with 0.9 µg/ml CsA, dropping the EC60 dose of MPA from 3.4 µg/ml to 2.0 µg/ml. When MPA or CsA where combined with 0.5 µg/ml Tacrolimus, 3 µg/ml Rapamycin or 5 nM Prednisolone, no synergistic or additive inhibition was observed (data not shown).

MPA and CsA were tested in combination with IFN-α. When MPA was combined with suboptimal doses of IFN-α, inhibition of HCV replication was increased (Fig. 6A). When CsA was combined with suboptimal doses of IFN-α, increased inhibition of HCV replication was also seen (Fig. 6B). A significant shift (p<0.001) from expected (additive) to the observed synergistic line was seen for both MPA (Fig 6C) and CsA (Fig. 6D). For MPA, the maximal concentration shift occurred with 0.4 IU/ml IFN-α, dropping the EC60 dose of MPA from 3.4 to 0.8 µg/ml. The maximal concentration shift for CsA occurred with 0.1 IU/ml IFN-α, dropping the EC60 dose from 0.9 to 0.2 µg/ml.
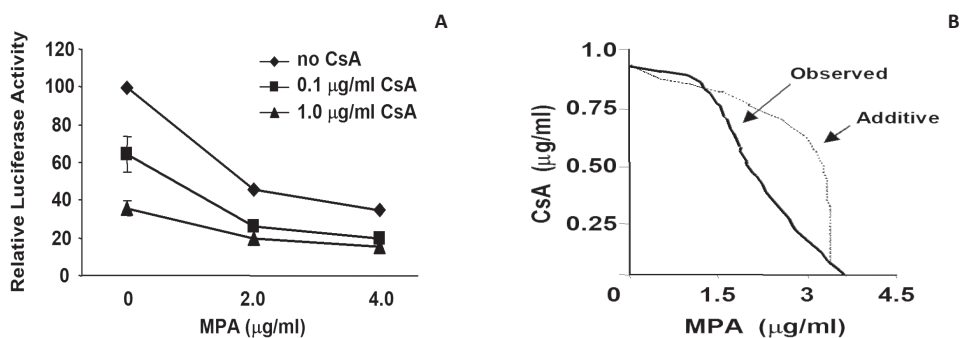
Fig 5. MPA and CsA act in synergy on HCV replication.Huh7 replicon cells were incubated for 18 hrs with different concentrations of MPA, alone, or in combination with 0.1 or 1.0 µg/ml CsA. A) The relative luciferase activity was calculated as a percentage of the vehicle control. Significant synergistic inhibition was observed with any combination of MPA and CsA (p<0.05, Wilcoxon test). Maximum inhibition (~90%) was observed at highest concentrations. Shown is one representative experiment of five. B) EC50 isobologram of the combination of MPA with CsA. The dotted line indicates the expected effect of the two drug combinations, assuming an additive relationship. The solid line indicates the observed effect of the combination of the two drugs. Any point lower than the additive line indicates a synergistic combination. These findings suggest that inhibition of HCV by MPA and CsA is not merely additive but truly synergistic.
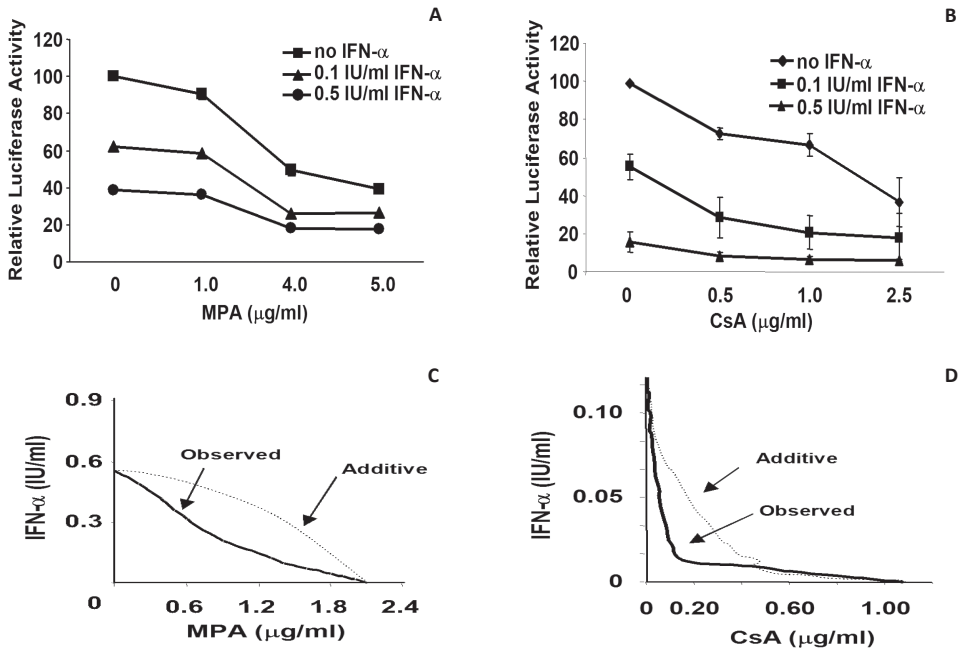
Fig 6. Synergistic inhibition of HCV replication by MPA or CsA combined with IFN-α. Huh7 replicon cells were incubated for 18 hrs with suboptimal doses of IFN-α alone or in combination with different concentrations of MPA (A) or CsA (B). The relative luciferase activity, as a measure of HCV replication, is represented as a percentage of DMSO (A) or EtOH (B) controls. Significant synergistic inhibition was observed with any combination (p<0.05, Wilcoxon test) (N=3). EC50 isobologram of the combination of MPA with IFN-α (C) or CsA and IFN-α (D). The dotted line indicates the expected effect of each two combinations, assuming an additive relationship. The solid line indicates the observed effect of the combination of the two drugs. Any point lower than the additive line indicates a synergistic combination. These findings suggest that inhibition of HCV by IFN-α and these immunosuppressants is synergistic, not merely additive.

MPA, CsA and IFN-α show different individual or combined anti-viral kinetics

The kinetics of the anti-HCV response was determined by real-time luciferase activity in live Huh-7 replicon cells. Cells were treated and luciferase activity monitored for 20 continuous hours. In untreated (dashed line) and tacrolimus treated cells (Fig. 7A), luciferase activity increased over time with the expansion of the replicon cells. The earliest effects were seen with IFN-α, beginning at 7 hrs and reaching near complete inhibition within 12 hrs of treatment (Fig. 7B). Cells treated with CsA showed progressive inhibition of viral replication beginning at 8 hours and continuing throughout the course of the experiment (Fig. 7C). MPA had a distinct anti-viral kinetic profile. A steep reduction in replication was seen between 8 and 10 hrs after treatment followed by a sustained inhibition throughout the remainder of the experiment (Fig. 7D). Interestingly, when MPA and CsA were combined, the anti-viral profile was a near composite of the individual kinetics, showing both the initial steep decrease in replication at 8 hrs of MPA and the sustained inhibition of CsA (Fig. 7E). These distinct anti-viral profiles of IFN-α, CsA and MPA and their synergistic effects suggest different mechanisms of action for each of these agents.
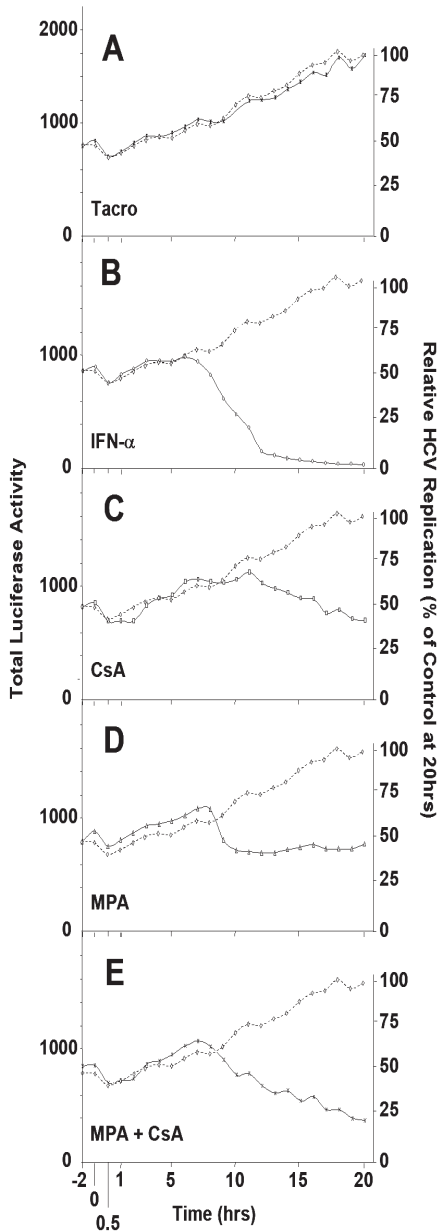
Total Luciferase Activity

Relative HCV Replication (% of Control at 20hrs)

Time (hrs)

Fig 7. Distinct kinetics of viral inhibition by MPA, CsA and IFN-a. Huh-7 replicon cells were treated with optimum doses of various agents (10 IU/ml peg-IFN-α 2a, 0.5 μg/ml Tacrolimus, 5 μg/ml CsA or 5 μg/ml MPA) or the combination of MPA and CsA (both at 5 μg/ml). The luciferase activity was read in live cells for 20 hrs to determine a time course of their anti-viral activities. Untreated Huh-7 replicon cells (dashed line) showed a steady increase in replicon activity reflecting increase in cell numbers. A) Cells treated with Tacrolimus showed no decrease in HCV replicon activity and followed the same upward trend as untreated cells. B) Treatment with IFN-α showed a rapid decline in HCV replicon, leveling out at 97% inhibition. C) Cells treated with CsA showed a progressive inhibition of replicon activity starting at 8 hrs. D) Cells treated with MPA showed a steep drop in replication between 8 and 10 hrs and then sustained inhibition throughout the remainder of the experiment. E) Cells treated with a combination of MPA and CsA showed a composite profile of both individual kinetics. Taken together, all anti-viral treatments have distinct kinetics and a combination of MPA and CsA results in combined kinetics. Shown is the mean of triplicates of one out of three independent experiments.

Discussion

In the current study we demonstrated that MPA, like CsA, specifically inhibits HCV replication in an *in vitro* replicon model. Inhibition was dose-dependent and resulted in a reduction of both HCV RNA and NS3 protein expression. The anti-HCV effect of MPA was not due to a reduction in cell proliferation as was suggested in a preliminary report[35]. In short-term cultures MPA did not inhibit cell proliferation, whereas the expected anti-proliferation effect of MPA was seen at 5 days. The kinetics of HCV inhibition by MPA, CsA and IFN-α were clearly distinct suggesting independent mechanisms of action.

Inhibition of replication by MPA was observed at clinically relevant concentrations. In liver transplant recipients receiving MMF or MPA, serum peak levels range from 0.6 to 11.5 μg/ml with the average trough level near 3.1 μg/ml[36]. In the replicon model we found a half-maximum inhibition at ~2.0 μg/ml MPA (Fig. 1). Thus, clearly effective anti-viral concentrations of MPA can be achieved in serum but also in the liver, as animal studies indicate that MPA accumulates in the liver[37]. For CsA, the blood level at 2 hours (C2-maintenance levels) ranges from 0.6 to 1.0 μg/ml[38]. In the replicon model

we observed a half-maximum inhibition at approximately 0.8 μg/ml (Fig. 1). It has been shown in human and rat studies that the concentration of CsA in the liver is at least seven-fold higher then in whole blood[39, 40]. The intrahepatic accumulation of both drugs indicates that it is feasible to reach effective anti-viral concentrations in the liver for MPA and CsA.

To our knowledge this is the first study that clearly demonstrates anti-viral activity of MPA against HCV. MPA's anti-viral activity is already known for a range of other RNA and DNA viruses, including HIV, HBV and West Nile virus. The current study contradicts a previous report addressing the *in vitro* anti-HCV properties of MPA[41]. Zhou et al showed no significant effect of scientific grade MPA (Sigma, St. Louis, USA) on HCV RNA, but did observe a greatly improved anti-viral effect when used in combination with ribavirin. We observed HCV inhibition using clinical grade MPA from Novartis Pharma (Basel, Switzerland). The production of clinical grade MPA and MMF generates several methylated intermediates[42] and it is conceivable that these impurities could contribute to the anti-viral effect against HCV.

Clinical studies that have addressed the issues of immunosuppressants in HCV positive liver transplant recipients focused on rejection and patient survival but lacked analysis of hepatitis C viral load[23, 43-47]. Five published studies addressed the effect of MPA on HCV viral load when compared to other immunosuppressants that lack anti-viral activity (e.g. Tacrolimus and steroids) three of which were prospective and none were randomized. Within the prospective studies, two reported a reduction of viral load in the MPA group[21, 24] (12 and 11 patients, respectively) and one did not[25] (13 patients). The two retrospective studies are contradictory, one showing a viral load reduction in 40 patients on MPA[22] and the other not showing any difference in 22 patients[27]. Four studies examined HCV-related fibrosis, two of which were prospective and randomized[23, 46] (278 and 50 patients on MPA, respectively) and two were retrospective[22, 44] (8 and 40 patients on MPA, respectively), all report beneficial effects of MPA. Though not decisive, most clinical studies support the anti-viral effect of MPA as reported here.

Despite the potential beneficial effects in transplantation patients, no clear anti-viral effect was observed with MPA monotherapy in chronic HCV patients outside the transplant setting[26]. Until recently the nucleoside analog ribavirin was considered to have no detectable anti-viral activity when given without IFN-α. A recent study clearly demonstrates anti-viral effects of ribavirin by analyzing the early viral kinetics during ribavirin monotherapy, though these effects were limited and transient[48]. When combined, ribavirin clearly increases the sustained virologic response of IFN-α by threefold. *In vitro*, we found that MPA, like ribavirin, can act in synergy with pegylated IFN-α (Fig. 6). There is now analogous clinical evidence that MPA potentiates IFN-α therapy[49]. In contrast to a previous study using an ineffective IFN-α therapy[50], Herrine et al demonstrated that combining MPA with pegylated IFN-α in chronic HCV patients resulted in a better end-of-therapy response rate (72%) over the current standard treatment (ribavirin/IFN, 59%) or amantadine/IFN group (42%). Though MPA exhibits clear anti-viral activity *in vitro*, the effect of MPA monotherapy on the HCV viral load, like for ribavirin monotherapy, is difficult

to study *in vivo* and might only be observed when looking at the early anti-viral kinetics. It is possible if MPA is used in combination with other anti-viral agents, that its anti-viral effect could give enough of an initial drop in HCV viral loads to actuate the effects of the other, pushing the virus below a threshold level that allows both the immune system and the anti-viral treatments to effectively clear the virus.

We observed an anti-viral effect of MPA in a HCV replication model that clearly lacks the element of a host immune response. In chronic HCV patients, viral replication evokes an ongoing immune response by virus-specific T-cells. Inhibition of this immune response by immunosuppressive drugs, like in HCV transplant recipients, is known to enhance viral replication and disease progression[51]. As MPA is a potent immunosuppressor it is conceivable that the gross effect on viral replication, as observed in the replicon model, is countered *in vivo* by the inhibition of the anti-viral immune response. Consistent with this idea, a study in chronic HCV patients treated with MPA/IFN combination therapy showed a superior on-therapy virologic response rate but had a reduced end-of-treatment response compared to ribavirin/IFN group[49] (17% versus 38%). Thus, their effects on the host immune response could mask changes in viral load by anti-viral activity of immunosuppressive drugs, like CsA or MPA.

Inhibition of cell proliferation by MPA acts via the de novo guanosine synthesis pathway[12]. MPA interferes with IMPDH that catalyzes the conversion of IMP to XMP via NAD-dependent oxidation[52]. By interfering with the precursors of GMP production, MPA can reduce cellular and viral replication[13, 14]. In the current study we have found that MPA could have a specific anti-viral mode of action, unrelated or at least partially removed from its guanosine depletion ability. The addition of exogenous guanosine did little to reverse the anti-viral activity of MPA on HCV replication (Fig. 4A), though MPA's reduction of West Nile virus was completely reversed (Fig. 4B). Inhibition of HCV replication by MPA was observed within 18 hrs without inhibition of cell proliferation (Fig. 3A, C). At later time points, cell proliferation was arrested (Fig. 3B, D), long after the initial anti-viral effect. This indicates that MPA has two effects on HCV replication, one that initiates rapidly and is independent of guanosine and one slower, guanosine-dependent, inhibition (not shown).

The exact mechanism by which MPA inhibits viral replication is still unknown. Avian reovirus has been reported to be inhibited by MPA through the minor core protein of the virus which has presumed nucleoside triphosphatase activity[20]. No such equivalent viral protein has been identified in the HCV genome, thus excluding this mode of inhibition. Recent studies by Watashi and Nakagawa demonstrate that CsA inhibits HCV replication through cyclophilins, particularly cyclophilin B, that acts as a functional regulator of the RNA-dependent RNA polymerase (NS5B)[9, 10]. To date there is no evidence that MPA acts on cyclophilins and thereby intervenes with the NS5b activity. This does not rule out the possibility that MPA affects NS5B activity via a cyclophilin-independent pathway or that it more specifically targets other components of the HCV replication machinery. One possibility is that MPA affects the NS5B activity by transiently

out competing nucleotides for the active site during RNA replication, similar to ribavirin. In this way MPA may act as a mutagen, reducing viral fitness and promote error catastrophe. Alternatively, some of the methylated intermediates in the clinical grade MPA preparation might be affecting viral replication.

In our short-term culture experiments, we did not observe complete inhibition of HCV replication by either MPA or CsA. Both drugs inhibited replication up to 75%. However, when combined, MPA and CsA show a significant synergistic effect, reaching almost complete inhibition of replication (Fig. 5). This combined effect was not merely additive, but was synergistic based on the Bliss Independence Zero Interaction model (Fig. 5B). The clinical relevance of these findings remains to be determined; however one can speculate that a combination of MPA and CsA may be more effective in reducing HCV recurrence after liver transplantation. Although still controversial, there is clinical evidence suggesting favorable effects of this combination of drugs in HCV-positive liver transplant recipients. In a prospective randomized double blind study by Wiesner et al[46], a marked reduction of histologically-proven HCV recurrence in patients treated was seen with a combination of corticosteroids, CsA and MPA (278 patients) versus a combination of corticosteroids, CsA and azathioprine (287 patients), a compound that has no profound effect on HCV replication *in vitro*[53].

In conclusion, we have demonstrated that the immunosuppressive drug MPA, like CsA, is a potent inhibitor of HCV replication *In vitro* and has a unknown mechanism of action, independent of guanosine depletion. The different anti-viral kinetics and synergy between MPA, CsA and IFN-α suggests that these drugs act via independent pathways on HCV replication. A combination of MPA and CsA could be beneficial to reduce HCV recurrence after liver transplantation and could be used as an adjuvant to IFN-α anti-viral therapy in non-transplant patients.
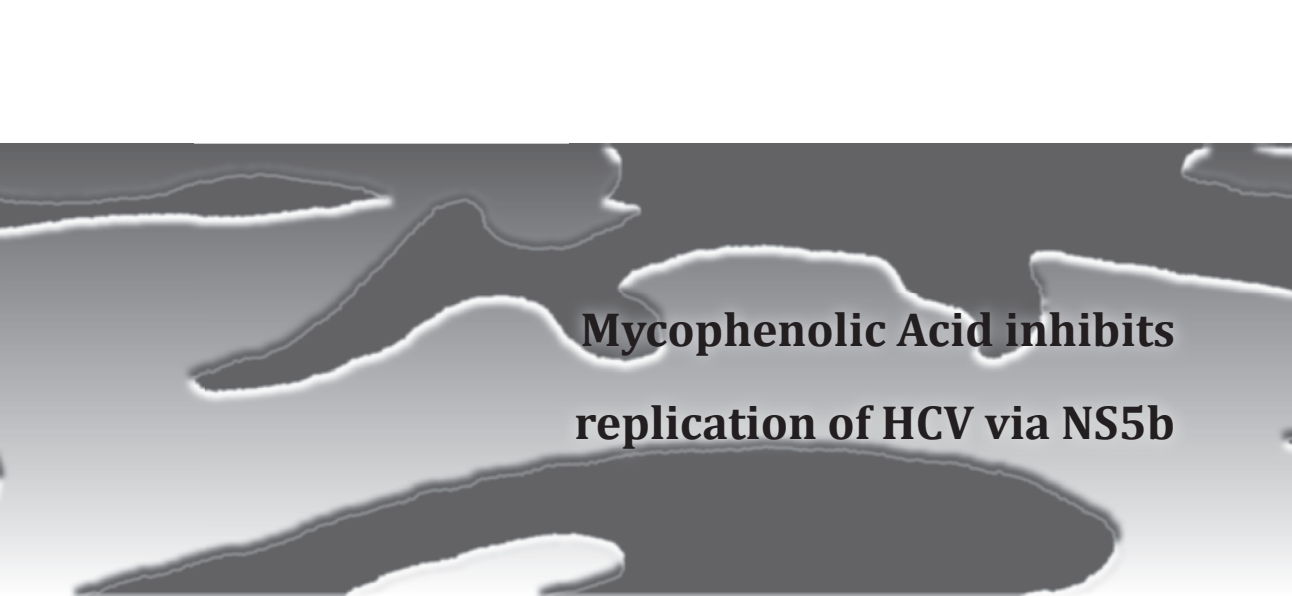
Acknowledgements

CHAPTER 5

Manuscript in Preparation

Mycophenolic Acid inhibits replication of HCV via NS5b

Scot D. Henry[1]

Antoine van der Sloot[1]

Herold J. Metselaar[2]

Hugo W. Tilanus[1]

Luc J. W. van der Laan[1*]

Departments of [1]Surgery , [2]Gastroenterology & Hepatology

Erasmus MC-University Medical Center, Rotterdam, The Netherlands.

Abstract

Mycophenolic acid (MPA) is a highly effective immunosuppressant, used to prevent rejection post organ transplantation. MPA blocks the enzyme inosine monophosphate dehydrogenase (IMPDH) which slows growth of cells due to a loss of cellular guanosine pools. MPA has been shown to affect the replication of various viruses through this mechanism, but is currently not used as an anti-viral. Though MPA is able to reduce HCV replication *in vitro*, independent of limiting de novo guanosine production, the exact mechanism in which it does so remains to be elucidated. Direct effects of MPA on HCV replication are expected to be due to interference to proteins required for replication, the RNA dependent RNA polymerase (NS5b) being a prime candidate, known to non-covalently bind non-nucleoside analogues. The current study explores the direct effects of MPA on the NS5b *in vitro*.

MPA was compared to other IMPDH inhibitors, Ribavirin (Rib) and Bredinin (Bred), in a HCV replicon cell line. In 18hr cultures MPA reduced the ability of HCV to replicate by a maximum of 75% of controls whereas Rib and Bred had little effect. Proliferation was unaffected at 18hr by any inhibitor, though at 72hr, all inhibitors slowed cellular growth when compared to controls.

The direct effects of MPA on the NS5b was tested *in vitro* using variations of a two plasmid NS5b/Luciferase expression system; the first plasmid expressing full length, functional NS5b and the second expressing a negative strand Luciferase RNA cassette (rLuc). The presence of both plasmids creates a functional luciferase protein, ensuring that only interference with the NS5b would result in a reduction in luciferase expression. Variations include; bacterial two plasmid isolated NS5b assay, T7 driven RNA transfections to Huh-7 and Huh-7 replicon cells and lentiviral driven rLuc expression in replicon cells.

At 30μM MPA inhibited the bacterial dual plasmid expression of luciferase by approximately 32%±6SEM (p=0.002) and did not induce bacterial cell death. Increasing concentrations of rLuc RNA transfections to replicon cell lines increased luciferase expression, with the addition of MPA at 15μM reducing expression to background levels.

The immunosuppressive drug MPA is a potent inhibitor of HCV replication. Preliminary results indicate that MPA has a direct effect on the NS5b polymerase of HCV.

Introduction

The activated form of Mycophenolate mofetil (MMF), Mycophenolic acid (MPA), is a highly effective immunosuppressant which lacks the nephrotoxicity associated with calcineurin inhibitors and is often used to prevent rejection post organ transplantation. MPA functions as a immunomodulatory agent due to its ability to inhibit inosine monophosphate dehydrogenase (IMPDH), the enzyme responsible for the conversion of inosine 5' monophosphate (IMP) into xanthosine 5' monophosphate (XMP) at the beginning of the guanosine triphosphate (GTP) and deoxy-guanosine triphosphate (dGTP) de novo synthesis pathways[1]. Inhibition of IMPDH decreases levels of intracellular guanosine nucleotide pools resulting in inadequate quantities for nominal DNA duplication. As such; MPA has been shown to have antiproliferative[2], immunosuppressive[3], and antiviral and anti-tumor effects[4]. Its antiviral effects have been shown *in vitro* for dengue virus[5], HBV[6], avian reovirus[7, 8], yellow fever[9], and West Nile virus[10], and most recently against HCV[11]. Inhibition of West Nile virus and yellow fever virus by MPA could be overcome by activating the nucleoside salvage pathway and bypassing the reduction in GTP pools caused by the blockade of IMPDH, by the addition of exogenous guanosine[9, 10]. This suggests that the antiviral effect of MPA for these viruses acts solely via IMPDH blockade and prevention of de novo guanosine nucleotide production, though data from our group suggests this is not the case for HCV[11] or the SARS corona virus (unpublished data). Therefore other mechanisms are responsible for anti-HCV effect of MPA.

Our group has shown previously that MPA has a direct effect on HCV replication that is independent of its ability to reduce the total GTP pools, though its exact mechanism was not determined. The HCV genome is comprised of 9 structural and non-structural proteins though only 5 are essential for replication; the NS3, NS4a, NS4b, NS5a, and NS5b[12]. Each of these proteins is a potential target for the antiviral mechanism of MPA as we described earlier[11], though no evidence, to date, supports any as specific targets. Potent inhibitors have been developed against the NS3 and NS3-NS4a complexes[13-16], though many more have been developed against the RNA dependant RNA polymerase (NS5b)[17-20]. Its active site as well as three allosteric binding pockets can be suitable targets for development of inhibitors, as such the NS5b could be a possible target for MPA. The current study explores the direct effects of MPA, specifically on the NS5b, *in vitro*, in an attempt to elucidate its molecular pathway against HCV.

Methods

Reagents

MPA (dissolved in DMSO) were provided by Novartis Pharma AG (Basel, Switzerland), Ribavirin and Bredinin (kind gifts from Johan Neyts, Rega Institute, Lueven, Belgium) were dissolved in PBS at 25mM and 3.6 mM respectively. All agents were stored in 15 µl aliquots and frozen at –20°C, excluding CsA which was stored at 4°C. Hygromycin (diluted in PBS) was purchased from Sigma and stored in 100x concentrated aliquots; Geneticin (G418-Sulfate dissolved in PBS) was purchased from Invitrogen-Gibco (Breda, The Netherlands) and stored in 100x concentrated aliquots.

Cell Cultures & Treatments

Cell monolayers of the human hepatoma cell line Huh-7 or Huh-6[21], or the human embryonic kidney epithelial cell line; 293T, were maintained in Dulbecco's Modified Eagle Medium (DMEM) complemented with 10% v/v fetal calf serum (PerBio Science), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-Glutamine (Invitrogen-Gibco) (cDMEM). Hepatoma cell lines containing subgenomic HCV monocistronic replicon (I_{389}/NS3-3'/Con1) (Huh-6) without reporter construct or bicistronic (I_{389}/NS3-3'/LucUbiNeo-ET) (Huh-7-ET) containing the luciferase reporter gene[22] were maintained with 250 µg/ml G418 (Sigma).

Replicon cells, Huh-7-ET, were plated in cDMEM at 70-80% confluence and treated with various concentrations of drugs and incubated at 37°C for 18 hrs. Concentration-range tested for MPA; 3-30 µM, Ribavirin; 25-75 µM, and Bredinin; 3.6-32 µM. Cells were retrieved from monolayers by Trypsin/EDTA (Invitrogen/Gibco) digestion (for luminescence) or directly lysed in the wells (for RT-PCR).

Luciferase Assay

Cells for endpoint assays were lysed and luciferin substrate added using Steady Glo Luciferase System (Promega, Leiden, The Netherlands) according to manufacturer instructions. Live cell experiments were performed as described above (excluding lysis step) with the addition of 100 µM luciferin potassium salt (Sigma). Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Cell Viability and Proliferation

Cell viability was determined by 7AAD staining (BD Pharmigen, San Diego, USA) to replicon cells followed by FACS analysis. 7AAD negative cells were considered viable, whereas apoptotic and necrotic cells were positive for 7AAD[23]. Proliferation profiling was achieved with Carboxy-fluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands) staining. In brief, 150,000 Huh-7 replicon cells were treated with 20 µM CFSE and incubated according to manufactures protocol and seeded in 12-well plates. After 18 hr or 3 days, cells

were treated with 7AAD at a 1:300 dilution and proliferation staining was measured via flow cytometry (BD FACS Calibur). Generational analysis was performed with ModFit LT v3.0 (BD Pharmigen) software and was gated to exclude 7AAD positive cells. Parent generation was assessed at highest CFSE concentration.

Plasmids

A non-isotopic two plasmid NS5b activity assay, developed by Lee et at[24] was maintain in *Escherichia coli* (DH5α). Briefly, one plasmid generated from the pBluescript SK plasmid contains a 21 amino acid (hydrophobic C-terminus) truncated functional NS5b (pSKNS5), under ampicillin (AmP) resistance. The second, pACLuc, was generated from the pACYC184 plasmid and contains the HCV 5' UTR (entire 341 nucleotide 5'-terminus plus 76 nucleotides from 5'-terminus of core-coding region of HCV genomic RNA) and firefly luciferase in the minus sense, under chloramphenicol (CmR) resistance. Both plasmids contain replication origins from different sources, allowing them to co-exist in the same cells.

Bacterial Isolated NS5b Assay

Plasmids pSKNS5 and pACLuc were transformed into DH5α and maintained under dual selection (AmP and CmR). Fresh cultures were grown for 2 hours and various concentrations of MPA added and incubated for 2-4 hours. Bacterial cells were collected with brief centrifugation, resuspended in lysis buffer (1 μg/ml lysozyme, 20% (w/v) sucrose, 30mM Tris–Cl, pH 8.0, 1mM EDTA) with vortexing. The relative luciferase expression was read as above.

Cell Culture Replicon NS5b assay

Huh-7-Hy replicon cells were electroporated with a Gene Pulser X-Cell (BioRad, Hercules, USA) as previously described[12] with 20μg RNA prepared from T7 driven pACLuc using a mMessage mMachine T7 Ultra Kit (Ambion, Austin, USA) and control RNA. Cells were incubated for 48 hrs post transfection, then treated with MPA at various concentrations for 18hrs and the relative luciferase expression was read as above.

Cell Culture Isolated NS5b assay

Huh-7 or 293T cells were electroporated with a Gene Pulser X-Cell (Bio-Rad, Hercules, USA) as previously described[12] with 20μg RNA prepared from T7 driven pSKNS5 and pACLuc using a mMessage mMachine T7 Ultra Kit (Ambion, Austin, USA) and control RNA. Cells were incubated for 48 hrs post transfection, then treated with MPA at various concentrations for 18hrs and the relative luciferase expression was read as above.

Real-time RT-PCR

Confluent monolayers of replicon cells were lysed by Trizol (Invitrogen-Gibco) and RNA precipitated with 75% EtOH and captured with a Micro RNAeasy silica column (Qiagen, Venlo,

The Netherlands). RNA was quantitated using a Nanodrop ND-1000 (Wilmington, Delware, USA) and adjusted to total cell numbers for each sample. cDNA was prepared using Promega's AMV reverse transcriptase following standard protocols. Real-time PCR (BioRad) was performed using primers NS5b (F-5'- CGG GAG AGC CAT AGT GG 3', R-5'- AGT ACC ACA AGG CCT TTC G -3'), rLuc (F-5'- AAT CGT CGT ATG CAG TGA AA-3', R-5'- TGA GCA ATT CAC GTT CAT TA -3') and GAPDH (F-5'- CCA TGG AGA CTG GGG-3', R-5'-CAA AGT TGT CAT GGA TGA CC-3'). Semi-quantitation of sample amplicons was performed with SybrGreen (Sigma) using Taq DNA polymerase (Invitrogen-Gibco) as previously described[25].

Results & Discussion

Cyclosporine A (CsA), Mycophenolic acid (MPA), tacrolimus (FK506) and rapamycin (Rapa) are the most widely used in transplantation today. Evidence based research has suggested that both CsA[26] and MPA[11] have direct effects on the replicative ability of HCV.

The predominate anti-viral effect of MPA, for viruses other than HCV, lies in its ability to block the enzyme IMPDH resulting in a reduction of total cellular GTP. To determine whether MPA's anti-HCV activity is related solely to the blockade of IMPDH, we compared two additional, nucleoside analogue competitive IMPDH inhibitors, Ribavirin (1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide)[27] and Bredinin (4-carbamoyl-1-ß-{scd}-ribofuranosylimidazolium 5-olate)[28, 29] in the same time period as we saw the anti-HCV effect of MPA. At concentrations that are effective to reduce cellular GTP pools, 25µM ribavirin significantly reduced HCV replication to 67%±9 SEM, bredinin reduced replication with all tested concentrations (3.6µM; 74%±13SEM, 18µM; 80%±9SEM, 36µM 52%±10SEM), whereas MPA significantly reduced HCV replication (5µM; 65%±13SEM, 10µM; 35%±4SEM, 30µM 24%±4SEM) of controls (Fig. 1) within the 18hr time period. When MPA was compared to the other IMPDH inhibitors, MPA's
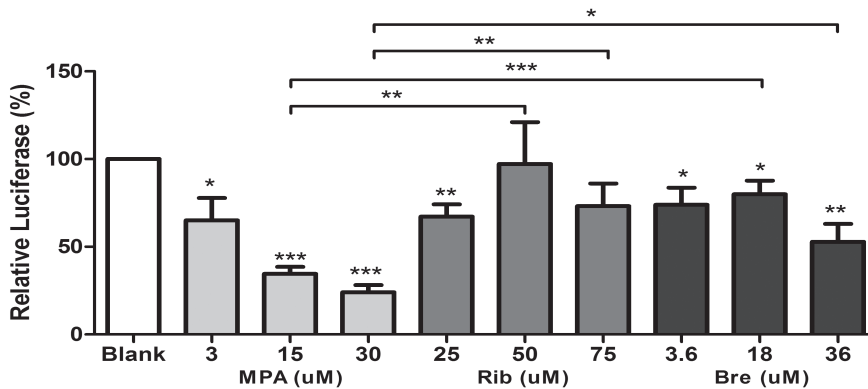
Fig 1. MPA reduces HCV replication significantly when compared to Ribavirin or Bredinin. Huh-7 replicon cells were treated for 18hrs with similar concentrations of IMPDH inhibitors; MPA, Ribavirin and Bredinin. MPA reduced HCV replication significantly in a dose response when compared to controls (3µM; 65%±13SEM, 15µM; 35%±4SEM, 30µM 24%±4SEM), whereas Ribavirin only reduced HCV replication to 67%±9SEM of controls with the lowest dose tested (25µM). Bredinin significantly reduced HCV replication with all tested concentrations (3.6µM; 74%±13SEM, 18µM; 80%±9SEM, 36µM 52%±10SEM). MPA significantly reduced HCV replication when compared at the median or high concentrations to the other IMPDH inhibitors. (* p<0.05, ** p<0.01, *** p<0.001).

reduction of HCV replication was significant at both the median and high doses used. To confirm the concentrations used were not affecting cellular proliferation within the 18hr time period, Huh-7 cells were labelled with CFSE and treated with the median concentrations of each IMPDH inhibitor. Cells were cultured in the presence of the inhibitors for 18hr or for 72 hours and the proliferation profile was determined. Within 18hr cultures, none of the inhibitors affected cellular growth, as no significant change was seen in the proliferation index (PI) of their profiles
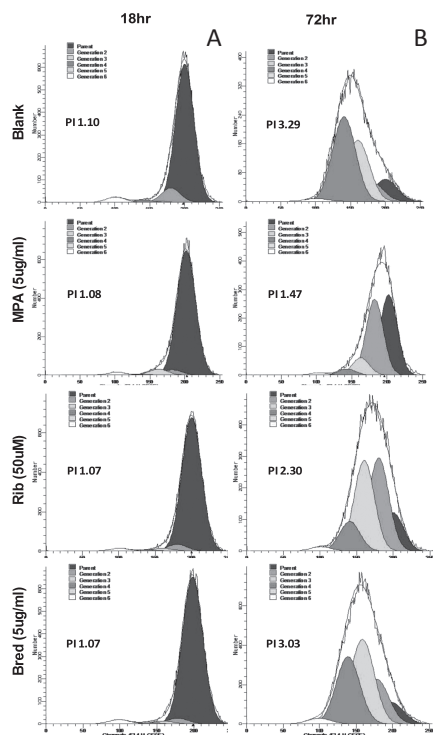
Fig 2. Cell proliferation was unaffected in the 18hr time period. Neither MPA, Ribavirin nor Bredinin reduced cell proliferation within the 18 hour testing period (A), though at 72 hours, all three affected cell proliferation to variable degrees (B).

(Fig. 2a). Within the 72hr cultures, all three reduced cell proliferation to varying degrees (Fig. 2b). This indicated that the reduction on HCV replication as seen in Figure 1 was not due to the reduction of replicon cell expansion.

As the anti-HCV effects of MPA were shown to be unrelated to cellular proliferation, its effects should be specific to viral replication processes. Within the HCV genome the NS3, the serine proteinase and the NS5b, the RNA-dependent RNA polymerase are the most important proteins involved in HCV replication. Hence, these two targets have been the main focus of targeted drug developments. The ability of the NS5b to copy negative strand RNA virus into translatable positive strand RNA, makes it's a particularly attractive component as any reduction in the activity of NS5b would drastically reduce the ability of HCV to replicate, as has been shown through replicon mutational studies[30, 31]. Many small, non-nucleoside analogue inhibitors have been developed against the NS5b[32, 33], many of which contain phenylalanine residues which has been shown to non-covalently bind to the hydrophobic

depression in the thumb domain of the NS5b[34-36]. As MPA has similar structural properties to the small compounds tested, it was reasonable to speculate whether MPA was acting on the NS5b in the same manner as these other non-nucleoside analogues.

To elucidate the specific anti-viral effect of MPA, a two plasmid bacterial system developed by Lee et al[24] was used to isolate and show specific effects on the HCV NS5b. The first plasmid contains a functional truncated NS5b and the second codes for a negative strand luciferase RNA coupled to a negative strand HCV internal ribosome entry side to facilitate its reverse coding into a functional RNA that can be translated by
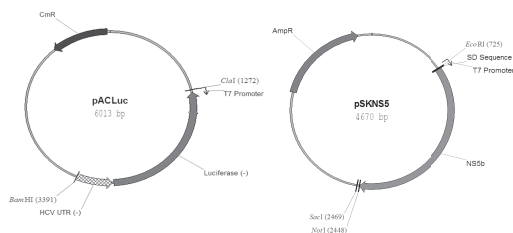

Fig 3. Schematic diagram of the two plasmid system. Each plasmid contains a distinct resistance gene (AMP for pSKN5 and CmR for pACLuc) as well as distinct replication origins that allow them be present and expressed effectively in the same bacterial cell.

host ribosomes into a functional luciferase reporter enzyme (Fig. 3). Bacterial cultures containing both plasmids were grown into log phase and then treated with MPA for 2 hours. Cells were lysed and active protein isolated. Luciferin potassium salt was added and relative light units were read via luminescent counter. As shown in Figure 4, MPA significantly reduced the ability of the NS5b to code for functional positive strand luciferase RNA, lowering the overall relative luciferase expression to 70%±6 SEM (p=0.002) of controls. This reduction was not as significant as the earlier results with the replicon cell lines, though this bacterial assay may not be as robust as the cell culture method, as well, the bacterial cells were only exposed to 2 hours of MPA, whereas the replicon cells were treated for 18h or more hours. Though preliminary, this result suggested that MPA was affecting the functionality of the NS5b in some manner.
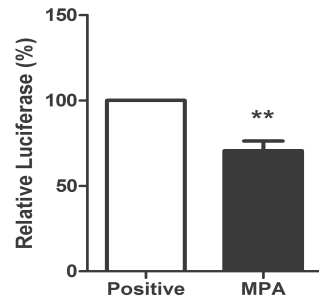
Fig 4. MPA significantly reduced luciferase protein levels in an isolated two plasmid NS5b functional assay. MPA at 5μM significantly reduced expression luciferase to 70%±6 SEM (p=0.002) with a two hour incubation.
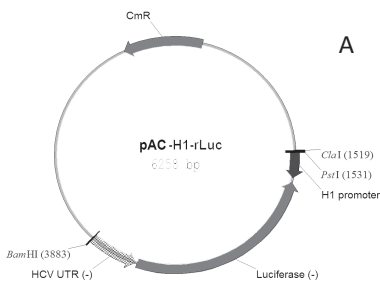
Fig 5A. Schematic diagram of the new H1 driven rLuc plasmid system.
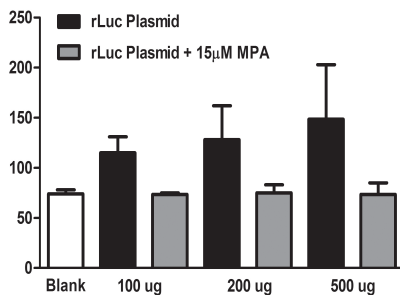
Fig 5B. MPA reduced luciferase protein levels after a two hour incubation with increasing concentrations of H1-rLuc expression plasmid were transfected to Huh-7 replicon cells (n=1).

As the bacterial model system more than likely does not represent the *in vitro* or the *in vivo* situation of human liver cells, the model was modified to allow for expression in a eucaryotic system. To show the effect in a more robust *in vitro* system, the reverse coding luciferase plasmid was modified to include a human H1 promoter that would facilitate its expression in human cell lines (Fig. 5A). This new plasmid termed pAC-H1-rLuc or the RNA generated from the original T7 promoted pACLuc plasmid was electroporated in increasing concentrations to replicon cells (Huh-7-Hy) and treated with MPA for 18 hours. Figure 5B shows the expression level of the plasmid expressing under the H1 promoter in increasing concentrations and the resulting knockdown of NS5b mediated luciferase expression.

Specific effects of MPA on the ability of NS5b to produce positive strand RNA copies from a negative strand template were tested in a cell free active protein PCR like assay. E. Coli expressing soluble NS5b were lysed by sonication and total protein was combined with
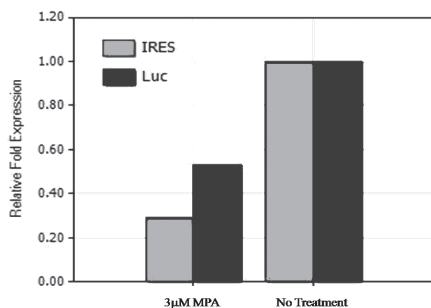
Fig 6. MPA reduces positive strand RNA production by impeding NS5b activity. RT-PCR was performed on products of a cell free NS5b assay. Two primer sets (Luc and IRES) detected positive strand RNA after 90min incubation with negative strand rLuc template RNA, with or without MPA (n=1).

NTPs and negative strand RNA from pACLuc. With the addition of MPA at 15µM, total production of positive strand rLuc RNA was reduced to 30% (IRES primers) and 50% (Luc primers) of controls. This indicated that MPA interferes with the ability of HCV's NS5b to copy negative strand RNA (Fig. 6).

Mycophenolic Acid has clear effects as an immunosuppressant due to its ability to inhibit the reaction of XMP to GMP by IMPDH, though its ability to down regulate HCV has only recently been elucidated. We show here the exploration of the possible mechanisms behind the antiviral effects in short term MPA treated replicon cell cultures. MPA significantly reduced the ability of HCV to replicate *in vitro*, whereas other IMDPH inhibitors Bredinin and Ribavirin were less potent. Within 18hr cultures, MPA had no effect on the ability of replicon cells to proliferate, indicating that MPA has a direct effect on the virus itself and is unrelated to the blockade of IMPDH and the subsequent reduction in cellular GTP pools as the addition of exogenous guanosine nucleosides did not reverse MPA's effect on HCV replication[11]. Though preliminary, this study reveals more evidence confirming that MPA has a direct effect on HCV and its ability to replicate *in vitro*. Data presented here suggests that MPA acts directly on the NS5b. This is mostly likely in a manner similar to that of other non-nucleoside inhibitors, by binding non-covalently to the hydrophobic shallow pocket in the thumb domain, near the polymerase active site[33], though this has yet to be confirmed.

Acknowledgements

Part III: The Future

Do not follow where the path may lead.

Go instead where there is no path and leave a trail.

~ Ralph Waldo Emerson ~

CHAPTER 6

New therapeutic opportunities for Hepatitis C based on small RNA

Qiuwei Pan[1,2]

Scot D. Henry[1]

Bob J. Scholte[3]

Hugo W. Tilanus[1]

Harry L.A. Janssen[2]

Luc J.W. van der Laan[1]

Departments of Surgery[1], Gastroenterology[2], Cell Biology[3]
Erasmus MC-University Medical Centre, Rotterdam, The Netherlands

Abstract

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease, including cirrhosis and liver cancer and is therefore, the most common indication for liver transplantation. Conventional antiviral drugs such as pegylated interferon-alpha, taken in combination with ribavirin, represent a milestone in the therapy of this disease. However, due to different viral and host factors, clinical success can be achieved only in part of patients, making urgent the requirement of exploiting alternative approaches for HCV therapy.

Fortunately, recent advances in the understanding of HCV viral replication and host cell interactions have opened new possibilities for therapeutic intervention. The most recent technologies, such as small interference RNA mediated gene-silencing, anti-sense oligonucleotides (ASO), or viral vector based gene delivery systems, have paved the way to develop novel therapeutic modalities for HCV. In this review, we outline the application of these technologies in the context of HCV therapy. In particular, we will focus on the newly defined role of cellular microRNA (miR-122) in viral replication and discuss it's potential for HCV molecular therapy.

Introduction

Hepatitis C virus (HCV), first identified in 1989, is a single-stranded positive-sense RNA flavivirus with 6 major genotypes and over 70 subtypes[1, 2]. According to the estimation of the World Health Organization, approximately 170 million people, 3% of the world population, are HCV positive with 3 to 4 million de novo infections each year. Unfortunately, 55–85% of those infected fail to clear the virus and progress to develop chronic infection. Over a period of 20 to 30 years cirrhosis develops in about 10% to 20% and hepatocellular carcinoma (HCC) develops in 1% to 7% of persons with chronic infection[3]. Currently, no safe and effective vaccine is available to prevent HCV infection. Conventional treatment, such as interferon taken alone or in combination with ribavirin, is only effective in part of the patients, but is often financially inaccessible for people in developing countries[4, 5].

To explore the potential of new therapeutic strategies, it is critical to better understand the viral and host factors involved in virus cell entry, replication and virus-cell interaction. An apparent two-way dialogue exists in which the virus apparently takes advantage of the cell's own signal transduction systems to facilitate virus entry and support replication[6]. Indeed, remarkable progress has been achieved in understanding the properties of the HCV genome and viral proteins. Contributions have come through several different sources including the vaccination of chimpanzees, structural studies, binding studies with recombinant envelope proteins, and the use of clinical isolates, HCV-like particles (HCV-LPs), HCV pseudotyped particles (HCVpp), and cell culture-derived HCV particles (HCVcc) in infectivity assays[7, 8]. Cellular pathways or molecules involved in viral entry, such as CD81, scavenger receptor class B type I (SR-B1), LDL receptor, L-SIGN, DC-SIGN and asialoglycoprotein receptor (ASGPR), could be putative therapeutic targets[9-12].

New technologies, particularly RNA interference (RNAi) induced by small interfering RNA (siRNA), are gaining favour as effective therapeutic entities for HCV infections. RNAi works at a posttranscriptional level by degrading cognate mRNA. As HCV is a single-stranded RNA that functions as both a messenger RNA and a template for replication, it is a prime candidate for RNAi. Moreover, previous reports have shown that by blocking cellular determinants of viral entry and replication, such as CD81, HSP90, or p68 either by RNAi, antisense oligonucleotides or chemically engineered 'antagomirs', leads to significant reduction of viral invasion[13-15]. In this review, we outline the novel small RNA based technologies in designing therapeutic approaches for HCV treatment, according to the mechanism of viral entry, replication and virus-cell interaction. In particular, we will discuss emerging evidence that a liver-specific, small non-coding microRNA (miRNA) is involved in replication of HCV through a novel mechanism and outline its therapeutic potential.

Molecular characteristics of HCV entry and replication

HCV, contains a single-stranded RNA genome of about 9400 nucleotides in length, composed of a 5' and 3' non-coding region (NCR) with a single open reading frame encoding a polyprotein precursor of approximately 3,000 amino acids that is cleaved into three structural (core, E1, E2) and seven non-structural (p7, NS2–NS5B) proteins[16, 17].

Since the discovery of HCV, numerous studies have demonstrated its mechanism of cell entry, but it is still unclear how the virus penetrates cell membranes. In order to elucidate the infection pathway, it is first required to identify and understand both the putative viral and cell factors involved in this process. The viral envelope glycoproteins E1 and E2, cleaved from the polyprotein by the endoplasmic reticulum (ER)-resident host enzymes signal peptidase and signal peptide peptidase, have been widely regarded as the critical determinants for virus cell entry. To date, several models have been designed to investigate E1/E2 function. These include HCV-LPs expressing E1-E2 heterodimers instead of glycosylated individual E1 and E2[18-21], HCVpp consisting of unmodified HCV envelope glycoproteins E1 and E2 assembled onto retroviral or lentiviral core particles[22-26], vesicular stomatitis virus (VSV)/HCV pseudotypes expressing HCV E1 or E2 chimeric proteins containing transmembrane and cytoplasmic domains of the VSV G glycoprotein, or HCVcc neutralization assays with E1 or E2 antibody[27-30]. These models have shown that both envelope glycoproteins E1 and E2 are essential for host cell entry. The lack of either E1 or E2 significantly decreases HCV infection activity whereas deletion of the whole envelope protein coding sequence abolishes the particle infectivity. Additionally, several cell surface molecules have been identified using these models and are now considered as critical components in mediating HCV attachment and entry.

Similar to viral entry, HCV replication requires both viral and cellular factors. Although our current knowledge of the HCV life cycle is still mainly at the hypothetical level, several minimum viral components and host cell factors have been proposed. The HCV 5'NCR, in particular the IRES sequence, plays an important function in ribosomal assembly and the NS3 to NS5B coding region are necessary for function of the replicase complex[31-35]. Found as interaction partners of NS5A and NS5B, human vesicle-associated membrane protein-associated proteins VAP-A and VAP-B were first identified from the host cell[36, 37]. More recently the geranylgeranylated protein FBL-2, the immunophilins cyclophilin B and FKBP8 have been identified as important host factors for HCV replication[38-40]. Furthermore, the host enzyme IMPDH, essential for the de novo synthesis of GTP nucleotides, may be involved in HCV replication as the IMPDH inhibitors ribavirin and mycophenolic acid suppresses replication[41, 42]. Interestingly, the mammalian liver-specific miRNA (miR-122) has been recently defined to facilitate HCV replication, indicating that this small RNA may present a novel target for antiviral intervention[43].

miR-122 and HCV replication

MiRNAs are approximately 22 nucleotide noncoding RNAs that can downregulate various gene products by inducing either cleavage or a reduction in the translational efficiency of the target mRNA[44, 45]. In the last 5 years, over 3,000 miRNAs have been identified in vertebrates, flies, worms, plants and even viruses. Most miRNAs have been shown to participate in essential biological processes, such as cell proliferation, apoptosis, differentiation and metabolism[46]. The 22 nucleotide mature miR-122, derived from a noncoding polyadenylated RNA transcript of the HCR gene, is a liver-specific developmental regulator. It can be detected as early as 12.5 days post-gestation and reach a plateau immediately before birth, then slowly increase up to 70% of the total miRNA population in adult liver[47-49]. MiR-122 is the first identified host miRNA linked to HCV viral replication. A further novelty to these findings is the fact that miR-122 upregulates, rather than downregulates, viral RNA by interaction with the 5' NCR of the viral RNA. Previous work had suggested that miRNA can only negatively regulate gene expression through targeting the 3' NCR of mRNA.

Interestingly, Jopling et al[43] have observed that though both Huh7 and HepG2 cells are derived from human hepatocytes, HCV RNA can only replicate in Huh7 cells. This may link to the fact that Huh7 is miR-122 positive, while HepG2 is miR-122 negative. To determine if miR-122 is required to regulate HCV replication, they transfected antisense oligonucleotides into Huh7 liver cells to suppress miR-122 function. The results showed that the amount of viral RNA was reduced by about 80% when miR-122 was silenced, but it is still unclear whether it is simply a direct or indirect interaction through cellular factors. Thus, to further address this issue, two putative binding sites, located in each of the viral NCR, were tested as possible targets for miR-122. It was found that only the binding sequence located in the 5' NCR was responsible for miR-122 targeting. This is notably very different from the common observation that miRNA target the 3' NCR, leading to suppression or degradation of target mRNA. Recently, a study in mice has shown synthesized antisense single-stranded 23-nucleotide RNA molecules can effectively inhibit production of miR-122 *in vivo*[50]. Therefore miR-122 seems a potential target for HCV treatment, although the mechanism for this new miRNA role is still very much unclear.

Therapeutic strategies based on gene silencing technology

As current antiviral regimens have proven largely unsatisfactory, particularly for patients with genotype 1 infection, it is important to explore novel therapeutic strategies. Small interfering RNAs and antisense oligonucleotides (ASO) have emerged as efficient nuclei acid-based gene silencing tools to target highly conserved or functionally important regions within the HCV genome or essential host cell factors for entry or replication (Fig. 1).

RNAi, induces gene silencing at a post-transcription level by double-stranded small interference RNA (siRNA) and represents an exciting new technology that could have applications in the treatment of viral diseases. Particularly, HCV could be an attractive target
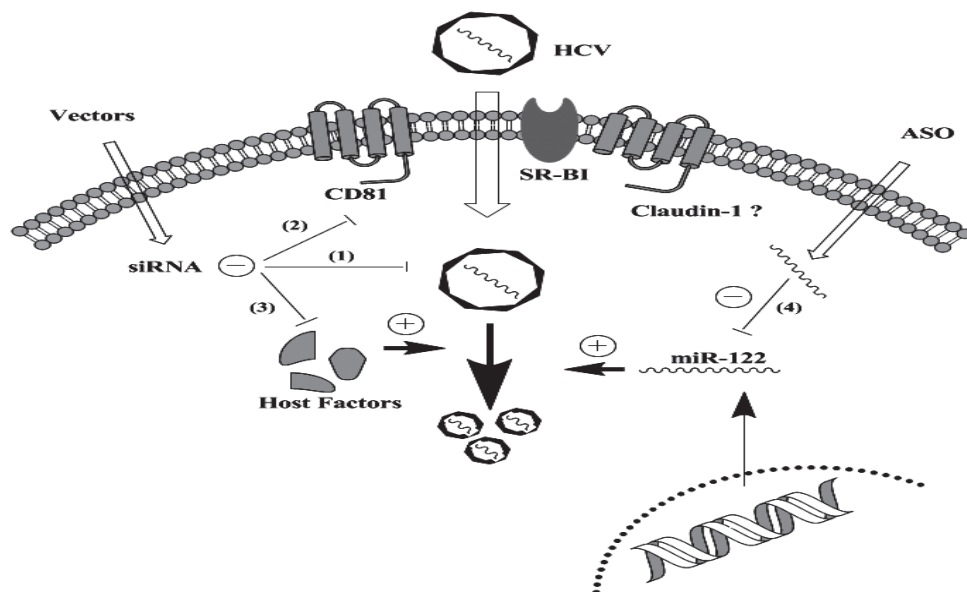
Fig 1 Novel anti-viral strategies based HCV life cycle. RNAi technology, inducing gene silencing at posttranscriptional level mediated by siRNA, can be applied to prevent HCV replication and infection by targeting either viral RNA (1) or host factors, such as surface receptor (2) or cellular molecules (3). miR-122 is a liver specific miRNA that is involved in HCV replication and therefore silencing of miR-122 by antisense oligonucleotides (ASO) could be considered as a potential therapeutic modality (4).

for RNAi therapy, as it is a RNA virus. The HCV genome is a positive single-stranded RNA that functions both as the viral messenger RNA and a template for RNA replication via a negative-strand intermediate. Instead of a 5' cap, the IRES, located at the 5' NCR, plays an essential role to bind eucaryotic ribosomal subunits and initiates the assembly of the translationally active 80S complex. Consequently, this sequence is more conserved than any other part of the viral genome, at least among the six known HCV genotypes[51, 52]. Thus, IRES seems an ideal target for RNAi mediated anti-HCV therapy and several groups have demonstrated efficient inhibition of HCV replication by designing siRNAs toward this region[53-55]. In addition, RNAi directed against the viral core, NS3, NS4B, NS5A and NS5B regions can suppress HCV infection. McCaffrey et al[56] was the first to demonstrate feasibility of siRNA targeting HCV NS5B *in vivo*. By co-expression of an NS5B-luciferase fusion gene with an anti-NS5B siRNA expression plasmid they found a significant reduction of luciferase expression in the mouse liver indicating selective degradation by the NS5B siRNA. Additionally, several other groups have observed suppression of HCV replicon by siRNA-mediated targeting either NS5B or NS3 region[57-59].

Besides these viral elements, numerous host cellular factors, such as CD81, SR-B1, HSP90, p68 or USP18, could be typical targets for potentiating RNAi antiviral therapy. CD81, expressed in most human cells, is able to bind to HCV E2 protein and is therefore considered an essential receptor for HCV entry. Further investigation, by either ectopic expression of CD81 in Huh7-Lunet cells (low expression of CD81) or modulation of CD81 cell surface density in Huh-7.5 cells (high expression of CD81) by RNAi, revealed that density of cell surface-exposed CD81 is

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a key determinant for HCV entry into host cells[60]. SR-B1, primarily expressed in the liver and steroidogenic tissues, was identified as another potential HCV receptor based on coprecipitation with recombinant E2. A 90% down-regulation of SR-B1 expression in Huh7 cells by RNAi caused a 30–90% inhibition of HCVpp infection, depending on the HCV genotype[61, 62]. However, either CD81 or SR-B1 alone is not capable of virus binding indicating that at least one additional host protein, possibly the recently identified co-receptor, Claudin-1[63], is required for cell entry of enveloped virions via the CD81/SR-B1 pathways.

Although using siRNA to target either viral or host factors could be considered effective tools to significantly block HCV infection and replication, an advanced method by knockdown both viral and cellular factors may further improve the therapeutic efficacy. Work by our group has shown that both entry and replication can be simultaneously targeted using shRNAs directed against two regions of the HCV RNA and one region of the host cell receptor, CD81. The triple shRNA expression vector was effective in concurrently reducing HCV replication, CD81 expression, and E2 binding, comparable to conventional single shRNA anti-HCV vectors[64].

Antisense oligonucleotides represent an alternative gene-silencing tool that can be employed as HCV therapy. ASO-based inhibition of HCV have been demonstrated extensively in the past[65-71]. Currently ASO is the most promising method to block the function of miRNA, such as miR-122. For instance, a 2'-O-methylated RNA oligonucleotide with exact complementarity to miR-122 was introduced to inactivate its function in Huh 7 cells, in order to determine the relationship between miR-122 and HCV replication. Subsequently, Krutzfeldt et al[50] developed a pharmacological approach for silencing miRNA *in vivo*, by chemically modified, cholesterol-conjugated single-stranded RNA analogues to complimentarily target miR-122. By injection of these 'antagomirs' into the tail veins of mice, efficient and specific suppression of endogenous miR-122 was observed. Hence, designing ASO based molecular medicines would provide new agents for human major diseases, because upregulation of certain miRNAs linked to a set of diseases such as cancer, diabetes or HCV.

Liver-targeted viral delivery systems

Obviously, RNAi or ASO technologies could be regarded as potentially effective novel modalities for anti-HCV treatment. Nevertheless, the success depends on developing effective delivery systems, to target therapy to the liver. Regarding to treat a liver-hosted and long-term persistent hepatitis virus, an ideal vector would be able to transfer genetic material efficiently and specifically into the target cells/tissues, resulting in high level, properly regulated and prolonged expression, without toxic and immunogenic side effects. Since viruses have many advantages as transgenic vehicles, we will discuss two of the most promising delivery systems: lentiviral and adeno-associated viral (AAV) vectors.

Lentiviral vectors, are mainly based on human immunodeficiency virus type 1 (HIV-1) and have been shown to effectively transduce liver, muscle, and hematopoietic cells. These vectors

integrate their payloads into the host genome ensuring transmission to progeny cells[72]. Although lentiviral-mediated short hairpin RNA (shRNA, precursor of siRNA) delivery has been widely developed for therapeutic application, there are few reports referring to HCV treatment[57, 64]. These are currently some limitations for the use of lentiviral vectors: (1) production efficiency limits *in vivo* transfection; (2) possibility of insertional mutagenesis or generation of wild-type virus lead to safety considerations. To circumvent these drawbacks, the following strategies may be required to achieve further improvement: firstly, newer generations, such as the gutted third generation, relatively high titers of VSV-G pseudotyped HIV-1 vectors, other types such as HIV-2 and simian immunodeficiency virus (SIV) vectors, or even immunodeficiency viruses derived from nonprimates, including felines and equines, are also being developed to overcome conventional problems[73-76].

Analogically, with the superiority of low pathogenicity and long-term gene expression, AAV could be another ideal viral vector for siRNA delivery, although no reference of AAV-mediated anti-HCV RNAi therapy has been reported so far. Particularly AAV serotype 8, a new member of the AAV family isolated from rhesus monkeys, is an attractive candidate for hepatic-directed shRNA transfer because of 10- to 100-fold increased transduction efficiency in mouse liver models, compared with the previous AAV2 based vectors[77]. Since derived from nonhuman primate, AAV8 is less prone to recognition by prevailing antibodies that generate side immunological effects in human[78]. Moreover, the safety and transgenic delivery efficacy could be further improved by conjugating other strategies, such as utilizing liver-specific promoters, hybridization of AAV8 with other serotypes, or modification of viral capsids.

Furthermore, since miRNA context based siRNA cassette (second-generation shRNA) can be driven by a regulatable pol-II promoter instead of conventional pol-III promoters[79], liver-targeted expression of shRNA could be achieved by employing a liver-specific pol-II promoter in viral delivery system.

Discussion

The treatment of HCV remains a challenge that requires further elucidating the process of viral life cycle and developing novel therapeutic approaches. In fact, recent progress has provided the possibilities of identifying novel antiviral targets and designing new therapeutic strategies. According to the previous description, miR-122 is one of the most emergent targets for HCV therapy that is commonly abundant in human livers and thus promotes viral replication. Therefore, downregulation of miR-122 by antisense based 'antagomirs' or oligonucleotides significantly suppressed viral replication. However, before such a method can be applied in the clinic, the role of miR-122 in maintaining normal hepatic function must be further investigated. Krutzfeldt et al[50] have demonstrated that silencing of miR-122 by 'antagomirs' do not show any apparent toxicity to mice, but the more recent study has shown that miR-122 is downregulated in the rodent and human hepatocellular carcinomas (HCC). Using the animal model of diet-induced hepatocarcinogenesis, Kutay et al[80] have observed that the reduced expression

of miR-122 probably occurs between 36 and 54 weeks when neoplastic transformation occurs. These findings suggest that the downregulation of miR-122 might be associated with hepatocarcinogenesis and therefore further investigation into these function of miR-122 is required before therapeutic application can be commenced. In conclusion, the recent progress of understanding the viral life cycle and identification of novel targets, in combination with the newly developed ASO and RNAi technology, may pave the way for new anti-HCV therapy.

Acknowledgements
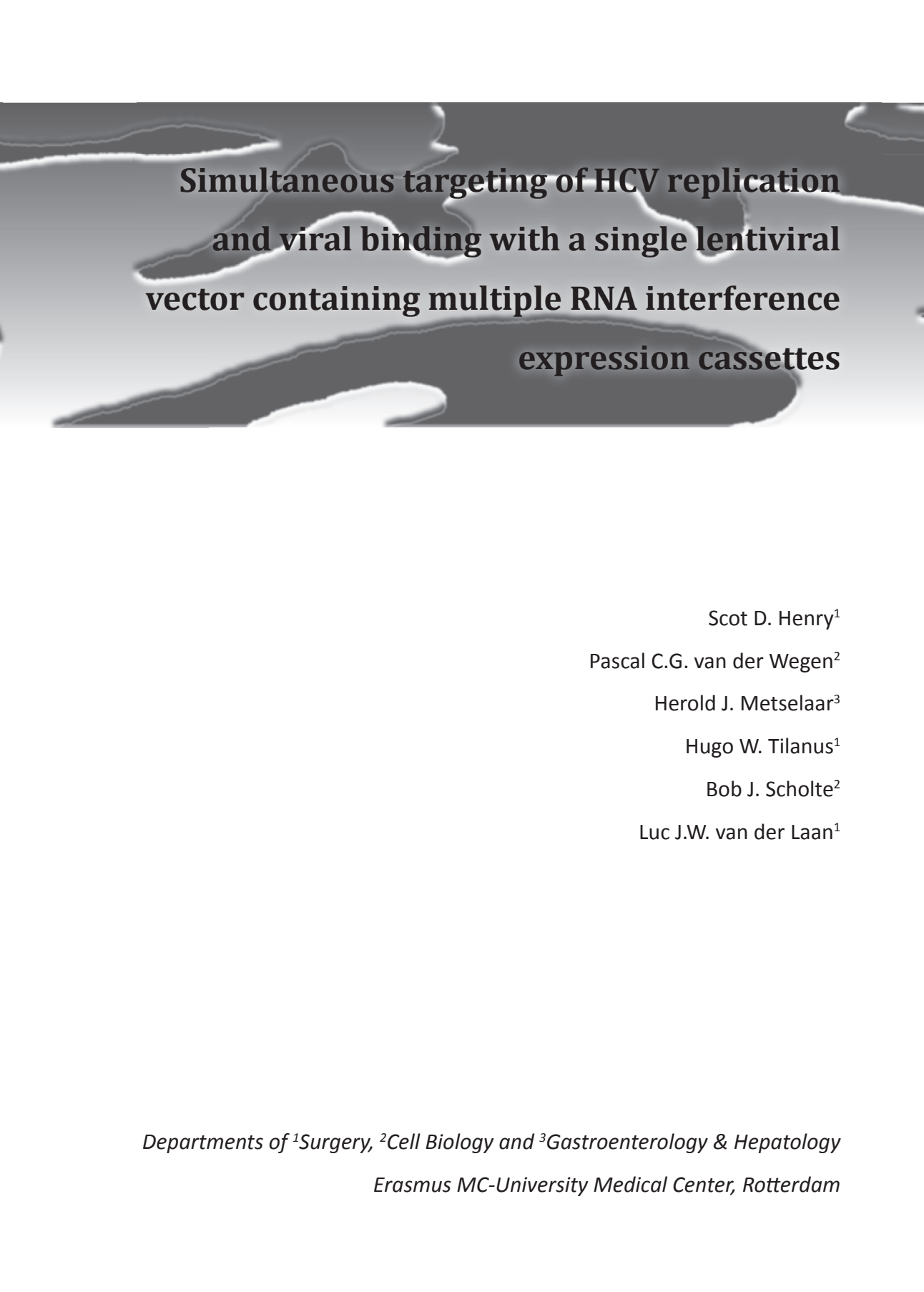
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CHAPTER 7

Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes

Scot D. Henry[1]

Pascal C.G. van der Wegen[2]

Herold J. Metselaar[3]

Hugo W. Tilanus[1]

Bob J. Scholte[2]

Luc J.W. van der Laan[1]

Departments of [1]Surgery, [2]Cell Biology and [3]Gastroenterology & Hepatology

Erasmus MC-University Medical Center, Rotterdam

Abstract

Chronic Hepatitis C Virus infection has a major medical impact and current treatments are often unsuccessful. RNA interference represents a promising new approach to tackle this problem. The current study details the design and testing of self-inactivating lentiviral vectors delivering RNA interference to prevent HCV replication and infection.

Vectors were constructed with single, double and triple cassettes expressing short hairpin RNA (shRNA) simultaneously targeting two regions of the HCV 1b genome and the host-cell receptor, CD81.

The shRNA directed against HCV IRES or NS5b regions were shown to be effective in inhibiting HCV replication *in vitro* (82 and 98%, respectively). No evidence of shRNA-related interferon production was observed. Vectors containing CD81 shRNA reduced cell surface expression up to 83% and reduced cell binding of HCV surface protein E2 up to 82% while not affecting levels of unrelated surface protein (Ber-EP4) or HCV replication. Double or triple shRNA vectors were independently effective in simultaneously reducing HCV replicon, CD81 expression and E2 binding.

This study demonstrates lentiviral delivery of multiple shRNA, inhibiting HCV in a specific, IFN-independent, manner. A gene therapy based on RNAi of multiple viral and host cell targets could reduce the risk of mutational escape by HCV.

Introduction

The hepatitis C virus (HCV), together with the hepatitis B virus, now accounts for 75% of all cases of liver disease around the world. HCV is an enveloped positive-strand RNA virus classified as the sole member of a distinct genus called hepacivirus in the family *Flaviviridae*[1]. The genome of HCV encompasses a single ~9600 nucleotide RNA molecule carrying one large open reading frame (ORF) that is flanked by non-translated regions (NTRs)[2]. The polyprotein created from this ORF is cleaved co- and post-translationally by cellular and viral proteinases into ten different products, with structural proteins located in the amino-terminal one-third and the non-structural replicative proteins in the remainder[3, 4]. The structural proteins include two envelope proteins, E1 and E2 which determine HCV tropism. E2 binding to cell surface tetraspanin CD81 is instrumental to viral entry[5-7].

RNAi based anti-viral therapies have begun to show great promise for HCV and other viral infections. RNAi is a process of sequence-specific, post-transcriptional gene silencing in plants and animals triggered by double-stranded RNA[8, 9]. Many viruses produce a transitory double-stranded RNA intermediate during replication that can be processed by enzyme termed Dicer into 21 nucleotide short interfering RNA (siRNA)[10], beginning the vestigial RNAi anti-viral mechanism. One strand of the siRNA associates with RNA-induced silencing complex (RISC)[10, 11] which guides the siRNA to its homologous mRNA target resulting in cleavage of the target sequence[8, 11]. To date RNAi has been used effectively to reduce infection in many *in vitro* viral systems including human immunodeficiency virus (HIV)[12, 13], poliovirus[14], influenza virus[15], and hepatitis B[16] with recent studies showing that RNAi can be effective in blocking HCV replication[17-19]. In addition to viral RNAs, RNAi can be an effective tool to downregulate host cell genes involved in viral infection such as CD4 and CXCR4 in HIV[13, 20]. In analogy, down-regulation of CD81 was shown to be effective in preventing HCV envelope binding[21].

If RNAi therapies are to be utilized as an effective treatment or prevention of disease, long term, stable siRNA expression needs to be achieved. Raw or plasmid siRNA transfections elicit only short term silencing, whereas. integrating self-inactivating lentiviral vectors[22, 23] that encode for short hairpin RNAs (shRNA), can produce long-term, continuous silencing[24, 25]. Vector-derived shRNA are single strand RNA containing complementary sequences separated by a loop sequence that can fold into a predicted double stranded hairpin. These structures are recognized by Dicer and cleaved into biologically active siRNA.

Many viruses mutate at high rates[14, 26] and can therefore rapidly develop resistance to monotherapies. HCV has been shown to generate mutants with monotherapy of ribavirin or RNAi[27-29]. Host cell factors involved in infection are not prone to mutation and are therefore good therapeutic targets for RNAi to help prevent resistance[13]. We present here the construction of lentiviral vectors producing single and multiple shRNAs that are capable of inferring protection by the downregulation the host cell receptor CD81 as well as simultaneously targeting multiple HCV sequences. Efficacy of these therapeutic vectors was demonstrated in an HCV replication model[30-32].

Methods & Materials

Cell Culture

Cell monolayers of the human embryonic kidney epithelial cell line; 293T and human hepatoma cell line; Huh-7[40], were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) and complemented with 10% v/v fetal calf serum (PerBio Science), 100IU/ml penicillin, 100mg/ml streptomycin, and 2mM L-Glutamine (Invitrogen-Gibco) (cDMEM). Huh-7 cells containing subgenomic HCV monocistronic replicon (I_{389}/NS3-3'/HygUbi/5.1) or bicistronic replicon (I_{389}/NS3-3'/LucUbiNeo-ET)[41] were maintained with 25µg/ml Hygromycin (Sigma, Zwijndrecht, The Netherlands) or 250µg/ml G418 (Sigma) respectively.

shRNA Annealing and Plasmid Construction

shRNA's oligo sequences used were derived from various sources (Table 1). All were purchased as 60 nt ssDNA oligomers (Invitrogen-Gibco) comprised of both forward and reverse sequences with 9 bp loop structures[42] and 3' BglII and 5' HindIII self inactivating overhangs (Fig. 1A). 20mM of both sense and antisense oligomers were incubated in annealing buffer (as previously described)[8] for 3 min at 90°C, temperatures were lowered in 2°C/min increments until 5°C above their respective T_m, then dropped to 4°C at maximum ramp rates.

pSuper (Oligoengine, Seattle, Washington, USA) was digested with PstI and HindIII, the fragment including the human H1 promoter and a portion of the multiple cloning site (MCS) was gel extracted and ligated in pSP72 (Promega, Leiden, The Netherlands)). This new pSP72-H1 plasmid was digested with SmaI and HpaI to remove the second BglII site and 83bp of the newly inserted MCS, in preparation for later ligations. Annealed shRNA oligomers were inserted into pSP72-H1 digested with BglII and HindIII via unidirectional insertion (Fig. 1B). The H1-shRNA fragments were then removed with either PstI/XhoI or SalI/XhoI and cleaned via gel extraction. This study uses the self-inactivating lentiviral transfer construct pND-CAG/GFP/WPRE, containing the composite CAG promoter (consisting of the cytomegalovirus immediate early enhancer, chicken β-actin promoter, and rabbit β-globin intron) that drives transcription of the enhanced green fluorescent protein (GFP) cDNA and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). The HIV-1 central polypurine tract (cPPT) was reintroduced to streamline nuclear import of the pre-integration complex as well as ensure efficient infection of hepatocytes[43]. The H1-shRNA cassettes were ligated to the XhoI site of the pND-CAG/GFP/WPRE in opposition to the CAG reading direction (Fig. 1C). New shRNA plasmids were checked for insertion and direction with restriction analysis using NdeI/XhoI. The resulting plasmids left one downstream XhoI site available for multiple cassette insertions. Plasmids containing double and triple shRNA cassettes were created by repeating this process for each additional shRNA cassette (Fig. 1D). Final pND plasmid constructions included singles: Mock (no shRNA GFP lentivector), Con (scrambled HBV shRNA), IRES, NS5b, CD81, doubles; IRES-NS5b, IRES-CD81, NS5b-CD81, triple; IRES-NS5b-CD81. These plasmids each express a GFP reporter

gene as well as all of the inserted self-expressing shRNA cassettes, each under the control of their own H1 promoter (Fig. 1E). All plasmid constructs were sequenced (Baseclear, Leiden, The Netherlands) in the region of the shRNA cassettes to ensure direction and that no mutations occurred during cloning steps.

Lentiviral Production

A 3rd generation lentiviral packaging system originally described by Dull et al[22] in combination with pND-CAG/GFP/WPRE containing either single, double or triple shRNA expression cassettes was used to produce high-titer VSV-G pseudotyped shRNA-GFP expression vectors, as previously described[44]. Briefly, 293T cells were grown in cDMEM to 80% confluency in $500cm^2$ flasks and transfected with pND, VSV-G, GagPol, and Rev plasmids in a 4:1:2:1 ratio using 3:1 (264mg) polyethylamine in serum free DMEM and replaced with cDMEM after 6 hours. Vector supernatants were removed 36 and 48hr post transfection, passed through a 0.45 mm filter and concentrated 1000 fold by ultracentrafugation. Concentrated virus stocks were titrated using 293T cells 24 h after infection, with transduction efficiency based on the number of GFP positive cells as determined by flowcytometry (FACS Caliber, BD BioSciences, Mountain View, CA). Vector titers ranged from $7x10^6$ to $3x10^7$ transducing units per mL.

Real Time RT-PCR

Confluent monolayers of replicon cells were lysed by Trizol (Invitrogen-Gibco) and RNA precipitated with 75% EtOH and captured with a Micro RNAeasy silica column (Qiagen Venlo, The Netherlands). RNA was quantitated using a Nanodrop ND-1000 (Wilmington, Delware, USA) and adjusted to 1mg for each sample. cDNA was prepared using Promega's AMV reverse transcriptase following standard protocols. Real Time PCR (MJ Research Opticon, Hercules California, USA) was performed using primers NS3 (F-5'-GGT TCT GTG CGA GTG CTA TG-3', R-5'-TCT CCT GCC TGC TTA GTC TG-3'), CD81 (F-5'- AGT-GGA-GGG-CTG-CAC-CAA-GT, R-5'-GTG-AGC-GGG-TCT-CTG-AGT-CGA-A) and GAPDH (F-5'-CCA TGG AGA CTG GGG-3', R-5'-CAA AGT TGT CAT GGA TGA CC-3'). Semi-quantitation of sample amplicons was performed with SybrGreen (Sigma) using Taq DNA polymerase (Invitrogen-Gibco) as previously described[45].

Luciferase Assay

Luciferase activity in cells was quantified using a Top Count scintillation/luminescence counter (Packard BioSciences Top Count-NXT, Milan, Italy). Cells were lysed and luciferin substrate added using Steady Glo Luciferase System (Promega) according to manufacturer protocols.

Flow Cytometry and Immunocytochemistry

Surface expression of CD81 and Ber-EP4 was determined via FACS after incubation with antibody conjugated to each; mouse a-human CD81 monoclonal IgG1 (BD Pharmingen, San Diego, USA) followed by a fluorescently labeled rat a-mouse r-phycoerythrin (PE) (BD Pharmingen), and

mouse a-human Ber-EP4/FITC (Dako Cytomation, Fort Collins, USA) using standard immuno-florescence staining techniques. Mouse monoclonal IgG1 (Dako Cytomation) was used for negative isotype controls.

Cells for immunocytochemistry were fixed in 2% para-formaldehyde (PFA) and labeled with antibodies conjugated to HCV-non-structural protein 3 (NS3) (Novo Castra, New Castle, UK), and CD81 (BD Biosciences). Secondary antibody binding and amplification of signal was accomplished with EnVision™ horse radish peroxidase and visualized with 3'-Amino-ethyl-carbozole (Sigma). Images were created on a Zeiss Axioskop microscope (Sliedrecht, The Netherlands) fitted with Nikon's (USA) Digital Sight DS-U1 imaging system. Images were captured and formatted using Eclipse Net digital software.

E2 Binding Assay

To determine whether CD81 downregulation prevents binding of HCV E2, an HCV E2 binding assay was performed[5, 46]. Huh-7 cells were transduced with CD81 shRNA containing vectors (CD81, NS5b-CD81, and IRES-NS5b-CD81) or control vector (Con). At seven days, transduced Huh-7 cells were trypsinized and incubated with recombinant E2 protein (Austral Biologicals, San Ramon California, USA) at a concentration of 8mg/ml for 30min at 4ºC in PBS/0.1% BSA. Cells were washed and incubated with 1mg/ml E2 detection antibody HuhMab108 (Genmab, Utrecht, The Netherlands) for 45min at 4°C. Cells were washed and incubated with a donkey anti-human PE (BD Pharmingen) labeled secondary antibody, and binding detected by flowcytometry.

IFN bioassay

To determine the IFN response to shRNA, HygUbi replicon cells were transduced with shRNA constructs and incubated for 72hr. 100ml supernatants harvested and added to LucUbiNeo replicon cells. After 24hr incubation, luciferase activity was determined as described above. IFN levels were calculated using the method described by Vrolijk et al[38]. 10 IU/ml (40pg/ml) of pegylated IFN-α was used as a positive control.

Statistical Analysis

Statistical analysis was performed using a non-parametric ANOVA with Dunn multiple comparison post test using GraphPad InStat software.

Results and Discussion

Effects of single shRNA vectors

TABLE 1: shRNA sequences, targets, and their references			
shRNA	Sequence	Target	Reference
Con	GCGCGCUUUGUAGGAUUCG	None (scrambled)	[16]
IRES	AGGUCUCGUAGACCGUGCA	Region IV (321–340) HCV IRES	[17]
NS5b	GACACUGAGACACCAAUUGAC	HCV NS5B (6367–6388)	[18]
CD81	GGAUGUGAAGCAGUUCUAU	Human CD81 (594–614)	Ambion siRNA 14501

Effects of single shRNA vectors. Lentiviral vectors containing shRNA sequences (Table 1) directed against HCV and CD81 were constructed by insertion of the shRNA oligonucleotides (Fig.1A) downstream of the H1-promoter into pSP72 plasmid (Fig. 1B). The H1-shRNA cassettes were removed and ligated in opposing direction to the CAG promoter within the lentiviral packaging plasmid (Fig.1C).

In order to determine effects of shRNA constructs on HCV replication and CD81 expression, Huh-7 replicon cells were incubated for 24 h with concentrated LV at similar titers. The level of transduction at 3 days, as determined by flowcytometry for GFP expression, ranged from 85 to 97% for all vectors. Single HCV shRNA vectors very significantly reduced HCV replication as compared to Mock transductions with empty GFP vector (Fig. 2A). The NS5b shRNA reduced HCV replication by 97%±2 SEM (p<0.001), and IRES shRNA by 82%± 8 (p<0.05). The Con shRNA (10%±3) and CD81 (20%±3) shRNA showed a slight reduction in HCV replication but was not significant as compared to Mock control. Results obtained with luciferase activity correlated with significant reductions in HCV mRNA (IRES, 72%±2, p<0.05; NS5b, 97%±0.1 (p<0.01)) (Fig. 2B) as well as a clear reduction of NS3 protein levels

Fig 1. shRNA vector design. A) Each shRNA is composed of both a forward and reverse sequence, interrupted by a hairpin loop, flanked by a downstream termination sequence and restriction enzyme overhangs. B) shRNA sequences were ligated into BglII/HindIII cut sites downstream of the H1-promoter of the generated pSP72-H1 plasmid, leaving the upstream SalI and downstream XhoI sites intact. C) The H1-shRNA cassette were removed by SalI/XhoI and placed into the XhoI site upstream and in opposing direction to the CAG promoter within the packaging plasmid pND-CAG/GFP/WPRE, destroying the SalI site while leaving an intact upstream XhoI site. D) Multiple shRNA pND plasmids were created by opening the newly formed XhoI site and inserting additional shRNA cassettes, each time leaving an intact XhoI site allowing further insertions of shRNA cassettes. E) Predicted hairpin structure of fully formed NS5b shRNA.

Fig 2. Single shRNA vectors effectively inhibit HCV replication and CD81 expression. A) Specific shRNAs significantly reduced HCV replication (n=5, left axis) and significantly reduced surface expression of CD81 (n=4, right axis) at 3 days post transduction (p<0.05, as compared to Mock). Control shRNA (Con) showed no inhibitory effect. B) Semi-quantitation of HCV (NS3) and CD81 mRNA normalized to GAPDH (ΔΔCT calculation). Specific shRNAs significantly reduced HCV and CD81 mRNA (n=3) (p<0.01, as compared to Con). C) HCV NS3 and D) CD81 expression in Huh-7 replicon cell line after 3 day transduction with single shRNA vectors. Clear reduction of protein expression was seen with the corresponding shRNA and not with the non-related shRNAs (1 of 3 representative experiments). E) Representative FACS profile of total GFP expression and surface expression of CD81 and EP4 in Huh-7 replicon cells after transduction with CD81 shRNA lentiviral vector (LV). Isotype matched controls are included to demonstrate specificity of staining. CD81 and EP4 expression is shown for GFP positive cells only (R1 gate). Specific reduction was seen in CD81 expression levels but no effect on EP4 surface staining was seen in transduced (GFP positive) cells.
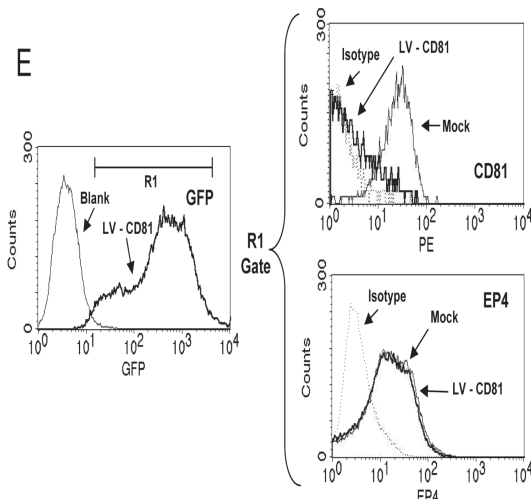
with both vectors when compared to Con vector (Fig. 2C).

CD81 shRNA vector reduced CD81 mean fluorescence intensity by 83%±3 (p<0.05) as compared to Mock, while other non-CD81 vectors had no effect on the expression levels of CD81 (IRES, 8%±3, NS5b 12%±2) (Fig. 2A). CD81 mRNA levels were not significantly affected by non-CD81 shRNA vectors, whereas significantly reduced by CD81 vector (91%±1, p<0.01) (Fig. 2B). A reduction

in cell surface CD81 expression was seen in only the CD81 shRNA containing vector as shown by immunocytochemistry (Fig. 2D). CD81 shRNA was shown to be specific with no effect on an unrelated surface protein Ber-EP4, as confirmed by FACS profiles of cells treated with CD81 vector (Fig. 2E). Together, these results demonstrate the effectiveness of single shRNA expression vectors to silence HCV replication and CD81 surface expression.

Double and triple shRNA vectors inhibit CD81 and E2 binding

Double and triple shRNA vectors inhibit CD81 and E2 binding. Plasmids containing double and triple shRNA cassettes were created by sequential insertion of H1-shRNA cassettes. Each insertion results in a unique downstream restriction site that is available for additional H1-shRNA insertions. Based on the packaging limitation of lentiviral vectors (~7000bp), this method could potentially be used to insert up to 21 self expressing shRNA cassettes within a single vector. As shown in Figure 3, double and triple vectors containing CD81 shRNA created with this method were effective in reducing CD81 expression levels (NS5b-CD81, 77%±5; IRES-NS5b-CD81, 78%±1). CD81 mRNA was reduced by all these vectors (NS5b-CD81, 71%±1; IRES-NS5b-CD81, 80%±1) but not with double HCV shRNA (IRES-NS5b, 10%±4) (Fig. 3B). Multiple target vector NS5b-CD81 downregulated NS3 protein expression (Fig. 3C) as well as CD81 surface expression (Fig. 3D).

A possible complication of the presence of multiple shRNAs is the potential of competition for RNAi resources. However, we and others[24,]
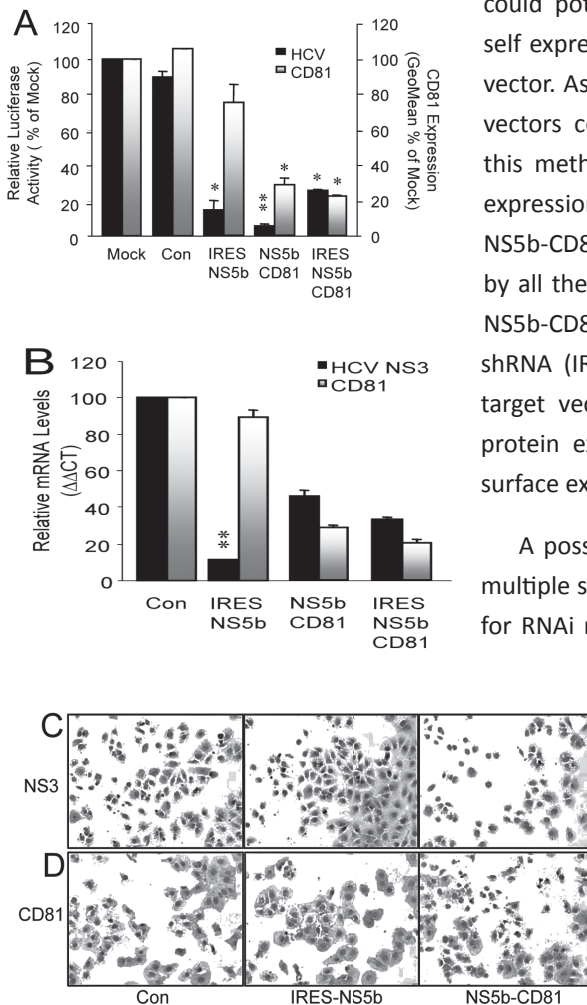
Fig 3. Double and Triple shRNA vectors effectively inhibit HCV replication and CD81 expression. A) Double and triple shRNAs vectors significantly reduced HCV replication (n=5, left axis) and significantly reduced surface expression of CD81 (n=5, right axis) at 3 days post transduction (p<0.001, as compared to Mock). Control shRNA (Con) showed no inhibitory effect. B) Semi-quantitation of HCV (NS3) and CD81 mRNA normalized to GAPDH (ΔΔCT calculation). Double and triple shRNAs significantly reduced HCV and CD81 mRNA (n=3) (p<0.001, as compared to Con). C) HCV NS3 and D) CD81 expression in Huh-7 replicon cell line after 3 day transduction with double shRNA vectors. Clear reduction of protein expression was seen with the corresponding shRNA and not with the non-related shRNAs (One of three representative experiments).

[33, 34] have found no significant competitive inhibition by the inclusion of multiple shRNAs. The presence of other shRNAs in the same vector did not clearly interfere with the inhibitory activity of the other. Single shRNA CD81 reduced surface expression by 83%±3 (p<0.05), while double and triple vectors reduced CD81 by 77%±5 (NS5b-CD81), 78%±1 (IRES-NS5b-CD81) respectively. This confirms that it is possible to introduce several shRNAs to silence many genes simultaneously with little or no loss is efficacy. Recent reports have suggested that non-specific effects can occur by siRNAs, both at the level of mRNA and protein[35]. In the current study we did not observe this. The Con, IRES and NS5b shRNAs had no effect on CD81 (Fig. 2) or Ber-EP4 surface expression (data not shown). Conversely, Con and CD81 shRNAs had no significant effect on HCV replication or EP4 surface expression (Fig. 2).

In order to determine the biological relevance of reducing cell surface CD81 by shRNA vectors an E2 binding assay was performed. As shown in Figure 4, the reduction of bound E2 was seen with all vectors containing CD81 shRNA within the transduced cell population. As expected, the non-transduced cells (GFP negative) showed no reduction in E2 binding (data not shown). The reduced binding of E2 was directly correlated with a reduction in the surface expression of CD81 (Fig. 4C). This suggests that even if CD81 cell surface density is reduced; low levels of bound HCV E2 can be observed. Therefore, we can not exclude that residual CD81 is sufficient for some viral binding and infection by HCV.
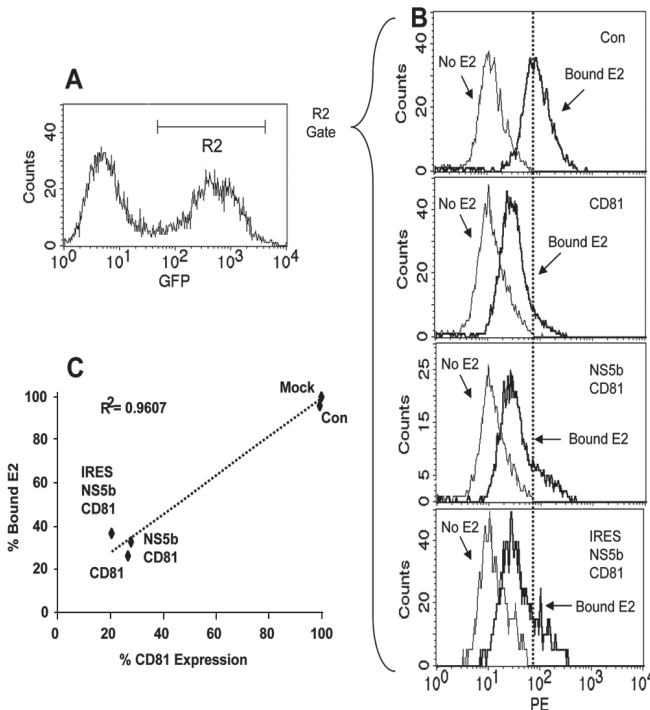
Fig 4. shRNA mediated reduction of CD81 inhibited HCV E2 binding. A) E2 binding was measured in GFP positive cells (R2 Gate), 7 days after transduction (left panel). B) Single, double and triple vectors containing CD81 shRNA effectively reduced binding of E2 (black line). Specificity of detection was confirmed with no E2 protein control (grey line). Peak channel (bound E2) in control cells is indicated by dotted line. C) A reduction in the percentage of cells binding E2 was directly proportional to the percentage of cell surface CD81 expression, as determined by flowcytometry. One of three representative experiments is shown.

Double and triple shRNA vectors inhibit HCV replication

Double and triple shRNA vectors inhibit HCV replication. Double and triple vectors containing HCV specific shRNA significantly inhibited HCV replication (IRES-NS5b, 85%±5SEM, p<0.05; NS5b-CD81, 90%±4, p<0.01; IRES-NS5b-CD81, 74%±1) (Fig. 3A). As with the single vectors, we saw also reduction in HCV mRNA levels when cells were treated with any of the double vectors (Fig. 3B) (IRES-NS5b, 97%±1, p<0.01; NS5b-CD81, 54%±3; IRES-NS5b-CD81, 66%±2). Double HCV vector IRES-NS5b showed similar downregulation of NS3 intracellular staining when compared to single HCV vectors (Fig. 3C).

There are several advantages to using single vectors containing multiple shRNAs. The probability of hitting a single hepatocyte with more than one vector, in the setting of clinical gene therapy, is very small. With the inclusion of several different shRNAs in one vector, the probability of one cell receiving the appropriate combination of shRNAs is assured. Our design theoretically overcomes the problem of mutational escape by targeting two or more regions within the viral genome. HCV is particularly mutational due to the low fidelity of its RNA polymerase. Treating HCV with monotherapies like ribavirin has been shown to rapidly select for resistant mutants, making therapy impotent[27, 28]. Even the most powerful siRNAs, given singly, will not prevent selection of viral mutants, rapidly reducing efficacy, and finally rendering it obsolete. A mathematical model by Leonard and Schafter predicts that prevention of viral escape can be achieved when a combination of four different suboptimal siRNAs are used[36]. The model also predicts that if more individually effective siRNAs are used in combination, fewer are needed. We calculated that, based on the individual efficiencies, a combination of IRES and NS5b shRNAs, could theoretically, eliminate HCV viral escape.

Long-term effects of double and triple shRNA vectors

In order to be effective as a therapy, RNAi needs to have long-term expression. Transfection of raw siRNA is not a viable gene therapy to persistent viral infection, as they lose their effectiveness within approximately 5 days[37]. We show that by using integrating lentiviral vectors to provide sustained shRNA mediated RNAi; our therapy is effective for at least two weeks. Replicon cells were treated with shRNA vectors and kept in culture without selection for up to 17 days. Integrated lentiviral shRNA constructs continued to reduce HCV replicon activity 17 days post infection (longest culture period). We found that cells transduced with vectors containing a single HCV shRNA (NS5b-CD81),
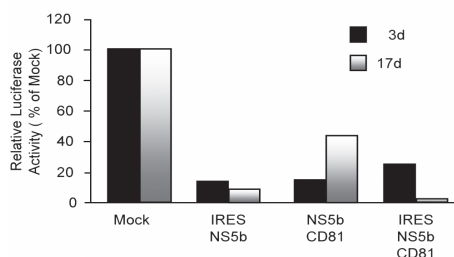
Fig 5. Long-term downregulation of HCV replication by double and triple shRNA vectors. Double vectors were effective at reducing HCV replication at three days post transduction. Long-term cultures showed the double vector, containing a single HCV shRNA (NS5b-CD81), showed a decrease in effectiveness over the course of 17 days. Vectors containing both HCV shRNAs (IRES-NS5b and IRES-NS5b-CD81) continued to reduce HCV replication to 17 days (One of three representative experiment shown).

efficacy was reduced at later time points. In vectors containing double HCV shRNA (IRES-NS5b or IRES-NS5b-CD81), efficacy was sustained over the same time points (Fig. 5).

Lack of IFN response to shRNA vectors

Lack of IFN response to shRNA vectors. Some evidence suggests that the use of siRNA under certain circumstances can invoke a host cell interferon response. To address this in our model, the production of IFN by replicon cells containing shRNA, an IFN bioassay, with Huh-7 replicon cells, was used as a sensitive indicator of secreted IFN[38]. No IFN was produced in response to any shRNA, regardless of sequence (Fig. 6). In fact, a recent paper has shown that Huh-7 containing HCV replicons have a strongly reduced capacity to produce IFN-α[39]. These findings support that a host cell IFN response does not contribute to the anti-viral effect of these shRNA.

Fig 6. No interferon production in response to shRNA. IFN bioassay using Huh-7 replicon cell line as a sensitive indicator of secreted IFN. Supernatants from Huh-7 replicon cells 3 days post transduction had no effect on luciferase activity indicating absence of interferon response (n=3). 10 IU/ml pegIFN-a completely inhibited HCV replication.

In conclusion, RNA interference offers new opportunities to control and eliminate viral infections. In the present study we developed lentiviral vectors that express multiple shRNAs. To our knowledge, this is the first report of a triple shRNA expression vector being used to simultaneously down-regulate independent targets involved in viral replication and viral binding. Integration of lentiviral vectors ensures long-term stable expression of RNAi based therapy. These vectors could be used to transduce hepatocytes in order to attack existing HCV infections and to make cells resistant to infection by down-regulating viral binding co-receptor CD81. The shRNAs were shown to be independently active, without clear evidence of cross-interference. Specifically, we show that vectors can be used to target multiple regions of the HCV genome as well as the cellular co-receptor CD81. This simultaneous targeting theoretically reduces the risk of viruses developing RNAi resistance. Though these vectors are effective in reducing viral replication and infection, the use of multiple shRNAs could be effective in treating many multifactorial diseases such as diabetes or cancer where several genes are involved in disease progression.

Acknowledgments

Chapter 7
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Hydroxyethyl starch based preservation solutions enhance gene therapy vector delivery under hypothermic conditions

Scot D. Henry[1]

Pascal van der Wegen[2]

Herold J. Metselaar[3]

Hugo W. Tilanus[1]

Bob J. Scholte[2]

Luc J.W. van der Laan[1]

Departments of [1]Surgery, [2]Cell Biology and [3]Gastroenterology & Hepatology

Erasmus MC-University Medical Center, Rotterdam

Abstract

Isolated liver perfusion offers a unique prospect for safe, effective targeting of gene therapies that can be directed against allograft rejection or recurrent diseases like reinfection by hepatitis C virus (HCV). We aimed to examine the effect of organ preservation solutions on vector based gene therapy delivery under hypothermic conditions.

University of Wisconsin (UW), Histadine Tryptophan Ketoglutarate (HTK), EloHaes (EH), Na-Peg-UW Solution (IGL-1) or DMEM culture medium (control) were tested at 2° or 37°C for lentiviral vector transduction efficiencies to the hepatoma cell line Huh-7, primary human or mouse hepatocytes. Lentiviral vectors expressing a short hairpin RNA were used to target HCV replication.

With a potent shRNA vector, transductions are directly correlated to the therapeutic effect, with low transduction yielding low knockdown and conversely. GFP reporter gene expression was observed with vector incubation times as short as 10 minutes. The highest transductions were seen, after two hour 37°C incubation, in UW (62%°±6 SEM), significantly higher than those in HTK (21%±7 SEM). Neither adenosine nor glutathione, present in UW, provided any increase in transduction when supplemented to HTK, though the addition of hydroxyethyl starch (HES) significantly improved transductions. To rule out size-exclusion as a mechanism of HES, IGL-1 was tested but did not result in better transductions than with HTK or cDMEM. When supplemented to UW, anionic compounds reduced transduction, indicating a charge interaction mechanism of HES.

In conclusion, this study demonstrates that effective vector delivery can be achieved under conditions of hypothermic liver perfusion. UW provides superior transduction to hepatocytes over non-starch solutions.

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Introduction

Various gene therapies, to date, have been successful *in vitro* as well as in small clinical studies. Several studies have shown that gene therapies can be applied to protect a graft against acute rejection[1-3] or recurrent diseases like HCV[4]. Though, the current inability to narrow the tropism of viral vectors as well as the safety concerns regarding the infection of patients with viral vectors has hampered the progress of new studies. Genetic therapy treatments for hematopoietic disorders are now being performed *ex vivo*, with the target cells being mobilized, treated in culture and re-implanted[5]. This represents a vast improvement for specific targeting with vectors that have high multiple tropisms. The liver or specific cell populations can be removed and treated without worry of non-target transductions to areas deemed sensitive or dangerous to vector deliveries.

During the process of liver transplantation, the donor undergoes multiple perfusions to clear the liver of blood, preserve and prepare it for transplant. Hypothermic liver perfusion is most commonly performed with University of Wisconsin (UW) or Histadine Thymadine Ketogluterate (HTK) preservation solutions[6]. After the liver is recovered from the donor within the minimum warm ischemic time (<30min), a cold perfusion is performed to clear the vasculature and cool the liver for storage. The graft is then placed on ice, ready for up to 12-18 potential hours for allocation to the receiving hospital. Generally, a second perfusion is performed with cold human albumin at the receiving hospital, in part to remove transportation outflow and also to dilute the high potassium levels in some perfusion solutions. This time between donor and patient open a window of opportunity in which pre-transplant gene therapies can be delivered to the donor graft, safely and efficiently with minimal perturbance in the existing protocols for liver transplantation.

Within experimental settings, third generation lentiviral vectors can achieve high transductions with wide tropisms due to the incorporation of the vesicular stomatitis virus glycoproteins (VSV-G) in their envelope. These VSV-G pseudotyped lentivectors enter the cell through receptor mediated endocytosis followed by low pH-induced membrane fusion in the endosome[7, 8]. Though the molecules involved in VSV-G binding are largely unknown, previous work has suggested that a specific receptor as well as an interaction between phosphatidylserine residues on the membrane surface is essential for VSV-G-membrane interaction[9, 10]. We have previously shown the development of a VSV-G pseudotyped lentivirus capable of downregulating multiple HCV or HCV related targets simultaneously[4]. Lentiviral directed transduction of RNA interference (RNAi) has been proven an effective method to silence HCV gene expression[4, 11-13]. Through the delivery of therapy during perfusion, before transplantation, one can impart RNAi based protection to the new graft before it enters an HCV infected patient, thus reducing or even eliminating the chance of recurrence. It is currently unknown whether these vectors are capable of transduction within the hypothermic conditions of liver perfusion and storage, thus the transduction potentials of the pre-transplant environment will need to be determined.

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The aim of this study was to determine the effect of organ preservation solutions on the transduction efficiency of lentiviral vectors, at temperatures used for liver preservation and perfusion. We show that lentiviral binding and subsequent transduction to hepatocytes can occur within normo- as well as hypothermic conditions. It was found that the type of perfusion solution used makes a distinct impact on the levels of transduction.

Methods & Materials

Cell Culture

Cell monolayers of the human embryonic kidney epithelial cell line (HEK 293T) and human hepatoma cell lines; Huh-714 and Huh-7 ET cells containing subgenomic HCV replicon (I389/NS3-3'/LucUbiNeo-ET)15, were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen-Gibco, Breda, The Netherlands) and complemented with 10% v/v fetal calf serum (PerBio Science), 100IU/ml penicillin, 100mg/ml streptomycin, and 2mM L-Glutamine (Invitrogen-Gibco) (cDMEM). Huh-7 ET cells were maintained under 250µg/ml G418 (Sigma, Zwijndrecht, The Netherlands) selection. Cryo-frozen primary neonatal mouse hepatocytes (a kind gift from Suzanne van de Nobelen, Erasmus MC, Rotterdam) were plated at near confluence and maintained in Williams E Medium w/ Gluatamax (Gibco) supplemented with 100IU/ml penicillin, 100mg/ml streptomycin, 10nM Dexamethasone (Sigma), 1mM Insulin (Sigma) and 10% v/v fetal calf serum (PerBio Science). Fresh primary human hepatocytes (Lonza, Verviers, Belgium) were maintained in Hepatocyte Maintenance Medium with Ultra-Glutamax (Clontetics, Verviers, Belgium) and supplemented with 10µM Insulin, 10µM Dexamethasone, 50mg/ml Gentamicin and 50ng/ml Amphotericin as supplied as mini-quot additives (Lonza).

Reagents

Cells were transduced with GFP expression vectors (LV-GFP) in various solutions to determine the optimal solution for hypothermic transduction: UW (ViaSpan)(Barr Laboratories, Pomona, USA), HTK (Pharmapal, Athens, Greece), EloHaes (EH)(Fresenius Kabi, Bad Homburg, Germany), Na-PEG-UW (IGL-1); (Laboratoire Clair LGL, Madrid, Spain. A kind gift from Prof. R. Ploeg, University of Groningen, Netherlands). Various components contained within UW solution were added to HTK to investigate their individual influences on lentiviral transduction. HTK was supplement with various concentrations of Viastarch (250kda Hydroxyethyl Starch (HES), a kind gift from Prof. R. Ploeg, University of Groningen, Netherlands), 3 mM Glutathione (Sigma, Zwijndrecht, Netherlands), or 5 mM Adenosine (Sigma) and transduction efficiencies determined. UW or HTK was also supplemented with anionic polymers; 100 µg/ml Dextran Sulfate (Sigma), or 20 mM Heparin (LeoPharma, Breda, Netherlands) to determine the dependency of charge interactions in lentiviral transduction.

Lentiviral Production and Transduction

A third generation lentiviral packaging system originally described by Dull et al[16] in combination with pND-CAG/GFP/WPRE was used to produce high-titer VSV-G pseudotyped lentivirus expression GFP reporter construct (LV-GFP) or both GFP and short hairpin RNA targeting the HCV NS5b (LV-shNS5b), as previously described[4, 17].

To test the effect of LV-shNS5b vectors on HCV replication, Huh-7 replicon cells were plated in cDMEM at 70-80% confluence. After a period of 3-4 hours, LV-shNS5b, at increasing multiplicity of infection (MOI), was added and incubated in normothermic conditions for 120 minutes. The cells were washed with warm PBS, fresh cDNA was added and cells were returned to 37°C and cultured for 48 hrs. Luciferin substrate was added and counts per minute were measured by scintillation counter.

Huh-7 cells were plated in cDMEM at 70-80% confluence. After a period of 3-4 hours, the medium was removed and cells were suffused in a variety of solutions; UW, HTK, EH or cDMEM. Cells, in all solutions, were incubated in either hypo- (2°C) or normothermic (37°C) conditions and transduced with LV-GFP at matched MOI for either 10, 30, 60 or 120 minutes. After incubation with LV-GFP, the solutions were removed and cells washed with warm PBS and replaced by cDMEM. The cells were then returned to 37°C and cultured for 48 hrs. Cells were harvested via trypsin/EDTA digestion.

All transduction efficiencies; percentage of GFP positive cells and geometric mean fluorescence intensity (MFI), were determined by flow cytometry (FACSCalibur, BD BioSciences, Mountain View, USA).

Isolated Liver Perfusion

Protocols for the isolated liver perfusion (IHP) in rat were approved by the local Erasmus MC ethics committee and performed under strict biological containment. The lentiviral vector used is based on pRRL.PPT.SF.EGFP.WPRE4*.SIN (G. Wagemaker et al., unpublished). To generate pRRL.PPT.SF.LUC.WPRE4*.SIN (LV-SF-LUC), the eGFP cDNA was replaced by luciferase cDNA excised from vector pGL3-Basic (Promega). Luciferase is expressed by a ubiquitous, non-liver specific transcription unit consisting of the Spleen Focus Forming Virus (SSFV) LTR promoter/ Murine Embryonic Stem cell Virus (MESV) enhancer.

The IHP procedure was performed as described earlier[18] with minor modifications. In brief, male Wistar rats, >280 g body weight and ~25 weeks of age were subjected to oxygenated IHP by cannulation of the inferior vena cava (via the femoral vein) and only the gastroduodenal side branch of the common hepatic artery. To isolate the liver vascular bed the portal vein, hepatic artery, suprahepatic caval vein and mesenteric artery were temporarily clamped. At an orthograde perfusion rate of 5 ml/min, the perfusate containing 2.5 x10^8 transducing units (TU) of LV-SF-LUC diluted in cold (4°C) heparinized UW (Viaspan, DuPont Pharma) was

circulated for 10 minutes, followed by a 15 ml wash out with warm (38-39°C) heparinized blood plasma substitute (Gelofusin, B.Braun, Germany) to wash out non-adhered vector particles and to prevent hyperkalemic cardiac arrest due to the high potassium concentration in UW (125 mmol/L, Rat reference value is ~5 mmol/L). At the end of the procedure, clamps and cannulas were removed to restore the blood flow to the liver and the operation wound was sutured. The complete procedure takes less than 1 hr.

For real time *in vivo* bioluminescence imaging, rats were anaesthetized with isoflurane and intraperitoneally injected with 25 μl per 10 gram body weight D-Luciferin potassium salt (Caliper Life Sciences) in PBS (30mg/ml). During a 10 minute incubation time the abdominal fur was trimmed and subsequently luciferase expression was quantified for 5 minutes from the ventral side of the animal using an IVIS Imaging System 200 Series (Caliper Life Sciences).

Luciferase Assay

Luciferase activity in Huh-7 ET cells was quantified using a Top Count scintillation/luminescence counter (Packard BioSciences Top Count-NXT, Milan, Italy). Cells were lysed and luciferin substrate added using Steady Glo Luciferase System (Promega) according to manufacturer protocols.

Cell Viability and Proliferation

Cell viability was determined by 7AAD (BD BioSciences, Mountain View, USA) staining followed by flow cytometric analysis (data not shown). 7AAD negative cells were considered viable, whereas late-apoptotic and necrotic cells were positive for 7AAD[19]. Cell quantification was assessed using Giemsa (Merck, Darmstadt, Germany) total protein staining[20]. Adherent cells were fixed with methanol followed by 20 min staining with 40% Giemsa solution. After five washes with PBS, the cell bound Giemsa was resolved in methanol and quantified using a spectrophotometer at 655 nm with a Model 680 Microplate Reader (BioRad, Hercules, USA). The cell count per well is directly related to the intensity of the OD, as such, expansion of cultures can be measured by comparing ODs of treated cells to cells fixed at time zero (before transduction).

Statistical Analysis

Statistical analysis was performed using non-parametric matched pair test (Wilcoxon) or unpaired T-test, when comparing individual conditions, or one way ANOVA with Tukey Multiple Comparison post test when comparing across time points, using GraphPad Prism 4.0 software.

Results

Vector concentration is proportional to transduction and to inhibition of HCV replication

The success of any viral vector based gene therapy is limited to the effectiveness of the treatment itself, but perhaps more importantly, by the number of cells that can be reached during administration. Thus, even with a very effective RNAi vector, such as the LV-shNS5b, therapeutic success is limited to the level of transduction that can be achieved to the target cells. To determine a correlation between the level of transduction and the effectiveness of the LV-shNS5b, Huh-7 replicon cells were treated with increasing MOI of LV-shNS5b and both GFP and HCV content was determined.
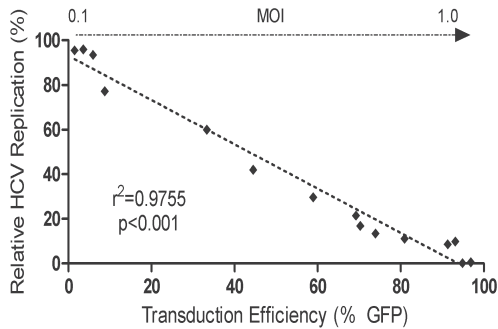
Fig 1: Increasing MOI increases transduction efficiency and HCV inhibition. Huh-7 replicon cells were treated with shNS5b containing lentivectors through a range of MOI's for 2 hours. Cells were washed and incubated for 48 hours to allow for integration and expression. Luciferase (HCV replication) and GFP (transduction efficiency) content was measured and a correlation drawn. Increasing vector input increases transduction of hepatocytes and correlates inversely to the ability of the shNS5b to knockdown HCV. (r2=0.9755, p<0.001).

The correlation is shown in Figure 1: increasing the vector MOI increases transduction, which, in turn, decreases the level of HCV content of the replicon cells (r2=0.9755, p<0.001), by delivering more RNAi to more cells.

Lentiviral transduction is achieved at hypo- or normothermic perfusion solutions

In order to determine if lentiviral vectors could be delivered to hepatocytes under the conditions of cold liver perfusion and storage, Huh-7 hepatoma cells were suffused in different preservation solutions containing sub-maximal MOI of LV-GFP in either hypothermic (2°C) or normothermic (37°C) conditions.

Figure 2 shows a representative FACS profile of the four conditions, in normothermic conditions (Fig. 2A) and hypothermic (Fig. 2B) conditions. The use of normothermic UW (62%±6 SEM) showed a significantly increased transduction percentage over that of both HTK
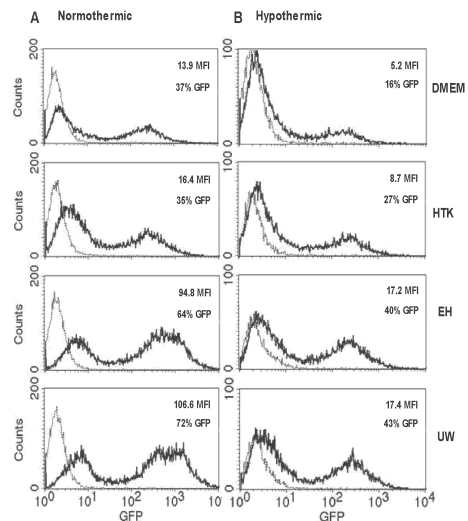
Fig 2. UW promotes lentiviral transduction, at normo- and hypothermic conditions. GFP positive Huh-7 cell percentages and geometric mean florescence intensity (MFI) were measured 24 hours after 2 hour lentiviral incubation in either A) normothermic (37°C) and B) hypothermic (2°C) solutions (cDMEM, HTK, UW, and EH). UW gave a higher transduction than other solutions (72% GFP, normothermic and 43% GFP, hypothermic). One representative FACS plot is shown of 5-8 independent experiments. The number of GFP positive cells was significantly greater than both cDMEM or HTK in both C) normothermic (n=5) and D) hypothermic (n=5) conditions. (* p<0.05, ** p<0.01).
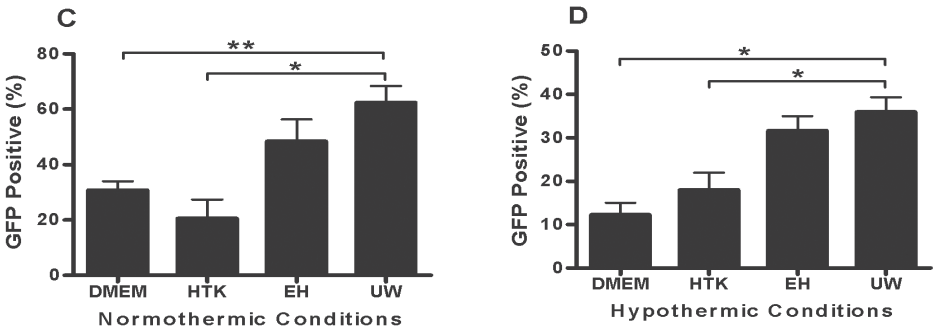
(21%±7 SEM; p=0.03) and cDMEM (31%±3; p=0.007) (Fig. 2C). Within hypothermic conditions (Fig. 2D), the best transductions were seen with the UW solution (36%±3 SEM), which showed significantly improved transduction over that of HTK (18%±3.9; p=0.03) or cDMEM (12%±3 SEM; p=0.03).

Kinetics of hypothermic and normothermic transductions

To determine the time dependency of transduction in perfusion solutions, sub-confluent Huh-7 cells were suffused for different incubation times with LV-GFP under normo (Fig 3A)- or hypothermic (Fig 3B) conditions.
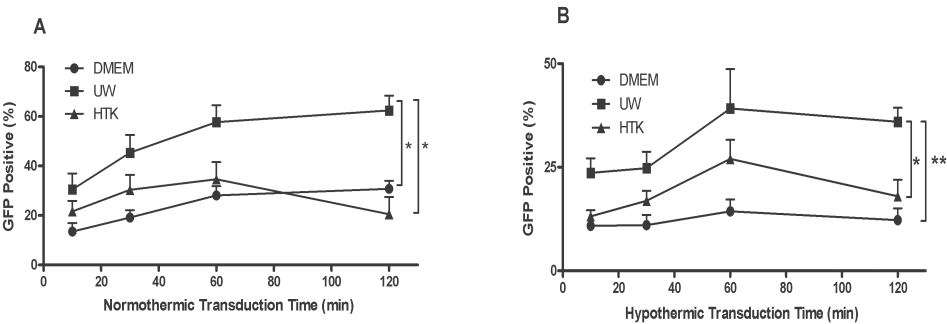
Fig 3. Transduction increases with incubation vector exposure time. Huh-7 cells were incubated with matched input MOI of LV-GFP and incubated in normothermic (37°C) or hypothermic (2°C) solutions for a range of times (10 min – 2 hrs). GFP positive cells were measured via flowcytometry 48 hrs post vector incubation. GFP positive cells increased with vector incubation time. A) In normothermic conditions (n=6), UW gave significantly higher transductions over those of HTK or cDMEM at all time points. B) As well, in hypothermic (n=6) conditions, UW gave significantly higher transductions over that of HTK or cDMEM. A transduction plateau was observed at 1 hr with all solutions, though HTK transductions were reduced at longer than 1 hr incubation (* p=0.05, ** p=0.01).

In hypothermic conditions GFP expression could be observed 48 hrs after with LV-GFP incubation times as short as 10 minutes and, expectably, increased with longer (2 hr) time periods. The transduction levels appeared to reach a maximum at one hour vector exposure and dropped, though not significantly, at 2 hours with HTK. Similar trends were found in normothermic conditions. UW solution provided a significantly better LV-GFP transduction environment over that of cDMEM or HTK solutions in both hypothermic (p<0.01, p<0.05 respectively) and normothermic (p<0.05, p<0.05 respectively) environments across all time points.

Cell viability and Growth

High tissue survival is one of the main aims of pre-transplant graft perfusion and as such protocols used are optimized to ensure maximum tissue viability post reperfusion. We determined whether cells remained viable under hypo- and normo-thermic conditions during the longest lentiviral incubation time point (120 min). Overall, cells remained vital (data not shown) and as shown in Figure 4, cells continued to proliferate after both normo- (Fig. 4A) and hypothermic (Fig 4B) transduction incubations.
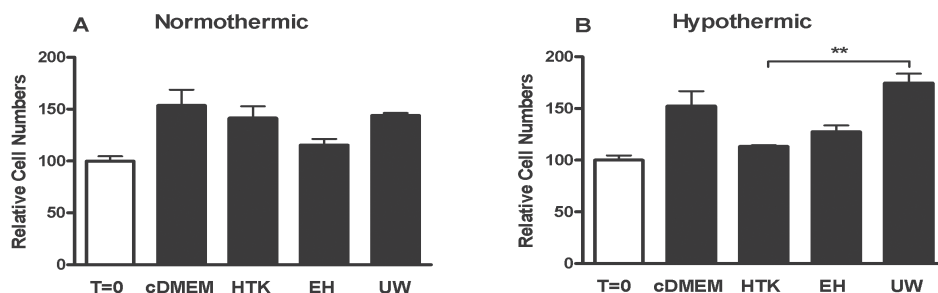
Fig 4. UW does not affect cell proliferation after normo- or hypothermic incubations. Giemsa total protein stain was used to determine total protein content, an indicator of cellular growth. Giemsa staining was determined at T=0 and 24 hr post incubation. Within A) normothermic conditions, cell growth was not significantly different across all solutions (n=3), though after B) hypothermic incubation, cells in UW had increased in numbers significantly more than those that were incubated in HTK (n=3) (** p=0.01).

Interestingly, after hypothermic conditions, cells incubated in UW (75%±9 SEM increase over T=0) showed more proliferation than those in HTK (13%±1 SEM increase over T=0; p=0.016), possibly due in part to the colloid (HES) and superior impermeant (Lactone) components of UW.

UW	
Poly (2-0 hydroxethyl) starch (250Kda)	Colloid: reduction of interstital edema and endothelial cell swelling
Lactobionic Acid (as Lactone)	Impermeant: suppression of hypothermic cell swelling
Potassium Phosphate monobasic	pH Buffer: maintenance of intracellular sodium and potassium concentrations: restoration of high energy phosphate
Magnesium Sulfate heptahydrate	preservation of intracellular magnesium concentration
Raffinose pentahydrate	Impermeant: suppression of hypothermic cell swelling
Adenosine	restoration of high energy phosphate
Allopurinol	Inhibition of xanthine oxidase activity and purine metabolism/reduction of oxygen free radicals
Total Glutathione	Antioxidant
Potassium Hydroxide	preservation of intracellular potassium concentration
H_2O	
pH 7.4 (37C)	
Osmolaity 320 mosmol/kg	
HTK	
Natrium chloride	isotonic balance
Potassium chloride	preservation of intracellular potassium concentration
Potassium-Hydrogen-2-oxoglutaraat	substrate for aerobic energy production
Magnesium Chloride -6H2O	preservation of intracellular magnesium concentration
Calcium Chloride-2H2O	isotonic balance
Histidine HCL-H2O	pH buffer: entends capacity
Histidine	pH buffer: entends capacity
Tryptophaan	membrane integrity
Mannitol	sugar alchohol : Impermeant: suppression of hypothermic cell swelling, maintainence of intracellular glucose levels
H_2O	
pH 7.4 (37C)	
Osmolality: 310 mosmol/kg	
EloHaes	
Poly (2-0 hydroxethyl) starch (200Kda)	Colloid: reduction of interstital edema and endothelial cell swelling
Sodium Chloride	isotonic balance
Sodium Hydroxide	pH control
Hydrochloric Acid	pH control
H_2O	
pH 3.5-6.0 (37C)	
Osmolality: 308 mosmol/kg	

Table 1: Annotated components.

HES is responsible for superior transduction efficiency by UW

Though UW and EH gave similar transductions these solutions differ in their component makeup as detailed in Table 1. As UW is used in the clinic as a perfusion and a preservation solution, the solution is much more complex than that of EH, which is used mainly as vascular volume expander during cardiovascular surgery. The two solutions contain hydroxyethyl starch, with UW containing 5% Viastarch (250kda HES pentafraction), and EH containing 6% (200kda) HES pentafraction (Table 1).

HTK and UW are similar in that the solutions have comparable concentrations of cations and anions; however, HTK contains no starch, but instead contains mannitol to reduce hypothermic swelling. As well, UW contains numerous active agents such as to replenish energy and scavenge
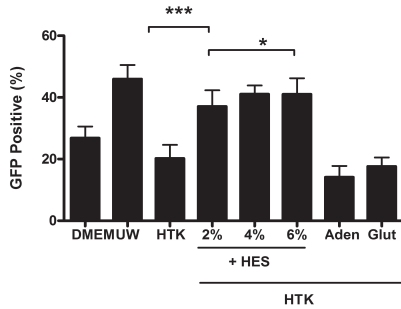
Fig 5. Increased transduction was dependant on the presence of HES but not adenosine or glutathione. Huh-7 cells were incubated for 2 hrs in normothermic perfusion solutions with LV-GFP. Transduction was determined in HTK supplemented with additional components found in UW (Adenosine, Glutathione, and HES). The addition of adenosine (n=5) or glutathione (n=3) did not significantly increase transduction of LV-GFP over that of HTK alone (n=12). When HTK was supplemented with HES (n=3-8), at percentages lower and higher than that of UW (5% HES), a general increase was observed, with the 4% HES/HTK solution showing a significant increase in transduction was observed over that of HTK alone (* p=0.05).

oxygen radicals (Table 1) neither of which is found in HTK or EH. To determine whether any or all of these component differences could account for the increase in transduction efficiency, HTK was supplemented with UW comparable concentrations of Adenosine, Glutathione or HES. After a two hour normothermic LV-GFP incubation and a nominal 48 hrs post transduction, flowcytometric analysis for GFP positive cells was performed. As seen in figure 5, no increased transduction was observed after HTK was supplemented with glutathione or adenosine, indicating that neither of these components was responsible for the additional transduction potential of UW.

However, when HTK was supplemented with increasing concentrations of Viastarch, significant improvement in transductions, nearing that of UW alone (Fig. 5) were observed. This indicated that HES is the most important component responsible for the increased transduction potential of UW.

Enhanced transduction by HES is due to charge interaction and not size exclusion

The presence of Viastarch in UW helps to minimize the free liquid while maintaining osmotic
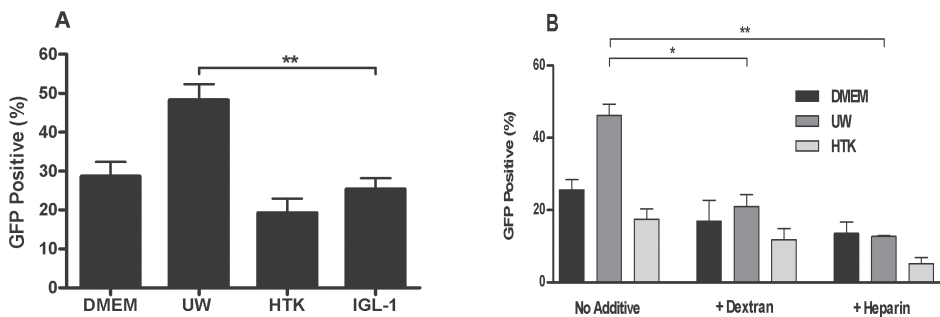
Fig 6. Increased transduction in UW solution is due to a charge interaction and not size exclusion concentration of vector. Huh-7 cells were incubated for 2 hrs in normothermic perfusion solutions with LV-GFP, transductions were measured by flowcytometry 48 hr post incubation then compared to transductions in machine perfusion solution IGL-1. A) Transduction percentages were non-significantly increased in IGL-1 (n=5) over that of HTK, but remained significantly lower than the transductions observed in UW. B) Huh-7 cells were incubated in normothermic solutions (cDMEM, HTK, or UW), supplemented with anionic polymers (Dextran Sulfate or Heparin Sulfate) and LV-GFP at matched MOI for 2 hrs. Transduction was significantly decreased with both Dextran sulfate (n=6) or Heparin (n=3) supplementation to UW, when compared to UW alone (* p=0.05, ** p=0.01).

pressure, allowing a more concentrated ion flow during perfusion. It could be postulated that the greater transduction potential of UW could be, in part, due to the nature of HES to exclude total free liquid, thereby concentrating the initial input of vector. To test this, UW was compared to machine perfusion solution IGL-1, a solution very similar to UW with the main exception that HES is replaced by polyethylene glycol (PEG 35) (see Maathuis/Ploeg for detailed differences[6]). As shown in Figure 6A, transductions in IGL-1 (25%±3 SEM) were not significantly different than those in HTK (19%±4 SEM), but were significantly lower than the transduction achieved with UW solution (48%±4 SEM, p<0.009).

Charged polymers alter the kinetics of viral adsorption and thereby modulate transduction efficiency independent of their tropism. To determine if a HES/vector charge dependant interaction was present in our setting, cDMEM, HTK or UW was supplemented with anionic polymers before the addition of matched MOI LV-GFP and then determined the level of transduction. Figure 6B shows the effects of the addition of heparin or dextran sulfate on the transduction potentials. Both heparin (13%±0.1 SEM) and dextran sulfate (21%±3 SEM, p<0.009) supplementation to UW significantly (p=0.07 and p=0.01 respectively) reduced the percentage of GFP positive cells when compared to UW alone (46%±3 SEM), whereas their addition to cDMEM or HTK resulted in non-significant reductions.

UW enhances transduction in Primary Hepatocytes
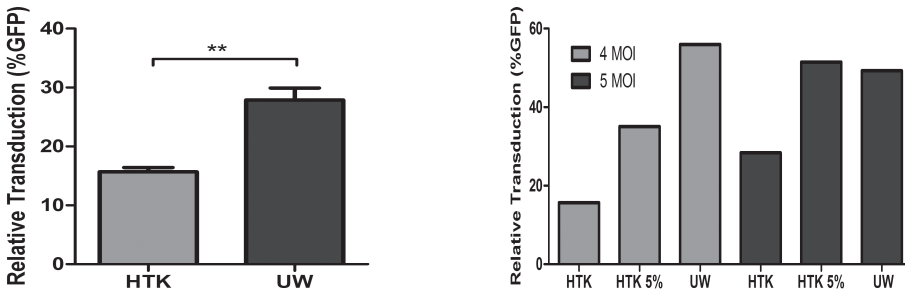
Fig 7. UW enhances transduction in primary hepatocytes. Primary hepatocytes were incubated for 2 hrs in normothermic perfusion solutions with LV-GFP. A) UW significantly increased the transduction of primary mouse hepatocytes over that of HTK (27.87%±0.7SEM, 15.67%±0.7SEM respectively, p=0.0078, n=4). B) In human primary hepatocytes similar results were obtained when transductions were performed in normothermic solutions for 2 hours. With the addition of 5% HES to HTK, transductions increased to near levels obtained with UW alone.

Though high transductions were achieved in a hepatoma cell line, these cells may not be representative of primary hepatocytes. To show the effectiveness of UW to deliver lentiviral vectors to primary hepatocytes, mouse and human, were subjected to the same perfusion conditions as the hepatoma cell lines and transduced with LV-GFP for 2 hours. In primary mouse hepatocytes, significantly higher relative transductions were achieved in UW (28%±2.1SEM) over that of HTK (15.7±0.7SEM) (p=0.0047, n=4) with a relatively low dose of 3 MOI (Fig. 7a). Primary human hepatocytes were transduced for 2 hours in similar conditions as the primary

mouse hepatocytes, with the added condition of HTK supplemented with 5% HES and increased the MOI to 4 and 5. Similar relative differences over controls were seen at both MOI's (4 and 5) between UW (55 and 49%) and HTK (16 and 28%), as well the addition of 5% HES to HTK brought transduction to near UW alone (35 and 55%) bringing the relative transductions comparable to that of UW alone. Though transductions to primary hepatocytes were lower than the results obtained with hepatoma cell lines, transductions were possible and were notably increased with the use of UW or the addition of 5% HES.

Transduction of lentiviral vectors to hypothermic isolated hepatic perfusion is possible with UW solution in rats

Isolated hepatic perfusion was used to determine if the effect we had observed with the *in vitro* perfusion settings would be applicable to deliver lentiviral vectors directly to the liver *ex vivo*. The liver was isolated from the body by cannulation and a full perfusion performed with cold UW so a predictive model could be assessed. After perfusion with lentiviral vectors expressing the luciferase gene, rats were surveyed for luciferase expression 8 (Fig. 8A), 19 (Fig. 8B) and 69 (Fig. 8C) days. In figure 8a, though a hair scatter effect (animal was not shaved at this time point) confounds this reading, there is clear deposition in the liver. This time point appears to have expression levels within non-target organs, suggesting that not all vector was washed or that other scatter effects, such as cavity effects, are creating non-specific light expression. At later time points (fig. 8b and 8c), the luciferase expression level is restricted to the liver area and seems to be increasing with time, suggesting that expansion of transduced liver cells is beginning to occur.
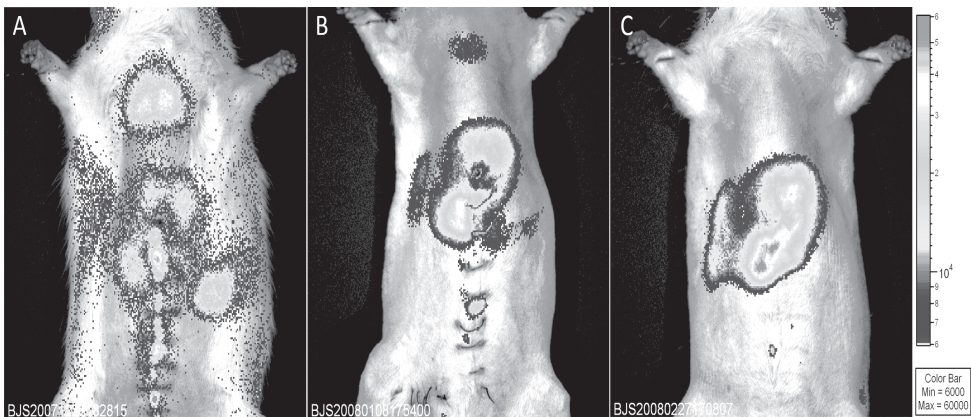
Fig 8. Hypothermic isolated rat liver perfusion deposits lentiviral vectors specifically to the liver. VsV-g pseudotyped, SSFV promoter driven luciferase expression cassettes were delivered directly to the liver during a 10min hypothermic perfusion with UW solution. A) Luciferase expression was measured after 8 days, 19 days (B) and 69 days (C) post perfusion. Non-liver specific expression of luciferase was notably from cavity scatter as well as hair related light scatter. Liver specific deposition of vector was clearly seen and seemed to increase over time as transduced liver cells continued to undergo mitosis.

Discussion

In the current study we have demonstrated that transduction is inversely correlated to the effectiveness of an RNAi therapy (Fig. 1) and that UW perfusion solution creates a superior transduction environment, improving the transduction of hepatocytes by lentiviral vectors. UW provided a much better environment that facilitated a greater number of transduced cells, in both normothermic and hypothermic conditions (Fig. 2). Therefore, liver preservation before transplantation may provide a unique opportunity; a safe application of viral vector based gene therapy that targets, for instance, recurrent liver diseases after liver transplantation, such as hepatitis C.

Recurrent HCV rapidly infects a new liver graft after transplantation and will thrive under the immunosuppression needed to prevent graft rejection. In order to prevent HCV from overwhelming the new graft a substantial proportion of hepatocytes need to express the given genetic therapy imparting a selective advantage over non treated cells. With only a small window of opportunity to deliver therapeutics to a liver graft, it is important that high transductions be achieved in not only the shortest period of time, but also under the conditions of cold perfusion. Using the parameters of a post procurement liver graft, we determined that this window was available for times ranging from ten minutes to two hours, during the second cold perfusion of the graft, with longer incubation times producing higher transductions. UW's superior transduction potential was observed across the range of times, in hypothermic as well as normothermic conditions. Surprisingly, we were able to achieve reasonable transductions with as little as 10 minutes incubation time, indicating a rapid interaction between vector particles and the cell monolayer (Fig. 3).

Cell culture conditions, though informative, are only a model for the *in vivo* environment. As this study sought to determine the effectiveness of transduction to hepatocytes under the conditions of a liver perfusion, primary hepatocytes were subjected to similar conditions as the cell cultures. Though lentiviral vectors are capable of transducing mitotically quiescent cells[21, 22], the levels attainable *in vivo* are generally lower than *in vitro*[23], possibly due to lentiviral vectors needing some level of cell cycling *in vivo* to effectively transduce hepatocytes[24]. Given this, it is not surprising that we saw lower transductions in both primary mouse hepatocytes (Fig. 7a) as well as in primary human hepatocytes (Fig. 7b). The trends were similar, in that, UW gave better transductions than other solutions used, indicating that UW could be a viable transduction agent for transfer of a genetic therapy to liver grafts during perfusion, though in order to increase transductions to therapeutic levels, additives may be required such as hyper-IL-6[23] or growth factors such as hepatocyte growth factor or epidermal growth factor[25].

Interestingly, when we attempted transductions *In vivo*, during isolated hepatic perfusion, we saw, what appeared to be a therapeutically viable transduction level as well as an increasing luciferase expression to 69 days post perfusion (Fig. 8c). This was an encouraging result in that during a cold perfusion, UW could be used to successfully transfer lentiviral vectors directly

to the liver, with minimal transfer and escape to other organs. *Ex vivo* delivery of adenovirus mediated gene therapy using perfusions solutions has been attempted to some success with heart transplantation animal models[26-28], though the effect was transient and often invoked an inflammatory response against transduced cells, reducing the effectiveness of treatment. To our knowledge this is the first account of the use of UW to transfer lentiviral gene therapy vectors directly to the liver during an isolated hepatic perfusion. The use of lentiviral vectors allows for lower transduction to still be therapeutically viable, as transduced cells will propagate therapeutically active daughter cells. With time, due to immune mediated selection pressure on non-transduced, HCV permissible cells, may over populate wild type hepatocytes, thus protecting the graft from the damage of HCV recurrence.

UW solution has become the standard solution for the preservation of most organs in transplantation since its introduction by Belzer et al[29] in the late 1980's. Preservation solutions have been designed to help reduce the physiological and biochemical effects of ischemia due to cold storage. One of the main purposes of these solutions is to prevent cell swelling and interstitial edema formation by including substances that are osmotically active and impermeable to the cell[30]. Compounds such as colloids, are used in perfusion solutions to counteract the hydrostatic force during the initial washout, thereby creating a homeostatic environment that precludes the need for the osmotic regulation. A major component of UW is HES (Viastarch), a colloid used to prevent hypothermic induced cell damage during major surgery. HES has been shown to decrease transvascular fluid flux and edema formation, maintain colloid osmotic pressure and preserve the microvascular barrier[31]. As EH, a 6% HES solution, showed similar transductions to that of UW, it was logical that this shared component was, at least in part, responsible for the increase in transduction that was observed. Indeed, the addition of HES to HTK solution increased transductions to levels nearing UW alone (Fig 5).

Traditionally, charged polymers have been used to increase the transduction potentials of viral vectors[32-35]. Charged polymers alter the kinetics of viral adsorption thereby modulating transduction efficiency, independent of their tropism[36]. Cationic polymers such as Dextran DEAE or Polybrene can enhance negatively charged adenovirus binding and transduction by reducing electrostatic repulsion between negatively charged viruses and cells[33, 37], though, can almost always be inhibited by the addition of anionic polymers through the increase in electrostatic repulsion[36, 38]. Most retroviruses, including lentiviruses, contain positively charged surface domains which probably create a repulsion force that prevents natural aggregation, similar to that which that prevents the natural aggregation of erythrocytes[39]. Though, in the presence of HES, human[30] and rat[39] red blood cells (RBC) aggregate, into large stacks known as rouleaux formations. The exact mechanism for this RBC aggregation is not known, though Morariu et al shows a strong correlation between colloids with high molecular weights and rouleaux formation. They suggest that the effect can be explained by the theory of macromolecular bridging[40], wherein polymers and plasma proteins with large molecular mass insert between adjacent erythrocytes, decreasing the electrostatic repulsive forces of erythrocytes. HES may

influence vector electrostatic charges in this way as well. This reduction in the normal repulsive state may force vector aggregation[34]; increasing the number of vector to individual cell contacts, or increase the speed at which vectors are able to sediment and bind to cell surfaces; by neutralizing repulsive forces between vectors and cells[41]. Most probably, both situations occur.

Though HES is a non-ionic compound, at pH 7.4 (such as in UW), it contains weak negative charges in flux. This flux possibly creates temporary surface dipoles altering charge distribution across the large irregular surface of the starch. Lentiviruses, being positively charged, are attracted to the negative regions and form temporary aggregation complexes with the starch. The vector/HES complex sediments to the cell surface, where it shields against the positively charged residues, exposing more negatively charged residues, and the vector shifts from vector/HES to vector/cell, attracted by the more negatively charged phospholipids. These two effects produce much greater transductions in solutions with HES when compared to solutions without.

The ability to impart a proactive protective treatment to liver grafts prior to transplantation is an attractive option to reduce the incidence and severity of HCV recurrence. The use of UW in liver transplantation opens not only a distinct window of opportunity to provide effective gene transfer *ex vivo*, but due to HES, increases the ability of the vector to reach more cells, even in cold conditions. As the use of vector based RNAi therapies in the clinics gain popularity, the need for *ex vivo* delivery becomes increasingly obvious. Targeting specific cells or whole organs, avoiding off-target integrations and producing smaller amounts of vector are specific goals and concerns of gene therapy. The use of UW, under the context of organ transplantation, allows for *ex vivo* transduction in both hypothermic and normothermic conditions, allowing for a customized approach to gene delivery, in which all three concerns can be addressed. Though effective in an *in vitro* setting, *in vivo* data will need to be collected to confirm these results.
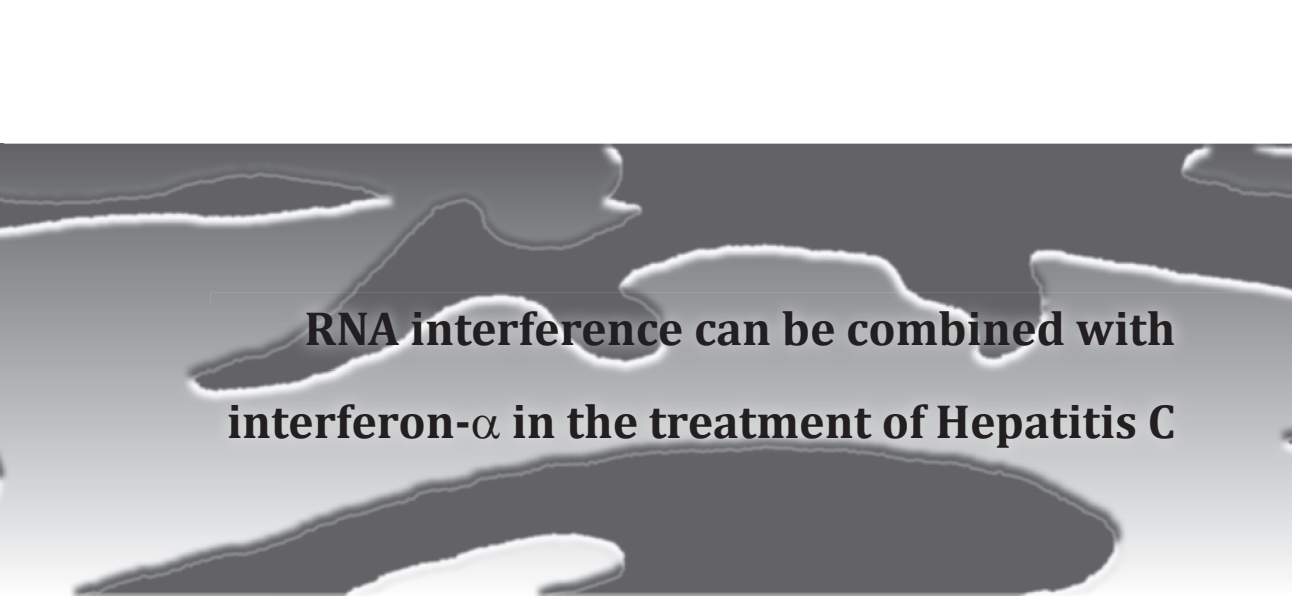
Acknowledgements

CHAPTER 9

Manuscript in Preparation

RNA interference can be combined with interferon-α in the treatment of Hepatitis C

Qiuwei Pan[3]

Scot D. Henry[1]

Herold J. Metselaar[3]

Bob J. Scholte[2]

Jaap Kwekkeboom[1]

Hugo W. Tilanus[1]

Harry L.A. Janssen[3]

Luc J.W. van der Laan[1]

Departments of [1]Surgery, [2]Cell Biology and [3]Gastroenterology & Hepatology

Erasmus MC-University Medical Center, Rotterdam

Abstract

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease with nearly 200 million carriers worldwide. The current standard treatment of Pegylated-interferon-alpha (IFN-α) administered in combination with ribavirin is only effective in approximately half of the patients, prompting the need for alternative treatments. RNA interference (RNAi) represents an attractive new approach to combat HCV, allowing direct knockdown of viral RNA or host factors involved in the viral life cycle. RNAi monotherapies might ultimately fail to control viruses that can escape silencing by mutation. Based on their distinct antiviral mechanism, we propose that combining lentiviral mediated RNAi with IFN-α may avoid therapeutic resistance and exhibit enhanced antiviral activity. However, there is some concern about the negative effect of IFN-α on retroviral transductions.

In this study, we used a lentiviral vector containing two small hairpin RNAs (LV-sh2) that can simultaneously modulate both viral RNA and a proposed viral entry receptor, CD81, in a HCV replication model. Exogenous IFN-α had no influence on vector transduction efficacy. In the presence of IFN-α LV-sh2 still retains CD81 silencing efficacy. Encouragingly, lentiviral mediated RNAi and IFN-α act independently on HCV replication showing combined efficacy without very clear evidence of cross-interference.

Combination of IFN-α with RNAi significantly enhanced anti-viral effects. Therefore, this novel combinatorial strategy may offer potential to eliminate HCV infection in chronically infected patients and could prevent mutational escape from therapy.

Introduction

Hepatitis C virus (HCV), first identified in 1989, remains a major cause of chronic liver disease concern with nearly 200 million carriers worldwide. HCV is a single-stranded positive-sense RNA flavivirus with 6 major genotypes and over 70 subtypes[1, 2]. Interferon alpha (IFN-α) therapy led to a rapid decline in viral genomic RNA levels in serum, and long-term responses were marked by sustained loss of HCV RNA from the serum and liver and resolution of the chronic infection[3]. Further improvement has achieved sustained response in about half of patients, by using pegylated IFN-α combined with ribavirin[4 5], which is now the standard of care. However, half of the patients remain non-responsive to this therapy. Thus, exploitation of alternative anti-HCV therapies is urgently required.

RNA interference (RNAi), the degradation of cognate mRNA by small interfering RNA (siRNA), has emerged as a novel therapeutic entity for viral infection. Since the HCV genome is a single-stranded RNA that functions as both a template for transcription and template for a negative strand replication intermediate, it is a prime candidate for RNAi. Host cellular factors involved in the viral life cycle, such as CD81, Claudin-1, SR-B1, HSP90, p68 or USP18, could also be candidate targets for RNAi antiviral therapy, resulting in either abolished viral entry or replication[6-9]. The success of RNAi in therapeutic application also depends on an efficient delivery system, which can support long-term siRNA production and continuous gene silencing. Integrating self-inactivating lentiviral vector can achieve this criterion by encoding small hairpin RNA (shRNA), a precursor of siRNA which is automatically cleaved into biologically active siRNA by Dicer in cells[10, 11].

However, RNAi monotherapies might ultimately fail due to viral mutation and the development of resistance. RNA viruses like HIV and HCV are highly adaptive in this context and mutational escape from therapy has been documented against single RNAi treatments[12-14]. Thus, a combinatorial strategy would be necessary for eliminating HCV infection. An advanced method reported by our group and others, has shown that simultaneously targeting both viral and host cell elements by RNAi could increase the potency of antiviral therapies[14, 15]. IFN-α possesses indirect antiviral activity by stimulating genes that can lead to a non-virus-specific antiviral response, whereas RNAi can directly interfere with viral entry and replication through targeting viral RNA genome or mRNA of cellular factors. Based on their dissimilar antiviral mechanisms, we propose that combining RNAi with IFN-α may avoid therapeutic resistance and exhibit enhanced antiviral activity.

To date, very little is known about the interaction of exogenous IFN-α with lentiviral vector delivery and the anti-viral action of RNAi. Reports have suggested that the administration of lentiviral vectors (LV) to mice triggers a rapid and transient IFN-α/β response. In animals that lack the capacity to respond to IFN-α/β, dramatic increases in transduction to hepatocytes were seen, indicating that endogenous IFNs interfere with lentiviral transduction in a cell-type specific

manner[16]. In the current study, we investigated the effect of combining lentiviral mediated RNAi with IFN-α for the treatment of HCV. The effect of exogenous IFN-α on the transduction efficiency of lentiviral vectors as well as the anti-viral RNAi activity was investigated in an HCV replication model.

Methods And Materials

Cell culture

Cell monolayers of the human embryonic kidney epithelial cell line 293T and human hepatoma cell lines Huh-7 and Huh-6 (kindly provided by prof. R. Bartenschlager, Heidelberg, Germany) were maintained in Dulbecco's modified Eagle medium (DMEM) (Invitrogen–Gibco, Breda, The Netherlands) and complemented with 10% v/v fetal calf serum (PerBio Science), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine (Invitrogen–Gibco) (cDMEM). Huh-7 cells containing a subgenomic HCV bicistronic replicon (I_{389}/NS3-3V/LucUbiNeo-ET, Huh-7 ET) were cultured under selection with 250 µg/ml G418 (Sigma, Zwijndrecht, The Netherlands)[17].

Construction and production of lentiviral vectors

Lentiviral vectors (LV), LV-Green Fluorescent Protein (GFP) and LV-sh2 containing double shRNA cassettes were constructed and produced as previously reported[15]. Briefly, a third-generation lentiviral packaging system pND-CAG/GFP/WPRE containing double shRNA expression cassettes targeting the IRES region of HCV and CD81 was used to produce high-titer VSV-G-pseudotyped lentiviral vectors in 293T cells. Vector supernatants were removed 36 and 48 hr post transfection, passed through a 0.45mM filter, and concentrated 30000-fold by ultracentrifugation.

Vector transduction assay

Concentrated virus stocks were titrated using 293T cells 24h after infection, with transduction efficiency based on the number of GFP-positive cells as determined by flow cytometry (FACSCalibur; BD BioSciences, Mountain View, CA, USA). Transduction efficiency of LV-GFP in the presence of IFN-α was tested using this method in Huh-7 cells 3 days post-infection.

Real-time RT-PCR

Confluent cell monolayers were lysed in Trizol (Invitrogen-Gibco) and RNA precipitated with 75% EtOH and captured with a Micro RNAeasy silica column (Qiagen, Venlo, The Netherlands). RNA was quantitated using a Nanodrop ND-1000 (Wilmington, Delware, USA) and adjusted to total cell numbers for each sample. cDNA was prepared using Promega's AMV reverse transcriptase following standard protocols. Real-time PCR (MyIQ, BioRad) was performed using primers CD81 (F': 5'-ACC TTC CAC GAG ACG CTT-3'; R: 5'-CAG GAT CAT CTC GAA GAT CAT G-3'), and CyB (F': 5'-ATG TAG GCC GGG TGA TCT TT-3'; R: 5'-TTT ATC CCG GCT GTC TGT CT-3'). Semi-quantitation of sample amplicons was performed with SybrGreen (Sigma) using Taq DNA polymerase (Invitrogen–Gibco).

Flow cytometry analysis for CD81 expression

CD81 expression was determined after incubation with phycoerythrin (PE) conjugated mouse anti-human CD81 monoclonal IgG1. Mouse IgG1 was used as isotype control (BD Pharmingen, San Diego, CA, USA). Flowcytometeric analysis was performed using a FACSCalibur (BD BioSciences).

Luciferase assay

Cells for endpoint assays were lysed and luciferin substrate added using Steady Glo Luciferase System (Promega, Leiden, The Netherlands) according to manufacturer instructions. Live cell experiments were performed as described above (excluding lysis step) with the addition of 100 µM luciferin potassium salt (Sigma). Luciferase activity was quantified using a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

Statistical analysis

Statistical analysis was performed by using either matched pair nonparametric test (Wilcoxon signed-rank test) or the nonpaired, nonparametric test (Mann–Whitney test) (GraphPad Prism software). $P<0.05$ was considered as significant.

Results And Discussion

LV-sh2 simultaneously inhibits CD81 expression and HCV replication

Since integrating lentiviral vectors have shown great advantages in transgenic delivery, we used a third-generation lentiviral vector (LV-sh2) for shRNA expression, which contains two independent shRNA cassettes targeting the HCV IRES region and host cell surface receptor CD81 as well as containing the GFP reporter gene (Fig. 1A). Increasing the dose of vector resulted in
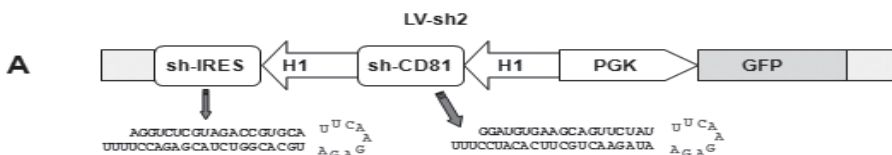
higher levels of transduction efficiency and inhibition of HCV replication, as monitored by GFP positive population and luciferase activity (MOI 5-30, Luciferase 75% to 41% of controls, GFP; 82-97% respectively). Though a threshold for the level of silencing was seen, suggesting that increasing vector dose from an MOI of 20 to 33 could not further inhibit HCV replication (Fig. 1B). Within the GFP positive population (transduced cells) CD81 expression was significantly down regulated. The percentage of CD81 positive cells was reduced from 72% in LV-GFP transduced cells to 3% in LV-sh2 transduced cells (Fig. 1C). These results demonstrate that lentiviral vector driving double shRNAs can simultaneously modulate host factorCD81 expression and viral
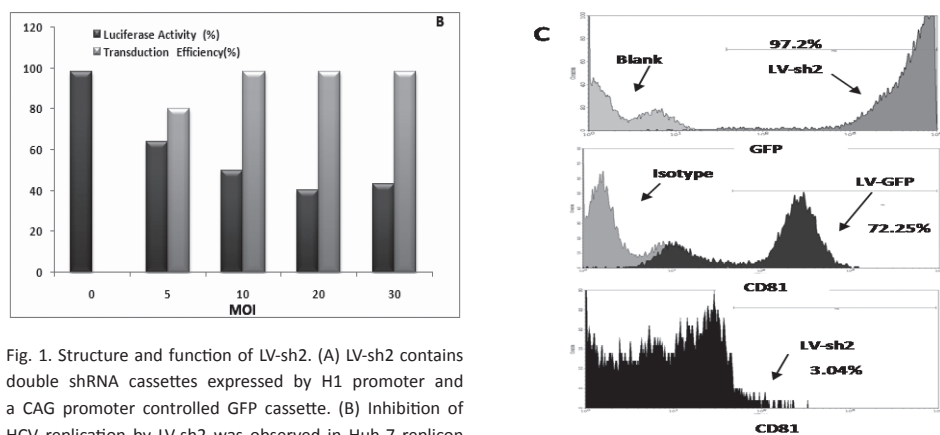
Fig. 1. Structure and function of LV-sh2. (A) LV-sh2 contains double shRNA cassettes expressed by H1 promoter and a CAG promoter controlled GFP cassette. (B) Inhibition of HCV replication by LV-sh2 was observed in Huh-7 replicon cells by monitoring luciferase activity. (C) Simultaneously, LV-sh2 can efficiently knockdown CD81 expression in Huh-7 replicon cells, resulting in a sharp reduction of CD81 positive population from 72.25% to 3.04%.

RNA. Down regulated expression of viral entry receptor, CD81, was shown to be effective in preventing HCV envelope binding[18]. It is conceivable that LV-sh2 has the potential to not only inhibit viral replication but could also be used to prevent infection, though the effects of downregulating important cellular factors like CD81 in humans are largely unknown, though CD81 knockout mice have reduced fertility[19].

Exogenous IFN-α has no negative impacts on lentiviral transduction

Studies have demonstrated an interferon response occurs by exposing human dendritic cells (DCs) in culture to wild-type HIV, the origin of many lentiviral vectors[20, 21]. A more recent study has demonstrated that lentiviral vector triggers a type I interferon (IFN-α/β) response that restricts hepatocyte gene transfer and promotes vector clearance in mice[16]. However, the effect of exogenous IFN-α on lentiviral transduction has not been investigated to our knowledge.
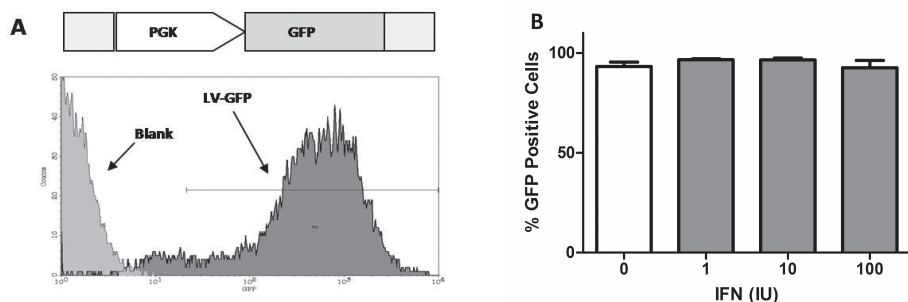
Fig. 2. Effect of exogenous IFN-α on LV-GFP transduction. (A) LV-GFP expressed GFP under control of PGK promoter. GFP positive population was determined by FACS 3 days after transducing LV-GFP. (B) No significant differences of transduction efficiency was observed at the presence of 1, 10 or 100 IU/ml concentration of IFN-α (96.61 ± 0.43 %, 96.52 ± 0.86 % and 92.57 ± 3.67 % respectively, mean ± SD, n=3, P>0.05), compared with non-treated control group (93.14 ± 2.22 %).

To address this question, a vector transduction assay was performed by infecting Huh-7 cells with LV-GFP (Fig. 2A) in the presence of 1, 10 or 100 IU/ml concentration of IFN-α. At day 3, transduction efficiency was assessed by FACS analysis for GFP positive cells. When compared with non-treated control group (93%± 2SD, n=3), 1, 10 or 100 IU/ml concentration of IFN-α had no significant effects on vector transduction (97%±0.4SD, 97%± 0.9SD and 93% ±4 SD respectively, p>0.05) (Fig. 2B).

The achievement of a high transduction is essential for therapeutic application of viral vectors and these *in vitro* observations have shown that exogenous IFN-α does not have apparent impact on lentiviral vector transduction, so the use of both these agents in a combinatorial therapy seems possible. To fully clarify this effect, *in vivo* studies are still required.

LV-sh2 retains CD81 silencing efficacy at the presence of IFN-α

Some studies suggest that siRNA, under certain circumstances, can activate the innate immune system and invoke a host cell interferon response, which may lead to nonspecific antiviral activity and off-target effects[22,23]. However, in the setting of combining RNAi with IFN-α, the most critical issue would be the influence of exogenous IFN-α on gene silencing efficiency, rather than interaction with an innate immune response towards either the vector or the expression of siRNA.

To evaluate this issue, Huh-7 cells were treated with 1, 10 or100 IU/ml IFN-α, then transduced with LV-sh2 and maintained in culture for 3 days. CD81 expression level was profoundly inhibited with each concentration. As shown in figure 3, LV-sh2 efficiently silenced CD81 in Huh-7 cells (0.8% CD81 positive in GFP positive population), when compared with non-transduced Huh-7 (86%) or LV-GFP transduced control (67%). INF-α alone seems slightly up-regulate CD81 expression at 1 and 10 IU/ml treated groups (91 and 89%, respectively), but at the highest dose (100 IU/ml) CD81 was down regulated to 65% of controls. Regardless of the complicated influence of IFN-α on CD81, LV-sh2 can retain robust gene silencing efficacy at each IFN-α treated group (0.9%, 1.7% and 2.7%, respectively).
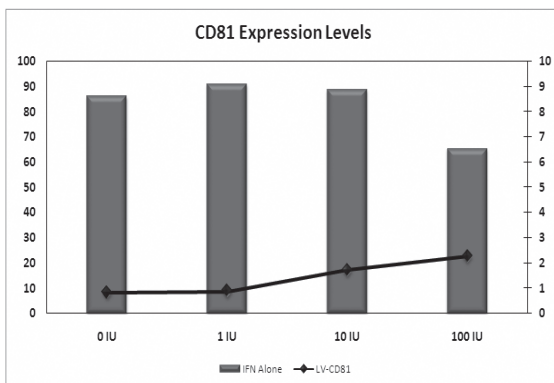
Fig. 3. LV-sh2 efficiently silenced CD81 in Huh-7 cells at the presence of IFN-α. CD81 protein level was examined by FACS analysis under the different conditions as indicated.

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Combination of LV-sh2 with IFN-α enhances anti-HCV activity

Since IFN-α alone or in combination with ribavirin is only successful in half of patients with chronic HCV infections, RNAi has begun to be considered as a potential alternative for HCV therapy. However, owing to rapid viral mutation and sequence-restricted recognition of RNAi, HCV may quickly escape and develop resistance to monotherapy. Therefore, we propose that RNAi armed with IFN-α may be a viable option to completely cure HCV infection by attacking the virus from two distinct angles.
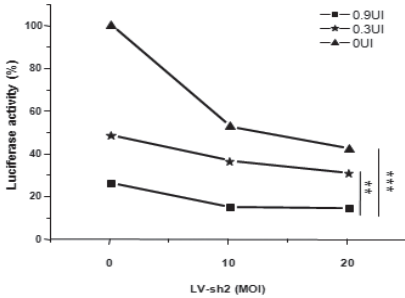
Fig 4. Co-antiviral effects of combining LV-sh2 with IFN-α in Huh-7 ET model. Different dose of LV-sh2 and IFN-α were treated either alone or in combination. Luciferase activity was measure to indicate viral replication. Enhanced antiviral effects were homogeneously observed at each combined group compared with single.

The combination of these two agents significantly enhanced anti-viral effects in the Huh-7 ET model (Fig. 4), without very clear evidence of cross-interference. For instance, the combination of LV-sh2 (MOI 20) with a suboptimal dose (0.9UI/ml) of IFN-α significantly

Fig 5. LV-sh2 down regulated CD81 expression in Huh-6 cell. Real time RT-PCR was used to determine mRNA level after 4 days treatment.

inhibited HCV replication (85% inhibition), when compared with LV-sh2 alone (58%, p<0.001) or IFN-α alone (69%, p<0.05). The same pattern was observed for each combination doses as shown in figure. 5, when analyzing HCV replication by quantitative RT-PCR. These results clearly suggest that RNAi and IFN-α act independently on HCV replication with combinatorial strategies showing a collective anti-viral effect.

In conclusion, in this study we found that exogenous IFN-α had no significant negative influence on lentiviral transduction. In the presence of IFN-α, LV-sh2 mediated knockdown of CD81 surface expression was unaffected and encouragingly, a combination of LV-sh2 and IFN-α significantly enhanced their individual anti-viral effects in the replication model. Therefore, this novel combinatorial strategy may offer the potential to eliminate HCV infection in chronically infected patients. In particular, this approach possesses unique advantages in preventing and treating HCV recurrence in the liver transplantation setting. Lentiviral mediated RNAi could be used to modify a donor graft and prevent or slow HCV recurrence after transplantation; meanwhile, low dose IFN-α can be used to systemically treat HCV enhancing the therapeutic effects of both.

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Part IV: Discussion

Science is nothing but developed perception, interpreted intent, common sense rounded out and minutely articulated.

~ George Santayana ~

CHAPTER 10

Summary and Discussion

Scot Henry

$\mathcal{T}$he face of medicine has indeed changed. Translational research has become a very important aspect in both the advance of science as well the development of therapeutic options for doctors and patients alike. Discovery and advancement move at a staggering pace, predominantly due to goal oriented translational research, which challenges existing paradigms and creates change through understanding.

Figure 1 details the most significant advancements over the years in liver transplantation (Fig. 1a), HCV therapy and research (Fig. 1b) and gene therapy research (Fig. 1c). Each of these discoveries and developments contributed to the collective knowledge we have today, beginning in 1963 with the first human liver transplantation and continuing on into the future. This thesis aimed to explore specific aspects of a select few of these advancements and how they are related to combating HCV recurrence post liver transplantation and hoped to contribute to each with new insights and data that supports or rejects the previous paradigms of understanding.
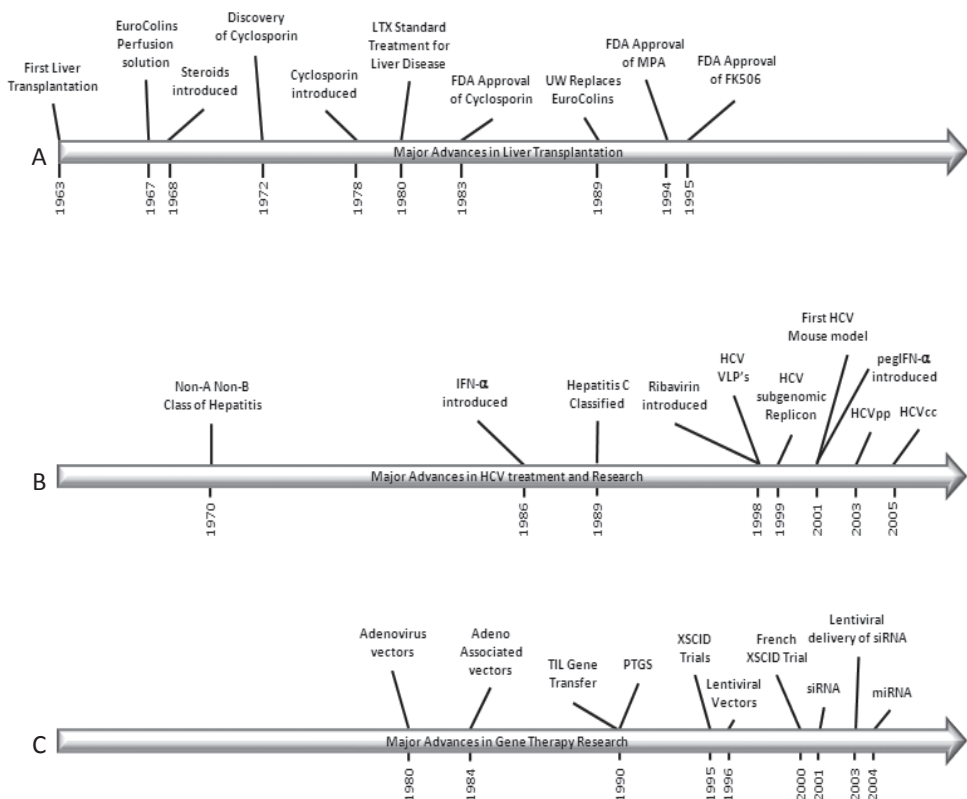
Figure 1

The Past

~ Study the past if you would divine the future ~

As everything we do as scientists hinges on the ability to draw from experience, understanding or misunderstandings that have occurred in the past, we should always be humble in knowing where our discoveries stem from. Though a foundation of the past is necessary, we must remain open and aware that this foundation can and should be challenged and updated as new paradigm shifts understanding in unexpected ways and allow us to revisit data with new eyes. As accurate as any past data appears, information may be lacking from the understanding due to technique, method or even oversight. Never should scientists admit anything as a given truth, without establishing those facts for themselves. With this in mind, scientific information ceases to be a unidirectional outgrowth of immobile branches and becomes instead a fluid evolution of information.

The past in the context of this thesis is represented by the use of corticosteroids in liver transplantation. Introduced to liver transplantation in 1968[1], steroids helped to prevent rejection by modulating cellular and inflammatory responses via stimulation or inhibition of gene transcription. With their use, transplantation rejection rates dropped dramatically when compared to patients that did not receive steroids as part of a maintenance protocol. Though high dose steroid treatments help to reverse the acute rejection episodes within a liver transplant patient, they may be doing more harm than good in patients transplanted for hepatitis C related disease. Classical information about steroids in hepatitis C virus (HCV) positive liver transplant patients has suggested that increases in viral load were due to the use of steroids. These studies have been controversial, with independent groups reporting contradicting effects, though no direct effects were ever established. In chapter 3 we examined various steroids in an *in vitro* model of HCV replication and found that steroids had no direct effect on the ability of HCV to replicate.

We concluded that the increased viral loads after high-dose steroid treatment are more likely due to a down-regulation of the immune response. In these patients, a dampened immune response allows viruses, like HCV, to replicate free of immune-mediated elimination of infected hepatocytes. When a change occurs, such as a tapering or an alteration of immunosuppressive drugs, the immune system reinitiates and vigorously attempts to control the virus, resulting in the acceleration of liver damage. With this in mind we suggested that either steroid avoidance or maintaining low levels, coupled with a slow tapering of corticosteroids may be beneficial to HCV-infected transplantation recipients.

The Present

~ The future influences the present just as much as the past ~

Indeed the past plays an important role in the design of the future; as well as determining the course of the present. Past experience with steroid immunosuppression and the benefits seen in liver transplantation has prompted the need to develop new and more powerful immunosuppressive agents. One of the most important improvements in liver transplantation success rates occurred when cyclosporine (CsA) was discovered in 1972. Its powerful immunosuppressive capacity was recognized when Sir Roy Calne used it to improve experimental pig heart transplantations[2], renal transplants in humans[3] in 1978 and liver transplants in rats[4] in 1979. Cyclosporine saw its first use in human liver transplant patients in 1980 after which it was rapidly approved by the FDA for clinical use. Dr. Thomas Starzl described cyclosporine as the "key which unlocks the door to transplants" with one year survival rates for his liver patients at the University of Colorado's Health Sciences Center nearly doubling from 35 to 60%, with rates as high as 80% at another transplant center in Stanford. As good as cyclosporine was as an immunosuppressive, new research indicated that CsA was a powerful antiviral as well[5]. After the development of the HCV cell culture replicon system in 1999[6], CsA was shown to have a blocking effect on cyclophilin B[7], a functional regulator of the HCV RNA dependant RNA polymerase (NS5b)[8].

This discovery prompted an exploration of the potential of other currently used immunosuppressive drugs for their effect on HCV replication. In chapter 4 we showed that like CsA, Mycophenolic acid (MPA), an immunosuppressive agent approved for use in 1994, has specific antiviral properties against HCV. This is a controversial statement. MPA's mechanism is known to block inosine monophosphate dehydrogenase (IMPDH)[9], the enzyme responsible for a conversion reaction downstream of guanosine nucleotide production. In blocking this enzyme, a reduction in total guanosine triphosphate (GTP) occurs. This limits *de novo* DNA and RNA production, in turn preventing the proliferation of cells, lymphocytes, in particular and as such MPA has immunosuppressive capacity. It stood to reason that if MPA was reducing *de novo* production of DNA and RNA, then naturally it would also impede the ability of viruses to replicate as well. This was shown to be true in many viruses, but MPA, in our hands, was found to be specifically suppressive to HCV, unrelated or at least partially removed from its guanosine depletion ability. This indicated that MPA has two effects on HCV replication, one that initiates rapidly, independent of guanosine and one that is slower and guanosine-dependent.

Though MPA exhibits clear anti-viral activity *in vitro*, the effect of MPA monotherapy on the HCV viral load, like for ribavirin monotherapy, is difficult to study *in vivo* and might only be observed when looking at the early anti-viral kinetics. It is possible if MPA was used in combination with other anti-viral agents, that its anti-viral effect could give enough of an initial drop in HCV viral loads to actuate the effects of the other, pushing the virus below a threshold level that allows both the immune system and the anti-viral treatments to effectively clear the virus. We

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observed an anti-viral effect of MPA in a HCV replication model that clearly lacks the element of a host immune response. In chronic HCV patients, viral replication evokes an ongoing immune response by virus-specific T-cells. Inhibition of this immune response by immunosuppressive drugs, like in HCV transplant recipients, is therefore expected to enhance viral replication and disease progression. As MPA is a potent immunosuppressor it is conceivable that the gross effect on viral replication, as observed in the replicon model, is countered *in vivo* by the inhibition of the anti-viral immune response (Fig. 2). Thus, their effects on the host immune response could neutralize the changes in viral load by anti-viral activity of immunosuppressive drugs, like CsA or MPA.
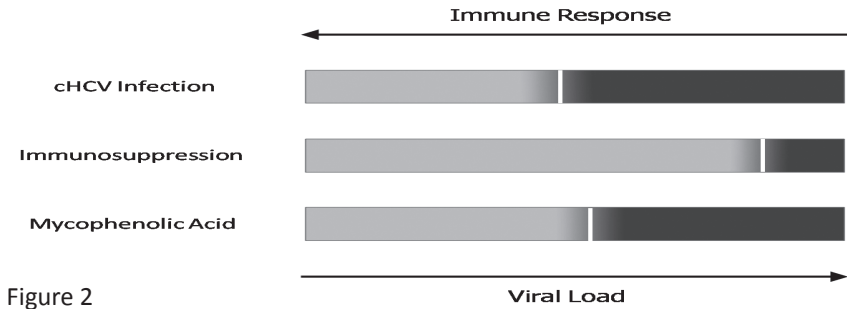
Figure 2

The exact mechanism by which MPA inhibits viral replication is still unknown. Avian reovirus has been reported to be inhibited by MPA through the minor core protein of the virus which has presumed nucleoside triphosphatase activity[10, 11]. No such equivalent viral protein has been identified in the HCV genome, thus excluding this mode of inhibition. One possibility is that MPA affects the NS5B activity by transiently out competing nucleotides for the active site during RNA replication, similar to ribavirin. In this way MPA may act as a mutagen, reducing viral fitness and promote error catastrophe.

The anti-HCV effect of MPA still lacks an elucidation of the exact mechanism. In Chapter 5 we explored the possible mechanisms behind the anti-HCV effects of MPA, with a specific focus on the NS5b polymerase.

We found, through comparison with other IMPDH inhibitors that the reduction in HCV replication due to the presence of MPA was unrelated to a reduction in cellular proliferation. As well, its reduction was not due to a limiting of the nucleotide pool, required for cellular replication.

With assays designed to show blocking effects on the NS5b, we found that MPA reduced the capacity of the NS5b to function as it would in normal viral replication. Though this reduction was not as significant as the earlier results with the replicon cell lines, it indicated a specific effect of MPA on the function of the NS5b, possibly in a manner similar to that of other non-nucleoside inhibitors, by binding non-covalently to the hydrophobic shallow pocket in the thumb domain, near the polymerase active site, though this has not been confirmed.

The Future

~ Do not follow where the path may lead.

Go instead where there is no path and leave a trail ~

The future is always uncertain, but to follow a specific path, only because that specific path exists, to me, leads only to places already visited. To explore into the unknown, to create a new path remains the only option. The future plays an important role in the development of this thesis, as it was the basis for the development of a new treatment option to prevent HCV recurrence in liver transplant patients as well as the creation of a new path towards an unknown destination.

The treatment of HCV remains a challenge that requires further elucidation of the processes of viral life cycle and the development of novel therapeutic approaches. Recent progress has provided the possibilities of identifying novel antiviral targets and designing new therapeutic strategies. Chapter 6 explored these strategies as they apply to Hepatitis C. Micro RNA (miRNA) molecules are rapidly becoming the most emergent focus for researchers for both treatment targets as well as treatments themselves. A prime example is the miR-122, a liver specific developmental regulator that has been linked to HCV replication[12], and interestingly, it promotes viral replication, the first miRNA to be shown regulating a non-self element. Work has begun to regulate the expression of miR-122, as a potential for anti-viral therapy. Providing downregulation of miR-122 shows no toxicity, it could be possible to reduce the ability of HCV to functionally replicate to near zero, though the fact that miRNA-122 is downregulated in rodent and human hepatocellular carcinomas[13], indicating the potential dangers of its loss of function, might confound its uses in humans.

As well as endogenous miRNA regulation, exogenous small interfering RNAs (siRNA) and antisense oligonucleotides (ASO) have emerged as efficient nucleic acid-based gene silencing tools to target highly conserved or functionally important regions within the HCV genome or essential host cell factors for entry or replication. siRNA's have received a particular amount of attention for their capacity to regulate gene expression, seemingly with unlimited potential. Though, as a caution, new discoveries that travel too quickly to clinical trials can spell disaster for not only the technique but for patients as well, as was witnessed in the French XSCID trial[14, 15]. Recent evidence has shown downsides to this magic bullet of biology; off target effects[16] and siRNA overloading can occur[17]. Even the most meticulously developed siRNA can be homologous to non-specific or non-target genes and down regulate their expression as well, sometimes to deleterious effects. Overloading the expression of siRNA is of particular concern as vector driven promoters become more efficient, too much of a good thing is often simply too much, with expressed siRNA's competing with *in situ* mRNA for resources and causing cell function to shut down. So as with all new discoveries, we should not be hasty in rushing its use, until a thorough and complete understanding of the potential benefits as well as the side effects are fully recognized.

The problem of HCV recurrence was detailed in chapter 1, and what remains clear is that there are no or few options for the treatment of HCV recurrence related disease progression. The use of current anti-viral therapies is not well tolerated and often has to be discontinued due to the risk to the patient and the possibility of complications leading to a loss of graft[18]. Utilizing the information gained from chapter 6, we set out to develop a new, technically sophisticated approach to solve the problem of HCV recurrence post liver transplantation.

As HCV is a positive strand RNA virus, the genome itself is sensitive to degradation by interfering RNA (RNAi). Many groups have developed specific siRNA sequences that target various regions of the genome, to significant result, some capable of reducing HCV replication to near undetectable levels. We explored the literature and found specific candidates to downregulate HCV within a new liver graft. Chapter 7 detailed the construction and testing of a 3rd generation lentiviral vector capable of knocking down multiple HCV targets simultaneously. This novel vector was designed to accommodate the insertion of multiple small hairpin RNA (shRNA) cassettes, each driven under their own promoter (H1), allowing separate, high levels of expression of each shRNA. Cassettes were inserted in such a way that each insertion results in a unique downstream restriction site that is available for additional H1-shRNA insertions. Based on the packaging limitation of lentiviral vectors (~7000bp), this method could potentially be used to insert up to 21 self expressing shRNA cassettes within a single vector. There are several advantages to using single vectors containing multiple shRNAs. The probability of hitting a single hepatocyte with more than one vector, in the setting of clinical gene therapy, is very small. With the inclusion of several different shRNAs in one vector, the probability of one cell receiving the appropriate combination of shRNAs is assured. HCV is particularly mutational due to the low fidelity of its RNA polymerase[19], as such treating HCV with monotherapies has been shown to rapidly select for resistant mutants[20, 21], making therapy impotent. Even the most powerful shRNAs, given singly, will not prevent selection of viral mutants, rapidly reducing efficacy, and finally rendering it obsolete. Multiple targeting of a viral genome should slow or eliminate the chance that escape mutants are able to grow out against combination therapy strategies. To this end, we developed constructs that included single targeting shRNA's and multiple targeting of the HCV genome as well as combinatorially targeting HCV and a putative HCV entry co-receptor, CD81. The results demonstrated the effectiveness of these shRNA expression vectors to effectively silence HCV replication and CD81 surface expression simultaneously. Though these vectors are effective in reducing HCV viral replication and infection, the use of multiple shRNAs could be effective in treating many multifactorial diseases such as diabetes or cancer where several genes are involved in disease progression.

As with any disease, the key to successful therapy often lies in the delivery of the therapy. In terms of gene therapy, two options exist; *in vivo* and *ex vivo*. *In vivo* indicates that treatments are applied directly to the patient, whereas *ex vivo* involved the removal of cells or organs from the recipient which are then treated with the therapy and re-implanted to the patient. Recurrent HCV rapidly infects a new liver graft after transplantation and will thrive under

the immunosuppression needed to prevent graft rejection. In order to prevent HCV from overwhelming the new graft a substantial proportion of hepatocytes need to express the given genetic therapy imparting a selective advantage over non treated cells. In the case of liver transplantation, a unique opportunity exists; to therapeutically treat the graft before transplantation. With only a small window of opportunity to deliver therapeutics to a liver graft, it is important that high transductions be achieved in not only the shortest period of time, but also under the conditions of cold perfusion during the procurement phase of transplantation. In chapter 8, we determined, in cell culture experiments, that this application window was available for times ranging from ten minutes to two hours, possibly during the second cold perfusion of the graft, with longer incubation times producing higher transductions. University of Wisconsin solution (UW)[22], the standard solution for the preservation of most organs in transplantation, gave superior vector transductions as observed across a range of times, in hypothermic as well as normothermic conditions.

Compounds such as colloids are used in perfusion solutions to counteract the hydrostatic force during the initial graft washout perfusion, thereby creating a homeostatic environment that precludes the need for osmotic regulation. A major component of UW is Hydroxyethyl starch (HES), a colloid used to prevent hypothermic induced cell damage during major surgery[23]. It was determined that this component was, at least in part, responsible for the increase in vector transduction due to HES/vector and HES/cell interaction dynamics.

The use of UW in liver transplantation opens not only a distinct window of opportunity to provide effective gene transfer *ex vivo*, but due to HES, increases the ability of the vector to reach more cells, even in cold conditions. As the use of vector based RNAi therapies in the clinics gain popularity, the need for *ex vivo* delivery becomes increasingly obvious. Targeting specific cells or whole organs, avoiding off-target integrations and the use of smaller amounts of vector are specific goals and concerns of gene therapy. The use of UW, under the context of organ transplantation, allows for *ex vivo* transduction in both hypothermic and normothermic conditions, allowing for a customized approach to gene delivery, in which all three concerns can be addressed.

HCV recurrence is universal and can be difficult to treat in liver transplant patients due to the complications associated with immunosuppression and antiviral drugs like interferon alpha (IFN-α). It was of interest to determine if low dose IFN-α could be combined with lentiviral delivered RNAi to better treat recurrent HCV in liver transplant patients. Previously to the development of chapter 9, the effect of exogenous IFN-α on vector transduction and RNAi gene expression from lentiviral vectors was unknown.

The *in vitro* observations from chapter 9 have shown that exogenous IFN-α does not have apparent impact on lentiviral vector transduction, so the use of both these agents in an *in vivo* combinatorial therapy seems possible. However, in the setting of combining RNAi with IFN-α, the most critical issue would be the influence of exogenous IFN-α on gene silencing efficiency,

rather than interaction with an innate immune response towards either the vector or the expression of siRNA. We found that RNAi can retain robust gene silencing efficacy even in the presence of high doses of IFN-α. As well, this combinatorial approach significantly enhanced the anti-viral effect in a cell culture replicon model without clear evidence of cross interference. This novel combinatorial strategy may offer the potential to eliminate HCV infection in chronically infected patients. In particular, it possesses unique advantages in preventing and treating HCV recurrence in the liver transplantation setting. Lentiviral mediated RNAi could be used to modify a donor graft and prevent or slow HCV recurrence after transplantation; meanwhile, low dose IFN-α can be used to systemically treat HCV, enhancing the therapeutic effects of both.

General Conclusions

This thesis aimed to explore to the past, present and future of the way we look at and treat HCV recurrence, to help in the development of a modern therapeutic agent. Through the exploration, some controversial arguments were raised. This thesis attempted to quell debate about some of the possible factors involved in the process of HCV recurrence post liver transplantation, and did, in part, but also fueled a long smoldering debate about the anti viral properties of mycophenolic acid. Interestingly, though having been published in a well read journal, these data have yet to be challenged. The realization that the current standard of care was not generally very efficacious led to the development of a modern approach to therapy. In the process of development, construction, utilization and refinement, an effective gene therapy based treatment for HCV recurrence was created that could be delivered to an *ex vivo* liver graft, without the complication of systemic treatment. In the process of validation, the therapy was challenged against other elements (IFN) that could be present during HCV standard of care and was found to be as effective, if not more effective than alone.

In conclusion, scientific study should be rooted in the foundation of the past, implemented in the present, while envisioning the future. This has never been more apparent than in translational research; the end goal of curing disease is achieved through understanding, implementation and exploration into possible futures. We are in a technically marvelous world, in which the tools are available to develop as far as our imaginations can take us.

It is hard to speak of the past of HCV research as the disease is a relatively modern one, but in the short time that doctors have been aware of its presence, advances in understanding and therapeutic developments have been staggeringly fast. We have moved from HCV's characterization in 1989 with only the observed progression of its disease, to the development of a standard of care, effective in more than 60% of patients. Now we have an almost complete understanding of its replication machinery as well its pathology.

At the very least these data can be used by new researchers as a foundation for their studies, contributing to the progression of our understanding. With tools like gene therapy, RNAi and better models, both *in vitro* and *in vivo*, we can look forward to a future where HCV related liver disease is a thing of the past.

CHAPTER 11

Nederlandse samenvatting en discussie

Scot Henry

$\mathcal{G}$eneeskunde is de afgelopen decennia behoorlijk veranderd. Klinisch-wetenschappelijk heeft een belangrijke bijdrage geleverd aan de wetenschappelijke vooruitgang en de ontwikkeling van behandelmogelijkheden voor artsen en patiënten. Ontdekkingen en ontwikkelingen volgen elkaar in hoog tempo op, voornamelijk dankzij dat doelgerichte onderzoek waardoor bestaande paradigma's in twijfel zijn getrokken, en dat heeft gezorgd voor een steeds verder voortschrijdend inzicht.

Figuur 1 toont de belangrijkste ontwikkelingen van de afgelopen jaren op het gebied van levertransplantatie (Fig. 1a), de behandeling van en het onderzoek naar HCV (Fig. 1b) en onderzoek naar gentherapie (Fig. 1c). Al deze ontdekkingen en ontwikkelingen hebben een bijdrage geleverd aan de kennis die we tegenwoordig hebben, te beginnen met de eerste menselijke levertransplantatie in 1963 en van daaruit de toekomst in. In dit proefschrift zijn specifieke aspecten van een klein aantal van deze ontwikkelingen onderzocht. Bekeken is wat zij te maken hebben met het bestrijden van HCV-reïnfectie van de donorlever na levertransplantatie. Dit proefschrift poogt met nieuwe inzichten en onderzoeksgegevens, die eerdere paradigma's ondersteunen of verwerpen, een bijdrage te leveren aan die ontwikkelingen.
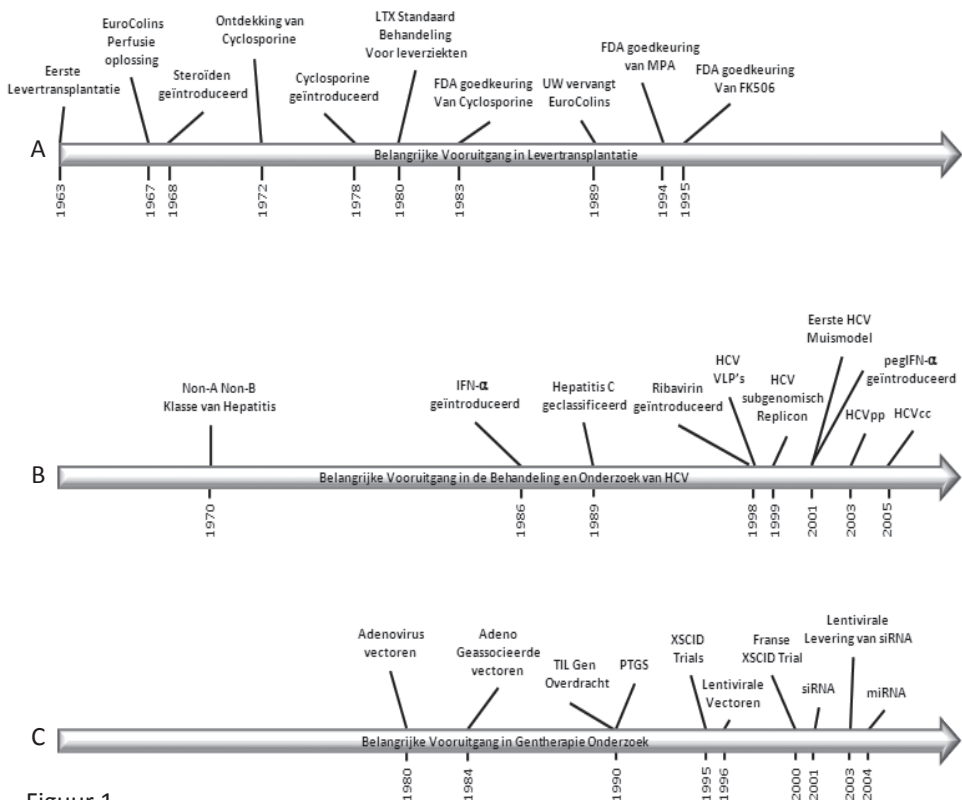
Figuur 1

Verleden

~ Bestudeer het verleden als je de toekomst wilt doorgronden ~

Aangezien alles wat we als wetenschappers doen, afhangt van ons vermogen om te leren van ervaringen, begrip en ook misvattingen uit het verleden, zouden we altijd een zekere mate van nederigheid moeten betrachten – we mogen niet vergeten waar onze ontdekkingen in geworteld zijn. Maar, hoewel die basis in het verleden noodzakelijk is, moeten we ook open blijven staan en ons ervan bewust zijn dat deze basis steeds betwijfeld en geactualiseerd moet worden; nieuwe paradigma's leiden tot onverwachte verschuivingen in ons begrip van de dingen en zorgen dat we gegevens opnieuw kunnen bekijken met een frisse blik. Hoe nauwkeurig gegevens uit het verleden ook mogen lijken, ons begrip ervan kan vertroebeld worden door de technieken en methoden die we gebruiken, of simpelweg doordat we iets over het hoofd zien. Wetenschappers mogen dan ook nooit iets voor waar aannemen als ze het niet met eigen ogen hebben geconstateerd. Als we dit in ons achterhoofd houden, hoeft wetenschappelijke data geen ongericht woekerend samenraapsel van op zichzelf staande onderdelen meer te zijn; het kan een vloeiend, zich gestaag ontwikkelend geheel van gegevens worden.

Binnen de context van dit proefschrift wordt het verleden vertegenwoordigd door het gebruik van bijnierschorshormonen in levertransplantaties. Sinds ze in 1968[1] voor het eerst werden ingezet na levertransplantatie, hebben steroïden afstotingsreacties helpen voorkomen: doordat ze gentranscriptie kunnen stimuleren of tegenhouden, veranderen ze cel- en ontstekingsreacties. Het gebruik van steroïden zorgde voor een enorme afname van het aantal afstotingsreacties na een transplantatie, vergeleken met het aantal afstotingsreacties bij patiënten die geen steroïden toegediend kregen. Hoewel behandelingen met hoge doses steroïden bijdragen aan het onderdrukken van acute afstotingsepisodes bij reguliere levertransplantatiepatiënten, doen ze wellicht meer kwaad dan goed bij patiënten die een levertransplantatie hebben ondergaan vanwege een Hepatitis C-gerelateerde aandoening. Eerdere onderzoeken over steroïden in levertransplantatiepatiënten met Hepatitis C-virus (HCV) stellen dat een toename van viruslading wordt veroorzaakt door steroïden. Deze gegevens zijn echter discutabel: niet alleen spreken de resultaten van verschillende onderzoeksteams elkaar tegen, ook kon tot nu toe nooit met zekerheid worden vastgesteld dat steroïden een rechtstreeks effect op het virus hebben. In hoofdstuk 3 onderzochten we het effect van verscheidene steroïden op HCV-replicatie in een *in vitro* model. We ontdekten dat steroïden geen rechtstreeks effect hadden op het replicatievermogen van HCV.

We constateerden dat de toename in viruslading na een behandeling met hoge doses steroïden meer waarschijnlijk te wijten is aan een onderdrukte immuunreactie van de patiënt. Door die verminderde immuunreactie is hun lichaam niet in staat om geïnfecteerde levercellen te vernietigen en kan een virus als HCV onbelemmerd repliceren. Als zich vervolgens een verandering voordoet, zoals bijvoorbeeld het in lagere doseringen toedienen of veranderen van de immuunsuppressieve medicijnen, komt het immuunsysteem weer op gang en probeert uit alle macht om het virus te beheersen – wat vervolgens resulteert in versnelde leverschade. Op basis van deze gegevens, stelden wij dat het voor levertransplantatiepatiënten die met HCV besmet zijn, misschien beter is om steroïden helemaal te vermijden of ze slechts in lage doses toe te dienen.

PART IV: Summary

Heden

~ De toekomst heeft net zo veel invloed op het heden als het verleden ~

Het verleden speelt een belangrijke rol in het ontwerpen van de toekomst en in het bepalen van de koers van het heden. Door ervaringen uit het verleden met hormonale immuunsuppressie en de bewezen waarde daarvan voor levertransplantaties, werd de noodzaak om nieuwe en krachtiger immuunsuppressieve stoffen te ontwikkelen, duidelijk. Een van de belangrijkste bijdragen aan het vergroten van de slaagkans van levertransplantaties lag in de ontdekking van cyclosporine (CsA) in 1972. Sir Roy Calne ontdekte de krachtige immuunsuppressieve werking van CsA toen hij het gebruikte voor de verbetering van experimentele varkenshartransplantaties[2], niertransplantaties bij mensen[3] in 1978 en levertransplantaties bij ratten[4] in 1979. Cyclosporine werd in 1980 voor het eerst in levertransplantaties bij menselijke patiënten gebruikt, waarna het al spoedig door de Amerikaanse Food and Drug Administration werd goedgekeurd voor klinisch gebruik. Dr. Thomas Starzl omschreef cyclosporine als de "sleutel die de deur naar transplantaties opent": de overlevingskansen van een jaar van zijn leverpatiënten in het Health Sciences Center van de University of Colorado verdubbelden bijna, van 35 naar 60%. In een ander transplantatiecentrum in Stanford de waren overlevingskansen zelfs 80%. Niet alleen had cyclosporine een geweldige immuunsuppressieve werking, nieuw onderzoek wees uit dat CsA ook een krachtig medicijn was tegen virusinfecties[5]. Toen in 1999[6] het HCV celkweek replicatiesysteem was ontwikkeld, kwam aan het licht dat CsA een blokkerend effect heeft op cyclophilin B[7], een functionele regulator van het RNA-afhankelijke RNA-polymerase in HCV (NS5b)[8].

Deze ontdekking leidde tot onderzoek naar de mogelijkheden van andere immuunsuppressieve medicijnen die tegenwoordig worden ingezet vanwege hun effect op HCV-replicatie. In hoofdstuk 4 lieten we zien dat, net als CsA, ook mycophenolic acid (MPA), een immuunsuppressieve stof die in 1994 werd goedgekeurd voor gebruik, antivirale eigenschappen heeft die specifiek gericht zijn tegen HCV. Dit is een controversiële stelling. We weten dat MPA het inosine monofosfaat dehydrogenase (IMPDH)[9] blokkeert, het enzym dat zorgt voor een verandering in het proces downstream van de productie van guanosine nucleotides. Doordat dit enzym geblokkeerd wordt, wordt er veel minder guanosine trifosfaat (GTP) aangemaakt. Dat beperkt *de novo* productie van DNA en RNA, wat op zijn beurt woekergroei van cellen verhindert – met name van lymfocyten – en op die manier heeft MPA een immuunsuppressief vermogen. Het was zonneklaar dat als MPA *de novo* productie van DNA en RNA beperkte, het uiteraard ook het vermogen van virussen om te vermenigvuldigen, zou verhinderen. Dit was inderdaad het geval bij vele virussen, maar in onze handen bleek MPA specifiek HCV te onderdrukken, los – of in ieder geval gedeeltelijk losgekoppeld – van het vermogen van MPA om guanosine te decimeren. Dat duidde erop dat MPA twee soorten effect heeft op de replicatie van HCV: een effect dat snel op gang komt, onafhankelijk van guanosine, en een langzamer effect, dat wel afhankelijk is van guanosine.

Hoewel MPA duidelijk antiviraal werkt *in vitro*, is het – net als bij een behandeling met alleen ribavirine – ingewikkeld om het effect van een *in vivo* behandeling met alleen MPA op de viruslading in HCV te bestuderen. Dat effect kan slechts worden waargenomen als gekeken wordt naar de antivirale kinetica in een vroeg stadium. Als MPA zou worden gebruikt in combinatie met andere antivirale stoffen, is het mogelijk dat het antivirale effect van MPA de viruslading in HCV zo ver zou kunnen reduceren dat de antivirale werking van de andere stoffen zou worden geactiveerd, waardoor het virus tot onder een bepaald minimumniveau zou worden gereduceerd. Als dat gebeurt, kunnen het immuunsysteem en de antivirale behandeling het virus gezamenlijk ontakelen. We hebben een antiviraal effect van MPA geconstateerd in een replicatiemodel van HCV, waar uiteraard geen immuunreactie van een gastheer optreedt. Bij chronische HCV-patiënten roept de virusreplicatie doorlopend immuunreacties op bij virusspecifieke T-cellen. Het afremmen van die immuunreactie door immuunsuppressieve medicijnen die deze reactie onderdrukken, wat gebeurt bij HCV-patiënten die een donorlever hebben ontvangen, zal dan ook naar alle waarschijnlijkheid de virusgroei en de ziekteprogressie verergeren. Aangezien MPA een krachtige immuunsuppressor is, is het voorstelbaar dat het effect van MPA op virusreplicatie, zoals we dat hebben waargenomen in het repliconmodel, *in vivo* grotendeels teniet wordt gedaan doordat de immuunreactie wordt onderdrukt (Fig. 2). Het effect dat immuunsuppressieve medicijnen zoals CsA en MPA op de immuunreactie van de gastheer hebben, doet dus mogelijk de verandering in viruslading weer teniet.
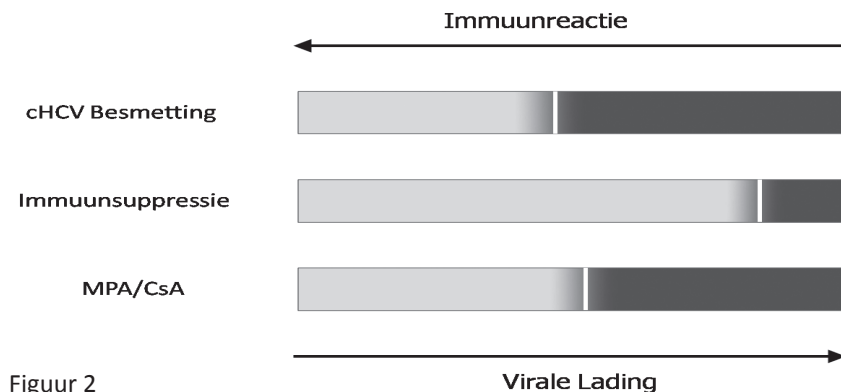
Figuur 2

Hoe MPA virusreplicatie precies tegenhoudt, is nog steeds niet bekend. Van avian reovirus is bekend dat het afgebroken wordt door MPA via een klein kerneiwit van het virus, waarvan verondersteld wordt dat er sprake is van nucleoside triphosphatase.[10, 11] In het HCV-genoom is echter geen vergelijkbaar viruseiwit aangetoond, waarmee deze manier van remwerking is uitgesloten. Het is mogelijk dat MPA de NS5b-activiteit in het gedeelte van het enzym dat de katalytische functie herbergt, beïnvloedt door nucleotides kortstondig buitenspel te zetten tijdens RNA-replicatie, net zoals ribavirine. Op deze manier werkt MPA wellicht als mutageen, dat de conditie van het virus aantast en catastrofale fouten bevordert.

Hoe het anti-HCV-effect van MPA precies werkt, moet nog worden opgehelderd. In hoofdstuk 5 onderzochten we de manieren waarop dat anti-HCV-effect van MPA mogelijk in zijn werk kon gaan. We richtten ons daarbij specifiek op het NS5b-polymerase.

Door een vergelijking te maken met andere IMPDH-remmers ontdekten we dat de afname van HCV-replicatie door de aanwezigheid van MPA, los stond van vermindering van celwoeker. Ook was de afname van HCV-replicatie geen gevolg van het beperken van de nucleotidevoorraad die nodig is voor celvermenigvuldiging.

Met behulp van assays die ontworpen waren om het blokkerende effect van NS5b aan te tonen, ontdekten we dat MPA het vermogen van NS5b om te functioneren zoals het bij normale virusreplicatie doet, beperkt. Hoewel die beperking niet zo omvangrijk was als in eerdere resultaten met de replicon cellijnen, liet ze wel een bepaald effect zien van MPA op het functioneren van NS5b. Dat effect komt mogelijk tot stand op een manier die vergelijkbaar is met die van andere niet-nucleoside remmers, namelijk door zich niet-covalent te koppelen aan het hydrofobe, ondiepe gebied in het duimdomein, dichtbij het gedeelte van de polymerase dat de katalytische functie herbergt. Dit is echter nog niet bevestigd.

Toekomst

> ~ Volg niet in het spoor van een bestaand pad.
>
> Ga waar nog geen pad is en laat een spoor na ~

De toekomst is altijd onzeker, maar een bestaand pad te volgen, enkel en alleen omdat het er is, brengt je volgens mij alleen maar op plekken waar je al geweest bent. Wat ons rest, is het verkennen van het onbekende en zo een nieuw pad te maken. De toekomst speelt een belangrijke rol in de totstandkoming van dit proefschrift. Ze ligt ten grondslag aan de ontwikkeling van nieuwe behandelmethoden om HCV-reïnfectie in levertransplantatiepatiënten te voorkomen, en ze staat aan de wieg van een nieuw pad, naar een vooralsnog onbekende bestemming.

De behandeling van HCV blijft een uitdaging. De processen die spelen bij de levenscyclus van het virus moeten verder worden opgehelderd en nieuwe invalshoeken voor behandeling moeten worden ontwikkeld. Recente vorderingen hebben het mogelijk gemaakt om nieuwe antivirale doelen vast te stellen en nieuwe behandelstrategieën te ontwerpen. In hoofdstuk 6 werd de toepassing van die strategieën op Hepatitis C onderzocht. Micro RNA (miRNA) moleculen zijn hard op weg om een van de voornaamste aandachtspunten voor onderzoekers te worden, zowel voor behandeldoelen als de behandelingen zelf. Een uitstekend voorbeeld hiervan is miR-122, een leverspecifieke ontwikkelingsregulator, die men heeft kunnen koppelen aan HCV-replicatie[12]: het is zeer interessant om te zien dat miR-122 virusreplicatie bevordert. miR-122 is daarmee het eerste miRNA waarvan is aangetoond dat het een niet-eigen element reguleert. Men is begonnen met werken aan het reguleren van miR-122, als mogelijke antivirale behandeling. Onder voorwaarde dat de vermindering van miR-122 geen nadelige gevolgen heeft, zou het mogelijk kunnen zijn om het functionele replicatievermogen van HCV bijna tot nul terug te brengen. Echter, het feit dat de hoeveelheid miR-122 vermindert wanneer er bij knaagdieren en mensen sprake is van leverkanker[13], wijst op de mogelijke gevaren van functieverlies van miR-122. Dat kan het gebruik ervan bij mensen beperken.

Naast miRNA-regulering van binnenuit, is ontdekt dat van buitenaf klein interfererend RNA (siRNA) en antisense oligonucleotiden (ASO) efficiënte instrumenten zijn voor genrepressie. Op basis van nucleïnezuur zetten ze de aanval in op goedbeschermde of functioneel belangrijke gebieden binnen het HCV-genoom en op elementen van gastheercellen die noodzakelijk zijn voor toegang of replicatie van het virus. Er is extra aandacht voor siRNA's vanwege hun naar het lijkt ongelimiteerde vermogen om genexpressie te reguleren. Enige waakzaamheid is echter geboden: we hebben bij de Franse trial van XSCID[14, 15] gezien dat een premature klinische trial van nieuwe ontdekkingen rampzalig kan uitpakken voor zowel de gehanteerde werkwijze als de betrokken patiënten. Recent zijn de negatieve kanten van dit biologische wondermiddel aan het licht gekomen: er kunnen onbedoelde bijwerkingen[16] en overexpressie van siRNA[17] optreden. Zelfs het meest zorgvuldig ontwikkelde siRNA kan homoloog zijn aan niet-specifieke of niet-beoogde genen en ook hun uiting verminderen, met soms schadelijke gevolgen. De overbelasting

an siRNA-uiting wordt nog zorgwekkender, naarmate door vectoren aangedreven promoters efficiënter worden: te veel van het goed is vaak eenvoudigweg te veel. Er onstaat dan een situatie waarin tot expressie gebracht siRNA met *in situ* mRNA strijdt om middelen, waardoor cellen niet langer kunnen functioneren. Dus, net als bij alle nieuwe ontdekkingen, moeten we ook siRNA niet overhaast willen inzetten, maar wachten tot we gedegen en volledig inzicht hebben in de mogelijke waarde ervan en we alle bijverschijnselen in kaart hebben gebracht.

Het probleem van HCV-reïnfectie is uiteengezet in hoofdstuk 1. Duidelijk is dat er weinig of geen mogelijkheden zijn voor de behandeling van ziekteprogressie die gerelateerd is aan HCV-reïnfectie. De huidige antivirale behandelingen worden niet goed verdragen en moeten vaak worden afgebroken vanwege het risico voor de patiënt en de mogelijkheid dat er complicaties optreden die kunnen leiden tot het verlies van het transplantaat[18]. We maken gebruik van de informatie die we in hoofdstuk 6 hebben opgedaan om een nieuwe, technisch geavanceerde methode te ontwikkelen om het probleem van HCV-reïnfectie na een levertransplantatie op te lossen.

Aangezien HCV een positief strengig RNA-virus is, is het genoom zelf gevoelig voor afbraak door RNA-interferentie (RNAi). Vele teams hebben specifieke siRNA-sequenties ontwikkeld die verschillende gebieden van het genoom aanvallen, met significant resultaat. Sommige siRNA-sequenties zijn in staat om HCV-replicatie te reduceren tot een bijna onwaarneembaar niveau. We onderzochten de literatuur en vonden kandidaat siRNA-sequenties die HCV in een nieuw levertransplantaat verminderen. Hoofdstuk 7 verdiepte zich in het bouwen en testen van een derde generatie lentivirale vector die in staat is om meerdere HCV-doelen tegelijkertijd uit te schakelen. Deze nieuwe vector werd ontworpen om haarspeld-RNA (shRNA) cassettes gemakkelijker in te brengen, ieder aangedreven door zijn eigen promoter (H1), waardoor afzonderlijke, hoge niveaus van ieder shRNA mogelijk werd gemaakt. De cassettes werden zodanig ingebracht dat iedere insertie resulteert in een unieke downstream restrictie site die beschikbaar is voor aanvullende H1-shRNA-moleculen.

Uitgaand van de maximale inhoud van lentivirale vectoren (~7000bp), zou met deze methode mogelijk tot 21 expressiecassettes met shRNA kunnen worden ingebracht met een enkele vector. Er zijn verschillende voordelen verbonden aan het gebruik van enkelvoudige vectoren die meerdere shRNA's bevatten. Het is zeer onwaarschijnlijk dat, in het kader van klinische gentherapie, een enkele levercel geraakt wordt door meerdere vectoren. Wanneer verschillende shRNA's in een enkele vector zijn opgenomen, is de kans dat één cel precies de geschikte combinatie van shRNA's ontvangt, verzekerd. HCV is bijzonder mutatiegevoelig door de lage betrouwbaarheid van zijn RNA polymerase[19]. Behandeling van HCV met een enkel medicijn leidt dan ook in rap tempo tot het ontstaan van resistente mutanten[20, 21], waardoor de behandeling krachteloos wordt. Zelfs de krachtigste shRNA's kunnen het ontstaan van virusmutaties niet voorkomen als ze als monotherapie worden gebruikt – hun werking wordt dan al gauw minder efficiënt en uiteindelijk zijn ze onherroepelijk verouderd. Een meervoudige aanval op een viraal

genoom zou de kans moeten vertragen of uitsluiten dat ontglipte mutanten kunnen ontkomen aan gecombineerde therapiestrategieën. Om dat te bereiken, hebben we constructies gebouwd die bestaan uit enkelvoudig aanvallende shRNA's, meervoudige aanvallen op het HCV-genoom en een gecombineerde aanval op HCV en CD81, een vermeende coreceptor voor de toegang van HCV. De resultaten tonen dat deze shRNA expressievectoren tegelijkertijd zowel HCV-replicatie als oppervlakte-expressie van CD81 doeltreffend dempen. Hoewel deze vectoren specifiek de replicatie van het Hepatitis C-virus en de HCV-infectie effectief verminderen, zouden meervoudige shRNA's mogelijk ook effectief kunnen worden ingezet bij de behandeling van vele multifactoriële aandoeningen, zoals diabetes of kanker waarin verschillende genen gemoeid zijn met de ziekteprogressie.

Zoals bij elke aandoening, ligt de sleutel tot een succesvolle behandeling vaak in de manier waarop de behandeling wordt aangeboden. Bij gentherapie zijn er twee opties: *in vivo* en *ex vivo*. *In vivo* impliceert een behandeling die rechtstreeks wordt toegepast op de patiënt; met *ex vivo* gaat het verwijderen van cellen of organen van de ontvanger gepaard, die vervolgens behandeld worden en teruggeplaatst in de patiënt. Gerecidiveerd HCV infecteert een nieuw levertransplantaat kort na de transplantatie en gedijt goed bij de onderdrukking van de immuunreactie die nodig is om de afstoting van het transplantaat te voorkomen. Om te voorkomen dat HCV het nieuwe transplantaat overmeestert, moet een aanzienlijk deel van de levercellen expressie tonen van de betreffende gentherapie, waarmee hun een voordeel wordt toebedeeld boven niet-behandelde cellen.

In het geval van levertransplantatie doet zich een unieke gelegenheid voor: je kunt een transplantaat behandelen voordat het getransplanteerd wordt. De gelegenheid om een levertransplantaat aan behandeling te onderwerpen is zeer kortstondig. Daarom is het belangrijk dat zo veel mogelijk transductie tot stand komt in zo weinig mogelijk tijd, en dat onder de condities van koude perfusie, tijdens de fase van orgaanwerving. In hoofdstuk 8 stelden we vast dat in celweekexperimenten de gelegenheid voor transductie zich voordoet vanaf tien minuten tot twee uur na aanvang van de transductieprocedure, mogelijkerwijs tijdens de tweede koude perfusie van het transplantaat. Daarbij geldt: hoe langer de incubatietijd, hoe hoger de transductie. De standaard bewaarvloeistof voor de meeste transplantatieorganen, de University of Wisconsin-oplossing (UW)[22], zorgde voor uitstekende vectortransductie. Dat werd waargenomen bij uiteenlopende tijdsspannen, zowel in hypothermische als in normothermische omstandigheden.

Verbindingen zoals colloïden worden gebruikt in perfusieoplossingen om de hydrostatische kracht van de eerste perfusie waarmee het transplantaat wordt doorgespoeld, teniet te doen. Daarbij creëren ze een homeostatische omgeving, waarmee de noodzaak van osmotische regulatie verdwijnt. Hydroxyethylzetmeel (HES), een colloïde die wordt gebruikt om celbeschadiging door hypothermie te voorkomen tijdens operaties onder algehele verdoving[23], is een groot bestanddeel van UW. Vastgesteld werd dat dit bestanddeel ten minste deels

verantwoordelijk was voor de toename van vectortransductie als resultaat van HES/vector- en HES/celinteractie.

Het gebruik van UW in levertransplantaties maakt niet alleen doeltreffende genoverdracht *ex vivo* mogelijk, maar vergroot dankzij HES ook het vermogen van de vector om meer cellen te bereiken, zelfs in koude omstandigheden. Nu het gebruik van op vectoren gebaseerd RNAi-behandeling in klinieken aan populariteit wint, wordt de noodzaak van *ex vivo* behandeling steeds duidelijker. De drie doelen waar gentherapie zich op richt, zijn het geïsoleerd kunnen treffen van specifieke cellen of complete organen, het vermijden van onbedoelde insertie en het inzetten van kleinere vectorhoeveelheden. Binnen de context van orgaantransplantatie wordt *ex vivo* transductie onder zowel hypothermische als normothermische omstandigheden mogelijk gemaakt door het gebruik van UW. Dat zorgt voor een op maat gesneden aanpak van genoverdracht waarin alle drie de doelen kunnen worden meegenomen.

HCV-reïnfectie is universeel. Het verschijnsel is moeilijk te behandelen bij levertransplantatiepatiënten omdat zich complicaties voordoen die te maken hebben met de onderdrukking van de immuunreactie en antivirale medicijnen zoals interferon alpha (IFN-α). Het was belangrijk om te bepalen of lage doses IFN-α gecombineerd zouden kunnen worden met lentiviraal overgedragen RNAi, zodat HCV-reïnfectie in levertransplantatiepatiënten beter kan worden voorkomen. Voordat hoofdstuk 9 tot stand kwam, was het effect van exogeen IFN-α op transductie en de genexpressie van RNAi van lentivirale vectoren, onbekend.

De *in vitro* observaties in hoofdstuk 9 hebben aangetoond dat exogeen IFN-α geen zichtbaar effect heeft op lentivirale vectortransductie, dus het lijkt mogelijk om deze twee stoffen te gebruiken in een *in vivo* gecombineerde behandeling. Echter, wanneer we RNAi combineren met IFN-α, ligt het grootste punt van zorg in de invloed van exogeen IFN-α op de effectiviteit van genrepressie, en niet zozeer in de interactie met een immuunreactie op de vector of de expressie van siRNA. We hebben ontdekt dat RNAi zeer effectief blijft op gebied van genrepressie, zelfs wanneer er hoge doses IFN-α aanwezig zijn. Daarbij komt dat deze gecombineerde aanpak het antivirale effect in het replicon model aanzienlijk heeft versterkt, zonder dat duidelijk kan worden aangetoond dat ze elkaars werking verstoren. Deze nieuwe, gecombineerde aanpak kan het misschien mogelijk maken om HCV-infectie in chronisch geïnfecteerde patiënten te vernietigen. De unieke voordelen schuilen met name in het voorkómen en behandelen van HCV-reïnfectie in het kader van levertransplantaties. Lentiviraal gemedieerd RNAi zou gebruikt kunnen worden om een donortransplantaat te modificeren en HCV-reïnfectie na een levertransplantatie te voorkomen of vertragen. Tegelijkertijd kunnen lage doses IFN-α gebruikt worden om HCV systematisch te behandelen, waardoor de behandeleffecten van beide middelen worden vergroot.

Algemene conclusies

In dit proefschrift is onze kijk op en behandeling van HCV-reïnfectie verkend voor het verleden, het heden en de toekomst, om zo een bijdrage te leveren aan de ontwikkeling van een geavanceerde behandelmethode. In deze verkenning zijn een aantal controversiële stellingen geponeerd. In dit proefschrift is geprobeerd om een aantal discussies over de mogelijke factoren die een rol spelen bij het proces van HCV-reïnfectie na een levertransplantatie, af te sluiten. Dat is deels ook gelukt, maar er is ook een lang lopende discussie aangezwengeld, namelijk die over de antivirale eigenschappen van mycophenolic acid. Het is interessant dat deze gegevens, hoewel ze gepubliceerd zijn in een gerenommeerd medisch tijdschrift, nog steeds niet zijn betwist. Het besef dat de huidige zorgstandaard over het algemeen niet heel effectief was, heeft geleid tot de ontwikkelingen van een geavanceerde kijk op behandeling. In het proces van ontwikkeling, constructie, toepassing en verfijning kwam een effectieve behandeling voor HCV-reïnfectie tot stand, gebaseerd op gentherapie. Deze behandeling kon worden losgelaten op een *ex vivo* levertransplantaat, zonder de complicaties die een systematische behandeling met zich meebrengt. In het valideringsproces lieten we de behandeling samen met andere zaken (IFN) die een rol kunnen spelen bij de standaardbehandeling van HCV de strijd aangaan. We ontdekten dat ze gezamenlijk minstens zo effectief waren als alleen.

Concluderend kunnen we stellen dat wetenschappelijk onderzoek gegrond moet worden in het verleden en toegepast in het heden, en alvast vooruit moet kijken naar de mogelijkheden in de toekomst. Dit is nergens zo duidelijk gebleken als in klinisch-wetenschappelijk onderzoek: het einddoel, namelijk het genezen van ziektes, wordt bereikt door begrip van het verleden, dat toepassen in het heden en het verkennen van een mogelijke toekomst. We leven in een technisch fantastische wereld met alle mogelijke middelen die ons in staat stellen om zo ver te gaan als onze verbeeldingskracht het toelaat.

Het is lastig om iets te zeggen over HCV-onderzoek uit het verleden, aangezien het een betrekkelijk 'jonge' ziekte is. Sinds artsen van het bestaan ervan op de hoogte zijn, hebben het begrip van de ziekte en ontwikkelingen in behandelmethoden echter een hoge vlucht genomen. In 1989 kon HCV enkel nog gekarakteriseerd worden, en dat alleen door te kijken naar de ziekteprogressie; nu hebben we een zorgstandaard ontwikkeld die voor 60% van de patiënten effectief is. Tegenwoordig hebben we een bijna volledig inzicht in de aard van het virus en de manier waarop het repliceert.

De gegevens in dit proefschrift kunnen in ieder geval door andere wetenschappers als basis dienen voor hun onderzoek en daarmee een bijdrage leveren voor ons steeds verder voortschrijdend inzicht. Dankzij instrumenten als gentherapie en RNAi en de ontwikkeling van betere *in vitro* en *in vivo* modellen, kunnen we vooruitkijken naar een toekomst waarin HCV-gerelateerde leveraandoeningen verleden tijd zijn.

Part V: Appendices

The ability to quote is a serviceable substitute
for wit.

~ Maugham ~

APPENDIX i

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Chapter 1

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APPENDIX II

List of Abbreviations

AAV	Adeno-associated virus
ADA	Adenosine amintotransferase
ALT	Alanine aminotransferase
AmP	Ampicillin
ASGPR	Asialoglycoprotein receptor
ASO	Anti-sense oligonucleotides
Ber-EP4	Epithelial cell surface glycoprotein monoclonal antibody
CFSE	Carboxy-fluorescein diacetate, succinimidyl ester
CmR	Chloramphenical
cPPT	Central polypurine tract
Csa	Cyclosporine A
DC	Dendritic cells
Dex	Dexamethasone
dGTP	Deoxy-guanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
dsRNA	Double stranded RNA
EH	EloHaes
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HCVcc	Cell culture-derived HCV particles
HCV-LPs	HCV-like particles
HCVpp	HCV pseudotyped particles
HEK293T	Human embryonic kidney epithelial cell line
HES	Hydroxyethyl Starch
HIV	Human immunodeficiency virus
HTK	Histadine Thymadine Ketogluterate
Huh-7-ET	Huh-7 cells w/ HCV bicistronic replicon
Huh-7-Hy	Huh-7 cells w/ HCV monocistronic replicon
Igl-1	Na-Peg-UW Solution
IMP	Inosine 5' monophosphate
IMPDH	Inosine monophosphate dehydrogenase
INF-α	Interferon alpha
LV-GFP	Lentiviral GFP expression vector
LV-sh2	Lentiviral vector w/ two small hairpin RNAs
LV-shNS5b	Lentiviral vector w/ short hairpin RNA targeting the HCV NS5b

MCS	Multiple cloning site
MFI	Mean fluorescence intensity
miRNA	MicroRNA
MMF	Mycophenolate mofetil
MOI	Multiplicity of infection
MPA	Mycophenolic acid
NCR	Non-coding region
NIH	National Institutes of Health
NS3	HCV-non-structural protein 3
NS5B	HCV RNA dependent RNA polymerase
NTR's	Non-translated regions
ORF	Open reading frame
PE	Phycoerythrin
PEG	Polyethylene glycol
pIFN-a	Pegylated Interferon alpha
PFA	Para-formaldehyde
PI	Proliferation index
Pred	Prednisolone
PTGS	Posttranscriptional gene silencing
Rapa	Rapamycin
RBC	Red blood cells
RBV	Replication inhibitor ribavirin
RdRp	RNA dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SLO	Liver Research Foundation
SR-B1	Scavenger receptor class B type 1
SVR	Sustained virological response
TCID	Tissue culture infectious dose
TIL	Tumor-infiltrating lymphocytes
UW	University of Wisconsin
VSV-G	Vesicular stomatitis virus glycoproteins
WPRE	Woodchuck hepatitis virus post-transcriptional regulatory element
XMP	Xanthosine 5' monophosphate
XSCID	X-linked severe combined immunodeficiency

APPENDIX

List of Publications

Publications

Scot D Henry, Pascal CG van der Wegen, Herold J Metselaar, Hugo W Tilanus, Bob J Scholte and Luc JW van der Laan "Enhanced Gene Therapy Vector Delivery to Hepatocytes under Hypo- and Normothermic Conditions." Liver Transplantation September 2007 (submitted).

Qiuwei Pan, **Scot D. Henry**, Bob J. Scholte, Hugo W. Tilanus, Harry L.A. Janssen, Luc J.W. van der Laan. "New therapeutic opportunities for Hepatitis C based on small RNA" *World Journal of Gastroenterology*. 2007 Sept; 13 (33):4431-4436

Scot D. Henry, Herold J. Metselaar, Jeroen van Dijck, Hugo W. Tilanus, Luc J.W. van der Laan. "Impact of Steroids on Hepatitis C virus replication in vivo and in vitro." *Annals of The New York Academy of Sciences*.2007 Sept;1110:439-437.

Scot D Henry, Herold J Metselaar, Richard CB Lonsdale, Alice Kok, Bart L Haagmans, Hugo W Tilanus and Luc JW van der Laan "Mycophenolic acid inhibits hepatitis C virus replication and acts in synergy with cyclosporin A and interferon-α." *Gastroenterology*. 2006 Nov; 131(5):1452-62.

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Christopher Power, MD, **Scot Henry, BSc**, Marc R. Del Bigio, MD, PhD, Peter H. Larson, MSc, Dale Corbett, PhD, Yumi Imai, MD, Voon Wee Yong, PhD, and James Peeling, PhD. "Intracerebral Hemorrhage Induces Macrophage Activation and Matrix Metalloproteinases". *Annals of Neurology*. 2003 Jun;53(6):731-42.

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Grants

"Preclinical Testing of Hepatitis C Antivirals" 2007 Erasmus Medical Center Pilot Grant. 50000€. Applicants: Bart L. Haagmans (Project Leader, Virology), Luc J.W. van der Laan (Surgery), Rob de Knegt (Gastroenterology and Hepatology). Advisors: T. Kuiken (Virology), S.L. Smits (Virology), A.D.M.E. Osterhaus (Virology), H.L.A. Janssen (Gastroenterology and Hepatology), S.D. Henry (Surgery).

Awards

Publication of the Year (2006) Award, March 2007. Dutch Society for Organ Transplantation. 1500€. Scot D Henry, Herold J Metselaar, Richard CB Lonsdale, Alice Kok, Bart L Haagmans, Hugo W Tilanus and Luc JW van der Laan "Mycophenolic acid inhibits hepatitis C virus replication and acts in synergy with cyclosporin A and interferon-α." Gastroenterology. 2006 Nov; 131(5):1452-62.

Poster of Distinction Award, July 2006. American Transplant & World Transplant Joint Congress. Boston, USA. "Mycophenolic Acid Inhibits Hepatitis C Virus Replication: Independent Of Guanosine Depletion And Acts In Synergy With IFN-α."

Rising Star Award. Top 6 Abstracts, May 2006. International Liver Transplantation Society. $3000 US. "Ex Vivo Gene Therapy in Liver Transplantation: Future Strategy to Prevent Hepatitis C Recurrence."

Student Research Award, Nov 2005. American Association for the Study of Liver Diseases. $500 US. "Simultaneous targeting of HCV replication and viral entry by a single gene therapy vector containing multiple RNA interference cassettes: prevention of mutational escape."

Biotest Basic Research Award, Oct 2005. European Society of Liver Transplantation. 2500€. "Ex Vivo Gene Therapy in Liver Transplantation: Future Strategy to Prevent Hepatitis C Recurrence."

NVH-Altana-Voordrachtsprijs (Top Abstract), Oct 2005. Netherlandse Vereniging Voor Hepatologie. 250 €. "Simultaneous targeting of HCV replication and viral entry by a single gene therapy vector containing multiple RNA interference cassettes: prevention of mutational escape."

Rising Star Award. Top 6 Abstracts, July 2005. International Liver Transplantation Society. $3000 US. "Direct and Synergistic Inhibition of Hepatitis C Replication by Cyclosporin A and Mycophenolic Acid."

Best Presentation Award, March 2005. 500 €. BootCongress, Netherlands. "Ex Vivo Gene Therapy in Liver Transplantation: Future Strategy to Prevent Hepatitis C Recurrence."

APPENDIX iv

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APPENDIX V

Curriculum vitae

Scot Henry was born June 2, 1971, in Ottawa, in south eastern Ontario, Canada's federal capitol, to parents Jeanne and Gary Henry, the third born, brother to Keith and Lisa. He attended high school in the middle of the country, the flat wide open spaces of Brandon, Manitoba, where the wheat extends shoulder high and fields of sunflowers trace the arc of the sun across the prairies.

After a ski-bum hiatus he renewed his interest in education and began university in 1996 at the newest university built in North America in 25 years, The University of Northern British Columbia, in the department of Environmental Sciences. During the four years of study Scot took on many additional side projects. During his second year he helped determine the population structures of an invading mussel species on the coast of British Columbia and received a summer internship where he assisted a graduate student with a massive salmon cross-breeding study. During his third year, he taught a first year biology lab and as part of an advanced genetics course, detailed a project to modify native bacterial species with plasmids coding substrate triggered enzymes that would enable the bacteria to degrade long chain hydrocarbons in the event of an oil spill. In his fourth year, he conducted an independent study to show dual uniparental inheritance in the mussel species *Modiolus rectus*. Scot graduated in the summer of 2000 with a Bachelor of Environmental Sciences.

He soon found work at the University of Calgary's Health Sciences in Calgary, Alberta. Here Scot focused on inflammatory brain diseases, particularly HIV, MS and stroke. He spent two years researching these diseases and their effects on brain cytokine profiles and the evolution of viral and host factors contributing to disease progression or treatment evasion.

In 2002 Scot worked sporadically in what he terms 'medical mercenary work'; consulting for various labs and departments at the University of Calgary. In 2003, he travelled to Ecuador to begin a population study based on genetic mark recapture methods of an endangered bear species in South America. For 6 months he made camp on the edge of the cloud forest and collected bear hair and scat. The samples were to be analyzed for micro-satellites and other genetic features to determine individuality and population structures, though this portion of the study has yet to be completed.

Early in 2004 Scot travelled to Holland for what was originally to be a short visit and some possible consulting work. During which time his CV was spread around the research departments of Erasmus MC. Though obscure and diverse, his CV caught the attention of Dr. Luc van der Laan, who had an idea that incorporated all of his skills and interests; a chance to develop a gene therapy based treatment of a persistent viral infection, genetic engineering, viral populations, it was perfect in all aspects and he leapt at the offer.

Four years, three job descriptions (research analyst, AIO, OIO), and much travel later; Scot would defend this PhD thesis under the supervision of Prof. Hugo Tilanus, Prof. Herold Metselaar and Dr. Luc van der Laan.

Dr. Henry's current whereabouts are unknown though it is wildly rumored that he now lives and works in his parent's basement in Moose Jaw, Saskatchewan. He is penniless and quite, quite insane.