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Novel Agents Inhibiting Hepatitis C Virus; Application to Prevention of Re-infection after Liver Transplantation

by

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Abstract

Recurrent Hepatitis C virus infection drives inferior outcomes experienced by patients undergoing liver transplantation due to HCV-associated liver disease. Existing therapies exhibit increased toxicity, poor efficacy, and profound patient intolerability in the immediate transplantation period. Liver transplantation provides a window of opportunity to prevent re-infection of the allograft and drastically improve patients' post-transplant outcomes. The anti-HCV activity of novel monoclonal antibodies(AR4a) and herbal extracts (Epigallocatechin-gallate, Silibinin) were studied *in-vitro* using HCV cell culture system and *in-vivo* using a humanized liver mouse model capable of supporting HCV replication. Alone these agents exhibit reliable cross-genotype HCV inhibition *in-vitro*. Combination therapy can completely prevent HCV infection. *In-vivo* EGCG alone fails to reliably protect against HCV-genotype1a challenge. AR4a alone and combined with EGCG robustly protects against the establishment of HCV infection. In common these agents have low toxicity potential and are thus applicable for use in complex transplant cohorts to prophylax against HCV re-infection.

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Abbreviations:

Apo-B: apolipoprotein-B

BSA: bovine serum albumin

CD81: cluster of differentiation 81

cDNA: complementary deoxyribonucleic acid

Cmax: peak drug concentration

DAA: direct acting antivirals

DC-SIGN: Dendritic cell Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

DMEM: Dulbecco's modified eagles medium

DMSO: dimethyl sulfoxide

EGCG: epigallocatechin-gallate

EGFR: epidermal growth factor receptor

ELISA: enzyme linked immunosorbent assay

FBS: fetal bovine serum

FFU: focus forming units

hAAT: human alpha-1-antitrypsin

HBV: Hepatitis B virus

HCV: Hepatitis C virus

HCVcc: HCV cell culture

HDL: high density lipoprotein

HIV: human immunodeficiency virus

HPLC: high performance liquid chromatography

HRP: horseradish peroxidase

HSV: Herpes simplex virus

Huh: human hepatoma

HVR: hypervariable region

IC_{50}: half maximal inhibitory concentration

IFN-α2a: interferon alpha- 2a

IP: intraperitoneal

IG:intragastric

JFH: Japanese fulminant hepatitis

JFH-1-Luc: JFH-1 construct encoding a luciferase reporter sequence

kb: kilobases

LDL: low density lipoprotein

mAb: monoclonal antibody

mIgG: murine immunoglobulin G

miR: microRNA

MOI: multiplicity of infection

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NEAA: non essential amino acids

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NS5a: non-structural protein 5a

PBS: phosphate buffered saline

PCR: polymerase chain reaction

Peg-IFN: pegylated interferon

RBV: Ribavirin

RdRp: RNA dependent RNA polymerase

RNA: ribonucleic acid

SCID/uPA mouse: severe combined immunodeficient mice/ albumin/urokinase plasminogen activator (uPA)

SR-B1: scavenger receptor class B1

STAT: signal transducers and activators of transcription

SVR: sustained virological response

$t_{1/2}$: half-life

TCID50: 50% tissue culture infective dose

T_{max}: time of maximum plasma concentration

TNF: tumour necrosis factor

Chapter 1:

Hepatitis C virus and Liver Transplantation

1.1 Hepatitis C Virus

Worldwide Hepatitis C virus (HCV) continues to maintain it's status as a major public health burden. Current estimates propose that from 130 up to 200 million people are chronically infected with HCV, the prevalence being disproportionately high in certain regions which reflects many different internal drivers of epidemics eg. Egypt, Asia, Africa [1-3]. Throughout the world HCV is a significant utilizer of healthcare resources and this is set to increase considerably in the years ahead [4].

HCV was first identified in 1989 as the aetiological agent responsible for the clinical disease entity then termed 'transfusion associated non-A, non-B hepatitis' [5, 6]. Since then huge resources have been consumed by efforts to further understand the virus and curtail the associated disease manifestations. HCV is a small, (55nm in diameter) spherical, positive sense, single-stranded RNA virus. It is the sole member of the *Hepacivirus* genus of the family *Flaviviridae* [7, 8]. HCV replicates primarily in hepatocytes, however the existence of extrahepatic sites of HCV replication has been suggested by some groups [9-11]. The HCV genome consists of a single-stranded RNA molecule, 9.6 kilobases (kb) long with a single open reading frame which encodes for a large polyprotein approximately 3000 amino acids in length. The polyprotein is co- and post-translationally processed by cellular and viral proteases to yield the final gene products. These comprise structural and non-structural proteins. The structural proteins include core, which forms the viral nucleocapsid, and

the two surface envelope proteins called E1 and E2 (see expanded discussion later). The non-structural proteins NS2-NS5b are involved in the processing of the HCV polyprotein (NS2 and NS3/NS4a proteases) and formation of the membrane-associated replication complex (NS3, NS4A, NS4B, NS5A and NS5B) [7, 12, 13].

Following acute infection HCV has a high propensity to establish chronic infection [14]. Only an estimated 20-30% of individuals will spontaneously clear acute HCV infection, the remainder developing chronic disease [15]. Recovery from acute infection is associated with a vigorous and broad cellular and humoral immune response [16-18]. In contrast, progression to chronic disease is associated with weak, narrow and short-lived host cellular immune responses and low or absent neutralizing antibody titers. [19] HCV itself has also evolved specific means of subverting the host response to favour the establishment of chronicity [20, 21].

In the setting of chronic infection, HCV replicates at an extraordinary rate. A high daily turnover has been estimated with the production of upwards of 10^{12} viral particles per day [22]. Combined with the absence of a proofreading activity of the RNA dependent RNA polymerase (RdRp), this culminates in enormous genetic diversity [23]. In addition HCV displays remarkable tolerance for the many mutations generated which often display no negative impact on viral fitness or replication capacity. Evasion of the host immunological response itself contributes further to the generation of considerable sequence diversity [24]. Thus, within an

individual HCV exists as a quasispecies with the myriad different strains displaying approximately 90% identity [25]. Seven known genotypes are recognised whose nucleotide sequences differ from each other by an estimated 31-33%. Within a genotype there are further divisions into subtypes which share 75-80% identity [26]. The vast diversity of species within and between individuals constitutes a significant barrier to the formulation of universally effective HCV prevention and treatment strategies.

Chronic HCV infection is a major cause of morbidity and mortality due primarily to hepatic disease. Progressive hepatic fibrosis ensues, and between 5-25% of individuals will develop cirrhosis and end stage liver disease after approximately 20 years [14, 27]. Following the establishment of cirrhosis, 10-20% of patients will manifest overt consequences of end stage liver disease within five years (eg. oesophageal varices, ascites, hepatic encephalopathy, and coagulopathy) [28]. In addition cirrhotic liver disease as a consequence of chronic HCV is associated with a markedly increased risk of hepatocellular carcinoma [14, 29].

Given the duration of time to presentation with end stage disease and the mainly asymptomatic nature of chronic HCV infection clinicians are now poised for a huge increase in the burden of disease due to HCV [4, 30]. Increasing numbers of individuals infected 20-30 years ago will now be seeking healthcare as a consequence of impaired liver function. This has prompted the recent recommendation by the Centers for Disease

Control and Prevention for routine HCV testing of new immigrants and ‘baby-boomers’, individuals born between 1945-75. These groups are most at risk of ‘silent’ HCV disease and must be identified and engaged in care [31].

Combination therapy with pegylated interferon alpha (Peg-IFN) and Ribavirin (RBV) has long provided the fundamental basis for HCV treatment [32-35]. This treatment is however undermined by variable efficacy, the requirement for lengthy treatment durations, patient intolerance, serious adverse effects, high costs and the requirement for close medical supervision throughout treatment [36]. A successful outcome from treatment is termed a sustained virological response (SVR) and is defined as remaining HCV RNA negative in blood samples by polymerase chain reaction (PCR) at six months post discontinuation of combination treatment. SVR rates differ according to HCV genotypes; individuals harbouring HCV genotype 1 or 4 achieve SVR only 50% of the time following a treatment duration of 48 weeks [33, 37].

For patients with genotype 1 disease (the most common genotype in North America and Europe) the rates of SVR have been improved to 70% with the addition of the first generation viral protease inhibitors Telaprevir and Bocepravir. However, this increased rate of SVR comes with considerable financial cost and further exacerbates the Peg-IFN/RBV associated adverse effects [38, 39]. More pronounced haematological toxicity (anaemia, thrombocytopenia), gastrointestinal disturbances, and skin

rashes are frequently observed with triple therapy. Many novel direct acting antiviral agents (DAAs) are in advanced stages of clinical trials and demonstrate excellent efficacy but accessibility to such agents for the majority of the HCV infected population worldwide, the threat of resistant strains emerging, and the potential for adverse effects when used in combination represent considerable hurdles to be overcome in the future [40-42].

1.2 Liver Transplantation in HCV Disease

HCV associated liver disease (cirrhosis or hepatocellular carcinoma) is now firmly established as the leading indication for liver transplantation throughout the world [43]. Unfortunately despite this life-saving intervention re-infection of the new liver allograft is almost universal. The viral kinetics of this re-infection have been very clearly delineated. There is an initial decline in the levels of HCV RNA following explantation of the native liver during the anhepatic phase of the transplant procedure. There is a further decline during reperfusion of the newly implanted graft reflecting the binding and entry of circulating HCV virions into the allograft. Thereafter the HCV RNA levels rise briskly to reach pre-transplant levels by day 3/4 post transplant and often exceed that level by one week post transplant. The HCV RNA levels ultimately plateau at a level higher than

that observed pre-transplant and histological evidence of recurrence is often evident by 6 months - 1 year post transplantation [44-46].

The severity of HCV re-infection varies considerably between individuals and numerous contributory factors have been identified which directly impact the post transplant course (HCV RNA titer at the time of transplant, older age of donor, use of corticosteroid immunosuppression, insulin resistance, hepatic steatosis) [43, 47, 48]. The immunosuppressed state contributes considerably to the accelerated course of HCV associated liver disease observed in patients with HCV who undergo liver transplantation. An estimated 15-20% will develop cirrhosis within five years. Overall graft and patient survival is significantly inferior in this patient group when compared to individuals undergoing liver transplantation for non-HCV related disease [49].

Efforts to treat HCV re-infection post-transplantation further magnify the shortcomings in the currently available HCV treatment options. Overall SVR rates approximate only 25-30% often despite employing even longer durations of treatment [50]. Treatment efficacy is considerably compromised by the complex co-morbidities and polypharmacy in this patient group. The frequency of adverse effects is considerably increased and Peg-IFN therapy itself can precipitate episodes of allograft rejection [47]. Drug-drug interactions further potentiate toxicity (eg. haematological and renal toxicity) and very close clinical supervision is warranted. Three

quarters of patients will ultimately require dose reduction or premature discontinuation of HCV therapy.

The management and outcomes for patients with chronic Hepatitis B virus infection (HBV) undergoing liver transplantation are in stark contrast. Highly effective therapy exists which is now utilised routinely following liver transplant to prevent HBV re-infection. Combination prophylaxis with hepatitis B immunoglobulin and lamivudine has effectively eradicated the risk of HBV recurrence in this cohort [51, 52].

No such strategy exists to prevent HCV recurrence. Many approaches have been tried but none have been successful in achieving their goal. Strategies to employ existing therapies (Peg-IFN/RBV) in a pre-emptive role pre-transplant or early post transplant were largely unsuccessful. The intolerability and contraindications of these agents in highly complex and medically unstable patients severely limits the applicability of such approaches [53]. A randomised trial using hepatitis C immune globulin concluded that this was a safe and tolerable agent in liver transplant recipients but no beneficial effect on the rate of HCV recurrence was observed [54].

Clearly, as exemplified by HBV patients undergoing liver transplantation, an effective preventative approach for HCV liver transplant recipients stands to have an enormous beneficial impact for both the patient and society. HCV related liver disease is the single largest indication for liver

transplant but patient and graft survival lag behind their non-HCV counterparts. Recurrent HCV disease is the primary driver of this inequity. Additionally it is projected that despite the recent therapeutic advances considerable numbers of patients with HCV infection now of 20-30 years duration will be seeking healthcare as a consequence of progressive liver fibrosis and cirrhosis [30]. Currently liver transplantation is a primary modality of therapy for such patients with advanced disease. Preventing HCV recurrence will improve outcomes from a scarce and valuable resource and enable patients to obtain the maximum benefit from such life-saving treatment. Any successful approach to the prevention of HCV recurrence will need to be safe and tolerable in this complex patient cohort. It also requires durability in the face of a dynamic virus with high replicative capacity in the setting of impairment of host cell mediated and humoral immunity. During liver transplantation there exists a theoretical window whereby therapeutic agents could be employed to prophylax the 'naïve' allograft and prevent HCV entry into hepatocytes and the resulting establishment of infection.

1.3: Study Aims and Hypothesis

Liver transplantation is a life saving intervention for individuals with end stage liver disease. HCV related liver disease is the leading indication for liver transplant but HCV re-infection drives inferior outcomes. The demand for liver transplantation is projected to rise still further in the coming years prior to the widespread availability of more effective HCV therapies.

The aim of this research proposal was to examine novel strategies capable of preventing HCV infection and subsequently applying these strategies to a liver transplant model.

Specific aims include:

1) To describe the *in-vitro* cross-genotype anti-HCV efficacy of herbal extracts in combination with cross-neutralising anti-HCV monoclonal antibody (AR4a)

2) To examine for the first time the ability of EGCG and AR4a alone and in combination to protect against HCV challenge *in vivo*.

We hypothesise that combining safe, tolerable and effective HCV therapies acting at different points of the HCV life cycle can reliably protect against HCV challenge *in vivo*. Clearly this research undertaking carries the prospect of yielding novel translational data of immediate clinical relevance; the provision of new and safe therapeutic options to improve outcomes in patients undergoing liver transplantation due to HCV associated liver disease.

Chapter 2:
HCV Cell Entry as a Therapeutic Target

2.1: Hepatitis C Virus Cell Entry

The sequence of events whereby HCV binds to and enters target cells has been the subject of intense study in recent years. Hepatocytes are the primary target cell of HCV. HCV entry into hepatocytes follows a well-orchestrated and complex series of interactions between the host cell and virus [8, 12, 55]. Multiple steps in this process represent potential therapeutic targets which can theoretically be exploited by treatments to prophylax hepatocytes against the entry of HCV [56, 57].

HCV viral factors

In humans HCV circulates in different forms. It can circulate as free virions or in forms associated with lipoproteins and immune complexes [58]. The association with lipoproteins has been reported to enhance infectiousness of the particles and also may confer immune evasion properties [59]. Virion associated lipoprotein and the HCV E1/E2 surface glycoprotein complex both play an integral role in HCV cell entry.

HCV E1/E2 surface glycoprotein complex

The HCV surface glycoprotein complex has a number of roles which are essential for the life cycle of HCV. It mediates binding to the host cell, fusion between the viral envelope and endosomal membrane, and also is

involved in the process of viral particle assembly. Following transcription and processing HCV E1 and E2 form an intracellular non-covalent heterodimeric complex. However in it's native form on the surface envelope E1/E2 exists as a heavily glycosylated covalent complex linked by disulphide bridges [60-62].

The structure and function of E2 has been described in much more detail than that of E1. The functionality of glycoprotein E2 is highly conserved across genotypes, this despite considerable genetic diversity. E2 contains a number of hypervariable regions (HVR) and the purported binding site for cluster of differentiation 81 (CD81), one of the primary host receptors mediating HCV cell entry [62, 63]. HVR1 located on the E2 ectodomain is one of the most immunogenic HCV epitopes and is also the region felt to interact with another key HCV surface receptor; scavenger receptor class B1 (SR-B1) [64]. Despite marked sequence variability the conformation of HVR1 remains quite well conserved. Interestingly despite being highly immunogenic, antibodies targeting HVR1 often exhibit inefficient neutralizing capacity. It appears that *in-vivo*, these antibodies then drive the variation observed within HVR1 by continuously selecting for HCV species capable of escaping neutralization [24, 65]. Furthermore, it is in fact likely that HVR1 functions to shield more conserved HCV epitopes, such as the CD81 binding site, from immune recognition. These conserved epitopes are likely to be capable of generating antibodies with both superior and cross-genotype neutralizing activity [66, 67].

Host cell Factors

The process of HCV cell entry is initiated following attachment of the HCV viral particle to the surface plasma membrane of the host cell. A number of putative host cell factors are implicated in assisting this initial attachment step. C type lectins (L-SIGN, DC-SIGN) expressed by macrophages, dendritic cells and liver sinusoidal endothelial cells can bind HCV surface glycoproteins to sequester circulating virus [68]. Surface glycosaminoglycans of hepatocytes (eg. heparin sulphate proteoglycans) interact with the HCV envelope glycoprotein complex to mediate initial attachment [55, 57]. The low-density lipoprotein (LDL) receptor on hepatocytes is also implicated in facilitating initial attachment. Apolipoprotein-B (Apo-B) or Apo-E containing HCV particles interact with the LDL receptor to further concentrate virions on the cell surface [69, 70].

Subsequent to attachment, HCV virions bind specifically to a number of cell surface receptors which facilitate entry of the virus particle into the cell. The first such HCV specific receptor to be characterized was CD81 [71]. CD81 is a widely expressed tetraspanin molecule and functions as an adaptor protein to sort and modulate localisation and interactions of membrane resident proteins. It is involved in a number of important cellular processes such as adhesion, proliferation, and differentiation. HCV E2 contains a CD81 binding site and the E2-CD81 interaction is known to take place post attachment [72, 73]. A second cell surface molecule, SR-B1 has been identified that specifically interacts with HCV viral particles

post cell attachment. SR-B1 is a lipoprotein receptor functioning to take up cholesterol ester from Apo-A containing lipoproteins such as HDL. The HVR1 region of HCV E2 has been shown to specifically interact with SR-B1 [74]. It has been proposed that this interaction may in fact precede the CD81-E2 interaction. Zeisel at al reported that the SR-B1 and HCV interaction alters the conformation of the HCV particle facilitating the specific interaction between CD81 and HCV E2 [74].

Two other entry factors have more recently been identified which play a key role in the HCV cell entry process. Claudin-1 and occludin are located at the junction of basolateral and apical cellular membranes at sites referred to as tight junctions [73, 75]. Tight junctions regulate paracellular transport of solutes, water and ions. These cell surface proteins do not interact directly with HCV but are necessary for efficient internalisation of HCV [56]. CD81 essentially acts as a shuttle to translocate the bound HCV particle to tight junctions, where CD81 and claudin-1 interact to form a HCV receptor complex [76, 77]. Lupberger et al reported that the receptor tyrosine kinases epidermal growth factor receptor (EGFR), and ephrin receptor A2, mediate the CD81-claudin-1 interaction via intracellular signalling pathways and actin reorganisation, which modulate the cellular trafficking of these entry factors [78].

Following localisation to tight junctions, clathrin mediated endocytosis occurs [79]. Subsequent endosomal fusion and viral uncoating is a pH dependent process. Low pH induces a conformational change in E2

facilitating fusion of the viral envelope with the endosomal membrane. Sainz et al have recently described a new host protein which plays an important role in this latter fusion step of HCV cell entry. Blockade of Niemann-Pick C1-like 1 cholesterol uptake receptor reduced HCV infection by impairing virion and hepatocyte fusion [80].

HCV is also capable of entering cells in a CD81 independent manner. Direct cell to cell transmission has been described and this will influence therapeutic approaches to preventing cell entry. This alternative mode of cell entry appears to be refractory to antibodies which target E2 or the host cell factors involved in HCV entry such as CD81 [81, 82].

The multiple steps involved in HCV cell entry present many possible therapeutic targets for novel agents capable of inhibiting HCV infection. Complete protection against HCV re-entry into hepatocytes is the ultimate goal of strategies to prevent recurrent HCV post liver transplant. Experimental work *in vitro* and *in vivo* has yielded a number of potential candidates.

2.2: Neutralizing antibodies and HCV

Studies addressing the immune responses of individuals who successfully eradicate acute HCV infection indicate that a robust host response requires both a broad, potent T cell response and an effective humoral

response. Viral clearance was dependent upon the rapid induction of neutralizing antibody exhibiting broad anti-HCV activity. In contrast chronic HCV infection is associated with low or absent neutralizing antibody titers throughout the early period of infection [16, 17]. Indeed, individuals who spontaneously resolve acute infection were shown to have considerably reduced variability in HVR1 sequences compared to the marked diversity detected in patients with chronic infection where HCV antibodies generated against HVR1 exert selective pressure contributing to the emergence of escape mutants capable of evading neutralization [83].

As mentioned earlier the HVR1 of E2 may function as an immunological decoy protecting conserved epitopes from immune recognition. Deletion of HVR1 was shown to increase susceptibility to neutralization by mAbs targeting the CD81 binding site of E2 [66]. In addition heavy glycosylation of E1/E2, the incorporation of lipid moieties, and non-neutralizing antibodies bound to the circulating viral particle all combine to restrict the access of antibodies to more conserved target epitopes.

The HCV E1/E2 surface glycoprotein represents the natural target of the protective antibody response [63, 84]. Many monoclonal (mAb) and polyclonal antibodies targeting linear or conformational epitopes within E2 have been described that can mediate virus neutralization *in vitro* [85-87]. Antibodies exhibiting broad cross-genotype activity have in general been reported to recognise conformational epitopes which contain conserved residues within the CD81 binding site on E2 [88, 89]. Additionally

numerous monoclonal antibodies (mAbs) targeting the host cell factors utilised by HCV have demonstrated ability to inhibit HCV entry. Blockade of CD81, SR-B1, claudin-1, and the LDL receptor all inhibited HCV infection [70, 90-93]. These have greatly facilitated the study of HCV entry; however, the targeting of host cell proteins can culminate in undesired adverse effects limiting their clinical application.

The *in vivo* performance of HCV neutralizing antibody has been more variable. Patient serum containing HCV antibodies have demonstrated a variable ability to protect against HCV challenge [94]. Chimpanzees administered serum from HCV infected individuals were protected against homologous HCV challenge [95]. Additionally a retrospective review published in 1998 concluded that hepatitis B immunoglobulin utilised in the era prior to HCV antibody screening conferred some protection against the development of HCV infection in liver transplant recipients [96]. Osburn et al have reported that intravenous drug users repeatedly exposed to HCV harbour neutralising antibodies capable of protecting against HCV infection [97]. In contrast a hepatitis C immune globulin preparation provided no protection against HCV re-infection in patients undergoing liver transplantation due to HCV related liver disease [54]. Similarly two groups have reported the failure of anti-E2 monoclonal antibodies to provide durable protection against HCV recurrence following liver transplantation [98]. In one of the studies monotherapy with a mAb was

associated with the selection of mutant variants in all patients administered the mAb [99].

Law et al characterised a number of antigenic regions on E2 and identified numerous human mAbs with cross neutralizing activity. Neutralizing mAbs targeting antigenic region 3 (AR3, contains the CD81 binding site) efficiently neutralized a number of different HCV genotypes in vitro and protected against a heterologous HCV quasispecies challenge *in vivo* [88]. This group have identified further antigenic regions of E1/E2, and generated a human mAb which targets a discontinuous epitope outside the CD81 binding site on HCV E1/E2. This mAb (AR4a) targets a region containing a very highly conserved residue and demonstrates the most potent cross neutralizing activity *in vitro*. Using an *in-vivo* genetically humanised mouse capable only of studying HCV entry, AR4a inhibited cellular entry of both HCV 1b and 2a genotypes [89].

Employing an anti-HCV mAb (HCV-1 mAb) in different therapeutic strategies in chimpanzees produced very informative results. A single high dose of the mAb prior to administration of the HCV inoculum protected against the establishment of HCV infection. Administering the mAb to chronically infected animals resulted in a decline in viral load followed by a rebound. Interestingly *in vitro* sera from chronically infected chimpanzees competitively inhibited the ability of the mAb to neutralize HCV [100]. Thus akin to the situation as it pertains to HCV individuals undergoing liver

transplant, circulating HCV antibodies may compete with and impair the efficacy of mAbs administered.

In patients with HCV undergoing liver transplant the prevailing HCV variants pre and post transplant differ considerably. Sequence diversity post transplant is less pronounced and the predominant species in circulation clearly can escape neutralization by the patients' pre-existing HCV antibodies [101, 102]. These pre-existing antibodies however could theoretically negatively impact immunotherapy strategies [103]. Whilst immunotherapy using antibodies with broad neutralizing activity is clearly attractive in terms of safety and tolerability, optimising *in vivo* cross-genotype neutralizing potential and protection from the emergence of resistance mutations are of key importance. Account must also be made for the possibility of direct cell to cell transmission of HCV which is capable of evading neutralization [81].

2.3: Herbal Extracts, Silibinin and Epigallocatechin-gallate

Silibinin

Silibinin is the major active constituent of silymarin. Silymarin is a mixture of flavonolignans extracted from milk thistle, *Silibum marianum gaertneri* [104, 105]. Silymarin has long been regarded as a 'hepatoprotectant' and has a very long history of use by patients with chronic HCV infection and

liver disease in general [106, 107]. Silibinin is widely available, safe, cheap, well tolerated and no significant drug-drug interactions have been reported [108, 109]. Silibinin has been demonstrated to exhibit a number of interesting properties. It exhibits anti-inflammatory, anti-oxidant, anti-proliferative, and anti-fibrotic activity [105, 110]. All these properties are of potential benefit in a post-transplant setting.

In particular it has been shown that silibinin can inhibit the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and thus reduce NF-κB dependent gene transcription [104]. Additionally it mediates a reduction in a number of pro-inflammatory cytokines such as TNF-α, Interferon-γ and interleukin-2 from stimulated peripheral blood mononuclear cells [104, 110].

Despite it's widespread use for many years definitive data on the anti-HCV activity of silibinin has only emerged in recent years. *In vitro* both the parent compound and the water soluble derivative (Legalon-SIL®) inhibit HCV infection [110, 111]. The compounds function primarily to decrease HCV infection of cells; an inhibitory action on the HCV RNA dependent RNA polymerase (RdRp) requiring very high *in vitro* drug concentrations [112]. The predominant mode of inhibiting HCV infection is felt to be mediated by an inhibition of endosomal fusion [111]. By incorporating into endosomal membranes, these become stabilised and thus are less prone to fusion. By virtue of this mechanism of action silibinin is capable too of inhibiting the direct cell to cell transmission of HCV.

Milk thistle products are mainly available for oral use. Many different formulations exist and the variable concentrations of flavonolignans, and a limited and erratic bioavailability have been suggested to explain the lack of any definitive evidence supporting an anti-HCV effect in clinical practice [106]. Legalon-Sil®, a water soluble version of silibinin (formulated to enable parenteral administration) is used in the management of acute liver failure due to amanita phalloides mushroom poisoning [113]. Silibinin is modified to generate Legalon-Sil® by virtue of the addition of two succinate moieties. This compound has clearly demonstrated antiviral efficacy by causing a dose dependent decline in HCV RNA levels when administered daily to patients by intravenous injection [114]. Further it was employed in two case reports to successfully prevent HCV re-infection post transplantation. Both patients had low HCV RNA levels at the time of transplant and tolerated 14-25 days of Legalon-Sil® therapy without significant adverse effects [115, 116].

Two small pilot studies in Spain administered intravenous Legalon-Sil® monotherapy to patients with HCV undergoing liver transplantation. All patients tolerated the treatment well whether administered in the immediate pre or post-transplant period. A significant decline in HCV viral RNA levels was observed during therapy compared with those untreated or receiving placebo however all recipients experienced relapse following discontinuation after three to four weeks of therapy. The relatively short

duration of therapy and the use of monotherapy are obvious limitations to these experimental approaches [117, 118].

Epigallocatechin gallate (EGCG)

EGCG is the most abundant catechin present in green tea extract. Green tea extracts are derived from the leaves of *Camellia Sinensis* and like silibinin have a long history of safe human consumption. Multiple purported benefits have been ascribed to green tea extracts and it's primary active component EGCG. Anti-inflammatory, anti-oxidant, anti-tumorigenic, anti-proliferative and anti-viral effects have all been reported [119-122]. These effects are mediated directly, via interaction with a myriad of proteins implicated in carcinogenesis, and indirectly via it's effects on transcription factors (eg NF-κB) and STAT (signal transducer and activator of transcription) proteins [121, 123-125].

Green tea extracts are very safe, widely available, and cheap [126, 127]. Numerous formulations exist which can vary in their stated EGCG content. Polyphenon E®, the formulation employed in clinical trials funded by the National Cancer Institute in the USA contains 65% EGCG, Teavigo® on the other hand contains 94% EGCG.

Benefits of EGCG have been demonstrated in numerous animal models of disease. Despite limited *in vivo* bioavailability green tea extracts and

EGCG have demonstrated efficacy against autoimmune disease and tumour progression [128-130]. With regard to specific anti-viral activity EGCG has been shown to demonstrate activity against HIV, Influenza, Adenovirus, HBV, and Herpes simplex (HSV). EGCG acts in different ways against the listed viruses and in some cases exhibits activity at more than one point of the viral life cycle. Against HIV EGCG blocks the interaction between glycoprotein 120 and CD4, and can inhibit the reverse transcriptase enzyme [131, 132]. Likewise against influenza EGCG acts to inhibit haemagglutination, neuraminidase activity, and RNA synthesis [133]. EGCG interrupts the HBV life cycle by reducing the synthesis of replicative intermediates of DNA [134, 135]. It is important to note however that a number of these *in vitro* anti-viral effects occur at concentrations far exceeding that which can be physiologically achieved by oral consumption.

Two groups independently published data recently reporting an *in-vitro* anti-viral effect of EGCG against HCV [136, 137]. Both concluded that at relatively low concentrations of EGCG, HCV infection could be inhibited. The predominant mechanism of action was via a prevention of initial attachment of the viral particle to hepatocytes. As with Silibinin this mechanism of action also inhibits direct cell-to-cell transmission. This effect of EGCG was HCV specific (no effect on vesicular stomatitis virus or other flaviviridae) but genotype independent and it was suggested that EGCG may be acting directly on the HCV viral particle effecting an

alteration in it's biophysical properties [137]. Neither group found any ability of EGCG to directly inhibit HCV replication, however Chen et al did report an ability to inhibit RNA replication but only using very high *in vitro* EGCG concentrations [138]. *In vivo* evidence of the anti-HCV effect of EGCG is lacking.

Silibinin and EGCG have in common favourable characteristics for application to the prevention of HCV re-infection in liver transplant recipients. Both act primarily at early stages to inhibit entry of HCV to the cell, and both also exhibit immunomodulatory activity. More importantly they are extremely safe and well tolerated facilitating their administration to this complex patient cohort who are often exceptionally unwell and receiving many other medications. These agents are readily available throughout the world and are cheap enabling broader patient accessibility throughout the world when compared to that of new direct acting antiviral agents.

Chapter 3:

Experimental Methodology

3.1: Reagents

Human hepatoma (Huh) 7.5 cells (provided by Dr. C Rice, The Rockefeller University, New York) were maintained in complete dulbecco's modified eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS), 0.1mM non-essential amino acids (NEAA), 100U/mL penicillin and streptomycin 100 mg/mL. Cells were incubated at 37°C in conditions of 5% CO_2.

Silibinin and EGCG were obtained from Sigma-aldrich (St Louis MO). Silibinin (Cat number: S0417) is water insoluble, thus a stock concentration was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. For all silibinin experiments the end concentration of DMSO was diluted to less than 1%. Samples containing the equivalent concentrations of DMSO were also used as controls. EGCG (Cat. number: E4143, 95% purity) was dissolved in double distilled H_2O and stored at -20°C.

Recombinant human interferon alpha 2a (IFN-α2a, positive control) was obtained from PBL interferon source, (Piscataway, NJ, cat. number: 11100-1) and stored as instructed.

Antibodies:

Human anti-E1/E2 mAbs AR3a and AR4a were provided by Dr. Mansun Law, The Scripps Research Institute (La Jolla, CA). These mAbs have

previously been characterised in detail [88, 89]. Murine IgG obtained from BD biosciences (Franklin Lakes, NJ) was used as an isotype control antibody for *in vitro* assays. Mouse monoclonal anti-human CD81 antibody from BD biosciences (Franklin Lakes, NJ, Cat number 555675) represented a positive antibody control during in-vitro experiments. An isotype human anti-HIV1 IgG (B6, from M. Law) was administered to the control group of mice during *in-vivo* studies. All antibodies were stored at 4°C until use.

3.2: HCV cell culture (HCVcc)system

For the *in-vitro* assessment of the ability of mAbs and herbal extracts to inhibit HCV infection we used the HCVcc system. The HCVcc system was first described in 2005 and constitutes an invaluable advance in the study of HCV [139, 140]. In this system the complete life cycle can be completed with the production of infectious virus capable of infecting naïve Huh cell lines. The parent HCV strain used in this system derives from a Japanese patient with fulminant hepatitis (JFH-1) and is a genotype 2a strain. Chimeric infectious clones have been successfully generated *in vitro* which consist of the JFH-1 non-structural genes fused to the structural genes (core, E1, and E2) of different genotypes [141]. These chimeras are particularly valuable in the study of agents acting to inhibit target cell entry and allow the assessment of efficacy across the range of HCV genotypes. Additionally a recombinant HCV construct harbouring a renilla luciferase reporter gene (JFH-1-Luc) has been created allowing assessment of

infectivity by means of luciferase activity. JFH-1 constructs utilised in these experiments were kindly provided by Dr C Rice, The Rockefeller University.

Generation of Infectious HCVcc

The protocol used to generate infectious HCVcc chimeras is adapted from Lindenbach et al [139]. Briefly, HCVcc complementary DNA (cDNA) cloned into a plasmid containing a T7 polymerase reporter was linearized via digestion. Following purification of the linear DNA (minelute PCR purification columns, Qiagen, Valencia, CA, Cat number: 28004) it served as a template to generate viral genomic RNA by *in vitro* transcription (T7 RiboMAX™ Express large scale RNA production system, Promega, Madison, WI, Cat number: P1320). This RNA was purified (RNeasy kit, Qiagen, Valencia, CA, Cat number: 74106) prior to delivery to Huh 7.5 cells by electroporation. Trypsinised cells were washed in ice cold PBS and resuspended at the required cell density. Five to ten micrograms of RNA were then mixed with Huh 7.5 cells in a 4mm cuvette and 5 pulses at 860V were delivered using BTX Electrosquare Porater ECM 830. Freshly transfected cells were allowed to rest for ten minutes prior to mixing with fresh cell culture media and seeding in culture flasks for incubation. Cells were incubated for 48-72 hours at 37°C, 5% CO_2. HCVcc virus in the supernatant was then harvested via filtration, frozen and stored at -80°C.

Titration of infectious HCV

The titer of the HCVcc (generated as above) was determined by the limiting dilution method. Cell supernatants harvested as outlined were serially diluted ten-fold in cell culture media and inoculated onto naïve Huh7.5 cells plated in a 96 well plate (BD Falcon™). After 10-12 hours at 37°C, 5% CO_2, fresh media was exchanged and incubation continued to 48 or 72 hours. Infection at this time point was determined by immunohistostaining for HCV NS5a (outlined below).The 50% tissue culture infective dose (TCID50) was determined by visualising wells with infective foci present and the titer was calculated using the Reed-Muench calculation and expressed in focus forming units (FFU) /mL.

HCV inhibition assays

Initially 96 well plates are prepared as follows. Wells were coated with Poly-L-Lysine (Trevigen, Gaithersberg, MD, Cat number: 3438-100-01) prior to plating with 10^4 Huh 7.5 cells in 100 µL of growth media and incubated overnight. Serially diluted concentrations of silibinin or EGCG were added as appropriate pre, simultaneous with, or after inoculation with HCVcc at a multiplicity of infection (MOI) of 0.01. For antibody neutralization assays the relevant antibody was serially diluted to the required concentrations and pre-incubated with HCVcc for one hour prior to addition to the Huh 7.5 cells. IFN-α2a 100 IU/mL and anti-human CD81

antibody were used as positive controls. Murine IgG was used as an isotype antibody control. After ten hours of incubation with HCV the cells were washed to remove unbound virus and fresh media or the relevant concentration of the investigational agents was added. HCV infectivity was determined at 48 hours using NS5a immunohistostaining or by measuring luciferase activity in the supernatant when using luciferase reporter viruses. Neutralisation/ inhibition of HCV activity was determined by a reduction in infectious foci or luciferase activity. Data shown in the results chapter is representative of between 2 and 5 independent experimental repeats conducted in triplicate.

Time of addition studies

Time of addition studies were conducted to assess the optimal conditions whereby silibinin and EGCG exerted maximal inhibition. Their activity was analysed by addition to Huh7.5 cells at differing timepoints with regard to the point of infection. Agents were added to cells for varying durations (1-16 hours) prior to viral inoculation (pre-treatment), simultaneous with the HCV inoculum, or following the 10 hour infection incubation. Additionally the HCV inoculum itself was pre-incubated with the agents for one hour prior to addition to Huh7.5 cells. Following the wash step silibinin or EGCG were, or were not re-added to wells for the duration of the experiment depending on the desired experimental conditions.

NS5a immunohistostaining

Following the required period of incubation the supernatant was removed from the wells. Cells were washed twice with phosphate buffered saline (PBS) and then fixed and permeabilized with ice cold 100% methanol for at least 30 minutes at -20°C. Following this the cells were washed twice with PBS and once with PBS/0.1% Tween 20 (this also represents the washing steps used below). Blocking buffer (1% bovine serum albumin (BSA), 0.2% skimmed milk in PBS/0.1% Tween 20) was added to the cells for thirty minutes at room temperature. The block was then removed and 3% hydrogen peroxide (H_2O_2) in PBS to block endogenous peroxidase activity was added for five minutes. The cells were then washed again. Anti-NS5a mAb 9E10 (provided by Dr T Tellinghuisen, The Scripps Research Institute, La Jolla, CA) diluted 1:25000 in PBS/0.1% Tween 20 was used as the primary antibody. Fifty µL was added per well and the sealed plate was stored overnight at 4°C. The secondary antibody is sheep anti-mouse HRP (GE healthcare biosciences, Pittsburgh PA, Cat number: NA931). Following another wash step, 50 µL of the secondary antibody diluted 1:200 in PBS/0.1% Tween 20 was added for 30 minutes at room temperature. The cells were again washed and DAB substrate (DAKO, Carpenteria, CA, Cat number: K3468) was reconstituted as per the manufacturer's instructions and added for 5 minutes at room temperature. The cells were finally washed twice in PBS and the foci were then visualised and counted under the microscope. The % infectivity of

HCVcc in the presence of the investigational agents was determined by the residual number of foci present at the relevant concentration with reference to the number of foci in the non-treated control wells.

Luciferase assay

The inhibitory effect of the novel compounds on HCVcc harbouring a luciferase reporter gene was assessed using the Renilla luciferase assay system (Promega, Madison, WI, Cat number: E2820). Following the required period of incubation 10 µL of supernatant was added to 10 µL of lysis buffer solution (Renilla luciferase lysis buffer 1:5 dilution in distilled H_2O) in a 96 well ELISA plate. Renilla luciferase assay reagent was prepared using renilla luciferase substrate diluted 1:100 in renilla luciferase buffer. Fifty µL of reagent was required per reaction. Using the Enspire® multimode plate reader (Perkin Elmer Walthin Ma), luciferase activity was analysed. The % infectivity was determined using the residual activity in the presence of compound with reference to the activity in the non-treated controls after subtraction of the background.

Initial experiments quantifying JFH-1-LUC infectivity using both NS5a immunostaining and luciferase activity showed a strong correlation between the two assay methods across differing MOIs and duration of inoculation (Figure 1).

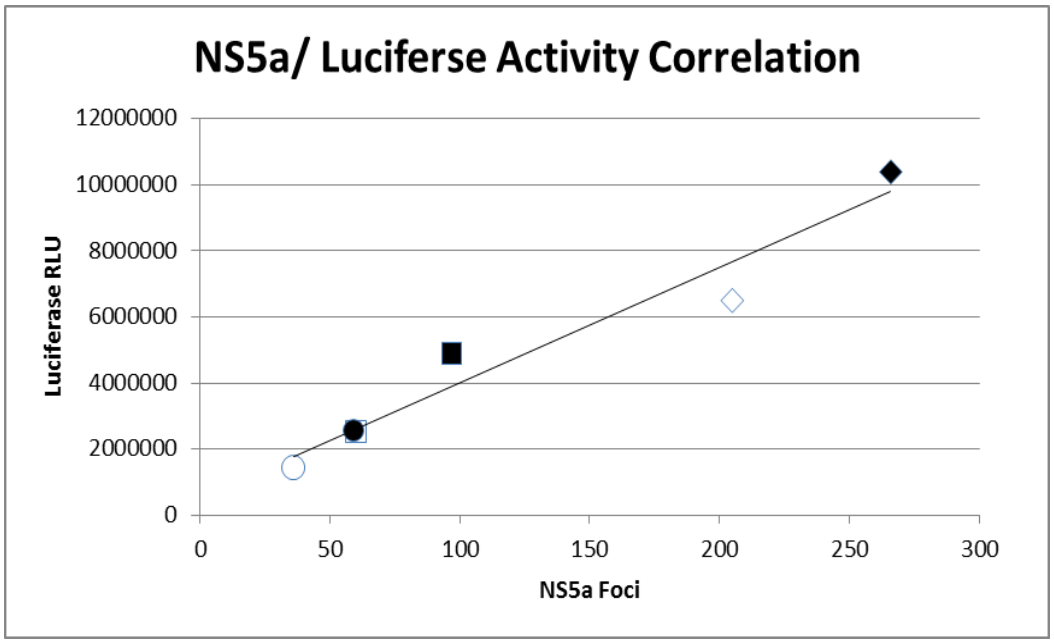

Figure 1: NS5a immunostaining (HCV foci) and luciferase activity of JFH-1-LUC correlate across different MOIs and Durations of Infection. Diamond: MOI 0.02; Square: MOI 0.01; Circle: MOI 0.005. Open shapes: 6 hr infection, Closed shapes: 10 hr infection

Quantification of Cell Viability/ Metabolic Activity

To quantify any *in vitro* toxic effects on cell activity and viability caused by silibinin or EGCG we used the Cell Proliferation Kit I assay (Roche, Basel, Switzerland Cat Number: 11465007001), which is based on the cleavage of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to purple formazan crystals by metabolically active cells. Ninety-six well plates were prepared and seeded with Huh7.5 cells in an identical manner to that outlined for the inhibition assays. The cells were allowed to incubate overnight. Serial dilutions of EGCG and silibinin, and the equivalent DMSO controls were then added to cells. After 24hrs

incubation, 10µL of MTT labelling reagent was added for 4 hours. Then 100 µL solubilisation solution was added and the plates were incubated overnight. Spectrophotometrical absorbance was measured using an ELISA reader. The % change in cellular metabolic activity was determined with reference to the non-treated cells and relevant controls.

3.3: SCID/uPA Animal model of HCV infection

To assess the *in-vivo* efficacy of mAbs and herbal extracts demonstrating anti-HCV activity *in vitro*, we utilised the chimeric mouse model of HCV infection pioneered at the University of Alberta. The generation of this small animal model of HCV infection was first reported in 2001 [142]. It has been extensively reported on since, and has proved a very powerful tool for *in-vivo* HCV studies [90, 94, 143]. The native liver of homozygous albumin/urokinase plasminogen activator (uPA)/ severe combined immunodeficient mice (SCID/uPA mice) undergoes accelerated death and transplanted human hepatocytes can repopulate the liver and subsequently are capable of supporting HCV infection and replication. By virtue of a complete lack of adaptive immunity (SCID trait) it is an ideal model to study the ability of mAbs to confer passive immunity against HCV and also to study the use of combination therapy to investigate means to prevent the initial establishment of HCV infection; the ultimate goal of strategies to prevent HCV recurrence after liver transplantation.

Animal Care

The animals were kept virus- and antigen-free and housed in the provincial laboratory vivarium at the University of Alberta. Animal care and manipulation was conducted by specialist animal technicians from KMT Hepatech, incorporated. Experimental protocols were approved by the health sciences animal welfare committee of the University of Alberta, and animals were cared for in accordance with the 1993 guidelines of the Canadian Council on Animal Care. Mice were anaesthetized for transfer of human hepatocytes via intrasplenic injection. Full ethical approval for the use of human tissue was obtained from the research ethics board of the University of Alberta Faculty of Medicine. Informed consent was obtained from all donors.

Human alpha-1-Antitrypsin levels (hAAT)

The extent and stability of human liver chimerism can be assessed by serial measurements of hAAT. High levels of human hepatocyte colonization correlate with high serum hAAT levels. Serum hAAT levels greater than 500µg/mL correspond to chimeric livers that consist of 70-80% of human liver cells [144]. Samples of mouse serum (2 µL) were diluted 1/100 in blocking buffer and analysed by sandwich enzyme linked immunosorbent assay (ELISA) using polyclonal goat anti-hAAT antibody (Diasorin, Stillwater MN, Cat number 81902) to capture. A portion of this

antibody was also cross linked to HRP (Pierce, Rockford, IL, Cat number 31489) and used as the secondary antibody. Signal detection was with 3.3',5,5'-tetramethylbenzidine (Sigma, St Louis, MO). Animals with low serum hAAT levels were used in tolerability/ toxicology studies, high hAAT mice were used in the HCV challenge studies.

Dosing and administration of agents:

Anti E1/E2 monoclonal antibody (AR4a)

For the *in-vivo* HCV challenge experiments we used an anti-E1/E2 mAb AR4a. The in-vivo performance of this mAb to date has not been characterized in an animal model of HCV infection. An initial dose of 200mg/kg was administered via intraperitoneal (IP) injection 24 hours prior to inoculation with HCV. Prior studies have shown that this dose comfortably yields mAb serum values in excess of *in vitro* 90% neutralization titers [88]. A further four mAb doses of 50mg/kg were administered IP at intervals of 5 days throughout the experiment. Mice in the control group received equivalent doses of an isotype antibody; human anti-HIV1 IgG.

Herbal extracts

A water soluble parenteral formulation of silibinin (the formulation demonstrating anti-HCV efficacy in human studies) was not available for

us to utilize in the animal experiments and thus we proceeded only with EGCG.

EGCG

Previous studies have demonstrated that the bioavailability of EGCG *in vivo* is limited. Lambert et al, in 2006, reported a linear dose response relationship when increasing doses of single administrations of EGCG were given to mice. Doses ranging from 50-2000mg/kg were safe in single doses. However, the maximum concentrations of EGCG achieved in plasma and liver tissue with this approach only ranged from 0.03-4.17 µg/mL and 0.09-18.3 µg/mL respectively [145]. The kinetics of EGCG in mice following intragastric administration in mice has also been characterized. EGCG is well absorbed but extensively glucuronidated limiting it's absolute bioavailability to approximately 12.4-25.6%. The maximum concentration of EGCG (C_{max}) in plasma is achieved around 90 minutes post administration (T_{max}). The elimination half-life ($t_{1/2}$) in mice is also quite short and estimated to be ~83 minutes [146]. The liver was amongst the tissues (outside of the intestinal tract) with the highest levels of EGCG and this represents a consistent finding in previous publications [147]. It has been suggested that repeated dosing further increases tissue and plasma levels of EGCG [147]. This phenomenon has also been reported in human studies using volunteers taking oral EGCG formulations [127].

With regards to toxicity in animal models Hsu et al reported that up to 2500 mg/kg of green tea extract could be administered to mice for 28 days without any adverse consequences [148]. In contrast, Lambert et al, when using a pure EGCG compound, reported 85% mortality in mice following a single administration of a dose of 1500mg/kg. Serum alanine aminotransferase (ALT) levels rose by 138 fold and moderate to severe hepatic necrosis was present on liver histology. In addition, more than two repeated doses at 750mg/kg/day and 500mg/kg/day was associated with a mortality of 75-80% and 20% respectively [149]. Hepatotoxicity of EGCG alone had previously also been reported by Galati et al [150].

Despite these apparent limitations numerous groups have demonstrated efficacy of EGCG using animal disease models. In an animal model of immune mediated hepatitis (concanavalin induced hepatitis) pre-administration of 10mg/kg/day by gavage (IG- intragastric) successfully reduced the severity of hepatitis induced by concanavalin [128]. Likewise in a model studying pancreatic tumorigenesis doses of 60, 80 and 100mg/kg/day inhibited pancreatic tumour growth [151].

Considering the available information on *in-vivo* EGCG pharmacokinetics, toxicity, and efficacy, we elected to use a dosing schedule of 100mg/kg twice daily by gavage. This dose was higher than that which had demonstrated efficacy in previous studies but lower than that with which toxicity had been observed with repeated dosing. This dosing schedule

was also within that tolerated by SCID/uPA mice in terms of volume and frequency of gavage.

To assess tolerability and toxicity of EGCG in the SCID/uPA mouse we administered 200mg/kg/day (100mg/kg 12 hours apart) of EGCG IG to three mice for 14 days. Control animals received an equivalent volume of H_2O twice daily IG. The health status of the animals was monitored by their general condition and weight change. At the end of the 14 day dosing period the treated animals were euthanized. Cardiac puncture was performed to provide sufficient serum to enable measurement of EGCG levels and liver tissue was snap frozen and stored at -80°C for later measurement of EGCG.

Experimental Conduct:

Screening of serum hAAT levels is undertaken six weeks following transplantation of human hepatocytes. Mice with serum hAAT levels (>500 µg/mL) reflecting high engraftment of human hepatocytes were selected to go forward into the HCV challenge studies. In total 8 mice were assigned to each of 4 study groups:

Group 1: Control mice receiving B6 antibody IP and water IG

Group 2: Mice receiving EGCG IG only

Group 3: Mice receiving AR4 mAb IP only

Group 4: Mice receiving both AR4a IP and EGCG IG

The study outline and protocol employed is illustrated in figure 2.

EGCG dosing began 48 hours prior to HCV inoculation and continued for 14 days. Antibody administration (200mg/kg) commenced 24hours prior to inoculation and was repeated 4 times (50mg/kg) at 5 day intervals. HCV inoculation was administered by intrajugular injection. The inoculum used (50 µL) was a patient derived genotype 1a sample. Blood sampling was conducted weekly by drawing 100 µL via tail bleeds for measurement of HCV titers and serum hAAT levels.

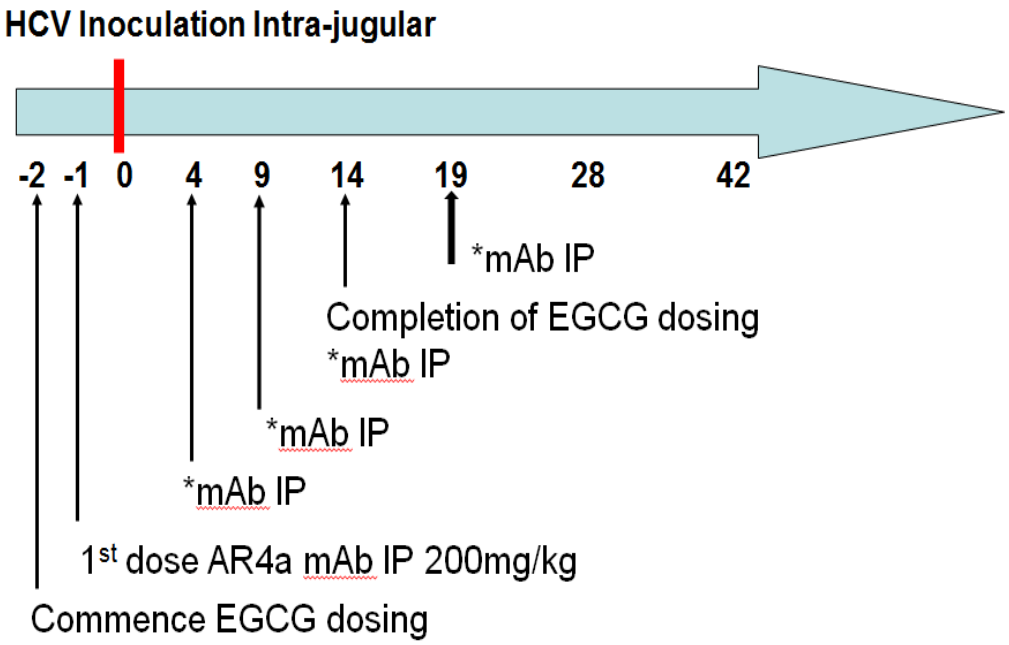

Figure 2: Schematic outline of SCID/uPA mouse HCV challenge experiments. mAb: monoclonal antibody, IP: intraperitoneal, * four doses of mAb at 50mg/kg.

HCV RNA quantification

Viral RNA was extracted from aliquots of mouse serum using a guanidine extraction method as per the manufacturer's instructions. (Buffer AVL. Qiagen, Valencia, CA, Cat number: 19073). Extracted RNA was transcribed to cDNA using an HCV specific primer (5'-AGGTTTAGGATTCGTGCTCAT) with a RNA to cDNA kit (Applied Biosystems, Foster City, CA, Cat number: 4387406). Polymerase chain reaction (PCR) was performed with a real-time PCR system (Model 7300, Applied Biosystems) and a Taqman assay. The HCV specific detection probe was 6-FAM-CACCCTATCAGGCAGTACCACAAGGCC-TAMRA; the primers used detected a conserved region of the 5' untranslated region (5'-TGCGGAACCGGTGAGTACA, 5'- AGGTTTAGGATTCGTGCTCAT). Internal controls containing known calibrated HCV amounts, and a standard dilution series using a plasmid containing the sequence of HCV strain H77c were performed in parallel. The lower limit of quantification of this assay is 300 IU/mL.

3.4: EGCG quantification

Plasma and liver tissue levels of EGCG were analyzed by Dr J Lambert, The Department of Food Science at The Pennsylvania State University. The experimental procedure employed to measure EGCG by this laboratory are outlined in brief below. Plasma (100 µL) was hydrolyzed

with 1U of sulfatase and 250U of β-glucuronidase at 37°C for 45 minutes. Extraction followed with methylene chloride and ethyl acetate. The ethyl acetate fractions were pooled and dried under vacuum. Samples were reconstituted and analysed by high performance liquid chromatography (HPLC). Liver tissue was homogenized in two volumes of ice-cold 2% ascorbic acid using a mechanical Dounce homogenizer, and 200µL aliquots were hydrolyzed and extracted as outlined above for plasma. The HPLC apparatus employed to measure EGCG levels consists of two ESA model 580 dual-piston pumps (ESA, Inc., Chelmsford, MA), a Waters model 717plus refrigerated autosampler (Waters, Milford, MA), and an ESA 5500 Coulochem electrode array system (CEAS). The CEAS potentials were set at -100, 100, 300 and 500 mV. Separation was achieved using a binary mobile phase of solvent A (0.03M NaH2PO4, pH 3.35, containing 1.75% acetonitrile and 0.125% tetrahydrofuran) and solvent B (0.015M NaH2PO4, pH 3.45, containing 58.5% acetonitrile and 6.25% tetrahydrofuran). The samples were separated on a 150 mm x 2.1 mm C18 column with 3.5 micron particle size at room temp.

3.5: Statistical analysis:

Statistical analyses were performed using Stata (Version 12.0, StataCorp LP, College Station, TX) and GraphPad prism (version 6.0, La Jolla, CA) software. Continuous variables were compared between groups using a Mann Whitney U test. A p value less than 0.05 was considered significant. The drug concentration at which HCV infection was inhibited by 50% (IC_{50}) was calculated using the following equations:

Best fit curve $y = a*e^{-slope*x}$

IC_{50} $x = Log_{10}(50/a) / slope*Log(EXP(1))$

Analysis of the animal HCV challenge experiments was conducted as follows: Animals with HCV RNA detectable above the threshold (1000 IU/mL) by PCR at day 7 or thereafter were considered ‘infected’. Animals not reaching this threshold were ‘censored’. A Kaplan-Meier survival curve (‘Survival free from infection’) was thus generated. Statistical significance between the groups was calculated using a two-tailed log rank test.

Chapter 4:
Results

4.1: Novel agents Inhibit HCVcc Infection

Silibinin demonstrates cross genotype HCV inhibition

To examine the effect of silibinin on HCV *in vitro* we used the HCVcc system. Chimeric constructs containing genotype specific genes coding for structural proteins fused to the parent JFH-1 (genotype 2a) genes coding for HCV non-structural proteins were utilized. Naïve Huh 7.5 cells were inoculated with HCV chimeras and the effect of different concentrations and exposure conditions of silibinin on subsequent infection was examined. Using the JFH-1 strain (J6, genotype 2a) increasing doses of silibinin were associated with a reduction in HCV infectivity. As silibinin is water insoluble it was initially dissolved in DMSO. Thus the corresponding vehicle controls were used containing equivalent DMSO concentrations, and residual infectivity in particular conditions of silibinin was determined relevant to the appropriate DMSO control (Figure 3a). The half maximal inhibitory concentration (IC_{50}) of silibinin against JFH-1 genotype 2a was approximately 44.62µg/mL. This inhibitory effect was observed across genotype constructs as silibinin demonstrated a similar ability to inhibit infection when tested against a genotype 1a chimeric construct (H77); (Figure 3b) the IC_{50} estimated at 44.88µg/mL. Furthermore, this anti-HCV activity *in vitro* was independently confirmed using a genotype 2a construct encoding a luciferase reporter sequence (Figure 3c). A dose

dependent reduction in HCV infection due to silibinin was again evident, the calculated IC_{50} of 32.5 µg/mL being somewhat lower when using the luciferase encoding construct.

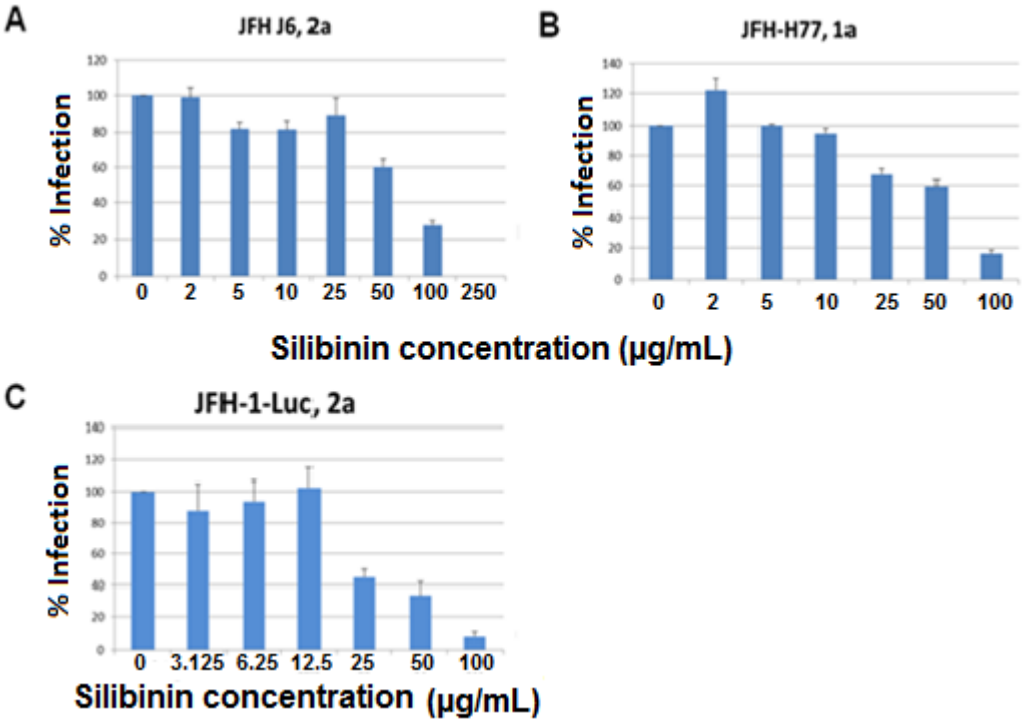

Figure 3: Silibinin dose dependently inhibits JFH-1 genotype 2a(A) and 1a(B) constructs. (C) JFH-1-Luc: genotype 2a construct encoding luciferase reporter gene. Mean % residual HCV infection (adjusted for DMSO vehicle concentration) is shown. Error bars indicate standard error of mean (sem).

To test the conditions by which silibinin exerted it's maximal effect we conducted time of addition studies. Using the luciferase reporter construct and silibinin at 50µg/mL it was clear that optimal HCV inhibition was observed when silibinin was added simultaneous with inoculation and allowed to remain present through the duration of the experiment (Figure 4). Extended silibinin pretreatment of cells for durations of two to 16 hours

in addition to these conditions did not confer any additive inhibitory activity (78% vs. 66% inhibition for two and 16 hour pretreatment respectively, p=0.7815). Pretreatment of cells alone, with removal of silibinin prior to HCV inoculation demonstrated only minimal inhibitory activity. Likewise the addition of silibinin to wells only following the 10 hour infection phase did not result in any reduction in HCV infection.

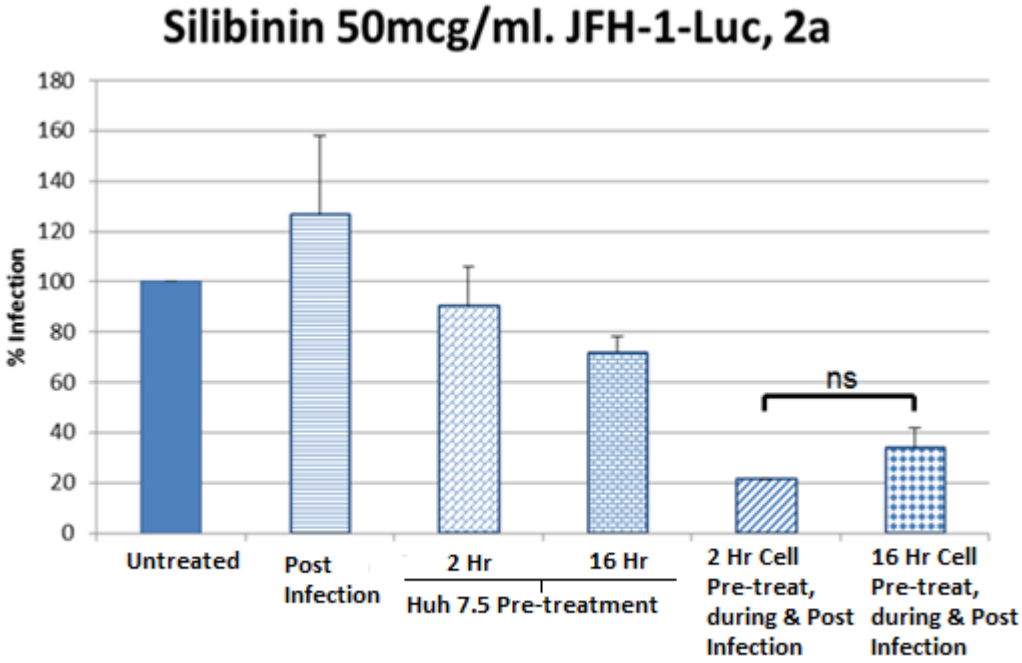

Figure 4: Silibinin time of addition studies. Varying *in vitro* Silibinin exposure conditions. ns: not significant. Mean % residual HCV infection is shown. Error bars indicate sem.

Silibinin displays a narrow *in vitro* therapeutic index

To evaluate the cytotoxic potential of silibinin we utilised an assay evaluating cellular metabolic activity (MTT assay). Serial dilutions of silibinin were added to Huh 7.5 cells. Again the corresponding vehicle

controls were used to correct for DMSO effects. At concentrations greater than 100 µg/mL of silibinin a steep decline in cell viability was observed (Figure 5). Cellular toxicity was also clearly apparent at concentrations of 250 µg/mL under direct microscopy. At this silibinin concentration during *in-vitro* experiments direct visualization of wells revealed cellular detachment and reduced cellular densities. The demonstrated cellular toxicity at these levels of silibinin is in keeping with that previously reported by other groups. Polyak et al, and Ahmed Belkacem et al both reported cytotoxicity above a concentration of 100 µg/mL of silymarin and silibinin respectively [105, 112]. In contrast the water soluble formulation, Legalon-Sil® exhibited very little cytotoxicity even at high doses [110]. Unfortunately this formulation was not available to us to study in these experiments.

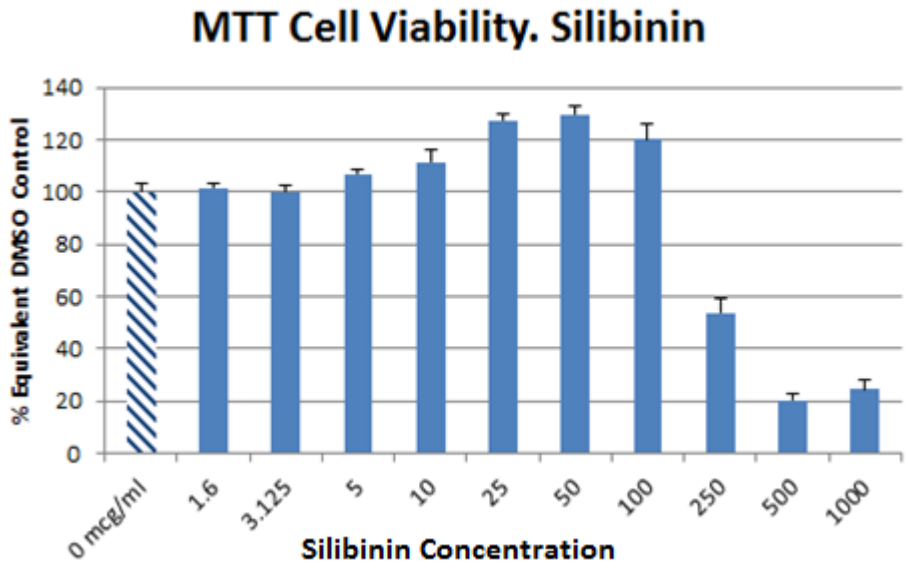

Figure 5: Silibinin exerts cytotoxicity at concentrations above 100 µg/mL. Mean % cellular metabolic activity adjusted to DMSO controls is shown.

Epigallocatechin-gallate (EGCG) potently inhibits HCV infection

Using the HCVcc we characterized the ability of EGCG to inhibit HCV infection. EGCG caused a dose dependent decline in HCV infection. As was observed with silibinin, this anti-HCV effect was demonstrable across HCV genotypes (Figure 6). When assayed against JFH-J6 (genotype 2a) the IC_{50} was approximately 5.6 µg/mL (Figure 6a). Against a genotype 1a chimeric construct the respective value was 6.6 µg/mL (Figure 6b). This dose dependent activity of EGCG was independently confirmed using the luciferase reporter construct (Figure 6c). At concentrations greater than 25 µg/ml, EGCG consistently achieved almost complete inhibition of HCV infection. Foci were absent when NS5a staining was undertaken, or luciferase activity was reduced to that observed in background non-treated wells.

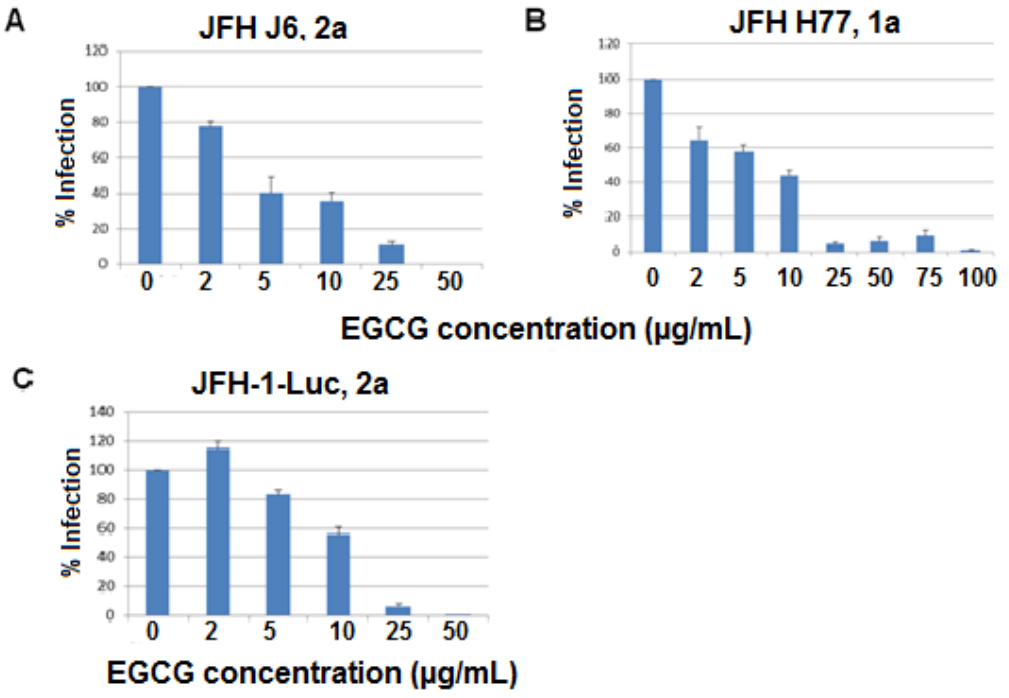

Figure 6: EGCG dose dependently inhibits JFH-1 genotype 2a(A) and 1a(B) and JFH-1-Luc(C) constructs. Mean % residual HCV infection is shown. Error bars indicate sem.

EGCG time of addition assays

To study the optimal conditions whereby EGCG exhibits maximum inhibition, time of addition assays were conducted. These experiments were conducted with a genotype 1a chimeric construct (JFH-H77, expressing genotype 1a E1/E2) using EGCG at 10 µg/mL. HCV genotype 1 is the most prevalent HCV genotype in North America, and a patient derived genotype 1a inoculum was to be employed during the SCID/uPA mouse HCV challenge experiments. Therefore the anti-HCV activity of EGCG against a genotype 1a construct was characterized in detail. As observed with silibinin, the presence of EGCG when Huh 7.5 cells were inoculated with HCV was the critical determinant of anti-HCV activity (Figure 7). EGCG 10 µg/mL added to the cells simultaneous with HCV and remaining present throughout the experiment reduced HCV infection by 56%. Additional pre-treatment of the cells with EGCG prior to inoculation did not confer any additive benefit (48% inhibition, p=0.1). Similarly pretreatment alone with removal of EGCG prior to HCV inoculation, or the addition of EGCG only following the 10 hour infection period demonstrated no significant ability to reduce HCV infection. Interestingly and in keeping with data reported by other groups the maximal inhibitory effect of EGCG was demonstrable following pre-incubation of EGCG with the viral

construct for one hour prior to addition to the cells. In such conditions HCV infection was reduced by a mean value of 75.9%. This was significantly superior to that achieved with the addition of EGCG to cells simultaneous with HCV, p=0.02. Clearly such conditions cannot be replicated *in vivo* however this finding supports the theory that the activity of EGCG is mediated in part by a direct targeting of the HCV viral particle itself.

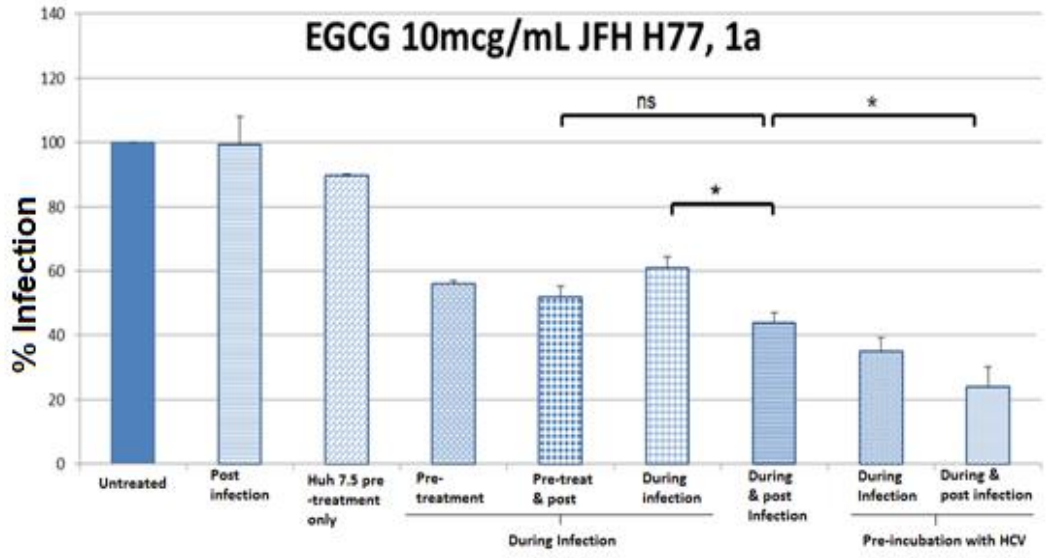

Figure 7: EGCG time of addition studies. Varying *in vitro* EGCG exposure conditions. * indicates statistical significance.

EGCG does not display significant cytotoxicity

Using the MTT cell viability assay EGCG did not demonstrate any reduction in cellular metabolic activity. Cellular metabolic activity remained stable across concentrations of 2-100 µg/mL (Figure 8). Indeed at higher concentrations the metabolic activity of Huh 7.5 cells was seen to increase

which is likely in keeping with the many effects on cellular metabolism attributable to EGCG. In keeping with the findings of other groups EGCG demonstrates low toxic potential and a wide index between IC_{50} and the concentration required to alter cellular metabolism significantly.

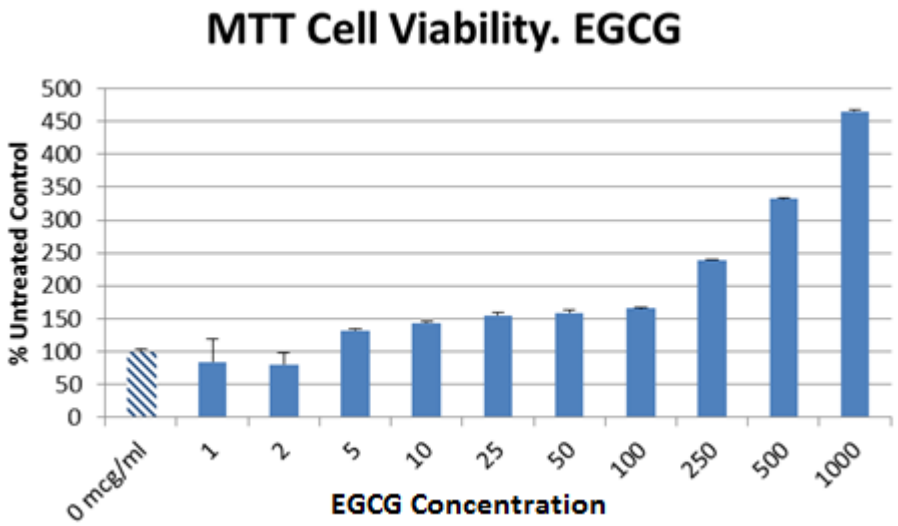

Figure 8: EGCG does not exhibit cytotoxic potential.

Anti E1/E2 monoclonal Antibodies (mAb) AR3a and AR4a

The in-vitro neutralizing capability of the anti-E1/E2 mAbs used in this study have been previously described [89]. An ability to cross neutralize different HCV genotypes has been demonstrated however the efficiency of neutralization differs considerably. AR4a mAb exhibited a more potent in-vitro cross neutralizing potential when compared with AR3a [89]. The efficacy of AR4a in an animal model of HCV infection and replication was not known. Thus we characterized the activity of AR4a in our HCVcc

system prior to proceeding to examine it's anti-HCV activity in the SCID/uPA mouse model. AR4a mAb demonstrated superior neutralizing activity against genotype 1a constructs compared to 2a constructs, with IC_{50} estimates of 1.28 µg/mL and 4.37 µg/mL respectively (Figure 9 a,b). This finding is consistent with that reported previously in the literature [84, 89].

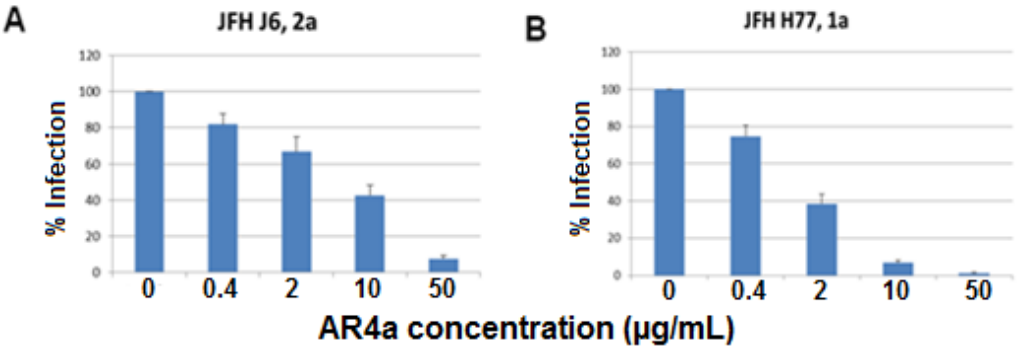

Figure 9: Anti E1/E2 mAb AR4a dose dependently inhibits infection with JFH-1 2a(A) and 1a(B) constructs.

4.2: Combination Inhibition of HCVcc

Additive reduction in HCV infection combining anti-E1/E2 mAbs and herbal extracts

Silibinin, EGCG, and anti-E1/E2 mAbs all act early in the HCV life cycle to inhibit HCV cell entry. Using the HCVcc system we investigated if these agents in combination were capable of additively inhibiting HCV infection. Using the genotype 1a construct, AR4a 2 µg/mL (pre-incubated with HCV for 1 hour) and silibinin 50 µg/mL (added simultaneous with infection)

significantly reduced HCV infectivity when compared with either agent alone (p=0.01, Figure 10a) A similar pattern of inhibition was observed against a genotype 2a construct (Figure 10b).

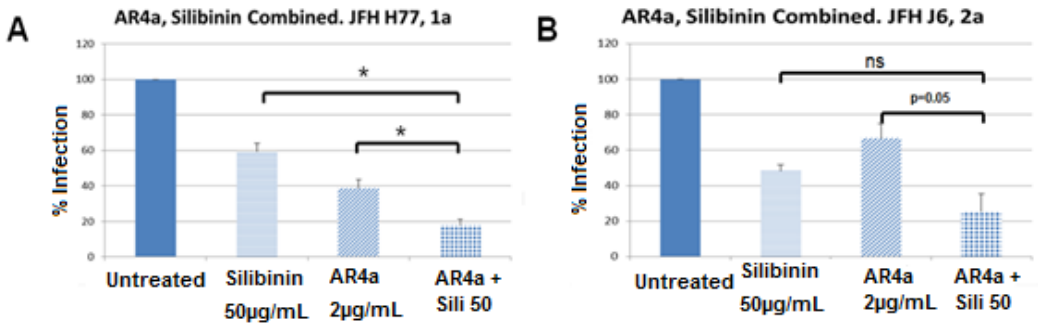

Figure 10: AR4a mAb and Silibinin additively inhibit infection with JFH-1 1a(A) and 2a(B) constructs.

Likewise EGCG at 10 µg/mL combined with AR4a 2 µg/mL demonstrated significantly increased inhibition compared to the agents alone. Using a genotype 1a construct the mean inhibition achieved by the combination was 88.7% compared to 61% for AR4a alone (p=0.01) and 56% for EGCG alone (Figure 11a). Additive efficacy was again demonstrated when using a genotype 2a construct (Figure 11b).

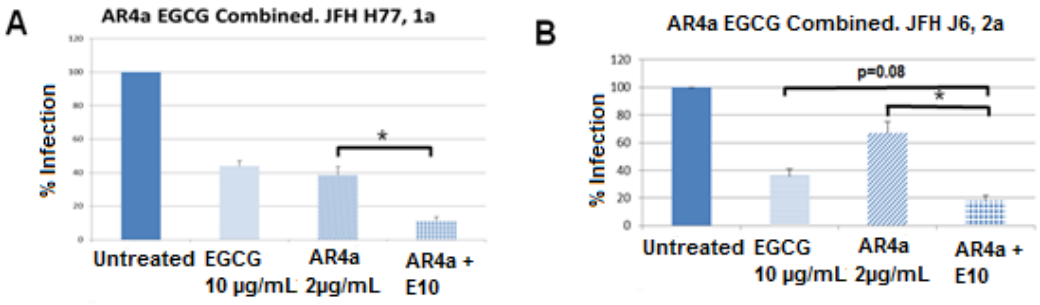

Figure 11: AR4a mAb and EGCG additively inhibit infection with JFH-1 1a(A) and 2a(B) constructs.

As silibinin demonstrated in vitro toxicity commencing at concentrations approximately twice the IC_{50}, and as the less toxic parenteral formulation (Legalon-SIL®) was not available for use in the animal model, further experiments characterizing the combination activity of these agents were only pursued with EGCG and AR4a mAb.

High dose anti E1/E2 mAb with low dose EGCG can completely inhibit HCV infection

To replicate the clinical scenario whereby therapeutic antibody products are administered at high concentrations, and knowledgeable of the limited *in vivo* bioavailability of EGCG, a combination of high dose AR4a mAb with serially titrated doses of EGCG was examined to identify the lowest EGCG concentration at which the combination could effectively completely inhibit HCV infection. Low concentrations of EGCG are eminently more achievable *in vivo* [129], and this approach provided a guide as to the likely minimum target concentrations required for EGCG efficacy when used in combination *in vivo*. We initially utilized the genotype 1a construct for these studies taking into consideration the fact that a patient derived genotype 1a inoculum would be used in the animal experiments. The mAb AR4a, at a concentration of 10 µg/mL, consistently achieved approximately 95% neutralization of genotype 1a HCV. Even at the highest dose of AR4a used (50 µg/mL), residual infection could be

detected. Despite 95% neutralization with AR4a (10 µg/mL) alone, the addition of EGCG at 10 µg/mL resulted in a significant further inhibition of HCV ($p<0.0001$ for AR4a alone vs. combination), with complete inhibition of HCV infection attained in a number of experimental repeats (Figure 12).

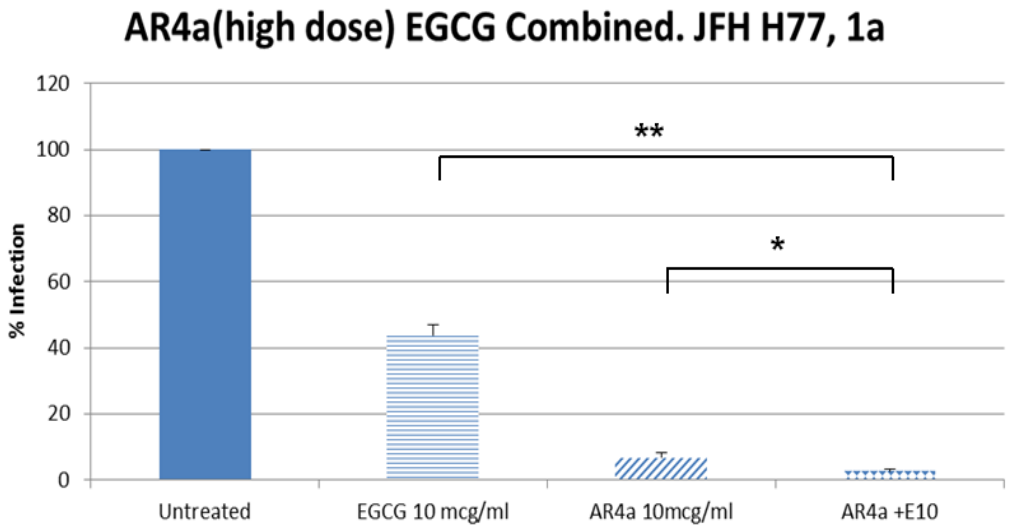

Figure 12: High dose AR4a mAb and low dose EGCG combined robustly inhibit infection with JFH-1 genotype 1a. Data from 5 independent experiments conducted in triplicate.

Efficacy for this combination was also demonstrated against a genotype 2a construct. Complete inhibition was not achieved against this genotype however the combination reduced HCV infection by 84% compared to AR4a (55%) or EGCG (60%) alone ($p<0.001$ and $p<0.0001$ respectively) (Figure 13).

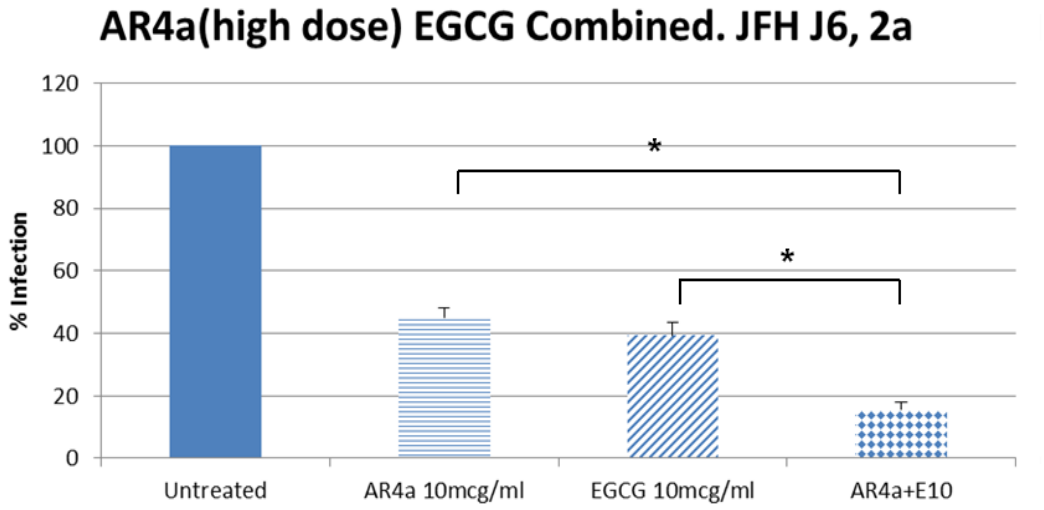

Figure 13: High dose AR4a mAb and low dose EGCG combined inhibit infection with JFH-1 genotype 2a. Data from 3 independent experiments

Combination anti-E1/E2 mAb and EGCG demonstrate cross genotypic anti-HCV activity

The activity of AR4a (10 µg/mL) and EGCG (10 µg/mL) in combination was further assessed against JFH-1 chimeric constructs expressing the structural proteins of genotypes 3-6. Clear efficacy was demonstrated against all genotype constructs tested. This high titer of AR4a strongly neutralized genotypes 4a, 5a and 6a, a finding in keeping with prior reports. Despite high neutralizing capacity the additive effect of low dose EGCG in facilitating complete inhibition of infection remained apparent (Figure 14).

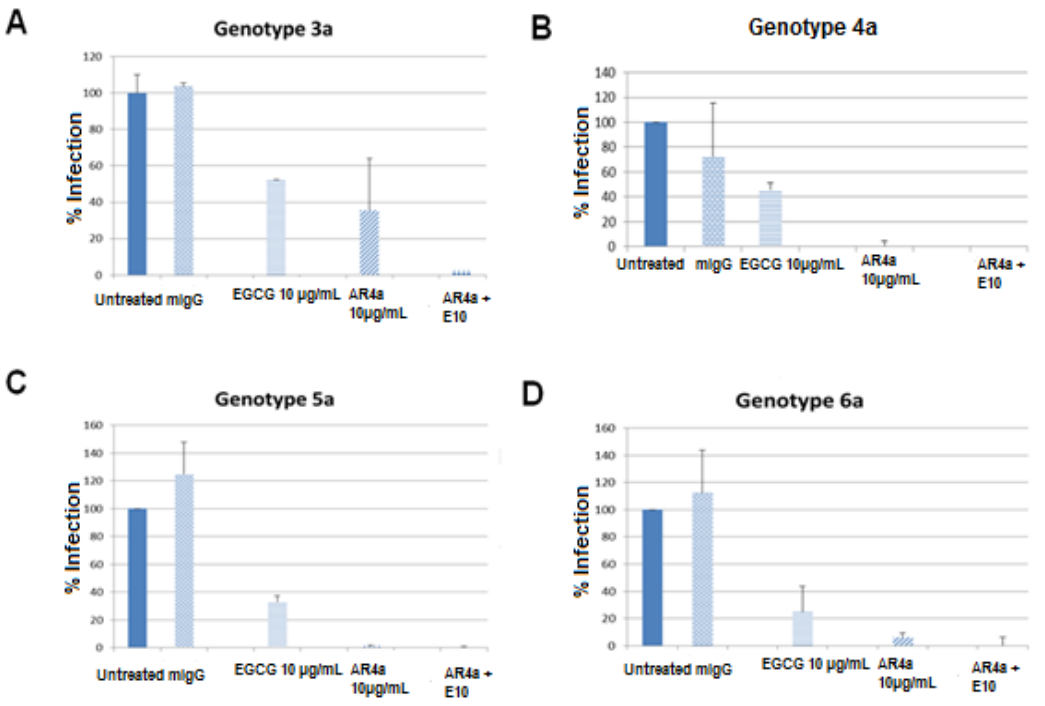

Figure 14: Cross genotypic activity of combined high dose AR4a mAb and low dose EGCG. **A**: genotype 3a, **B**:4a; **C**:5a; **D**:6a. mIgG: murine IgG.

4.3: High dose EGCG exhibits no toxicity in SCID/uPA mice

Prior to conducting the HCV challenge experiments we undertook an EGCG tolerability/ toxicity study in the SCID/uPA humanized liver mouse model. A high dose of EGCG (200mg/kg/day) was administered to mice daily by gavage for 14 days in this study. The dose employed was below that for which toxicity in mice has been previously reported. Additionally it was considerably higher than EGCG doses which have demonstrated efficacy in other animal models. The volume and frequency of administration tolerated by the animals was another limiting factor taken into consideration during the decision regarding the EGCG dosage to

proceed with. All mice who received EGCG tolerated it very well and no significant weight loss and or signs of illness were apparent.

4.4: Anti E1/E2 mAb AR4a robustly protects against HCV infection in SCID/uPA mice, EGCG alone demonstrates low efficacy.

To assess the *in vivo* anti-HCV activity of EGCG and AR4a mAb alone, and in combination, SCID/uPA mice with high levels of human liver chimerism (hAAT > 500 µg/mL) were pretreated with the investigational agents and challenged with a patient derived HCV genotype 1a inoculum. Animals were assigned to four study groups as outlined previously: Control, EGCG alone, AR4a alone, and combination AR4a/EGCG. Two separate HCV challenge experiments were conducted, five animals were assigned per group in the first experiment, three per group in the second. The inoculum for the first experiment was a patient sample with a titer of 1.5×10^7 IU/mL. In the second a mouse passaged viral specimen derived from the same patient source was used. This was mixed with human serum prior to inoculation and had a titer of 1×10^5 IU/mL. As observed in the prior tolerability study all animals treated tolerated the investigational agents very well.

Serial bleeds were conducted on all mice weekly commencing on day seven and continuing until day 42. These samples were used to determine the HCV titer and serum hAAT levels. The dynamics of serum hAAT are

shown according to intervention groups in figure 15. Serum hAAT levels remained broadly stable in most mice over the course of the experiment with levels overall gradually trending downwards.

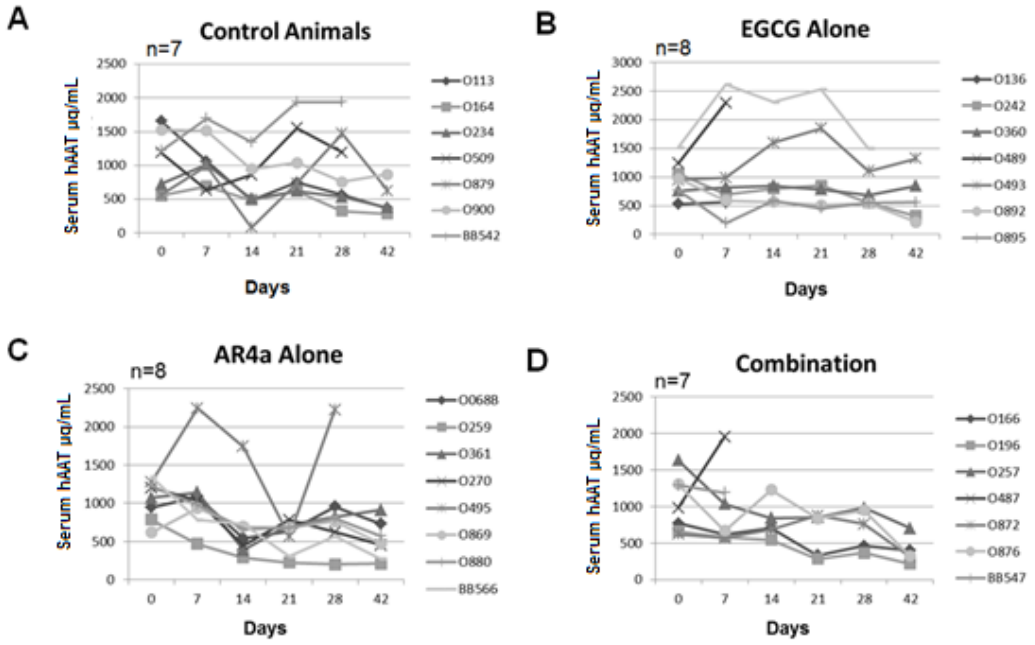

Figure 15: Serum hAAT levels according to intervention groups

Some mice did not recover following the intrajugular HCV inoculation procedure. One animal in each of the control and combination group were lost. Additionally two mice from the EGCG alone, and the combination groups became morbid after the first timepoint.

HCV viral kinetics: (Figure 16)

Control group:

At the first timepoint (Day 7) five of seven mice had HCV titers detectable above the threshold of 1000IU/mL demonstrating the establishment of HCV infection and replication within hepatocytes. The remaining two control animals reached this pre-determined threshold at day 14, and 21 respectively. Thus all control animals reached the HCV titer threshold indicating the establishment of HCV infection. However, only five of seven mice demonstrated consistent viremia above 1000IU/mL, with four maintaining high titers to the end of the study.

EGCG alone:

By day 7 in animals receiving EGCG alone four of eight had HCV RNA detectable above the threshold, two of these progressing to demonstrate sustained infection. Interestingly all animals developing detectable viral replication in this group received the patient derived HCV 1a inoculum. No animal treated with EGCG alone had HCV RNA detectable by PCR following challenge with the mouse passaged inoculum.

AR4a containing groups:

In contrast to the control and EGCG alone groups, just one of five mice in the AR4a alone group had a HCV RNA titer greater than 1000IU/mL, this

being detected at day 14. In the combination arm there was no detection of HCV RNA in any animal at any of the timepoints tested.

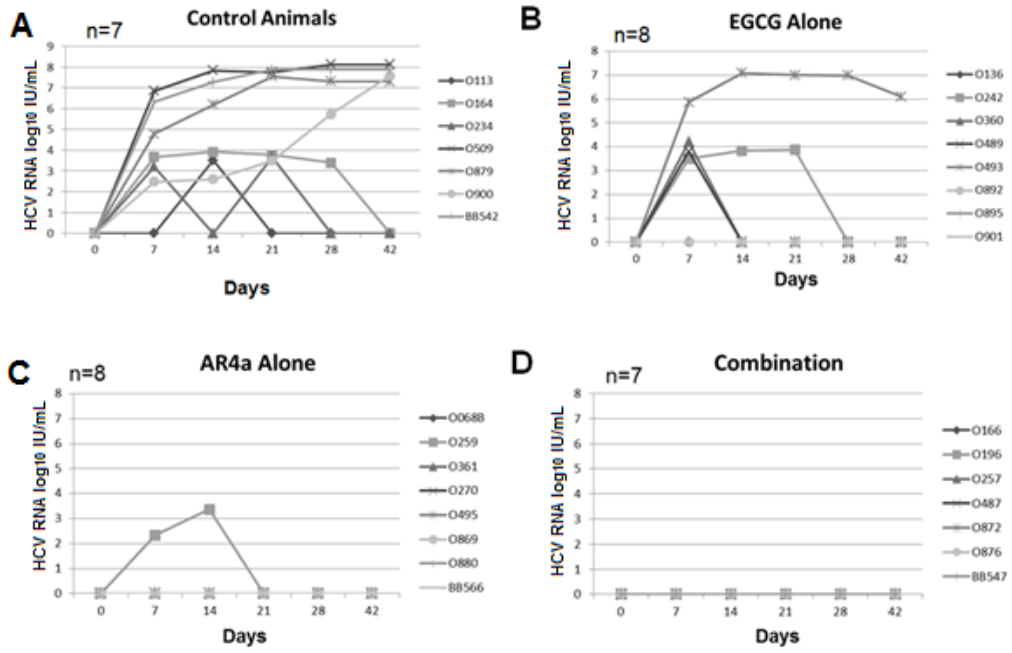

Figure 16: HCV viral kinetics according to intervention group

Kaplan-Meier Estimate: 'Survival Free from Infection'

Animals who reached the predetermined HCV RNA threshold level were categorized as 'infected'. Those who did not reach this threshold point were censored. The resulting survival curve is shown in Figure 17. The log-rank test was used to compare outcomes (Survival free from infection) between groups.

In this animal model of HCV infection, both AR4a mAb containing arms of the study demonstrated efficacy and a significant difference in HCV infection rate was detected between the control group and both the AR4a alone, and the combination therapy groups, $p<0.001$. In this study no statistically significant difference was demonstrable between the group receiving AR4a mAb alone and the combination of AR4a mAb and EGCG, $p=0.43$.

EGCG alone conferred no statistically significant benefit in terms of the prevention of the establishment of HCV infection, $p= 0.07$. The activity of EGCG was evidently dependent upon the HCV inoculum used in the challenge experiments (Figure 18).

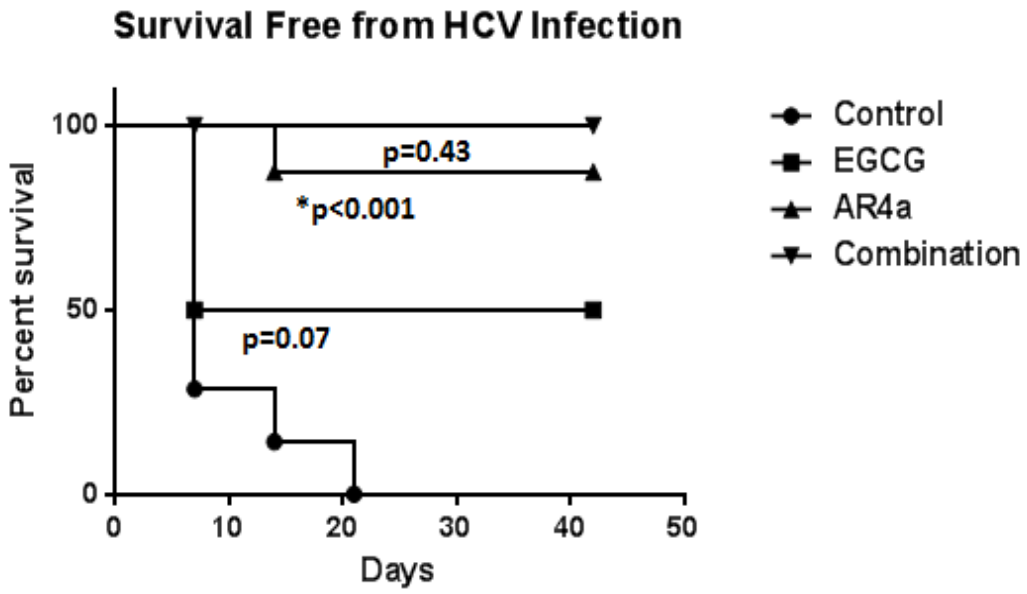

Figure 17: Kaplan-meier 'Survival Free From HCV Infection' curve

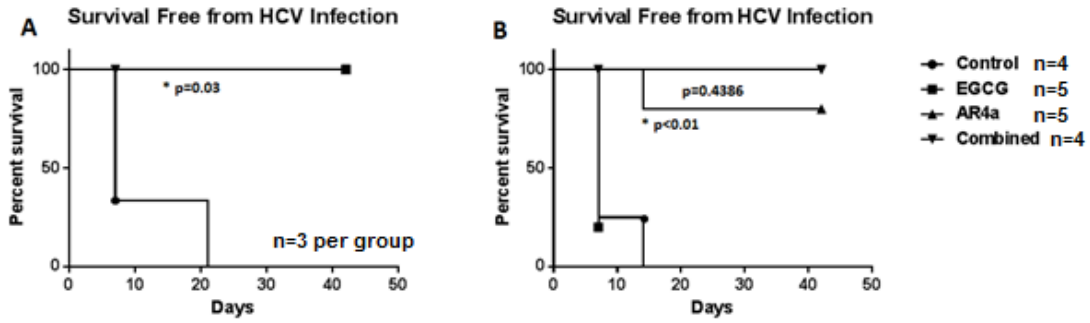

Figure 18: Kaplan-meier 'Survival Free From HCV Infection' according to HCV inoculum. A: mouse passaged inoculum, n=3 per group, total 12 animals; B: patient derived inoculum, n=4/5 per group, total animals 18.

4.5: EGCG levels in plasma and liver tissue of mice following 14 consecutive days of administration

The bioavailability of EGCG in mice is known to be limited. In order to assess if repeated doses of EGCG 200mg/kg/day were capable of achieving sufficient levels of EGCG in plasma and liver we collected samples after 14 consecutive days of dosing. For this analysis samples were obtained four hours following the final dose, providing an estimate of the *in vivo* sustainability of EGCG concentrations after administration of high doses. Plasma and liver tissue samples underwent analysis for the levels of EGCG, mono- and di-methylated EGCG, and levels of their

respective glucuronide and sulfated metabolites. A representative HPLC chromatogram from an analysis of liver tissue is provided in figure 19.

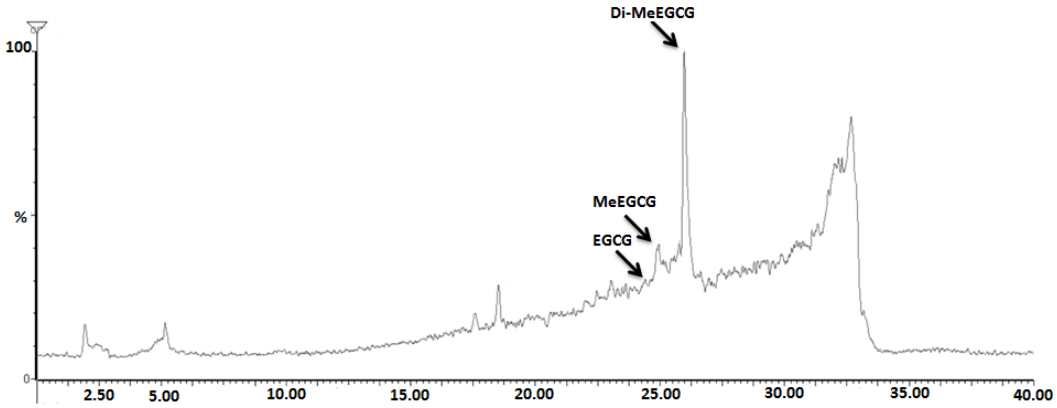

Figure 19: Chromatogram from HPLC analysis of EGCG and metabolites in a liver tissue specimen. MeEGCG: Monomethylated EGCG; Di-MeEGCG: Dimethylated EGCG.

Figure 20 illustrates the levels of detection of EGCG and it's metabolites in plasma and liver tissue. In all mice treated with EGCG, the parent compound itself or it's metabolites were detectable four hours after administration. Although detectable in treated mice, the levels in both plasma and liver tissue were low (in the nanomolar range) when measured at this time post administration. Of note EGCG itself remained detectable in liver tissue at four hours whilst it had been completely cleared from the plasma. There was no detection of EGCG or related compounds in control animals receiving sterile water by gavage. EGCG evidently undergoes brisk metabolism *in vivo* and within four hours of administration residual levels of EGCG and it's metabolites are very low.

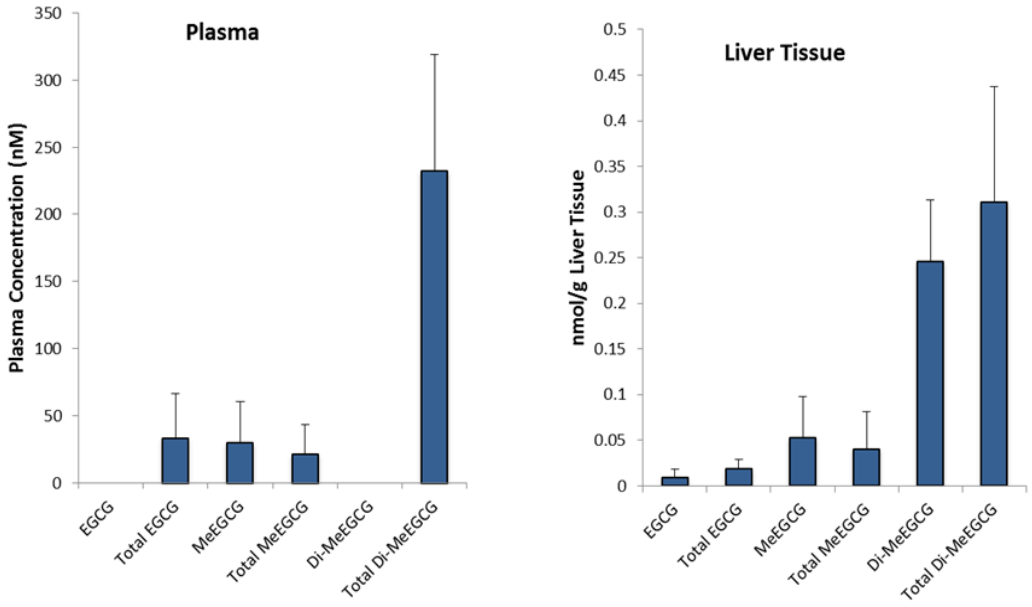

Figure 20: Detection of EGCG, Monomethylated EGCG (MeEGCG) and Dimethylated EGCG (Di-MeEGCG) in plasma and liver tissue of treated mice. The mean value of 3 treated mice are presented, bars indicate sem. Total: includes estimates of parent compound, in addition to the glucuronide and sulfated metabolites.

Chapter 5:

Discussion and Conclusion

Hepatitis C virus persists as a major cause of morbidity and mortality worldwide. This burden of disease is projected to increase even further in the years ahead [4]. The adequate control and management of HCV has presented many unique challenges to clinicians and scientists alike. Despite major advances in the relatively short time since the first identification of HCV, significant challenges remain in areas of paramount importance. Considerable resources have been invested in HCV research, and successes are now emerging in relation to HCV prevention (development of candidate vaccines [152]), and treatment, with many effective and better tolerated therapies to successfully treat chronic infection in late phases of clinical trials (direct acting antivirals, DAA) [40, 41, 84]. However, there remains a growing need to improve the clinical care pathways that strive to manage the consequences of HCV associated end-stage liver disease. The long and asymptomatic natural history of disease from acquisition through the development of symptoms means clinicians are now poised for a potentially marked rise in the number of patients who will be seeking healthcare as a consequence of severe HCV associated liver disease [30].

For this patient group at late stages of disease, liver transplantation represents a primary modality of care. HCV-associated liver disease is presently the leading indication for liver transplantation worldwide. However, the benefits of this life saving intervention are hugely undermined by the universal recurrence of HCV and the associated

accelerated disease progression. Outcomes in terms of graft and patient survival lag considerably behind those observed following liver transplantation conducted for other indications [49].

To date many groups have attempted to address this obvious area of discrepancy, whereby the leading indication for liver transplantation has considerably inferior outcomes. These attempts have primarily focused on therapeutics trying to treat HCV before or early after liver transplantation. Such approaches have proved largely unsuccessful.

Employing anti-HCV therapy in the immediate period pre transplantation has been favored by the observation that HCV RNA levels at the time of transplantation significantly influence post transplant outcomes. Almost one third of patients who become HCV RNA negative by PCR 'on treatment' pre transplant will not develop recurrent HCV infection of the new allograft [53]. SVR rates traditionally achieved by treating patients such as these who have advanced liver disease are known to be poor (approximate 20%) [33, 153], however almost half will respond 'on therapy' to become PCR negative, and in doing so increase their chances of averting re-infection. Everson et al, from a randomized controlled trial of HCV treatment to prevent recurrent HCV infection after transplantation (Adult to Adult Living Donor Liver Transplantation Cohort study) reported an overall prevention rate of 25% using a low accelerating dose regimen of Peg-IFN and Ribavirin (RBV) pre transplantation. The rate of serious adverse events ranged from 55-68% and was not different between

treated groups and those receiving placebo. This rate of adverse events clearly portrays the high complexity of this patient group awaiting liver transplant. Rates of drug discontinuation and dose reduction were also high [154]. Even the very modest success observed can't be generalized to patients with HCV awaiting transplant as prior non-responders to Peg-IFN/ RBV were excluded and only the most stable patients were eligible for inclusion. Thus this is a risk laden modality likely only a potential option in a minority of well selected patients.

Pre-emptive strategies, employing treatment in the first few weeks post liver transplant, were based upon theoretical benefits associated with an easier to treat phase of re-infection, and embarking upon treatment prior to the development of histological damage in the allograft. A number of authors have again reported globally poor outcomes with such approaches [53, 155]. Approximately half of patients will need to discontinue treatment early and the median SVR rates reported are only in the order of 16%. The application of pre-emptive therapy is limited to patients with an uncomplicated post transplant course, receiving stable immunosuppression, and with no ongoing significant co-morbidities such as cytopenias, renal impairment or infection.

Whilst treating HCV in the immediate pre and post transplant phase carry theoretical benefits, in practice the high toxicity of the available anti-HCV agents (Peg-IFN and RBV) coupled with a highly complex patient group removes these strategies from consideration in almost all patients. Thus at

present the treatment of HCV after liver transplantation is restricted to a later phase post transplantation when re-infection and histological disease has already been well established [47, 50, 156].

Clearly there is a need for safe, effective and tolerable therapies that can avail of the window of opportunity provided by liver transplantation to prevent HCV re-infection. Whilst a precedent exists with regard to the prevention of chronic Hepatitis B re-infection after transplantation, HCV provides a unique set of challenges. Genetic diversity, high replication rate, and a lack of highly effective therapies all require due consideration. To be successful one must use the vast knowledge generated on this evasive pathogen and create therapeutic combinations capable of inhibiting the life cycle at different stages. To be clinically applicable such combinations need to be safe for use in patients with complex co-morbidities.

Fundamental to this work's research hypothesis was the goal of identifying novel anti-HCV therapies which when acting in combination are potentially capable of prophylaxing against HCV re-infection after liver transplantation. Robust inhibition of HCV entry into the 'naïve' hepatocytes of the transplanted liver allograft represents the ultimate goal of any preventative strategies. Agents acting primarily to inhibit HCV cell entry were investigated; their optimal *in-vitro* activity was defined and their

anti-HCV efficacy was challenged using a small animal model of HCV infection.

Owing to a narrow *in vitro* therapeutic index between the IC_{50} and concentrations conferring cytotoxicity, and the non-availability of the less toxic water soluble formulation (Legalon-Sil®), silibinin was only included in the preliminary *in vitro* experiments. High concentrations of silibinin *in vitro* have demonstrated toxicity in other reports and conclusive data exists that oral silibinin-containing formulations whilst beneficial in long-term liver disease, do not specifically display any significant anti-HCV activity [157]. Legalon-Sil® a parenteral formulation of silibinin has demonstrated anti HCV activity in when administered intravenously to chronic HCV patients, and it has been used with some success peri liver transplantation in pilot studies conducted in Europe [117, 118]. It may remain an agent worthy of inclusion in future prophylactic strategies, but unfortunately the non-availability of this formulation precluded us from including it in our study models.

EGCG, the main active constituent of green tea extract, on the other hand is widely available and to date demonstrates a very favorable toxicity profile. The *in vitro* anti HCV activity of EGCG is clearly demonstrated in this work and corroborates that which has been reported previously. The observed dose dependent inhibition of HCV is genotype independent. Also noteworthy is that the maximal inhibitory effect of EGCG is reliant upon it being present at the time of addition of HCVcc to hepatocytes.

Interestingly pre-treatment of the cells did little to improve the inhibitory capacity, whereas pre-incubation of HCVcc with EGCG was in fact capable of achieving further inhibition. These findings indicate that the primary anti-HCV activity of EGCG is dependent upon direct interruption of the early steps involved in cell entry, and raise the possibility that EGCG could directly interact with the viral particle thus altering it's biophysical properties. Previous authors have demonstrated an interruption at the stage of primary attachment [136, 137].

These findings have important implications for the future potential clinical application. Whereas animal models of cancer and inflammatory disease have been used to demonstrate the anti-tumorigenic and anti-inflammatory activity of EGCG, in these models EGCG acts primarily on, and alters, cellular constituents and metabolism. In such models the consistent maintenance of high local concentrations may not be essential as the EGCG mediated effects are more long lasting. In keeping with this, relatively low doses of EGCG administered to animals have demonstrated efficacy. In contrast if the anti-HCV activity primarily arises from a direct interaction with the virion, or direct disruption of viral attachment, the consistent and durable maintenance of sufficient concentrations in the local environment will be a prerequisite for reliable efficacy *in vivo*.

EGCG is rapidly metabolized in animals and humans. Administration of very high doses via the oral route achieve rather modest maximum serum concentrations. In humans repeated oral administration of 800mg daily

(equivalent to 16 standard Japanese cups of green tea per day) yielded a maximum mean serum concentration of 0.4 µg/mL [127]. In mice a single dose of 2000 mg/kg yielded a maximum mean concentration of 4.17 µg/mL in serum and 18.3 µg/mL in liver tissue [145]. The elimination half-lives are also short; means of 160 and 83 minutes have been reported in human and mice studies respectively [127, 146]. Clearly in favor of EGCG however, are the advantages of tolerability and safety. Specific to a liver transplant population exploitation of the immunomodulatory properties could also prove very useful.

Despite potent *in vitro* activity it is conceivable that the anti-HCV activity of EGCG *in vivo* (experimental animal models or in the clinic) would be less profound. Indeed in this study using a daily dose of 200 mg/kg, EGCG monotherapy, whilst well tolerated, failed to significantly prevent the establishment of HCV infection in the SCID/uPA humanized liver mouse model. Interestingly the observed activity of EGCG appeared dependent upon the challenging inoculum. EGCG primarily failed to protect against a higher titer, direct patient-derived HCV inoculum.

Numerous potential reasons exist to explain this finding of impaired *in vivo* anti-HCV activity. The pharmacokinetics/ pharmacodynamics of EGCG in mice when administered by gavage are of particular interest. Despite twice daily dosing of high doses of EGCG it is clear levels of EGCG fall quickly below that expected to exhibit anti-HCV activity. The data derived from measuring the serum and hepatic tissue levels of EGCG (and it's

metabolic derivatives), conclusively demonstrate that EGCG is absorbed but very quickly metabolized and eliminated. Thus during the HCV challenge experiments there were almost certainly periods between doses where trough EGCG concentrations in plasma and liver tissue were likely considerably lower than that displaying activity *in-vitro*. With optimal efficacy reliant on a direct interruption of HCV attachment, this represents a significant hurdle to be overcome.

Reliable success of EGCG therapy to prevent HCV infection in this animal model may also be dependent upon the maintenance of consistently high concentrations at the time of inoculation and for the early period of time following challenge. Given the brisk pharmacokinetics, and the results from the *in vivo* measurement of the levels of EGCG and it's metabolites in the mice, even in the most manipulated experimental conditions this would prove a difficult objective with EGCG alone.

Unfortunately the conduct of detailed EGCG pharmacokinetic analysis in SCID/uPA mice was not feasible during this proposal. The minimum volume of serum required for the analysis of EGCG levels is 100 µL. This represents the maximum amount of blood allowable by ethics to be drawn on these mice once per week. Detailed kinetic data would therefore require the sacrifice of large numbers of mice which given the complexity and cost involved in their production was not practical. However, from the results of the analysis of levels in our group of treated mice, and knowing the kinetics of EGCG in mice as previously published, we can deduce that

initial levels post dosing peak early but subsequently decline relatively quickly to quite a low level. Administering EGCG more frequently is one means whereby the limited bioavailability of EGCG could be addressed. In this animal model however gavage more than twice daily leads to a reduction in caloric intake and a consequent decline in health status.

It is also possible that the native viral particle in cell culture systems is physically different to that which circulates in animal models. *In vivo* HCV is known to variably associate with lipoprotein elements and antibody and these associated elements may act to interfere with the effectiveness by which EGCG alters the virion or the process of cellular attachment. The inclusion of lipoprotein and antibody in viral particles circulating in human serum may also explain the differences observed in EGCG efficacy between the 'direct' patient-derived HCV inoculum and the 'mouse-passaged' inoculum. An altered virion composition following passage in the mouse model may result in a viral particle more susceptible to the action of EGCG.

Furthermore data has been published regarding the efficacy of pure EGCG compounds when compared with other green tea extracts which contain a number of green tea catechins. Using animal tumour models it was suggested that the maximum efficacy was demonstrable when EGCG was administered in a formulation containing other minority catechin constituents. Pure EGCG alone manifest a reduced effect [123, 158].

Thus, the formulation employed for *in vivo* studies, may also have an impact upon the observed effectiveness.

EGCG alone (like any anti-HCV agent used as monotherapy) fails to reliably prevent the establishment of HCV infection. The limited bioavailability of herbal extracts often hinders the translation of findings from *in vitro* to *in vivo* settings. EGCG is therefore more likely to exert a significant contribution when used in combination, where it's benefits can be availed of whilst minimizing it's limitations.

The success of HCV treatment approaches employing immunotherapy have generally been quite limited. The enormous genetic diversity of HCV species has undermined the applicability of this approach. Recently however there have been significant advances in the generation of human monoclonal antibodies capable of exerting cross neutralizing activity against HCV [84, 87, 89]. These advances have renewed hopes of not only realizing a successful HCV vaccine but also of successfully using these mAbs to prophylax against HCV re-infection following liver transplantation. Human anti-E1/E2 mAb (AR4a) targeting highly conserved epitopes exhibited neutralizing activity against all the HCVcc genotype constructs tested (genotype 1-6). SCID/uPA mice receiving AR4a mAb by intraperitoneal injection were also significantly less likely to develop established infection (as measured by HCV RNA positivity by PCR after

day seven) following HCV challenge irrespective of the inoculum used. This is the first data reporting the anti-HCV efficacy of AR4a in an animal model capable of sustaining HCV replication. AR4a certainly robustly protects against the initial establishment of infection.

As the sustainability of HCV infection in control mice was variable (4 of 7 animals progressed to high titer replication) it is more difficult to accurately comment on the durability of this protection. The half-life of human antibody (IgG) in SCID mice has been estimated to be between 6-9 days [159, 160]. In previous animal studies using anti-E2 mAbs late breakthrough was evident in some animals possibly due to the emergence of resistant mutants, or by virtue of cell to cell transmission evading neutralization [88]. In the animal experiments reported herein, the consistent lack of detectable viremia over six weeks of surveillance may be solely attributable to the mAb therapy and the inclusion of repeated administrations in this protocol. The experimental findings, whilst strongly suggestive of durable protection by AR4a mAb, are such that it is difficult to definitively confirm this assertion.

Breakthrough infection was evident in one animal receiving AR4a therapy alone. This animal experienced low level viremia transiently at day 7 and 14. Whether this occurred as a consequence of evasion of neutralization or the evolution of resistance mutations is an important consideration. To definitively address this issue cDNA will be synthesized from HCV RNA extracted at these timepoints. Nested PCR will be used to amplify the

E1/E2 region, and the product will undergo sequence analysis to detect the presence of any new mutations.

Prior clinical studies administering immunotherapy to HCV patients undergoing liver transplantation have been largely disappointing. Polyclonal immunoglobulin failed to prevent HCV re-infection [54]. Similarly an anti-E2 mAb (HCV-AbxTL68), whilst effecting some decline in titers, did not prevent HCV recurrence in patients undergoing liver transplantation [98]. Another group administered an anti-E2 mAb (MBL-HCV1) to six HCV transplant recipients. All eventually experienced re-infection with viral species harboring mutations in the target epitope [99]. Reasons purported for the disappointing clinical results with these mAbs include the fact that culture adaptive mutations in JFH-1 can render isolates more susceptible to neutralization, thus overestimating efficacy [161, 162]. In addition circulating viral subspecies and viral particles may differ from those predominating *in vitro*. Another key consideration is the suggested role for circulating host derived non neutralizing antibody competing with the administered mAbs and inhibiting their activity [100, 103]. This is clearly not a consideration *in vitro* or in the SCID/uPA mouse model of infection. Thus again the actual efficacy could be overestimated using these experimental models.

In these experiments however AR4a consistently demonstrated a robust ability to protect against a HCV challenge with a patient derived genotype

1a inoculum, and it's *in vitro* characteristics compare very favorably with those of other anti-HCV mAbs.

Antibody derived anti-viral therapeutics often possess a low to moderate genetic barrier to resistance. In the face of a dynamic viral pathogen such as HCV they are evidently highly susceptible to the selection and emergence of resistant mutants. Optimal therapeutic strategies employing mAbs thus need to protect these agents with combination therapy. Identifying combinations which can be used safely in patients undergoing liver transplantation has proven challenging to date however new agents have been identified/ developed that may considerably alter this widely held belief.

Combination therapy is well accepted as the fundamental requirement for effective treatment of chronic HCV infection. Combinations of agents with different mechanisms of action target multiple points of the HCV life cycle, and in doing so efficiently inhibit the virus and protect against the emergence of resistant quasispecies. This issue is now to the fore of HCV clinical care pathways as numerous direct acting antivirals are in late stages of development and require careful incorporation into new durable combination regimens. Combining agents can also enhance the efficacy at respective doses and on occasion yield synergistic anti-viral activity.

In-vitro high dose AR4a (10 µg/mL) displayed high neutralization against all HCVcc genotype constructs tested; genotypes 1, and 4-6 being

comparatively more susceptible to neutralization than genotypes 2/3. However, even the highest dose of AR4a failed to completely inhibit *in vitro* infection with 1a and 2a chimeras. Despite the high levels of neutralization observed with AR4a alone, the addition of EGCG at concentrations more conceivably attainable *in vivo* (10 µg/mL) resulted in a significant further inhibition of HCV infection. This additive effect was observed across genotypes and was capable of completely blocking infection against all genotype constructs tested aside from 2a, where the combination achieved 84% inhibition. Thus AR4a mAb in high concentration combined with a lower dose of EGCG successfully achieved our goal *in vitro*.

Given the limited bioavailability of EGCG these experiments identified the minimum concentration of EGCG capable of achieving complete inhibition in combination with high dose mAb. Both agents act to inhibit HCV entry and it is conceivable that in the presence of high circulating mAb titers, EGCG can exert it's additive effect at lower concentrations, and that fluctuations in EGCG levels may not be as critical an influence on HCV outcome as they are with EGCG monotherapy. EGCG by virtue of it's mechanism of action can also help combat the evasion of mAb neutralization mediated by direct cell to cell transmission of HCV. In the SCID/uPA mice treated with both AR4a and EGCG no animal developed detectable HCV RNA by PCR at any point in the six week period of surveillance following HCV inoculation. Applying a Kaplan-meier curve and

log-rank test, ‘survival free from HCV infection’ was significantly higher in the combination group compared to both control animals, and the animals receiving EGCG alone ($p<0.001$; $p=0.03$ respectively). There was no statistical difference in infection prevention between the combination therapy group and the group administered AR4a alone. This could simply reflect the impressive activity of high dose AR4a, but may also reflect an impaired power to detect a difference owing to the limitation in numbers of animals available to study. Aside from protection against the initial establishment of infection, combination therapy would also be expected to outperform mAb monotherapy in the prevention of late viral breakthrough. Again owing to limited numbers of animals and the somewhat variable extent of sustained HCV replication in control animals, it is more difficult to definitively conclude this.

The SCID/uPA humanized liver mouse model is an extremely useful tool for conducting *in vivo* HCV studies. Whilst production of the mice is technically complex, the availability of a small animal model capable of supporting HCV replication provides a highly practical means of analyzing the anti-HCV activity of novel compounds. The sustainability of HCV replication is highly dependent upon high levels of chimerism in the animals. Durable HCV replication requires mice with extensive and sustained human hepatocyte engraftment. In these experiments only mice expressing high hAAT levels (>500 µg/mL) were used and these levels remained broadly stable for the duration of follow up. However, although

all animals in the control group developed detectable HCV RNA above the threshold consistent with infection, only four progressed to sustained replication. Thus while we can definitively answer the questions addressing the ability to prevent initial HCV infection, conclusions relating to durability of protection are more limited. The SCID/uPA mouse also lacks an adaptive immune response. This can be exploited to facilitate the pure assessment of the ability of administered human mAbs to neutralize HCV *in vivo*. However caution is required when translating findings to the clinical situation, especially when applying to a cohort undergoing liver transplantation. Pre-existing host antibody can act to inhibit anti-HCV mAbs, and this interaction may contribute to the disappointing findings from clinical trials using such an approach to date.

In this study the investigational agents were only challenged with a patient derived genotype 1a inoculum. Genotype 1 is the predominant HCV genotype in Europe and North America, however this does limit somewhat the immediate generalizability to the population of individuals with HCV of diverse genotypes awaiting liver transplant. Clear *in vitro* cross genotype activity was however demonstrated, and the patient inoculum provides a heterologous HCV species challenge. In this context AR4a and the AR4a/EGCG in combination demonstrated impressive preventative capacity.

Despite these limitations this research undertaking has yielded a number of important and novel findings. EGCG, a potent *in vitro* inhibitor of HCV, demonstrates a lack of definitive efficacy to protect SCID/uPA mice against HCV infection when used alone. Undoubtedly a primary reason for this deficiency is the limited *in vivo* bioavailability coupled with it's proposed mechanism of action which appears reliant on consistent high EGCG concentrations. The anti-E1/E2 mAb AR4a on the other hand demonstrates a powerful ability *in vitro* and *in vivo* to cross neutralize diverse HCV genotypes. This is the first demonstration of the activity of AR4a in an animal model capable of sustaining active HCV replication. The activity of AR4a provides considerable hope for the future successful application of HCV immunotherapy.

This body of work also demonstrates the proof of concept that HCV infection can be efficiently prevented using combination therapy *in vitro* and *in vivo* and that this could be availed of to address the major deficiency in the use of liver transplantation for HCV associated liver disease. Combination therapy can avail of agents with favorable safety profiles but that alone are expected to demonstrate inferior efficacy. This is particularly important when applying these findings to clinical cohorts of patients with HCV awaiting liver transplantation. Intolerability to the currently available anti-HCV agents precludes their use to prevent HCV re-infection in almost all patients. Moreover the wide availability of natural

compounds means many more individuals worldwide with HCV associated liver disease could avail of their benefits.

Combination therapy for chronic HCV is a prerequisite. Clearance of HCV confers marked benefits to patients both pre and post transplant [47, 163, 164]. With the potential for considerable increases in the demand for a resource as valuable as liver transplants, there is a clear need for novel approaches to avert recurrent HCV disease. It is likely that combined approaches and combined therapies will be required to overcome the significant challenges presented by both the virus, and the often severely compromised patients awaiting transplantation. Safe and tolerable agents are needed which can be administered pre, during and post transplantation.

The results from these preclinical experiments identify that anti E1/E2 monoclonal antibodies represent a real therapeutic advance and may form the backbone of any future prophylactic strategy. Future clinical studies are warranted to conduct a detailed evaluation of AR4a kinetics, efficacy and safety in HCV patient cohorts. Pilot phase 1 dose finding studies could be undertaken in patients with chronic HCV infection to establish kinetics and safety. Such a study could also assess for any ability of AR4a to reduce HCV viral loads in this setting. The half-life of human immunoglobulin when administered to humans approximates 21 days. Thus single administrations of a range of doses to patients with chronic HCV infection could provide preliminary characteristics upon which to

base further pilot studies in patients with HCV undergoing liver transplantation.

This data could then be used to design a pilot study in HCV liver transplant candidates. Outcomes could be compared with patients who receive standard perioperative care. A peri-transplant schedule of AR4a infusions could follow that employed for hepatitis B immunoglobulin with an initial infusion during the anhepatic phase followed by daily infusions for one week, three weekly infusions to week 4, and two further infusions at week 8 and 12 post transplant if HCV RNA remains negative. An important consideration for the design of any such study would be the measurement of AR4a mAb levels at regular timepoints and to correlate these with the post-transplant HCV kinetics.

It is likely however, that to be fully effective the mAb will require additional agents to protect against virologic breakthrough and resistant mutants. Pre-transplant therapy can be employed to reduce the burden of viral infection, a known prognostic indicator of post transplant HCV outcomes. Intravenous silibinin pre-transplant in pilot European studies has been used successfully and safely in this regard but the unpredictability of graft availability is a limiting consideration [117, 118]. Pre-transplant silibinin may optimize the chances of success with immunotherapy when employing mAb therapy later peri- and post -transplant.

Pairing the mAb with novel agents will provide additive efficacy and further protect against virological escape. A role for EGCG whilst safe and tolerated may be hindered by the limited bioavailability. The development of more potent and bioavailable derivatives of EGCG may yet yield an incredibly powerful agent to block HCV cell entry [165]. In addition the emerging DAAs if tolerated in this patient group could be successfully paired with mAbs essentially replicating the successful regimen employed to prevent hepatitis B re-infection after liver transplant [51]. Clinical studies using combination therapies will follow on from the preliminary studies characterizing AR4a efficacy in HCV liver transplant recipients.

Of most intrigue is a possible approach which can take advantage of the very nature of liver transplantation. Availing of the opportunity afforded by the procedure to treat the allograft *ex-vivo* represents a truly unique therapeutic approach. Priming the liver allograft with anti-viral agents *ex-vivo* could prove a powerful means to definitively reduce the risk of HCV re-infection. Safe and non-toxic herbal extracts such as silibinin and EGCG can be availed of either to bath the graft in, or to infuse directly into the graft prior to implantation. Indeed the many anti-oxidant, and anti-inflammatory effects of these extracts may well provide ancillary benefits. High local concentrations of these cell entry inhibitors could therefore be readily achieved. A further therapeutic advance which has only recently been reported could also be incorporated into potential prophylactic

strategies. Janssen et al, presenting data from a phase 2 clinical trial, report for the first time the anti-HCV activity of a novel agent targeting human microRNA. Miravirsen can sequester microRNA-122 (miR-122) which is expressed abundantly in hepatocytes and is known to bind HCV at well conserved sites and protect it from recognition and degradation by the host. It demonstrates cross genotype activity, and had no significant attributable adverse effects [166]. This agent also exhibits a prolonged duration of action. Thus direct administration to the liver *ex-vivo*, overcoming the traditional microRNA delivery challenges, could theoretically paralyse the early precipitous uptake and replication of HCV virions. Miraversen could therefore provide an effective and durable background level of protection for a number of weeks when used in combination post transplantation.

Clearly successful approaches capable of robustly preventing HCV infection post liver transplantation will require innovative approaches drawing upon the many advances in modern medicine whilst also seeking to exploit the benefits of more traditional herbal remedies. Addressing worldwide health disparities and ensuring access to therapies for those who require them is a considerable and growing challenge for the HCV community. Significant numbers of individuals with HCV reside in regions with under-resourced healthcare services. In these regions incorporating natural herbal remedies with both anti-HCV and hepatoprotectant

properties may prove particularly important pending the more widespread availability of new agents. Novel proposals such as the *ex-vivo* treatment of the liver allograft obviously need to be carefully studied in terms of safety and efficacy; the viability of such a valuable resource cannot be compromised. Furthermore the durability of prophylactic strategies in highly immunosuppressed individuals will also require careful evaluation.

Significant advances are now being made in tackling key HCV issues such as vaccination and improved therapeutics. This has provided renewed hope for ultimately achieving the upper hand over this highly dynamic pathogen. Therapeutic advances have also renewed interest in conceiving fresh approaches to tackle the major weakness of liver transplantation for HCV related liver disease; HCV re-infection. This research undertaking demonstrates that protection against HCV infection is eminently achievable and that anti-HCV agents exist that are safe and tolerable. AR4a robustly protects against HCV infection and EGCG can act to enhance this protection whilst also safeguarding against virological escape and the emergence of resistant mutants. Future clinical studies of anti-E1/E2 mAbs incorporating the new DAAs stand to deliver effective, durable and tolerable HCV prophylactic combinations. There is now considerable optimism that routine prevention of HCV re-infection after liver transplantation is in fact attainable. In achieving this goal, outcomes for patients with HCV undergoing liver transplant will be brought back in

line with that of their non-HCV counterparts. We have thus moved one step closer to realizing an effective strategy to addressing the glaring deficiency which exists in the management of HCV related liver disease.

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Appendix A:

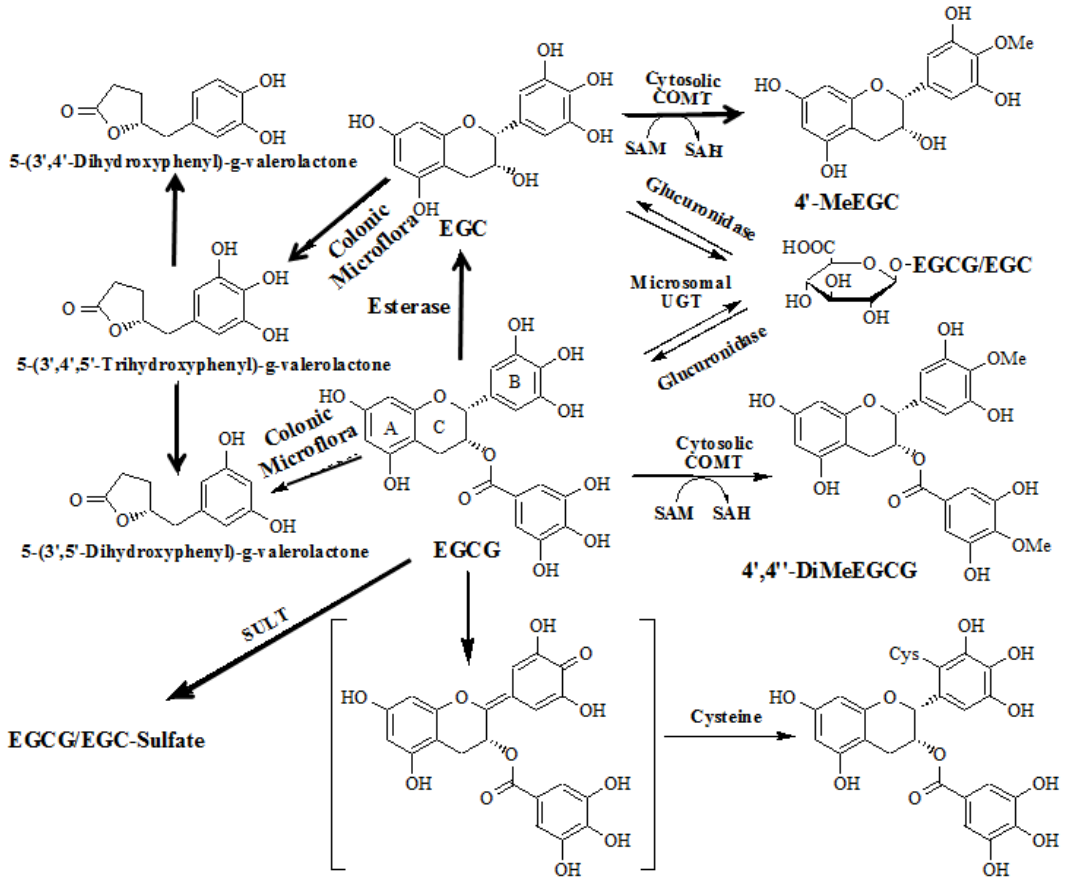

Figure 21: Biotransformative Pathways of Tea Polyphenols. EGCG: epigallocatechin gallate; EGC: epigallocatechin; DiMeEGCG: di-methyl-EGCG; SULT: sulfotransferase; COMT: catechol-O-methyl transferase; UGT: Uridine 5'-diphospho-glucuronosyltransferase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine. Courtesy Dr J Lambert, Pennsylvania State University.