OPINION

Virus–drug interactions—molecular insight into immunosuppression and HCV

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Abstract | Liver transplantation is an effective treatment for end-stage liver disease that is attributable to chronic HCV infection. However, long-term outcomes are compromised by universal virological recurrence in the graft. Reinfection that occurs after transplantation has increased resistance to current interferon-based antiviral therapy and often leads to accelerated development of cirrhosis. Important risk factors for severe HCV recurrence are linked to immunosuppression. Owing to the lack of good randomized, controlled trials, the optimal choice of immunosuppressants is still debated. By contrast, much progress has been made in the understanding of HCV biology and the antiviral action of interferons. These new insights have greatly expanded our knowledge of the molecular interplay between HCV and immunosuppressive drugs. In this article, we explore the effect of different immunosuppressants on the complex cellular events involved in HCV infection and interferon signalling. Potential implications for clinical practice and future drug development are discussed.

Pan, Q. et al. Nat. Rev. Gastroenterol. Hepatol. 9, 355–362 (2012); published online 17 April 2012; doi:10.1038/nrgastro.2012.67

Introduction | End-stage liver diseases that are caused by chronic HCV infection are currently the leading indications for liver transplantation. However, HCV reinfection of the graft occurs universally and often results in accelerated recurrence of liver fibrosis and early development of cirrhosis. HCV infection is also the most frequent cause of liver disease after kidney transplantation and is an independent risk factor for graft loss. In general, the current standard therapy for patients with HCV (PEG-IFN-α in combination with ribavirin) is much less effective in patients who have had a liver transplant than in those who have not had a transplant. An aggravated course of infection and increased resistance to antiviral therapy have been attributed to several host and viral factors, in particular the application of specific immunosuppressive medication. Over the past five decades, the immunosuppressive regimen has evolved from being mainly based on glucocorticosteroids, azathioprine and ciclosporin A to steroid-sparing regimens involving anti-IL-2 receptor antibody induction therapy, calcineurin inhibition by tacrolimus and the use of new-generation immunosuppressants—mycophenolate mofetil and rapamycin. The changes in drug types and drug combinations together with increases in the age of donors have probably contributed to the reported worsening of HCV recurrence since the early 1990s. Although enormous efforts have been devoted to clinical research, the choice of immunosuppressants in HCV-positive recipients is still a subject of debate.

The lack of good model systems hampered HCV research in its early stages. However, this deficiency has improved dramatically in the past decade with the development of various in vitro systems of cell culture (Box 1). The HCV pseudoparticles, the HCV replicons and the JFH-1-derived infectious HCV cell culture systems have all demonstrated their importance in studies on different aspects of the viral life cycle and pathophysiology, including viral entry, trafficking in the cell and assembly and release of de novo virus particles. These models have enabled basic research to make considerable contributions to an improved understanding of how immunosuppressive compounds can actually influence HCV infection and the antiviral action of interferons. In this article, we explore the potential molecular interaction of different immunosuppressants with HCV and interferon signalling. We also discuss how to extrapolate these laboratory findings into clinical practice and drug development.

Calcineurin inhibitors | The primary cellular targets of the calcineurin inhibitors are immunophilins. Cyclosporin A binds to cyclophilins, and tacrolimus (also known as FK506) binds to FK binding proteins (FKBPs); both events result in a profound inhibition of the phosphatase activity of calcineurin. Immunophilin-dependent signal transduction via calcineurin represents a key event in the activation of T-cell proliferation by regulating expression of the gene that encodes IL-2 (Figure 1a). Apart from their role in calcineurin signalling, immunophilins are catalysts of protein folding and as such have a supportive role in viral infection. Viruses, including HIV, herpes simplex virus, vaccinia virus, vesicular stomatitis virus and coronaviruses, take advantage of immunophilins for their replication, and this virus–host interaction is inhibited by cyclosporin A. Moreover, cyclophilin A can actually incorporate itself into these viral particles. For HCV, it is well established that cyclophilins have an essential role in viral replication and de novo virus production. Early studies suggest that HCV replication is dependent on the interaction between cyclophilin B and nonstructural protein 5B (NS5B, HCV RNA polymerase) to stimulate its RNA binding activity and thereby promote the de novo synthesis of positive and negative stranded RNA. Cyclosporin A blocks the binding of cyclophilin B to NS5B RNA and thereby inhibits its viral replication. Cyclophilin A also directly interacts with NS5B and is
Box 1 | HCV culture models

- The first full-length HCV clones could infect chimpanzees but replication in vitro remained ineffective. By creating antibiotic-resistant HCV genomes, selection of replication-competent viral clones became possible. This advance led to the development of subgenomic or bicistronic (two cistrons) replicons, which mimic HCV replication. HCV replicons are most efficient in a human hepatoma cell line (Huh7), and can be linked to fluorescent or luminescent reporter genes.

- Early studies of HCV viral entry used pseudoparticles incorporating HCV envelope proteins E1 and E2 onto a retrovirus or lentivirus.

- The highly effective HCV infectious model was based on the first genotype 2a clone called JFH-1, isolated from a Japanese patient with fulminant hepatitis C. JFH-1-derived HCV models can be used to study the complete viral life cycle and chimeric genomes have been constructed of all seven known genotypes.

involved in modulating the polyprotein cleavage activity of NS2. In addition, cyclophilin A interacts with NS5A and stimulates its binding with HCV RNA.

As ciclosporin A interacts with both cyclophilin A and cyclophilin B, it is conceivable that it affects multiple steps in the virus life cycle (Figure 1b). The anti-HCV activity of ciclosporin A is not mediated by an interferon response and is independent of calcineurin signalling. Although studies showed a role for FKBP8 in HCV replication through interaction with NS5A, FKBP8 lacks several amino acid residues thought to be important for tacrolimus binding. No evidence exists that tacrolimus inhibits HCV replication.

Several in vitro studies have shown that ciclosporin A can act in synergy with IFN-α, but clinical evidence to support these findings is still limited. The results of a prospective, randomized, controlled pilot study published in 2010 demonstrated that a switch from tacrolimus to ciclosporin A resulted in a modest drop in serum levels of HCV RNA and an enhancement of the response to antiviral interferon therapy in patients who had received a liver transplant. This finding supports those of a previous study that observed a benefit of converting from tacrolimus to ciclosporin A during pre-emptive IFN-α and ribavirin antiviral therapy in patients who were HCV positive and had undergone liver transplantation.

The mechanism by which ciclosporin A can promote the response to interferon is an intriguing issue. Hirano et al. suggested that tacrolimus, but not ciclosporin A, interferes with interferon signal transduction. However, a later study did not confirm this suggestion, instead demonstrating that two separate studies of the role of ciclosporin A in HCV replication by promoting NS5B association with the viral replication complex, the role of other glucocorticoid receptors is largely unknown. A study using the HCV subgenomic replicon model found that both prednisolone and dexamethasone have no stimulatory effects on RNA levels, but rather have minor inhibitory effects. Using an infectious HCV model (the JFH1-derived chimeric Jc1 in Huh-7.5 cells), similar results were obtained showing an ~50% inhibition of viral replication at high doses of prednisolone. However, in this infectious model, prednisolone stimulated HCV infection by enhancing virus entry. This specific enhancement was mechanistically linked to the upregulation of the two essential HCV entry factors—occludin and scavenger receptor class B type I—at both mRNA and protein levels. On the basis of their observations, the authors speculated that treatment with a steroid bolus (>250 mg prednisolone per day) in patients with HCV might foster virus dissemination through facilitation of virus entry into hepatocytes, thus aggravating HCV recurrence. Moreover, treatment with prednisolone probably suppresses the patient’s immune control of the HCV-infected cells, by effects on T cells and plasmacytoid dendritic cells, and thereby might indirectly accentuate infection. Plasmacytoid dendritic cells are trials and the first results are encouraging. In a cohort infected with HCV and HIV, 2 weeks of treatment with Debio-025 decreased HCV viral load by more than 3-log, including in patients infected with HCV genotypes 1, 3 and 4. By contrast, the anti-HIV effects were much less pronounced (1-log reduction of viral load). In treatment-naive patients infected with just HCV, treatment with Debio-025 alone resulted in a more than 2-log reduction of viral load in those with genotypes 1 and 4, and a more than 4-log reduction in those with genotypes 2 and 3. When combined with PEG-IFN-α and ribavirin, additive antiviral effects were demonstrated.

Glucocorticosteroids

Glucocorticosteroids have been used since the early years of organ transplantation. Prednisolone and its close analogue dexamethasone are potent suppressors of the immune system, as they modulate cellular and inflammatory responses via stimulation or inhibition of gene transcription. Although evidence indicates that the oestrogen receptor is functionally involved in HCV replication by promoting NS5B association with the viral replication complex, the role of other glucocorticoid receptors is largely unknown. A study using the HCV subgenomic replicon model found that both prednisolone and dexamethasone have no stimulatory effects on RNA levels, but rather have minor inhibitory effects. Using an infectious HCV model (the JFH1-derived chimeric Jc1 in Huh-7.5 cells), similar results were obtained showing ~50% inhibition of viral replication at high doses of prednisolone. However, in this infectious model, prednisolone stimulated HCV infection by enhancing virus entry. This specific enhancement was mechanistically linked to the upregulation of the two essential HCV entry factors—occludin and scavenger receptor class B type I—at both mRNA and protein levels. On the basis of their observations, the authors speculated that treatment with a steroid bolus (>250 mg prednisolone per day) in patients with HCV might foster virus dissemination through facilitation of virus entry into hepatocytes, thus aggravating HCV recurrence. Moreover, treatment with prednisolone probably suppresses the patient’s immune control of the HCV-infected cells, by effects on T cells and plasmacytoid dendritic cells, and thereby might indirectly accentuate infection. Plasmacytoid dendritic cells are
of particular interest in HCV infection, as they produce large amounts of IFN-α; however, the capability of plasmacytoid dendritic cells to produce IFN-α seems to be reduced in patients with chronic HCV.43 Experimental data from our group have shown that treatment with prednisolone abolishes the anti-HCV activity of plasmacytoid dendritic cells in vitro (P. E. de Ruiter, personal communication).

In liver transplantation, glucocorticosteroids are often given as an induction protocol during surgery and low doses in combination with other immunsuppressants are used as maintenance immune suppression after transplantation. In cases of acute cellular rejection episodes, patients receive several boluses of methylprednisolone to reverse the rejection. The most compelling evidence that steroids affect HCV viral load actually came from studies in patients with HCV who did not undergo a liver transplant.44–46 In a transplant setting, the general consensus is that steroid avoidance47 or slow tapering of the dose48 is associated with reduced disease recurrence, whereas boluses for treating acute rejection can increase the viral load.49,50 However, a randomized multi-centre study comparing steroid-free therapy and standard immunosuppression showed no clear advantage of not using steroids in liver transplant recipients who had HCV.51
Two systematic Cochrane reviews with meta-analyses of randomized, controlled trials have been performed in the settings of chronic HCV and HBV infection to comprehensively assess the effects of glucocorticosteroids on interferon antiviral therapy. No statistically significant effect of steroids on the virological response at the end of treatment was found in patients with HCV. By contrast, pretreatment with steroids was associated with a considerably higher frequency of loss of hepatitis B e antigen and HBV DNA, although no statistically significant effect on clinical outcomes was found. However, mechanistic studies have shown that glucocorticosteroids can interfere with signal transduction of the interferon receptor. The glucocorticosteroid receptor and type I interferon receptor signalling pathways share the cofactor glucocorticoid receptor-interacting protein 1 (GRIP1). Glucocorticosteroids suppress interferon responses by antagonizing the heterotrimeric STAT1–STAT2–IRF9 (ISGF3) activity via GRIP1 or attenuation of STAT1 activation through induction of SOCS1, which is a suppressor of cytokine signalling 1 (Figure 1). However, these events probably differ between cell types and no direct link between these molecular interactions and viral infection has been well established. Therefore, it is of particular relevancy to further investigate the actual effects of steroids on interferon antiviral response in HCV models.

**MMF and MPA**

Mycofenolic acid (MPA) is the activated form of the prodrug mycophenolate mofetil (MMF). MMF and MPA are part of the class of antimetabolite immunosuppressive agents that also includes azathioprine. The effect of azathioprine on HCV has not been extensively studied, despite one early study suggesting that this agent has antiviral effects on two *Flaviviridae* viruses. MMF is commonly used in kidney transplantation, but it is also approved by the FDA for the prophylaxis of allograft rejection after heart or liver transplantation. In addition to its potent immunosuppressive capacity, MPA also has a broad spectrum of antiviral activity in vitro against numerous DNA and RNA viruses, including dengue virus, West Nile virus, yellow fever virus, Chikungunya virus, HBV and HCV. MPA is an uncompetitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), in particular the isoform IMPDH2. Inhibition of IMPDH decreases intracellular levels of guanosine nucleotide pools resulting in inadequate quantities for nominal DNA duplication, which was thought to be the antiproliferative and immunosuppressive mechanism of MPA (Figure 2a). This mechanism is probably also responsible for its antiviral effects against at least the West Nile, yellow fever and Chikungunya viruses, as supplementation by exogenous guanosine almost completely overcomes these inhibitory effects. By contrast, supplementation of guanosine had little effect on the inhibition of HCV replication. Consistently, ectopic expression of IMPDH2 mutants lacking the binding site for MPA largely restored its antiproliferative effect, but with only minor effects on HCV replication, suggesting other mechanisms are involved in its anti-HCV action. Unexpectedly, MPA was found to induce the expression of important antiviral interferon-stimulated genes in HCV cell culture models, including interferon regulatory factor (IRF) 1, IRF9 and interferon-induced transmembrane protein 3 (IFITM3). Using an RNAi-based loss-of-function approach, IRF1 was demonstrated to be directly involved in the anti-HCV activity of MPA.

The expression of a panel of interferon-stimulated genes was also considerably higher in peripheral blood mononuclear cells of patients who had received a kidney transplant and were maintained on MMF immunosuppression, compared with peripheral blood mononuclear cells of healthy control individuals. Similar results have been reported for ribavirin, which also induces expression of a broad spectrum of interferon-stimulated genes. Interestingly, like MPA, ribavirin is a known inhibitor of IMPDH. Inhibiting IMPDH and inducing the expression of interferon-stimulated genes probably simultaneously contribute to the anti-HCV action of MPA (Figure 2b). Whether IMPDH by itself is involved in the induction of the expression of interferon-stimulated genes is not fully clear. However, supplementation with exogenous guanosine did not interfere with inducing the expression of these genes.

Although the safety and efficacy of MMF as an immunosuppressive medication in patients with HCV who received a transplant has been demonstrated, the exact effects on HCV recurrence have not been clearly studied in prospective, randomized and double-blinded trials. Results from prospective nonrandomized or retrospective studies remain controversial at this point of time. However, the *in vivo* efficacy of many antiviral compounds (such as ribavirin) can be observed only when combined with other antivirals. Ribavirin has shown substantial anti-HCV activity *in vitro* but is generally considered to have little or no detectable antiviral activity as a monotherapy in patients. However, when combined with IFN-α, ribavirin increases the sustained virologic response by threefold. Similarly, a previous clinical study showed that combined treatment with MMF and PEG-IFN-α in patients with chronic HCV resulted in a considerably improved end-of-therapy response rate in a difficult-to-treat population of previous nonresponders to standard therapy. The sustained virologic response of the IFN-α and MMF combination group, however, was lower than that of the group treated with IFN-α and ribavirin. A possible explanation for these limited sustained virologic responses is that MMF interferes with a robust antiviral immune response required to eliminate HCV. Notably, MPA works in synergy with IFN-α on HCV replication *in vitro*. When combined with IFN-α, MPA augments the transcription of multiple interferon-stimulated genes that are mediated by interferon-stimulated response elements. These latest findings have shed some light on the molecular basis of how MPA works in synergy with IFN-α.

**Rapamycin**

Rapamycin, a new-generation immunosuppressant, has been gaining increasing favour in the transplantation context, mainly attributable to its low nephrotoxicity and potential anticancer properties. Rapamycin engages the cytosolic protein FKBP12 to form a complex. This complex inhibits the mammalian target of rapamycin (mTOR) pathway by directly binding to the mTOR complex 1, resulting in blockage of cell cycle progression at the G1 to S phase and thereby causing inhibition of T-cell proliferation (Figure 2).

One particular interesting feature of rapamycin is that it induces autophagy by inhibition of mTOR. Autophagy is a process for catabolising organelles and other cytoplasmic components to balance cellular metabolism and to promote cell survival during stressful conditions. Autophagy is also an important event in regulation of the cellular response against viral infections. The role of autophagy is complex and can have both antiviral and proviral effects in different virus infections. During infection,
autophagy can be a surveillance mechanism that delivers viral antigens to endosomal compartments to stimulate innate immune signalling and to provide processed antigens for presentation in the major histocompatibility complex. Virus infection can trigger the induction of autophagy, but can also block the function of the autophagy pathway or even subvert autophagy. By contrast, viruses can directly exploit the autophagy machinery to facilitate replication or to promote their maturation and the dissemination of virions.\textsuperscript{75}

Several studies have shown that HCV infection induces autophagy in hepatocytes via the unfolded protein response.\textsuperscript{76,77} However, the induced autophagy appears incomplete, as the maturation of autophagosomes to autolysosomes is blocked. Therefore, autophagosomes are not degraded but instead support viral replication.\textsuperscript{77} HCV viral proteins, such as NS5B, directly interact with the host proteins that are required for the induction of autophagy.\textsuperscript{78} In addition, induction of autophagy by HCV seems to facilitate infection by reducing the innate immunity of host cells, including interferon responses\textsuperscript{79,80} Indeed, accumulating evidence exists for a direct link between the mTOR signalling pathway and interferon-activated JAK-STAT signalling.\textsuperscript{81–83} Type I interferon induces phosphorylation and activation of mTOR in a PI3-kinase-dependent manner.\textsuperscript{83} In addition, mTOR complex 1 was found to stimulate the production of type I interferons through activation of IRF5 and IRF7.\textsuperscript{82} On the basis of these mechanistic insights, it is conceivable that rapamycin could affect HCV recurrence and antiviral interferon therapy, though clinical evidence to support this theory is still limited.\textsuperscript{84,85}
Novel immunosuppressants

Despite the success of current immunosuppressive agents in reducing the incidence of acute cellular rejection, the toxicity associated with these regimens has now become a major obstacle for positive long-term outcomes of liver transplantation. Currently, novel agents, including small molecules, are at various stages of clinical development.66 These novel small molecules include the new generation ciclosporin A analogue, ISA247, the protein kinase C (PKC) inhibitor, AEB071, and the selective Janus kinase inhibitor, CP-690550.

Given the evidence for an important role of cyclophilins in the life cycle of HCV,12,15,16 investigation of the effects of ISA247 on HCV infection would be of particular interest. ISA247 only differs from ciclosporin A by a single amino acid residue. The structural change induced by this substitution is considered to result in a greater calcineurin inhibitory potency and a more predictable pharmacokinetic profile than ciclosporin A. An X-ray crystal structure study has revealed the basis for the cyclophilin A binding affinity and immunosuppressive potency of ISA247.67 As cyclophilin A is a host factor that modulates infection of a number of viruses, it will be important to study the potential antiviral activity of ISA247 compared with ciclosporin A.

AEB071 is a small-molecule inhibitor that targets multiple members of the PKC family. PKC inhibition results in decreased T-cell activation. Several members of the PKC family have been linked to both HCV infection and interferon signalling.68,69 Using cell culture models, however, no stimulatory effect of AEB071 on HCV infection was observed; instead, at high drug concentrations a reduction in viral replication was found.70 This reduction might be related to inhibition of cell proliferation by AEB071. Therefore, it was concluded in this study that AEB071 had no direct effect on HCV infection.70 Further investigation is required to draw firm conclusions.

CP-690550 was developed as a selective inhibitor of JAK3, which is predominantly expressed in immune cells and is only bound by y-chain-bearing cytokine receptors involved in the JAK–STAT signalling pathway.91 Some of the immunosuppressive mechanism of CP-690550 can be explained as the result of IL-2 inhibition, as JAK3 functions in the receptor signalling pathway for this cytokine.92 However, CP-690550 has also been shown to inhibit other members of the JAK family, including JAK1, JAK2 and TYK2.93 These pathways have considerable implications in both defence of virus infection and interferon responses. In addition, expression and activation of JAK3, which is involved in defence against dengue virus infection, have been reported in hepatic cells.94 Therefore, studying the effects and mechanisms of CP-690550 on both HCV infection and interferon signalling would be highly relevant.

Conclusions

The development of subgenomic HCV cell culture models has greatly contributed to the discovery of the antiviral effects of ciclosporin A and MPA on viral replication. The use of a recently developed infectious culture model has revealed the negative effects of steroids, specifically on HCV entry.95 However, a common question from transplant clinicians is why these clear in vitro effects of HCV are not univocally observed in clinical practice. For instance, the clear antiviral activity of ciclosporin A and MPA in vitro is yet to be confirmed in proper prospective clinical studies, and retrospective data sketch a murky picture. One probable answer to this in vivo and in vitro discrepancy is that tempering of the host immune system against the virus masks the direct antiviral effects of these immunosuppressive agents. The antiviral potency of particular immunosuppressants might not be strong enough to result in a dramatic effect on viral load, but could potentially be reflected in other parameters, such as slowing down fibrosis progression.96–98 Perhaps the potent effects of the derivates of ciclosporin A, such as Debio-025, in patients were achieved by both deprivation of immunosuppressive properties and enhancement of cyclophilin binding affinity.94,95

A major new milestone for HCV treatment is the approval of two protease inhibitors by the FDA in 2011. Although boceprevir and telaprevir are only approved in combination with PEG-IFN-α and ribavirin for treating adults with chronic HCV genotype 1 infection who have compensated liver disease, trials are expected to be designed to treat patients both before and after transplantation. As a caution, a phase I study found that telaprevir interferes with the metabolism of both ciclosporin A and tacrolimus by inhibition of cytochrome P450 3A enzymatic activity.99 Telaprevir caused a tremendous increase in blood concentrations of both these calcineurin inhibitors and could potentially lead to serious or even life-threatening adverse effects. Therefore, the interaction of new antiviral compounds and immunosuppressants requires extensive studies of drug–drug interactions before designing any antiviral therapy trial in the post-transplantation setting.

In summary, progress in understanding HCV biology is important in evaluating the complex effects of immunosuppressive medication on HCV recurrence. Despite clear effects of ciclosporin A and MPA in HCV culture models, reliable clinical evidence for their effect on HCV recurrence is still lacking. Inhibition of the immune response against HCV by immunosuppressants probably counteracts the direct antiviral effects. The effectiveness of interferon-based antiviral therapy is reduced after transplantation, but, to date, no concrete evidence from HCV culture models that immunosuppressive drugs interfere with interferon signal transduction exists. Immunosuppressive medication for transplant recipients who have HCV should be further improved. The application of newly registered anti-HCV compounds in transplant recipients should be carefully tested because of potential concerns regarding adverse drug–drug interactions with immunosuppressants.

Although basic science alone will never resolve the clinical debate, it already provides mechanistic insight into how particular immunosuppressants can affect the course of infection and outcomes of antiviral therapy. The knowledge gained in a liver-transplant setting will also definitely be a valuable reference for the management of patients who are HCV positive and receive a kidney transplant. Conceivably, these mechanistic understandings will promote future investment in the initiation of randomized, controlled studies and development of new-generation immunosuppressants for HCV-positive transplantation.

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Acknowledgements

The authors would like to thank Petra de Ruiter for critically reading the manuscript and sharing unpublished data. The authors received financial support from the Erasmus MC Translational Research Fund and the Liver Research Foundation Rotterdam (SLO).

Author contributions

Q. Pan and L. J. van der Laan contributed equally to discussion of the content and review/editing of the manuscript before submission.