Medical Hypotheses 102 (2017) 51–55

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy

Hepatitis C virus may have an entero-hepatic cycle which could be blocked with ezetimibe

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ARTICLE INFO

Article history:
Received 14 February 2017
Accepted 8 March 2017

ABSTRACT

Hepatitis C virus can lead to chronic infection, cirrhosis and hepatocellular carcinoma. With more than 170 million people infected worldwide, eradication remains a challenge even with the revolutionary current direct antiviral agents (DAAs). The risk of resistance, the safety profile in some populations, the genotype specificity and the high price of current DAAs explain why there is still interest in developing host targeting agents (HTA) that may help overcome some of these difficulties. Specifically, targeting the entry of HCV to the cell seems like a promising strategy. Recently it has been shown that the cholesterol transporter NPC1L1, a protein located in the small bowel epithelium and in the canalicular membrane of the hepatocyte is also an HCV receptor. Just as this protein is key in the entero-hepatic cycle of cholesterol, we hypothesize that there is an entero-hepatic cycle of HCV that could be disrupted by blocking NPC1L1 with ezetimibe, an already approved and readily available safe drug. Ezetimibe, either alone or in combination with DAAs, could decrease relapse rates, reduce resistance and even make treatments cheaper.

Introduction

Chronic hepatitis C affects more than 170 million individuals who are at risk of developing cirrhosis and hepatocellular carcinoma [1]. The impact of this disease is evident as mortality due to hepatitis C virus (HCV) infection superseded mortality from HIV infection in the US [2]. Since the earlies 1990s, the mainstay of therapy was interferon, but unfortunately sustained virological response (SVR) was achieved in only 55% of patients who were eligible for interferon-based treatments [3,4]. Moreover, interferon needs subcutaneous injections for administration and is associated with innumerable side effects. Because of these reasons, better strategies and new approaches for treatment of hepatitis C infection were urgently need and over the last 15 years a much clear understanding of the viral structure and replication cycle allowed for developing direct acting antivirals (DAAs), leading to a new era of improved results with high SVR higher than 90% in most patient populations with shorter duration of therapy and an improved security profile.

Nevertheless, DAAs have shortcomings. The first wave of DAAs – boceprevir and telaprevir – had significant adverse effects, a high pill burden and substantial risk for developing resistance [5]. Even the current generation of DAAs, with improved security and simplification of therapeutic schemes has problems high price, security in special populations (decompensated patients) and in some cases resistance. These limitations have led to a renewed interest in antiviral strategies aimed at the host [6]. Host targeting agents (HTA) have the advantage of not inducing resistance. Moreover, there is evidence that combination of DAAs with HTA could lead to a synergistic effect [7], decreasing resistance, increasing the security profile or decreasing the price of the combination. One of the most interesting host targeting strategies aim at blocking HCV entry to the cell [8]. In this article, we focus on the role of NPC1L1 (Niemann-Pick C1-like 1), the cholesterol transporter and a newly discovered HCV entry factor as a potential target for blocking HCV entry to the cell and the possible role of ezetimibe in interrupting a putative entero-hepatic cycle of the virus.

Hypothesis

Given the canalicular and intestinal localization of NPC1L1, we hypothesize that this transporter (in parallel to the cholesterol cycle) may be either preventing the biliary escape of the virus at the canalicular level or hampering its fecal loss by reabsorption at the intestinal level, thus having an enterohepatic cycle. This cycle could be blocked by using ezetimibe (Fig. 1).

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http://dx.doi.org/10.1016/j.mehy.2017.03.016
0306-9877/

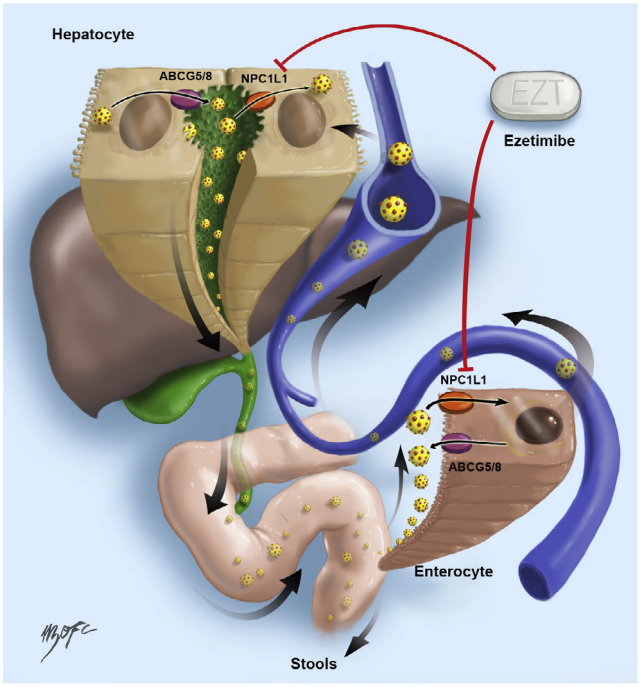

Fig. 1. Proposed model for NPC1L1 function as an HCV entry receptor and the possible effects of ezetimibe. An increase in either biliary or fecal HCV excretion would support the existence of an enterohepatic cycle of the virus. ABCG5/8 has not (yet) been involved in HCV transport. (Figure by Dr. Nicolás Triantafilo).

Evaluation of the hypothesis

Targeting host factors for hepatitis C treatment

Given the aforementioned shortcomings of DAAs and the greater understanding of virus-host interactions, there has been a growing interest in developing antiviral strategies that target host factors required for viral replication. The advantages of targeting these host factors include: 1) Broad spectrum of activity, as these are pangenotypic. 2) High barrier of resistance relative to compounds targeting viral functions. 3) Increasing the drug armamentarium against HCV with diverse mechanisms of action. 4) Probably faster drug development. The main problem of targeting these host factors is the risk for mechanism-related toxicity.

Among potential host factors involved in the HCV viral cycle and available for antiviral treatment are cyclophylline, miRNA-122 and HCV receptors. Cyclophyllines belong to a family of cellular proteins with different functions which include protein processing, mitochondrial function and immune response. The HCV replication complex is formed by viral proteins (NS5A and NS5B) together with host cyclophylline A and B, which modulate trafficking of viral proteins. Cyclosporine has been shown to reduce HCV replication *in vitro* and *in vivo* by means of its inhibitory activity on cyclophyllines [9,10]. Alisporivir (Debio-025) is a synthetic derivative of cyclosporine with no immunosuppressive activity which has shown good antiviral activity in phase 1 and 2 clinical trials combined with peginterferon and ribavirin [11]. This drug has pangenotypic activity. This compound has been generally well tolerated, with mild conjugated hyperbilirubinemia as the main side effect, but recently, six cases of acute pancreatitis (one fatal) led to stopping the development program of alisporivir [12].

It has been shown that micro RNA 122 (miRNA-122), a liver specific small cellular non-coding RNA, binds directly to two sites in the 5′ untranslated region (UTR) of HCV RNA and stimulates viral replication. It is not yet clear how miR-122 regulates HCV replication, but sequestration of miR-122 by a complementary oligonucleotide results in long-lasting suppression of HCV viremia in primates [13]. Specific miRNA-122 inhibitors are promising therapeutic targets, but still in early phases of development [14]. Other cellular factors have been described as important for HCV replication, like phosphatidylinositol 4-kinase (PI4KA) [15].

HCV entry to the cell. Targeting HCV receptors as an antiviral strategy

HCV circulates in human serum associated with various lipoproteins, including HDL, LDL and VLDL, forming lipo-viral particles (LVP) [16–18]. LVPs formation take place during lipoprotein synthesis in hepatocytes. These lipoproteins shield the virus from neutralizing antibodies [19]. Entry of HCV to the cell is dependent on interaction of viral envelope glycoproteins E1 and E2 with the host cell. The entry of HCV to the host cell is a complex multiple steps process that can be mediated by several cellular receptors and entry factors. HCV access the liver through the sinusoidal blood requires DC-SIGN (dendritic cell-specific intercellular adhesion molecule three grabbing non integrin) and L-SIGN (DC-SIGNr, liver and lymph node specific), which are expressed in Küpfer cells, sinusoidal endothelial cells and hepatocytes [20,21]. In the liver cell, heparan sulphate glycosaminoglycans (GAGs) mediates low affinity interaction with the cell membrane, allowing further interaction with other entry factors [22,23]. Tetraspanin CD81 seems to be critical for viral entry [24,25] by interaction with specific residues of E2 protein [26]. The LDL receptor (LDL-R) acts as an HCV co-receptor [27]. The receptor of HDL [28], scavenger receptor class B type I (SR-BI, also known as CLA-1 and now officially designated SCARB1) was described as an HCV entry factor 10 years ago, interacting with E2 at the hypervariable region 1 (HVR1) and indirectly by lipoprotein binding [29]. SR-BI is expressed in the liver and steroidogenic glands and is involved in bidirectional transport of cholesterol. This receptor seems to act together with CD81 in viral entry [30]. Claudin-1 (CLDN1) [31] and occludin (OCLDN) [32] have been also implicated as HCV entry factors. Interestingly, these two membrane proteins are located at the tight junction of hepatocytes, and not in the basolateral membrane as the previously described entry factors, and could act at a later postbinding step of viral entry. The virion undergoes clathrin-mediated endocytosis and a pH-dependent membrane fusion occurs to release its genomic RNA [33]. More recently, using siRNA screening technology, it has been shown that epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2), which are highly expressed in human liver, are required for HCV entry, promoting CD81-CLDN1 association and membrane fusion [34]. Early this year (2012), the role of NPC1L1 in viral entry was described.

Among the previously mentioned entry factors, we will briefly discuss SR-BI and EGFR blocking as potential therapies for HCV infection. ITX-5061 is a small molecule antagonist of SR-BI, inhibiting uptake of HDL-cholesteryl ester that prevents HCV infection *in vitro* at a post-binding step, with no *in vivo* data available so far [35,36]. Unfortunately, blocking SR-BI may have potential side effects, including altered adrenal glucocorticoid and platelet function, as shown in humans carrying the P297S SR-BI mutation [37] and further implied by experiments knocking-down the gene showing a phenotype of thrombocytopenia, impaired platelet aggregation and increased susceptibility to arterial thrombosis in mice [38]. Erlotinib is an approved tyrosine kinase EGFR inhibitor used for several types of cancer [39] which has been shown to delay HCV infection in uPA-SCID chimeric mice [34], but with limited efficacy, very common and cumbersome adverse effects (diarrhea, fatigue, severe rash) and at a very high cost.

NPC1L1, a newly discovered HCV entry factor

Niemann-Pick C1-like 1 (NPC1L1) cholesterol uptake receptor is a recently identified HCV entry factor [40]. NPC1L1 is a cholesterol-sensing receptor expressed in enterocytes in humans and mice and in the liver in humans, but not in mice. Interestingly, in humans the presence of NPC1L1 is 15 to 30-fold higher in duodenum than in liver [41]. NPC1L1 is expressed in the canalicular (apical) membrane of the hepatocyte, not in the basolateral membrane [42]. Infection of Huh7 cell lines with cell cultured HCV (HCVcc) down-regulates NPC1L1 expression [43]. A similar effect is observed for other HCV entry factors as HCV down regulates these molecules as a specific viral strategy that prevent superinfection [43]. Sainz et al. (2012) show that NPC1L1 silencing does not affect HCV replication and secretion and that the large extracellular loop 1 (LEL1) of the protein is the required domain for HCV interaction. This is the same domain that binds cholesterol [44]. They also demonstrate that NPC1L1 functions late in the viral entry process, probably after binding, but before virion-host cell fusion. NPC1L1 mediated entry seems to be dependent on the concentration of viral-associated cholesterol. Even with the description of this novel entry factor, the HCV uptake is still incompletely understood, particularly, the role of NPC1L1 during viral entry given its canalicular localization has not been clarified. It could therefore be hypothesized that liver cell to cell transmission of HCV occurs at the canalicular level. Moreover, NPC1L1 is highly expressed in the intestinal enterocytes. The biological meaning of NPC1L1 distribution in terms of the HCV cycle has not been addressed.

Role of NPC1L1 in cholesterol homeostasis and cholesterol enterohepatic cycle

Cholesterol is an essential component for maintaining structural integrity, permeability and fluidity of cell membranes [45]. It also serves as a signaling molecule and as a precursor of bile salts and steroids [46]. Synthesis of bile acids from cholesterol has emerged as an evolutionary advantage, as bile acids are critical for absorption of dietary fat and cholesterol itself [47]. Synthesis of cholesterol requires significant energy expenditure, so a number of mechanisms have evolved to allow the absorption of dietary (300–500 mg/d) and biliary (800–1200 mg/d) cholesterol. Excess cholesterol is excreted in the feces from biliary cholesterol and probably through direct intestinal secretion [48]. Cholesterol homeostasis is maintained, thus, by intestinal absorption, *de novo* synthesis and biliary/fecal excretion. NPC1L plays a key role in dietary cholesterol absorption and biliary cholesterol reabsorption.

NPC1L1 is expressed in brush border of enterocytes in the duodenum, jejunum and ileum, where is serves as the primary cholesterol transporter for absorption. Unsterified cholesterol is incorporated in mixed micelles containing bile acids and phospholipids [49]. In humans and primates, NPC1L1 is also expressed in the canalicular membrane of the hepatocyte. Here, NPC1L1 functions reabsorbing biliary cholesterol. Another cholesterol transporter, ABCG5/G8, localizes in similar distribution, serving as the primary cholesterol secretion transporter. NPC1L1 cholesterol uptake involves several lipid rafts proteins, like flotilin-1 and 2, inducing cholesterol-rich membrane microdomains which are subsequently internalized *via* clathrin-coated vesicles [50]. Noteworthy is the close parallel of this process to the current model of HCV entry described before. The strategic localization of the cholesterol transporters allow an enterohepatic cholesterol cycle with high efficiency (50% or more) of intestinal cholesterol reabsorption.

Ezetimibe as a potential anti-HCV treatment

One of the most interesting projections associated to the discovery of NPC1L1 as an HCV entry factor is the availability of an antagonist to this molecule current in clinical use ezetimibe. We now understand that the mechanism of action of ezetimibe is by disrupting the enterohepatic cycle of endogenous cholesterol [47]. Ezetimibe binds to the extracellular loop C, in a different domain than cholesterol and HCV virion, blocking the internalization of the NPC1L1/cholesterol complex [51]. Ezetimibe is rapidly absorbed and metabolized (glucuronidation), recirculating in the enterohepatic circuit, having a half-life of 16–31 h. Steady state concentration in plasma is reached after 10 days [52].

There are several characteristics that make ezetimibe a very attractive candidate for HCV treatment. It is a very safe drug [53,54], and can be used even in liver transplant patients [55]. Ezetimibe has no effect on the activity of CYP450 drug metabolizing enzymes. In the paper describing NPC1L1 as an HCV entry factor, authors show *in vitro* dose-dependent inhibition of HCVcc infection [40]. They also show that ezetimibe delays the establishment of HCV infection in uPA-SCID mice transplanted with human hepatocytes, a model of humanized liver mice [56]. We are aware that the *in vitro* dose was ~100 higher than the maximal plasma concentration achieved in patients [57], but the pharmacodynamic/pharma cokinetic properties of ezetimibe (luminal effect and enterohepatic circulation), do not allow to predict the *in vivo* effect. Moreover, if our hypothesis proves true, ezetimibe could demonstrate an antiviral effect even if not absorbed.

An additional reinforcing argument is our description of an HCV infected patient that, despite failing to three treatments with interferon and ribavirin, achieved sustained virological response after ezetimibe treatment. This is the first published evidence of an effect of ezetimibe in HCV infection in humans [58].

It is interesting to note that intestinal cholesterol absorption efficiency is variable (from 29 to 80%) [59], which is due to genetic variations in NPC1L1 [60]. Moreover, sensitivity to ezetimibe has been associated with several polymorphisms of NPC1L1, with both, super-responders and non-responders [61,62]. In this project, we propose to study different NPC1L1 polymorphisms associated with ezetimibe response and correlate them with the virological response to the drug.

The notion that HCV may be absorbed in the intestine might sound naïve and senseless for a virus that has a parenteral route of transmission, as opposed to hepatitis A and E viruses. In fact, there is no clinical or epidemiological evidence for an oral transmission of HCV, but it is interesting to note that HCV is detectable in bile, although it has not been quantified [63–65]. In addition, HCV RNA has not only been detected in stools from chronically infected individuals [65], but also it has been quantified in high amounts, up to 2.8×10^5 copies/mL feces [66]. Remarkably, the "stool viral load" did not correlate with plasma RNA titers. This evidence even though not definitive would give partial credence and support the here proposed hypothesis. Moreover, there is ample evidence that there is an increased risk of HCV transmission in men who have sex with men [67] and we cannot exclude the possibility that HCV in stools has a role in this method of spread.

The setting of HCV infection and liver transplantation

HCV infection is the leading reason for liver transplantation in Western countries [68]. With lower response to antiviral treatment in cirrhotics, liver transplantations is still the only life-saving procedure for decompensated cirrhosis and hepatocellular carcinoma due to HCV infection. Unfortunately, reinfection of the graft is universal after transplantation [69], occurring early at reperfusion [70]. Detailed analysis of HCV viral kinetics after transplantation

has been described [71], showing that HCV viral load decreased during the anhepatic phase, but in the hours following implantation of the new graft, the decline in HCV viral load was faster, indicating massive infection of the new graft. After 12 h, HCV RNA level normally increases, to reach pre-transplant levels around day 4 and reaching in most patients a new plateau, 1–2 logs higher than pre-transplantation level, 4 weeks after transplant. These data indicate that the new graft is permissive to HCV entry and very effectively infected. Strategies aiming at avoiding or delaying graft reinfection are not currently available, but are eagerly awaited.

We have described the effect of ezetimibe in patients undergoing liver transplantation [72]. In this pilot study, we could show in two patients that HCV viral load decreased after liver transplant in patients undergoing liver transplantation, but returned to baseline level after a week. Even though this experience is difficult to interpret with just two patients, it shows that probably blocking just one HCV entry factor is not enough to completely prevent hepatocyte re-infection.

Projections

If HCV has an enterohepatic cycle, even if the relevance of this cycle on overall HCV infection is low, this could further our understanding of the mechanisms by which the virus establishes a persistent infection, resulting in a paradigm shift in the field. Additionally, strategies aimed at disrupting this enterohepatic cycle could lead to the identification of new targets for HCV treatment, either alone or in combination with other antiviral agents. Using ezetimibe seems a particularly interesting alternative, given that it is an already approved compound and it is generally well-tolerated. We don't expect a very potent effect of ezetimibe in patients with an already established infection, but even a mild effect in the steady state of the infection in this group may allow to use ezetimibe as an addition to current therapies, probably decreasing relapse rates. As for patients undergoing liver transplant, the effect could be much more pronounced, even preventing reinfection in some patients with an already approved and very safe drug. Our own preliminary experience in the setting of liver transplantation has proved that ezetimibe is safe, but of limited value in preventing infection after transplantation. Different treatment strategies and combinations with DAAs could be tried nevertheless.

Funding

This study was funded by grant FONDECYT #1130357 from the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT – Chile) to AS.

Conflict of interest statement

A.S. has received fees as a speaker for MSD, Roche, BMS, he has participated in Advisory Board Meetings for MSD, Abbvie, Gilead, Vertex, Roche, and Janssen and he has stock ownership of Gilead and Achillion. The others authors have nothing to disclose.

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