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Ribavirin ante portas: Uptake transporters into hepatocytes dissected

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See Article, pages 486–492

Ribavirin is a key antiviral component in the treatment of hepatitis C virus (HCV) infection. Although it is only minimally effective as a monotherapeutic agent [2,7], in combination with Interferon it increases the sustained virological response rate (SVR) upon antiviral treatment compared to Interferon alone from 13% to 40% [11]. Thus, the combination therapy with Ribavirin and Interferon is currently recommended for the treatment of chronic HCV infection. Of note, high doses of Ribavirin and the presence of Ribavirin-associated side effects (e.g., anemia) are positive predictors of SVR, underlining the pivotal role of Ribavirin in the success of the combination therapy [10,12]. New antiviral therapies specifically targeted to HCV will further improve treatment success rates but a combination with Ribavirin will most likely continue to be required to reduce viral relapse and increase SVR [6,9].

Despite the clear effectivity of Ribavirin in the treatment of chronic HCV infection, the underlying mechanisms of action are still unclear. Ribavirin is a purine nucleoside analog that is structurally related to Guanosine [3]. It is converted to Ribavirinmonophosphat (RMP) and Ribavirintriphosphat intracellularly (RTP). Four potential mechanisms of Ribavirin's anti-HCV activity have been suggested [3,4] (Fig. 1): first, direct inhibition of HCV RNA replication due to the erroneous incorporation of RTP into replicating RNA strands by viral polymerases. Second, increase of HCV mutagenesis to the level of error catastrophe. Third, inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH) required for GTP synthesis. Fourth, immunomodulation resulting in a stronger antiviral T cell response. These hypotheses are derived mainly from *in vitro* data that support each of the proposed mechanisms. However, it is unclear how Ribavirin exerts its anti-HCV activity *in vivo* and why it is only effective in combination therapy with Interferon [3].

It is reasonable to assume that the uptake of Ribavirin into virus-infected hepatocytes is a prerequisite for its antiviral activity. In addition, the level of Ribavirin uptake may define its antiviral effectivity and thus, differences in Ribavirin uptake may account for differential treatment outcomes. However, there is limited data available regarding Ribavirin uptake in human hepatocytes.

Synthetic nucleosides such as Ribavirin can be imported into the cell by nucleoside transporters, that can be classified into two families: equilibrative nucleoside transporters (e.g., ENT1–4) are proteins that facilitate the bidirectional diffusion of nucleotides across the cell membrane in a concentration-dependent manner, whereas concentrative nucleoside transporters (e.g., CNT1–3) use the Na^+-gradient as a driving force to import nucleosides against a concentration gradient. Previous studies in animal cell lines and immortalized human hepatoma cells have shown that Ribavirin may be potentially transported into host cells by ENT1, ENT2, CNT2 and CNT3 [8,13]. It was unclear, however, which of the transporters are responsible for the import of Ribavirin into human hepatocytes.

A study published in this issue of the *Journal of Hepatology* addresses this important question. Fukuchi et al. analyzed the contribution of putative Ribavirin transporters in human hepatocytes derived from three deceased donors without underlying liver disease [5]. For the functional analyses, the authors used radioactive-labeled Ribavirin uptake assays in the presence or absence of the ENT inhibitor nitrobenzylmercaptopurine riboside (NBMPR) and in Na^+-containing and Na^+-free buffers. They found that Ribavirin uptake is primarily mediated by a Na^+-independent, NBMPR-sensitive system that shows the typical characteristics of ENT1. Next, they analyzed the mRNA expression of putative Ribavirin transporters. Indeed, ENT1 was also found to be expressed in all human hepatocytes analyzed, and high levels of ENT1 expression in hepatocytes correlated with high Ribavirin uptake rates, underlining the major role of ENT1 in mediating Ribavirin uptake. In contrast, ENT2, which was also expressed in human hepatocytes, did not mediate Ribavirin uptake in the hepatocytes nor in control hepatoma cells that overexpressed ENT2. This was an unexpected finding since previous reports had shown an ENT2-mediated Ribavirin uptake in *Xenopus* oocytes [13]. The Na^+-dependent CNT2 and CNT3 transporters were expressed only at lower levels compared to ENT1 and ENT2. Although the contribution of Na^+-dependent Ribavirin uptake was neglectable compared to ENT1 in two out of three hepatocyte donors analyzed in this study, in hepatocytes derived from one particular donor, 27–43% of Ribavirin uptake was Na^+-dependent. Hence, Fukuchi et al. cannot exclude the possibility that Na^+-dependent transporters, such as CNT2 and CNT3 or yet unidentified additional Ribavirin transporters may contribute to substantial amounts of Ribavirin uptake in some individuals. In summary, using an experimental system that closely mimics the *in vivo* situation, Fukuchi et al. demonstrate that ENT1 is the major Ribavirin uptake transporter in human hepatocytes.

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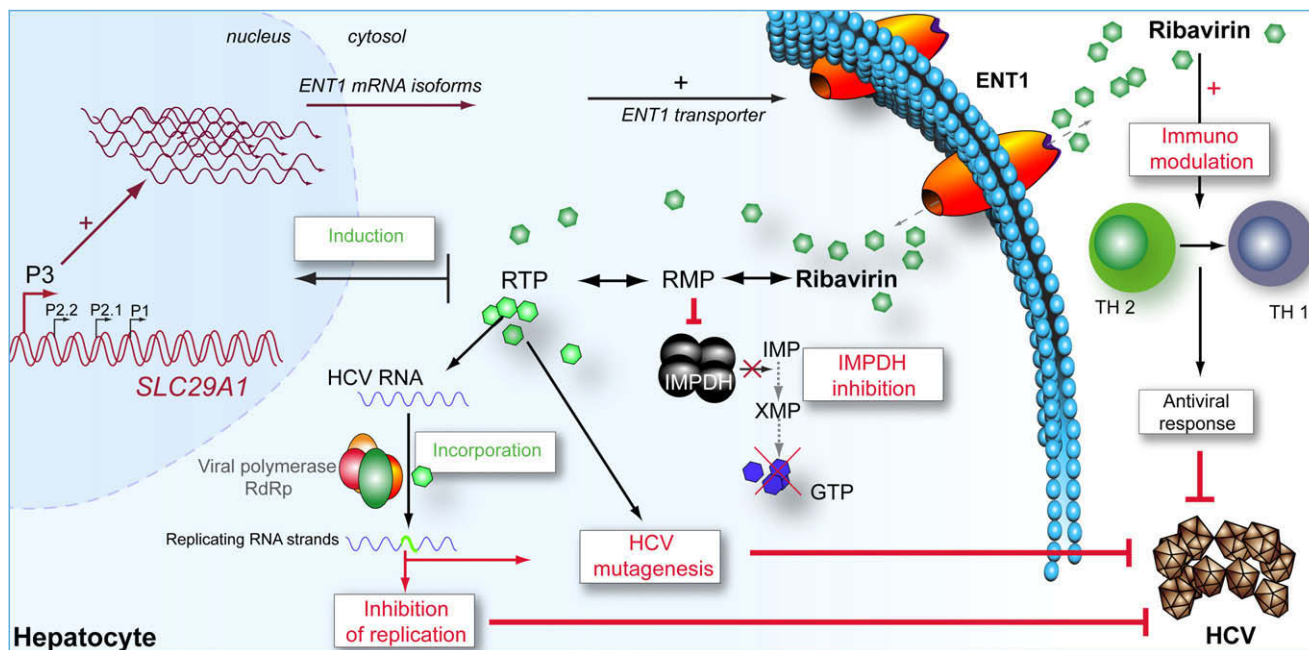
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Fig. 1. The putative antiviral mechanisms of action of Ribavirin during the treatment of HCV infection. Upon Ribavirin uptake into hepatocytes through the ENT1 transporter, it gets modified by cellular kinases to RMP and RTP. RTP causes enhanced HCV mutagenesis due to erroneous integration of RTP into newly synthesized viral RNA and may also directly inhibit HCV replication. RMP inhibits IMPDH which converts IMP to GTP, thus effectively depleting the intracellular GTP pool required for viral replication. In addition, Ribavirin treatment may enhance the antiviral T cell function. TH, T helper cells; RdRp, RNA-dependent RNA polymerase.

In an attempt to understand why ENT1 expression levels varied among the donor cells, the authors analyzed the 5′ region of the *SLC29A1* gene which codes for ENT1. Interestingly, by analyzing human liver cDNA with the RLM-5′-RACE technique, Fukuchi et al. identified four alternative promoter regions (P1, P2.1, P2.2 and P3) that regulate SLC29A1 gene transcription and generate at least 12 ENT1 mRNA isoforms. The authors then analyzed which ENT1 isoforms were expressed by human hepatocytes and found that the majority of ENT1 mRNAs was dependent on the P3 promoter region. Importantly, the expression of these mRNA isoforms (named ENT1 d1–4) also correlated with increased Ribavirin uptake of donor hepatocytes. These results demonstrate the existence of multiple ENT1 mRNA isoforms in human hepatocytes that may potentially explain differences in Ribavirin uptake by hepatocytes and might thus influence the outcome of antiviral therapy in chronic HCV patients.

In agreement with this hypothesis, a reduced Ribavirin antiviral efficacy against a model RNA virus was recently found to be associated with a reduced Ribavirin uptake in Ribavirin resistant cell lines [8]. Importantly, the Ribavirin resistance in this model could be overcome by ENT1 overexpression, indicating that alterations in the expression of Ribavirin transporters may confer resistance to antiviral therapy [8]. It is tempting to speculate that a similar mechanism might be active during the treatment of chronic HCV patients with Ribavirin. However, until now the role of Ribavirin transporters has not been addressed in HCV-infected hepatocytes. The recent establishment of infectious replicating HCV cell culture systems [1] may provide the adequate tools for future and more precise analyses of the anti-HCV mechanisms exerted by Ribavirin.

Fukuchi et al. have clarified that ENT1 is the major Ribavirin transporter in human hepatocytes. Furthermore, they have showed that ENT1 expression is regulated by at least four pro-

moter regions, that multiple ENT1 mRNA isoforms can be detected in human hepatocytes, and that specific ENT1 isoforms correlate with Ribavirin uptake. These are important new insights leading to additional questions that will have to be addressed in the future, e.g., whether the different ENT1 mRNA isoforms indeed translate into differences in ENT1 transporter function and how ENT1 mRNA isoform expression is regulated. Since Fukuchi et al. have analyzed Ribavirin uptake in human hepatocytes from non-HCV infected donors, it also remains unclear whether the expression of Ribavirin transporters is altered by HCV infection and whether Ribavirin transporters are influenced by Interferon and Ribavirin treatment.

Clearly, the identification of the major Ribavirin uptake transporter in human hepatocytes is an important step towards a conclusive answer to the puzzling enigma of the mechanism of action of Ribavirin in the treatment of HCV infection.

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