Short Communication

Differential effect of p7 inhibitors on hepatitis C virus cell-to-cell transmission

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Inhibitors targeting the hepatitis C virus (HCV) encoded viroporin, p7 prevent virus release in vitro. HCV can transmit by cell-free particle infection of new target cells and via cell-to-cell dependent contact with limited exposure to the extracellular environment. The role of assembly inhibitors in preventing HCV transmission via these pathways has not been studied. We compared the efficacy of three published p7 inhibitors to inhibit cell-free and cell-to-cell transmission of two chimeric HCV strains encoding genotype 2 (GT2) or 5 (GT5) p7 using a recently developed single cycle co-culture assay. The inhibitors reduced the infectivity of extracellular GT2 and GT5 virus by 80–90% and GT2 virus cell-to-cell transmission by 50%. However, all of the p7 inhibitors had minimal effect on GT5 cell contact dependent transmission. Screening a wider panel of diverse viral genotypes demonstrated that p7 viroporin inhibitors were significantly more effective at blocking cell-free virus than cell-to-cell transmission. These results suggest an altered assembly or trafficking of cell-to-cell transmitted compared to secreted virus. These observations have important implications for the validation, therapeutic design and testing of HCV assembly inhibitors.

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1. Results and discussion

Hepatitis C virus (HCV) is a global health problem, affecting approximately 170 million, and results in a chronic degenerative liver disease that is characterised by hepatic fibrosis, cirrhosis and in 10% of cases hepatocellular carcinoma. Therapeutic regimens of pegylated-interferon and the nucleoside analogue ribavirin are used to treat HCV disease that is characterised by hepatic fibrosis, cirrhosis and in 10% of cases hepatocellular carcinoma. Therapeutic regimens of pegylated-interferon and the nucleoside analogue ribavirin are used to treat HCV.

In recent years, many advances have been made in understanding how viral infection can lead to liver disease. This is due to the discovery of HCV cell contact transmission (Tedbury et al., 2011; Wozniak et al., 2010). However, cell-free transmission remains an important target for new antivirals. Inhibition of cell-free transmission is important as cell-free virus is present in high concentrations that can be achieved in man, consistent with in vitro observations (Fong et al., 1999; Griffin et al., 2008; Juba et al., 2000; Steinmann et al., 2007a, b). A recent study by OuYang et al., elucidated an NMR structure of HCV p7 strain EUH1480 (GT5A) and predicted the amantadine binding domain. Both amantadine and rimantadine are suggested to hinder the p7 channel from opening by restricting movement of helical segments in the p7 hexamer. The authors report variations in the amantadine-binding pocket which may explain the broad range of responses to inhibitors reported for diverse HCV genotypes (Ouyang et al., 2013).

The majority of in vitro studies on p7 inhibitors have characterised the effect of compounds on virus assembly and the infectivity of secreted particles. However, these studies did not address the ability of HCV to transmit via cell-to-cell contacts, a dominant route of viral transmission for several HCV genotypes (Brimacombe et al., 2011; Catane et al., 2013; Meredith et al., 2013; Timpe et al., 2008). We therefore assessed the efficacy of several known p7 inhibitors to prevent HCV cell-to-cell transmission, including the amantadine-derivative Rimantadine, the long alkyl-chain iminosugar NN-DNJ (StGelais et al., 2007; Wozniak et al., 2010) and the small molecule inhibitor BIT225 (Luscombe et al., 2010). We previously reported that diverse strains of HCV can transmit effectively via the cell-to-cell route, with J6/JFH (GT2A/2A) showing a
Fig. 1. Differential effect of p7 inhibitors on hepatitis C virus cell-free and cell-to-cell transmission. (A) Schematic representation of co-culture assay. HCV strain J6/JFH (B) or SA13/JFH (C) infected Huh-7.5 cells or producers were treated for 24 h with p7 inhibitors, washed thoroughly, labelled with CMFDA and co-cultured at a 1:1 ratio with naïve Huh-7.5 target cells. Extracellular infectious virus was neutralised by the inclusion of anti-HCV IgG (150 µg/mL), parallel infections performed in the presence of a neutralising anti-HCV Ig or control IgG allowed us to quantify the frequency of cell-free and cell-to-cell infection events. 2 h post contact of infected and naïve cells a sample of media was collected to measure the effect of p7 inhibitors on extracellular infectious virus levels prior to the addition of neutralising anti-receptor CD81 mAb (2s131) (10 µg/mL) to block all further HCV infection events. Co-cultures were incubated for a further 20 h and the cells stained for viral encoded non-structural protein NS5A. Newly infected target cells (NS5A+/CMFDA−/CD30−) were quantified per 10⁵ producer cells by flow cytometry. Results are the mean and standard deviation of three experiments and statistical significance determined using unpaired T-test with corrections for multiple comparisons (Significance **P < 0.01, ***P < 0.001). (D) Additional viral genotypes were tested for their sensitivity to BIT225 (30 µM), Rimantadine (3 µM), NN-DNJ (30 µM) and NN-DGJ (30 µM) using the same assay protocol as described in (A). Significant differences were observed between inhibition of cell-to-cell and cell-free inhibition of infection for all drugs tested (**BIT225, Rimantadine (P < 0.01), ***NN-DNJ, NN-DGJ (P < 0.05)).
distinct preference for cell-to-cell infection, while SA13/JFH (GT5A/2A) transmitted with equal efficiency by either route (Brimacombe et al., 2011; Meredith et al., 2013). Furthermore, HCV SA13/JFH is the only published infectious GT5 strain and has a closely related sequence to EUH1480, the subject of the recent p7 NMR study (OuYang et al., 2013).

To determine the sensitivity of HCV J6/JFH and SA13/JFH to p7 inhibitors BIT225, NN-DNJ and rimantadine, infected Huh-7.5 cells were treated overnight with increasing concentrations of compound. The drug was removed by repeated washing, conditioned media was collected over a 2 h period and infectivity measured. All compounds were effective against both strains, although J6/JFH was more sensitive than SA13/JFH, with IC₉₀ values of 10, 3 and 0.3 μM for BIT225, NN-DNJ and Rimantadine, respectively, compared to IC₉₀ values of 30, 30 and 1 μM for SA13/JFH (data not shown). The higher IC₉₀ values reported here compared to previous studies most likely reflect differences in the duration of treatment, with earlier studies treating infected cells for up to 72 h before measuring extracellular virus infectivity. Since NN-DNJ can affect glycosylation of viral proteins we limited the duration of treatment to minimise such off-target effects.

The efficacy of the inhibitors to limit HCV cell-to-cell transmission was tested using a recently developed single-cycle co-culture assay (Meredith et al., 2013). Since p7 has been reported to play a role in viral internalisation (Griffin et al., 2008) it is important to discriminate the effect of p7 inhibitors on virus assembly and entry. This assay allows one to assess the effect of p7 inhibitor treatment on infected ‘producer’ cells and enables the quantification of new infection events within 2 h of culturing infected and naïve hepatoma cells, which is essential given the reversible nature of p7 targeted compounds (Pavlovic et al., 2005, 2003). HCV J6/JFH or SA13/JFH infected Huh-7.5 cells were treated with 30 μM of either BIT225 or NN-DNJ and 3 μM Rimantadine for 24 h, concentrations previously shown to inhibit the level of infectious extracellular virus by 80–90%. The cells were washed to remove the compounds, labelled with 5-Chloromethylfluorescein diacetate (CMFDA Cell Tracker Green, Invitrogen), and cultured with naïve Huh-7.5 targets at a 1:1 ratio as detailed in Fig. 1A. We confirmed that all compounds reduced the level of extracellular infectious virus in the co-culture (Fig. 1B and C), consistent with a reduction in J6/JFH and SA13/JFH cell-free transmission events. Although all three compounds inhibited 50–70% of J6/JFH cell-to-cell transmission, they had no detectable effect on SA13/JFH cell-to-cell transmission (Fig. 1C). To determine how wide ranging this effect was, we screened a panel of diverse chimeric viruses expressing the structural proteins from genotype 1–7 for their sensitivity to all existing compounds that specifically target the function of p7 as in mediating capsid assembly and envelopment (Gentzsch et al., 2013), HCV assembly complex formation (Shanmugam and Yi, 2013) or other aspects of the viral life cycle. This study has important implications for the therapeutic design and evaluation of agents targeting HCV p7, or other assembly inhibitors, that may inhibit the secretion of virus detected in the periphery but have minimal effect on viral spread within the liver, limiting their therapeutic value.

Authors contributions

L.W.M. designed experiments, acquired the data and co-wrote the manuscript. N.Z. supplied reagents and contributed to experimental design. J.A.M. provided study supervision and co-wrote the manuscript. All authors contributed to the final version of the manuscript.

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