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Short Communication

Differential effect of p7 inhibitors on hepatitis C virus cell-to-cell transmission[☆]L.W. Meredith^{a,*}, N. Zitzmann^b, J.A. McKeating^a^a Centre for Human Virology, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom^b Oxford Antiviral Drug Discovery Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

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ABSTRACT

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 only active in about 50% of cases with varying efficacy across different genotypes. More recently direct acting antivirals (DAAs) targeting the viral encoded protease (telaprevir and boceprevir) and polymerase (sofosbuvir) (Chang et al., 2012; Gane et al., 2013; Matthews and Lancaster, 2012) have been developed and show increased viral clearance rates. However, genotype-dependent differences in drug sensitivity and viral resistance highlight the need for additional drugs for future combination therapy.

The HCV encoded viroporin p7 is an attractive target for therapeutic intervention since it is essential for viral assembly and egress (Tedbury et al., 2011; Wozniak et al., 2010). However, clin-

ical trials of p7 inhibitors, including the adamantane-derivatives amantadine and rimantadine, have showed limited efficacy at concentrations that can be achieved in man, consistent with *in vitro* observations (Fong et al., 1999; Griffin et al., 2008; Jubin 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 adamantane-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; Catanese 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 adamantane-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

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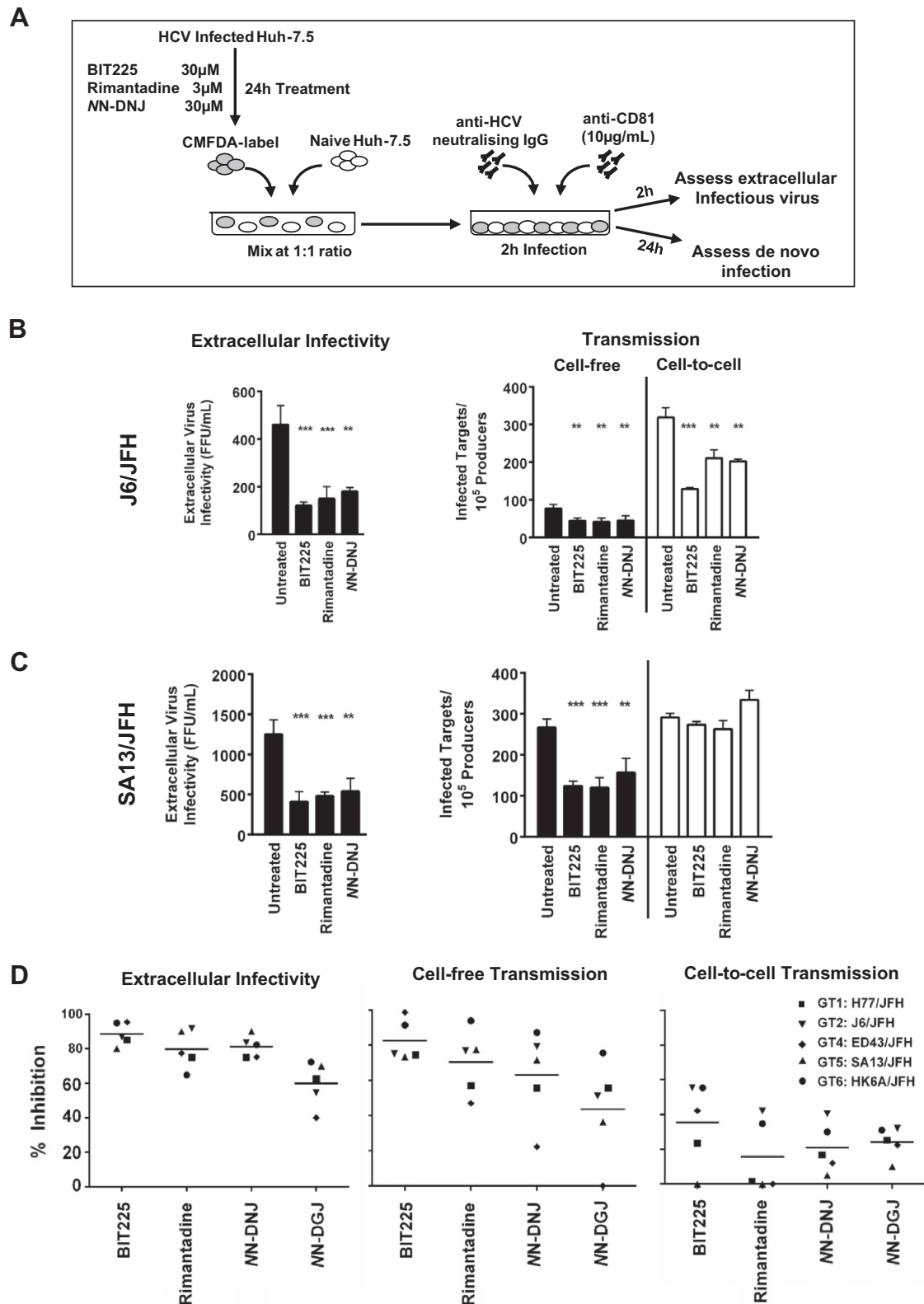


Fig. 1. Differential effect of p7 inhibitors on hepatitis C virus cell-free and cell-to-cell transmission. (A) Schematic representation of co-culture assay. HCV strain J6/JFH (B) or SA13/JFH (C) infected Huh-7.5 cells or producers were treated for 24 h with p7 inhibitors, washed thoroughly, labelled 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⁺) 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* < 0.001, ***P* < 0.01). (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 IC_{90} values of 10, 3 and 0.3 μ M for BIT225, NN-DNJ and Rimantadine, respectively, compared to IC_{90} values of 30, 30 and 1 μ M for SA13/JFH (data not shown). The higher IC_{90} 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 μ M of either BIT225 or NN-DNJ and 3 μ M 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 currently available p7 inhibitors, including NN-DGJ that does not affect host cell glycosylation pathways (Chapel et al., 2006a,b,c). Three viruses (JFH-1 – GT2; ED43/JFH – GT3 and QC69/JFH – GT7) showed limited transmission and were excluded from the analysis. The results show that all of the p7 inhibitors were significantly more effective at inhibiting cell-free infection than cell-to-cell transmission when all genotypes are considered (Fig. 1D).

The recent study by OuYang et al., suggest that amantadine binds the p7 ion-channel and locks it into a closed position (OuYang et al., 2013), preventing the de-acidification of the intravesicular compartments and leading to the secretion of non-infectious virus. Our study suggests that virus transmitting via cell-to-cell contact may have differential sensitivity to low pH compared to the secreted virus. Further experiments are needed to investigate the role of vesicular pH in HCV cell-to-cell transmission, as these results may indicate that p7 activity may be dispensable for this mode of infection. However the studies presented here focus on existing compounds that specifically target the function of p7 as a viroporin, and do not take into account roles that p7 may play 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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