Pentagalloylglucose, a highly bioavailable polyphenolic compound present in Cortex moutan, efficiently blocks hepatitis C virus entry

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Abstract

Approximately 142 million people worldwide are infected with hepatitis C virus (HCV). Although potent direct acting antivirals are available, high costs limit access to treatment. Chronic hepatitis C virus infection remains a major cause of orthotopic liver transplantation. Moreover, re-infection of the graft occurs regularly. Antivirals derived from natural sources might be an alternative and cost-effective option to complement therapy regimens for global control of hepatitis C virus infection.

We tested the antiviral properties of a mixture of different Chinese herbs/roots named Zhi Bai Di Huang Wan (ZBDHW) and its individual components on HCV. One of the ZBDHW components, Penta-O-Galloyl-Glucose (PGG), was further analyzed for its mode of action *in vitro*, its antiviral activity in primary human hepatocytes as well as for its bioavailability and hepatotoxicity in mice.

ZBDHW, its component Cortex Moutan and the compound PGG efficiently block entry of HCV of all major genotypes and also of the related flavivirus Zika virus. PGG does not disrupt HCV virion integrity and acts primarily during virus attachment. PGG shows an additive effect when combined with the well characterized HCV inhibitor Daclatasvir. Analysis of bioavailability in mice revealed plasma levels above tissue culture IC_{50} after a single intraperitoneal injection.

In conclusion, PGG is a pangenotypic HCV entry inhibitor with high bioavailability. The low cost and wide availability of this compound make it a promising candidate for HCV combination therapies, and also emerging human pathogenic flaviviruses like ZIKV.

Key words:

Hepatitis C virus, Natural compounds, antivirals, Cortex Moutan, Penta-O-Galloyl-Glucose, entry inhibitor, bioavailability

Abbreviations

Traditional Chinese Medicine (TCM)

Cortex Moutan (CM)

Penta-O-Galloyl-Glucose (PGG)

hepatitis C virus (HCV)

half maximal effective inhibitory concentration (IC_{50})

Zika virus (ZIKV)

Concanamycin A (ConcaA)

direct-acting antivirals (DAAs)

Epigallocatechin-Gallate (EGCG)

1.0 Introduction

Worldwide, approximately 142 million people are infected with HCV which can result in liver damage, the development of cirrhosis and an increased risk for hepatocellular carcinoma (Disease et al., 2016; Lavanchy, 2011; Levrero, 2006). Hence, end-stage liver disease due to HCV is the most common indication for liver transplantation (Brown, 2005). However, re-infection of the graft occurs in almost all transplanted individuals and can lead to the need of re-transplantation (Rubin et al., 2011). While the treatment of choice against HCV consisted of interferon/ribavirin for more than a decade, now an array of direct-acting antivirals (DAAs) have been licensed by the Food and Drug Administration (FDA) and show drastically improved potency in almost all infected individuals (Sarrazin, 2015). Despite of this, these new treatments face several obstacles that need to be overcome in the near future. While treatment of genotype 1 infected individuals with the new DAAs displayed improved response rates relative to interferon/ribavirin-based therapy, treatment efficacy is lower in genotype 3 infected individuals (Petta and Craxi, 2015). Another limitation is that special cases demand alternative therapies, such as when individuals have end-stage renal/liver disease or have recently undergone liver transplantation (Lam et al., 2015). Furthermore, drug-to-drug interactions between DAAs and certain anticonvulsants, antiarrythmics, antibiotics, antifungals, immunosupressants and even HIV antiretrovirals have been described and narrow treatment options in patients taking these drugs (Lam et al., 2015). Most importantly, accessibility to DAAs is limited due to their high costs, in particular in developing countries, which host the majority of chronically infected people (Messina et al., 2015).

ZBDHW is a mixture of eight different herbs/roots used in Traditional Chinese Medicine (TCM) to treat diseases of the liver, kidney and heart. Cortex Moutan (CM) is the root bark of tree peony and one component of ZBDHW. It is used in TCM as a hepatoprotective and anti-inflammatory

herb (Poon et al., 2011). PGG is one constituent of CM that has previously been shown to prevent oxidative DNA damage in particular (Okubo et al., 2000), although anti-diabetic, anti-cancer and antiviral properties have also been reported (Cao et al., 2014).

In this study, we evaluated the antiviral properties of ZBDHW against HCV. We show that out of the different constituents of ZBDHW, an extract of CM revealed the strongest antiviral potency. Testing of different bioactive ingredients of CM revealed that PGG is a potent inhibitor of HCV entry in Huh-7.5 hepatoma cells as well as primary human hepatocytes. Further mode of action analyses showed that PGG acts at an early stage of virus entry into the target cells. Intraperitoneal administration of PGG in mice lead to compound plasma levels above tissue culture half maximal effective concentration (IC_{50}).

In conclusion, we report that an extract of CM and in particular the constituent PGG harbor potent anti-HCV properties. Thus, PGG could be a promising alternative for the generation of a more affordable antiviral treatment or as an add-on to current treatment options in specific cohorts, including transplanted individuals.

2.0 Material and Methods

2.1 Cell culture: Huh-7.5 cells (Blight, 2000), Huh-7.5 cells stably expressing firefly luciferase (Gentzsch et al., 2011) and Vero B4 cells (kindly provided by M. Müller, University of Bonn Medical Centre) were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 10 % fetal calf serum. Human hepatocytes were isolated by a modified 2-step collagenase perfusion technique (Kleine et al., 2014).

2.3 Viruses: The chimeric 2a/2a Jc1 virus (pFK-Jc1), the monocistronic renilla luciferase reportervirus JcR2a (Haid et al., 2012; Pietschmann et al., 2006), the bicistronic firefly Jc1 virus (pFK-Luc-Jc1)(Koutsoudakis et al., 2006) and the monocistronic renilla luciferase reporter chimeric virus genomes H77c/1a/R2a, J4/1b/R2a, JcR2a, J8/2b/R2a, S52/3a/R2a, ED43/4a/R2a, SA13/5a/R2a, HK6a/6a/R2a and QC69/7a/R2a (Haid et al., 2012) have been previously described. Virus stocks were produced by electroporation of cells with *in vitro* transcribed RNA as described (Perin et al., 2015). The circulating Zika virus (ZIKV) strain from Puerto Rico (PRVABC59) has been obtained from Culture Collections of Public Health England (UK) and passaged twice on A. albopictus C6/36 before used in experiments.

2.4 Virus titration by immunohistochemical staining and luciferase assays: Virus titration by limiting dilution assay was determined according as previously described (Vieyres and Pietschmann, 2013). For immunohistochemical detection we used the monoclonal antibody targeting NS5A (9E10) (Lindenbach et al., 2005).

2.5 Quantitative detection of viral RNA: RNA was isolated from cells using the Nucleo Spin RNA II kit (Macherey-Nagel; Düren, Germany) according to the manufacturer´s instructions. A one-step RT-PCR LightCycler 480 RNA Master Hydrolysis Probe kit (Roche; Mannheim, Germany) was used. The PCR protocol and primers were previously reported (Menzel et al.,

2012). ZIKV RNA was isolated using the NucleoMag® VET kit (Macherey-Nagel; Düren, Germany) according to the manufacturer´s instructions. Viral RNA was transcribed into cDNA using the M-MLV Reverse Transcriptase (Promega) according to the manufacturer´s instructions. The Taqman Fast Universal Master Mix 2X (Applied Biosystems) was used for RT-PCR using the following primers and probes: forward primer CGYTGCCCAACACAAGG, reverse primer 5'-CCACYAAYGTTCTTTTGCABACAT-3', probe 5'-FAM-AGCCTACCTTGAYAA-GCARTCAGACACYCAA-BHQ1-3'.

2.6 Binding and fusion of R18 labeled viruses: HCV JFH-1 virions were labeled with 0.5 μM R18 as recently described (Colpitts and Schang, 2014; Colpitts et al., 2013). Fusion with Huh-7.5 cells was triggered by increasing the temperature and lowering the pH and measured by R18 dequenching as previously described(Anggakusuma et al., 2013; Colpitts et al., 2013).

2.7 Fusion at the plasma membrane assay: The assay was performed as previously described (Tscherne et al., 2006). As controls, the CD81 antibody JS-81 and the fusion inhibitor flunarizine (Perin et al., 2015) were used.

2.8 Pharmacokinetic and cytotoxicity study of PGG in mice: Evaluation of bioavailability of PGG was conducted in a small proof-of-concept study in SCID mice. Five animals were intraperitoneally injected with a single dose of PGG (50 mg/kg), dissolved in PBS containing 5% ethyl alcohol. Blood was taken at 2, 4 or 8 as well as 24 and 96 hours post treatment and analyzed for PGG concentrations. The concentration of PGG in mouse plasma was determined by LC-MS/ MS as described before (Li et al., 2011). Epigallocatechin-Gallate (EGCG) was used as internal standard. For evaluation of cytotoxicity, ten Balb/c mice were similarly treated with PGG. Blood was drawn before treatment as well as day 1, 2 and 6 after treatment and analyzed for ALT as a marker of liver cytotoxicity. Six mice were treated with vehicle alone as control.

Further supporting "Material and Methods" can be found in the supplementary.

3.0 Results

3.1 The extract of CM inhibits HCV

ZBDHW pills, composed of a mixture of eight roots/herbs, are used in TCM to treat a diversity of ailments. In particular, it has been claimed to balance liver functions. In this study, we aimed to elucidate whether this TCM inhibits HCV propagation *in vitro*.

To this end, we tested the impact of ZBDHW on the infectivity of the efficient HCV chimera, Jc1, by a limiting dilution infection assay ($TCID_{50}$) in the presence of different dilutions of an extract ZBDHW. A dose-dependent decrease of infectivity was observed, with a reduction of approximately 3-logs at a 1:50 dilution of the herbal supplement (Fig. 1A). In order to determine the active ingredient, extracts of the different roots/herbs of ZBDHW were prepared and assayed similarly (Fig 1B). Addition of CM during virus titration notably decreased infectivity of virus particles, leading to a reduction of more than 3-logs when diluted to 1:50 in DMEM while the other extracts displayed mild to no antiviral activity. Anemorrhena resulted in significant cytotoxicity and did not allow further analysis in this assay set-up (data not shown).

3.2 The extract of CM and its component PGG inhibit cell entry of HCV

To determine at which step of the viral life-cycle the extract of CM inhibits HCV, dilutions of this extract in DMEM were added at 4 hours before, during, or after inoculation with a renilla luciferase reporter virus (Fig. 1C). After 48 hours, the effects on infection were assessed by measuring the luciferase reporter activity. Simultaneous treatment of the cells with the extract and the virus resulted in a reduction of infection by more than10-fold (Fig. 1C), whereas no antiviral activity was observed when the extract was added prior to or post-inoculation (Fig. 1C). This indicated that the CM extract inhibits HCV cell entry. CM consists of a variety of bioactive compounds which have been identified in previous studies (He et al., 2006; Xiao et al., 2015). Out of these, selected compounds (ingredients bencoic acid, gallic acid, methyl gallate,

paeoniflorin, paenol and PGG) were tested by the same experimental setup indicated in Figure 1C (data not shown). Only the polyphenolic compound PGG was antiviral against HCV (Fig. 2). Similarly to CM, PGG solely inhibited infection of Jc1 if added during virus inoculation of Huh-7.5 cells (Fig. 2A-C). The IC_{50} of PGG was 2.2µM. In order to exclude a significant cytotoxicity of PGG in our assay set-up we conducted two different experiments. First, we treated Huh-7.5 cells for 4 hours with different concentrations of PGG, followed by addition of normal medium. After 48 hours an MTT-assay was performed to determine cell viability (Fig. 2D). In this assay the CC_{50} was calculated at 114.3µM. Second, we used Huh-7.5 cells which stably express a firefly luciferase in the same assay set-up as in Fig 2B. In addition to viral infection, this allowed us to determine cell viability by detection of luciferase activity in the cell-lysates (Fig. 2E). However, this assay lead to a calculated CC_{50} value of 63.2µM.

3.3 PGG inhibits all HCV genotypes and acts antiviral on ZIKV

Next, we examined if PGG inhibits infection of different HCV strains derived from all major HCV genotypes. To this end a panel of chimeric viruses carrying the structural proteins of genotypes 1a, 3a, 4a, 5a, 6a or 7a was used to infect Huh-7.5 cells in the presence of increasing concentrations of PGG for 4 hours. After 48 hours, measurement of luciferase reporter activity indicated that PGG inhibited entry of all of the genotypes to similar extent (Fig. 2F).

To test if PGG also inhibits other enveloped viruses, we examined if Zika virus (ZIKV), an emerging member of the family *Flaviviridae* is also susceptible to inhibition by PGG. To this end, we infected Vero B4 cells with ZIKV (Puerto Rico strain) in the presence of different concentrations PGG. After 48 hours released viruses were detected via qRT-PCR. We could observe a dose-dependent decrease of virus titers in the supernatant with an IC_{50} of 4.1µM (Fig. 2 G). This argues that PGG is not an HCV-specific antiviral but is also active against other *Flaviviridae,* like for instance ZIKV.

3.4 PGG does not act by disrupting the virion integrity or by modifying particle density

To explore if PGG acts by disruption of virus particles, undiluted virus, or 10-fold pre-diluted HCV were incubated with PGG at a concentration of 10µM (undiluted virus) or 1 and 10µM (diluted virus) for 1 hour at room temperature (Fig. 3A). Afterwards, the undiluted PGG/virus mixture was diluted 1:10 in medium and the mixtures were used to infect permissive cells. Compounds, which act via disruption of the viral structure would cause an inhibition of the infection in the initially treated undiluted virus comparable to the inhibition of infection of the diluted virus stock at the highest treatment concentration. As depicted in Fig 3A, PGG loses most of its inhibition by dilution (initial PGG concentration of 10µM) as compared to the diluted virus control. As an additional negative control we performed the same experiment with ITX 5061, a well characterized HCV receptor antagonist (Haid et al., 2012), that similarly showed a reduced antiviral activity upon dilution of the virus/compound mixture (Fig 3A).

We next evaluated if PGG disrupts the viral lipid envelope of HCV. To this end, we conducted a proteinase K (PK) protection assay, in which Jc1 particles are incubated with PGG or vehicle control for 4 hour at room temperature, followed by incubation with the proteinase. Since HCV is an enveloped virus, PK can only digest the capsid protein core if the protecting lipid envelope is absent. Viruses were either pre-treated with PBS, with PK or with PK combined with Triton X-100, a detergent that dissolves the viral lipid membrane. Following a 1-hour treatment at 4°C, capsid protein levels were measured by ELISA. PK was not able to digest core protein from solvent or PGG-treated particles, pointing to equally intact envelopes of particles treated with vehicle or compound (Fig. 3B).

HCV is associated with lipoproteins, which plays an important role during the HCV entry process, including attachment, receptor interaction and fusion (Jiang and Luo, 2012; Zeisel et al., 2011). Due to differential lipoprotein association, HCV particles in the bloodstream show diverse

buoyant density (Andre et al., 2002; Nielsen et al., 2006; Thomssen et al., 1992). PGG might alter lipoprotein association and, therefore, density distribution of Jc1 viruses was assessed. Therefore, particles pre-incubated with vehicle control or PGG for 4 hour at room temperature were subjected to ultracentrifugation in an iodixanol density gradient. Infectivity and density distribution of particles remained unchanged when particles were incubated with PGG (Fig. 3C). Collectively, PGG does not disrupt HCV particles nor does it change the buoyant density of virions.

To check for viral RNA integrity, PGG or solvent control was added to cell free Jc1 virus particles and incubated for 4 hours at 37ºC (Fig. 4A). In parallel, viruses were heat-treated to denature viral proteins. Following compound or heat treatment, viral RNA was extracted and quantified by qRT-PCR. To assess HCV RNA integrity, the extracted viral RNA was also used to transfect naïve cells. At 4 and 72 hours post-electroporation, the viral RNA amount was measured in supernatants of transfected cells. Infectivity of the supernatant of transfected cells at 72 hours post-electroporation was determined by limiting dilution assay. The total amount of virus particle associated HCV RNA was unchanged directly after PGG treatment and after heat inactivation (Fig. 4B). Heat inactivation did compromise the integrity of viral RNA, as the amount of HCV RNA following electroporation of cells with viral RNA extracted from heat treated virions was substantially reduced compared to the control at both 4 and 72 hours post electroporation (Fig. 3B). Accordingly, transfection with viral RNA extracted from heat inactivated particles yielded supernatants with 100- 1,000-fold reduced infectivity (Fig. 4C). In contrast, incubation of virus particles with PGG did not affect RNA integrity, as infectivity and RNA levels at 4 and 72 hours post-electroporation with RNA extracted from PGG-treated viruses were comparable to control treated particles (Fig. 4B and Fig. 4C).

3.5 PGG inhibits early steps of HCV entry, primarily by inhibition of viral attachment to the target cells

To further understand how PGG inhibits entry of HCV, we conducted a series of time-of-addition experiments. We inoculated Huh-7.5 cells with firefly reporter Jc1 viruses at 4ºC for 1 hour, so viruses bind to cells and infection can proceed in a synchronized manner once cells are transferred to 37ºC. PGG or control compounds known to inhibit attachment (heparin) or endosomal acidification (Concanamycin A; ConcaA), an important prerequisite for HCV membrane fusion, were added at different times throughout the infection (Fig. 5). In each case, compounds were present for a total of 4 hours. After 48 hours, we assessed infection efficiency by measuring luciferase activity of cell lysates. As expected, heparin was antiviral when added during viral binding. Inhibition was substantially reduced if heparin was added after attachment (Fig. 5). ConcaA maintained almost complete antiviral activity up to 60 minutes after the temperature shift (Fig. 5). Loss of antiviral acidity over time with PGG had similar kinetics as compared to heparin, pointing to inhibition of early steps of viral entry (Fig. 5).

In order to more specifically narrow down the entry step during which PGG impedes HCV infection, a fusion at the plasma membrane assay was performed (Perin et al., 2015; Tscherne et al., 2006). In this assay, ConcaA is added before and throughout inoculation to prevent virus infection via low pH-triggered membrane fusion in endosomes. In this setting, HCV infection through the natural endosomal route is blocked, and can be triggered by addition of a low pH buffer which induces viral membrane fusion at the plasma membrane (Perin et al., 2015; Tscherne et al., 2006). Notably, low pH-triggered HCV plasma membrane fusion can only be elicited after an incubation of virus inoculated cells for one hour at 37°C indicating that virus receptor interactions are necessary to prime HCV for low pH-triggered fusion (Tscherne et al., 2006). Thus, by adding drugs at different time points throughout the experiment it is possible to

distinguish if a compound primarily blocks attachment, HCV receptor interactions or membrane fusion. Thus, PGG was added at different time points during the experiment (Fig. 6A) and its inhibition was assessed by measuring renilla luciferase reporter virus activity after 48 hours. JS81 antibody (anti-CD81) was added during the receptor interaction stage and the known membrane fusion inhibitor flunarizine (Perin et al., 2015) was added during the pH shift as controls (Fig. 6A).

Addition of PGG throughout the entire time course resulted in over ten-fold reduction of HCV infection. More than a 5-fold reduction of infectivity was observed when PGG was added only during initial virus binding. No inhibition of infection was observed, when PGG was added only after virus binding (protocol 5 in Fig. 6A), during fusion (protocol 6) or after fusion (protocol 7). Thus, these results support the notion that PGG primarily inhibits virus attachment and not receptor-triggered conformational changes and/or membrane fusion (Fig. 6A).

To support this model directly, we incubated R18 fluorescent labeled JFH-1 virions with DMSO or PGG for 10 minutes at 37ºC. Pre-treated viruses were adsorbed on to Huh-7.5 cell monolayers at 4°C for 1 hour. Cell monolayers were extensively washed to remove unbound viruses and lysed. Bound viruses were measured by reading R18 fluorescence signals of cell lysates. We observed a dose-dependent decrease of binding relative to control, supporting the conclusion that PGG indeed acts during virus attachment (Fig. 6B).

3.6 Combination of PGG with a known HCV inhibitor increases potency in vitro

We next addressed the potential clinical use of PGG by examining the antiviral activity of combination of PGG with previously characterized inhibitors of viral replication. To this end, we titrated the antiviral activity of PGG in Huh-7.5 cells infected with Jc1 renilla luciferase reporter viruses in the presence of fixed concentrations of the inhibitor Daclatasvir, which inhibits the

viral NS5A protein. PGG increases antiviral activity of this drug, indicating an additive effect (Fig. 7).

3.7 PGG prevents infection of primary human hepatocytes and is highly bioavailable in mice

Finally, we examined if PGG also prevents HCV infection of primary human hepatocytes. To this end, primary human hepatocytes were infected with Jc1 for 4-6 hours at 37°C in the presence of vehicle control, ConcaA or PGG and washed thereafter. After 48 hours, supernatants were harvested, filtered and infectivity was assayed by limiting dilution assay. Both, ConcaA and PGG reduced infection of primary human hepatocytes compared to the control (Fig 8A). Next we evaluated the bioavailability of PGG when administered to SCID mice. To this end, we treated 5 animals with 50mg/kg PGG intraperitoneally and analyzed blood samples for PGG content after 2, 4 or 8 as well as 24 and 96 hours post-treatment. As depicted in figure 8B, we could observe plasma levels above tissue culture IC_{50} 2 hours after administration. Plasma levels decreased slowly over time (Fig. 8B). To test hepatotoxicity of PGG *in vivo*, we administered PGG to 10 Balb/c mice intraperitoneally (50mg/kg PGG) and measured plasma levels of ALT over time. Six animals were treated with vehicle as control. Overall, 6 of the PGG treated animals survived the treatment. When quantifying ALT plasma levels as a marker of hepatocellular cytotoxicity, we observed a drastic increase of ALT plasma levels in those animals that did not survive, while the other animals displayed only moderately elevated ALT levels (Fig 8C).

4.0 Discussion

Treatment of HCV infected patients has considerably improved over the last few years. Following the approval of second-generation DAAs such as sofosbuvir, a one-pill, interferon-free therapy is finally available. Clinical data show that new treatments combine high efficiency - including against the previously difficult to treat genotype 1 - with minimal side effects. In spite of that, some challenges remain. In particular, high treatment costs of these new drugs warrant development of cost-effective regimens to facilitate broad access to treatment. Natural products and medicinal plants are a potential source of new antivirals against HCV to complement current therapies. In fact, several reports demonstrate phytochemicals act as antivirals not only against HCV, but also against other viruses (Ashfaq and Idrees, 2014; Ciesek et al., 2011; Haid et al., 2012). With that in mind, we selected a complex of medicinal plants that is used in TCM and believed to improve liver function to analyze its antiviral activity against HCV. An extract of ZBDHW reduced HCV titers by more than 300-fold at the highest tested concentration. The antiviral activity of this mixture of roots/herbs was mainly derived from the properties of one of the ingredients, CM. When testing different bioactive compounds of CM we observed that a dose of ca. 8µM PGG inhibited infection of cell-culture derived HCV in hepatoma cell line Huh-7.5 as well as primary human hepatocytes by approximately 10-fold with no toxicity within the used concentrations. Thus, PGG might be one of the major antiviral components in ZBDHW and CM extracts. However, variations in terms of the presence of bioactive molecules within this mixture of natural compounds can be observed from one batch to the other.

Reports of the biological activity of PGG are numerous and comprise anti-oncogenic, anti-diabetic, anti-inflammatory, anti-oxidant, anti-angiogenic and anti-bacterial activities (Zhang et al., 2009). As would be expected for such a bioactive compound, PGG has been described to be antiviral by inhibition of viral entry also against other viruses, including respiratory syncytial

virus (RSV) (Haid et al., 2016; Yeo et al., 2002), hepatitis B virus (HBV) (Lee et al., 2006), herpes simplex virus (HSV-1) (Pei et al., 2011) and influenza A viruses (Liu et al., 2011). This points to a broad mode of action, common to enveloped viruses. In fact, we could observe antiviral effects also for ZIKV, another member of *Flaviviradae,* further supporting the broader antiviral activity of this compound. It is possible that the relatively polar PGG interacts with charged residues on the surface of virions (e.g. sugar moieties on glycosylated viral envelope proteins) and thus competes for natural virus-receptor interactions necessary for virus particle binding.

Before this work, it was unknown how PGG exerts its antiviral activity against HCV. PGG had been identified in a bioassay screening for NS3 protease inhibition (Duan et al., 2004). However, NS3 has a role in RNA replication and in our experiments, addition of PGG after infection causes no inhibition, indicating PGG does not block the RNA replication step. Herein, we showed PGG acted very early during infection with HCV, mainly during attachment of viruses to cells and independently of direct effects on virus particle integrity.

Currently, treatment recommendations against HCV differ depending on the genotype of infection (European Association for Study of, 2015). As an example, genotype 3-infected patients usually need longer treatment courses and would, therefore, also benefit from more effective therapies (McQuaid et al., 2015). Drugs with broad genotype activity are, therefore, needed. In this study, we demonstrated that PGG shows pan-genotypic activity against HCV, potentially contributing to simplifying therapy. Indeed, we could show that combination of PGG with previously known antivirals improved inhibition of HCV infection *in vitro*.

Recently, the potential of interfering with HCV entry was demonstrated by efficient *in vivo* inhibition of HCV infection by neutralizing monoclonal antibodies targeting viral glycoproteins or host receptors or by small molecule fusion inhibitors (Desombere et al., 2016;

Fofana et al., 2010; He et al., 2015; Mailly et al., 2015; O'Shea et al., 2015; Vercauteren et al., 2014) . In addition, adding an entry inhibitor to a DAA therapy prevents on-therapy breakthrough of drug-resistant viral variants (Vercauteren et al., 2015). PGG displayed good bioavailability in SCID mice and resulted in plasma levels above tissue-culture IC_{50} 2 hours after a single dose i.p. injection. However, PGG lead to cytotoxic effects in approximately half of the animals when dosed at 50mg/kg. This data could serve as a guide for future studies addressing dosing of PGG in animal models or for evaluation of PGG-related formulas. Future studies should also address if combination of PGG with second generation DAAs also improves infection outcomes to allow shorter treatment courses or improved responses.

In summary, we identified a natural compound that is easily accessible and shows pan-genotypic antiviral activity. Hence, PGG or PGG-related compounds would be a good candidate as an anti-HCV drug, most probably in combination with current DAAs to shorten therapies. Alternatively, PGG or PGG-related compounds have the potential to be administered to post-liver transplanted patients to prevent graft reinfection by directly inhibiting virus entry.

5.0 References

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