Trachelogenin, a novel inhibitor of hepatitis C virus entry through CD81

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Although much progress has been made in antiviral agents against hepatitis C virus (HCV) in recent years, novel HCV inhibitors with improved efficacy, optimized treatment duration and more affordable prices are still urgently needed. Here, we report the identification of a natural plant-derived lignan, trachelogenin (TGN), as a potent entry inhibitor of HCV without genotype specificity, and with low cytotoxicity. TGN was extracted and purified from Caulis trachelospermi, a traditional Chinese herb with anti-inflammatory and analgesic effects. A crucial function of TGN was the inhibition of HCV entry during a post-binding step without affecting virus replication, translation, assembly and release. TGN blocked virus infection by interfering with the normal interactions between HCV glycoprotein E2 and the host entry factor CD81, which are key processes for valid virus entry. In addition, TGN diminished HCV cell-to-cell spread and exhibited additional synergistic effects when combined with IFN or telaprevir. In conclusion, this study highlights the effect of a novel HCV entry inhibitor, TGN, which has a target that differs from those of the current antiviral agents. Therefore, TGN is a potential candidate for future cocktail therapies to treat HCV-infected patients.

INTRODUCTION

Hepatitis C virus (HCV) belongs to the family Flaviviridae and affects approximately 3% of the world’s population (Thomas, 2013). Until recently, there were no effective prophylactic or therapeutic vaccines available for the treatment of HCV infections. The current antiviral therapy against HCV consists of pegylated IFNα and ribavirin, together with directly acting antivirals (DAAs) such as the protease inhibitors telaprevir, boceprevir and simeprevir, and the nucleotide analogue sofosbuvir (Cox, 2015; Kohli et al., 2014; Pelosi et al., 2012). However, the use of currently available DAAs is limited because of a series of problems including safety profiles, virus breakthroughs and comparatively higher medical costs. Moreover, preventive antiviral drugs and strategies are still unavailable to eradicate virions before their entry, resulting in the high graft re-infection rate in liver transplantation surgeries (Crespo et al., 2012; Liang & Ghany, 2013). Therefore, novel anti-HCV drugs with better response rates, shorter treatment durations, fewer concomitant adverse reactions and cases of drug resistance, and better affordability are still needed.

Most of the current anti-HCV agents such as simeprevir and sofosbuvir target the maturation of HCV non-structural proteins or viral RNA synthesis (Gane et al., 2013; Lawitz et al., 2014). However, the major obstacle with these antivirals is the low fidelity of the virus replication machinery. Entry inhibitors that mostly target host components or key enzymes required for HCV entry have unique properties and promising prospects (Burlone & Budkowska, 2009). HCV entry requires the involvement of essential host entry factors including the tetraspanin molecule CD81, scavenger receptor class B type 1 (SRB1) and tight junction proteins claudin-1 (CLDN1) and occludin (OCLN). Recent studies have identified novel receptors of HCV entry, including transferring receptor 1 (TRR1), the receptor tyrosine kinase epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and cholesterol uptake receptor Niemann-Pick C1-like 1 (NPC1L1), all of which have become optimal anti-HCV targets for HCV...
treatment. In recent years, the discovery of novel and diverse entry inhibitors has accelerated. HCV entry inhibitors such as the virion attachment blocker lactoferrin, the small-molecule compound ITX5061, which targets entry factor SRB1, and the clinically approved anti-cancer compound erlotinib (an EGFR inhibitor) have been evaluated in treating HCV-infected patients in the clinical stage (El-Fakahany et al., 2013; Lupberger et al., 2011; Redwan et al., 2014; Sulkowski et al., 2014). These advances provide new insights into and prospects for the treatment of HCV.

Caulis trachelospermi refers to the stems and leaves of the plant Trachelospermum jasminoides (Lindl.) Lem., which is widely used in East Asia for the treatment of rheumatic arthralgia, aching joints and traumatic injuries. It has also been evaluated for its anti-cancer and anti-inflammatory properties in vitro and in vivo (Lee et al., 2007; Nishibe & Han, 2002). C. trachelospermi is rich in lignans, which are reported to have various biological actions including antiviral activity against human immunodeficiency virus (HIV) (Gnabre et al., 1995), Japanese encephalitis virus (JEV) (Swarup et al., 2008) and dengue virus (Fang et al., 2015). Among the purified lignans from C. trachelospermi, trachelogenin (TGN) has exhibited the most effective antiviral activity. In this study, we confirmed that TGN inhibits HCV entry by interfering with the interactions between HCV glycoprotein E2 and the host entry factor CD81. We evaluated the mode of action of the antiviral TGN and tested its potential as a candidate for future HCV therapy.

RESULTS

Identification of TGN as an HCV entry inhibitor

The lignan compounds were isolated and purified from extracts of C. trachelospermi to evaluate their antiviral activities against HCV in both cell culture-derived HCV (HCVcc) and HCV pseudo-particles (HCVpp) (Fig. 1a). Among these, TGN, a lignanolide, showed the most potent inhibitory effect (Fig. 1a, b). The purity and structure of TGN were confirmed by high-performance liquid chromatography (HPLC) and mass spectrometry (MS) (Fig. 1c, d) and a series of other chemical identification methods (Fig. S1, available in the online Supplementary Material). It was subsequently evaluated for its antiviral potential in HCVcc and HCVpp in a dose-response mode. As shown in Fig. 1(e), this compound inhibited HCVcc infection and HCVpp cell entry in a dose-dependent manner with an IC50 of 0.325 and 0.259 μg ml⁻¹ in HCVcc and HCVpp models, respectively. TGN showed no obvious cytotoxicity in the effective concentration range (Fig. 1f).

TGN does not affect HCV replication, translation, assembly or release

After entering the host cells, the virus takes control of the host machinery to complete post-entry steps, which include viral protein translation, RNA replication and virion assembly and release. TGN inhibited HCV infection more potently during the initial 4 h of viral infection but did not show obvious antiviral activity when added 8 h post-infection (p.i.) (Fig. 2a). To further confirm that TGN did not interfere with the post-entry steps of viral infection, human hepatoma Huh7 cells were transfected with subgenomic Japanese fulminant hepatitis type 1 (JFH-1) RNA, and TGN was added 4 h after transfection. The replication efficiency was assessed 72 h later by examining HCV RNA levels, and the translation efficiency was evaluated by detecting the expression levels of HCV core and NS3 and NS5A proteins. Interferon (IFN) and VX-950 (telaprevir) were used as positive controls. No significant change in HCV RNA level (Fig. 2b) or viral proteins expression (Fig. 2c) was observed after TGN treatment. An HCV BB7 replicon cell line is Huh7 cells that support viral replication of genotype 1b HCV replicon subgenome, and was also utilized to test the influence of TGN on virus replication, and no significant change was observed after TGN treatment (Fig. 2b). To determine whether TGN affects virus assembly or release, the supernatant of the transfected Huh7 cells was collected and intracellular virions were obtained by repeated freeze–thaw lysis of the transfected cells after TGN treatment. The intracellular and extracellular virus infectivity were quantified by infection of naïve Huh7 cells. As shown in Fig. 2(d), TGN did not reduce either intracellular or extracellular virus infectivity, even at a dose of 5 μg ml⁻¹, while naringenin, an assembly inhibitor, reduced both intracellular and extracellular virus infectivity. These results indicated that TGN does not interact with viral polypeptide translation, RNA replication, virus assembly or release.

The inhibitory effect of TGN is pan-genotypic and TGN also inhibits HCV entry into primary human hepatocytes

To test whether the antiviral activity of TGN on HCV entry is genotype dependent, HCVpp harbouring different strains of E1E2 proteins was produced. Vesicular stomatitis virus (VSV) which harboured glycoprotein G (VSV-G), a different fusion protein from HCV, was used as a control. As shown in Fig. 3(a), TGN was found to block HCVpp entry of different genotypes and subtypes in a dose-dependent manner, while for the VSV pseudo-particles no obvious inhibitory effect was observed. Moreover, TGN was able to prevent entry of HCVpp (genotype 1a strain H77 or 1b strain Con1) into primary human hepatocytes, which are thought to be the main reservoir for HCV in the infected host, in a dose-dependent manner without obvious cytotoxicity (Fig. 3b). Thus, TGN is able to inhibit pan-genotypic HCV entry into host cells with a consistent antiviral effect in primary human hepatocytes.

TGN inhibits HCV cell entry during the post-binding stage

To investigate the potential of TGN to perturb virion integrity or lipoprotein association directly, concentrated HCVcc was incubated in the presence of TGN for 4 h before loading on to a 10–40 % iodixanol gradient for
Fig. 1. Identification of TGN as an entry inhibitor of HCV. (a) The lignan compounds in Caulis trachelospermi were screened for their antiviral activities in inhibiting HCVcc infection and HCVpp entry, and also for cytotoxicity. The compounds (0.5/2 µg ml\(^{-1}\)) were added with HCVcc of strain JFH-1 or HCVpp of strain H77 in cell culture for 4 h. VX-950 (0.5 µM) or dasatinib (2 µM) was used as a positive control for HCVcc infection or HCVpp entry, respectively. Results are shown as the percentage relative to the solvent control (DMSO) (521 colonies and 328 positive cells per well for HCVcc infection and HCVpp entry, respectively). **\(P<0.01\), compared to the control group. (b) Chemical structure of TGN. (c) HPLC analysis of TGN. (d) MS analysis of TGN. (e) Huh7 cells were infected with HCVcc JFH-1 (m.o.i. = 1) or HCVpp H77 in the presence of the indicated concentrations of TGN or DMSO for 4 h. At 48 h post-infection (p.i.), the HCVcc-infected cells underwent immunofluorescence staining for the viral NS5A protein. At 72 h p.i., HCVpp-inoculated cells were quantified by flow cytometry. The IC\(_{50}\) values in both experiments were calculated by GraphPad Prism 6 (IC\(_{50}\) HCVcc: 0.325 µg ml\(^{-1}\); IC\(_{50}\) HCVpp: 0.259 µg ml\(^{-1}\)). Results are shown as the percentage of infection or entry compared to the untreated group (589 colonies and 402 positive cells per well for HCVcc infection and HCVpp entry, respectively). (f) Huh7 cells were treated with TGN or DMSO of different concentrations for 24 h before determining cell viability using a Cell Counting kit-8. Results are shown as relative viability compared to the untreated group. Results are means±SD for three independent experiments.
ultracentrifugation. Similar profiles of density distribution and viral HCV RNA levels of the fractions were observed in TGN- and solvent-treated groups, while 0.5 % deoxycholic acid altered the HCV RNA distribution from low density to intermediate density. These results indicated that TGN did not impair viral particle density or lipoprotein association (Fig. 4a). Therefore, TGN may impede HCV cell entry by acting on the interactions with host cells.

Next, we evaluated whether TGN inhibited HCV entry by interfering with virus binding. Concentrated HCVcc was incubated with Huh7 cells at 4 °C for binding in the presence of TGN. The cells were then lysed to determine the amount of bound HCV RNA. Compared with heparin, which strongly impaired HCV binding, TGN did not significantly affect virus binding to the host cells (Fig. 4b).

To further characterize the mechanism of action of TGN, a kinetic test was performed. Purified HCVcc was incubated with Huh7 cells at 4 °C to allow the viral particles to concentrate on the host cells. The plate was then placed in a 37 °C incubator for synchronized virus entry. The cells were treated with TGN for 4 h at different time points of virus entry. Heparin was used as a positive control for virus binding, and significantly blocked HCVcc infection when added prior to HCVcc binding but not when added shortly after virus infection (Fig. 4c). However, TGN exhibited an inhibition pattern similar to chloroquine (an endosomal acidification inhibitor) and JS-81 (a CD81-specific mAb), and inhibited HCV infection potently, even when added 2 h after inoculation (Fig. 4c). These results indicated that lignanolide TGN blocks HCV entry during a post-binding process.
Internalization is an essential process of the post-binding step in HCV. Therefore, we determined whether TGN blocked virus entry by interfering with this process. Cells were inoculated with the purified HCVcc at 4°C in the presence of TGN, and the amount of bound virions was determined by quantifying HCV RNA. For internalization, the cells were incubated at 37°C for 30 min and non-internally virions were removed by trypsinization at 4°C for 1 h.

As expected, heparin strongly reduced the binding of HCV to the cell surface, while no obvious effect was observed for TGN (Fig. 4d). Similarly, the internalized virus ratio was not altered by TGN treatment (Fig. 4d).

TGN impairs interaction between HCV E2 and CD81

After binding, HCV interacts with the host entry factors on the surface of host cells to continue the entry process. Therefore, we analysed whether the expression levels of the essential HCV entry factors CD81, SRB1, CLDN1 and OCLN were altered after TGN treatment. Huh7 cells were incubated with TGN at 2 µg ml⁻¹ for 4 h, and the expression level of entry factors was assessed by Western blotting or flow cytometry. As shown in Fig. 5(a), the expression levels of the four known essential HCV entry factors were unchanged, ruling out the possibility of TGN acting by downregulating the entry factors.

The interactions between HCV E2 and entry factors including SRB1 and CD81 are essential to initiate virus entry. Therefore, we evaluated whether these interactions were diminished by TGN treatment. Chinese hamster ovary (CHO) cells stably expressing human SRB1 or CD81 (CHO-SRB1 or CHO-CD81) (Fig. S2) were established, and incubated with cell lysate containing HCV E2 proteins in the presence of TGN. The efficiency of bound E2 was quantified by flow cytometry. Compared with naïve CHO cells, E2 protein was robustly concentrated on the surface of CHO-CD81 or CHO-SRB1 cells (Fig. 5b). However, this phenomenon in CHO-CD81 cells was largely diminished following treatment with TGN (Fig. 5b). As for CHO-SRB1 cells, TGN did not show any inhibitory effect (Fig. 5b).

In order to investigate further the inhibitory effect of TGN, we performed co-immunoprecipitation experiments between HCV E2 and CD81. Purified CD81 large extracellular loop–His fusion protein (CD81 LEL–His) was incubated with HCV E2 proteins in the presence of TGN. We used an anti-His antibody to precipitate CD81 and, as shown in Fig. 5(c), the TGN-treated group showed less precipitation with E2 proteins. In contrast, we also utilized an HCV E2–His fusion protein of strain H77 to precipitate with CD81 LEL, and the blocking property of TGN was consistent with the above experiment (Fig. 5d). These observations indicated that TGN interferes with the interaction between HCV E2 and CD81 proteins.

Additionally, molecular docking analysis was used to investigate possible associations between TGN and CD81.

Fig. 3. Pan-genotypic inhibitory effect of TGN and antiviral effect on primary human hepatocytes. (a) The antiviral effect of TGN on HCVpp harbouring different strains of HCV E1/E2. Huh7 cells were treated with different concentrations of TGN, together with HCVpp of different genotypes or glycoprotein G of vesicular stomatitis virus (VSV-G) for 4 h. At 72 h post-incubation, positive cells were quantified by flow cytometry. Results are shown as the percentage relative to the control (DMSO) (336, 298, 406, 268, 196, 187 or 149 positive cells per well for VSV-Gpp entry or HCVpp entry of genotypes 1a, 1b, 2a, 4a, 5a or 6a, respectively). (b) Primary human hepatocytes were treated with the indicated concentrations of TGN and HCVpp of genotype 1a strain H77 or genotype 1b strain Con1 for 4 h. At 72 h post-incubation, the entry rate was measured by flow cytometry. Results are shown as the percentage relative to the control (DMSO) (128 or 207 positive cells per well for HCVpp entry of H77 or Con1, respectively). The viability of the primary human hepatocytes at 24 h after TGN treatment was determined using Cell Counting kit-8 and is shown as relative viability. Results are means ± SD for three independent experiments.
The data suggested that TGN can form hydrogen bonds with several amino acid residues (Thr166, Asn184, Lys187 and Glu188) on CD81 LEL, the high-affinity binding site for viral envelope glycoprotein E2, thereby disrupting the normal interactions between HCV E2 and CD81 (Fig. S3a). We constructed mutations of CD81–His according to the docking hypothesis and tested the inhibitory effect of TGN on the interaction between HCV E2 and CD81 with specific mutations. The results showed that mutation of Lys187 or Glu188 on CD81 largely reversed the original antiviral activity of TGN, suggesting that the hydrogen bond formed between TGN and amino acid residue Lys187 or Glu188 might contribute to the blocking activity of this compound (Fig. S3b).

Fig. 4. TGN inhibits HCV cell entry during the post-binding stage. (a) Concentrated HCVcc JFH-1 was incubated with TGN (2 μg ml⁻¹), solvent or 0.5 % deoxycholic acid at 37 °C for 4 h followed by the removal of TGN using an Amicon Ultra-15 Centrifugal Filter Unit. HCVcc was then subjected to 10–40 % iodixanol gradient ultracentrifugation. Each of the 12 gradient fractions was collected, weighed to calculate the density (left graph) and RT-qPCR was performed to test the HCV RNA levels of each fraction (right graph). (b) Huh7 cells were incubated with HCVcc at 4 °C for 2 h to facilitate virus binding with either 2 μg TGN ml⁻¹ or 200 μg heparin ml⁻¹. The culture was then removed and the cells were washed three times with cold PBS, and the quantity of bound viral particles was determined by RT-qPCR. (c) Kinetic assay of TGN on virus infection. Huh7 cells were incubated with HCVcc at 4 °C for 90 min to allow the viral particles to concentrate on the host cells. The virus was removed and the plate was placed in a 37 °C incubator for synchronized virus entry. TGN (2 μg ml⁻¹), and heparin (200 μg ml⁻¹), chloroquine (10 μM) or the CD81-specific mAb JS-81 was added at the indicated time points to allow 4 h of treatment during different periods of virus infection (dotted lines in panel). At 48 h p.i., infected cells were quantified by immunofluorescence. Results are shown as the percentage relative to the control (DMSO) (545, 572, 533, 524, 589, 577, 584 or 596 colonies per well for HCVcc infection in the respective time periods). (d) Huh7 cells were incubated with HCVcc at 4 °C for 90 min for virus binding in the presence of TGN (2 μg ml⁻¹) or heparin (200 μg ml⁻¹). A fraction of the cells was washed with cold PBS and further lysed to detect bound HCV RNA levels by RT-qPCR. Another fraction of cells was placed in a 37 °C incubator for 30 min to allow internalization of viral particles followed by trypsinization at 4 °C for 1 h to remove the non-internalized virions. The RNA levels of the internalized viral particles were determined by RT-qPCR.
TGN exhibits synergistic inhibitory effects and hinders cell-to-cell transmission

IFN-α is an essential component of current HCV treatment, and VX-950, also known as telaprevir, is an inhibitor of HCV NS3-4A protease. The synergistic effect of TGN combined with IFN-α or VX-950 was investigated to explore the potential of the compound for use in cocktail therapy. We found that the addition of TGN to IFN or VX-950 increased the efficacy of these agents in inhibiting HCV infection (Fig. 6a, b). The median effect and combination index of TGN when combined with IFN or VX-950 were also evaluated by CalcuSyn (Fig. S4a, b). The results suggested that TGN exhibited a synergistic effect when combined with IFN or VX-950, as the combination index value of both combination therapies was <1.0 (Fig. S4).

Cell-to-cell spread is an important transmission mode of HCV infection, resulting in refractory infection or graft reinfection in post-transplant HCV patients. This mode of transmission is resistant to anti-E2 neutralization antibodies or agarose (Brimacombe et al., 2011). We performed both experiments to determine whether TGN hindered cell-to-cell transmission. While being treated with 2 μg TGN ml⁻¹, infected Huh7 cells, which were overlaid with agarose-containing medium or anti-E2 neutralizing antibody AR3A, displayed much smaller foci sizes compared with the control group, as shown in immunofluorescence images (Fig. 6c, d) and relevant quantification graphs (approx. 52 and 34 cells per focus for controls, and seven and eight cells for TGN treatment, respectively) (Fig. 6e, f). Together, these results suggested that TGN inhibits HCV cell-to-cell transmission.

DISCUSSION

Current advancements in biomedical technology have enhanced our understanding of the molecular mechanisms of HCV entry, which has helped to promote the
Fig. 6. TGN exhibits a synergistic effect on virus infection and blocks HCV cell-to-cell transmission. (a, b) HCVcc was incubated with Huh7 cells for 4 h in the presence of the indicated concentrations of TGN. At 12 h p.i., the indicated concentrations of IFN-α or VX-950 were added to the respective cultures. The infected cells were quantified at 48 h p.i. by immunofluorescence. Results are shown as the percentage of infection compared to the untreated group (633 colonies per well for HCVcc infection). Results are means ± SD for three independent experiments. (c, d) Huh7 cells were incubated with HCVcc for 2 h before overlaying with 1% agarose dissolved in culture medium or AR3A anti-E2 neutralizing antibody in the presence of 2 μg TGN ml⁻¹. At 48 h p.i., the infected cells were quantified by immunofluorescence, and their nuclei were stained with DAPI. (e, f) The numbers of cells per positive colony were counted in 15 foci.
development of entry inhibitors that focus on the treatment of the early phase of HCV infection. Over the last few years, a series of entry inhibitors have been used, increasing the genetic barrier as well as optimizing the antiviral efficacy against HCV infections. In our study, we identified TGN, a major lignan constituent in C. tracheliopercetti, as a potent inhibitor of HCV entry. TGN inhibited virus entry with no genotype specificity, and the antiviral activity was also confirmed in primary human hepatocytes, which resemble human HCV disease more closely. This compound was found to block viral entry at a post-binding step, most probably by interfering with viral E2 protein interactions with the host CD81, an essential factor for the entry of HCV entry into host cells. TGN also prevented HCV cell-to-cell transmission, which is a main route of virus transmission for graft reinfection.

It is known that the tetraspanin CD81 plays an important role during HCV entry into host cells. Of the identified entry factors, only CD81 and SRB1 have been reported to interact directly with the soluble form of HCV envelope glycoprotein E2, which is a key viral protein throughout the life cycle and disease progression of HCV infections (Qin et al., 2015; Vieyres et al., 2010). Specific anti-CD81 mAbs effectively interfere with E2–CD81 interactions and inhibit HCV infections in humanized mice (Li et al., 2015; Meuleman et al., 2008). However, as CD81 is a ubiquitously expressed protein in almost all tissues, the toxicity issues limit its use. Small-molecule compounds like TGN selectively abolish the function of CD81 during HCV entry while sparing the physiological functions (Fig. S5), such as the cellular association between CD81 and CD151. The current study showed that TGN significantly reduced the binding of E2 to CHO-CD81 cells, implying that CD81 is an antiviral target of TGN. CD81 LEL was found to be the high-affinity binding domain for the viral protein E2 (Drummer et al., 2002; Petracca et al., 2000). E2/CD81 LEL interactions have been reported to show a significant contribution to HCV-associated liver damage (Zhang et al., 2010). Co-immunoprecipitation analysis of CD81 LEL and HCV E2 showed a significantly decreased interaction between these two proteins with TGN treatment, and computational simulation analysis predicted that TGN can bind to four sites on CD81 LEL. Collectively, these results suggest that the LEL of CD81 may be the target for TGN.

Lignans are a class of compounds consisting of two phenylpropanoid derivatives. The active constituents of lignans have been reported to have antiviral activity against several viruses including influenza virus, IAV and HIV. They have been reported to effectively suppress the expression of HIV as well as inhibiting localization of the virus to host cells (Lee et al., 2010; Seal et al., 2011; Xiao et al., 2010). In this study, most of the lignans from C. tracheliopercetti showed inhibitory effects on HCV entry, of which TGN was the most potent. Whether the lignanolide structure-based compounds have universal anti-HCV properties needs further investigation. In addition, studies comparing the antiviral efficacy of different modifications on the lignanolide structure would be useful.

TGN differs from protease or polymerase inhibitors of HCV regarding its mode of action, and therefore has potential to be an optimal antiviral agent for the early stage of HCV infection. Combination assays of TGN with IFN or VX-950 showed synergistic antiviral effects. In cases of liver transplantation in end-stage HCV patients, graft reinfection remains one of the main issues preventing significant recovery. The inhibitory effects of TGN on cell-to-cell transmission suggest the possibility of its use in preventing graft reinfection in HCV patients.

Thus, the natural lignanolide product TGN is a promising HCV entry inhibitor for future antiviral therapy development and exploration.

**METHODS**

**Cell culture.** Human hepatoma (Huh7) cells, human embryonic kidney (HEK 293T) cells and CHO cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS, 1× non-essential amino acids, 100 IU streptomycin/penicillin ml⁻¹ and 2 mM l-glutamine (Gibco, Invitrogen). Primary human hepatocytes were purchased from ScienCell Research Laboratories and cultured according to the manufacturer’s instructions (Nahmias et al., 2006).

**Chemicals and antibodies.** Heparin and chloroquine were purchased from Sigma-Aldrich. VX-950, dasatinib and naringenin from Selleck and DAPI from Invitrogen were also used in this study. Anti-HCV NS5A mAb was purchased from ViroGen Corp. Anti-HCV NS3 or core antibody, anti-TAPA1 polyclonal antibody and anti-SRB1 mAb were obtained from Abcam. Anti-CD81 mAb JS-81 was from BD Biosciences. Anti-CLDN1, anti-OCLN antibodies, Alexa Fluor 488- and HRP-conjugated anti-goat, anti-rabbit or anti-mouse IgG were from Invitrogen. Anti-His tag mAb was purchased from Proteintechn. Anti-HCV E2 antibodies (H52, H53 and H48) were kindly provided by J. Dubuisson (Institute Pasteur de Lille, Lille, France).

**HCV infection assay.** Huh7 cells were seeded on a 96-well plate overnight and then infected with HCVcc strain JFH-1 together with the indicated concentrations of TGN for 4 h at 37 °C. The cells were cultured for 48 h before the infection was measured using an immuno-fluorescence assay according to a previous study (Zhong et al., 2005).

**HCV entry assay.** Huh7 cells were seeded on a 96-well plate overnight and then inoculated with HCVpp strain H77 together with the indicated concentrations of TGN for 4 h at 37 °C. The cells were cultured for 72 h before the entry of HCVpp was measured using flow cytometry as described previously (Bartosch et al., 2003).

**Indirect immunofluorescence assay.** Infected Huh7 cells were washed with PBS and fixed with cold methanol followed by the detection of NS5A expression with mAb 9E10 to check infectivity (Tong et al., 2011).

**Viability assay.** A viability assay was performed after 24 h of TGN treatment using a Cell Counting kit-8 (Beyotime Biotechnology).

**Freeze–thaw lysis of HCV-transfected cells.** Huh7 cells were transfected with JFH-1 RNA and seeded on six-well plates for 4 h before treatment with TGN for 24 h. After 48 h of transfection, the cell supernatant was collected to measure the extracellular virus.
infectivity. The intracellular virions of the HCV-electroporated cells were collected by three freeze–thaw cycles in liquid nitrogen and a 37 °C incubator as described previously (Haid et al., 2012). Virus infectivity was detected by inoculating naïve HuH7 cells.

**Iodixanol density-gradient fractionation.** Concentrated and purified HCVcc was incubated with TGN at 37 °C for 4 h followed by the removal of TGN using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore). The density-gradient centrifugation and fractionation were performed according to previously described protocols (Bankwitz et al., 2010).

**HCVcc binding and internalization assay.** HuH7 cells were infected with purified HCVcc in a 24-well plate for 90 min at 4 °C with TGN. The cells were washed three times with ice-cold PBS and further lysed to extract the total RNA. The bound HCV RNA levels were quantified by quantitative real-time PCR (qPCR). For the internalization assay, purified HCVcc was bound to HuH7 cells according to the above description, followed by a 30 min incubation at 37 °C for virus internalization of the viral particles. The cells were then trypsinized at 4 °C to remove non-internalized virions, and internalized virions were measured by qPCR as described in a previous study (Vausselin et al., 2013).

**Western blotting and flow cytometry.** Western blotting and flow cytometry were performed to analyse the expression level of HCV entry factors as described previously (Zhu et al., 2012). Briefly, HuH7 cells were incubated with TGN for 4 h before they were lysed to determine the expression levels of SRB1, CLDN1 and OCLN by Western blotting. The expression level of CD81 was measured by flow cytometry.

**HCV E2 binding to human SRB1 or CD81.** The production of CHO cells expressing human SRB1 or CD81 and HCV E2 protein is given in Supplementary Methods. The binding of E2 to the cell surface SRB1 or CD81 was determined by flow cytometry as described in a previous study (Guan et al., 2012). Briefly, 1 × 10⁵ CHO cells expressing human SRB1, CD81 or empty vector were incubated with cell lysate containing H77 E2 protein in the presence of TGN for 4 h at 37 °C. The cells were washed three times with PBS, and the bound E2 on the cells was quantified by flow cytometry using anti-E2 mAb H53.

**Immunoprecipitation.** Immunoprecipitation was performed according to a protocol described previously (Dubuisson et al., 1994). The CD81 LEL–His protein or E2–His protein was incubated with cell lysate containing H77 E2 protein or CD81 LEL protein in the presence of TGN for 4 h at 37 °C. The supernatants were centrifuged at 12 000 × g for 10 min, and then incubated with anti-His antibody at 4 °C overnight. Protein G–agarose beads (Roche Diagnostics) were then added and incubated for 3 h at the same temperature. Immune complexes were collected by centrifugation and washed three times with washing buffer. The samples were analysed by Western blotting.

**Cell-to-cell transmission assay.** An HCV cell-to-cell transmission assay was performed in two ways (Calland et al., 2012; Law et al., 2008). Briefly, HuH7 cells were incubated with concentrated HCVcc for 2 h before overlaying with 1% agarose dissolved in culture medium or AR3A anti-E2 neutralizing antibodies in the presence or absence of TGN. At 48 h p.i., the infected cells were quantified by immunofluorescence and their nuclei were stained with DAPI.

**Statistical analysis.** Bar and line graphs showing the means ± SD of at least three independent experiments were plotted. Statistical analyses were performed using srs 17.0. A P-value of <0.05 in Student’s t-test was considered statistically significant.

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