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# Benzhydrylpiperazine compounds inhibit cholesterol-dependent cellular entry of hepatitis C virus

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ABSTRACT

Hepatitis C virus (HCV) remains a serious global health problem that lacks an effective cure. Although the introduction of protease inhibitors to the current standard-of-care interferon/ribavirin therapy for HCV infection has improved sustained virological response of genotype 1-infected patients, these inhibitors exacerbate already problematic side effects. Thus, new HCV antivirals are urgently needed. Using a cell-protection screen previously developed in our laboratory, we evaluated 30,426 compounds for inhibitors of potentially any stage of the HCV life cycle and identified 49 new HCV inhibitors. The two most potent hits, hydroxyzine and chlorcyclizine, belong to the family of benzhydrylpiperazines and were found to inhibit the entry of cell culture-produced HCV with $IC_{50}$ values of 19 and 2.3 nM, respectively, and therapeutic indices of >500 and >6500. Both compounds block HCV entry at a late stage of entry prior to viral fusion and their inhibitory activities are highly dependent on the host's cholesterol content. Both compounds are currently used in the clinic for treating allergy-related disorders and the reported peak plasma level (160 nM) and estimated liver concentration (1.7 μM) of hydroxyzine in humans are much higher than the molecule's anti-HCV $IC_{90}$ in cell culture (64 nM). Further studies are therefore justified to evaluate the use of these molecules in an anti-HCV therapeutic regimen.



## 1. Introduction

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA enveloped virus belonging to the *Flaviviridae* family (Simmonds, 1999). After initial infection, 75–85% of patients develop chronic hepatitis and at least one-fifth of chronically infected patients develop cirrhosis within 20 years (Seeff, 2002). It is estimated that 3–4 million people contract HCV each year with more than 300,000 deaths attributed to HCV-end stage liver disease annually (Bukh, 2012; Davis et al., 2010). Until recently, a non-specific antiviral therapy – pegylated interferon α in combination with ribavirin – has been the only standard of care (SOC) therapy for HCV infection (Hoofnagle and Seeff, 2006). Although the recent addition of protease inhibitors – telaprevir and boceprevir – to the SOC has significantly increased the sustained virological response, improvement is accompanied by a more severe side effect profile (Jacobson et al., 2011; Lee et al., 2012; Poordad et al., 2011). Moreover, both inhibitors are not very effective against patients who were previously unresponsive to the SOC, and monotherapy with either molecule causes the rapid emergence of drug-resistant mutants (Susser et al., 2011). Thus, compounds with novel modes of action and low toxicity are still urgently needed.

The entry of HCV into hepatocytes is a highly coordinated process involving the viral envelope glycoproteins and multiple host cell factors. Heparin sulfate glycosaminoglycans and low density receptor proteins are the first attachment sites for HCV (Albecka et al., 2012; Barth et al., 2003; Molina et al., 2007; Shi et al., 2013). Although the exact sequential order of receptor engagement is still unclear, some evidence suggest that viruses interact with scavenger receptor class B type 1 (SR-B1), CD81, tight junction proteins claudin-1 (CLDN1), occludin (OCLN) (Sourisseau et al., 2013) and possibly other factors (Meredith et al., 2012). Virions are later internalized through clathrin-mediated endocytosis and fuse with the host membrane following endosomal acidification (Meertens et al., 2006). More recently the host factors Niemann Pick C1 Like 1 (NPC1L1), transferrin receptor 1 (TfR1), epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) have also been implicated in HCV entry, but their specific roles remain to be elucidated (Lupberger et al., 2011; Martin and Uprichard, 2013; Sainz et al., 2012).

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Previously, our lab developed a cell protection screen based on an engineered hepatoma cell line – n4mBid – that undergoes pronounced apoptosis upon HCV infection (Chen et al., 2010; Chockalingam et al., 2010). This assay is not only highly amenable for the high throughput screening of HCV antivirals but allows for the simultaneous identification of inhibitors targeting potentially any stage of the viral life cycle. In the current study, we screened 30,426 compounds using this cell protection assay at The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB) and identified 49 compounds with previously unknown anti-HCV activity. The best two inhibitors – hydroxyzine and chlorcyclizine – belong to the benzhydrylpiperazine family of $H_1$-antihistamines and displayed anti-HCV $IC_{50}$ values in the nanomolar range and therapeutic indexes >500. Importantly, hydroxyzine is approved by the Food and Drug Administration (FDA) for human therapeutic use and is able to achieve plasma concentrations higher than its anti-HCV $IC_{90}$ in cell culture. Both compounds strongly blocked HCV entry at a step immediately prior to virus fusion. Inhibition was highly dependent on host cholesterol content, suggesting that benzhydrylpiperazines may exert their effects through a Niemann–Pick C1-like 1 cholesterol absorption receptor (NPC1L1)-dependent pathway.

## 2. Materials and methods

### 2.1. HCV infection assay

To determine the anti-HCV activities, Huh-7.5 cells seeded in poly-L-lysine treated 96-well plates ($2 \times 10^4$ cells/well) 4–6 h earlier were infected with Jc1 Gluc HCVcc (MOI 0.01, virus was dialyzed into phosphate buffered saline (PBS) buffer) in complete growth medium (DMEM + 1× non-essential amino acid + 10% FBS) in the presence of increasing concentrations of the compounds. Supernatant Gluc activity was measured 72 h post-infection using a BioLux Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA). Viability of the remaining cells was quantified using the CellTiter-Glo assay (Promega, Madison, WI) to gauge the drug toxicity.

### 2.2. HCV entry assay

Huh-7.5 cells ($2 \times 10^4$ cells/well) seeded in poly-L-lysine-treated 96-well flat-bottom plates the previous day were inoculated with Jc1 Gluc HCVcc (MOI 1), HCVpp (5-fold final dilution) or pseudoparticles devoid of any envelop protein ($Env^-$pp; 5-fold final dilution) in the presence of the compounds at 37 °C/5% $CO_2$. Three hours post infection cells were thoroughly washed and replenished with 100 μL of fresh compound-free growth medium. Supernatant Gluc activities were quantified 72 h post infection using a BioLux Gaussia Luciferase assay kit (New England Biolabs, Ipswich, MA) and expressed as a percentage of the 0.015% DMSO treatment control.

### 2.3. Effect of cellular cholesterol

To examine the effects of cellular cholesterol on the anti-HCV activity of benzhydrylpiperazines, we infected cells with different amount of cholesterol with HCVcc in the presence of the compounds. For the cholesterol depletion assays, Huh-7.5 cells were seeded in poly-L-lysine treated 96-well plate at $2.8 \times 10^4$ cells/well. The next day, these cells were incubated with 0, 1, 5 or 10 mg/mL of methyl-beta-cyclodextrin (mβcd) (Sigma Aldrich, St. Louis, MO) for 1 h at 37 °C, washed thoroughly with complete growth medium to remove mβcd and infected with Jc1 Gluc HCVcc (MOI ~1) in the presence of the compounds for 1 h at 37 °C. Cells were thoroughly washed to remove residual virus and the compounds, and replenished with fresh media. Supernatant Gluc activity was quantified 72 h post infection using a BioLux Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA) and normalized to cell viability gauged by the CellTiter-Glo assay (Promega, Madison, WI). Values are expressed as percentage of that from 0.015% DMSO-treated cells. For the cholesterol replenishment experiments, Huh-7.5 cells seeded in 48 well plates at $4.8 \times 10^4$ cells/well the previous day were treated with 0 or 10 mg/mL mβcd in serum-free growth medium (DMEM containing 1× non-essential amino acid) for 1 h at 37 °C, washed thoroughly and then incubated with 0 or 150 ng/μL of water soluble cholesterol (Sigma Aldrich, St. Louis, MO) in the same medium for 1 h at 37 °C. Cells were then thoroughly washed and infected with Jc1 Gluc HCVcc (MOI ~1) in growth medium containing 5% FBS in the presence of the compounds for 1 h at 37 °C. Viral infectivity was quantified 72 h post infection by measuring supernatant Gluc activity using a BioLux Gaussia Luciferase Assay Kit (New England Biolabs, Ipswich, MA) and normalized to that of 0.015% DMSO-treated cells.

### 2.4. Effect of virion cholesterol

To evaluate the effect of virion cholesterol content on the anti-HCV activity of benzhydrylpiperazines, we compared the efficacy of the compounds against two strains of HCVcc with different cholesterol content – JFH-1 WT ($HCVcc^{WT}$) and the high-cholesterol-content mutant strain G451R HCVcc ($HCVcc^{G451R}$). Briefly, Huh-7.5 cells were seeded in poly-L-lysine treated 48-well plates at $2 \times 10^4$ cells/well and incubated at 37 °C and 5% $CO_2$ for 5–6 h to allow attachment. Cells were exposed to the compounds for one hour prior to infection with $HCVcc^{WT}$ or $HCVcc^{G451R}$ ($TCID_{50}$/mL $5.62 \times 10^3$) for 14–16 h at 37 °C and 5% $CO_2$. The next day, cells were washed thoroughly and replenished with 300 μL of inhibitor-containing medium. Seventy-two hours post inoculation, cells were washed once with Dulbecco's phosphate-buffered saline (Thermo Scientific HyClone, Logan, UT) and subjected to one freeze–thaw cycle at −80 °C/ambient temperature before RNA extraction using the EZNA Total RNA kit (Omega Bio-Tek, Norcross, GA). The amount of HCV RNA was quantified via TaqMan quantitative reverse transcriptase-PCR (qRT-PCR) (qScript One-Step Fast kit; Quanta Biosciences, Gaithersburg, MD), using previously described primers (Takeuchi et al., 1999). As an internal control, mRNA levels of phosphoglycerate kinase 1 (PGK) (qScript One-Step SYBR Green qRT-PCR kit; Quanta Biosciences, Gaithersburg, MD) were determined for each RNA template using previously described primers (Chockalingam et al., 2010).

Additional material and methods can be found in the Supplementary Information.

## 3. Results

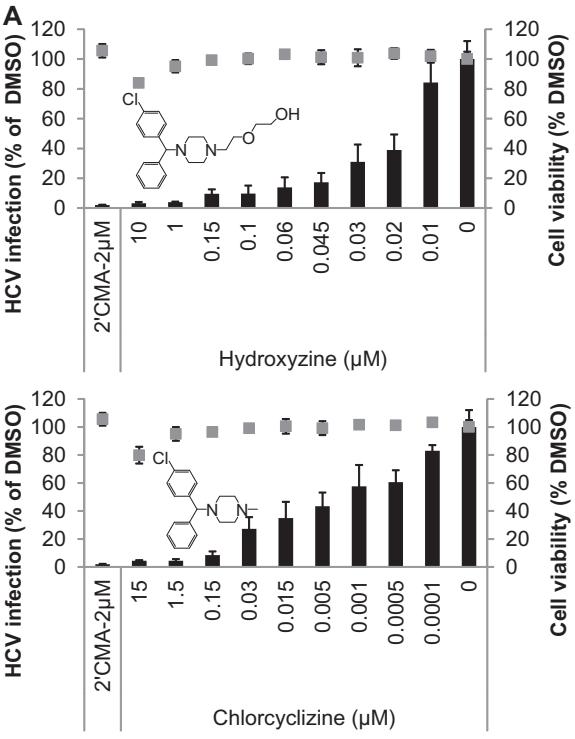
### 3.1. Identification of anti-HCV compounds using a cell protection screen

Previously we engineered a human hepatoma cell line – n4mBid – that sensitively reports HCV infection via a cell death phenotype (Chen et al., 2010). Using this reporter cell line, we screened seven libraries of known bioactive compounds, commercial compounds and natural extracts, totaling 30,426 compounds. The primary screening was performed in duplicate 384-well plates at a single-compound dosage range of 5–12.5 μg/mL. Ninety-seven compounds exhibited ⩾40% rescue of cells from HCV-mediated cell death, including the known HCV inhibitors cyclosporine (Ciesek et al., 2009; Fernandes et al., 2010), flunarizine (Chockalingam

et al., 2010), and tamoxifen (Watashi et al., 2007). To confirm the results of the cell protection assay, hits were subjected to a secondary HCV infectivity screen using PBS-dialyzed Jc1 Gluc HCVcc. Forty-nine of the candidates reduced HCV infection by at least 40% when compared to mock-treated cells (Table S1). Dose responses of the top 10 hits were determined using the same assay (Fig. S1). Among these, four compounds belong to the benzhydrylpiperazines family, including candidates 65 and 73, hydroxyzine and chlorcyclizine, respectively. The anti-HCV $IC_{50}$ and $CC_{50}$ values for hydroxyzine and chlorcyclizine are 19 and 2.3 nM and >10 and >15 μM, respectively, corresponding to therapeutic indices of >500 and >6500 (Fig. 1). These two compounds were selected for further characterization.

### 3.2. Benzhydrylpiperazines do not inhibit HCV replication or production

To evaluate the effect of the hydroxyzine and chlorcyclizine on HCV replication and production, Huh-7.5 cells were electroporated with RNA from the HCV reporter strain Jc1 Gluc HCVcc and exposed to the compounds. HCV replication levels were quantified 72 h post compound addition by measuring the Gluc reporter activity in the supernatant. Slight-to-no inhibition was observed for either of the compounds at the tested concentrations (Fig. 2A). To determine the effect of the compounds on virion production, supernatants from drug-treated cells were diluted 100-fold and used to infect naïve Huh-7.5 cells. Supernatant Gluc activity was quantified 72 h post inoculation, normalized to that of control cells infected with viruses from DMSO-treated cells in the presence of the corresponding drug at 100-fold diluted concentration and used as indication of the amount of infectious virus in the supernatant. No inhibition was observed for either of the compounds even at concentrations at or above 10 μM (Fig. 2B). Collectively these results suggest that viral replication and production are not inhibited by benzhydrylpiperazines and that their anti-HCV activity may derive from their ability to block the entry step.




B

| Compound | $IC_{50}$ | $IC_{90}$ | $CC_{50}$ | $CC_{50}/IC_{50}$ |
|---|---|---|---|---|
| Hydroxyzine | 19nM | 64nM | >10μM | >526 |
| Chlorcyclizine | 2.3nM | 394nM | >15μM | >6,521 |

**Fig. 1.** Benzhydrylpiperazines inhibit HCVcc infection. (A) Huh-7.5 cells were infected with Jc1 Gluc HCVcc (MOI 0.01) in the presence of increasing concentrations of hydroxyzine or chlorcyclizine. Supernatant Gluc activity was measured 72 h post infection, normalized to that of 0.15% DMSO treated cells and used as indication of viral infection. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate. 2′CMA: 2′-C-methyladenosine (Carroll et al., 2003) (B) $IC_{50}$ and $IC_{90}$ values of hydroxyzine and chlorcyclizine were calculated using the sigmoidal fit function in Origin Lab (OriginLab, Northampton, MA).

### 3.3. Benzhydrylpiperazines suppress HCVcc entry

Since benzhydrylpiperazines clearly inhibit HCVcc infection but did not appear to inhibit viral replication or production, we tested the hypothesis that these compounds block HCV entry. Huh-7.5 cells were infected with HCVcc or HCVpp/Env⁻pp in the presence of the compounds at 37 °C/5% $CO_2$ for 3 h. Supernatant Gluc activities were quantified 72 h later and used as an indication of viral infection. The short contact time between the compounds and the cells/viruses is designed to limit the effect of the compounds to the entry step alone. Both compounds strongly blocked the entry of HCVcc but not HCVpp (Fig. 3). A modest inhibition of entry for HCVpp (<2-fold), but not VSV-Gpp, was only observed at 10 and 15 μM of hydroxyzine and chlorcyclizine, respectively (Fig. S2). The ability of both benzhydrylpiperazines to selectively block the entry of HCV but not VSV-Gpp suggests that cellular processes such as clathrin-mediated endocytosis and endosomal acidification are likely not impaired by these compounds at the tested concentrations, and that hydroxyzine and chlorcyclizine likely inhibit an aspect of HCV entry that is not strongly represented in the lentiviral HCVpp model.

Next, we investigated the stage of entry affected by the benzhydrylpiperazines. Huh-7.5 cells were incubated with Jc1 Gluc HCVcc (MOI 5) at 4 °C for 1.5 h, thoroughly washed to remove unbound virions and shifted to 37 °C/5% $CO_2$ to initiate virus entry. Compounds were added at different time points post temperature shift as illustrated in Fig. 4A. Both benzhydrylpiperazines retained inhibitory activity for up to 100 min after the temperature shift to 37 °C (Hydroxyzine $t_{½}$ ~53 min, Chlorcyclizine $t_{½}$ ~63 min), displaying a similar time-dependent inhibition profile to that of the endosomal acidification inhibitor bafilomycin ($t_{½}$ ~57 min) (Fig. 4B). Thus, these compounds inhibit a post binding step of HCVcc prior to virus-cell fusion.

### 3.4. Entry inhibition by benzhydrylpiperazines is host cholesterol-dependent

The ability of benzhydrylpiperazines to block the entry of HCVcc but not HCVpp prompted us to examine the differences between these systems. One of the major differences lies in their distinct cholesterol profiles. HCVpp contains 94% less cholesterol than authentic HCVcc (Sainz et al., 2012) when produced from 293T cells that do not have cholesterol-associated lipoproteins (Farquhar and McKeating, 2008). On the other hand, cell culture-adapted $HCVcc^{G451R}$, which contains a G451R point mutation in the viral E2 glycoprotein, was found to have ~50% more cholesterol than the wild-type HCVcc ($HCVcc^{WT}$) (Zhong et al., 2006). To evaluate whether the entry inhibition by benzhydrylpiperazines is dependent on virion cholesterol content, Huh-7.5 cells were infected with $HCVcc^{WT}$ or $HCVcc^{G451R}$ in the presence of the compounds, and the viral RNA from infected cells was quantified 48 h post infection. As shown in Fig. 5A, $HCVcc^{G451R}$ was more

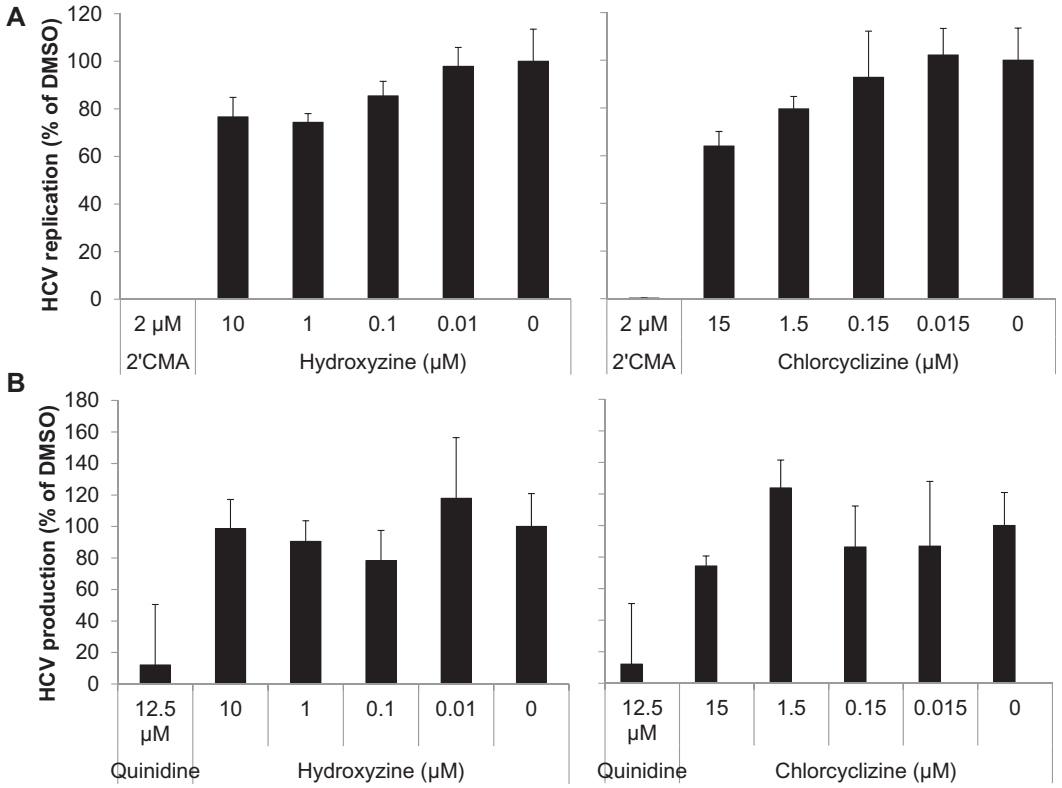



**Fig. 2.** Benzhydrylpiperazines do not inhibit HCV replication or virus production. (A) Huh-7.5 cells were electroporated with Jc1 Gluc HCVcc RNA. After cell attachment (4–6 h post seeding), cells were exposed to the indicated compounds. Supernatant Gluc activity was measured 72 h post compound addition, normalized to 0.15% DMSO treated cells and used as an indication of viral replication. (B) Virus-containing supernatants from (A) were diluted 100-fold in complete growth medium and used to infect naive cells. Supernatant Gluc activity was quantified 72 h post inoculation, normalized to that of control cells infected with viruses from DMSO-treated cells in the presence of the corresponding drug at 100-fold diluted concentration and used as indication of the amount of infectious virus in the supernatant. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate.

sensitive to inhibition by benzhydrylpiperazines than $HCVcc^{WT}$, while the Js81 antibody which binds to the HCV entry receptor CD81 was equally effective at blocking the entry of both viruses at the tested concentration, suggesting dependence of the entry inhibitory activity of benzhydrylpiperazines on the cholesterol content of virions. However, it is also noted that there are a number of other differences between $HCVcc^{WT}$ and $HCVcc^{G451R}$, including loss of SR-B1 and CD81 dependency and an altered relationship between particle density (lipoprotein association) and infectivity (Grove et al., 2008). Thus, the observed increased sensitivity of G451R towards benzhydrylpiperazines may derive from any of these other factors. Removal of viral cholesterol via mβcd renders HCV significantly less infectious (data not shown (Aizaki et al., 2008)). HCVcc with slightly reduced cholesterol content by low concentration of mβcd treatment retained similar sensitivity toward benzhydrylpiperazines compared to untreated virus (Fig. S3).

Next, we examined the effect of host cholesterol content on viral entry inhibition by benzhydrylpiperazines. Huh-7.5 cells were exposed to different concentrations of a cholesterol depletion agent (mβcd) for 1 h at 37 °C/5% $CO_2$, washed and then infected with Jc1 Gluc HCVcc (MOI ~1) for 1 h in the presence of the compounds. Mβcd treatment significantly reduced the ability of benzhydrylpiperazines to block HCV entry (Fig. 5B). Next, Huh-7.5 cells treated with 10 mg mβcd were incubated in medium containing 150 ng/μL of water-soluble cholesterol for 1 h at 37 °C/5% $CO_2$ to replenish cholesterol, followed by infection with Jc1 Gluc HCVcc (MOI ~1) for 1 h in the presence of the compounds. Replenishment of cholesterol partially restored the antiviral activity of chlorcyclizine (Fig. 5C). Taken together, these data suggest that the potency of benzhydrylpiperazine is dependent on host cholesterol content. Similar results were obtained with Js81, as cell-surface CD81 levels are affected by the host's cholesterol content (Kapadia et al., 2007; Voisset et al., 2008).

## 4. Discussion

We carried out a high-throughput screen of 30,426 compounds for inhibitors of potentially any stage of the HCV life cycle using an unbiased cell-based assay (Chockalingam et al., 2010) and discovered 49 compounds with previously undocumented anti-HCV activity. We categorized these hits into 16 groups based on their chemical structure. The two most potent inhibitors, hydroxyzine and chlorcyclizine, were shown to suppress the infection of cell culture-produced HCV (HCVcc) with estimated $IC_{50}$ values of 19 and 2.3 nM, respectively, and $CC_{50}$ values of >10 and >15 μM, corresponding to therapeutic indices of >500 and >6500. Hydroxyzine and chlorcyclizine are members of the first generation $H_1$ antihistamines belonging to the family of benzhydrylpiperazines (Brunton et al., 2011). Other activities of hydroxyzine and chlorcyclizine include blockade of muscarinic, serotonin and α-adrenergic receptors (Simons and Simons, 2011). Chlorcyclizine (trade name: Ahist (chlorcyclizine) Oral), an over-the-counter drug for treating allergy-related symptoms including stuffy nose, runny nose, itching and sneezing, has been reported to have virucidal/virustatic (Chakrabarty et al., 2000), antimetabolic (Hossain et al., 1987) and anticarcinogenic (Dastidar et al., 2006) properties. Hydroxyzine was approved by the U.S. FDA in 1956 for the treatment of allergy-related disorders under the trademark name Atarax

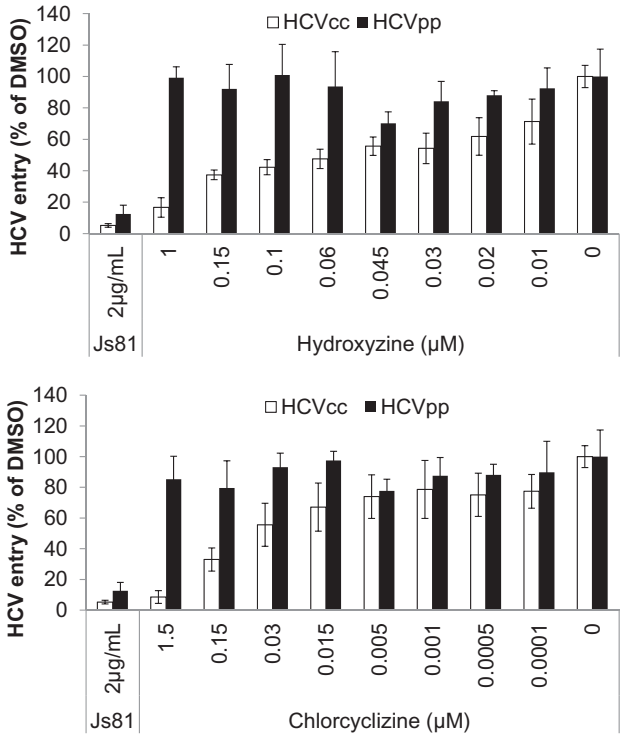



**Fig. 3.** Benzhydrylpiperazines block HCVcc but not HCVpp entry. Huh-7.5 cells were infected with Jc1 Gluc HCVcc (MOI 1), H77 HCVpp (5-fold diluted supernatant) or pseudoparticles devoided of envelop protein (Env⁻pp; 5-fold diluted) in the presence of compounds for 3 h at 37 °C/5% $CO_2$ prior to washing to remove virus and replenishment with compound-free growth medium. Supernatant Gluc activity was measured 72 h post infection. Values are expressed as a percentage of the control cells treated with 0.015% DMSO. For HCVpp, raw Gluc values were subtracted from Env⁻pp prior to DMSO normalization. Values and error bars represent the mean and standard deviation, respectively, of two independent experiments carried out in duplicate.

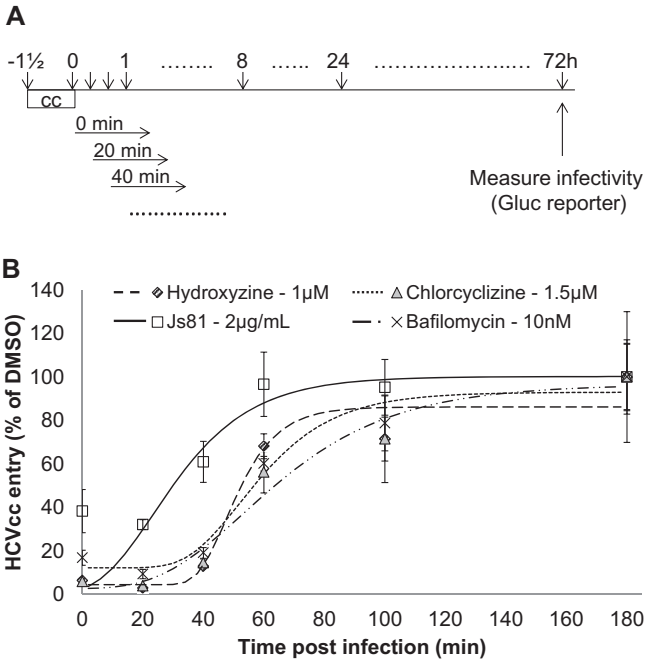



**Fig. 4.** Benzhydrylpiperazines inhibit a late stage of HCV entry. (A) Schematic representation of experiment. (B) Huh-7.5 cells were incubated with Jc1 Gluc HCVcc (MOI 5) at 4 °C for 1.5 h, thoroughly washed, replenished with virus-free growth medium, and shifted to 37 °C/5% $CO_2$. The compounds were added to the cells at the indicated time points post temperature shift and removed 5 h later. Supernatant Gluc activity was measured 72 h post temperature shift and expressed as a percentage of activity from the final compound addition time point to indicate viral entry. Values and error bars represent mean and standard deviation, respectively, of two independent experiments carried out in duplicate.

(Jellinek et al., 1995). The serum half-life of oral dosages of hydroxyzine (0.7 mg/kg) ranges from 21 to 29 h with a peak plasma level of 72–77 ng/mL (160–171 nM) (Simons et al., 1989, 1984) and an expected liver concentration of up to 1.7 μM (Hamelin et al., 1998). The anti-HCV $IC_{90}$ of hydroxyzine in cell culture is 64 nM (Fig. 1), which is well within the range of reported plasma levels, pointing to a strong anti-HCV clinical potential of this compound. Moreover, hydroxyzine is currently prescribed for the alleviation of adverse dermatological effects experienced by hepatitis C patients undergoing treatment with telaprevir (Cacoub et al., 2012; Nguyen and Morgan, 2012).

Neither hydroxyzine nor chlorcyclizine is virucidal against HCVcc (Fig. S4) or significantly affects HCV replication or virus production (Fig. 2). Both compounds were instead found to block the entry of HCVcc at a step immediately prior to the fusion process (Figs. 3 and 4). Interestingly, these compounds did not significantly block the entry of lentiviruses pseudotyped with HCV envelope proteins (HCVpp), which have a much lower cholesterol content than HCVcc (Sainz et al., 2012) (Fig. 3), and more potently inhibit the entry of HCV mutant G451R (Fig. 5A), which was found to have ~50% more cholesterol than the wild-type HCVcc (Zhong et al., 2006). However, since $HCVcc^{G451R}$ also display an altered relationship between particle density (lipoprotein association) and infectivity and are less dependent on CD81 and SR-B1 (Grove et al., 2008), we cannot rule out the possibility that the increase potency of our compound against $HCVcc^{G451R}$ is impacted by these other factors. On the other hand, the potency of benzhydrylpiperazine appears to be strongly dependent on host cholesterol content, with significantly reduced HCV inhibition in cells treated with cholesterol-depletion agent mβCD and partially restored inhibition upon replenish of cellular cholesterol (Fig. 5B and C).

To date, three groups of HCV-host fusion inhibitors have been reported. Since HCV fusion is triggered by the acidic environment in the late endosome, the first group of compounds are endosomal acidification inhibitors such as concanamycin A, bafilomycin A, chloroquine and ammonium chloride (Koutsoudakis et al., 2006). The second group of compounds target the viral and/or host lipid membrane and include arbidol (Teissier et al., 2011), silymarin (Wagoner et al., 2010), phenothiazines (Chamoun-Emanuelli et al., 2013), RAFIs (Colpitts et al., 2013; St Vincent et al., 2010) and LJ001 (Wolf et al., 2010). The third group fusion inhibitors are of yet unclear mechanism including ferroguine, a chloroquine analog (Vausselin et al., 2013), and phosphorothioate oligonucleotides, amphipathic DNA polymers (Matsumura et al., 2009). Our studies showed that benzhydrylpiperazine compounds inhibit HCV entry at a step prior to HCV fusion inhibitors although further studies are needed to demonstrate whether these compounds indeed inhibit HCV fusion.

Cholesterol plays an important role in HCV fusion. Depletion of host cholesterol can significantly reduce viral entry by altering the localization of entry factors CD81 and NPC1L1 (Aizaki et al., 2008; Ge et al., 2011; Kapadia et al., 2007). Cholesterol was also found to enhance the fusion efficiency between HCVpp/cc and receptor-free liposomes (Haid et al., 2009; Lavillette et al., 2006, 2007). Previously, Sainz et al. identified Niemann–Pick C1-like 1 (NPC1L1), a cholesterol adsorption receptor, as a new HCV entry factor (Sainz et al., 2012). NPC1L1, a homolog of Niemann–Pick 1 receptor, is a polytopic transmembrane protein important for cholesterol adsorption (Davies and Ioannou, 2000). It is highly expressed in the small intestine and liver (Altmann et al., 2004). Depending on the extracellular cholesterol concentration, NPC1L1 is localized

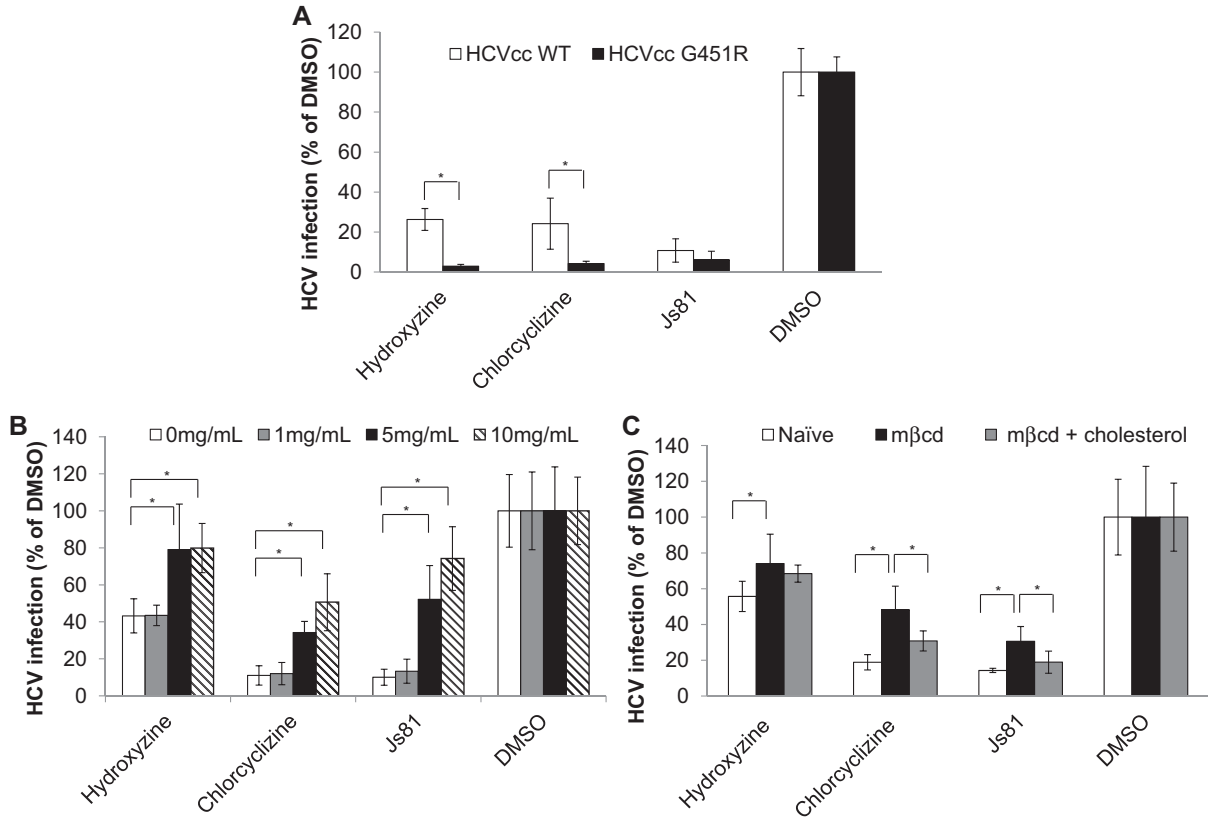



**Fig. 5.** Benzhydrylpiperazine entry inhibition is cholesterol dependent. (A) Huh-7.5 cells were infected with $HCVcc^{WT}$ or the high-cholesterol-content mutant virus $HCVcc^{G451R}$ (MOI 0.04) in the presence of hydroxyzine (1 μM), chlorcyclizine (1.5 μM), Js81 antibody (2 μg/mL) or DMSO (0.015%) for 14–16 h. Intracellular HCV RNA was quantified 72 h post inoculation. Values and error bars represent the mean and standard deviation, respectively, of two independent experiments carried out in duplicate. (B) Huh-7.5 cells were exposed to the indicated concentrations of mβcd for 1 h at 37 °C/5% $CO_2$. After treatment, cells were thoroughly washed and inoculated with Jc1 Gluc HCVcc (MOI ~1) for 1 h at 37 °C/5% $CO_2$ in the presence of hydroxyzine (1 μM), chlorcyclizine (1.5 μM), Js81 antibody (2 μg/mL) or DMSO (0.015%). Supernatant Gluc activity was quantified 72 h post infection. Values and error bars represent the mean and standard deviation, respectively, of at least three independent experiments carried out in duplicate. (C) Huh-7.5 cells were treated with 0 or 10 mg/mL of mβcd for 1 h at 37 °C/5% $CO_2$. After treatment, some of the cells were incubated in serum-free growth medium containing 150 ng/μL of exogenous cholesterol for 1 h at 37 °C/5% $CO_2$ to replenish cholesterol. Excess cholesterol was removed and the cells were infected with Jc1 Gluc HCVcc (MOI ~1) in the presence of hydroxyzine (1 μM), chlorcyclizine (1.5 μM), Js81 antibody (2 μg/mL) or DMSO (0.015%) for 1 h at 37 °C/5% $CO_2$. Supernatant Gluc activity was quantified 72 h post infection. Values and error bars represent the mean and standard deviation, respectively, of at least two independent experiments carried out in duplicate. Statistical significance was determined by the Student's *t* test (*$p < 0.1$).

either on the plasma membrane or in intracellular compartments (Altmann et al., 2004; Davies et al., 2005). Ezetimibe (trademark name Zetia) blocks the internalization of NPC1L1 and thus inhibits cholesterol adsorption and lowers serum cholesterol level (Davis and Veltri, 2007; Ge et al., 2008). Interestingly, ezetimibe was also found to potently block the entry of HCVcc, but not HCVpp, at a post-binding, prefusion step and its inhibitory potency was also higher against $HCVcc^{G451R}$ (Sainz et al., 2012), similar to that of benzhydrylpiperazines (Fig. 5A). Neither hydroxyzine nor chlorcyclizine was found to affect NPC1L1 mRNA expression level (data not shown). However, it is possible that benzhydrylpiperazines may affect the cellular localization of NPC1L1. Oral administration of hydroxyzine and chlorcyclizine in mice were found to result in reduced serum, but increased liver, cholesterol content (Schmidt and Martin, 1965), thus changes in the host cholesterol homeostasis by benzhydrylpiperazines may ultimately impact HCV entry efficiency.

It is worth mentioning that both hydroxyzine and chlorcyclizine were able to inhibit the fusion of both HCVcc and HCVpp with liposome in the *in vitro* fusion assay (Fig. S5). Although the *in vitro* fusion assay has greatly augmented our understanding of HCV fusion, there are a number of key differences between viral fusion in cell culture and *in vitro*. For example, fusion of Jc1 HCVcc with liposomes requires a lower pH than that for HCVpp. A plausible explanation would be that fusion *in cellulo*, to be optimal, would require additional factors or receptors that are lacking in our *in vitro* fusion assay (Haid et al., 2009) but could be compensated at lower pH values through a high protonation level. In addition, no fusion of HCVcc with the plasma membrane was observed upon exposure to low pH (Meertens et al., 2006; Sharma et al., 2011). Therefore fusion inhibition observed in the *in vitro* assay does not necessarily translate to the exact same mechanism in cell culture.

In summary, we carried out a screen for inhibitors of potentially any stage of the HCV life cycle and identified several novel inhibitors, including the benzhydrylpiperazine class of compounds. The anti-HCV activity of the two leading screening hits from this class, hydroxyzine and chlorcyclizine, were further characterized. Both compounds were found to block a post attachment stage of HCV entry at a time point prior to viral fusion with the plasma membrane. The antiviral effect of both compounds is dependent on the host cholesterol content. Both hydroxyzine and chlorcyclizine have already been used in humans for the treatment of allergy-related symptoms, justifying further studies to evaluate these molecules for HCV therapy.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.06.014.

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