Research Article
Viral Hepatitis

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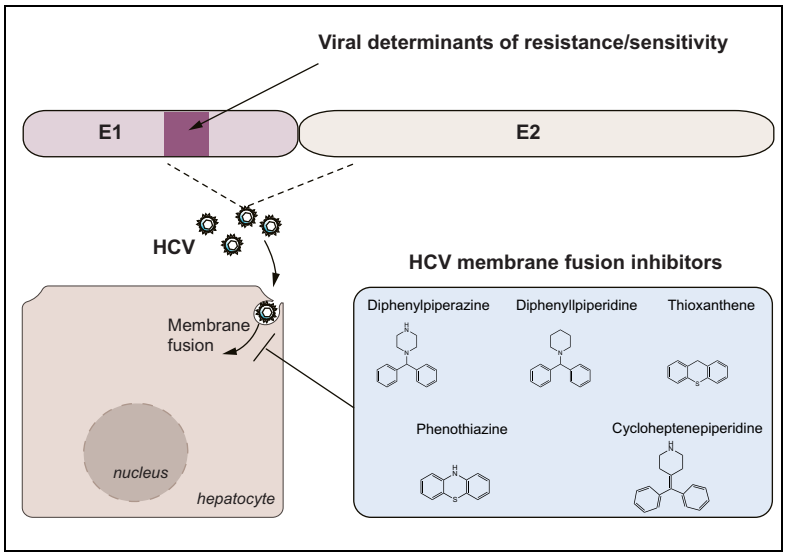
A central hydrophobic E1 region controls the pH range of hepatitis C virus membrane fusion and susceptibility to fusion inhibitors

Graphical abstract



Highlights

- Diverse compounds from different chemotypes preferentially inhibit HCV genotype 2 membrane fusion.
- Viral determinants controlling susceptibility to these compounds map to a central hydrophobic region of E1.
- Four conserved residues within this region govern sensitivity to these compounds and pH-dependence of membrane fusion.
- The hydrophobicity of this region, proximal to the putative HCV fusion loop, predicts susceptibility to these compounds.
- Resistance to these compounds correlates with more relaxed requirements for pH-triggering of membrane fusion.

Authors

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Lay summary

This study describes diverse compounds that act as HCV membrane fusion inhibitors. It defines viral properties that determine sensitivity to these molecules and thus provides information to identify patients that may benefit from treatment with membrane fusion inhibitors.

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A central hydrophobic E1 region controls the pH range of hepatitis C virus membrane fusion and susceptibility to fusion inhibitors

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Background & Aims: Hepatitis C virus (HCV) infection causes chronic liver disease. Antivirals have been developed and cure infection. However, resistance can emerge and salvage therapies with alternative modes of action could be useful. Several licensed drugs have emerged as HCV entry inhibitors and are thus candidates for drug repurposing. We aimed to dissect their mode of action, identify improved derivatives and determine their viral targets.

Methods: HCV entry inhibition was tested for a panel of structurally related compounds, using chimeric viruses representing diverse genotypes, in addition to viruses containing previously determined resistance mutations. Chemical modeling and synthesis identified improved derivatives, while generation of susceptible and non-susceptible chimeric viruses pinpointed E1 determinants of compound sensitivity.

Results: Molecules of the diphenylpiperazine, diphenylpiperidine, phenothiazine, thioxanthene, and cycloheptenepiperidine chemotypes inhibit HCV infection by interfering with membrane fusion. These molecules and a novel *p*-methoxy-flunarizine derivative with improved efficacy preferentially inhibit genotype 2 viral strains. Viral residues within a central hydrophobic region of E1 (residues 290–312) control susceptibility. At the same time, viral features in this region also govern pH-dependence of viral membrane fusion.

Conclusions: Small molecules from different chemotypes related to flunarizine preferentially inhibit HCV genotype 2 membrane fusion. A hydrophobic region proximal to the putative fusion loop controls sensitivity to these drugs and the pH range of membrane fusion. An algorithm considering viral features in this region predicts viral sensitivity to membrane fusion inhibitors. Resistance to flunarizine correlates with more relaxed pH requirements for fusion.

Lay summary: This study describes diverse compounds that act as HCV membrane fusion inhibitors. It defines viral properties that determine sensitivity to these molecules and thus provides information to identify patients that may benefit from treatment with membrane fusion inhibitors.

Introduction

Hepatitis C virus (HCV) is a highly variable, enveloped virus of the family *Flaviviridae*. According to sequence analyses, viral isolates are classified into 7 genotypes and 86 subtypes.[1] HCV particles harbor a plus-strand RNA genome of positive polarity that encodes a polyprotein comprising structural proteins, the p7 ion channel and various non-structural proteins. Virus particles are composed of the core protein that encases the viral RNA, and of the envelope 1 and envelope 2 (E1-E2) glycoproteins, which are embedded in the viral lipid membrane. Virion-associated E1-E2 glycoproteins coordinate interactions with cellular receptors, cell uptake and membrane fusion which is triggered by the low pH in cellular endosomes.

If left untreated, chronic infection by HCV can cause severe liver disease including liver fibrosis, cirrhosis and hepatocellular carcinoma. Approximately 71 million individuals are chronically infected and at risk of developing disease. The licensing of multiple classes of direct-acting antivirals (DAAs) has in recent years revolutionized antiviral treatment. These drugs either target the viral NS3 protease, the NS5A phosphoprotein or the NS5B polymerase. Combination therapies involving these drugs are active against viruses from genotype 1 to 6 and reach cure rates of greater than 95%.[2]

Resistance-associated substitutions (RASs) have been observed for all classes of DAAs and associated with treatment failure.[3,4] Although viral resistance and treatment failure occur infrequently, due to the relative short history of these new

Keywords: Hepatitis C virus (HCV); Membrane fusion; Fusion inhibitors; Antivirals; Resistance.
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treatments it is difficult to predict to which level salvage therapies including novel drug classes will be required in the future.

Cell entry inhibitors comprise part of HIV-1 combination therapies,[5] and are in clinical development for treatment of hepatitis B and hepatitis delta virus infections.[6] Cell entry inhibitors which either target viral components or cellular factors have also emerged as an interesting class of inhibitors in HCV.[7] Preclinical studies have confirmed their efficacy *in vitro* and *in vivo* and have shown that combined with DAAs they prevent breakthrough of antiviral-resistant HCV.[8] In the course of several independent screening campaigns involving various compound libraries, including for instance the NIH clinical compound (NCC) collection or the library of pharmacologically active compounds (LOPAC1280), a number of HCV entry inhibitors were discovered.[9] These HCV entry inhibitors have diverse but related chemical scaffolds including molecules from the group of diphenylpiperazines, diphenylpiperidines, phenothiazines, thioxanthenes, and cycloheptenepiperidines (Fig. 1A). This group includes licensed drugs with neuroleptic (flunarizine) or anti-histamine (chlorcyclizine) activity.[10,11] Antiviral activity in the nanomolar range and proven *in vivo* efficacy render some of these compounds attractive candidates for further development as HCV entry inhibitors. However, it remains unclear if these diverse compounds share their mode of action, if they target a common viral site, and which virus strains and protein(s) are susceptible to these types of inhibitors.

Herein we compared the mode of action of these diverse chemical scaffolds, screened for derivatives with improved anti-HCV activity, and determined the viral target in the glycoprotein E1 290–312 region. Interestingly, amino acids in this region also modulate the pH range required for virus-host membrane fusion.

Materials and methods

HCV infection assay

Huh-7.5 cells stably expressing firefly luciferase (Huh-7.5-fluc) were seeded in 12 or 96 well plates. The following day cells were inoculated with infectious viral particles in the presence of the test compounds and incubated at 37 °C. After 4 h, the inoculum was aspirated, cells were washed with PBS, supplied with fresh medium and incubated at 37 °C. *Renilla* and firefly luciferase activity were quantified 48 h later to determine infection efficiency and cell viability, respectively.

Fusion at the plasma membrane assay

We conducted the assay as previously described.[10] Briefly, Huh-7.5 cells were seeded in 6 well plates (3×10^5 cell/well) and incubated at 37 °C overnight (18 h). Subsequently, cells were pre-treated with 5 nM concanamycin A (Con A) for 1 h at 37 °C (additional steps were carried out in presence of 5 nM Con A to block acidification of endosomes). Cells were inoculated with virus preparations at 4 °C for 2 h. Subsequently, cells were washed twice with PBS, fresh medium with or without test compounds was added and cells were incubated at 37 °C for 1 h. After this, cells were treated with pH 5.0 citric acid buffer for 5 min at 37 °C in presence or absence of the test compounds. Cells were washed with PBS again, and fresh medium with or without test compounds was added and cells were incubated at 37 °C for 3 h. Finally, cells were washed with PBS, fresh medium without Con A and test compounds were added. Cells were further incubated for 48 h at 37 °C. After this, cells were lysed and *Renilla* luciferase activity, indicative of infection efficiency, was assessed.

HCV whole life cycle compound screening assay

Flunarizine derivatives were screened for their antiviral activity in the HCV whole life cycle compound screening assay as previously described.[10] Briefly, Huh-7.5-fluc cells were transfected with genomic RNA of a GT2a *Renilla* luciferase reporter construct JcR2a.[12] Cells were seeded in 96 well plates and incubated at 37 °C. After 4 h, media containing serially diluted compounds was added to the cells which were further incubated at 37 °C. After 48 h, media from the transfected cells was harvested and inoculated onto a new set of naïve target Huh-7.5-fluc cells which were incubated at 37 °C for 48 h. The transfected cells were lysed with water and the cell lysates were analyzed for *Renilla* and firefly luciferase output to assess viral genome replication and cell viability respectively. The target cells were lysed after 48 h to assess *Renilla* luciferase reporter activity for the whole life cycle.

Results

Flunarizine resistance mutations confer cross-resistance to diphenyl piperazine, diphenyl piperidine, phenothiazine, thioxanthene and cycloheptene piperidine HCV entry inhibitors

Recently, we and others identified flunarizine, chlorcyclizine (CCZ), pimozide, chlorpromazine, fluphenazine, trifluoperazine, mequitazine, cis-flupentixol and cyproheptadine, which are from the diphenyl piperazine, diephenyl piperidine, phenothiazine, thioxanthene and cycloheptene piperidine families, respectively, as potent HCV entry inhibitors (Fig. 1A;[9] and references therein). Passaging of an HCV genotype 2a virus (Jc1) in the presence of flunarizine yielded a combination of 3 mutations that map to the E1 and E2 proteins and which confer an approximately 50-fold resistance to flunarizine.[10] These mutations also conferred cross-resistance to pimozide, fluphenazine, and trifluoperazine.[10] To explore if susceptibility to other phenothiazine-, thioxanthene- or cycloheptene piperidine-derived entry inhibitors was also affected by these residues, we infected cells in the presence of increasing doses of these compounds with either the parental Jc1 *Renilla* luciferase virus (JcR2a) or the flunarizine-resistant variant carrying 4 mutations (M267V and Q289H in E1 and M405T in E2 and I757T in p7; JcR2a-Flun-R). Note that among these 4 mutations only the 3 changes in E1 and E2 contribute to flunarizine resistance. Each of them independently confers partial resistance which is additive when all 3 changes are combined.[10] Curcumin, a structurally unrelated HCV entry inhibitor, was used as control (Fig. 1B;[13]). As expected, all compounds inhibited JcR2a infection in a dose-dependent manner (Fig. 1C). Except for curcumin, which equally inhibited JcR2a and the flunarizine-resistant Jc1 variant JcR2a-Flun-R, all other compounds were much less active against JcR2a-Flun-R (change in IC50 18 to 275-fold). These results therefore indicated that M267V, Q289H and M405T mutations confer cross-resistance to diphenyl piperazine, diphenylpiperidine, phenothiazine, thioxanthene and cyloheptene piperidine based HCV entry inhibitors and suggest that these molecules inhibit HCV cell entry through a common mechanism.

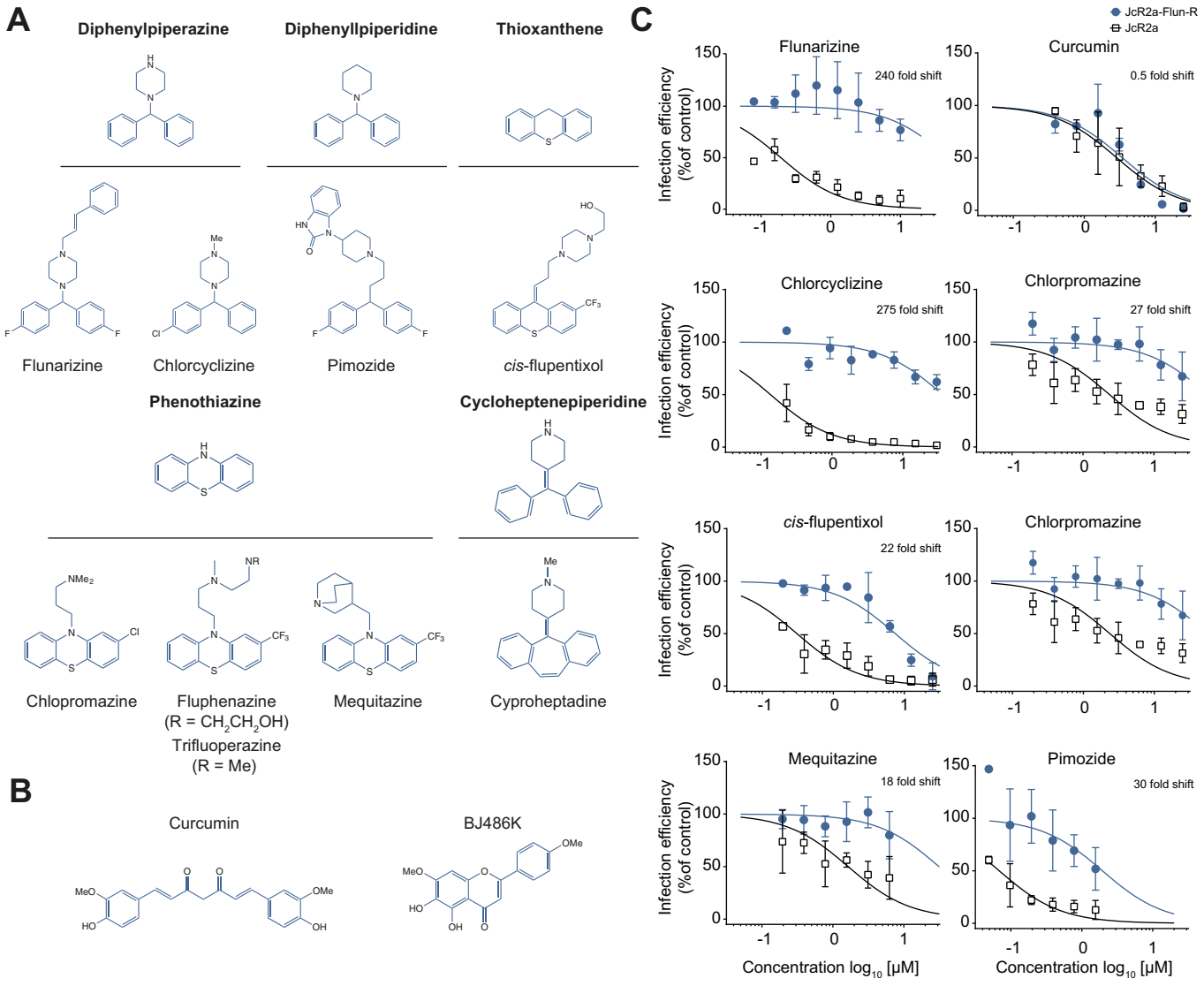

Fig. 1. Resistance mutations to flunarizine confer cross-resistance to structurally related HCV entry inhibitors. (A) Chemical scaffolds of recently described HCV entry inhibitors of the diphenylpiperazine, diphenylpiperidine, thioxanthene, phenothiazine, and cycloheptenepiperidine families[9] and references therein). (B) Curcumin and BJ486K, 2 HCV entry inhibitors[13,16] with grossly different chemical scaffold. (C) HCV infection of Huh-7.5-fluc cells by JcR2a or JCR2a-Flun-R in the presence of increasing doses of given compounds. Infection efficiency is expressed relative to the DMSO control and the fold shift of the IC50 of compounds against the 2 viruses is given. Mean values of 3 biological replicates ± SD are given. HCV, hepatitis C virus.

Diphenyl piperazines, diphenyl piperidines, phenothiazines, and thioxanthenes inhibit low pH-triggered HCV cell membrane fusion

HCV infects liver cells by way of virus-cell membrane fusion, which is essential to deliver the viral genomic RNA into hepatocytes for initiation of infection.[7] Fusion occurs after the virus has interacted with receptors and is internalized into endosomes. These receptor interactions prime the E1/E2 proteins to respond to the acidified pH in endosomes with conformational changes that mediate virus-host cell membrane fusion.[10,15] When acidification of endosomes is inhibited – for instance by treatment with concanamycin A that inactivates endosomal ATPases needed for acidification of these organelles – HCV infection is ablated.[14] Under these conditions, infection can be rescued by briefly washing virus-exposed cells with a low pH buffer that triggers virus membrane fusion and allows infection.[10,15] Using this setup, we recently provided evidence that flunarizine specifically inhibits HCV membrane fusion since application of this compound during the 5 min low pH wash was sufficient for its full antiviral activity (Fig. 2 and[10]). Here we compared the activity of a panel of HCV entry inhibitors in this membrane fusion assay. Compounds tested included a flunarizine-related diphenyl piperazine (chlorcyclizine), a diphenyl piperidine (pimozide), a phenothiazine (mequitazine), and a thioxanthene (cis-flupentixol). In addition, we used BJ486K which is a flavonoid that is structurally unrelated to flunarizine (Fig. 1, and[16]) and likely inhibits HCV entry by a different mechanism. In line with this assumption, and in contrast to flunarizine, BJ486K did not inhibit HCV infection when administered only during the low pH washing step (Fig. 2). In contrast, all tested flunarizine-related compounds from the family of diphenyl piperazines, diphenyl piperidines, phenothiazines, and thioxanthenes inhibited HCV infection when added only during the low pH wash. This finding supported the conclusion that these related compounds selectively inhibit low pH-triggered HCV cell membrane fusion.

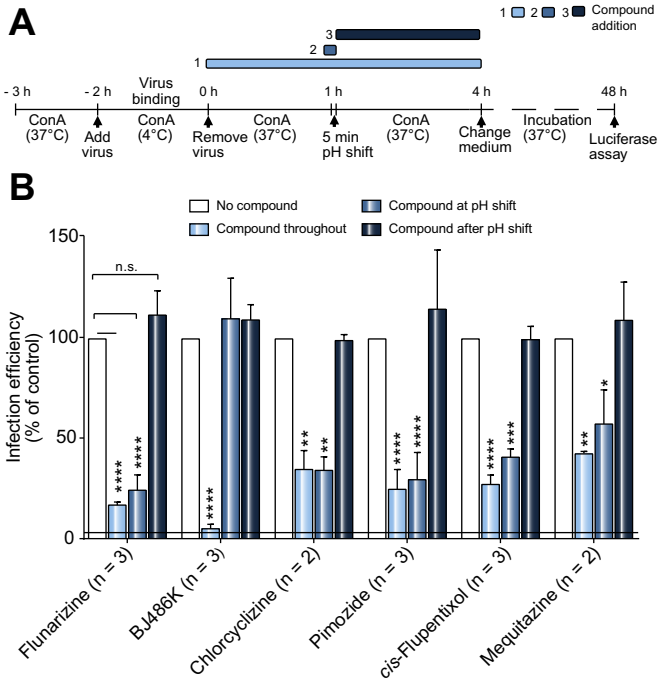

Fig. 2. Compounds structurally related to flunarizine inhibit HCV membrane fusion at the plasma membrane. (A) A schematic representation of the experimental procedure is given at the top. Huh-7.5 cells were incubated with 5 nM Con A 1 h before virus inoculation and throughout the experiment until 4 h post virus inoculation. Additional drugs or DMSO were applied as indicated by green, red or blue bars according to protocols denominated I, II or III, respectively. JcR2a particles were inoculated for 2 h at 4 °C. Virus membrane fusion at the plasma membrane was triggered by washing cells with a pH 5 buffer for 5 min, 1 h after inoculated cells were shifted to 37 °C. In all treatments, cells were incubated another 48 h at 37 °C before infection efficiency was quantified by luciferase assays. (B) Infection is expressed relative control infections in absence of compound (white bars). Mean values of two to three biological replicates ± SD are given. Con A, concanamycin A, HCV, hepatitis C virus. Significance was tested using an ordinary 2-way ANOVA test followed by Dunnett's multiple comparison tests. *p* values below the level of 0.05 were considered significant (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001).

Diphenyl piperidines preferentially inhibit of HCV GT2 membrane fusion

Among the compounds tested by He *et al.* and us, flunarizine and chlorcyclizine emerged as most potent HCV membrane fusion inhibitors (*i.e.* lowest IC50 value).[10–11] Therefore, we focused our analysis on these 2 molecules. Previously we observed that flunarizine preferentially inhibits HCV GT2 viruses including viral strains from subtypes 2a, 2d, 2e, 2k, 2m and 2q and that it was less active against other viral genotypes.[10] To explore if cholorcyclizine exhibits a similar preference for GT2 over other HCV genotypes we tested inhibition of HCV strains Con1 (GT1b), J8 (GT2b), S52 (GT3a), ED43 (GT4a), SA13 (GT5a), HK6a (GT6a) and QC69 (GT7a). In contrast to curcumin which was similarly active against all tested viruses, both flunarzine and cholorcyclizine preferentially inhibited Jc1, a G2a virus chimera, and they were less active against a GT2b strain (J8) and all other strains from non-GT2 genotypes tested (Fig. 3). Thus, both flunarizine and chlorcyclizine exhibit a similar strain-dependent antiviral activity suggesting that they inhibit HCV membrane fusion through a common mechanism, possibly involving a similar viral target site.

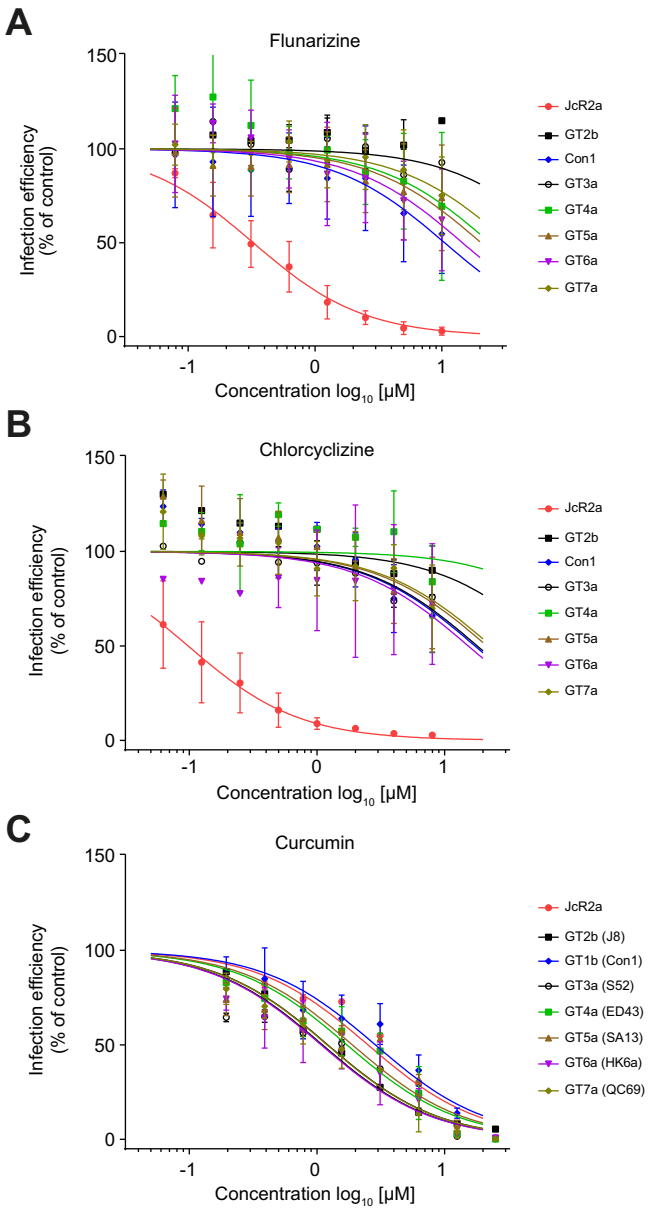

Fig. 3. Inhibition of HCV infection by flunarizine and chlorcyclizine is strain-dependent. JcR2a and given *Renilla* luciferase reporter viruses carrying structural proteins of indicated viral strains were inoculated with increasing doses of (A) flunarizine, (B) chlorcyclizine, or (C) curcumin. Infection is expressed relative to control infections in presence of solvent (DMSO). Mean values of 3 biological replicates ± SD are given. HCV, hepatitis C virus.

Structure-activity relationship-studies with new flunarizine derivatives

To explore if the antiviral activity of flunarizine or the spectrum of targeted viral chimeras can be further improved, we used computational chemical modeling to identify flunarizine and chlorcyclizine-related molecules that exhibit anti-HCV activity (supplementary materials and methods). This approach identified -(Bis(4-Fluoropenyl) Methyl)-4-(3-Pyridinylmethyl) Piperazine which exhibited an antiviral activity comparable to flunarizine (Fig. S1). Moreover, we synthesized a series of novel flunarizine derivatives (Table S1). The design of this compound library focused on structural changes in the allylic element and the adjacent arene substituent. We deliberately excluded structural changes in the piperazine ring and the bis(4-fluorophenyl)

methane unit. All derivatives were prepared from 4,4′-difluorbenzophenone according to the synthetic protocol reported in reference.[17,18] Moreover, a series of molecules bearing structural similarities to flunarizine were selected, which are commercially available. With this compound library in hand we tested their antiviral activity in a whole life cycle compound screening assay[10] showing that 30 exhibited antiviral activity against the Jc1 reporter virus (Table S1). Clearly, the (*E*)-configured allyl element is essential as demonstrated when comparing entries 16 and 17 in Table S1. An additional methyl substituent at the alkene is also not beneficial (entry 20). Several additional substituents at the phenyl ring, particularly in the para position to the allyl element modulate and in selective cases increased the antiviral activity. Particularly, differently substituted amino substituents including the free amino group (entries 3, 6. 7 and 10), triazoles (entries 11–13) need to be mentioned. Noteworthy, substituents that can serve in photo affinity labeling studies (entries 4 and 14) only lead to a moderate loss of activity. Methoxy substituents located in the para and meta positions were found to be beneficial (entries 2 and 19). *p*-Methoxy-flunarizine (entry 2) exhibited an IC_{50} of 28 nM in the whole life cycle infection assay while maintaining the typical preference for JcR2a (Fig. 4A–E, and Table S1). Cell viability of *p*-methoxy-flunarizine treated cells was reduced by 50% at a dose of 9.6 μM. Thus, *p*-methoxy-flunarizine emerged as the most powerful compound, with an IC50 ca. 2-fold lower than chlorcyclizine and ca. 8-fold lower than flunarizine (entry 1) and a therapeutic index (IC50/CC50) of 335, which is a 1.8 and 8.8-fold improvement relative to chlorcyclizine and flunarizine, respectively.

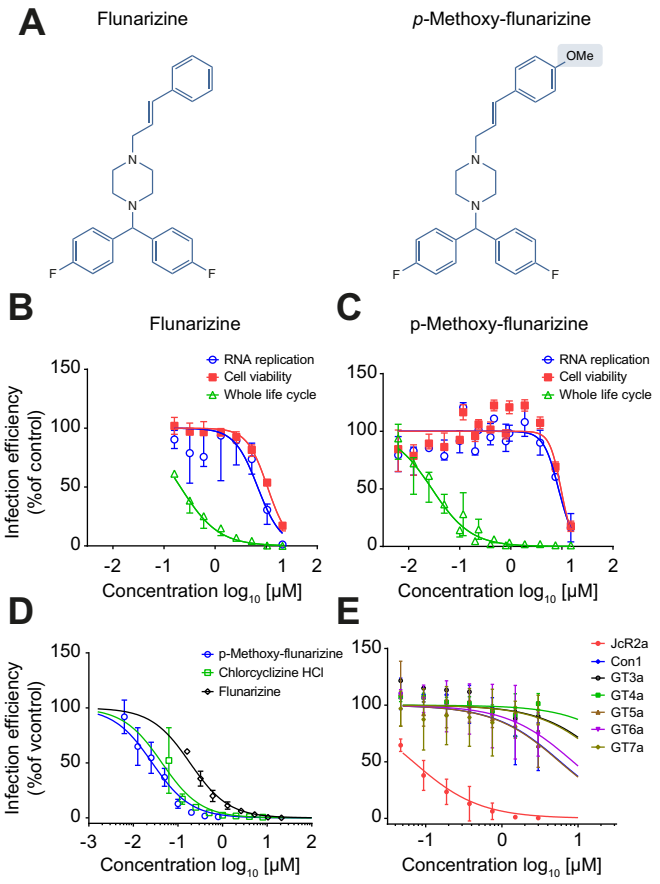

Fig. 4. *p*-Methoxy-flunarizineexhibits enhanced antiviral activity and improved therapeutic index. A library of new flunarizine derivatives were synthesized and screened for antiviral activity in a viral whole life cycle assay,[10] Table S1). (A) Structure of flunarizine, of *p*-methoxy-flunarizine and summary of structure–activity relationship of flunarizine-related molecules. Whole life cycle JcR2a infection assay in presence of increasing doses of (B) flunarizine and (C) *p*-methoxy-flunarizine, respectively. Cells are transfected with JcR2a and compounds are added 4 h after transfection. HCV RNA replication is determined by quantifying *Renilla* luciferase activity in transfected cells after 48 h (blue). Cytotoxicity of compounds is quantified at the same time point using cellular fluc expression (red). Culture fluid of the transfected cells is passed on to naïve cells where *Renilla* luciferase activity is determined 48 h later (green). (D) Comparison of antiviral activity of *p*-methoxy-flunarizine, chlorcyclizine and flunarizine as determined by the HCV whole life cycle infection assay. (E) Antiviral activity of *p*-methoxy-flunarizine was examined using given chimeric reporter viruses. Mean values of 3 biological replicates ± SD are given. DMSO was used as vehicle control. HCV, hepatitis C virus.

Viral determinants of susceptibility to HCV membrane fusion inhibitors map to 4 residues in E1

Flunarizine, *p*-methoxy-flunarizine and chlorcyclizine share a preference for inhibiting JcR2a membrane fusion over other HCV strains. While most GT2 strains are susceptible to flunorizine,[10] J8 (GT2b) exhibits pronounced resistance to flunarizine, chlorcyclizine and *p*-methoxy-flunarizine (Figs. 3 and 4). Thus, to map viral determinants responsible for sensitivity to these HCV membrane fusion inhibitors, we took advantage of this difference and created chimeras between the susceptible J6 (GT2a) and the resistant J8 (GT2b) strain. Replacement of Jc1 reporter virus glycoproteins E1 by J8-derived E1 rendered chimeric viruses resistant to flunarizine showing that primarily E1 determines susceptibility to this drug (Fig. 5A). Of note, replacement of Jc1-E1 for J8-E1 did not affect virus RNA replication and release of HCV particles as evidenced by comparable accumulation of luciferase reporter gene activity in lysates of transfected cells and by similar accumulation of released core protein (Fig. S2). However, virus infectivity was much decreased suggesting that exchange of E1 selectively impaired cell entry. To compensate for this, virus stocks of the Jc1R2a/J8-E1 chimera were concentrated by ultrafiltration and virus stocks with luciferase transduction efficiency comparable to parental Jc1 were used for the drug resistance testing. We then created Jc1 chimeras where only specific regions of E1 were replaced by the homologous J8 region. This did not affect RNA replication and virus release, but infectivity of the chimeras was partially reduced (Fig. S2) and was again compensated for by using concentrated virus preparations. While Jc1 chimeras carrying the N-terminal E1 region of J8 (GT2b) (R1; Aa 192–230) or the most C-terminal region (R4; 313–383) displayed partial resistance to flunarizine, a chimera with the internal segments of E1 (R2; 231–266) remained fully susceptible (Fig. 5B). In contrast, chimera Jc1/J8-E1_R3 (R3; Aa 267–312) exhibited a resistance profile comparable to Jc1 encoding the flunarizine resistance mutations or to Jc1 encoding the entire E1 protein of J8 (Fig. 5B and[10]). Notably, 2 of the 3 observed flunarizine resistance mutations map to E1 region 3 (M267Y and Q289H; Figs. 5 and 6A) and this portion of E1 encompasses a putative fusion loop.[19–21]

To find out which amino acids in this region are critical for conferring susceptibility to HCV membrane fusion inhibitors, we prepared a sequence alignment between 4 susceptible GT2a strains and 4 resistant GT2b isolates (Fig. 6A and[10]). We identified 8 amino acids which are fully conserved among the sensitive strains and where the resistant strains encode a

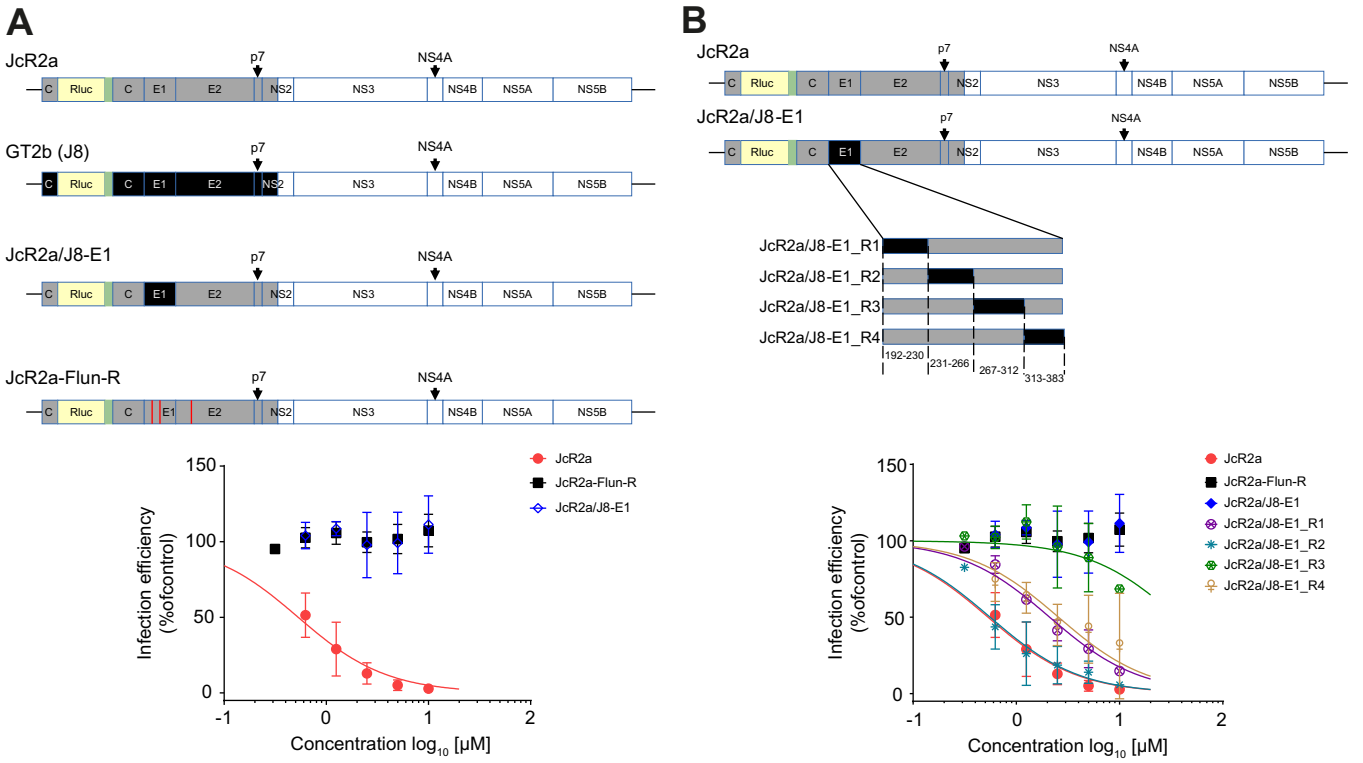

Fig. 5. Viral determinants within E1 control sensitivity to flunarizine. (A) A schematic representation of chimeric reporter viruses with differential susceptibility to flunarizine is given at the top. Infection efficiency of given reporter viruses in presence of increasing doses of flunarizine. (B) Susceptibility of chimeric JcR2a reporterviruses with J8-(GT2b) derived E1 regions. Infection is expressed relative to control infections in presence of solvent (DMSO). Mean values of 3 biological replicates ± SD are given.

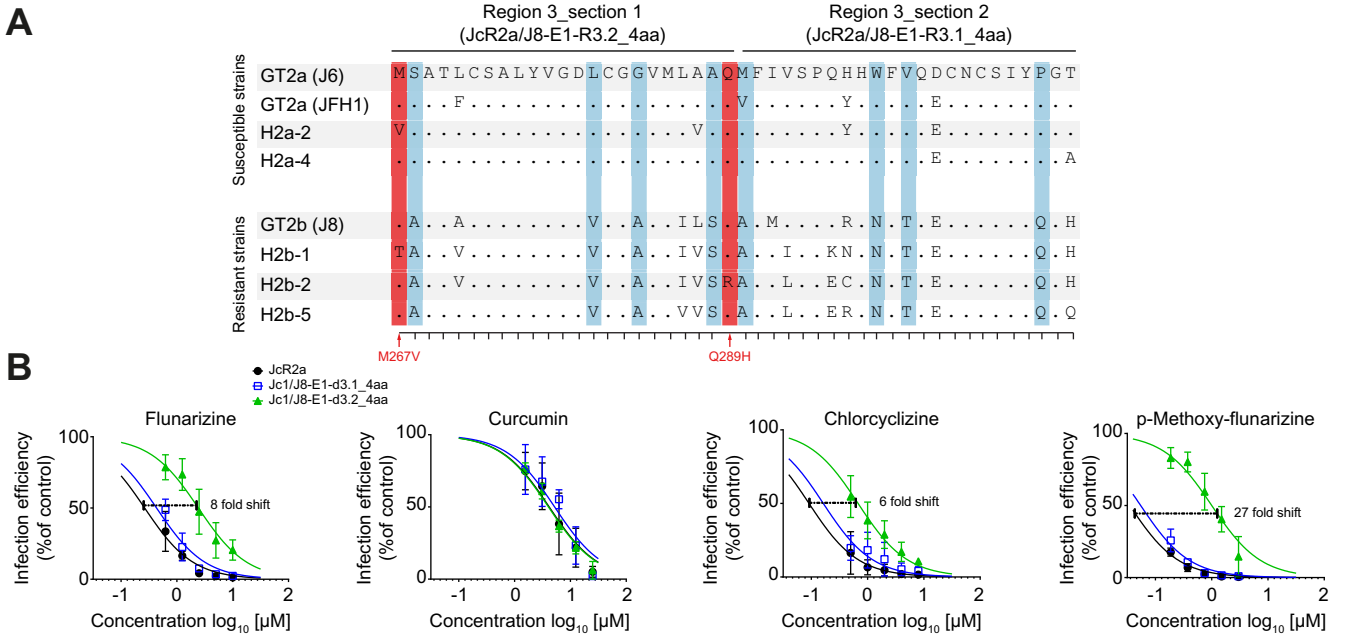

Fig. 6. Four conserved residues proximal to flunarizine resistance mutations and the putative fusion loop govern susceptibility to HCV membrane fusion inhibitors. (A) Sequence alignment of E1 protein region covering the putative fusion loop[19–21], flunarizine resistance mutations and region 3 of E1. Four GT2a-derived strains susceptible to flunarizine are given at the top, and 4 GT2b-derived isolates resistant to flunarizine are plotted below. Susceptibility of these strains to flunarizine was examined in Perin *et al.*[10]. Flunarizine resistance mutations are highlighted in red, fully conserved residues that distinguish sensitive and resistant strains are shown in blue. (B) Inhibition of JcR2a reporter viruses by given entry inhibitors. Infection is expressed relative to control infections in presence of solvent (DMSO). Mean values of 3 biological replicates ± SD are given. HCV, hepatitis C virus.

divergent residue that was fully conserved among the resistant strains. Thus, 2 additional JcR2a constructs were created encoding either these 4N-terminal (*i.e.* S268A, L280V, G283A, A288S) or these 4C-terminal (M290A, W299N, V301T, P310Q) residues of the GT2b strains instead of the cognate GT2a-typical residues. These mutant viruses exhibited RNA replication, virus release and infectivity comparable to parental JcR2a (Fig. S2). When we analyzed sensitivity of these virus mutants to entry inhibitors, JcR2a/J8-E1-R3.1_4aa displayed comparable sensitivity to all tested inhibitors like parental JcR2a. In contrast, JcR2a/J8-E1-R3.2_4aa was much more resistant to flunarizine, chlorcyclizine, and *p*-methoxy-flunarizine (8-, 6-, 27-fold, respectively) but not to curcumin. Thus, one or more of the 4 polymorphic residues encoded in the E1 region 291 to 312 (M290A, W299N, V301T and P310Q) determine susceptibility of HCV GT2a and 2b isolates to these HCV membrane fusion inhibitors.

Susceptibility of HCV to membrane fusion inhibitors correlates with their pH requirements of fusion triggering

Many enveloped viruses initiate membrane fusion upon triggering of their fusion proteins in cellular compartments with low pH. Abundant protons in these acidic compartments mediate protonation of viral fusion proteins which in turn trigger conformational changes that bring the viral and host membrane into close proximity, allowing for injection of viral hydrophobic fusion peptides or fusion loops into the host cell membrane. This process also releases energy that is needed to overcome the energy barrier to virus-cell membrane fusion.[22]

To test if sensitivity of HCV to flunarizine correlates with pH requirements of fusion protein triggering, we used a plasma membrane fusion assay (Fig. 2 and[10]) and washed cell surface-bound HCV particles with buffers ranging from pH 5 to pH 7 (Fig. 7). Virus fusion and infection through the natural route of acidified endosomes was prevented by administration of Con A, thus allowing for quantification of virus fusion triggering and infection under defined pH conditions. In line with previously published studies describing the HCV pH requirements,[23–25] pH 5 efficiently triggered infection of all tested viruses whereas pH 7 did not. However, the tested viruses exhibited significantly different responsiveness to pH values between 5 and 7. Flunarizine sensitive JcR2a exhibited the most stringent pH requirement and efficiently infected cells only when fusion was triggered with a pH of 5.0 (Fig. 7B). In contrast, viruses that exhibited natural resistance to flunarizine (GT2b,

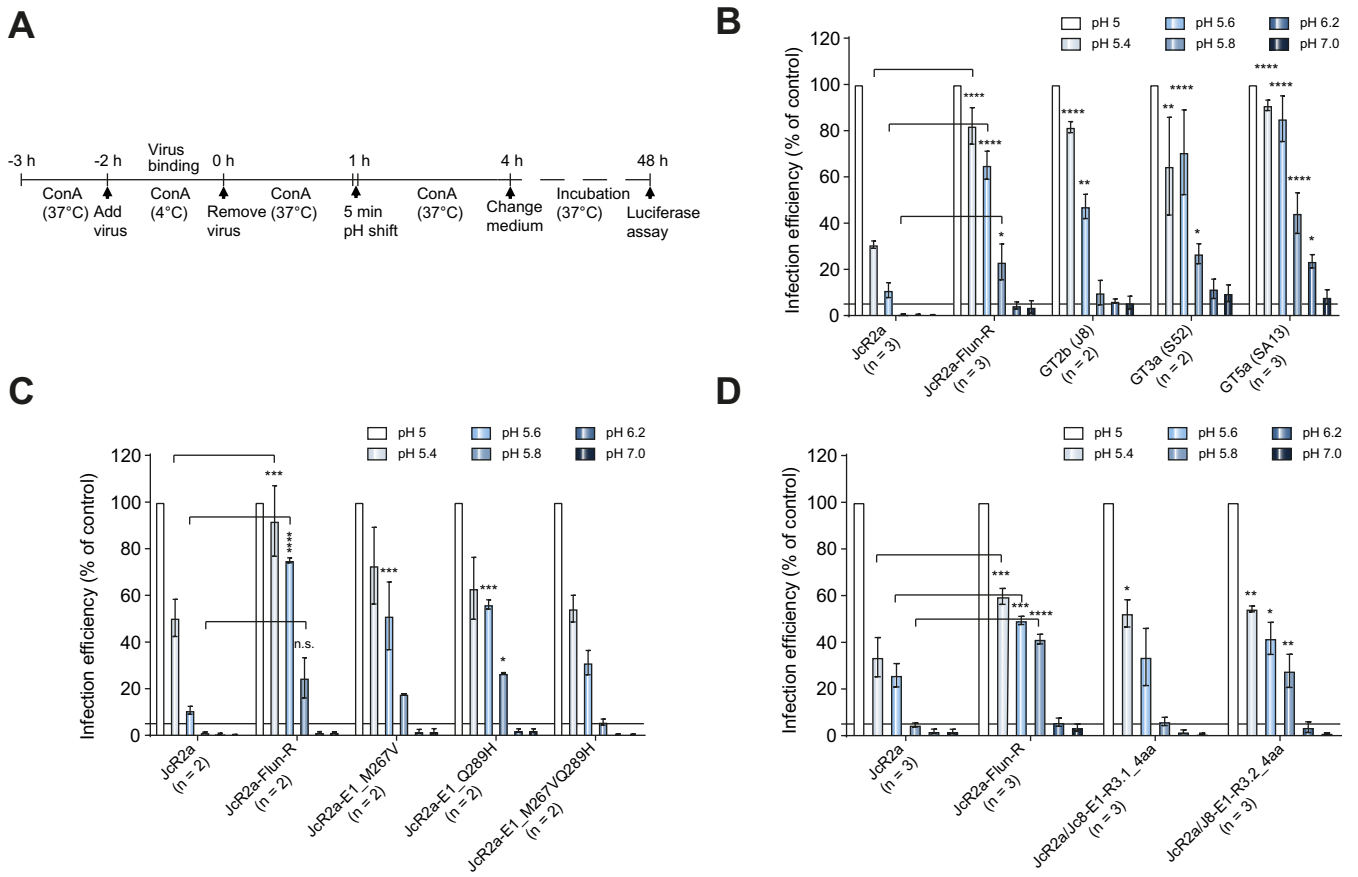

Fig. 7. Correlation between sensitivity to flunarizine and requirements for pH triggering of HCV infection. (A) A schematic representation of the experimental procedure used to determine viral requirements for pH triggering of HCV infection. Cells were treated with Con A to inhibit acidification of endosomes, and viruses were inoculated for 2 h at 4 °C to permit a synchronized infection. After incubation of cell associated virus with cells for 1 h at 37 °C, virus membrane fusion was triggered by administration of buffers with defined pH for 5 min. Subsequently, cells were washed, incubation with Con A was continued for 4 h and finally infection efficiency was quantified 48 h later by luciferase assays. (B, C, D) Given viruses with differential susceptibility to flunarizine (Figs. 1, 3, 6 and[10]) were used for infection of Huh-7.5 cells. For each virus, infection was triggered by application of a buffer with indicated pH ranging from pH 5.0 to 7.0. Infection efficiency was determined and is expressed relative to the results obtained upon treating virus-exposed cells with a pH of 5. Mean values of at least 2 biological replicates ± SD are given. Control treatment was conducted in the presence of DMSO and with pH 5 wash. Con A, concanamycin A, HCV, hepatitis C virus. Significance was tested using an ordinary two-way ANOVA test followed by Dunnett's multiple comparison tests. *p* values below the level of 0.05 were considered significant (**p* <0.05, ***p* <0.01, ****p* <0.001, *****p* <0.0001).

GT3a and GT5a) accepted a significantly broader range of pH including values approaching more neutral conditions (Fig. 7B). Therefore, natural resistance of HCV to membrane fusion inhibitors correlated with more relaxed requirements of HCV for pH-dependent induction of membrane fusion.

Resistance mutations to membrane fusion inhibitors relax pH requirement for HCV fusion

Notably, Jc1 carrying the 3 resistance mutations to flunarizine also displayed relaxed pH requirements compared to parental Jc1 (Fig. 7C). Moreover, single E1 mutations associated with flunarizine resistance (M267V and Q289H;[10]) extended the viral responsiveness towards more neutral pH. This phenotype was not further boosted by the combination of both changes. Finally, the JcR2a variants encoding the 4 E1/R3.2_4aa residues conserved among naturally resistant GT2b viruses instead of the cognate 2a-typical residues (M290A, W299N, V301T, P310Q) also fused upon triggering with more neutral pH compared to parental JcR2a and compared to the JcR2a variant encoding the 4 unique GT2b residues of E1/R3.1_4aa (S268A, L280V, G283A, A288S) (Fig. 7D). Thus, single or combined point mutations that conferred resistance to flunarizine at the same time conferred a more relaxed responsiveness to fusion triggering.

Resistance to flunarizine, and relaxed pH requirements for fusion correlate with modified hydrophobicity in a central E1 region proximal to the putative fusion loop

To investigate E1 protein features that may modify sensitivity to membrane fusion inhibitors and pH-dependent membrane fusion, we examined the interfacial hydrophobicitiy of HCV E1 from multiple strains using the Wimley and White interfacial hydrophobicity scale[26] (Fig. 8 and Table S2). This scale provides a basis for understanding membrane protein folding and membrane insertion.[26] Fig. 8A displays the Wimley-White hydropathy plot for the J6-E1 protein and highlights changes in predicted hydropathy in the central E1 region which encompasses the putative fusion loop and residues controlling sensitivity to flunarizine and pH-dependent membrane fusion (Figs. 5–7). J6 E1 encodes 2 histidine (His) residues (Aa 297, 298) within a large, continuous hydrophobic region downstream of the predicted fusion loop (Fig. 8A). His has a pK_a of ca. 6–7 and it is the only amino acid whose protonation state changes in a pH range where viral membrane fusion is commonly activated.[27] His is typically uncharged at neutral pH (prediction in Fig. 8A, and[27]). However, lowering of pH can lead to protonation of His, rendering it positively charged. Such a change can trigger conformational changes by repelling His away from positively charged residues towards negatively charged amino acids where it can engage novel salt bridges. Notably the local environment of His influences its pK_a value and in turn the threshold for protonation and membrane fusion.[27] All HCV strains analyzed here (Fig. 8 and Table S2) encode at least one His closely downstream of the putative fusion loop (Fig. 8). Interestingly, in the flunarizine sensitive J6-E1 which has stringent requirements for pH-triggered fusion, 2 His in proximity to the putative fusion loop are embedded within a large continuous hydrophobic region. In contrast, in strains with natural resistance to flunarizine and with more relaxed requirement for membrane fusion (*e.g.* J8 [GT2b], S52 [GT3a], and S13 [GT5a] the His residue proximal to the putative fusion loop is in a much more hydrophilic environment and the continuity of the predicted hydrophobic region is disrupted

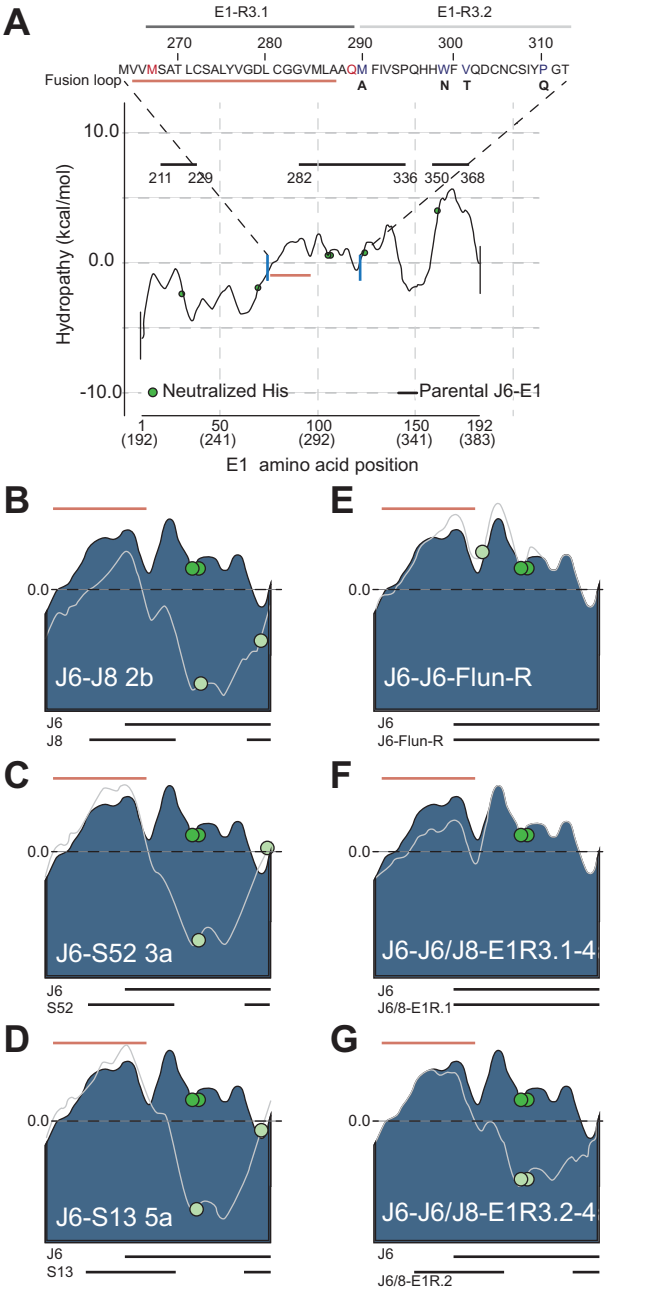

Fig. 8. Predicted hydropathy of HCV E1 proteins with a focus on the central region encompassing the putative fusion loop and residues governing sensitivity to flunarizine. (A) Wimley-White hydropathy plot[26] for the J6-E1 protein. Predicted hydrophobic regions are indicated by thick black bars on top of the smoothed predicted hydropathy (thin black line). The primary amino acid sequence of the central E1 region from residues 264 to 312 is provided above. The putative fusion loop is indicated by a red bar (Aa 265–287), E1 region 3 section 1 (Aa 267–289) and section 2 (290–312) characterized in this study are indicated by grey bars. Residues conserved among flunarizine-resistant HCV GT2b strains are plotted in bold face directly below the J6 sequence. Neutralized histidine residues are shown by green circles. (B-G) Comparison of predicted hydropathy of central E1 region (residues 264–312) between J6-E1 (filled in blue with black contour) and given alternative E1 proteins (light grey contour). Neutralized histidines are given in dark green for J6 and in light green for the alternative E1 proteins. HCV, hepatitis C virus.

(Figs. 7B and 8A–D). Replacement of 4 E1-R3.2 residues by amino acids conserved among flunarizine-resistant GT2b isolates (J6/J8-E1R3.2-4aa) also disrupts continuity of this

hydrophobic region and increases hydrophilicity close to these 2 His residues (Fig. 8G). Notably, the modified E1 protein (J6/J8-E1R3.2-4aa) not only displays resistance to flunarizine, it also has broadened requirements for low pH-triggered membrane fusion (Figs. 6 and 7D), thus reinforcing this correlation. In contrast, exchange of the 4 residues within the E1R.1 region (J6/J8-E1R3.1-4aa) slightly reduces hydrophobicity of the putative fusion loop but does not affect resistance to flunarizine nor alter low pH fusion triggering (Figs. 7D and 8F). Collectively, these observations support a model where a central hydrophobic region directly downstream of the putative fusion loop and containing at least one His residue controls pH-dependence of HCV membrane fusion and, correlating with this, sensitivity to HCV membrane fusion inhibitors. The level of predicted hydrophobicity of the most proximal downstream His and continuity of the central hydrophobic region predicts flunarizine sensitivity. All HCV strains showing a continuous central hydrophobic region and also a hydrophobicity greater than 0 around the proximal His are sensitive to flunarizine (Table S2;[10]). In contrast, strains with a disrupted central hydrophobic region and also hydrophobicity lower than 0 around the proximal His residue are resistant to flunarizine (Table S2,[10]). Thus, for all 11 strains with robust experimental data on flunarizine sensitivity, this simple algorithm stratifies strains into sensitive and insensitive strains. It should be mentioned that isolates from a few GT2 subtypes (*i.e.* 2e, 2k, 2m, 2q, 2r) recently analyzed by us cannot be properly assigned using this algorithm as the IC50 of flunarizine for these strains was not determined.[10] Moreover, flunarizine resistance mutations that occurred upon selection with the drug seem to confer resistance by an alternative mechanism, as these residues do not disrupt the J6-typical central hydrophobic region. Instead these changes slightly increase hydrophobicity of the C-terminal portion of the putative fusion loop and the adjacent N-terminal part of the central hydrophobic region. Moreover, the Q289H exchange introduces an additional His residue (Fig. 8E).

Discussion

In this study, we clarified the mode of action of several chemically related, HCV entry inhibitors, identified novel derivatives with improved efficacy, defined viral determinants of resistance, mapped the viral target site of these molecules and advanced our understanding of how HCV escapes this class of drugs. Moreover, this work also sheds light on E1 protein features that control pH-dependent membrane fusion.

Several laboratories reported a broad set of loosely related compounds from the families of diphenylpiperazines, diphenylpiperidines, phenothiazines, thioxanthenes, and cycloheptenepiperidines as potent HCV entry inhibitors[9] and references therein). Here we unite and extend these reports by showing that representatives of these chemical classes share a common mode of action which is specific inhibition of HCV membrane fusion. This conclusion is based on several observations: First, an HCV variant with resistance mutations to flunarizine exhibited pronounced cross-resistance to all tested representatives of these chemical scaffolds. In contrast these mutations did not confer resistance to a previously published entry inhibitor with grossly different scaffold (*i.e.* curcumin; Fig. 1). Second, all tested representatives of these compound families were uniquely able to fully inhibit HCV infection when administered only during low pH triggering of the HCV fusion process (Fig. 2). In contrast, BJ486K, an HCV entry inhibitor with different chemical properties and alternative mode of action only exerted antiviral activity when present throughout the viral entry process. Finally, flunarizine, *p*-methoxy-flunarizine, and chlorcyclizine, the most potent among these related molecules and also the related compounds pimozide, trifluoperazine and fluphenazine share a pronounced preference for inhibition of GT2-derived viral strains (Figs. 3 and 4 and[10]). These findings suggest that most GT2 strains (with the exception of the GT2b subtype; Fig. 3 and[10]) are particularly vulnerable to this novel class of HCV membrane fusion inhibitors. The exquisite sensitivity of GT2 viruses to these compounds seems to be linked with a uniquely tight regulation of pH-dependent membrane fusion and/or specific sequence properties of their E1 proteins (see below).

Based on these observations we completed a structure-activity relationship analysis and identified *p*-methoxy-flunarizine, as the most potent compound of this series of inhibitors. *p*-Methoxy-flunarizine exhibited an IC_{50} of 28 nM, which is 8-fold better than flunarizine and ca. 2-fold improved compared to chlorcyclizine. Notably, this modification does not alter cross-genotype coverage of the antiviral activity (Fig. 3). This is in line with our previous observation that flunarizine-related molecules with modifications in the tricyclic aromatic phenothiazine backbone exhibited somewhat improved cross-genotype antiviral activity (fluphenazine and trifluoperazine;[10]. This broad analysis of related compounds permits a more precise definition of the chemical space of these membrane fusion inhibitors. Our analysis particularly demonstrates the importance of the cinnamyl group as an attractive element for expanding the structure–activity relationship-studies for achieving improved antiviral properties. The possibility of synthesizing azido- and 3-(trifluoromethyl)-3*H*-diazirine derivatives (Table S1, entries 4 and 14) paves the way for initiating studies on target elucidation for this group of potential antiviral agents.

Identification of viral determinants for susceptibility to these compounds is important to select patients that may benefit from treatments involving such molecules. This information is also critical to guide development of improved membrane fusion inhibitors with regard to potency and possibly also cross-genotype coverage. By using a series of chimeras between the sensitive J6 strain and a resistant GT2b isolate J8 we pinpointed viral determinants of flunarizine sensitivity. We identified a central hydrophobic region in J6-E1 (J6-E1-R3.2; Aa 290–312) to be important for controlling sensitivity to this compound. In fact, 4 residues within this region and which are conserved among sensitive GT2a strains (M/V290, W299, V301, P310) and where resistant GT2b strains encode a conserved alternative residue (A290, N299, T301, Q310) cause an 8-fold, 6-fold or 27-fold shift of the IC50 of flunarizine, chlorcyclizin and *p*-methoxy-flunarizine, respectively. Presently it is not clear how these residues confer sensitivity or resistance to these membrane fusion inhibitors. One hypothesis is that these compounds bind to this region. However, it is also possible that these molecules inhibit infection indirectly by changing membrane properties. In this latter scenario, determinants of this E1 region may influence conformational changes during fusion that are more or less compatible with membrane changes induced by these compounds. Additional structural studies may help to resolve this question. Notably, these amino acids modulating resistance to fusion inhibitors also influence

responsiveness of J6 E1 to low pH-triggered membrane fusion. While parental J6 is sensitive to flunarizine and fuses only in the presence of very low pH (Fig. 7), J6/J8-E1R3.2_4aa is much more resistant and it accepts a more neutral pH for membrane fusion (Fig. 8). These differences correlate with a remarkably altered hydrophobicity in the central hydrophobic region directly downstream of the putative fusion loop between these 2 variants. In the mutant J6 protein (J6/J8-E1R3.2_4aa) the hydrophobic stretch is disrupted and the 2 proximal His residues end up in a more hydrophilic environment. Notably, HCV strains that have natural resistance to flunarizine also display a disrupted central area of hydrophobicity and their His residue most closely downstream of the putative fusion loop is in a more hydrophilic environment.

Taken together these findings suggest that analysis of the hydrophobicity within the E1 region proximal to the putative fusion loop including the assessment of the hydrophobicity around the adjacent His predicts viral sensitivity to flunarizine and related membrane fusion inhibitors. Beyond this, our data support the conclusion that viral determinants in this E1 region control requirements for low pH-dependent membrane fusion. Therefore, these findings provide an algorithm for selection of patients with HCV that may benefit from treatments with membrane fusion inhibitors. They also provide new perspectives for development of further improved inhibitors and they offer new insights into the molecular mechanisms that control HCV membrane fusion.

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Conflict of interest

The authors declare no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceived and designed experiments: D.B, A.K., P.M., T.P. Performed the experiments: DB, P.M.P., W.S., P.H., R.J.P.B. Analysed the data: D.B, A.K., R.-J.-P.B., R.M., P.M., D.T., T.P. Contributed reagents/material/analysis tools: K.K.S., M.H., D.T. Wrote the manuscript: DB, TP.

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Supplementary data

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