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Flunarizine Prevents Hepatitis C Virus Membrane Fusion in a Genotype-Dependent Manner by Targeting the Potential Fusion Peptide Within E1

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To explore mechanisms of hepatitis C viral (HCV) replication we screened a compound library including licensed drugs. Flunarizine, a diphenylmethylpiperazine used to treat migraine, inhibited HCV cell entry *in vitro* and *in vivo* in a genotype-dependent fashion. Analysis of mosaic viruses between susceptible and resistant strains revealed that E1 and E2 glycoproteins confer susceptibility to flunarizine. Time of addition experiments and single particle tracking of HCV demonstrated that flunarizine specifically prevents membrane fusion. Related phenothiazines and pimozide also inhibited HCV infection and preferentially targeted HCV genotype 2 viruses. However, phenothiazines and pimozide exhibited improved genotype coverage including the difficult to treat genotype 3. Flunarizine-resistant HCV carried mutations within the alleged fusion peptide and displayed cross-resistance to these compounds, indicating that these drugs have a common mode of action. *Conclusion*: These observations reveal novel details about HCV membrane fusion; moreover, flunarizine and related compounds represent first-in-class HCV fusion inhibitors that merit consideration for repurposing as a cost-effective component of HCV combination therapies. (HEPATOLOGY 2016;63:49-62)

See Editorial on Page 14

Hepatitis C virus (HCV) is a liver-tropic, enveloped virus of the family Flaviviridae. It possesses a plus-stranded RNA genome of 9.6 kb which encodes a single polyprotein. Proteolytic processing mediated by cellular and two viral proteases (NS2-3 and NS3-4A) liberates 10 distinct HCV polypeptides.[1] The structural core proteins, E1 and E2, compose the virion with core encasing the viral RNA and the glycoproteins E1 and E2 mediating receptor interactions and low pH–triggered

Abbreviations: CC_{50}, median cytotoxic concentration; DiD, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiI C18[5]); DMSO, dimethylsulfoxide; EEA1, early endosomal antigen-1; F-Luc, firefly luciferase; G-Luc, Gaussia luciferase; GT, genotype; HCV, hepatitis C virus; HCVcc, cell culture–derived HCV; HCVpp, HCV pseudoparticles; IC_{50}, median inhibitory concentration.

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membrane fusion. The p7 ion channel is essential for virus assembly and protection of E1 and E2 during virus release. NS3, NS4A, NS4B, NS5A, and NS5B assemble into a multiprotein complex critical for viral RNA replication. HCV is highly variable, and viral strains are classified into seven distinct genotypes (GTs), 67 confirmed and 21 unassigned subtypes.[2]

Chronic HCV infection frequently leads to liver fibrosis, cirrhosis, and hepatocellular carcinoma; and it has become the leading indication for liver transplantation.[3] Novel drugs have improved treatment options, and several efficacious combination therapies are available.[4] However, these regimens are expensive, limiting access particularly in medium-income to low-income countries where HCV is most prevalent.[5] To identify alternative, cost-effective treatment options for HCV and to explore mechanisms of viral replication, we screened a compound library including drugs approved to treat neuronal or heart diseases for antiviral activity against HCV.

Materials and Methods

Whole Life Cycle Screen. The initial screen of the compounds was performed as described.[6] Briefly, Huh7-Lunet/hCD81 cells constitutively expressing Gaussia luciferase (G-Luc) were transfected with firefly reporter Jc1 (pFK-Luc-Jc1) and seeded into 96-well plates. Media containing serial dilutions of each compound were added to the cells after 4 hours. After 48 hours, the supernatant containing G-Luc was collected for cell viability assessment and cells were lyzed and measured for firefly luciferase (F-Luc) activity as an indicator of HCV RNA replication. The supernatant of these cells was used to infect target cells. These were lyzed after 48 hours and their reporter levels measured for whole life cycle assessment.

Fusion at the Plasma Membrane Assay. The assay was performed as described.[7] Huh7-Lunet/hCD81 cells (3×10^5 cells/mL) were seeded into each well of a six-well plate 1 day before the experiment. The following day, cells were treated with concanamycin A (5 nM) for 1 hour at 37°C, before infection with concentrated reporter viruses in the presence of concanamycin A. The cells were washed twice with phosphate-buffered saline and incubated with medium containing concanamycin A for 1 hour at 37°C. Subsequently, cells were incubated for 5 minutes at 37°C with pH 7 or pH 5 citric acid buffer (McIlvaine buffer system). Fresh medium was added to the cells in the continuous presence of concanamycin A for 3 hours longer. Medium was changed, and infectivity was measured by assessment of reporter activity after 48 hours.

Virus Passaging in the Presence of Flunarizine. Huh7-Lunet/hCD81 cells were seeded in six-well plates (4×10^5 cells/well) 1 day before being infected with Jc1 virus stocks (pFK-Jc1) for 4 hours in the presence of two concentrations of flunarizine (5.25 μM or 10.5 μM) or 1% dimethylsulfoxide (DMSO) before addition of fresh medium. At 48 hours to 72 hours later, cells were split and flunarizine or DMSO was added after 4 hours. After 48 hours, virus-containing supernatants were used for infection of new Huh7-Lunet-hCD81 cells, according to the procedure performed for the first infection. After 10 cycles of infection of naive cells and splitting of virus-producing cells (approximately 10 weeks), cells were allowed to produce viruses in the absence of any compound for 16 hours and supernatant was harvested for core measurement by enzyme-linked immunosorbent assay. The stocks from DMSO, flunarizine 5.25 μM, and flunarizine 10.5 μM passaged viruses were normalized to the same core levels and used for infection of cells seeded in coverslips in 24-well plates. Infection efficiency was assessed by immunofluorescence using the NS5A-specific monoclonal antibody 9E10 at a dilution of 1:2000. Bound primary antibodies were detected using goat anti-mouse immunoglobulin G–specific secondary antibodies conjugated to Alexa Fluor 488 (Sigma) at a dilution of 1:1000. Nuclear DNA was stained using 4′,6-diamidino-2-phenylindole at a dilution of 1:3000.

Results

Flunarizine Inhibits HCV Entry Both* In Vitro *and* In Vivo *in a Genotype-Dependent Fashion. Licensed HCV drugs target either polyprotein processing (NS3-4A protease inhibitors) or RNA replication (NS5A inhibitors, NS5B polymerase inhibitors). To identify molecules that would complement these therapies and that may reveal novel insights into other life cycle steps, we aimed at identifying inhibitors with a novel mode of action. In addition, such molecules should not suffer from potential viral cross-resistance to established drug classes.

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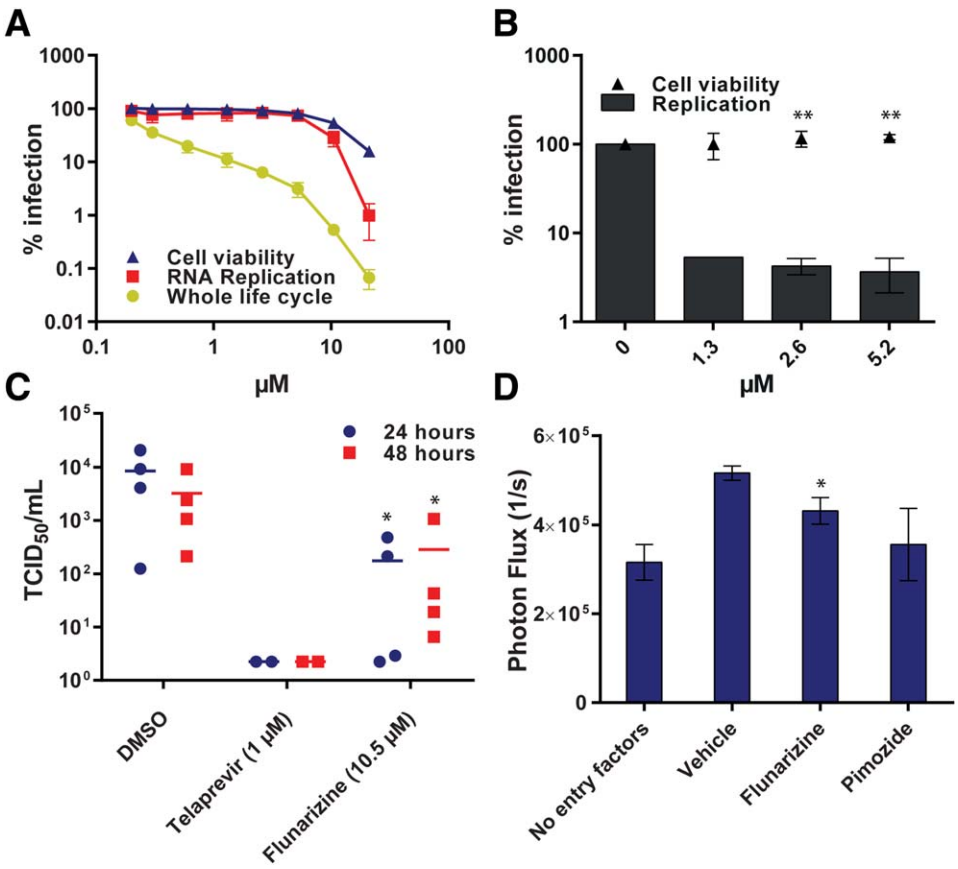

Fig. 1. Flunarizine inhibits HCV entry into human hepatocytes *in vitro* and *in vivo*. (A) Huh7-Lunet/hCD81/G-Luc cells expressing G-Luc were transfected with F-Luc-Jc1. After 4 hours, medium with two-fold dilutions of flunarizine was added. Measurements of G-Luc (cell viability) and F-Luc (RNA replication) were taken after 48 hours. Viruses produced at this time point were used to inoculate target cells where F-Luc activity was determined 48 hours later (whole life cycle). (B) Huh7-Lunet/hCD81/G-Luc cells were inoculated with F-Luc-Jc1 and flunarizine or solvent for 4 hours. Infection was measured 48 hours afterwards. (C) Primary human hepatocytes were incubated with the indicated compounds and Jc1 for 6 hours. The supernatant of inoculated cells was collected at 24 hours and 48 hours, and virus infectivity was determined by median tissue culture infective dose. (D) HCV entry reporter mice expressing firefly luciferase in a Cre-dependent manner were pretreated with the indicated compounds. Subsequently, they were challenged with a Jc1 variant expressing Cre recombinase. HCV-Cre-dependent luciferase expression, which reflects HCV cell entry efficiency, was determined 24 hours later. Abbreviation: $TCID_{50}$, median tissue culture infective dose.

Thus, we chose a whole life cycle screening assay based on the GT2a chimeric Jc1-luciferase reporter virus (JcR-2a),[8] which interrogates HCV entry, RNA translation, polyprotein processing, RNA replication, and virus assembly (Supporting Fig. S1).[6] Because inhibitors of the influenza A virus M2 ion channel have been used to treat humans and because HCV p7 is an ion channel protein essential for virus production, we screened a library of 23 compounds including several clinically approved ion channel inhibitors used to treat heart or neurologic conditions (Supporting Table S1). Fifteen compounds inhibited HCV entry, assembly, or release (Supporting Fig. S1). Flunarizine displayed a median inhibitory concentration (IC_{50}) value of 388 nM (Supporting Table S2) and a median cytotoxic concentration value of 10.85 μM, which is equivalent to a therapeutic index (median cytotoxic concentration/IC_{50}) of >25 (Fig. 1A). Flunarizine has recently emerged as an anti-HCV inhibitor in independent screening campaigns.[9-11] However, its mode of action remains unclear. Moreover, it has not been explored if this molecule is antiviral in primary human hepatocytes and *in vivo*.

To address these limitations, we inoculated Huh7-Lunet/hCD81/G-Luc cells with a Jc1-F-Luc virus in the presence of the compound and subsequently washed away unbound virus and compound. Virus-encoded F-Luc activity was decreased by more than 10-fold at a dose of 1.3 μM, whereas cell-encoded G-Luc expression was not affected, thereby indicating absence of cytotoxicity (Fig. 1B). In contrast, addition of flunarizine to HCV RNA–transfected cells did not reduce release of infectious virus particles as determined by a core-specific enzyme-linked immunosorbent assay and limiting dilution assay (median tissue culture infective dose) (Supporting Fig. S2). Interestingly, it did not inhibit entry of HCV pseudoparticles (HCVpp) carrying the J6-derived

glycoproteins (Supporting Fig. S3). Thus, flunarizine selectively inhibits entry of authentic, cell culture–derived HCV (HCVcc) but not of HCVpp. Moreover, it does not inhibit RNA replication or virus assembly (Fig. 1A; Supporting Fig. S2).

Next, we infected primary human hepatocytes with Jc1 particles in the presence of telaprevir, flunarizine, or DMSO solvent. Flunarizine significantly inhibited *de novo* production of infectious virus at both 24 hours and 48 hours after inoculation compared to inoculation in the presence of solvent (Fig. 1C). Finally, we administered flunarizine to HCV cell entry reporter mice, which express crucial human cofactors for HCV entry and carry a luciferase reporter gene that is activated by CRE recombinase.[12] When these animals were challenged with infectious Jc1 expressing a CRE recombinase, flunarizine serum levels reached an average of 133 nM, which is close to the *in vitro* median effective concentration value described above. Importantly, at this dose HCV-CRE-dependent activation of luciferase expression was significantly repressed (Fig. 1D). Thus, flunarizine inhibits HCV cell entry into both human primary liver cells *in vitro* and into humanized mouse hepatocytes *in vivo*.

Because flunarizine did not inhibit infection by vesicular stomatitis virus or human coronavirus (Supporting Fig. S4), we investigated if it prevents HCV entry across all seven HCV genotypes. Therefore, Huh7-Lunet/hCD81/G-Luc cells were inoculated with chimeric HCV viruses in the presence of increasing doses of flunarizine (Fig. 2A). All non-GT2 strains displayed resistance toward this drug with IC_{50} values more than an order of magnitude greater than Jc1. The GT2b isolate showed an intermediate phenotype, with an IC_{50} value of 3.69 μM; and both GT2a isolates, JFH1 and Jc1, were highly susceptible, displaying IC_{50} values of 0.37 μM and 0.22 μM, respectively. To map the determinants of flunarizine susceptibility, we shuffled viral core to NS2 proteins between the resistant Con1 (GT1b) and the susceptible Jc1 (GT2a). Transfer of Con1-derived p7 into Jc1 did not render the resulting virus (Jc1/Con1-p7) resistant to flunarizine (Fig. 2B). In contrast, insertion of the Con1-derived E1 and E2 proteins into Jc1 did confer flunarizine resistance to Jc1/Con1-E1-E2. Conversely, replacement of the Con1-derived E1 and E2 sequences by those of Jc1 rendered the Con1 chimera (Con1-/J6-E1-E2) susceptible to the drug (Fig. 2B). Therefore, susceptibility to flunarizine is governed by determinants resident in the E1 and E2 genes. Likewise, when we exchanged structural proteins, p7 or NS2 between J6 (GT2a) or J8 (GT2b), all viruses carrying the J6 E1 and E2 genes displayed greater susceptibility to flunarizine compared with those harboring the J8-derived glycoproteins (Supporting Fig. S5A). A chimeric virus carrying J8-derived E2 in the backbone of Jc1 produced infectious virus and exhibited partial resistance to flunarizine, indicating that determinants in both E1 and E2 influence susceptibility to this drug (Supporting Fig. S5B). Next, we used HCV particles trans-complemented with primary E1 and E2 gene sequences from GT2a-infected and GT2b-infected patients.[13] Also, these clinical GT2a isolates were susceptible to flunarizine, whereas the GT2b isolates were resistant (Fig. 2C). Finally, we created HCV trans-complemented particles carrying E1 and E2 proteins from six additional GT2 subtypes including d, e, k, m, q, and r. All of these strains were susceptible to inhibition by flunarizine, albeit to variable degrees (Fig. 2D). Collectively, this indicates that flunarizine interferes with HCV entry by targeting viral E1 and E2 protein function(s) of almost all GT2 subtypes tested and with a clear preference for GT2 over other viral genotypes.

Flunarizine Resistance Maps to Both E1 and E2 and Increases Susceptibility to Cross-Neutralizing Antibodies. To corroborate that E1 and E2 are targeted by flunarizine, we passaged HCV in the presence of flunarizine. After 10 weeks we observed drug resistance, as infection of Huh7-Lunet/hCD81/G-Luc cells by virus populations cultured in the presence of the drug was poorly inhibited by flunarizine compared to viruses cultured in the presence of DMSO (Fig. 3A). Sequencing revealed conserved amino acid mutations at positions M267V in E1 (A1140G in H77 genome), Q289H in E1 (A1208C in H77 genome), M405T in E2 (T1554C in H77 genome), and I757T in p7 (T2603C in H77 genome), which we engineered into the parental Jc1-luciferase virus either alone or in combination. Insertion of the p7 mutation did not change susceptibility to flunarizine (Fig. 3B). In contrast, the E2 mutation and both mutations in E1 increased virus resistance. A combination of all three envelope protein mutations conferred maximal resistance (~50-fold change in IC_{50}).

Interestingly, E1 residue Q289 is fully conserved among all HCV sequences deposited in the gene bank database (Fig. 3C). Additionally, 88.5% of all GT2 isolates carry M267, which correlates with susceptibility to flunarizine in our GT2a virus Jc1, whereas all non-GT2 sequences encode glycine at this position (Supporting Table S3). Finally, the E2 mutation resides in the hypervariable region, an important yet strain-specific viral neutralizing epitope. The full conservation of 289 in E1 and the high level of conservation of 267 in E1 may indicate important functional constraints that could limit viral escape *in vivo*. To explore this, we evaluated neutralization of the flunarizine-resistant virus by potent monoclonal antibodies. Neutralization by the E2-targeting HC-11 and AR4A

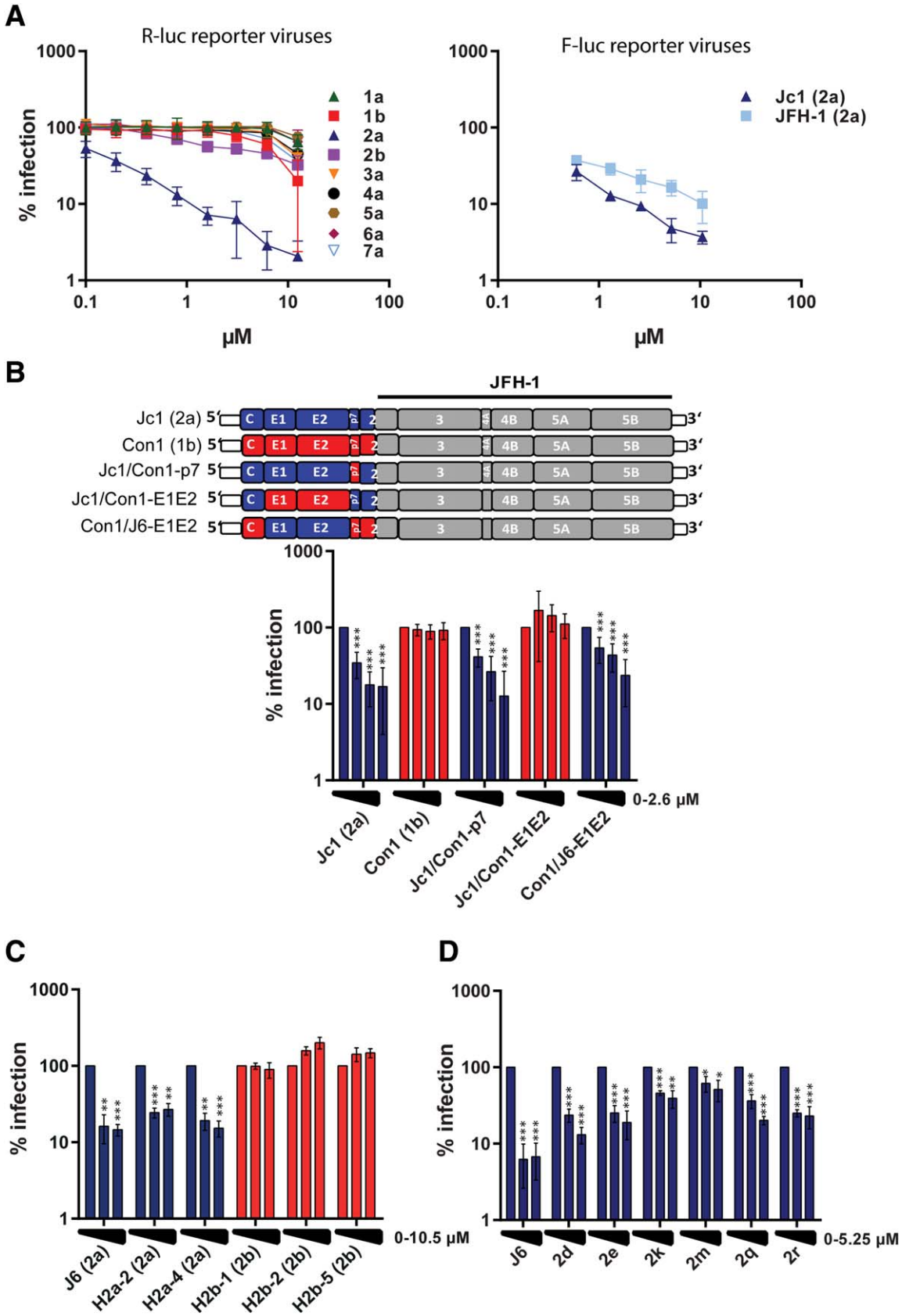

Fig. 2. The antiviral activity of flunarizine is HCV strain–dependent, and viral determinants governing susceptibility reside within the E1 and E2 genes. (A) Chimeric *Renilla* luciferase reporter viruses (left panel) or F-Luc-Jc1 or F-Luc-JFH-1 (right panel) were inoculated with two-fold dilutions of flunarizine into Huh7-Lunet/hCD81/G-Luc cells for 4 hours. Luciferase levels were measured 48 hours postinfection. (B) Chimeras between flunarizine-susceptible Jc1 (GT2a) and flunarizine-resistant Con1/C3 (GT1b) were created as indicated and tested for their susceptibility to flunarizine using a focus formation unit assay. (C) HCV trans-complemented particles harboring GT2a or GT2b E1 and E2 glycoproteins of given primary, patient-derived viruses[13] were used to inoculate cells in the presence (two-fold dilutions) or absence of flunarizine for 4 hours. Infection efficiency was determined 48 hours postinoculation using *Renilla* luciferase assays. (D) HCV trans-complemented particles harboring E1 and E2 glycoproteins of representatives of indicated GT2 subtypes[2] were used to inoculate cells in the presence of (two-fold dilutions) or absence of flunarizine. Infection efficiency was determined 48 hours postinoculation by focus formation unit assay.

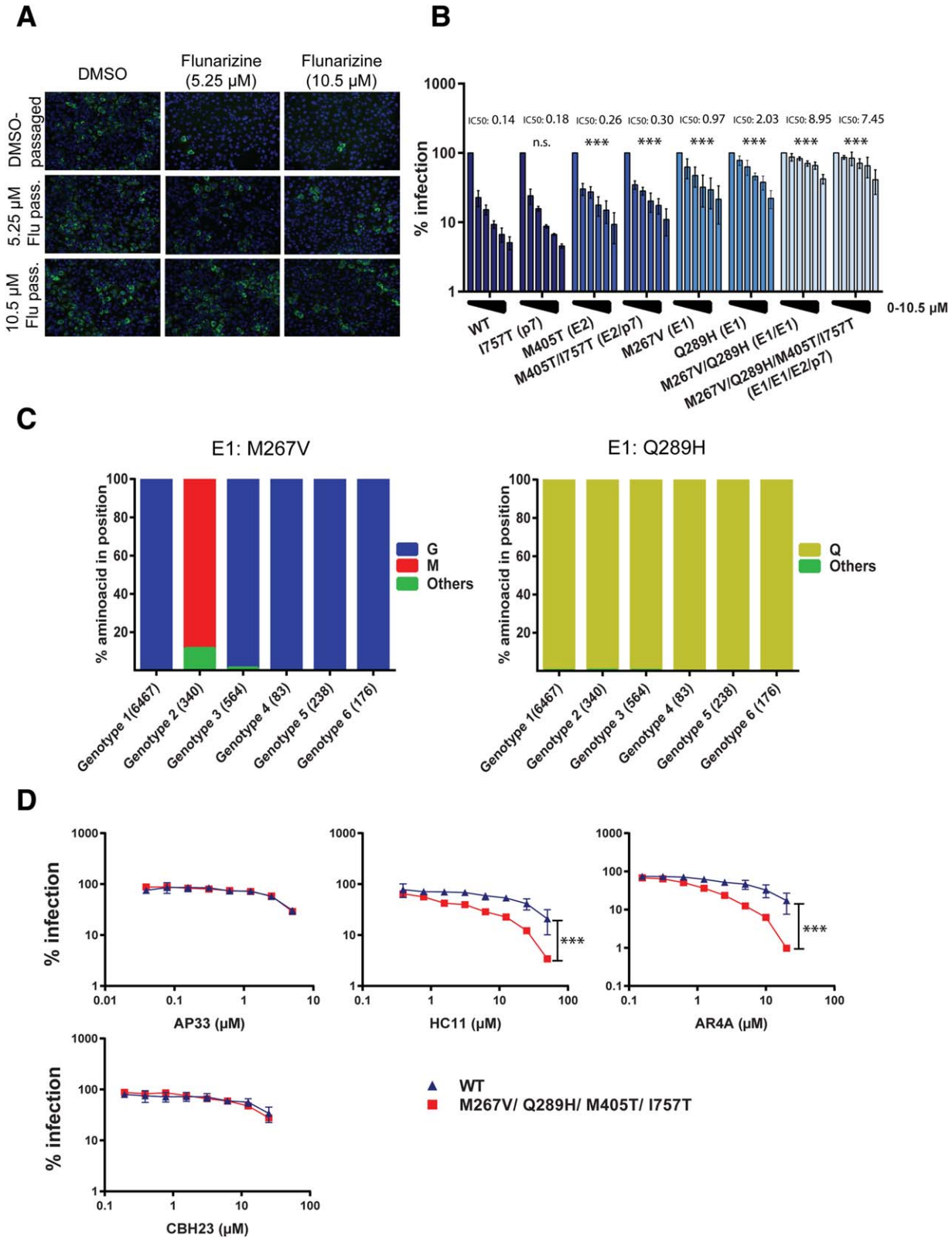

Fig. 3. Two mutations of conserved residues in E1 and one mutation in E2 confer resistance to flunarizine. (A) Jc1 was passaged in Huh7-Lunet/hCD81/G-Luc cells in the presence of given doses of flunarizine or DMSO. The resulting virus populations were used to inoculate naive Huh7-Lunet/hCD81/G-Luc cells for 4 hours in the presence of 5.25 µM or 10.5 µM of flunarizine or DMSO. The infection was assessed 48 hours later by immune staining of the NS5A protein (green). DNA in the nucleus was counterstained with 4′,6-diamidino-2-phenylindole (blue). (B) Jc1 luciferase reporter virus (WT) or derivatives thereof carrying the indicated point mutations were used to infect Huh7-Lunet/hCD81/G-Luc cells in the presence of 0 µM, 0.65 µM, 1.3 µM, 2.6 µM, 5.25 µM, or 10.5 µM of flunarizine for 4 hours. *Renilla* luciferase activity was measured 48 hours later. (C) Prevalence of indicated amino acids at positions 267 or 289 in HCV E1 across all GT1-GT7 strains deposited in the HCV database. The total number of analyzed sequences for each genotype is stated in parentheses. (D) Parental or flunarizine-resistant Jc1 was incubated for 1 hour at 37°C with serial dilutions of given monoclonal antibodies targeting E2 (AP33, CBH23, or HC11) or a discontinuous epitope of the E1 and E2 complex (AR4A) before inoculation of Huh7-Lunet/hCD81/G-Luc cells for 4 hours at 37°C. After 48 hours, cells were lysed and *Renilla* reporter activity was measured. Abbreviation: WT, wild type.

antibodies was significantly enhanced by the flunarizine resistance mutations (Fig. 3D). Collectively, a combination of three mutations—in part affecting highly conserved residues—is necessary to confer a 50-fold resistance to flunarizine. These mutations render HCV more susceptible to neutralizing antibodies, suggesting a high barrier to viral resistance.

Flunarizine Inhibits HCV Membrane Fusion. To precisely define the mode of action of flunarizine, we conducted time-of-addition experiments including HCV entry inhibitors which arrest HCV infection at distinct stages of cell entry (Supporting Fig. S6). Because flunarizine resistance was reached at a time point similar to resistance to inhibitors of endosomal acidification (e.g., concanamycin A), this suggested that flunarizine inhibits a late entry step. To explore this further, we used an HCV plasma membrane fusion assay where cells are pretreated with concanamycin A to prevent viral entry and membrane fusion by the normal route through acidified endosomes (Fig. 4).[7] Under these circumstances, HCV fusion and thus productive infection can be triggered by briefly exposing virus inoculated cells to a low pH buffer. Because HCV membrane fusion requires receptor interactions including binding to CD81,[14] the virus is receptive to this exogenous trigger (low pH buffer) only after incubation of approximately 1 hour at 37°C.[7] Thus, Huh7-Lunet/hCD81 cells were continuously treated with concanamycin A and additional drugs were added either directly after virus inoculation and throughout the experiment until 4 hours post–temperature shift (Fig. 4A, protocol I), only during the incubation with low pH buffer (protocol II), or only directly subsequent to the fusion triggering low pH wash (protocol III). A buffer with neutral pH 7 was used to control that productive infection fully depends on low pH-induced fusion. As expected, HCV infection only occurred when cells were exposed to low pH buffer because only then was luciferase expression above the background of uninfected cells detected (Fig. 4B). When DMSO was added to the cells according to protocol I, II, or III, the low pH wash resulted in high luciferase activity, approximately 50-fold above the background of mock infected cells or virus inoculated cells treated with the neutral pH buffer (Fig. 4B). Addition of bafilomycin A1, which like concanamycin A prevents acidification of endosomes, did not have an antiviral effect, regardless of the administration protocol. This confirms that HCV can only access cells by exogenous administration of low pH, independently of endosomal acidification. Moreover, all inhibitors had completely lost antiviral activity when applied directly after the low pH fusion trigger, indicating that they exert their antiviral activity by blocking HCV entry during or upstream of membrane fusion (Fig. 4B, protocol III). Both BJ486K and flunarizine reduced HCV infection essentially to background levels when administered directly after virus inoculation and thus when present during virus trafficking at the cell surface (1 hour 37°C) and the low pH fusion treatment. However, only flunarizine was fully antiviral when added selectively during the 5-minute low pH washing step (Fig. 4B, protocol II). Interestingly, viruses carrying the two E1 resistance mutations were no longer inhibited (Fig. 5C), whereas the E2 mutation alone was not sufficient to confer flunarizine resistance in this fusion assay. These results indicate that flunarizine specifically targets HCV membrane fusion and that resistance is primarily mediated by two mutations within E1.

To further corroborate that flunarizine targets HCV fusion, we used an imaging assay of fluorescent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiI C18[5]; DiD) HCV entry into polarized, three-dimensional hepatocyte cultures (Baktash et al., unpublished data), which enables quantification of HCV single particle cell surface trafficking to the tight junction complex, virus internalization into early endosomes, and ultimately fusion and uncoating. Following DiD-HCV fusion and uncoating, there is an increase in distribution of fluorescence due to mixing of DiD with cellular membranes, which can be quantified.[15] Huh-7.5 cells were grown in extracellular matrix to form hepatic spheroids, treated with either flunarizine or the vehicle control, infected with DiD-HCV, and imaged for colocalization with either the tight junction marker zona occludins-1 or the early endosomal marker early endosomal antigen-1 (EEA-1) over a time course of entry. We observed that flunarizine did not affect the localization of DiD-HCV to the tight junction (Fig. 5A,B), nor did it affect the internalization and localization of DiD-HCV with the early endosome (Fig. 5C,D). Treatment with flunarizine did decrease DiD-HCV fusion, which is evidenced by significantly lower DiD fluorescence volume at 360 minutes post–temperature shift compared to the vehicle control (Fig. 5E,F). Therefore, flunarizine does not affect HCV trafficking and endocytosis but inhibits fusion of HCV with the endosomal membrane.

Phenothiazines and Diphenylmethylpiperidines, but Not Ca^{2+} Channel Inhibitors in General, Prevent HCV Fusion in a Genotype-Dependent Manner. Flunarizine is a T-type Ca^{2+} channel inhibitor and belongs to the group of diarylmethyl piperazine drugs. A range of structurally related drugs (e.g., pimozide) are known which also block Ca^{2+} ion channels and are used as antipsychotic drugs.[16] Moreover,

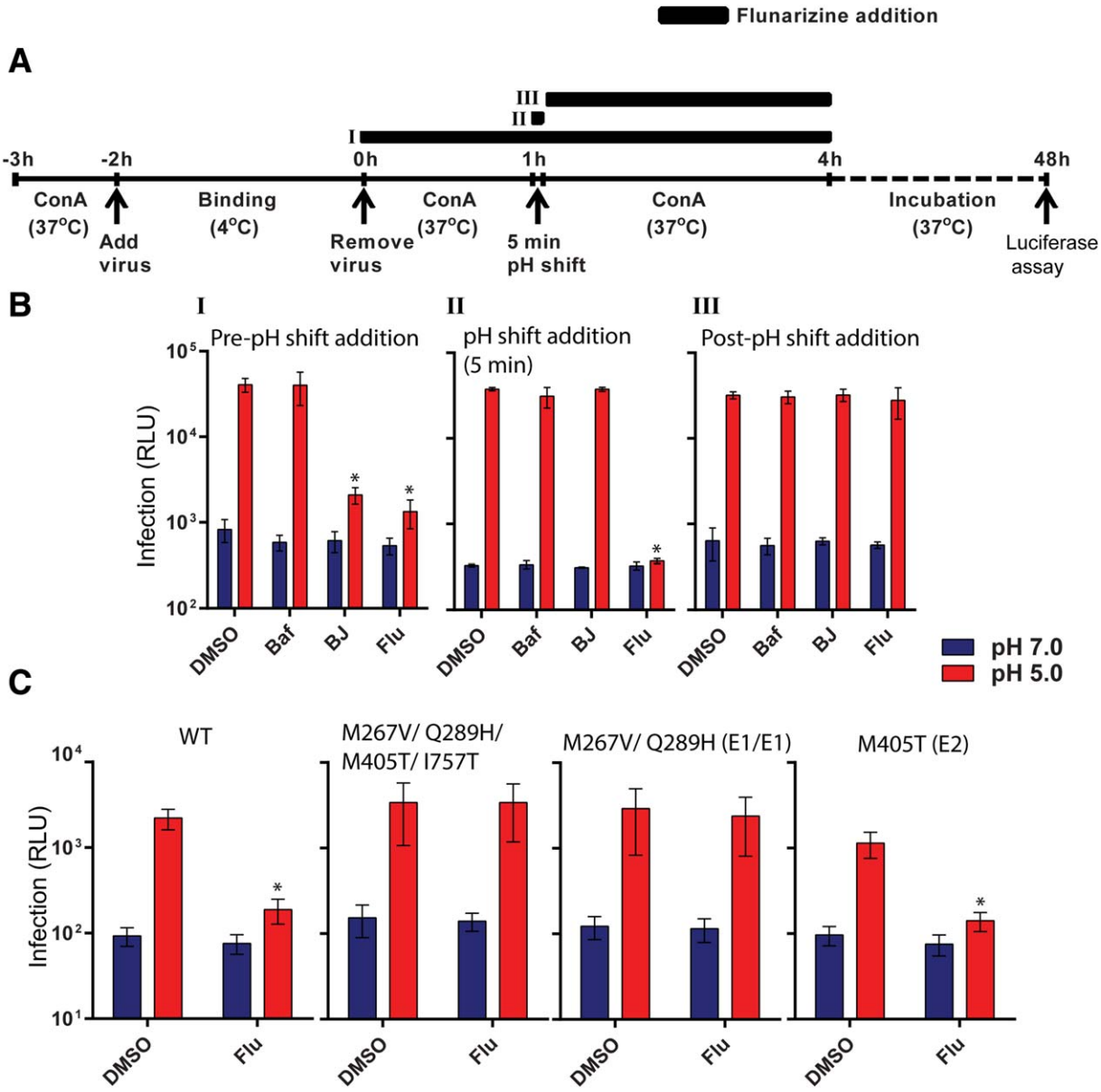

Fig. 4. Flunarizine inhibits HCV membrane fusion at the plasma membrane. (A) Schematic representation of the experimental procedure. Huh7-Lunet/hCD81/G-Luc cells were incubated with 5 nM concanamycin A 1 hour before virus inoculation and throughout the experiment until 4 hours post–virus inoculation. Additional drugs or DMSO were applied as indicated by black bars according to protocols denominated I, II, and III. F-Luc Jc1 particles were inoculated for 2 hours at 4°C. Virus membrane fusion at the plasma membrane was triggered by washing cells with a pH 5 buffer (or a pH 7 buffer as control) for 5 minutes 1 hour after inoculated cells were shifted to 37°C. In all treatments, cells were incubated another 48 hours at 37°C before infection efficiency was quantified by luciferase assays. (B) F-Luc-Jc1-dependent luciferase expression in cells inoculated according to protocol I, II, or III and with pH 5 or pH 7 buffer treatment. (C) R-Luc-Jc1-dependent luciferase expression in cells inoculated with parental Jc1 (wild type) or with Jc1 derivatives carrying indicated resistance mutations. Infection was conducted according to protocol II, depicted in (A). Abbreviations: ConA, concanamycin A; RLU, relative light units.

phenothiazines such as fluphenazine and trifluoperazine are also important drugs in which the phenothiazine group resembles the biarylmethyl group. Indeed, all of these have emerged as potent HCV inhibitors in our screening (Supporting Fig. S1). Therefore, we explored if inhibition of Ca^{2+} ion channels in general is essential for the antiviral activity of these molecules. Furthermore, we investigated if these drugs inhibit HCV through an antiviral mechanism comparable to flunarizine. To address this we explored the influence of 1,2-bis(o-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid and ethylene glycol tetraacetic acid, two Ca^{2+}-chelating agents, which sequester intracellular and extracellular Ca^{2+} pools, respectively, on the antiviral activity of flunarizine. Although both 1,2-bis(o-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid and ethylene glycol

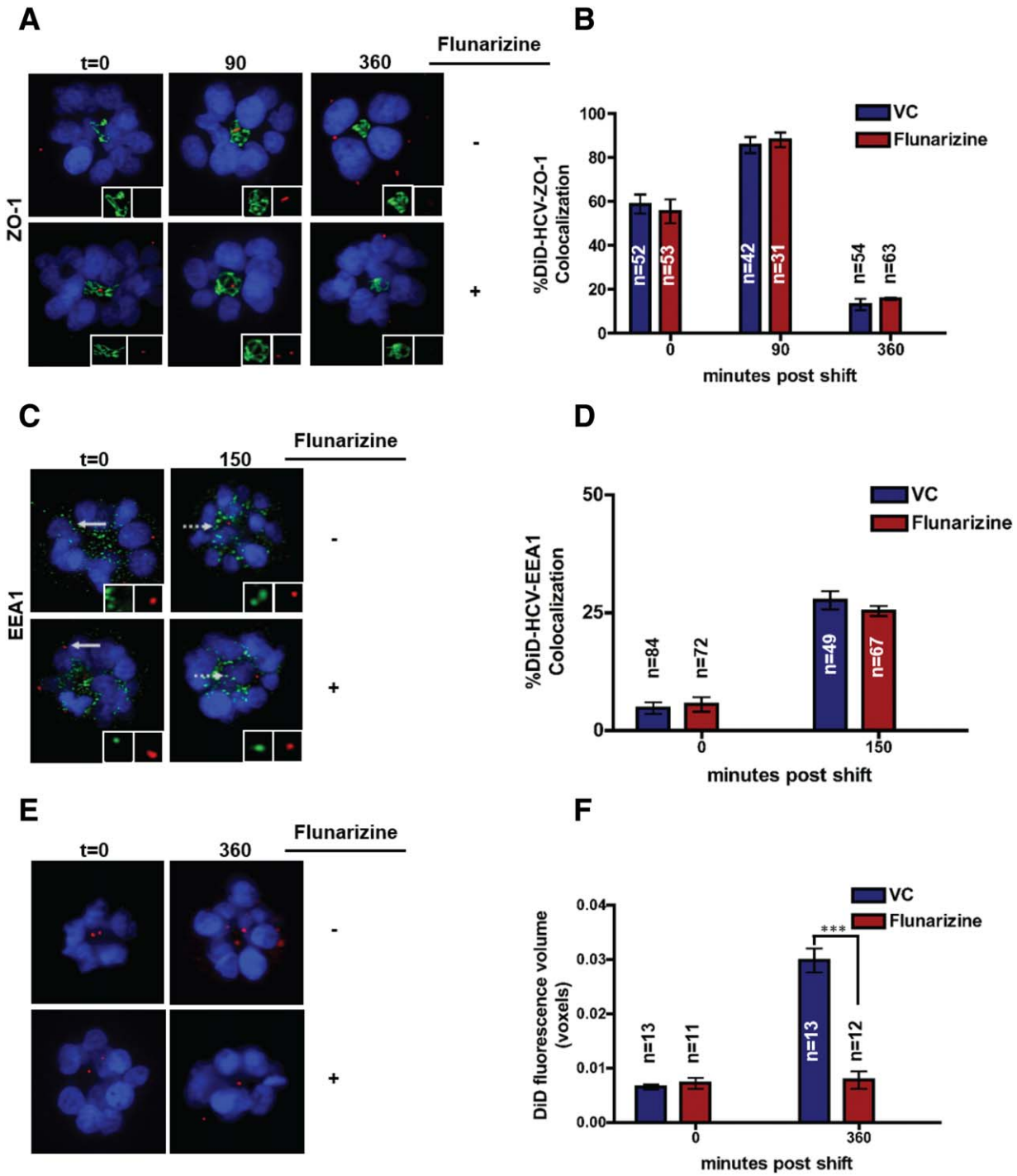

Fig. 5. Treatment with flunarizine perturbs DiD-HCV endosomal membrane fusion. Huh-7.5 organoids were incubated with 10 μM flunarizine or 10% Dulbecco's modified Eagle's medium alone for 1 hour, infected with DiD-HCV for 1 hour at 4°C, shifted to 37°C for the indicated times, fixed, and probed for tight junction protein zona occludins-1 (A) or early endosomal marker EEA1 (C). (A) Tight junction region is shown in the inset. (Left) Zona occludins-1 (green), (right) DiD-HCV (red). (B) Quantitation of (A). (C) Arrows indicate DiD-HCV particles enlarged in insets. (Left) EEA1 (green), (right) DiD-HCV (red). DiD-HCV colocalization with EEA1 antibody is indicated by dashed arrow. (D) Quantitation of (C). (E) DiD fluorescence over EEA1 time course. (F) Average DiD fluorescence per cluster in (E). n = total DiD signal (B,D) or total clusters (E); mean ± standard deviation. ***$P < 0.001$. Abbreviations: VC, vehicle control; ZO-1, zona occludins-1.

tetraacetic acid were administered at high doses, they did not modulate the antiviral activity of flunarizine, indicating that inhibition of HCV entry by flunarizine does not depend on availability of Ca^{2+} (Fig. 6A). Next, we explored if mibefradil,[17] penfluridol,[18] or $NiCl_2$,[19] three well-established inhibitors of T-type Ca^{2+} channels, interfere with HCV infection. None of these molecules inhibited infection of parental HCV or

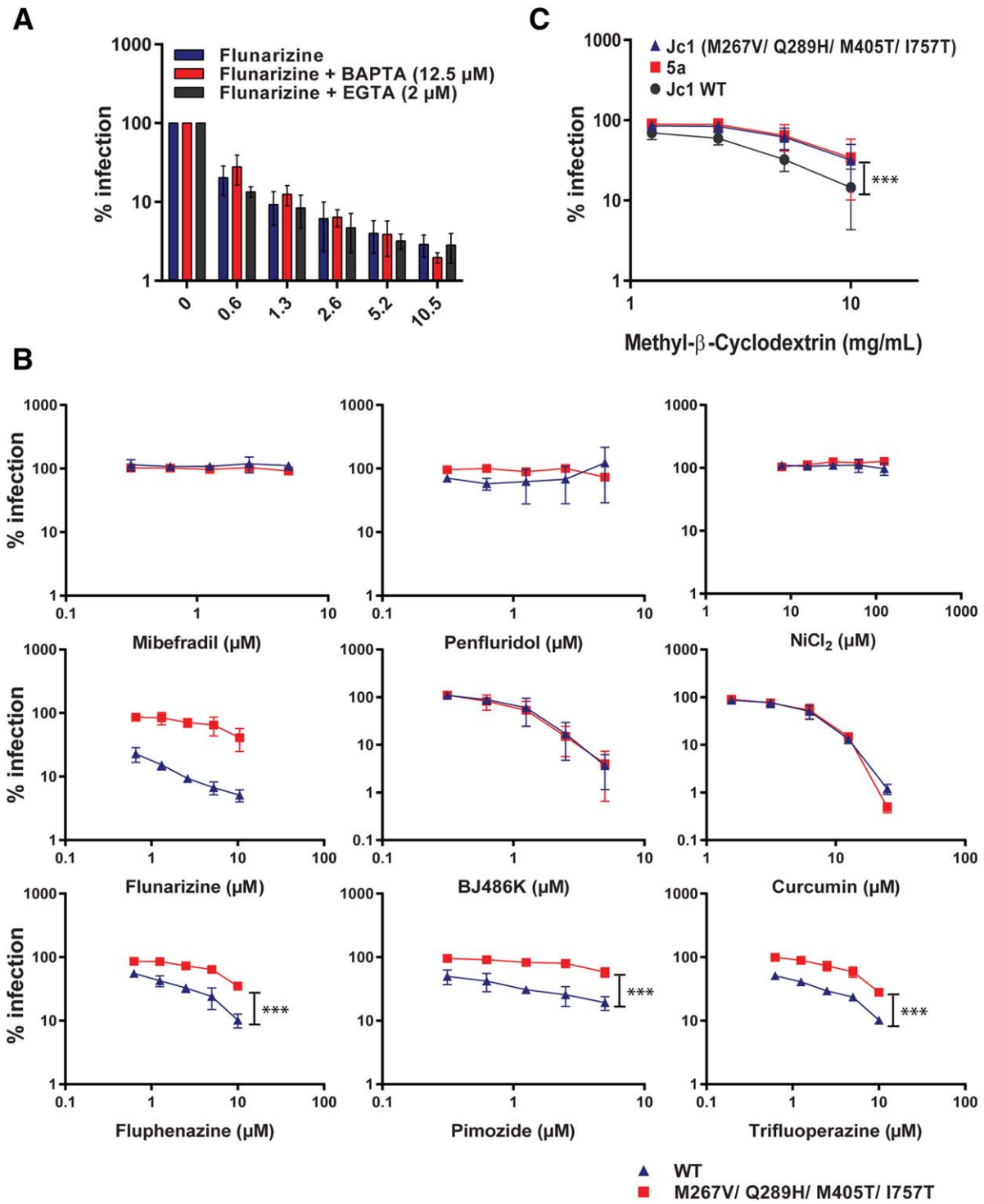

Fig. 6. Flunarizine-resistant HCV is cross-resistant to phenothiazines and pimozide, and antiviral activity is not modulated by Ca^{2+} sequestration. (A) Huh7-Lunet/hCD81/G-Luc cells were infected with F-Luc-Jc1 in the presence of increasing concentrations of flunarizine either alone or together with the intracellular calcium chelator 1,2-bis(o-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra-(acetoxymethyl)ester or the extracellular calcium chelator ethylene glycol tetraacetic acid. After 2 hours, the viruses and compounds were washed away with phosphate-buffered saline twice and fresh medium was added. Cells were lysed and the firefly levels measured 48 hours postinfection. (B) Huh7-Lunet/hCD81/G-Luc cells were infected with parental Jc1 *Renilla* reporter viruses (WT) or with the flunarizine-resistant Jc1 variant (M267V/Q289H/M405T/I757T) for 4 hours in the presence of serially diluted mibefradil, penfluridol, or $NiCl_2$. Medium was changed, and 48 hours later cells were lysed and measured for *Renilla* luciferase activity. (C) Huh7-Lunet/hCD81/G-Luc cells were pretreated for 1 hour with increasing concentrations of methyl-*β*-cyclodextrin to deplete membrane cholesterol. After washing with phosphate-buffered saline, cells were inoculated with given *Renilla* reporter viruses for 4 hours. Cells were lyzed and *Renilla* levels measured 48 hours postinoculation. Abbreviations: BAPTA, 1,2-bis(o-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetra-(acetoxymethyl)ester; EGTA, ethylene glycol tetraacetic acid; WT, wild type.

flunarizine-resistant HCV (Fig. 6B). In contrast, both phenothiazines (fluphenazine and trifluoperazine) and pimozide preferentially inhibited the parental virus and were less active against the variant with flunarizine resistance mutations. The antiviral activity of BJ486K[20] and curcumin,[21] two molecules that inhibit HCV cell entry by different molecular mechanisms, was not influenced by these mutations (Fig. 6B). While this work was in preparation, Chamoun-Emanuelli et al. reported that selected phenothiazines inhibit HCV entry potentially by increasing the fluidity of cholesterol-rich membranes.[22] This prompted us to explore if viral susceptibility to flunarizine correlates with susceptibility to modulation of membrane fluidity achieved by depletion of cholesterol using methyl-beta-cyclodextrin. Interestingly, we observed that Jc1 was significantly more susceptible to cholesterol depletion than a GT5a virus, which has high endogenous flunarizine resistance (Fig. 6C). Moreover, also the flunarizine-resistant Jc1 variant was significantly more resistant to depletion of cholesterol compared with the parental Jc1 (Fig. 6C). This suggests that differences in the dependence of the HCV fusion machinery on membrane properties may in part determine susceptibility to flunarizine, phenothiazines, and pimozide. However, in our experiments these drugs did not modulate membrane fluidity (Supporting Fig. S7) or cellular cholesterol content (Supporting Fig. S8), suggesting that other membrane changes are responsible for fusion inhibition.

Notably, pimozide was antiviral *in vivo* (Fig. 1D), and the individual mutations in E1 and E2 conferred partial resistance to pimozide, like they did to flunarizine (Supporting Fig. S9). Finally, fluphenazine, trifluoperazine, and pimozide displayed a preference for GT2a over the other GTs, very similar to flunarizine. However, pimozide and fluphenazine displayed a much improved cross-genotype coverage, with IC_{50} values below 10 μM for GT3a, GT5a, and GT7a in the case of pimozide and for GT3a, GT6a, and GT7a for fluphenazine (Fig. 7). In conclusion, the antiviral activity of flunarizine and related compounds is not directly linked with their ability to inhibit Ca^{2+} ion channels. Nevertheless, viral cross-resistance and comparable genotype specificity between flunarizine, these phenothiazines, and pimozide strongly argue for a shared antiviral mode of action.

Discussion

We screened a library of clinically licensed drugs and identified four related ion channel inhibitors that prevent HCV entry *in vitro* and *in vivo*. We provide evidence based on chimeric viruses and resistance mutations that these molecules selectively target HCV E1 and E2 functioning during entry. Moreover, time-of-addition experiments and single particle tracking indicate that these drugs specifically inhibit HCV membrane fusion. Finally, the domain of E1 carrying the resistance mutations likely plays a key role in membrane fusion.

Several scenarios for how these molecules prevent HCV membrane fusion are possible. Firstly, these drugs, which all inhibit cellular Ca^{2+} channels, may prevent fusion by changing Ca^{2+} fluxes in HCV target cells and/or by directly blocking the interaction of HCV with a Ca^{2+} channel critical for membrane fusion. However, not all tested Ca^{2+} channel targeting drugs also arrested HCV infection. Moreover, sequestration of Ca^{2+} did not modulate the antiviral activity of flunarizine. Therefore, we consider it unlikely that inhibition of Ca^{2+} channels by these drugs, which then would be used by HCV in a genotype-dependent fashion, is responsible for the anti-HCV activity of this class of molecules.

Secondly, it is possible that flunarizine, pimozide, and phenothiazines directly bind E1 and E2, thereby inhibiting membrane fusion. The genotype specificity of the antiviral activity supports this notion as sequence variation may prevent drug binding. However, HCVpp carrying the same viral envelope proteins as flunarizine-susceptible HCVcc particles are not inhibited by flunarizine, arguing against this. Also, Chockalingam et al.[10] and Hu et al.[11] reported resistance of HCVpp to flunarizine, leading them to conclude that flunarizine is likely not an entry inhibitor. Our data, however, show that flunarizine inhibits entry of authentic HCV at the stage of membrane fusion. This highlights an as yet unexplored difference between the mode of HCVpp and HCVcc membrane fusion, which may arise because HCVpp are produced in 293T cells rather than in human liver cells. Consequently, HCVpp could differ in lipid and lipoprotein composition, thus affecting cell entry. Notably, it has been reported that receptor usage can differ between HCVcc and HCVpp, with only the former using the Niemann-Pick C1-like 1 cholesterol absorption receptor for cell entry.[23] Furthermore, Meertens and colleagues observed that cholesterol depletion of the host cell had no effect on the entry of HCVpp, whereas it was antiviral for HCVcc.[24] Therefore, the resistance mutations identified by us could be part of a direct drug binding site, or they could modulate the conformation and function of E1 and E2 in membrane fusion, which seems to be exquisitely cholesterol-dependent for HCVcc particles. The E1 domain from residue 264-290 has previously been implicated in harboring an HCV fusion peptide based on phylogenetic comparison with related

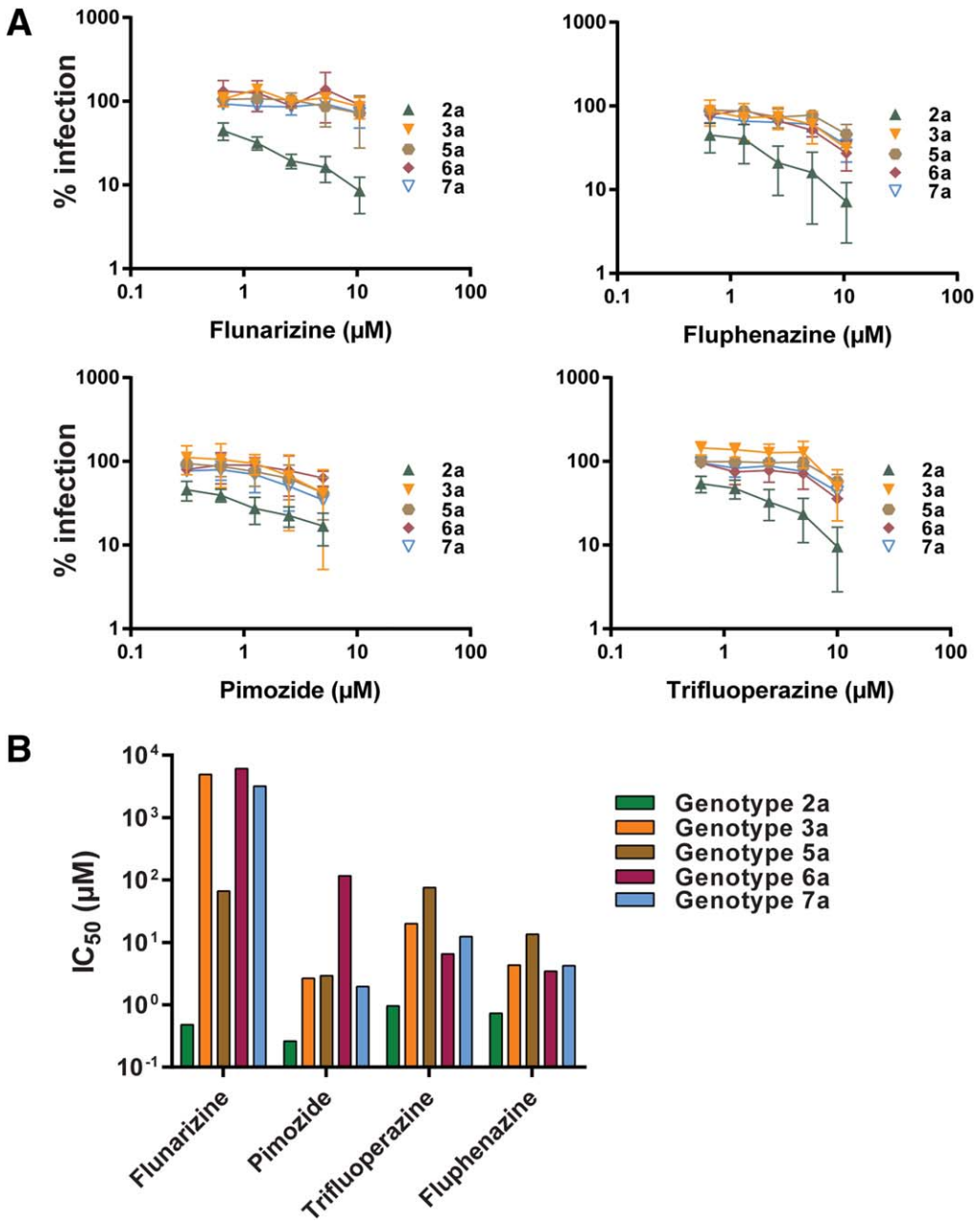

Fig. 7. Fluphenazine, pimozide, and trifluoperazine preferentially inhibit HCV infection in a genotype-dependent fashion but are more active against other genotypes than flunarizine. (A) Huh7-Lunet/hCD81/G-Luc cells were inoculated with *Renilla* luciferase reporter virus chimeras of GT2a (Jc1), GT3a (S52), GT5a (SA13), GT6a (HK6a), or GT7a (QC69) together with two-fold dilutions of flunarizine, fluphenazine, pimozide, or trifluoperazine. After 48 hours cells were lyzed and measured for *Renilla* luciferase activity. Means and standard deviations of three independent experiments are given. (B) The calculated IC_{50} of each compound against each indicated genotype represented in (A) was plotted for comparison.

flaviviruses, sequence conservation, and hydrophobicity.[25] The observation that drugs that specifically inhibit membrane fusion select for viral resistance in this region indirectly supports the notion that this peptide is critical for fusion. The crystal structures of E2 did not reveal evidence for a major role of E2 as a fusion protein, so E1 is now assumed to be the primary fusion protein of HCV.[26,27] This is supported by earlier studies.[28-30]

Finally, it is possible that these lipophilic drugs perturb membrane properties critical for virus membrane fusion. In fact, while this work was in preparation Chamoun-Emanuelli et al. reported that this was the case for specific phenothiazines including fluphenazine and trifluoperazine, also characterized by us.[22] Intriguingly, we observed a correlation between HCV susceptibility to flunarizine and susceptibility of HCV to

cholesterol depletion–induced changes in membrane properties. This result is compatible with the notion that flunarizine and pimozide, like the phenothiazines, modulate membrane fluidity and that this is the antiviral principle of these groups of compounds. In turn, the genotype-specific antiviral activity of these molecules implies that viral strains differ with regard to membrane requirements during fusion. The cholesterol depletion experiment reported here, which shows that Jc1 is more susceptible to cholesterol depletion compared to the flunarizine-resistant Jc1 and to a flunarizine-resistant strain (GT5a), supports this notion. However, unlike Chamoun-Emanuelli et al. but consistent with Thomas and Verkleij, we did not observe a modification of membrane fluidity by flunarizine and related drugs.[31] This discrepancy between studies may be due to differences in the assay setup including the composition of liposomes (Supporting Fig. S7). Moreover, flunarizine and related compounds did not extract cholesterol from Huh-7.5 cells, even upon prolonged incubation (Supporting Fig. S8).

Flunarizine also affects other biophysical membrane properties. For example, it inhibits the formation of hexagonal H_{II} phase,[31] which is homologous to the formation of the negative curvature required for fusion of viral and cellular membranes.[32,33] Notably, cholesterol depletion also affects the ability of a lipid bilayer to form the negative curvature required for fusion. Flunarizine may therefore inhibit HCV infectivity by affecting biophysical properties of the cellular membranes, which are also dependent on cholesterol content, other than fluidity.

A combination of three amino acid changes was necessary to obtain a 50-fold viral resistance to flunarizine. Moreover, the resistance mutation affected in part completely or highly conserved residues and significantly increased virus neutralization by two cross-neutralizing antibodies, suggesting that the barrier to viral resistance could be relatively high *in vivo*. Therefore, flunarizine and related compounds merit consideration for repurposing as potential adjunct therapy for GT2-infected patients and, due to broader GT coverage in case of pimozide, trifluoperazine and fluphenazine also for the difficult to treat GT3.

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