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Clinically Approved Ion Channel Inhibitors Close Gates for Hepatitis C Virus and Open Doors for Drug Repurposing in Infectious Viral Diseases

Thomas Pietschmann

Institute for Experimental Virology, TWINCORE Centre for Experimental and Clinical Infection Research, Hannover, Germany, and German Centre for Infection Research, Hannover-Braunschweig, Germany

ABSTRACT Chronic hepatitis C virus (HCV) infection causes severe liver disease and affects ca. 146 million individuals. Novel directly acting antivirals targeting HCV have revolutionized treatment. However, high costs limit access to therapy. Recently, several related drugs used in humans to treat allergies or as neuroleptics emerged as potent HCV cell entry inhibitors. Insights into their antiviral modes of action may increase opportunities for drug repurposing in hepatitis C and possibly other important human viral infections.

KEYWORDS cell entry, drug repurposing, fusion inhibitor, hepatitis C virus, infectious disease, ion channel inhibitors, membrane fusion

HCV AND HEPATITIS C: FROM THE UNKNOWN CULPRIT TO THE FIRST CURABLE CHRONIC VIRAL INFECTION

Forty years ago, hepatitis A virus (HAV) and hepatitis B virus (HBV) had been discovered as major viral causes of liver disease (hepatitis). Nevertheless, at this time numerous cases of blood transfusion-associated cases of chronic hepatitis occurred. The elusive pathogen responsible for that form of transmissible liver disease was termed non-A, non-B hepatitis (NANBH) until in 1989 Michael Houghton and his team cloned an RNA viral genome from a chimpanzee that had been inoculated with serum from a NANBH patient (1). The genome structure of the novel virus resembled that of a flavivirus, and the pathogen was named hepatitis C virus (HCV). It encodes a single polyprotein of roughly 3,000 amino acids which is cleaved into 10 distinct polypeptides. These include the core, envelope 1 (E1), and E2 proteins, which constitute the virus particle, the p7 ion channel protein, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 1). Within a very short time after the discovery of the virus, diagnostic methods were developed and implemented to screen blood products and to prevent virus transmission through this route.

In contrast, the development of efficacious and well-tolerated antiviral treatments took much longer and finally culminated in the licensing of telaprevir and boceprevir, the first directly acting antivirals (DAAs), in 2011 (2). These drugs target the viral protease NS3-4A, thus precluding polyprotein cleavage and, in turn, RNA replication (Fig. 1). The drugs were the vanguard of a phalanx of additional DAAs that address one of three viral targets, i.e., the NS3-4A protease, the viral phosphoprotein NS5A, and the RNA-dependent RNA polymerase NS5B. Since HCV is a plus-strand RNA virus that does not stably integrate into the host cellular genome, these drugs can eliminate the virus and cure the infection. Presently, highly efficacious and well-tolerated treatments for the entire spectrum of HCV genotypes are available (3). Nevertheless, some challenges remain. First, these drugs are costly, which may limit therapy uptake, particularly in low-income countries where the disease burden is greatest. Second, the majority of infected patients are not diagnosed and thus do not know that they are infected.
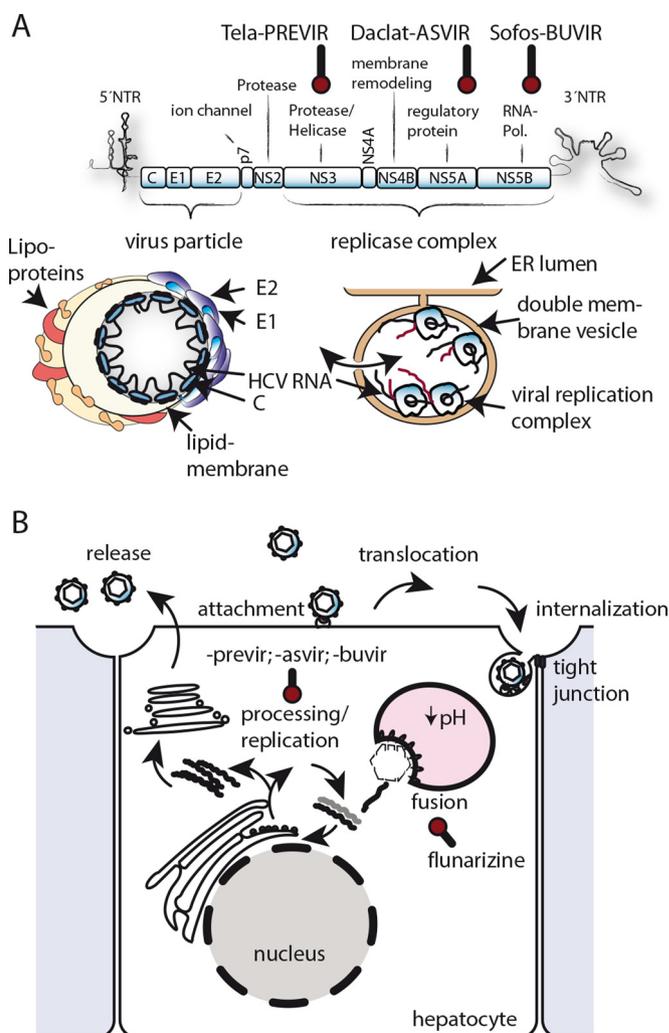
FIG 1 Schematic representations of the HCV genome organization, the virus and replication complex structures, and the replication cycle. (A) The HCV genome encodes highly structured 5′ and 3′ non-translated regions (NTRs) needed for polyprotein translation and RNA replication. Key functions of individual proteins are highlighted, and the three major drug targets addressed by licensed directly acting antivirals are indicated. The suffixes identifying the different drug classes are printed in capital letters. The HCV particle consists of viral factors and human lipoproteins, and RNA replication complexes are composed of viral NS3 to NS5B proteins and host factors enclosed in endoplasmic reticulum (ER)-derived double-membrane vesicles. RNA Pol., RNA polymerase. (B) HCV attaches to hepatocytes by binding to glycosaminoglycans and SR-B1, and it translocates to tight junctions, where it is endocytosed through clathrin-mediated endocytosis. The viral genome is delivered into the cytoplasm through a low-pH-triggered fusion process that involves the E1 and E2 glycoproteins.

Finally, treatment-induced viral clearance does not protect from reinfection with the virus. This is particularly of relevance for groups at high risk for virus transmission, such as people who inject drugs. Therefore, global public health efforts are needed to increase diagnosis and treatment uptake and consequently reduce the HCV-associated disease burden.

ION CHANNEL INHIBITORS AND RELATED ANTIHISTAMINES AS FIRST-IN-CLASS HCV MEMBRANE FUSION INHIBITORS

To enrich therapeutic options, several laboratories screened for compounds that could inhibit virus replication. In several independent campaigns, diverse compound libraries were screened; these included the NIH Clinical Compound (NCC) Collection (4), the Library of Pharmacologically Active Compounds (LOPAC[1280]) (5), a library of approved drugs that was assembled by the NIH Chemical Genomics Centre (NCGC) and

Basic Scaffolds

Diphenyl-piperazine   Diphenyl-piperidine   Pheno-thiazine

Thio-xanthene   Cycloheptene-piperidine

Diphenyl-piperazines/piperidines

	Flunarizine	Chlorcyclizine	Pimozide
Ref.	5,7, 52	6, 22	5, 7
Applicat.	Migraine	Allergy	Antipsychotic
Targets	Ca2+ chan. Calmodulin Histamine H1	Histamine H1	Dopamine r. Ca2+ chan.

Phenothiazines

	Chlorpromazine	Fluphenazine	Trifluoperazine	Mequitazine
Ref.	10	4, 5, 7, 21	4, 5, 7, 21, 52	6
Applicat.	Antipsychotic	Antipsychotic	Antipsychotic	Allergy
Targets	Dopamine r.	Dopamine r. D2	Dopamine r. Calmodulin	Histamine H1

Thio-xanthenes

	Cis-flupentixol
Ref.	21
Applicat.	Antipsychotic
Targets	Dopamine r. D1 and D2

Cycloheptene-piperidines

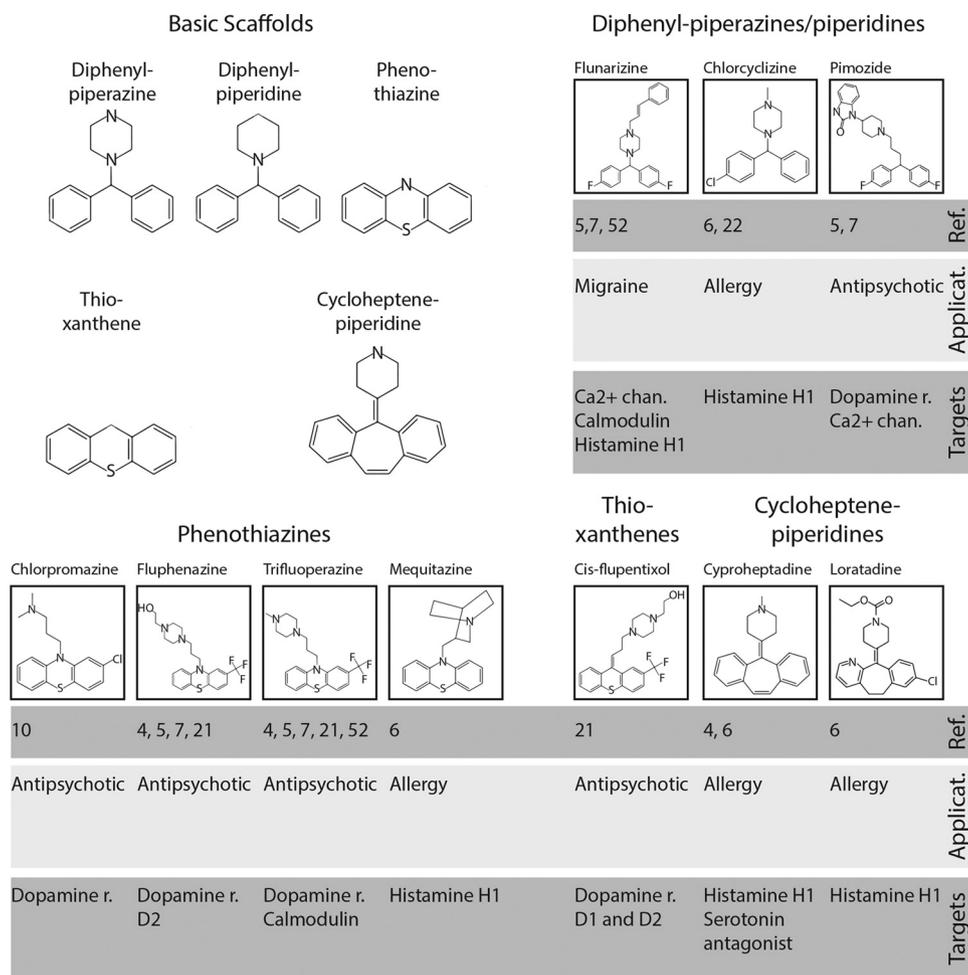|	Cyproheptadine	Loratadine
Ref.	4, 6	6
Applicat.	Allergy	Allergy
Targets	Histamine H1 Serotonin antagonist	Histamine H1

FIG 2 Overview of related chemical compounds that were recently identified as HCV entry inhibitors. Basic chemical scaffolds used to categorize the compounds highlighted here and mentioned in the text are depicted at the top left. The typical clinical uses and targets of these compounds (according to the respective PubChem entries) are given, and the publications that reported the anti-HCV activities of the given molecules are indicated. Ref., reference; Applicat., application; chan., channel; r., receptor.

called the NCGC Pharmaceutical Collection (NPC) (6), and a small group of clinically approved ion channel inhibitors used to treat cardiac or neurologic conditions (7). These independent studies identified several structurally related compounds as potent HCV cell entry inhibitors both *in vitro* and *in vivo*. HCV cell entry is a complex process that involves multiple entry factors, among which the scavenger receptor class B type 1 (SR-B1), the tetraspanin CD81, and the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) are absolutely required (8, 9). HCV is taken up, through clathrin-mediated endocytosis, into host cells (10, 11), where the viral glycoproteins E1 and E2 coordinate viral membrane fusion in a process that is triggered by the low pH in endosomes (12) (Fig. 1). Interestingly, several recent studies provided *in vivo* proof of the concept that interference with HCV cell entry by broadly virus-neutralizing antibodies or by host receptor-targeting antibodies is a viable therapeutic strategy capable of eliminating HCV (13, 14), which stimulated attempts to develop HCV entry inhibitors as antiviral agents. The molecules that emerged as HCV entry inhibitors in the above-mentioned screenings can be grouped into four basic chemical scaffolds, namely, the diphenyl-piperazines or diphenyl-piperidines, the phenothiazines, the thioxanthenes, and the cycloheptene-piperidines (Fig. 2). While the anti-HCV activity was revealed only recently, some of these molecules were discovered as virus inhibitors more than 30 years ago. For instance, phenothiazines like chlorpromazine emerged as inhibitors of influenza virus replication (15), and trifluoperazine, chlorpromazine, prochlorproma-

zine, and promethazine were reported to block Epstein-Barr virus infectivity (16). Also, members of the paramyxovirus and arenavirus families were shown to be susceptible to phenothiazines (17, 18), and HIV-1 was reported to be blocked by the phenothiazine trifluoperazine (19) and by chlorcyclizine, a diphenyl-piperazine (20). Thus, highly diverse, enveloped, DNA and RNA viruses are inhibited by representatives of these related chemical scaffolds. These molecules may inhibit these diverse viruses by common or different molecular mechanisms, and they can influence early cell entry (4–7, 15–17, 19, 21, 22) or late virus assembly and release steps (17, 18, 23). Although this cumulative evidence highlights the potential of these molecules as antivirals, their precise modes of action against these diverse viruses still remain elusive. Moreover, even though some of these compounds have been routinely used in humans, to date, none have been repurposed for treatment of viral infections. The recent discoveries in the HCV field have shed additional light on the modes of action of these interesting molecules, potentially bringing these or related drugs a few steps closer to clinical use to treat viral infections.

On the one hand, many diphenyl-piperazines (e.g., chlorcyclizine, cyclizine, and hydroxyzine), cycloheptene-piperidines (e.g., cyproheptadine, ketotifen, loratadine, and desloratadine), and phenothiazines (e.g., mequitazine and trimeprazine) that were recently shown to inhibit HCV cell entry (4, 6) are known to act as H_1 antihistamines (24). Thus, in clinics, these compounds are (or have been) used because of their ability to competitively inhibit the interaction between histamine and the H_1 histamine receptor. The histamine receptor is a typical G protein-coupled receptor (GPCR) that, upon binding to histamine, is activated and stimulates several signaling processes. These include the production of inositol 1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG), causing an accumulation of intracellular calcium (24, 25). Moreover, NF-κB-, phospholipase D-, and phospholipase A-dependent pathways can be stimulated by H_1 histamine receptor activation, and they are involved in the development of allergies (24, 25). H_1 antihistamines are grouped as first- and second-generation drugs, with the latter causing many fewer side effects (24). Unlike second-generation H_1 antihistamines, drugs of the first generation cross the blood-brain barrier. They influence the function of muscarinic, α-adrenergic, and serotonergic receptors and can modulate cardiac channels, resulting in a relatively broad spectrum of side effects, including sedation, hyperactivity, insomnia, and convulsions (24). Among antihistamines with anti-HCV activity are both first-generation (chlorcyclizine, cyclizine, hydroxyzine, cyproheptadine, ketotifen, mequitazine, and trimeprazine) and second-generation (loratadine and desloratadine) antihistamines (4, 6, 24), which indicates that both drug classes include molecules that can target HCV.

Numerous anti-HCV phenothiazines (trifluoperazine, fluphenazine, promazine, chlorpromazine, triflupromazine, mesoridazine, and thioridazine), diphenyl-piperazines (flunarizine), diphenyl-piperidines (pimozide), and thioxanthenes (cis-flupentixol) are used as neuroleptics in clinics to treat migraines or psychiatric diseases. These drugs preferentially target Ca^{2+} ion channels (e.g., flunarizine) or dopamine receptors. Like the H_1 histamine receptor, dopamine receptors belong to the superfamily of GPCRs, which includes multiple transmembrane proteins that share seven transmembrane helices as a common structural feature. Based on sequence and structural similarity, these proteins are grouped into five families, namely, the rhodopsin (family A), secretin (family B), glutamate (family C), adhesion, and frizzled/taste families (26). GPCRs coordinate a large spectrum of physiological reactions by receiving signaling input from the extracellular milieu and relaying this information into the cell through interactions with G proteins, thus permitting adequate responses to hormones, neurotransmitters, and environmental stimuli (26). GPCRs homo- or heteroligomerize constitutively or upon ligand binding (27), and they are the targets of almost one-third of prescribed drugs, including beta blockers and antipsychotics (28, 29). Accumulating structural information about GPCRs bound to ligands or antagonists is now disclosing the architecture of drug binding sites within GPCR transmembrane helices and guides modeling approaches to identify compounds that selectively target specific GPCRs (30–32). The H_1 histamine receptor

and dopamine receptors all belong to family A, the rhodopsin-like GPCRs (33; http://www.gpcrdb.org). Receptors of family A GPCRs usually bind their ligands within a transmembrane pocket involving contact residues within several GPCR transmembrane helices (27, 28). Ligand binding triggers conformational changes that relocate helices and unmask previously inaccessible binding sites for G proteins (28, 34).

Voltage-gated calcium channels are key regulators of brain, skeletal, and heart muscle functions (35). They control the influx of Ca^{2+} ions, thus serving electrogenic functions by mediating Ca^{2+}-dependent changes in membrane potential. Moreover, they modulate intracellular Ca^{2+} levels and thus influence the activity of Ca^{2+}-binding proteins like calmodulin and Ca^{2+}-dependent enzymes, thus regulating many cellular processes, including muscle contraction, gene transcription, and neurotransmitter/hormone release (reference 35 and references therein). In turn, dysfunction of these processes can cause hypertension, cardiac hypertrophy, and neurological problems, thus rendering Ca^{2+} channels important targets for therapeutic intervention (35). Ca^{2+} channels are grouped into high- and low-voltage-activated (HVA and LVA, respectively) channels, which are further subdivided into T-type LVA and L-, P-, Q-, and R-type HVA channels. Each of these channels possesses a specific $\alpha 1$ subunit (called Cav3.1, Cav3.2, or Cav3.3 in the case of T-type channels) that is ca. 200 kDa in size and is composed of four transmembrane domains, each containing six transmembrane helices (35). The majority of drug binding sites that have been characterized surround the channel pore, involving the fifth and sixth transmembrane helices, or they lie within the permeation pathway (reference 35 and references therein). Although there is no structural information regarding the precise binding site of flunarizine, it is well established that this neuroleptic is used to treat migraine (36) and vertigo (37) and that it inhibits all three T-type Ca^{2+} channels (38). Moreover, a moderate antagonistic activity toward dopamine receptors was reported (38). Thus, all of the above-mentioned drugs have in common the fact that they inhibit cellular transmembrane proteins with multiple transmembrane helices. Moreover, the structural information that is available for some GPCRs and their respective (ant)agonists highlights the fact that key drug and ligand interactions occur through direct binding within transmembrane pockets. This information has implications for understanding the antiviral modes of action of these molecules and is discussed below, with an emphasis on HCV.

It is possible that virus infection is blocked by these drugs through direct interference of these compounds with their primary target protein(s) (i.e., histamine receptors, dopamine receptors, or Ca^{2+} ion channels). In turn, this implies that HCV and the other above-mentioned viruses rely on these host factors for virus infection. However, based on transcriptional profiling, H_1 histamine receptors are not expressed in Huh-7.5 cells (39; T. Pietschmann, unpublished observation), a cell line routinely used in HCV research and the parent of the cell line used by He et al. to identify numerous antihistamines as HCV inhibitors (6). Moreover, primary human hepatocytes do not express H_1 histamine receptor mRNA (40), although the antiviral activity of chlorcyclizine in these cells was readily detectable (6). Finally, histamine itself does not affect HCV infection, and two related compounds with divergent antihistamine activities have similar anti-HCV activities (6). Along the same lines, dopamine receptors and T-type Ca^{2+} ion channels (CACNA1G, CACNA1H, CACNA1I) are not (or are poorly) expressed in Huh-7.5 cells (<0.18 reads per kilobase per million [RPKM]) (data not shown; T. Pietschmann, unpublished observation), in primary hepatocyte transcriptional profiles, and in human livers (the Human Protein Atlas [http://www.proteinatlas.org]). Moreover, pimozide inhibits T-type Ca^{2+} channels much more effectively than flunarizine (38) but inhibits HCV about as much as flunarizine (7). Beyond this, other potent Ca^{2+} channel inhibitors, like mibefradil (41), penfluridol (42), and $NiCl_2$ (43), do not inhibit HCV, and chelation of intracellular or extracellular Ca^{2+} does not influence the antiviral activity of flunarizine (7). Taken together, these observations indicate that the anti-HCV activities of chlorcyclizine and flunarizine (and likely also of compounds related to these molecules) are independent of the inhibition of H_1 histamine receptors, dopamine receptors, and Ca^{2+} ion channels. Connected with this, our observation that chelation of Ca^{2+}

does not inhibit HCV infection suggests that indirect effects based on the activities of Ca^{2+}-dependent enzymes or calmodulin are also unlikely to explain the antiviral activities of these compounds toward HCV. This may be different for other viruses targeted by such compounds. For instance, the phenothiazines trifluoperazine and chlorpromazine inhibit arenaviral multiplication at early or late steps of the viral replication cycle, and the effect of trifluoperazine on Junin virus is significantly reversed by the administration of recombinant calmodulin (17). Similarly, the effect of trifluoperazine on the late stage of influenza A virus infection is reversed by the addition of purified calmodulin (23), indicating that, for these viruses, Ca^{2+}- and/or calmodulin-dependent functions targeted by trifluoperazine may be responsible for the antiviral activity. Since calmodulin influences the cytoskeleton and phenothiazines have been reported to disrupt the cytoskeletal organization in different cell types (44, 45), it was proposed that these viruses require the integrity of actin microfilaments and the drugs modify these structures, thus exerting an antiviral effect (17).

In keeping with the notion that key cellular structures or processes are disrupted by these compounds, leading to inhibition of HCV and other viruses, it is worth mentioning that chlorpromazine, a phenothiazine, is an inhibitor of clathrin-mediated endocytosis (46). Since HCV infects cells by using the endocytic pathway (10), it is reasonable to assume that chlorpromazine and possibly also related compounds inhibit HCV and other viruses by disrupting this process (4, 10). While this may be the case for chlorpromazine and its antiviral activity toward HCV and other viruses, this is, however, unlikely to be the primary antiviral mechanism for related phenothiazines, like fluphenazine and trifluoperazine, and for flunarizine, a structurally similar diphenyl-piperazine (7). Endocytosis is thought to be critical for HCV infection across all strains and genotypes (10, 11). However, the latter drugs inhibit HCV in a highly strain-dependent manner, with 50% inhibitory concentration (IC_{50}) values varying more than 10-fold between individual strains (7). Moreover, at least flunarizine was shown to inhibit HCV cell entry independently of endocytosis. When HCV infection via endosomes was blocked by administration of bafilomycin, an inhibitor of ATPases needed for acidification of these organelles, and HCV was "forced" to infect cells by entering at the plasma membrane by using a brief low-pH pulse, flunarizine was still antiviral (7). In fact, administration of flunarizine only during a 5-min-long wash with low-pH buffer was necessary and sufficient to inhibit HCV infection (7). Moreover, using single-particle-tracking live-cell imaging, we observed that, in the presence of flunarizine, HCV particles readily reached cellular tight junctions and were internalized into endosomes but that only membrane fusion was inhibited (7). Since viral flunarizine resistance mutations, which map to residues M267 and Q289 of E1 and M405 of E2 (J6 isolate, GT2a), confer cross-resistance to pimozide, fluphenazine, and trifluoperazine, it can be assumed that these related diphenyl-piperidines and phenothiazines share a mode of action, which, given the findings described above, is unlikely to be inhibition of endocytosis (7). Taken together, these results support the conclusion that at least these drugs (and possibly other related compounds as well) inhibit HCV membrane fusion independently of their effects on virus endocytosis and cell surface trafficking.

Instead, at least two different, not mutually exclusive, mechanisms may account for the inhibition of HCV membrane fusion by these drugs. These compounds have lipophilic domains allowing them to integrate into cellular (or viral) membranes. This may perturb the alignment, orientation, and mobility of lipids and cholesterol and, in turn, affect viral membrane fusion (21). Alternatively (or in addition), the common structural properties of these drugs may allow them to directly bind viral or cellular transmembrane domains, thus inhibiting conformational changes that are critical for coordinating fusion between viral and cellular membranes (7). The latter mode of action would be reminiscent of the way in which ligands and antagonists of GPCRs contact and modulate histamine or dopamine receptors, for example. With regard to flunarizine and phenothiazines like fluphenazine and trifluoperazine, pretreatment of cells but not of virus particles is antiviral, suggesting that, when added in solution, these drugs do not sufficiently interact with virus particles to inhibit infection (7, 21).

However, even after the drugs are washed out, pretreated cells are protected from HCV infection, indicating that these drugs are taken up into the cells and block infection (7, 21). Using various biophysical assays, Chamoun-Emanuelli et al. provided evidence that phenothiazines inhibit HCV fusion by increasing cellular membrane fluidity (21). However, we and others did not observe changes in membrane fluidity upon administration of these compounds (7, 47), possibly due to differences in the experimental setups and the compositions of liposomes used in these studies. Nevertheless, flunarizine also influences other membrane properties, such as formation of the hexagonal II phase and thus the negative membrane curvature that is needed for membrane fusion (47–49). Moreover, we observed that susceptibility to flunarizine correlates with the sensitivity of HCV to the depletion of membrane cholesterol (7). Since the abundance of cholesterol influences the propensity of membranes to accept the negative curvature required for membrane fusion, this correlation supports the notion that flunarizine and related drugs act on HCV, and possibly other viruses, by modulating the ability of membranes to adopt the curvature conducive to membrane fusion. Clearly, such drug effects may also involve direct binding to transmembrane protein domains, which may impede conformational changes or protein-protein interactions needed to remodel membranes during fusion. In fact, the highly strain-specific antiviral activities of many of these drugs support the notion that direct drug-protein interactions may be relevant (7). Moreover, specific resistance mutations localized to a hydrophobic patch of the HCV E1 protein that has been implicated as a viral fusion loop support this idea and point to this domain as a possible drug interaction site (7). Alternatively, there may be subtle strain-specific differences regarding the membrane requirements to trigger fusion, and the resistance mutations observed may change these requirements. Either way, it is enigmatic that HCV pseudoparticles (HCVpp), i.e., retroviral particles carrying the HCV envelope proteins E1 and E2 at their surfaces (50, 51), in contrast to authentic cell culture-derived HCV particles (HCVcc) with exactly the same glycoproteins, are not inhibited by flunarizine (5, 7, 52). Moreover, chlorcyclizine inhibits HCVcc but not HCVpp (6). In contrast, some phenothiazines, like fluphenazine, trifluoperazine, and prochlorperazine, inhibit both particle types (4, 5, 21). These observations show that there may be subtle differences in the ways that these structurally related drugs inhibit HCV. Moreover, HCV strains and particle types (HCVcc versus HCVpp) apparently infect cells in slightly dissimilar manners, thus rendering these viruses either resistant or susceptible to these drugs. One possibility is that divergent particle compositions related to the abundance of E1-E2 complexes or lipoproteins (the latter are present in HCVcc but not HCVpp [Fig. 1]) may be responsible for this. Understanding these delicate variances, ideally at the structural level, between the compounds and the viral fusion machinery will be critical for developing molecules with improved antiviral efficacy and strain coverage and should help us to understand the molecular principles of HCV membrane fusion. In parallel, such activities may boost efforts to develop such molecules or related compounds for treatment of other important viral infections. To my knowledge, to date only enveloped viruses have been shown to be susceptible to these types of drugs. It will be interesting to find out whether this is generally true and whether other enveloped viruses are also susceptible. Considering the presumed mode of action for HCV (as described above), it is certainly conceivable that compounds interfering with entry and membrane fusion may also exhibit activity against other (enveloped) viruses. Moreover, it will be interesting to explore why some compounds inhibit early viral infection steps in one virus but late steps (e.g., budding) in others; this may be related to the fact that fusion and fission of membranes share common biophysical requirements. Clearly, more molecular work regarding the precise modes of action of these compounds in different viruses will be needed to determine whether changes in membrane properties and possible direct interactions with viral or host-derived transmembrane domains are important for this. The remarkable advances in the understanding of how GPCRs are contacted by ligands and antagonists, down to the level of atomic resolution, should help investigators to move forward. Whether any of the above-mentioned drugs will ever be repurposed for treatment of viral infections

is an open question. Considering the often relatively broad spectrum of targets addressed by these compounds and the resulting side effects, the risk/benefit ratios for repurposing may not be good enough. However, it is remarkable how, in the field of antihistamines, subtle chemical modifications have much improved the specificities of compounds and reduced their side effects. These successes highlight the fact that specificity can be improved, which should therefore motivate endeavors to identify antiviral scaffolds with acceptable side effects. Team efforts between chemists, structural biologists, and virologists will be needed here. Ultimately, there may be a gem among these multicyclic compounds.

ACKNOWLEDGMENTS

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