Viroporins: structure and biological functions

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Abstract | Viroporins are small, hydrophobic proteins that are encoded by a wide range of clinically relevant animal viruses. When these proteins oligomerize in host cell membranes, they form hydrophilic pores that disrupt a number of physiological properties of the cell. Viroporins are crucial for viral pathogenicity owing to their involvement in several diverse steps of the viral life cycle. Thus, these viral proteins, which include influenza A virus matrix protein 2 (M2), HIV-1 viral protein U (Vpu) and hepatitis C virus p7, represent ideal targets for therapeutic intervention, and several compounds that block their pore-forming activity have been identified. Here, we review recent studies in the field that have advanced our knowledge of the structure and function of this expanding family of viral proteins.

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The prevalence of pathogenic viruses and the shortage of effective treatment options for the diseases that they cause warrant further research into the basic physiology and pathogenic mechanisms of these viruses. Over the past decade, an expanding viral protein family has become the subject of much interest owing to its central role in the viral life cycle. These proteins, termed viroporins, are involved in viral genome replication and assembly, as well as virus particle entry into and release from infected cells. The functional activities of these proteins also have a profound impact on the host cell, disrupting a number of important physiological processes. Viroporins are encoded by a range of animal viruses of clinical interest, such as hepatitis C virus (HCV), HIV-1, influenza A virus (IAV), coronaviruses, picornaviruses such as poliovirus, and togaviruses[1–6].

The existence of viroporins was initially suggested following the observation that virus-infected cells become permeable to ions and small molecules[7]. One possibility put forward at that time implicated the exiting virus particles themselves as the permeabilizing agent[7,8]. However, cloning and expression of selected viral genes in animal cells supported the idea that these viruses encode at least one protein that could permeabilize cellular membranes[9–13]. On the basis of such observations, the idea of a viroporin family of proteins was subsequently accepted[1].

The discovery of viroporins and their common and unique structural and functional characteristics have been reviewed previously[2]. Viroporins share several features, including their small size and hydrophobicity as well as their ability to permeabilize membranes on oligomerization. In general terms, their main function is participation in the assembly of virus particles and their release from infected cells[2]. Typically, deletion of a viroporin-encoding gene from a viral genome drastically diminishes the formation of viral progeny and viral pathogenicity, underscoring the essential role of these proteins in the viral life cycle[2].

In its infancy, viroporin research concentrated on a limited number of proteins, such as picornavirus protein 2B (P2B)[10,11,13,14], IAV matrix protein 2 (M2)[15–18] and togavirus protein 6K[19–21]. More recently, the excellent research on HIV-1 viral protein U (Vpu) and HCV p7 has greatly expanded our knowledge of the structure and function of these viroporins[3,4,22–30], and we are only now beginning to appreciate the diversity of this protein family. Indeed, the architecture of the pores or ion channels that are formed varies between viroporins, as do the sequences of the protein regions that protrude from the pores, suggesting that each viroporin can interact specifically with other viral or cellular proteins[2] (FIG. 1). Furthermore, the effects on cellular metabolism vary widely according to the viroporin considered[2,3,5,6].

The central role of viroporins in promoting viral pathogenicity has stimulated particular interest owing to their potential as targets for antiviral agents[4], and a number of compounds that interfere with the permeabilizing ability of viroporins and thereby inhibit virus production have been identified[5,15,18,24,25,27,31–33]. Moreover, viroporin-defective viruses are being explored as live attenuated vaccines[34]. In this Review, we focus on viroporins from RNA viruses — although these proteins are also encoded by a number of DNA viruses (BOX 1) — and highlight
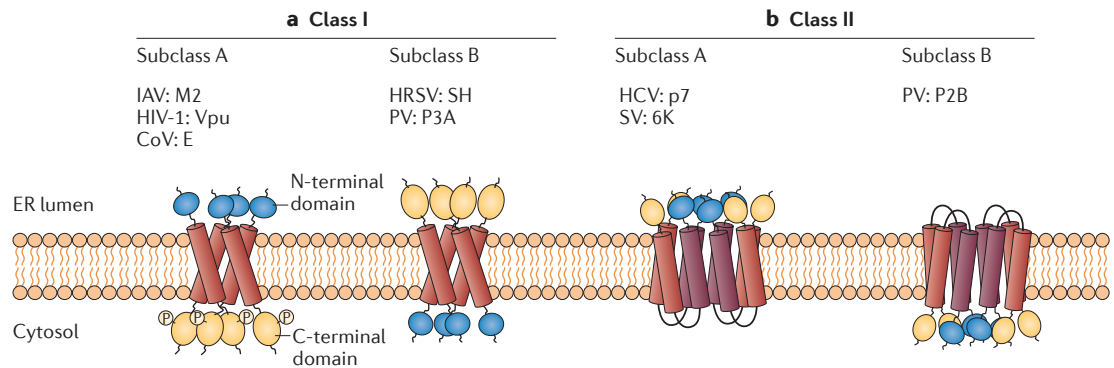
Figure 1 | **Classification of viroporins according to the number of transmembrane domains and the membrane topology of the constituent monomers. a** | Class I viroporins have a single membrane-spanning domain. The A and B subclasses contain proteins that are inserted into the membrane with either a lumenal amino terminus and cytosolic carboxyl terminus (class IA) or a cytosolic amino terminus and lumenal carboxyl terminus (class IB). In addition, class IA members are usually phosphorylated at the C terminus. Known viroporins of each subclass are shown. **b** | Class II viroporins form helix–turn–helix hairpin motifs that span the membrane. Subclass A members have lumenal N and C termini, whereas members of subclass B have cytosolic N and C termini. Known viroporins of each subclass are shown. CoV, coronavirus; E, envelope small membrane protein; HCV, hepatitis C virus; HRSV, human respiratory syncytial virus; IAV, influenza A virus; M2, matrix protein 2; PV, poliovirus; SH, small hydrophobic protein; SV, Sindbis virus; Vpu, viral protein U.

recent findings pertaining to the classification, structure and mode of action of viroporins as well as their potential as antiviral targets.

Classification by architecture

Over the past few years, substantial advances have been made in our understanding of viroporin architecture. Current hypotheses suggest that viroporins form aqueous channels following insertion into the host cell membrane and subsequent oligomerization. We propose that viroporins be classified into two major groups, class I and class II, depending on whether they contain one or two transmembrane (TM) domains, respectively (FIG. 1). These classes can be further divided into subgroups according to membrane topology. Class IA viroporins contain a small (9–25 amino acids) domain at the amino terminus, which resides in the ER lumen, and a long cytosolic tail (~50 amino acids) at the carboxyl terminus, which is prone to phosphorylation[18,35]. The class IB viroporins contain a short N terminus that is found in the cytosol and a long C-terminal tail that is located in the ER lumen. Class II viroporins have two TM domains that are connected by a loop of basic amino acids. The N and C termini of class IIA viroporins both face the ER lumen, whereas in class IIB viroporins the opposite orientation is adopted, with both termini located in the cytosol. Class II viroporins are usually derived from larger precursors by proteolytic processing[2], and both termini form part of the peptidase cleavage site. A putative third class of viroporins, for those that contain three hydrophobic domains, has been proposed; this class would include the coronavirus protein 3a[36], but further investigation is required to confirm that this protein is a genuine viroporin.

Viruses that are deficient in viroporins can be rescued in *trans* by their own viroporin and, in some instances, can be partially rescued by other, unrelated viroporins, although opposing results have been reported, probably owing to differences in the experimental approaches used[32,37,38]. However, in general, the interchange of viroporins between different species of animal viruses fails to completely rescue viral replication[38], supporting the idea that these proteins interact in a specific manner with other viral or cellular proteins. In some instances, chimeric viroporins can promote viral release; for example, the TM domain of HIV-1 Vpu can be functionally substituted by the corresponding domain of IAV M2 (REF. 39).

Finally, linking specific functional activities to either the aqueous channels or the cytosolic domain (or domains) of each viroporin is challenging. Throughout this Review, we make this distinction when possible, but it is important to note that this information is often unavailable. The use of selective inhibitors of these ion channels, as well as detailed analyses of viroporin variants, should help resolve this issue.

Cytopathic effects of viroporins

Membrane permeability and calcium homeostasis. Modification of host cell membrane permeability is a common feature of infections with most animal viruses[1,2]. Notably, transgenic expression of the individual viroporin genes of these viruses in host cells mimics this phenomenon[1,2,40], and this has greatly facilitated the identification of new viroporins. In addition, transgenic expression of viroporins in bacterial, yeast and mammalian cells has helped in analysing their mode of action, as their expression increases the membrane permeability of all these cell types[2,9,14]. Even neighbouring cells that do not express the viroporin become indirectly permeabilized in cell culture[41]. Purified viroporins also have the ability to form ion channels in planar lipid bilayers or in liposomes[6].

A range of assays are available to analyse changes in membrane permeability, including assays that are based on the entry of ions and small molecules into cells or their release into the culture medium. One of these assays — patch clamping of *Xenopus laevis* oocytes after

Patch clamping
A high-resolution electrophysiological technique that allows currents to be recorded through single ion channels. The technique uses glass pipettes to electrically isolate channel-containing membrane patches. Current flow can be recorded in cell-attached and excised membrane patches.

Box 1 | Viroporins of DNA viruses

Although viroporins were initially discovered in RNA viruses, they are also encoded by DNA viruses[46,129]. Complex DNA viruses can encode several membrane-active proteins, one or more of which can exhibit viroporin activity[1,2]. Recently, viroporins were identified in small DNA viruses belonging to the polyomavirus group, such as JC polyomavirus and Simian virus 40 (SV40)[46,129]. Progressive multifocal leuko-encephalopathy is a fatal demyelinating disease that results from lytic infection of oligodendrocytes by JC polyomavirus. This virus encodes the so-called agnoprotein, which participates in viral release and propagation[46,129]. In infected host cells, agnoprotein is initially located at the ER membrane and later appears at the plasma membrane, where it promotes the entry of extracellular Ca^{2+} and other small membrane-impermeable compounds such as hygromycin B[46]. Viroporin activity has also been detected for SV40 viral protein 4 (VP4), which is expressed during the late stage of the viral life cycle, when cell lysis occurs. The central hydrophobic domain of VP4 is essential for membrane binding and disruption, a process that is required for viral release and spread. Membrane disruption by VP4 involves the formation of pores with a diameter of approximately 3 nm[129].

Interestingly, human papillomavirus type 16 (HPV16) encodes a small protein of 83 residues that is known as E5 and forms ion channels[130]. This oncoprotein assembles hexameric pores with a diameter of approximately 1–2 nm. Rimantadine, an inhibitor of viroporin ion channels, abrogates the ion channel activity of E5 and its effects on cell signalling pathways[130]. Thus, HPV16 E5 represents the first known example of a viroporin that modulates cell transformation and the immune response.

Hygromycin B test
An experiment to test membrane permeability. Hygromycin, an inhibitor of translation, does not easily pass through the plasma membrane of control cells, but after cell permeabilization by viroporins, the inhibitor is able to reach its target (the ribosome) and block protein synthesis.

Ionophore
A lipophilic compound that inserts into membranes and transports ions in favour of the electrochemical gradient.

microinjection of viroporin mRNAs — provided the first evidence of ion channel formation by IAV M2 (REF. 15). Another approach, the hygromycin B test[1], has become a widely applied, easy-to-use assay for assessing membrane permeability[42–46]. Such assays have revealed that compounds below a molecular mass of 800–1,500 Da enter cells or liposomes containing viroporins[47]. In addition, ions are redistributed according to their concentration gradient, leading to depolarization as a result of disruption of the membrane potential[2] (FIG. 2a).

Viroporin-induced membrane permeabilization to ions and/or small solutes can occur through the formation of gated channels or size-limited pores[2,48]. For instance, the membrane-spanning IAV protein M2 forms tetrameric channels that selectively conduct protons[49]. Similarly, HIV-1 Vpu oligomers can function as ion-conducting channels[50] that open in a voltage-dependent or voltage-independent manner[51]. When expressed in bacteria and mammalian cells, these proteins also induce a certain degree of membrane permeabilization to small solutes[52,53]. Weak ion selectivity together with the capacity to induce leakage of solutes into and out of the cell across a sealed membrane (known as pore–channel dualism[48,51]) have been reported for other viroporins, such as HCV p7 (REFS 52,54), togavirus 6K[19,20] and coronavirus envelope small membrane protein (E)[55,56]. Viroporin activity is probably regulated by differences in the components and structures of organellar and plasma membrane lipid bilayers[48,57]. As the helices of viroporins form integral oligomeric bundles, they can adapt to changes in the lipid bilayer thickness or to bilayer deformations by tilting and/or bending. The ensuing pore opening and closing, coupled with helix motions, constitute the gating mechanism[57].

Another important aspect of viroporin function is the resultant increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) after entry of extracellular Ca^{2+} and/or Ca^{2+} leakage from intracellular stores, such as the mitochondria, ER and Golgi complex[12,58–60] (FIG. 2b). An increased $[Ca^{2+}]_i$ is observed when several viral proteins are expressed, including picornavirus P2B and rotavirus non-structural protein 4 (NSP4)[11,60–62]. Ca^{2+} is an important regulator of many cellular enzymes and processes, and an increase in $[Ca^{2+}]_i$ has substantial repercussions for many cellular and viral processes[63], including viral morphogenesis. An increased $[Ca^{2+}]_i$ can also induce apoptosis[64], a phenomenon that is observed when various viral proteins, including viroporins, are expressed in host cells[52,58,65]. Furthermore, many viral proteins interact with Ca^{2+}, which modulates their function. In this respect, Ca^{2+} regulates viral gene expression, post-translational processing of viral proteins, and virion maturation and release[63]. In conclusion, the alteration in $[Ca^{2+}]_i$ that is observed after infection with most animal viruses modulates several viral functions and plays a part in host cell death.

Membrane remodelling and glycoprotein trafficking.
To promote their own proliferation, animal viruses that replicate their genomes in the cytoplasm can induce profound remodelling of intracellular membranes. Thus, many animal viruses induce the formation of new cytoplasmic vesicles in which genome replication takes place[66]. For several viruses, including picornaviruses, inhibition of the vesicular system by brefeldin A or by inhibitors of phospholipid synthesis abrogates viral genome replication[40]. In general, viroporins are not necessary for viral genome replication; however, the rotavirus and picornavirus viroporins are exceptions. Indeed, transgenic expression of some picornavirus proteins, such as P2B, P2C and in particular their polyprotein precursor, P2BC, leads to remodelling of the membrane and formation of the 'viroplasm' (FIG. 2c), a multivesicular body that is generated from the ER[14,61,67–69]. The double-membraned vesicles of the viroplasm are necessary for the replication of viral RNA and have an autophagic origin[61,67]. Co-expression of picornavirus P2BC and P3A leads to the formation of intracellular vacuoles that are morphologically similar to those observed in picornavirus-infected cells[61]. Whether these membrane-remodelling events are due to ion channel formation, are mediated by another aspect of viroporin function or are dependent on interactions with cellular proteins is currently unknown.

The pioneering work of Doedens and Kirkegaard[10] revealed that the transgenic expression of poliovirus P2B or P3A not only increases membrane permeability to hygromycin B but also interferes with trafficking of vesicular stomatitis virus (VSV) glycoprotein. In cells that are infected with enveloped viruses such as VSV, viral structural glycoproteins travel through the vesicular system accompanied by viroporins[21]. Trafficking of viral and cellular glycoproteins can be delayed by viroporins such as IAV M2 or HCV p7 through a monensin-like activity[16,32]. Monensin is an ionophore that promotes proton redistribution in a manner that prevents acidification of intracellular compartments. This loss of acidification can prevent structural modifications or rearrangements
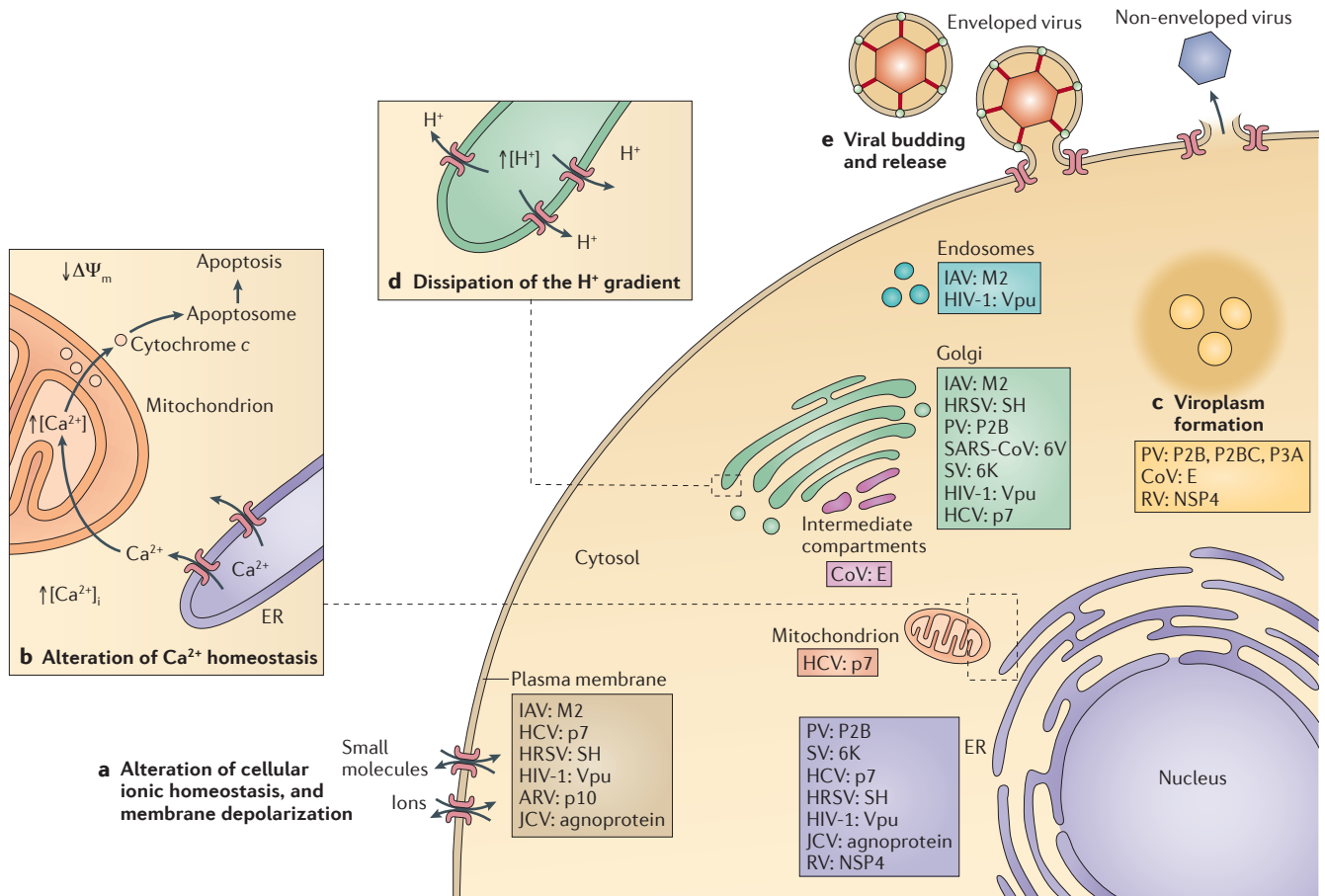
Figure 2 | **Cytopathic effects of viroporins and their functions during the viral life cycle.** The main host cell organelles that are targeted by viroporins, and the cytopathic effects that are induced by viroporins and their functions during the viral life cycle are represented. **a** | Alteration of plasma membrane potential. Viroporins that are located at the plasma membrane can dissipate the ionic gradient across the membrane, leading to depolarization. **b** | Alteration of cellular Ca^{2+} homeostasis. The poliovirus (PV) viroporin protein 2B (P2B) assembles pores in the ER membrane and induces the release of Ca^{2+} from the ER lumen into the cytosol. Uptake of Ca^{2+} by the mitochondria can lead to dissipation of the inner-mitochondrial-membrane potential ($\Delta\Psi_m$), permeabilization of the outer mitochondrial membrane and, finally, the release of cytochrome *c*. In the cytosol, cytochrome *c* promotes the formation of the so-called apoptosome, a molecular platform that is involved in apoptosis. **c** | Certain viroporins, such as P2B, polyprotein P2BC and P3A from PV or envelope small membrane protein (E) from coronavirus (CoV), induce intracellular membrane remodelling to generate new membrane vesicles (called the viroplasm) that serve as viral replication sites. **d** | Dissipation of the proton gradient in the Golgi and the *trans*-Golgi network. The viroporins influenza A virus (IAV) matrix protein 2 (M2) and hepatitis C virus (HCV) p7 reduce the acidification of vesicular acidic compartments by equilibrating the proton concentration with the cytosol. Alteration of the intracellular ionic gradient in the vesicular system impairs glycoprotein trafficking. **e** | During the viral replication cycle, viroporins play an essential part in assembly, budding and release of the viral progeny. ARV, avian reovirus; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HRSV, human respiratory syncytial virus; JCV, JC polyomavirus; NSP4, non-structural protein 4; RV, rotavirus; SARS-CoV, severe acute respiratory syndrome CoV; SH, small hydrophobic protein; SV, Sindbis virus; Vpu, viral protein U.

of viral glycoproteins. Indeed, HCV p7 and IAV M2 reduce the acidification of intracellular vesicles and even cellular organelles[16,32] (FIG. 2d). Thus, in some instances viroporin-mediated inhibition of intracellular-vesicle acidification may be important for the production of infectious viral particles[32]. Finally, HIV-1 Vpu also hampers trafficking of several cellular proteins, including tetherin (also known as BST2) and the receptor CD4, promoting their degradation by lysosomes (tetherin) or by the proteasome (CD4)[70–72]. Vpu impedes the trafficking of tetherin by interfering with protein sorting at

the *trans*-Golgi network (TGN), redirecting the protein from the cytoplasmic membrane to endolysosomal compartments[72]. Both the TM and cytosolic domains of Vpu are involved in directing the degradation of these cellular proteins[23,35,70,72] (see below).

Viroporins and the viral life cycle
Although viroporins participate in different steps of the viral life cycle, such as cell entry and genome replication, the main activity of viroporins is their involvement in virion assembly and the release from infected

Adamantane
A rigid molecule with a backbone based on three interconnected cyclohexane rings. Derivatives of adamantane (amantadine and rimantadine) have been shown to be effective inhibitors of viroporin activity in some cases.

cells (FIG. 2e). Viruses that are defective in viroporins are unable to accomplish proper assembly and release from cells[26,27,36,46,70,73,74].

In the case of non-enveloped animal viruses, the progressive increase in membrane permeability as a result of viroporin accumulation during infection leads to cell lysis and the subsequent release of the assembled virus particles[1,46]. The last step in the maturation of enveloped viruses is the budding of virus particles from the plasma membrane (for IAV, togaviruses and lentiviruses) or from intracellular vesicles (for flaviviruses and coronaviruses). In the case of both enveloped and non-enveloped viruses, the insertion of viroporins in the membrane leads to the dissipation of membrane potential. We propose that viroporin insertion breaks the chemoelectrical barrier by conducting ions across membranes, thus dissipating the membrane potential of the plasma membrane or of internal vesicles and thereby stimulating budding[21] (FIG. 3). Therefore, in enveloped viruses, the dissipation of membrane potential can be coupled to viral release. In the process of budding, membrane fission begins with the formation of a membrane neck, which is followed by self-merging of the inner monolayer of the neck. Subsequent self-merging of the outer monolayer of the neck results in the virus particle pinching off from the plasma or vesicle membrane. Membrane depolarization is accompanied by a reduction in the surface charge density of the membrane, and it has been argued that this reduces the repulsion between contacting monolayers and therefore enhances the contacts that are required for fission[75] (FIG. 3). In this manner, the

production of viruses and vesicles, even those of non-viral origin, is promoted[75,76]. IAV M2 increases proton conductance across the membrane and may act as a Na^{+} antiporter[77–79]; however, there is no pH gradient at the plasma membrane, which is the site of IAV budding. M2 localizes at the neck of budding virions and is necessary for this process[80].

There are reports linking IAV M2 to viral entry[15,17]. However, during entry, membrane pores are usually formed by viral glycoproteins or capsids, depending on whether the virions are enveloped or not. The formation of pores in endosomes dissipates the proton gradient and propels viral capsids into the cytosol, and we suggest that it is unlikely that the majority of known viroporins participate in this process[81]. In support of this, studies have shown that the entry and infectivity of IAV and HCV are not hindered by the presence of a defective M2 viroporin or in the absence of p7 viroporin, respectively (REFS 27,32,82). Furthermore, chimeric viroporins bearing the M2 TM domain are sensitive to rimantadine, an adamantane derivative that blocks viral budding but not entry into cells[39]. Conversely, a study that examined p7 inhibitor compounds demonstrated that HCV entry is partially impeded in the presence of some of these compounds, suggesting that HCV requires p7 for entry into the cell[83]. Although the data are conflicting, we propose that viroporin-mediated permeabilization is generally not required for viral entry, as this is induced by virus particles. Thus, the cell permeabilization that is induced by virus particles appears to have a different function to the permeabilization functions of viroporins[81,84].
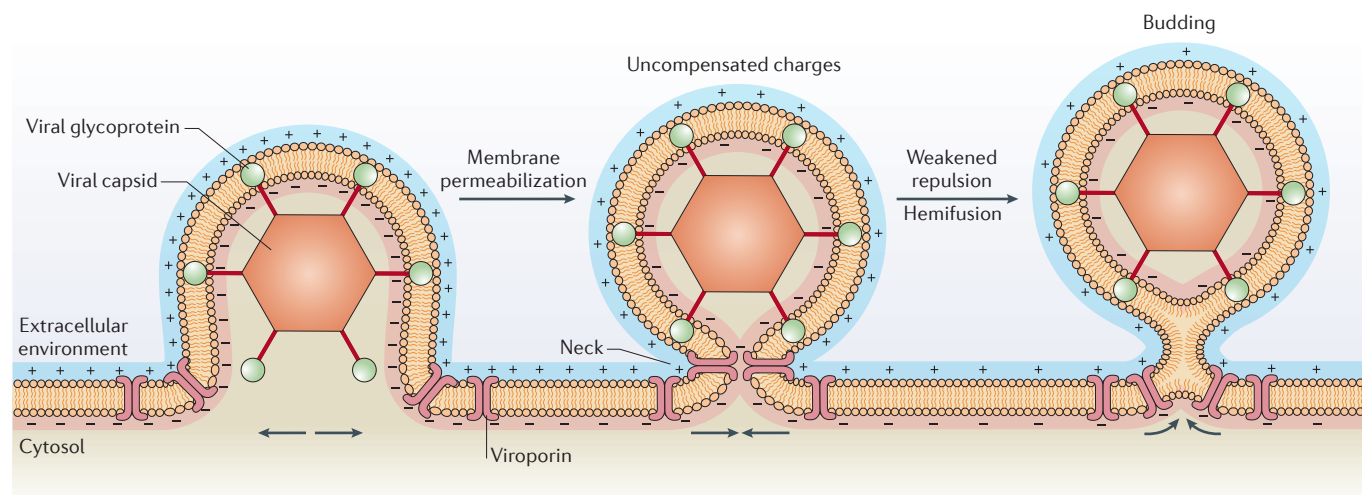

Figure 3 | **Model of a viroporin promoting viral budding at the plasma membrane.** Viroporins localize at the plasma membrane in specific sites surrounding the neck of the budding virus particle, as described for the influenza A virus matrix protein 2 (M2) viroporin[80]. Viroporins alter membrane permeability by conducting the flux of different ions (for example, Na^{+} and K^{+}) across the membrane in favour of their concentration gradients and so reducing the transmembrane potential, which is essentially determined by three factors: the concentration of ions inside and outside the cell; the permeability of the cell membrane to those ions (that is, the ion conductance) through specific ion channels; and the activity of electrogenic pumps (for example, the (Na^{+}+K^{+})ATPase and Ca^{2+} transport pumps) that require energy to maintain the ion gradients across the membrane. Depolarization of the membrane (that is, decreasing the imbalance of charges across the membrane) leads to a reduction in the charge density on the membrane surface. This phenomenon would result in a decrease in the electrical or contact repulsion between opposing monolayers of the membrane at the neck of the budding site (middle) and could provide the stimulus and even the energy to locally promote budding and release[21,75], as occurs in depolarization-dependent exocytosis[76].

Pore-forming activity of viroporins

Picornavirus P2B. The family *Picornaviridae* includes several viral species of clinical interest, including poliovirus, human rhinoviruses and hepatitis A virus. These viruses contain a single-stranded RNA genome of positive polarity with a single ORF that encodes a large polyprotein. All of the mature viral proteins are derived from this polyprotein by proteolytic cleavage. The mature picornavirus P2B viroporin, or its precursor, P2BC, is thought to be responsible for Golgi and mitochondrial membrane permeabilization and an increase in plasma membrane permeability to small solutes and ions, both of which effects are observed during the late phase of picornavirus infection[10,11,13]. As a member of the class IIB viroporins, the helical TM domains of picornavirus P2B are connected by a highly polar short stretch of amino acids, forming a hairpin α–loop–α motif across the bilayer[40,47,85–87]. Research on the biogenesis of viroporins and their mechanisms of membrane insertion has been scarce to date. Recent *in vitro* translation experiments support the suggestion that poliovirus P2B can be inserted into the ER membrane by the SEC translocon as a helical hairpin with a cytosolic orientation for the N and C termini[88]. Furthermore, mutagenesis studies of the hairpin motif have demonstrated the functional importance of the hydrophobic-residue distribution that forms a partially amphipathic helix and is followed by an overall hydrophobic TM domain[11,13,86,87,89,90]. Both of these domains are required for Golgi targeting and plasma membrane permeabilization[90].

Membrane-inserted P2B pores were first reconstituted and characterized using purified hybrid maltose-binding protein (MBP)–P2B and large unilamellar vesicles (LUVs)[47,85]. The observed LUV permeabilization pattern was consistent with the formation of P2B TM pores with a radius of ~6 Å, allowing the free diffusion of small solutes (<1 kDa). This pore-forming activity of P2B was therefore reminiscent of the plasma membrane permeabilization process that was detected during the mid phase of poliovirus infection[40]. In terms of the composition of P2B pores, detergent-resistant tetramers are formed in the presence of lipids[47], and additional evidence supports the formation of similar P2B homooligomers at cell membrane surfaces[91]. The biochemical data agree reasonably well with recent molecular dynamics simulations, which indicate that P2B can assemble in lipid bilayers as stable tetrameric aqueous pores with a diameter of 5–7 Å[92].

In the absence of a functional translocation machinery (for example, SEC or TIM), P2B can insert directly into membranes and form pores following a mechanism similar to that used by certain cytolytic toxins[93]. Work with peptides and recombinant proteins actually suggests that picornavirus P2B functions as an intracellularly delivered toxin during late phases of picornavirus infection[94]. The N-terminal amphipathic helices promote transfer of the protein from aqueous solution into the membrane and then act as the building blocks of the permeating, oligomeric pore structures that are subsequently assembled[94,95]. Quantitative assessments of P2B-induced permeabilization indicate that one membrane-bound tetramer can permeabilize a single vesicle with a diameter of 100 nm (~10^5 lipid molecules)[85]. Such low doses indicate that P2B and its derivatives are capable of forming true TM pores and are devoid of detergent-like membrane-destabilizing activity[85,94]. The ability of the N-terminal amphipathic helix to effectively conduct ions across natural membranes provides further support for the existence of P2B TM channels[94].

HCV p7. HCV is a hepatotropic single-stranded, positive-sense RNA virus belonging to the family *Flaviviridae* and the genus Hepacivirus. HCV is one of the fastest mutating viruses, and about 170 million individuals throughout the world are believed to be infected[96]. Persistent infection is associated with a chronic liver disease that often progresses to cirrhosis and hepatocellular carcinoma[96]. The viroporin p7 consists of 63 amino acids and is encoded at the junction between the structural and non-structural region of a viral polyprotein precursor, which is cleaved by cellular and viral proteases[97]. p7 is a member of the class IIA viroporins and possesses two TM α-helices that are slightly tilted relative to the membrane bilayer and are connected by a cytosolic loop (residues 33–39) containing a dibasic motif[38]. In human hepatocarcinoma cells transfected with the full-length genome of a JFH1 (Japanese fulminant hepatitis 1) isolate, p7 localizes primarily to the ER, and a small proportion of p7 is also detected in mitochondria and at the plasma membrane when it is ectopically overexpressed[98]. Topological analysis of epitope-tagged p7 showed that both the N and C termini are exposed to the ER lumen. However, when p7 is expressed as its precursor, E2p7, the C terminus can adopt a topology in which it faces the cytosol[98]. The oligomerization of p7 monomers in artificial membranes results in the assembly of cation-selective ion channels that can be specifically blocked by different drugs, such as adamantanes, with varying efficacies[4,24,25,99,100] (TABLE 1). Although p7 does not appear to be required for viral entry or RNA replication, it is needed for polyprotein processing[98] and is essential for a late step in viral assembly and release of infectious virions[26,27], as confirmed using a *trans*-complementation assay[38]. The essential role of p7 in the production of virus particles relies on both its viroporin activity and its interactions with other structural proteins (and possibly with NS2, a non-structural HCV protein)[27]. Indeed, IAV M2 can compensate for the ion channel activity of a fully intact but inactive p7, but cannot compensate for the activity of a p7 deletion mutant[32,38]. This discrepancy may be due to functions of p7 that are unrelated to ion channel formation; however, this has not yet been clarified. In hepatoma cells, active p7 channels dissipate the proton gradient of the acidic vesicular compartments, causing leakage of protons into the cytosol. This inhibition of acidification is required for the production of virus particles[32].

IAV M2. Influenza viruses are responsible for acute respiratory disease, leading not only to human suffering but also to economic losses. IAV contains a single-stranded, negative-sense RNA genome that is divided into eight segments. The smallest segment encodes two

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Molecular dynamics simulations
Computational simulations that calculate the time-dependent motions of atoms in a molecular system. This method is used to obtain microscopic-level information about the fluctuations and conformational changes of proteins.

Table 1 | **Viroporin inhibitors**

Viroporin (virus)	Inhibitor	Test systems used	Refs
M2 (IAV)	Adamantanes (amantadine and rimantadine)	Artificial membranes, cell culture	31,114,118
Vpu (HIV-1)	HMA	Cell culture	33
	BIT225	Cell culture	126
p7 (HCV)	Amantadine	Artificial membranes, cell culture, clinical studies	24,83,119,122
	HMA	Artificial membranes, cell culture	83,99
	Long alkyl chain iminosugar derivatives	Cell culture, clinical studies	25,28,121,123
	BIT225	Early stage Phase Ib/IIa trials	125
E (SARS-CoV)	HMA	Cell culture	124
P2B (EV71)	DIDS	*Xenopus laevis* oocytes, cell culture	127

BIT225, *N*-(5-(1-methyl-1*H*-pyrazol-4-yl)-napthalene-2-carbonyl)-guanidine; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid; E, envelope small membrane protein; EV71, enterovirus 71; HCV, hepatitis C virus; HMA, 5-(*N,N*-hexamethylene) amiloride; IAV, influenza A virus; M2, matrix protein 2; P2B, protein 2B; SARS-CoV, severe acute respiratory syndrome coronavirus; Vpu, viral protein U.

proteins, M1 and M2, that are synthesized from two different mRNAs generated by alternative splicing. M2 belongs to the class IA viroporins and is a 97 amino acid integral membrane phosphoprotein consisting of three distinct regions: an extracellular 23-residue fragment that is important for incorporation into the virion, a 19–20-residue TM helix and a 54-residue cytosolic tail. The tail includes an amphipathic helix that is involved in cholesterol binding, membrane localization, budding and scission, and its extreme terminus interacts with M1 (REFS 5,101). The TM helix is essential for tetramer-ization, proton channel formation and binding to ada-mantane drugs such as amantadine and rimantadine[5]. It has been proposed that M2 becomes activated in acidic endosomes during viral entry and triggers viral uncoating by conducting protons to the interior of the virus particle[5,18]. In spite of this, M2 channel activity is not essential for viral RNA replication in cell culture, but it is necessary for completion of the IAV life cycle in mice[82]. In some IAV strains, M2 channel activity also equilibrates the pH gradient between the TGN and the cytosol to prevent premature maturation of the viral glycoprotein haemagglutinin[17,18]. As a consequence of disrupting the ionic balance in the Golgi, M2 activates host inflammasomes, leading to the processing and release of pro-inflammatory cytokines[102].

M2 is also required for viral assembly and release; its cytosolic tail in particular participates in genome packaging and facilitates virus production[101]. Moreover, in IAV strains lacking M2, infectivity is attenuated in both cell culture and mice[34]. During viral release, M2 is located at the neck of the budding virion. During viral egress, the C-terminal amphipathic helix alters mem-brane curvature in a cholesterol-dependent manner, and the protein also assists the membrane scission process independently of the host ESCRT (endosomal sorting complex required for transport) machinery[80]. However, cellular factors that interact with M2 can modulate this function, as is the case for annexin A6, which negatively regulates IAV infection by specifically interfering with the budding process[103]. It has also been reported that M2 acts as an inhibitor of autophagosome maturation[104].

HIV-1 Vpu. HIV is the causative agent of AIDS, which affects approximately 35 million people worldwide. HIV-1, as well as some other primate lentiviruses (but not HIV-2), encodes Vpu, which is translated from a bicistronic mRNA that also encodes viral glyco-protein 160 (gp160)[35]. Vpu is a class IA viroporin that is mainly located at the ER, TGN, endosomes and, to a lesser extent, the plasma membrane. Depending on the viral isolate, Vpu consists of 77–86 amino acids comprising a short ER-lumenal N-terminal domain of approximately nine residues, a single TM helix and a cytosolic domain containing two shorter α-helices (residues 33–49 and 57–70, approximately)[35,70]. These two cytosolic helices are separated by a short flexible loop with two conserved Ser residues that are suscep-tible to phosphorylation by casein kinase II[35,70]. On oligomerization in membranes, Vpu is thought to form an oligomeric ion channel[105]. The cytosolic domain is crucial for protein trafficking through the vesicular system[3,35,72]. Thus, mutations in this region can cause Vpu to exhibit deficiencies in its interactions with other cellular proteins. For example, mutations located in the first α-helix revealed that this region is important for the interaction with CD4 and the subsequent degrada-tion of this receptor[35]. More recently, an EXXXLV motif in the second α-helix has been shown to be required for efficient tetherin degradation[106]. In addition, the TM domain of Vpu interacts with cellular proteins, including CD4 and tetherin[107,108]. The presence of CD4 at the cell surface impedes viral budding, but Vpu induces rapid degradation of newly synthesized CD4 molecules in the ER via the ubiquitin–proteasome system[70]. In addition, Vpu counteracts tetherin, a host restriction factor that is induced by interferon and strongly inhibits the release of virions from the host cell surface[23,30,72,109,110]. In this manner, this viroporin antagonizes the innate immune

Inflammasomes
Protein complexes that are involved in the processing and release of pro-inflammatory cytokines during the innate immune response to viral infection.

Autophagosome
A double-membraned vesicle that captures cellular components (including proteins, membrane fragments and whole organelles) and delivers them to lysosomes for degradation through a process called autophagy.

In addition to the examples mentioned in the main text, a number of other viroporins have been analysed in detail. Coronaviruses encompass several human and animal pathogens, such as severe acute respiratory syndrome coronavirus (SARS-CoV). These viruses encode a small protein named envelope small membrane protein (E protein), which ranges from 76 to 109 amino acids in size[44]. It consists of a short hydrophilic domain (about 21–29 residues) preceding the transmembrane region, which is followed by a longer hydrophilic carboxyl tail. The amino-terminal 40 amino acids of SARS-CoV E protein are sufficient for the formation of ion channels that are selective for monovalent cations[56]. Moreover, E viroporins of SARS-CoV and mouse hepatitis virus enhance membrane permeability in both bacterial and mammalian cells[44,55]. The expression of coronavirus E protein induces the formation of vesicles, and its co-expression with the viral glycoprotein M leads to the production of virus-like particles[44]. Coronavirus E protein influences the morphogenesis and budding of virus particles, and its transmembrane domain is necessary for this activity[131,132]. The transmembrane domain of the infectious bronchitis virus E protein is also required for efficient viral release. The expression of individual E proteins from different coronaviruses can induce apoptosis in a cell type-specific manner[133].

Other well-studied viroporins include protein 6K from togaviruses, small hydrophobic (SH) protein from paramyxoviruses, non-structural protein 4 (NSP4) from rotaviruses, p10 from avian orthoreovirus, p7 from pestiviruses and Kcv from *Paramecium bursaria* Chlorella virus 1 (PBCV)[2,19,21,134–136]. Viroporin candidates that exhibit the capacity to form ion channels include BM2 (also known as matrix protein 2) from influenza B virus[137], viral protein R (Vpr) from HIV-1 (REF. 138) and protein 3a from SARS-CoV[36]. Moreover, other viral proteins and glycoproteins, or even moieties derived from them, can exhibit viroporin-like activity[42,45,60,129,139].

response to facilitate morphogenesis and viral release. Moreover, Vpu may also enhance the release of other viruses, such as Sindbis virus or filoviruses[37,50,110,111]. Thus, the Vpu cytosolic and TM domains counteract several cellular proteins, and the ion channel activity of the TM domain is responsible for dissipating the membrane potential and disrupting the electrical barrier at the plasma membrane[75] (see below).

In addition to the viroporins of the RNA viruses discussed in detail thus far, a number of other RNA viruses encode well-characterized viroporins; however, a detailed account of these proteins is beyond the scope of this review (BOX 2).

Viroporin structure

IAV M2. The structures of some viroporins have been determined by X-ray crystallography, solution and solid-state NMR spectroscopy, and electron microscopy (FIG. 4). IAV M2 is the best characterized viroporin in terms of structure, and its TM domain has been studied by X-ray crystallography with sufficient resolution to facilitate mechanistic studies of its proton-conducting function[112]. In the tetrameric pore, side chains of the highly conserved residues His37 and Trp41 in each helix face the aqueous channel and are crucial for the transport of protons (FIG. 4a). The mechanism of M2-mediated proton conductance has been extensively studied in *X. laevis* oocytes, mammalian cells, lipid bilayers and vesicles[17,18,113]. M2 channel activation and proton selectivity are conferred by the four His37 residues, which interrupt the conductance pathway and can be protonated at low pH to enhance proton flow. Below the four His residues, the side chains of Trp41 are clustered at neutral pH and block the proton channel, thereby

acting as a gate to facilitate asymmetrical conductance[5] (FIG. 4a). A comparison of M2 structures solved at neutral (~pH 7.5)[77], mildly acidic (~pH 6.5)[112] and acidic (~pH 5)[114] pH suggests that the gate of aromatic residues opens at low pH, concomitant with a rearrangement of the TM helices. Accordingly, His protonation correlates with tilting and bending of the TM helices relative to the pore axis[112]. In addition, the structure of M2 TM helices in complex with adamantanes has been solved[31,77,114]. High-resolution studies identified a high-affinity binding site for the amantadine inhibitor close to the Ser31 cluster, a pore region surrounded by residues that are mutated in amantadine-resistant viruses[114]. More recent results from solid-state NMR spectroscopy suggest that amantadine binding physically obstructs the proton channel[31].

HIV-1 Vpu. The three-dimensional structure of the Vpu TM helix has been determined by solution and solid-state NMR spectroscopy in micelle and bilayer samples, respectively[22] (FIG. 4b). The data confirm that Vpu TM helix monomers comprise slightly kinked α-helices spanning residues 8–25 with an average tilt of 13° with respect to the bilayer normal. Although the structure of a functional, oligomeric Vpu has not been solved to atomic resolution, analytical ultracentrifugation and photochemical crosslinking experiments indicate the presence of a variety of coexisting oligomers ranging at the very least from tetramers to heptamers[115]. These studies are corroborated by computational modelling of Vpu oligomers, which suggests that the predominant Vpu channel is a pentamer or larger oligomer[105,116]. Using the three-dimensional structure and its known orientation within the bilayer as a template, oligomeric forms of the Vpu pore were modelled based on energy minimization protocols[22]. The most stable pentameric structure places Trp22 out towards the lipid headgroups, with a kink at Ile17 directly in the pore (FIG. 4b).

HCV p7. Experimentally solved, high-resolution three-dimensional structures of complete HCV p7 monomers or oligomers are not presently available in the Protein Data Bank (PDB). However, the possible oligomeric state and general organization adopted by this viroporin have been determined by single-particle electron microscopy[29]. Detergent-solubilized hexamers were subjected to high-contrast staining, which allowed an efficient three-dimensional image reconstruction of the complex. The density map at a resolution of ~16 Å, together with immunolabelling using Fab fragments specific for the N and C termini, revealed a conically shaped hexameric channel with protruding petals oriented towards the ER lumen[29] (FIG. 4c). The petals comprise the N- and C-terminal sequences, and the overall diameter of the cone ranges from 3.2 nm at its narrowest point to 8.1 nm at its widest point.

Furthermore, high-resolution data pertaining to the region comprising Arg35–Ala63 (that is, a reverting bent helical region that is not implicated in channel formation) were obtained by ¹H and ¹³C solution NMR spectroscopy in trifluoroethanol–water mixtures and

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Asymmetrical conductance
The capacity of an ion channel to conduct preferentially outward or preferentially inward ion fluxes.
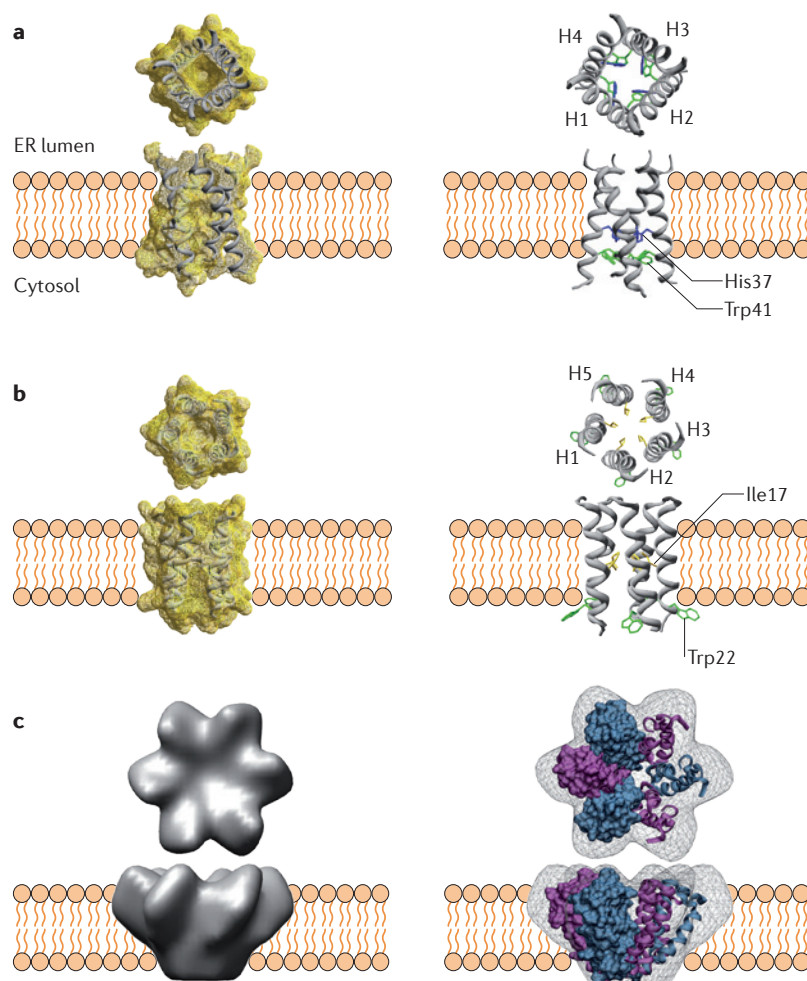
Figure 4 | **Three-dimensional structures of selected viroporins.** Each viroporin structure is shown both oriented in the membrane bilayer and from a down-top view. **a** | High-resolution (1.65 Å) X-ray structure of the oligomers formed by a peptide representing the transmembrane region (residues 25–46) of the class IA viroporin matrix protein 2 (M2) from influenza A virus. Crystals were obtained at pH 6.5 in the presence of *N*-octylglucoside (Protein Data Bank (PDB) accession 3LBW). In the ribbon representation (right), constituent helices (H1–H4) are indicated, and side chains of His37 and Trp41 are depicted in blue and green, respectively. Structures were generated using Swiss-PdbViewer. **b** | Solid-state NMR structure of the transmembrane region (residues 2–30) from the class IA viroporin viral protein U (Vpu) from HIV-1, in lipid bilayers (PDB accession 1PI7). The oligomeric form was calculated using energy minimization protocols, and side chains were added to a backbone structure that was generated from solid-state NMR spectroscopy data. Ribbon representations (right) display the constituent helices (H1–H5) and side chains of Ile17 and Trp22 in yellow and green, respectively. Structures were generated using Swiss-PdbViewer. **c** | Density map contours of oligomers of p7, a class IIA viroporin from hepatitis C virus, solubilized in detergent. Simulated p7 monomers were fitted with their amino and carboxyl termini oriented towards the petal tips[29] (right). Structures in part **c** are modified, with permission, from REF. 29 © (2009) US National Academy of Sciences.

that the protein contains helices that are tilted by ~25° and ~10° relative to the bilayer normal, with the helix consisting of residues 50–57 being tilted by ~10°. The observed smaller tilt angle of the second TM domain (helices 41–49 and 50–57) is consistent with the shorter length of this domain compared to that of the first TM domain (helices 6–16 and 17–27). A shorter TM domain would be expected to require a smaller tilt angle to span the lipid bilayer, whereas the longer (first) TM domain would accommodate a larger tilt angle, possibly in its second helical segment.

Viroporins as therapeutic targets

Viroporins are attractive targets for antiviral therapy, as they are essential for viral release. Furthermore, inhibition of their membrane-permeabilizing activity is possible in both artificial membranes and cell culture systems, thereby facilitating the drug discovery process. Thus, the use of classic viroporin inhibitors, as well as the development of new or highly specific drug derivatives, could potentiate the efficacy of current antiviral treatments (TABLE 1).

Amantadine was the first drug shown to block IAV uncoating by inhibiting the ion channel activity of M2 (REF. 118). It was later reported that amantadine interacts directly with HCV p7 in artificial membranes[24,119]. However, in cell culture amantadine induces only moderate and genotype-dependent inhibition of infectious HCV production[83]. A major concern regarding the use of amantadine as a monotherapy to treat influenza is the rapid emergence of amantadine-resistant IAV variants[120], and adamantanes were also recently shown to select for specific resistance mutations in HCV p7 (REF. 121). The results of clinical trials suggest that toxicity and resistance explain the failure of amantadine to enhance a sustained antiviral response in patients infected with HCV[4,122]. Using an alternative approach, recent studies have demonstrated that immunizing mice with an IAV strain that lacks M2 protects against lethal doses of the highly pathogenic H5N1 virus; these results suggest that this virus has potential as a live attenuated vaccine[34]. Long alkyl iminosugar derivatives, such as *N*-nonyl-deoxynojirimycin (*N*N-DNJ) and *N*-nonyl-deoxygalactonojirimycin (*N*N-DGJ), inhibit the function of the HCV p7 ion channel *in vitro* and reduce the production of infectious viruses[25,28,121]. However, UT-231B, a derivative of *N*N-DNJ with α-glucosidase inhibition properties, was used in a clinical Phase II study but did not demonstrate satisfactory efficacy[123]. Amiloride derivatives, especially 5-(*N*,*N*-hexamethylene) amiloride (HMA), can also inhibit HCV p7, HIV-1 Vpu and severe acute respiratory syndrome coronavirus (SARS-CoV) E channel activity[33,99,124]. HMA also impairs HIV budding and SARS-CoV replication. However, the concentration that is required to block the function of the p7 channel is cytotoxic in cell culture[83]. A new p7 ion channel inhibitor, BIT225 (*N*-(5-(1-methyl-1*H*-pyrazol-4-yl)-napthalene-2-carbonyl)-guanidine), is a potential candidate for future treatments against HCV. In a Phase Ib/IIa trial, administration of BIT225 for a week induced

have been deposited in the PDB (accession 2K8J)[100]. New experimental data confirm that the membrane-embedded section of p7 consists of two TM domains connected by a short interhelical basic loop[117]. Each protein monomer comprises seven distinct segments, including four dynamic helices (spanning residues 6–16, 17–27, 41–49 and 50–57) that differ in their segmental motions. Solid-state NMR analysis has revealed

a moderate reduction of the viral load in patients infected with bovine viral diarrhoea virus, an HCV-related virus[125]. It has also been reported that this compound can target HIV-1 Vpu and inhibit viral release from macrophages[126]. Recently, a classic anion exchanger inhibitor, DIDS (4,4′-diisothiocyano-2,2′-stilbenedisulphonic acid), was reported to partially suppress the chloride conductance that is mediated by the enterovirus P2B viroporin in *X. laevis* oocytes and to block virus production in human cells[127].

Concluding remarks and future prospects

This Review focuses on the biological structure and function of archetypal members of the viroporin family. With the advent of new bioinformatic tools and the availability of infectious clones, the identification of new members of the viroporin family is progressing at an increasing rate. Currently, viroporins are also emerging as new potential targets for clinical intervention. In this regard, the establishment of liposome-based assays and the development of high-throughput screening systems will facilitate the discovery of new and more potent inhibitors of viral infection[119,128]. We foresee that these systems could be optimized for drug screening, potentially leading to the identification and isolation of new antiviral compounds. Selective inhibitors of viroporins are useful tools to unveil the multifunctional activities of each viroporin during viral infection. Furthermore, advances in molecular biology techniques have facilitated the cloning and high-yield expression of viroporins, thereby allowing the implementation of crystallization protocols[114] and NMR techniques to elucidate the structure of these proteins and to aid in their molecular characterization[22]. We predict that the availability of high-resolution crystallographic pore structures will boost the rational design of specific viroporin inhibitors in the near future.

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