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Targeting the Channel Activity of Viroporins

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Abstract

Since the discovery that certain small viral membrane proteins, collectively termed as viroporins, can permeabilize host cellular membranes and also behave as ion channels, attempts have been made to link this feature to specific biological roles. In parallel, most viroporins identified so far are virulence factors, and interest has focused toward the discovery of channel inhibitors that would have a therapeutic effect, or be used as research tools to understand the biological roles of viroporin ion channel activity. However, this paradigm is being shifted by the difficulties inherent to small viral membrane proteins, and by the realization that protein–protein interactions and other diverse roles in the virus life cycle may represent an equal, if not, more important target. Therefore, although targeting the channel activity of viroporins can probably be therapeutically useful in some cases, the focus may shift to their other functions in following years. Small-molecule inhibitors have been mostly developed against the influenza A M2 (IAV M2 or AM2). This is not surprising since AM2 is the best characterized viroporin to date, with a well-established biological role in viral pathogenesis combined the most extensive structural investigations conducted, and has emerged as a validated drug

target. For other viroporins, these studies are still mostly in their infancy, and together with those for AM2, are the subject of the present review.



1. INTRODUCTION

The field of viroporins was born about 20 years ago from the realization that viruses inflict injuries in the membranes of cells during infection, which results in increased membrane permeability (Carrasco, 1995). This early observation heralded the current interest in viroporins, along with the therapeutic opportunities afforded by the modulation of their channel activity using small molecules (Fischer, Wang, Schindler, & Chen, 2012; Scott & Griffin, 2015), and the deeper understanding of the roles of viroporin ion channel activity on viral replication and pathogenesis (Nieto-Torres, Verdia-Baguena, Castano-Rodriguez, Aguilera, & Enjuanes, 2015).

This interest will likely increase with the discovery of new viroporins along with their ever-growing roles in the virus life cycle. Indeed, the number of medically important viroporins known is expected to rise as new human viruses continuously emerge from animal hosts. It is also possible that new viroporins may have novel features and structural motifs which will contribute in updating their classifications in terms of topology and number of transmembrane α -helical domains (Nieva, Madan, & Carrasco, 2012). In vertebrates alone, the number of viruses was estimated to be around one million (Morse, 1993). More recent estimates quantify the viral diversity in mammals, which are the reservoir hosts of the majority of emerging zoonoses, as 320,000 (Anthony et al., 2013). This strongly contrasts with the few thousand viruses identified to date, which means that more than 99% of viruses—and their viroporins, if any—are still unknown.

Most viroporins have been identified as virulence factors that lead to viral attenuation when deleted. This attenuation is attributed only in part to their channel activity, but nevertheless small-molecule channel inhibitors have been sought after. The vast majority of these channel inhibitors have been developed against the influenza A virus M2 (IAV M2 or AM2) protein (reviewed recently in Du, Cross, & Zhou, 2012; Gu, Liu, & Wei, 2013), which is the first viroporin discovered. This is not surprising since AM2 is the best characterized viroporin to date, with a well-established biological role in viral pathogenesis combined the most extensive structural investigations conducted, and has emerged as a validated drug target. For other

viroporins, these studies are still mostly in their infancy, although a high-resolution structure of the hepatitis C virus (HCV) p7 protein has been recently described (Ouyang et al., 2013) that may be useful for the rational design of p7 channel inhibitors in the future.

However, attempts to rationally design small-molecule inhibitors against viroporins will encounter several challenges. One is the potential for sequence variability in the viroporin. Indeed, it has been argued that since the number of genes susceptible to deleterious mutations increase with genome size, mutation rates are higher for small genomes (Drake, 1969). A related problem is that viral proteins, when compared to prokaryotic and eukaryotic proteins, have a higher degree of structural flexibility that may represent adaptation mechanisms to increase resistance to random mutations, especially in the case of RNA viruses, which mutate and evolve faster than DNA viruses. A less dense packing, and a concomitant smaller number of network interactions, translates not only to a smaller difference in energy between folded and unfolded states, but an also smaller percentual contribution of each mutation to stability (Tokuriki, Oldfield, Uversky, Berezovsky, & Tawfik, 2009). Additionally, this enhanced flexibility may have an impact on the ability of a single protein to perform a variety of tasks, consistent with the economical use of resources in viruses. Adding to this intrinsic flexibility, another problem in structure-based drug discovery concerning viroporins is the strong dependence of the protein structure on environment. Indeed, solution NMR can be applied to study small-membrane proteins in membrane-mimicking environments that allow rapid reorientation, but these artificial systems may also alter the intrinsic properties of the proteins (Cross, Dong, Sharma, Busath, & Zhou, 2012; Cross, Sharma, Yi, & Zhou, 2011). Last, some viroporins seem to form a protein-lipid complex rather than a purely proteinaceous pore, as shown for the VP4 protein in the triatoma virus (Sanchez-Eugenía, Goikolea, Gil-Carton, Sanchez-Magraner, & Guerin, 2015), and similar observations have also been reported for coronavirus envelope proteins (Verdia-Baguena et al., 2012).

In the quest to discover small molecules targeting viroporins, amantadine (Amt) can be considered to be the first viroporin channel inhibitor, and one of the first antivirals licensed for use in humans. Although it was known that this adamantane was active against IAV (Davies et al., 1964; Wingfield, Pollack, & Grunert, 1969), the mechanism of inhibition was described only in 1992 (Duff & Ashley, 1992; Pinto, Holsinger, & Lamb, 1992). The target itself, AM2, was not known until 1985 (Hay, Wolstenholme,

Skehel, & Smith, 1985). Even today, Amt, together with its methylated derivative rimantadine (Rim) which was licensed in the 1980s, are the only licensed antiviral drugs that target viroporins. This contrasts with the availability of hundreds of compounds that exert their effects with subnanomolar affinities on nonviral ion channels. Although this phenomenon could be explained in principle by a lack of structural data for viroporins, this is not the case for AM2, where precise structural data has been obtained, even for some of its Amt-resistant mutants (Pielak & Chou, 2011; Wang, Qiu, Soto, & Degrado, 2011). Unfortunately, an effective alternative to adamantanes has not yet been licensed.



2. THE INFLUENZA A VIRUS MATRIX PROTEIN 2 (IAV M2 OR AM2)

With our current knowledge, the AM2 channel provides yet another paradigm valid for other viroporins. It has a very simple structure compared to ion channels from higher organisms (such as K^+ and Ca^{2+} channels), but the mechanism of proton conduction and drugs binding and inhibition are not yet fully understood. Indeed, the structural and functional properties of the AM2 protein vary widely in changing experimental conditions, such as peptide length, drug binding, lipid composition, lipid thickness, or pH (Acharya et al., 2010; Cady, Wang, & Hong, 2011; Cross et al., 2012; Kovacs, Denny, Song, Quine, & Cross, 2000; Thomaston et al., 2013; Zhou & Cross, 2013). An important factor in this sensitivity is the direct contact of its TM domains with the lipid molecules, in contrast to higher organism ion channels (eg, K^+ channels) (Cuello, Jogini, Cortes, & Perozo, 2010), where pore-forming α -helices are surrounded and protected by additional transmembrane helices. However, while the AM2 channel exists in a variety of conformational states in the apo-form, inhibitor-binding significantly reduces the conformational flexibility of the channel. The structural dynamics of the channel represents another drawback in the use of high-resolution structures for rationally designed drug discovery.

Amantadine is just one of the four antivirals against IAV available: two neuraminidase inhibitors (oseltamivir and zanamivir) and two AM2 channel blockers (Amt and Rim) (Vanderlinden & Naesens, 2014). The seasonal flu caused by IAV is associated with high medical burden (Molinari et al., 2007) and sudden pandemics with high mortality rates (Hay, Gregory, Douglas, & Yi, 2001; Neumann, Noda, & Kawaoka, 2009). Currently, most circulating strains are Amt resistant (Bright, Shay, Shu, Cox, & Klimov, 2006;

Bright et al., 2005; Deyde et al., 2007; Hayden & De Jong, 2011) and therefore the use of Amt and Rim has been discontinued for the human population (Fiore et al., 2011).

The AM2 channel is a homotetramer (Sakaguchi, Tu, Pinto, & Lamb, 1997), where each monomer has 97 amino acids containing one α -helical TM domain (residues 25–46). The extramembrane C-terminal domain (residues 47–97) is exposed to the cytoplasmic side (Lamb, Zebedee, & Richardson, 1985) and contains an amphipathic helix (residues 51–59). Four of these amphipathic helices stabilize the tetrameric channel, forming a base on the membrane surface almost perpendicular to the TM bundle. The end of this C-terminal tail is disordered and interacts with the viral matrix protein 1 (M1). The M2 protein performs several critical roles for virus replication (Pinto & Lamb, 2006). For instance, M2-mediated acidification of the interior of the virus is required for the uncoating of genetic material inside the cell (Pinto et al., 1992). M2 is also required to regulate the intraluminal pH of the Golgi apparatus preventing the premature conformational change of hemagglutinin (Takeuchi & Lamb, 1994).

Influenza B, the closest relative of the influenza A virus, accounts for about 50% of all influenza disease in recent years (according to the US Centers for Disease Control and Prevention website, www.cdc.gov) and has a similarly essential pH-activated proton channel M2 viroporin, the BM2 (Hatta, Goto, & Kawaoka, 2004; Pinto & Lamb, 2006). Although AM2 and BM2 share almost no sequence identity, both have a single TM domain and form homotetramers (Paterson, Takeda, Ohigashi, Pinto, & Lamb, 2003; Wang, Pielak, McClintock, & Chou, 2009). In comparison, BM2 is completely insensitive to Amt or Rim (Mould et al., 2003; Pinto & Lamb, 2006) and no BM2 inhibitors have been identified yet.

The location of the binding site for the adamantanes Amt and Rim in AM2 was initially controversial. A crystallographic structure suggested a pore-binding model in the amino-terminal part of the TM (22–46), ie, the P-binding site (Stouffer et al., 2008) (Fig. 1A–C). In the same and in the following year, a solution NMR-based structure of a longer construct of AM2 (18–60) (Pielak, Schnell, & Chou, 2009; Schnell & Chou, 2008) proposed a surface-binding site (the S-binding site) for Rim, on the carboxy-terminal, lipid-facing surface of the helices (Fig. 1D–G), suggesting an alternative allosteric mechanism of inhibition. In this allosteric binding site, the adamantane group interacted favourably with the hydrophobic side chains of Leu-40, Leu-43, and Ile-42, whereas the positively charged ammonium group interacted with the polar patch formed by

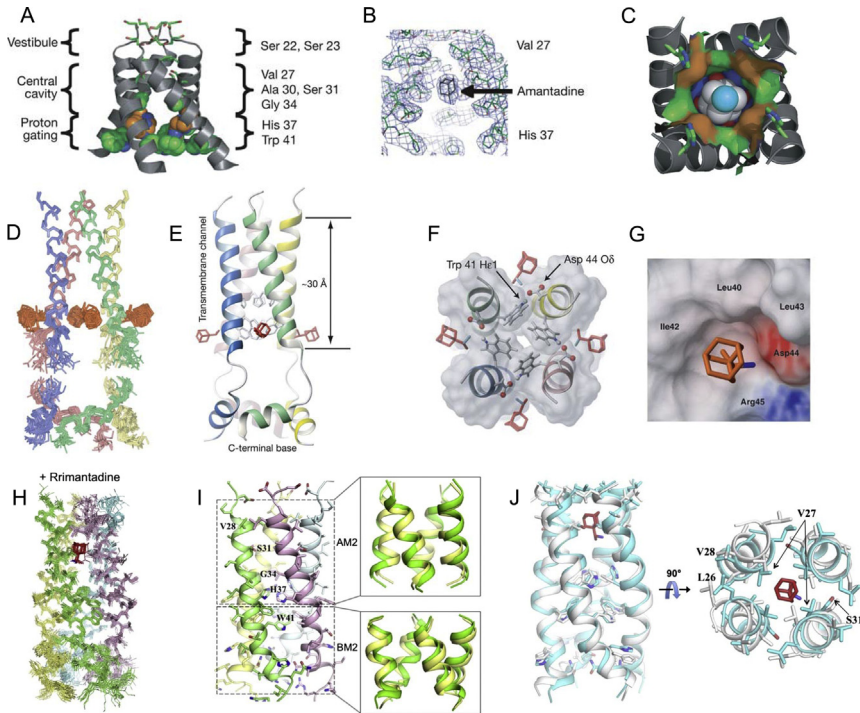


Figure 1 P- and S-binding sites for Amt and Rim to IAV M2. (A–C) AM2 TM domain crystallized in detergent, with the most critical residues identified by site-directed mutagenesis lining the AM2 pore (A); omit map showing electron density in the Amt binding region (B); structure of amantadine (nitrogen in cyan (light gray in the print version) and carbon in white) inside the binding site showing the surface associated with residues Val-27 (red (light gray in the print version) surface), Ala-30 (green (dark gray in the print version)), Ser-31 (blue (light gray in the print version)), and Gly-34 (orange (light gray in the print version)) (C); (D and E), an ensemble of 15 low-energy structures derived from NMR restraints, with TM α -helices (residues 25–46) and amphipathic helices (residues 51–59) superimposed separately (D); ribbon representation, showing the drug Rim (colored in red (light gray in the print version)) (E); (F and G), surface representation of the Rim-binding pocket, showing the Asp-44, the indole amine of Trp-41, and Arg-45, which form the polar patch, as well as the hydrophobic wall composed of Leu-40, Ile-42, and Leu-43; (H–J) solution NMR structures of the (AM2–BM2)TM channel in the absence and presence of Rim; ensembles of 15 low-energy structures of drug-bound chimera channels. Rim is highlighted in red (light gray in the print version) (H); ribbon representation of drug-free (AM2–BM2)TM tetramer (*left*), and overlay of its AM2 and BM2 regions (green (dark gray in the print version)) with the corresponding regions (yellow (light gray in the print version)) of the AM2 (PDB code: [2RLF](#)) and BM2 (PDB code: [2KIX](#)) structures (*right*) (I); overlay of the drug-free (white) and the drug-bound (cyan (light gray in the print version)) chimera structures, showing substantial differences in helical packing (J). Adapted by permission from Macmillan Publishers Ltd.: Stouffer, A. L., Acharya, R., Salom, D., Levine, A. S., Di Costanzo, L., Soto, C. S., et al. (2008).

Asp-44 and Arg-45 from the adjacent subunit (Fig. 1G). A dilemmatic feature of allosteric sites is that viral mutations at these sites are easily accommodated by viruses without dramatic loss of fitness. In HCV, for example, protease inhibitor monotherapy can rapidly select resistant viral populations within a few days (Schmidt et al., 2014).

However, clear support for the P-binding site was obtained from solid-state NMR in lipid bilayers (PDB ID: 2KQT) (Cady et al., 2010); the inhibitor was found in a hydrophilic pocket formed by Ala-30, Ser-31, and Gly-34, ie, occluding the AM2 channel. In this case, hydrophobic interactions were detected between the adamantane group and Val-27 side chains, whereas the ammonium group was hydrogen-bonded to pore-facing residues and water molecules. The latter work revealed that the S-binding site was in fact just a low-affinity site, and only observed at high concentrations of the drug in the membrane. Importantly, Amt was found to undergo significant motion in the N-terminal lumen, suggesting that its structure can be improved for a better fit to the AM2 channel. This pore-location (P-binding) was also further validated by Chou et al. by solution NMR (Pielak, Oxenoid, & Chou, 2011) using an M2 chimeric sample, where the N-terminal part of the M2 TM domain corresponded to that of the AM2, whereas the C-terminal part was from the Amt-insensitive BM2 (Fig. 1H–J). In that model, methyl groups of Val-27 and Ala-30 from four subunits form a hydrophobic pocket around the adamantane, with the amino group in polar contact with the backbone oxygen of Ala-30. This ended the controversy, with the acceptance of the initially proposed P-binding site.

There are 12 structures of the wild-type and drug-resistant mutant M2 channels available in the Protein Data Bank solved by different techniques and conditions (see summary in Gu et al., 2013). Among these, structure 2RLF solved in micelle (Schnell & Chou, 2008) and structure 2LOJ in the bilayer environment contain, in addition to the TM domain, short C-terminal intracellular amphipathic helices. In 2RLF, the C-terminal helices were connected to the transmembrane helices via a short loop (residue

Structural basis for the function and inhibition of an influenza virus proton channel. Nature, 451(7178), 596–599. Copyright (2008); Schnell, J. R., & Chou, J. J. (2008). *Structure and mechanism of the M2 proton channel of influenza A virus.* Nature, 451(7178), 591–595. doi: 10.1038/nature06531. Copyright (2008). Adapted by permission from Elsevier: Pielak, R. M., Oxenoid, K., & Chou, J. J. (2011). *Structural investigation of rimantadine inhibition of the AM2-BM2 chimera channel of influenza viruses.* Structure, 19(11), 1655–1663. doi: 10.1016/j.str.2011.09.003. Copyright (2011).

47–51) and were exposed to the solution. However, in 2L0J, the C-terminal helices were connected to the TM helices through a rigid turn (residue 47) and were positioned on the bilayer surface (Sharma et al., 2010), and this conformation was stabilized by extensive hydrophobic interactions among these helices, and polar residues Asp-44 and Arg-45 were buried by hydrophobic residues of the intracellular amphipathic helices, making the S-binding site more hydrophobic than that in micelles. Therefore, the S-binding site was absent in 2L0J (Sharma et al., 2010).

Molecular dynamics simulations of the M2 proton channel with inhibitors binding at different sites have shown that the P-binding site is more stable for drug binding, but a higher energy barrier needs to be overcome for binding to occur (Gu et al., 2011). The S-binding site was less stable for drug binding but it was nearly barrierless and was easily accessed. Therefore, the P-binding site represents the thermodynamic binding site where the drug molecule binds slowly and stably and dissociates even more slowly, whereas the S-binding site is a kinetic binding site where the drug molecule binds readily but less stably and dissociates easily.

Over the last four decades, systematic studies of amantadine analogues and library screening have elucidated structure–activity relationships (SARs) and helped to identify potent wild-type (WT) AM2 channel blockers (De Clercq, 2006; Lagoja & De Clercq, 2008). Several other molecules were not based on adamantanes. For example, the spirene guanidine analogue, 2-[3-azaspiro(5,5)undecan-2-ylideneamino]imidazole (BL-1743) (Fig. 2A) was discovered during a high-throughput screen based on the ability of inhibitors to reverse the toxicity associated with M2 channels expressed in the yeast *Saccharomyces cerevisiae* (Kurtz et al., 1995). Based on this non-adamantane, a SAR study using a combination of viral plaque assays and two-electrode voltage clamp (TEVC) (Wang, Cady, et al., 2009) generated 3-azaspiro[5,5]undecane hydrochloride (Fig. 2B), which lacks the

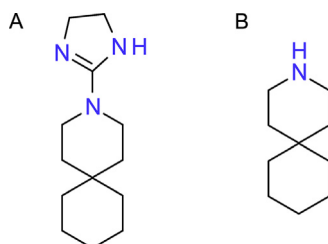


Figure 2 Example of IAV M2 inhibitors not based on the adamantane framework. (A) BL-1743; (B) spiro-piperidine developed from (A) (Wang, Cady, et al., 2009).

imidazoline group of BL-1743. This compound showed an IC_{50} of $0.92 \pm 0.11 \mu\text{M}$ against AM2, ie, more than an order of magnitude more potent than amantadine ($IC_{50} = 16 \mu\text{M}$) and more than 45-fold increase in potency relative to the parental BL-1743.

Compared to Amt, this spiro-piperidine induced a more homogeneous conformation of the AM2 peptide, reducing dynamic disorder near the water-filled central cavity of the helical bundle, suggesting a more extensive binding to the AM2 channel, thus leading to stronger inhibitory potency. However, this compound was only effective against the wild-type AM2. This is crucial because of the current prevalence of Amt-resistant variants of AM2, eg, L26F, V27A, and S31N, which comprise more than 99% of the reported resistance mutants (Furuse, Suzuki, & Oshitani, 2009; Suzuki et al., 2003). Of these three mutations, V27A is the only one known to originate from drug selection pressure (Furuse et al., 2009), and this mutant is the only one among the three that is completely resistant to Amt and Rim (Balannik et al., 2009). On the other hand, S31N is found in more than 98% of the mutated M2 channels in the IAV subtype H3N2 viruses (Bright et al., 2005), and causes a 10–20 times decrease in the IC_{50} , from low micromolar to $200 \mu\text{M}$ (Amt) and more than 2 mM (Rim) (Wang, Ma, et al., 2013).

The first non-adamantane inhibitor of the V27A mutant was a polycyclic pyrrolidine, a dual inhibitor of WT (IC_{50} of $3.4 \mu\text{M}$) and V27A (IC_{50} of $0.29 \mu\text{M}$) (Rey-Carrizo et al., 2013). Later, a compound with triple inhibitor efficacy was reported by the same authors, active against the WT, V27A, and L26F mutants, with IC_{50} of 18, 0.7, and $9 \mu\text{M}$, respectively (Rey-Carrizo et al., 2014). The inhibitory activity of the compounds was tested on AM2 channels expressed in *Xenopus laevis* oocytes using the two-electrode voltage clamp (TEVC) technique, but most of the compounds were cytotoxic (more than Amt and Rim), as tested in cell-based antiviral plaque reduction assays. Therefore, despite the encouraging results obtained in vitro, the toxic properties of these compounds still hinder their therapeutic potential.

The spiro-piperidine inspired the generation of another compound (Fig. 3C) that could inhibit not just the WT AM2 ($IC_{50} = 18 \mu\text{M}$) but also mutants L26F ($IC_{50} = 6 \mu\text{M}$) and V27A ($IC_{50} = 0.3 \mu\text{M}$) (Wang, Ma, Fiorin, et al., 2011). Direct interaction of this compound with the V27A mutant was assessed by solid-state NMR, and inhibition was further demonstrated in plaque reduction assays. This compound, however, was inactive against the S31N mutant. Such observation was rationalized by the fact that

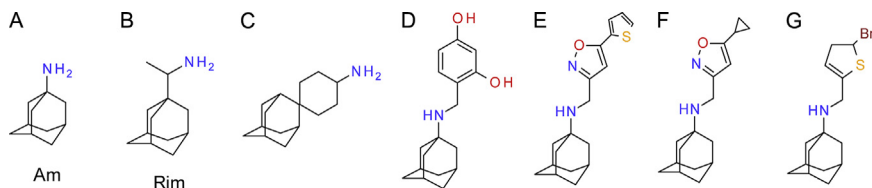


Figure 3 Inhibitors for IAV M2. (A) Amantadine (Amt); (B) rimantadine (Rim); (C) compound **9** in Wang, Ma, Fiorin, et al. (2011); (D) benzenediol (Wang, Ma, et al., 2013); (E and F) compounds with IC_{50} of $\sim 16 \mu\text{M}$ (Wang, Wu, et al., 2013); (G) compound found in Wu et al. (2014).

the S31N mutant is more dynamic and hydrated in the pore than the corresponding WT protein (Pielak et al., 2009), as shown by NMR studies. This led to a disruption in the size and polarity of the pore in precisely the region that accommodates the adamantane group, thereby hampering the discovery of inhibitors of S31N. This led to attempts to increase the polarity or the dimensions of the adamantane core in order to capture more interactions with the backbone of the channel.

A variety of scaffolds have been used to substitute for the hydrophobic adamantane group (Hu et al., 2010; Wang, Ma, Balannik, et al., 2011). These compounds showed excellent activity against the WT AM2, and some were highly active against V27A and L26F (Wang, Ma, Fiorin, et al., 2011). However, none was better than Amt against S31N. Attempts to introduce additional groups to the amine to enhance affinity for both S31N and WT via additional electrostatic interactions led to the discovery of benzyl-substituted amantadine derivatives (Fig. 3D) as dual WT and S31N inhibitors (Wang, Ma, et al., 2013) with IC_{50} of 35 and 59 μM for S31N and WT, respectively.

The DeGrado group later discovered small-molecule drugs, eg, M2WJ332, that locked the dynamic S31N mutant into a well-defined conformation, enabling high-resolution structure determination by solution NMR that showed the drug bound in the homotetrameric channel, threaded between the side chains of Asn-31. These compounds were formed by conjugation of a α -CH₂-heteroaryl group to the amine of Amt (Fig. 3E and F, IC_{50} of 16 μM) and inhibited S31N with potencies greater than Amt against AM2 (Wang, Wu, et al., 2013). The charged ammonium binds as a hydrate to one of three sites aligned along the central cavity that might stabilize hydronium-like species formed as protons diffuse through the outer channel to the proton-shuttling residue His-37 near the cytoplasmic end of the channel.

Based on structural and molecular dynamics data, the DeGrado group realized that inhibitors that are active against both WT and S31N channels bind in opposite orientations: the aromatic headgroup faces toward the C-terminus in WT, and toward the N-terminus in S31N. Investigation of substitutions led to the identification of halide-substituted thiophene compounds. The most potent of which, *N*-[(5-bromothiophen-2-yl)methyl]adamantan-1-amine (Fig. 3G), inhibited *both* WT and S31N mutant with comparable affinities as Amt against WT (Wu et al., 2014).

It would be desirable to develop the same paradigm found in IAV AM2 and their inhibitors for other viroporins, so that their channel inhibitors can be validated. For example, many amantadine-resistant influenza viruses can be selected in cell culture (Grambas, Bennett, & Hay, 1992; Grambas & Hay, 1992; Hay et al., 1985), and some of these are also found in infected patients undergoing treatment with amantadine (Shiraiishi et al., 2003). In turn, engineered viruses harboring these pore-lining mutations, while competent to replicate in mouse (Abed, Goyette, & Boivin, 2005), give rise to attenuated viruses that tend to revert to the WT in the absence of drug pressure (Grambas & Hay, 1992; Suzuki et al., 2003).



3. THE HEPATITIS C VIRUS p7 PROTEIN (HCV-p7)

Another very important viroporin is p7, found in the Hepatitis C virus (HCV), a member of the Flaviviridae family that has six genotypes (GTs). HCV is a positive-strand RNA virus that causes severe liver disease and is the major cause of hepatocellular carcinomas in the developed world. HCV has chronically infected 170 million people worldwide and is responsible for more than 300,000 yearly deaths, while no prophylactic or therapeutic vaccine is available.

HCV infection can be cured with drugs targeting viral enzymes and proteins, as well as host cellular proteins. One host-targeted antiviral agent (HTAs) is the type I IFN- α (Hoofnagle et al., 1986), which exerts antiviral activities against both RNA and DNA viruses through IFN-stimulated gene (ISG) products. Pegylated IFN has been used in combination with ribavirin (RBV), a synthetic guanosine analogue (Herrmann, Lee, Marinos, Modi, & Zeuzem, 2003), but this antiviral therapy can only last up to 1 year, associated with side effects, and is not always effective (Manns, Wedemeyer, & Cornberg, 2006; Pawlotsky, Chevaliez, & McHutchison, 2007). Other drugs target HCV proteins with high potency, such as those against the HCV NS3/4A protease, the NS5A protein or the NS5B RNA-dependent

RNA polymerase (RdRP) (Sulkowski et al., 2014) [see Pawlowsky, 2014; Schmidt et al., 2014, for recent reviews]. However, drug resistance creates a burden for patients, worsened by the rapid turnover of HCV replication, which produces up to 10^{12} virions daily (Neumann et al., 1998). This is compounded by the error-prone activity of the HCV RNA polymerase, which leads to the high genetic diversity of HCV and the formation of “quasi-species.” In this complicated context, the clinical niche of p7 inhibitors is unclear.

The viroporin HCV p7 is encoded within a single polyprotein precursor (Simmonds, 2013) processed by host and viral proteases (Moradpour & Penin, 2013). It separates the structural proteins (core and envelope glycoproteins E1 and E2) from the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Lindenbach & Rice, 2005). p7 results from cleavage of E2-p7-NS2 and E2-p7 by a signal peptidase at the ER (Lin, Lindenbach, Prágai, McCourt, & Rice, 1994; Mizushima et al., 1994). The p7 protein is 63 residues long with two TM domains, found mainly at the ER membrane. Although p7 is not necessary for RNA replication (Lohmann et al., 1999), it is essential for productive HCV propagation in vivo (Sakai et al., 2003). However, the precise role of the p7 in virus production was only determined when it was possible to replicate a genotype 2a isolate from a Japanese patient suffering from fulminant hepatitis (designated JFH-1) (Kato et al., 2003; Wakita et al., 2005). This isolate efficiently replicated in human hepatoma cell lines and produced infectious virus particles. This was used to demonstrate that p7 is essential for virus particle assembly and release (Jones, Murray, Eastman, Tassello, & Rice, 2007; Steinmann et al., 2007). The bovine viral diarrhea virus (BVDV) (Harada, Tautz, & Thiel, 2000) and the hepacivirus GB virus B (GBV-B) (Takikawa et al., 2006), HCV's closest relatives, also have a p7 protein crucial for virus replication.

The channel activity of p7 was examined more than 10 years ago in experiments involving artificial lipid bilayers. It was suggested that p7 has preference for cations vs anions, with permeability ratios in the range 7–11 (Griffin et al., 2003; Montserret et al., 2010; Premkumar, Wilson, Ewart, & Gage, 2004; Pavlovic et al., 2003) and more recently in *Xenopus* oocytes (Ouyang et al., 2013). This selectivity is rather low compared to other channels, eg, for cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, selectivity for anions over cations is 10–30 (Anderson et al., 1991; Bear et al., 1992). For potassium channels, which conduct K^+ ions near the diffusion limit, the selectivity for K^+ over Na^+ is about

10,000–1 (Doyle et al., 1998), and in aquaporins water to ion flux ratio is about 10^9 (Pohl, 2004). However, the biological importance of p7 cation channel activity may be relative, in the light of its recently found role to permeabilize membranes to protons, and acting to prevent acidification of intracellular vesicles (Wozniak et al., 2010). This proton channel activity was found to be crucial for the production of infectious viruses, and was observed in intracellular vesicles harboring p7. Recently, this proton channel activity has been directly confirmed in vitro using purified nontagged p7 protein in a liposome-based assay (Gan, Surya, Vararattanavech, & Torres, 2014), where the lipid composition used was the mixture PAESC (PA/PE/PS/PC 5:2:2:1 w/w) (Gervais et al., 2011).

Several initial papers also reported the inhibition of channel activity by some compounds. For example, His-tagged p7 from genotype 1b was inhibited by $1 \mu\text{M}$ amantadine (Griffin et al., 2003), whereas synthetic p7 from genotype 1a (strain H77) was inhibited by $100 \mu\text{M}$ hexamethylenamiloride (HMA, Fig. 4A), although this peptide was largely unpurified (Premkumar et al., 2004). Another synthetic p7, also unpurified, was inhibited by 50– $100 \mu\text{M}$ of the long-alkyl-chain imino sugar derivatives NN-DGJ, NN-DNJ (Fig. 4B) and *N*-7-oxanonyl-6-deoxy-DGJ (Pavlovic et al., 2003). However, the channel activity of *purified* synthetic p7 HCV-J genotype 1b in artificial lipid bilayers was inhibited by just 50% in the presence of $200 \mu\text{M}$ HMA, whereas Amt did not show any effect even at 1mM concentration (Montserret et al., 2010). In fact, the efficacy of Amt in HCV culture systems is low (Steinmann et al., 2007), and no clinical benefit of Amt-based combination therapy was found in comparison to standard of care (peg-IFN α plus ribavirin) (Castelain et al., 2007; Mihm et al., 2006). Some of these compounds have been studied in infected cells, where they reduced virus production up to 10-fold in a genotype-dependent manner (Foster et al., 2014; Gottwein et al., 2011; Steinmann et al., 2007), reducing

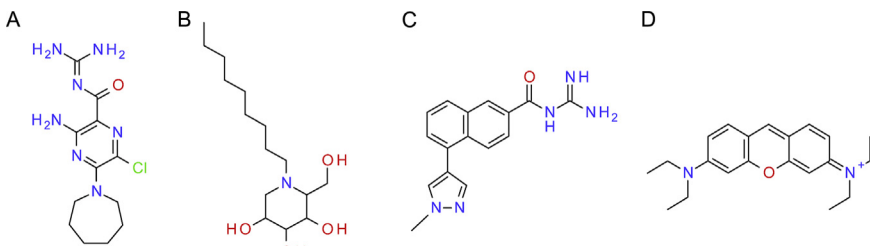


Figure 4 Some other inhibitors for viroporins reported in the literature. (A) HMA, (B) NN-DNJ, (C) BIT225, (D) pyronin-B.

secretion of HCV particles by inhibition of the acidification of virus-containing compartments (Wozniak et al., 2010), without impairment of the infectivity of intracellular virions (Foster et al., 2011; Griffin et al., 2008).

To discover more p7 channel inhibitors, a liposome-based dye (carboxy-fluorescein, CF) release assay has been proposed that involves the addition of p7 protein to preloaded liposomes (StGelais et al., 2007). The method has been claimed to be sensitive to inhibitors such as Amt and Rim, and proposed as a high-throughput functional assay to test drug sensitivity of p7 mutants, or to discover new p7 channel activity inhibitors (Foster et al., 2011; Gervais et al., 2011). These dye release assays have been previously performed with tagged p7 protein, either with FLAG (Li et al., 2012; StGelais et al., 2009) or flu-antigen (Foster et al., 2011). However, this method has been put into question since just the C-terminal half of p7, p7(27–63) was as efficient as full-length p7 in releasing CF, while a properly reconstituted α -helical sample of p7 was not able to elicit CF release (Gan et al., 2014).

Nevertheless, p7 inhibitors may be able to inhibit both the proton channel activity and dye release if the mechanisms are similar, eg, via an allosteric effect that increases the rigidity of the C-terminal half (TM2) of p7. This is supported by the fact that TM2 of p7 interacts with Amt and is the site of inhibition by adamantanes. Indeed, Amt interacts with leucines 51–57, and these leucines have been shown unequivocally to be part of a membrane inserted α -helix (Cook & Opella, 2009; Ouyang et al., 2013). Binding of Amt to p7 in dihexanoylphosphatidylcholine (DHPC) micelles led to resonance shifts in several residues, some of which are the leucines 51–57 (but not 53), ie, located in TM2 (Cook & Opella, 2009). Consistent with this, Leu to Ala mutations at this region produced an Amt-resistant p7 mutant revealed using the CF release assay (StGelais et al., 2009).

The imino sugar NN-DNJ and Rim inhibited secreted infectivity in Amt-resistant JFH-1 and CON-1/JFH-1c3 viruses, whereas mutation L20F conferred resistance to rimantadine (Foster et al., 2011), consistent with Rim resistance was found in GT1b patients not responding to the triple therapy IFN/Rib/Amt (Mihm et al., 2006). Using an oligomerization assay in DHPC gels, the GT1b J4 p7 oligomerization was abolished in presence of imino-sugars, whereas Rim did not affect oligomerization, suggesting two different mechanisms of action for these two compounds (Foster et al., 2011). The p7 of GT3a, but not GT1b, was found to be resistant to NN-DNJ both in vitro and in culture, and this was attributed to a F25A mutation (Foster et al., 2011; Griffin et al., 2008). However, direct

interaction between HMA or imino sugar derivatives with p7 reconstituted in a detergent or membrane has not been reported. Addition of Rim to GT1b J4 p7 monomer in methanol (Foster et al., 2014) showed disruption at several residues, but not especially at Leu-20. Thus, this observation is either inconsistent with the proposed role of L20F in acquired resistance, or the methanol environment is not a good system to test this interaction.

Structural data for p7 was obtained initially in solvents and micelles by solution NMR. For example, solution NMR data was obtained for synthetic p7 (C27A mutant) in 50% of the helix inducer trifluoroethanol (TFE) (Montserret et al., 2010), and for recombinant p7 (C27S mutant) in DHPC micelles (Cook & Opella, 2010). The prevalent model for the p7 monomer according to these studies was that of an α -helical hairpin with two α -helical TMs kinked in the middle (Cook, Zhang, Park, Wang, & Opella, 2010; Montserret et al., 2010), where the N-terminal TM helix (TM1) would face the lumen of a channel (Carrere-Kremer et al., 2002; Chew, Vijayan, Chang, Zitzmann, & Biggin, 2009; Patargias, Zitzmann, Dwek, & Fischer, 2006) formed by either six or seven monomers (Clarke et al., 2006; Griffin et al., 2003; Luik et al., 2009; Montserret et al., 2010).

In a more recently published structural model of p7 (Ouyang et al., 2013), the oligomeric size is still ambiguous since this data could not be obtained in the same environment used in electron microscopy. From that model, the “old” TM1 and TM2 domains would correspond to three helical segments H1–H3, ie, H1 and first half of H2 (TM1) and second half of H2 and H3 (TM2). There are concerns, however, that this model can be present in a physiological context since the folding implied is difficult to explain by our current understanding of protein folding in the cell (Madan & Bartenschlager, 2015). In the Ouyang et al. paper, the authors systematically tested p7 amino acid sequences from various HCV genotypes and found that the sequence from GT5a (EUH1480 strain) generated samples adequate for structure determination. Although the oligomeric size of p7 in dodecyl phosphocholine (DPC) micelles was not determined, this was combined with data from negative stain electron microscopy (EM), which showed hexameric, flower-shaped particles. The latter were similar to those p7 hexamers (of GT2aJFH-1) observed in EM in the presence of DHPC micelles used earlier for single-particle reconstruction (Luik et al., 2009). The NMR-based model showed that the C-terminal helix, p7(27–63), is not the pore-lining sequence but instead forms a “lipid facing” part of the molecule, whereas the N-terminal half would orient lining the lumen (Ouyang et al., 2013). This is in agreement with previous models based

on the partial inhibition of p7 channel activity by Cu^{2+} , but not by Mg^{2+} , which suggested exposure to a His residue located in the N-terminal half to the lumen of the channel (Chew et al., 2009). Although this interpretation may be correct, it is unfortunate that synthetic peptides corresponding to the N-terminal half “TM1,” eg, p7(1–34) or the C-terminal half “TM2,” eg, p7(35–63) aggregate easily, such that individual channel activity of these peptides cannot be properly measured (Montserret et al., 2010). In fact, neither of these helices separately forms α -helical structure in micelles or membranes, and peptide p7(1–26) was totally insoluble in TFE or in detergent (Gan et al., 2014), a difficulty also encountered for the N-terminal fragment of p7(1–34) (Montserret et al., 2010). In contrast, TM domains of other viroporins are completely α -helical, eg, the IAV M2 (Torres, Kukul, & Arkin, 2000), severe acute respiratory syndrome coronavirus (SARS-CoV) E (Torres et al., 2006), or respiratory syncytial virus (RSV) SH (Gan et al., 2012) produce sharp amide I bands in the infrared spectrum consistent with $\sim 100\%$ α -helix.

Nevertheless, the Amt binding site in the p7 channel was determined by identifying Nuclear Overhauser Enhancements (NOEs) between the protein backbone amide protons and drug protons (Ouyang et al., 2013) (Fig. 5). Although the full-scale structure determination of the complex could not be achieved, Amt (10 mM) showed NOE cross-peaks between

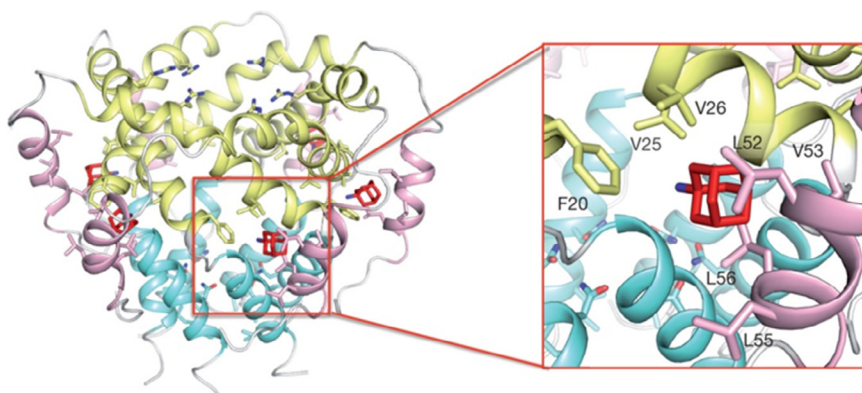


Figure 5 Interaction between HCV subtype 5a p7 and Amt. *Left*, Amt docked into the p7(5a) hexamer using restraints from NOEs contacts with backbone amide protons; *right*, a close-up view of Amt in the binding pocket. Adapted by permission from Macmillan Publishers Ltd.: Ouyang, B., Xie, S., Berardi, M. J., Zhao, X., Dev, J., Yu, W., et al. (2013). Unusual architecture of the p7 channel from hepatitis C virus. *Nature*, 498(7455), 521–525. doi: 10.1038/nature12283. Copyright (2013).

the adamantane protons and the amide protons of Val-26, Leu-55, Leu-56, and Arg-57, ie, between the pore-forming and peripheral helices, suggesting a pocket formed by Leu-52, Val-53, and Leu-56 from H3, and Phe-20, Val-25, and Val-26 from H2, with the polar amino group of Amt pointing to the channel lumen, with similar results also obtained for Rim. Amt and Rim have also been reported previously to interact with some residues in the N-terminal half (Cook & Opella, 2009; StGelais et al., 2009). Isothermal titration calorimetry and NMR chemical shift perturbation analyses of p7(GT5a)–Rim interaction produced a binding constant (K_d) from 50 to 100 μM at 3 mM detergent concentration. The authors proposed that large differences in drug efficacies observed between different HCV genotypes are probably due to variations in the hydrophobicity of the binding pocket among p7 variants, and that the adamantane derivatives inhibit channel activity by restricting the structural rearrangement of the channel, ie, an allosteric mechanism.

Perhaps the most efficient effect for a p7 inhibitor has been reported for BIT225 (*N*-[5-(1-methyl-1H-pyrazol-4-yl)-naphthalene-2-carbonyl]-guanidine) (Fig. 4C). This amiloride was identified in a bacterial assay that involved expression of p7 in *E. coli*; only in presence of inhibitors the normal ionic gradients are maintained and cells are able to grow despite the presence of p7. This compound had antiviral activity against the HCV model pestivirus bovine viral diarrhea virus (BVDV) with an IC_{50} of 314 nM. The addition of 100 μM of BIT225 blocked ion channel activity of synthetic HCV p7 protein of GT1a H77, although no data was reported in the context of HCV infection (Luscombe et al., 2010). While some docking efforts have been performed (Bichmann, Wang, & Fischer, 2014), direct interaction of BIT225 with p7 has not been proven using biophysical or structural assays, and therefore rational optimization of the compound is still not possible.

In addition to HCV p7, the human immunodeficiency virus (HIV) virion Vpu is also targeted; BIT225 was tested against the Vpu TM domain using a black lipid membrane (BLM) system, where the target was exposed to 40 μM of the drug (Khoury, Ewart, Luscombe, Miller, & Wilkinson, 2010). Although direct interaction with Vpu was not observed, this was assumed since the drug was not active against HIV-2, which has no Vpu gene.

One early clinical trial (phase Ib/IIa) involving the treatment of 24 naïve GT1 HCV patients indicated a significant reduction of viral load after 12 weeks treatment with BIT225 in combination with $\text{IFN}\alpha/\text{ribavirin}$ (www.biotron.com.au: biotron 2011). Currently, a phase 2a/2 clinical trial

is in progress and the first results indicate activity against several HCV genotypes, including the difficult-to-treat GT3a and GT1a (www.biotron.com.au; May 29, 2015). Since it is also active against the HIV-1 Vpu, BIT225 might be suitable for treatment of HCV/HIV coinfecting patients. In a Jul. 2015 update of a 3-month dosing trial with BIT225, there was a recommendation that future trials would focus on patients infected with HCV GT3 and to further study BIT225 in combination with other direct-acting antiviral drugs. In these trials, patients received 400 mg of BIT225 twice daily for 3 months. A phase 2a trial on HCV demonstrated that 100% of the patients infected with HCV GT1 who received BIT225 (400 mg) in combination with current standard of care therapies, IFN/RBV, had undetectable virus after 48 weeks. A phase 2 trial in HIV/HCV coinfecting patients showed that all HCV GT3 patients completing 28 days of treatment with BIT225 in combination with IFN/RBV have undetectable HCV load for 12 weeks (sustained viral response, SVR12) after completing all therapy. BIT225 is also in development for treatment of HIV, and is the pioneer in a new class of antiviral drugs that may provide a new approach to the eradication of this virus. It has shown clinical efficacy against HIV in reservoir cells, and has the potential to be combined with new or existing antiretroviral drugs to eradicate long-lived pools of virus that are not successfully eliminated with current treatments. Despite these promising results, however, it remains to be demonstrated that BIT225 targets p7 or Vpu in infected cells.



4. THE CORONAVIRUS ENVELOPE PROTEIN (CoV E)

The most studied viroporin in coronaviruses is the envelope (E) protein. Coronaviruses (CoV) typically affect the respiratory tract and gut of mammals and birds. CoVs belong to the subfamily *Coronavirinae* in the family *Coronaviridae*, and are organized into four genera ([Enjuanes et al., 2000](#)): α , β , γ , and δ . Approximately 30% of common colds are caused by two human coronaviruses—OC43 and 229E. Of particular medical interest is the virus responsible for the severe acute respiratory syndrome (SARS), which produced a near pandemic in 2003 ([Holmes, 2003](#)), and the recently emerged Middle East respiratory syndrome coronavirus (MERS-CoV), which after 3 years has caused hundreds of deaths ([Raj, Osterhaus, Fouchier, & Haagmans, 2014](#)). Other coronavirus viroporins, eg, the SARS-CoV3a ([Lu et al., 2006](#)), a 274-amino acid protein with three putative TM domains,

are also attracting increasing interest (Chien et al., 2013; Hsu & Fischer, 2012), but no inhibitors or structural data is available so far.

Currently, no effective licensed treatment exist against coronavirus infection (Kilianski & Baker, 2014; Kilianski, Mielech, Deng, & Baker, 2013; Lou, Sun, & Rao, 2014), although live vaccines consisting of attenuated viruses is a promising strategy (Enjuanes, Nieto-Torres, Jimenez-Guardeno, & DeDiego, 2011; Graham et al., 2012), along with fusion inhibitors (reviewed in Heald-Sargent & Gallagher, 2012). Even so, the possibility of reappearance of virulent phenotypes, drug side effects, and resistance calls for continued antiviral development.

The envelope (E) proteins are 76–109 residues long with one TM domain (Li, Surya, Claudine, & Torres, 2014; Parthasarathy et al., 2008, 2012; Pervushin et al., 2009; Torres et al., 2006; Torres, Wang, Parthasarathy, & Liu, 2005), and most of them have a cytoplasmic C-terminal domain and a luminal N-terminus (Corse & Machamer, 2000; Nieto-Torres et al., 2011; Raamsman et al., 2000; Ruch & Machamer, 2012).

Most CoV E proteins are present at low concentrations in virions (Corse & Machamer, 2000; Godet, L'Haridon, Vautherot, & Laude, 1992; Liao, Yuan, Torres, Tam, & Liu, 2006; Yu, Bi, Weiss, & Leibowitz, 1994), but found abundantly in internal membranes of infected hosts (Corse & Machamer, 2003; Liao et al., 2006; Raamsman et al., 2000; Tung et al., 1992), eg, the ER–Golgi intermediate compartment (ERGIC), where virions assemble (Lopez, Riffle, Pike, Gardner, & Hogue, 2008; Nieto-Torres et al., 2011).

CoV E proteins have been found to be critical for viral pathogenesis, while having a protective antiapoptotic effect on infected cells (DeDiego et al., 2011) which may help the virus evade premature death of host cells, thereby allowing viral replication. Interestingly, deletion of E protein reduced pathogenicity and mortality in animal models through a reduced inflammation (DeDiego et al., 2014)—as discussed later—and this has led to the development of live attenuated vaccines based on E-deleted or E-truncated virions (Almazán et al., 2013; Lamirande et al., 2008; Netland et al., 2010).

The only structural data available for a CoV E protein is for SARS-CoV E, where the TM domain (E-TM) has been characterized in some detail in lipid membranes (Torres et al., 2006). Later, solution NMR of the selectively labelled synthetic E-TM (residues 8–38) in DPC micelles produced a similar pentameric left-handed parallel bundle (Pervushin et al., 2009).

In these models, Asn-15 is facing the lumen of the channel (Torres et al., 2006) whereas Val-25 is involved in helix–helix interactions with other subunits (Pervushin et al., 2009). Mutations at these residues, N15A and V25F, abolished channel activity in vitro (Torres et al., 2007), and introduction of these mutations in a recombinant SARS-CoV resulted in in vivo attenuation in a mouse model. The integrity of the TM domain, and preservation of channel activity, were shown to be important for inflammasome activation and elevated production of the proinflammatory cytokine IL-1 β (Nieto-Torres et al., 2014). In the same study, revertant mutants of the channel-inactive E that regained fitness and pathogenicity also regained channel activity, as measured in black lipid membranes. Both E-TM and the full-length E protein form homopentameric channels with poor ion selectivity that can be partially inhibited by the drug HMA with micromolar affinity (Verdia-Baguena et al., 2012; Wilson, Gage, & Ewart, 2006).

The NOEs between HMA and E-TM suggested the presence of two binding sites at both ends of the TM domain. At the N-terminal domain, Leu-19 exhibited the largest chemical shift, and the relative intensities of the HMA proton cross-peaks indicated an HMA:E-TM stoichiometry of 1:5 at the N-terminal binding site near Asn-15, suggesting one HMA molecule per E-TM pentamer. This is consistent with a pore-blocking inhibition mode similar to Amt in AM2, where in this case the HMA molecule may be stabilized by a hydrogen bonding network to the Asn-15 side chains of the SARS-CoV E, with the cyclohexamethylene ring pointing away from the center of the channel (Fig. 6A). At the C-terminal of the TM domain, near Arg-38, the HMA:E-TM stoichiometry was 1:2, suggesting for this site a rapid exchange, in the chemical shift timescale, between E-TM-bound and micelle-bound forms of HMA. At this location, the amiloride group of HMA is likely to be involved in interactions with the guanidinium groups of Arg-38 (Fig. 6B). These results were confirmed using the extended peptide E (8–65) in mixed SDS/DPC (1:4 molar ratio) micelles (Li, Surya, et al., 2014), where the addition of HMA perturbed residues clustered near the N-terminal end of the TM domain, eg, Glu-8, Gly-10, Thr-11, Val-14, Asn-15, and Ser-16. At the C-terminal end of the TM, Leu-37 was the most affected, suggesting that the interaction of HMA at the H ϵ of Arg-38 reported previously may have been an artifact due to the use of an E-TM (8–38) peptide. In any case, these results suggest a similar behaviour of HMA-to-E protein and Amt-to-AM2 protein, ie, the presence of two binding sites, one pore-binding and one surface-binding in the TM domain.

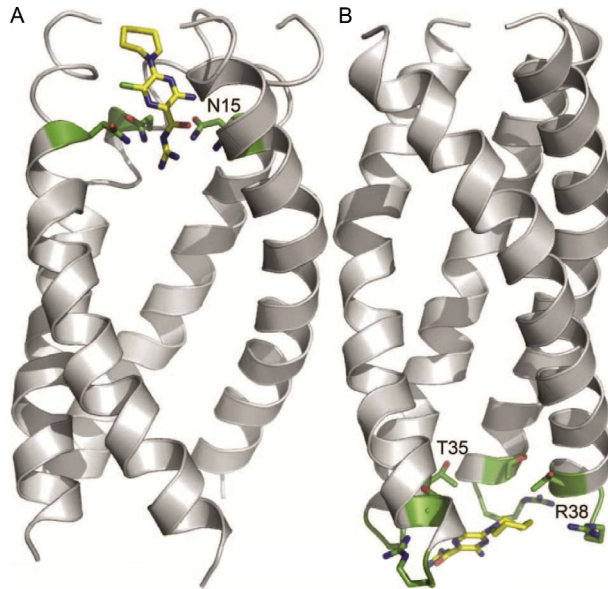


Figure 6 Binding of HMA to the SARS-CoV E-TM pentameric channel (Pervushin et al., 2009). (A) Side view of the binding of HMA to the vicinity of Asn-15. The side chains of amino acids interacting with HMA are shown using a stick representation; (B) binding of HMA to the C-terminal binding site of the channel, in the vicinity of Thr-35 and Arg-38. For clarity, one of the E-TM monomers has been removed.

This structure provided a possible rationale for inhibition and a platform for future structure-based drug design of this potential pharmacological target.



5. THE RESPIRATORY SYNCYTIAL VIRUS SMALL HYDROPHOBIC PROTEIN (RSV SH)

The small hydrophobic (SH) protein is found in the human respiratory syncytial virus (hRSV), an enveloped pneumovirus in the *Paramyxoviridae* family. hRSV is the leading cause of bronchiolitis and pneumonia in infants and elderly (Dowell et al., 1996), and the most frequent cause of hospitalization of infants and young children in industrialized countries. In developing countries, hRSV is a significant cause of death, with global estimates of more than 70,000 deaths in young children. hRSV is the third most important cause of deadly childhood pneumonia after *Streptococcus pneumoniae* and *Haemophilus influenzae* (Nair et al., 2010).

Several compounds target the RSV fusion (F) protein, which facilitates viral entry through the host cell membrane (Zhao, Singh, Malashkevich, &

Kim, 2000) through formation of a 6-helix bundle (Douglas et al., 2005; Lambert et al., 1996; Pastey, Gower, Spearman, Crowe, & Graham, 2000; Razinkov, Gazumyan, Nikitenko, Ellestad, & Krishnamurthy, 2001; Roymans et al., 2010; Shepherd et al., 2006). Other approaches have involved gene transfer to expose viral proteins to cells (Kumar et al., 2002), or siRNA against specific viral proteins (Zhang et al., 2005). Vaccines have been recently designed based on a stabilized RSV-F form which preserves a highly antigenic site in its prefusion state, yielding RSV-specific neutralizing antibodies in mice and macaques (McLellan, Chen, Joyce, et al., 2013; McLellan, Chen, Leung, et al., 2013). Recently, a vaccine candidate based on the extracellular domain (C-terminal) of the RSV viroporin, the small hydrophobic (SH) protein, has been reported (Schepens et al., 2014). In addition, the prevention of nasopulmonary infection in mice caused by RSV has been reported using stapled peptides targeting the fusogenic F-protein 6-helix bundle (Bird et al., 2014). However, despite all these efforts, new FDA-approved drugs have yet to emerge. The only licensed drug for use in infected individuals is ribavirin, a nucleoside analogue, but its efficacy is very limited (Hall et al., 1986).

The hRSV genome transcribes 11 proteins (Collins & Melero, 2011). One of these is the viroporin SH protein, which is 64–65 residues long (depending on subgroup, A or B) and has a single α -helical TM domain with N- and C-terminal extramembrane domains oriented cytoplasmically and lumenally/extracellularly, respectively (Collins & Mottet, 1993; Gan et al., 2012). Most SH protein accumulates at the membranes of the Golgi complex in infected cells, but it has also been detected in the ER and plasma membranes (Rixon et al., 2004).

RSV that lacks SH (RSV Δ SH) is still viable, and still forms syncytia (Bukreyev, Whitehead, Murphy, & Collins, 1997; Karron et al., 1997; Techarpornkul, Barretto, & Peeples, 2001), but is attenuated in vivo. In mouse, RSV Δ SH replicated 10-fold less efficiently in the upper respiratory tract (Bukreyev et al., 1997), whereas chimpanzees developed significantly less rhinorrhea than those infected with wild-type RSV (Whitehead et al., 1999). Other reports have shown that the lack of SH protein leads to an attenuated phenotype in children and in rats (Jin et al., 2000; Karron et al., 1997). Overall, these results indicate involvement of hRSV SH protein in replication and pathogenesis.

In common to the SARS-CoV E protein, SH protein blocks or delays apoptosis in infected cells (Fuentes, Tran, Luthra, Teng, & He, 2007), and a similar antiapoptotic effect of SH protein has been observed for other

members of the *Paramyxoviridae* family, eg, J Paramyxovirus (JPV) (Jack, Boyle, Eaton, & Wang, 2005; Li et al., 2011), mumps virus (MuV), and the parainfluenza virus 5 (PIV5), formerly known as simian virus 5 (SV5). In all these systems, the SH protein seems to block apoptosis during infection—at least in part—through inhibition of the TNF- α pathway (Fuentes et al., 2007; Li et al., 2011; Lin, Bright, Rothermel, & He, 2003; Wilson, Fuentes, et al., 2006).

The mutual orientation of the TM α -helices that form the ion channel was determined in lipid bilayers using site-specific infrared dichroism (Gan, Ng, Xin, Gong, & Torres, 2008). A description of the full-length RSV SH protein monomer has been obtained by solution NMR in DPC micelles (Gan et al., 2012) and later in bicelles, only for the extramembrane domains (Li, To, et al., 2014). Like SARS-CoV E, the RSV SH protein forms homopentameric channels (Gan et al., 2008, 2012) that have low ion selectivity (Li, To, et al., 2014). The TM domain of SH protein has a funnel-like architecture (Gan et al., 2012), as observed in other viroporins, eg, the IAV M2 (Schnell & Chou, 2008), SARS-CoV E (Pervushin et al., 2009) and HCV p7 (Ouyang et al., 2013). A narrower region (Gan et al., 2012) in the TM domain is lined with hydrophobic side chains (Ile-32, Ile-36, Ile-40, and Leu-44) whereas the more open N-terminal region is lined by polar residues, ie, His-22, Thr-25, and Ser-29. The TM α -helix extends up to His-51 in the C-terminal region, followed by a loop, whereas the N-terminal cytoplasmic extramembrane domain forms a short α -helix (residues 5–14) present both in micelles and in bicelles (Li, To, et al., 2014).

Only one compound, pyronin-B, has been reported to inhibit the channel activity of the SH protein. This compound was obtained from liposome-based fluorescence assay, and was tested against purified SH protein reconstituted in planar lipid bilayers (BLM). A concentration of 10 μ M led to a \sim 60% inhibition of channel activity, with a K_d of \sim 7 μ M. The effect of pyronin-B was tested against RSV replication in Vero cells, where the tissue culture infectivity (50% infective dose, TCID₅₀) was zero at a drug concentration of 0.25 μ M.

In the NMR studies, the binding of pyronin-B to the SH protein revealed backbone resonance perturbations at both ends of the TM domain. At the N-terminal cytoplasmic end, residues more affected were Ile-6 and Ile-21. At the luminal C-terminal end, the residue most affected was Ala-39, and a group of nearby residues, Ile-38, Ile-40, Leu-41, and Lys-43 (Fig. 7A). Interestingly, most of these juxtamembrane residues (residues 38–43) form a conserved motif in the SH protein “A₃₉ILNKL₄₃,”

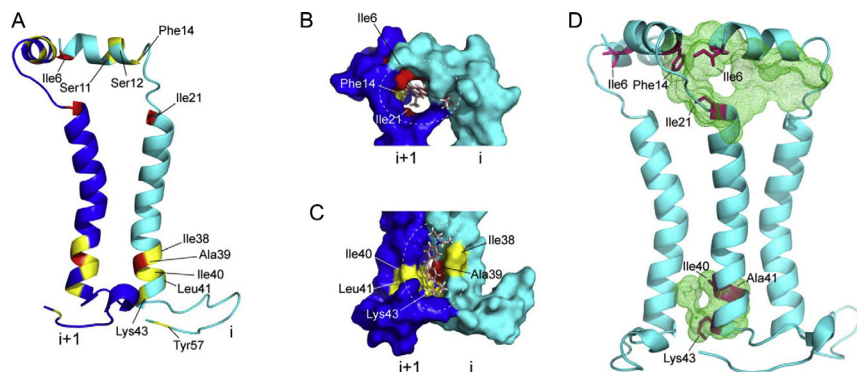


Figure 7 Mapping of pyronin-B binding to the RSV SH protein. (A) Chemical shift perturbation (CSP) values in presence of pyronin-B, mapped onto the structure of SH protein; larger ($\text{CSP} \geq 0.07$ ppm) and smaller ($\text{CSP} \geq 0.04$ ppm) shifts in red (dark gray in the print version) and yellow (light gray in the print version), respectively; (B and C) two predicted pyronin-B binding sites on the SH pentamer, at the N-terminal (B) and C-terminal (C) ends of the TM domain (*dotted circles*). Only two monomers of SH protein pentamer (*i* and *i* + 1) are shown for simplicity; (D) druggable pockets (*green* (dark gray in the print version) mesh) predicted by DoGSiteScorer (Volkamer, Kuhn, Rippmann, & Rarey, 2012). The main residues that showed largest NMR chemical shift changes are shown in red (dark gray in the print version). Two monomers have been removed from the pentamer for clarity.

which suggests that this is a critical region for SH protein with a high barrier to resistance. Determination of intermolecular nuclear Overhauser effects (NOEs) for the drug–protein complex was not possible, probably due to weak interaction.

Docking studies of pyronin-B to the SH pentameric model obtained in DPC micelles (Gan et al., 2012) produced two predicted binding sites at both ends of the TM domain. Indeed, a majority of structures showed the drug located near residues with largest chemical shift perturbation, ie, Ile-6, Ile-21, and Ala-39 (Fig. 7). The binding site near the N-terminus is formed by Phe-14, Ile-21 in one monomer (*i* + 1), and Ile-6 of the previous (*i*) monomer. The one near the C-terminus is formed by residues Ile-40, Leu-41, and Lys-43 in one monomer (*i* + 1) and Ile-38 and Ala-39 of the previous (*i*) monomer (Fig. 7A–C). Similar “druggable pockets” on the SH pentamer surface were identified using DoGSiteScorer (Volkamer et al., 2012) (Fig. 7D). However, the C-terminal end of the TM domain (residues 38–41) is sufficient for inhibition, since a conservative mutation A39S almost completely prevented inhibition (~10% inhibition).



6. THE HUMAN IMMUNODEFICIENCY VIRUS VIRAL PROTEIN U (HIV-1-Vpu)

Vpu is a 81-residue small-membrane protein encoded by the single-stranded positive-sense RNA human immunodeficiency virus 1 (HIV-1), and some simian immunodeficiency virus (SIV) isolates. Vpu is one of four HIV-1 accessory proteins involved in the release of new virions from host cell membranes (Cohen, Terwilliger, Sodroski, & Haseltine, 1988; Strebel, Klimkait, & Martin, 1988; Terwilliger, Cohen, Lu, Sodroski, & Haseltine, 1989). Without Vpu, HIV particles fail to detach from the plasma membrane and accumulate in large numbers in the cell surface (Klimkait, Strebel, Hoggan, Martin, & Orenstein, 1990).

The initial structural studies of Vpu were performed more than 20 years ago by solution NMR in TFE/water using synthetic peptides of various overlapping lengths, which led to a model of an N-terminal hydrophobic transmembrane helix (the TM domain) and a C-terminal cytoplasmic domain with a helix-loop-helix arrangement [reviewed recently in Opella, 2015]. Structural similarity of Vpu with AM2 suggested that Vpu could form pores (Maldarelli, Chen, Willey, & Strebel, 1993) and was later shown to have cation-selective channel activity (Ewart, Sutherland, Gage, & Cox, 1996; Schubert et al., 1996), but a unique structural model for the Vpu oligomer cannot be defined [reviewed in Radoicic, Lu, & Opella, 2014]. An analysis of the Vpu TM (residues 1–40) by solid-state NMR in membranes, combined with analytical centrifugation measurements in detergent, produced data compatible with a variety of oligomers coexisting in micelles and phospholipid bilayers (Lu, Sharpe, Ghirlando, Yau, & Tycko, 2010).

This led to the proposal that regulation of virus release by Vpu might involve an alteration of the plasma membrane potential, and this was supported by (i) the reduced rate of HIV-1 virus release after membrane depolarization (Hsu, Han, Shinlapawittayatorn, Deschenes, & Marbán, 2010), (ii) the observed reduction in viral load and viral pathogenicity when Vpu TM domain is scrambled in the context of a simian–human immunodeficiency virus (SHIV) (Hout et al., 2005), and (iii) the substitution of Ala-18 by a histidine (A18H) rendered HIV-1 infections susceptible to Rim (Hout et al., 2006). However, recent studies challenge this model and argue against a critical involvement of Vpu ion channel activity in Vpu-enhanced virus release. Indeed, mutations in the Vpu TM domain that affect channel

activity do not impair the ability to enhance virus release, whereas mutations that affect virus release retain ion channel activity (Bolduan et al., 2011). Thus, the functional significance of Vpu ion channel activity for HIV replication, and the role as a target for channel inhibitors, remains unclear.



7. 6K, AGNOPROTEIN, AND 2B

There are several other viroporins of which only very limited structural data is available, and no channel inhibitors have been reported. The following section will briefly describe three of such viroporins: the alphavirus 6K protein, polyomavirus agnoprotein, and the 2B protein found in picornaviruses.

7.1 The Alphavirus 6K Protein

The 6K protein is 58–61 amino acids long and has one predicted α -helical TM domain. This viroporin is found in several members of the alphavirus genus in the positive-sense RNA family *Togaviridae*. This includes, for example, equine encephalitis viruses, Chikungunya virus, Sindbis virus, Semliki Forest viruses (SFV), Ross River virus (RRV), and Barmah Forest virus (BFV). In these viruses, structural proteins are translated in the ER from a subgenomic mRNA as a single polyprotein later cleaved by proteases, in the order capsid-PE2-6K-E1 (Liljestrom & Garoff, 1991; Strauss & Strauss, 1994).

The 6K protein was first described as early as 1980 (Welch & Sefton, 1980). Its expression in *E. coli* caused an increase in membrane permeability, whereas in eukaryotic cells it promoted viral budding (Sanz, Perez, & Carrasco, 1994). Further, 6K proteins of RRV and BFV formed cation-selective ion channels in planar lipid bilayers (Melton et al., 2002). This suggested a role for viroporins in alphavirus infection, since an increased permeability of cells to monovalent cations is followed by budding of virions.

Whatever the mechanism, 6K proteins have an important role in virus assembly and budding. For example, in Sindbis virus, cysteine-less mutants dramatically reduced the release of virus particles, leading to aberrant enveloped particles containing multiple nucleocapsids (Gaedigk-Nitschko, Ding, Levy, & Schlesinger, 1990). Other results are consistent with an indirect role in the packing of the virus spike glycoproteins; mutation C39S resulted in defective budding and a revertant contained two additional mutations at the ectodomain of the virus glycoprotein (Ivanova, Lustig,

& Schlesinger, 1995). Further, deletion of 6K in a full-length cDNA clone of SFV resulted in a dramatic reduction in virus release (Liljestrom, Lusa, Huylebroeck, & Garoff, 1991), and BHK cells expressing a Δ 6K strain produced thermolabile particles, suggesting a disrupted glycoprotein packing (Loewy, Smyth, von Bonsdorff, Liljestrom, & Schlesinger, 1995). Despite these investigations, the specific role of 6K channel activity in these processes is still uncertain.

7.2 The Polyomavirus Agnoprotein

Agnoprotein is a 71-amino acids long membrane protein with one predicted α -helical TM domain required for the productive replication of the human polyomavirus JCV (John Cunningham virus). JCV causes progressive multifocal leukoencephalopathy, a fatal demyelinating disease resulting from lytic infection of oligodendrocytes. Agnoprotein is also found in other polyomaviruses, including BK virus, and simian virus 40 (Sariyer, Saribas, White, & Safak, 2011). Agnoprotein was shown to play important regulatory roles in the JCV replication cycle (Ellis, Dang, Norton, & Koralnik, 2012; Ellis, Norton, Dang, & Koralnik, 2013; Saribas et al., 2013), and possesses properties commonly associated with viroporins (Suzuki et al., 2013; Suzuki, Orba, Malcino, et al., 2010; Suzuki, Orba, Okada, et al., 2010). For example, a deletion mutant is defective in virion release and viral propagation. Also, agnoprotein localizes to the ER during early stages of infection, but is also found at the plasma membrane late in infection. Finally, agnoprotein, an integral membrane protein, forms homo-oligomers that enhance permeability of cells to hygromycin B and induce the influx of extracellular Ca^{2+} , leading to membrane dysfunction and enhancement of virus release. Only a solution NMR structure of a synthetic peptide corresponding to the TM domain (Thr-17 to Glu-55) is available, but with an uncertain oligomeric size in lipid bilayers (Coric et al., 2014). As with the 6K protein, characterization of this viroporin is still in its infancy.

7.3 The Picornavirus 2B Protein

Protein 2B is a \sim 100 residues long nonstructural protein found in enteroviruses [see Ao, Sun, & Guo, 2014 for a recent review], eg, poliovirus (Agirre, Barco, Carrasco, & Nieva, 2002). Enteroviruses belong to the single-stranded positive-sense RNA *Picornaviridae* family, and include

poliovirus, Coxsackie virus and human enterovirus 71 (EV71). The single open reading frame encodes a peptide chain divided into three regions, P1 to P3. The 2B protein is one of the products from the polypeptides in the P2 region, and has two TM domains, one more hydrophobic than the other (de Jong et al., 2003; Nieva, Agirre, Nir, & Carrasco, 2003). The C- and N-termini of the poliovirus 2B protein are exposed to the ER and Golgi lumen when expressed in cells (de Jong et al., 2003), but the 2B protein in Coxsackie virus adopts an opposite orientation (Abed et al., 2005, van Kuppeveld, Galama, Zoll, van den Hurk, & Melchers, 1996). Poliovirus 2B protein was found to increase the concentrations of free calcium in the cytosol, which has been proposed to promote viral replication and repression of the antiviral immune response of host cells (Aldabe, Irurzun, & Carrasco, 1997). However, precise structural data is not yet available for this protein or its oligomers.



8. HOST-CELL RESPONSES TRIGGERED BY VIROPORIN ACTIVITY

One common, specific effect of viroporin channel activity may be associated with the observed ability of viruses to activate the host inflammasome, which activates caspase-1 to produce proinflammatory cytokines, eg, IL-1 β . These effects have been shown in many cases to be caused by the disruption of cellular ion homeostasis by allowing ion leakage from intracellular organelles into the cytosol, through the expression of viroporins [reviewed in Guo, Jin, Zhi, Yan, & Sun, 2015]. For example, the IAV activates the NLRP3 inflammasome as a result of H⁺ or ion flux from Golgi mediated by its M2 ion channel (Ichinohe, Pang, & Iwasaki, 2010). The 2B protein from several picornaviruses, including the encephalomyocarditis virus (EMCV), poliovirus, enterovirus 71, and human rhinovirus (Triantafilou, Kar, van Kuppeveld, & Triantafilou, 2013) were shown to induce NLRP3 cytoplasmic relocalization and inflammasome activation in an intracellular Ca²⁺-mediated manner (Ito, Yanagi, & Ichinohe, 2012). The channel activity of the SARS-CoV E protein is required for the processing of IL-1 β (Nieto-Torres et al., 2014), which requires caspase-1 activation. Channel activity of the SH protein may also result directly or indirectly in activation of the NLRP3 inflammasome (Segovia et al., 2012; Triantafilou, Kar, Vakakis, Kotecha, & Triantafilou, 2013). In these cases, therefore, development of channel inhibitors should improve the pathological effects of host inflammation caused by these viruses.



9. MODULATION OF PROTEIN–PROTEIN INTERACTIONS INVOLVING VIROPORINS

Apart from the effects of their channel activity, viroporins are involved in many protein–protein interactions (PPIs) that are susceptible for therapeutic intervention, and may be increasingly important once these interactions are properly characterized [see recent reviews [Fischer, Li, Mahato, Wang, & Chen, 2014](#); [Fischer et al., 2012](#); [Nieva & Carrasco, 2015](#)].

For example, the cytoplasmic regions of AM2 and BM2 are important for proper virus assembly and interaction with M1 ([Chen, Leser, Jackson, & Lamb, 2008](#); [Imai, Kawasaki, & Odagiri, 2008](#); [Imai, Watanabe, Ninomiya, Obuchi, & Odagiri, 2004](#); [McCown & Pekosz, 2006](#)), which can be proposed as an attractive drug target.

Also, the HCV p7 interacts with other viral structural and nonstructural proteins that are important to promote virus assembly and release ([Gentzsch et al., 2013](#); [Haqshenas, Dong, Ewart, Bowden, & Gowans, 2007](#); [Pietschmann et al., 2006](#); [Sakai et al., 2003](#); [Yi, Ma, Yates, & Lemon, 2007](#)), and functions related to capsid assembly and localization of several viral proteins are not affected by either channel-inactivating mutations or treatment with Rim ([Tedbury et al., 2011](#); [Wozniak et al., 2010](#)), eg, the transfer of HCV core protein from lipid droplets to the ER depends on the interaction between p7 and NS2 ([Boson, Granio, Bartenschlager, & Cosset, 2011](#)). Therefore, the disruption of such interactions would require the action of PPI modulators rather than channel inhibitors.

The interaction partners of E and SH proteins have been reviewed recently ([Torres, Surya, Li, & Liu, 2015](#)). In the case of CoV E proteins, the interaction with M protein has long been reported to contribute to M localization and virion formation ([Corse & Machamer, 2003](#); [Hogue & Machamer, 2008](#); [Ilk, Egelseer, & Sleytr, 2011](#); [Lim & Liu, 2001](#); [Siu et al., 2008](#); [Vennema et al., 1996](#)). Recent reports have highlighted interactions with Protein Associated with Lin Seven 1 (PALS1) ([Teoh et al., 2010](#)) and syntenin through PDZ domains of E proteins, which are consistent with alterations of lung epithelia integrity and inflammation observed during CoV infection. Disruption of these pathways may have clear therapeutic implications, since in SARS-CoV-infected patients it is an exacerbated inflammatory response that leads to epithelial and endothelial damage, edema, and acute respiratory distress syndrome ([Smits et al., 2010](#)). Noticeably, several other viruses, eg, human papillomavirus and

influenza A, have been found to enhance pathogenesis through proteins containing PDZ binding motifs (reviewed in [Javier & Rice, 2011](#)), which suggests that this is a particular case of a widely used viral strategy.

Vpu is known for its ability to degrade the HIV-receptor CD4 ([Bour & Strebel, 2003](#)), which requires interaction between Vpu and CD4 cytoplasmic domains ([McNatt, Zang, & Bieniasz, 2013](#); [Singh et al., 2012](#)) and probably TM domains ([Do et al., 2013](#); [Magadán & Bonifacino, 2012](#)) of the two proteins. This may not even require formation of oligomers, and even less channel activity. Vpu also affects virus release via interaction with interferon-induced protein tetherin (BST-2) ([Miyagi, Andrew, Kao, & Strebe, 2009](#); [Neil, Zang, & Bieniasz, 2008](#); [Skasko et al., 2012](#); [Van Damme et al., 2008](#)), an interferon-induced host protein that inhibits the release of many enveloped viruses by tethering virions to the cell surface [reviewed in [Strebel, 2014](#)].

In another case, the interaction between RSV SH and G proteins has been reported previously in infected cells ([Low, Tan, Ng, Tan, & Sugrue, 2008](#); [Rixon, Brown, Murray, & Sugrue, 2005](#)), although the significance is not clear yet. The B-cell receptor-associated protein 31 (BAP31) has been recently reported to interact with the SH protein ([Li et al., 2015](#)). Although this interaction has not been reported in infected cells, it could prevent the cleavage of BAP31 and the formation of proapoptotic fragment p20, thus delaying apoptosis of infected cells. Interaction with BAP31 was also observed in the E5 viroporin from the high-risk human papillomavirus HPV-16 and HPV-31, where it is similarly thought to regulate apoptosis ([Regan & Laimins, 2008](#)).

JCV agnoprotein has been shown to interact with a number of cellular and viral proteins ([Gerits & Moens, 2012](#); [Gerits et al., 2015](#)). However, despite all these intense research, the exact regulatory function of agnoprotein in viral replication is not fully understood. JCV agnoprotein specifically interacts with the adaptor protein complex 3 through its delta subunit. This interaction interrupts adaptor protein complex 3-mediated vesicular trafficking. The protein is then not targeted to the lysosomal degradation pathway, while allowing transport of agnoprotein to the plasma membrane. The findings suggest that the viroporins of other viruses may also be highly regulated by specific interactions with host cell proteins ([Suzuki et al., 2013](#)).

These pathologically relevant interactions between viroporins and their associated viral and host proteins present an attractive therapeutic target for disruption by small molecules, as well as peptides stabilized by internal

covalent linkage (stapled peptides) (Walensky & Bird, 2014). In principle, both the transmembrane and extramembrane domains of viroporins can be potential templates for peptide-based modulators. For example, one could design a stapled peptide mimicking the α -helical cytoplasmic domain of the SH protein to disrupt the interactions between SH and a variant of the death effector domain (vDED) of BAP31, which would lead to enhanced apoptosis and cell death of infected cells. One could also mimic the transmembrane domain to disrupt monomer–monomer interactions, as shown recently for *Halobacterium salinarum* where efflux by a small multidrug resistance protein was inhibited with peptides targeting its TM domain (Bellmann–Sickert, Stone, Poulsen, & Deber, 2014). In the case of viroporins, the disruption of the oligomeric status would abolish channel activity, a classical strategy revisited with a different approach. However, more detailed mechanistic information should be available before these conceptual strategies can be widely used.

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