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# Journal of General Virology

## Viroporins: structure, function and potential as antiviral targets

--Manuscript Draft--

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<b>Abstract:</b>	<p>The channel forming activity of a family of small, hydrophobic integral membrane proteins termed "viroporins" is essential to the life cycles of an increasingly diverse range of RNA and DNA viruses, generating significant interest in targeting these proteins for antiviral development. Viroporins vary greatly in terms of their atomic structure and can perform multiple functions during the virus life-cycle, including those distinct to their role as oligomeric membrane channels. Recent progress has seen an explosion in both the identification and understanding of many such proteins encoded by highly significant pathogens, yet the prototypic M2 proton channel of influenza A virus remains the only example of a viroporin with provenance as an antiviral drug target. This review attempts to summarise our current understanding of the channel forming functions for key members of this growing family, including recent progress in structural studies and drug discovery research, as well as novel insights into the life cycles of many viruses revealed by a requirement for viroporin activity. Ultimately, given the successes of drugs targeting ion channels in other areas of medicine, unlocking the therapeutic potential of viroporins represents a valuable goal for many of the most significant viral challenges to human and animal health.</p>



## 20 **Summary**

21 The channel forming activity of a family of small, hydrophobic integral membrane proteins termed “viroporins”  
22 is essential to the life cycles of an increasingly diverse range of RNA and DNA viruses, generating significant  
23 interest in targeting these proteins for antiviral development. Viroporins vary greatly in terms of their atomic  
24 structure and can perform multiple functions during the virus life-cycle, including those distinct to their role as  
25 oligomeric membrane channels. Recent progress has seen an explosion in both the identification and  
26 understanding of many such proteins encoded by highly significant pathogens, yet the prototypic M2 proton  
27 channel of influenza A virus remains the only example of a viroporin with provenance as an antiviral drug  
28 target. This review attempts to summarise our current understanding of the channel forming functions for key  
29 members of this growing family, including recent progress in structural studies and drug discovery research, as  
30 well as novel insights into the life cycles of many viruses revealed by a requirement for viroporin activity.  
31 Ultimately, given the successes of drugs targeting ion channels in other areas of medicine, unlocking the  
32 therapeutic potential of viroporins represents a valuable goal for many of the most significant viral challenges  
33 to human and animal health.

34

35 (200 words)

36

## 37 Introduction

38 Amantadine was one of the first antiviral agents to be licensed for the treatment of influenza A virus (IAV) in  
39 the 1960s (1969; Couch, 1969; Davies *et al.*, 1964; Dawkins *et al.*, 1968; Sabin, 1967; Togo *et al.*, 1968;  
40 Wingfield *et al.*, 1969), yet it wasn't until the mid-1980s when the target for its mode of action was discovered  
41 to be the M2 protein (Hay *et al.*, 1985). Even then, it was several more years until the channel forming activity  
42 of M2 was demonstrated (Pinto *et al.*, 1992) and the mechanisms underpinning how its proton channel activity  
43 related to the requirement for M2 function at early, and in some strains, late stages of the virus life cycle  
44 became apparent. The identification of M2 as a virus-coded proton channel explained observations made many  
45 years previously whereby virus infection increased cell membrane permeability to both ionic flux as well as a  
46 variety of small molecules (Carrasco, 1978).

47 The 1990s saw rapid expansion of the viroporin family to include proteins encoded by many significant human  
48 pathogens, including human immunodeficiency virus type 1 (HIV-1) (Ewart *et al.*, 1996), picornaviruses (Aldabe  
49 *et al.*, 1996; Barco & Carrasco, 1995; Doedens & Kirkegaard, 1995; Lama & Carrasco, 1992; van Kuppeveld *et*  
50 *al.*, 1997), alphaviruses (Melton *et al.*, 2002; Sanz *et al.*, 1994) and paramyxoviruses (Perez *et al.*, 1997). More  
51 recently, viroporins have been identified in numerous other RNA viruses and this family has expanded to  
52 include DNA virus proteins (Suzuki *et al.*, 2010; Wetherill *et al.*, 2012) (Table 1). Whilst several viroporins  
53 functionally resemble M2 in mediating virus entry/exit, many do so via distinct mechanisms and, as the family  
54 grows, new and diverse viroporin functions continue to be identified. However, one unifying characteristic for  
55 viroporins is that their function is almost universally essential to the virus life cycle, making them ideal drug  
56 targets.

57

58 The majority of viroporins are small (~100 amino acids or less) and comprise one, two or three potential *trans*-  
59 membrane domains (TMD), often based on computer predictions. This requires that they oligomerise to form

60 an intact pore across the membrane, a process mediated in the main by hydrophobic interactions between  
61 TMDs. Examples ranging from tetrameric (e.g. IAV M2, (Sakaguchi *et al.*, 1997)) up to heptameric (e.g. hepatitis  
62 C virus (HCV) p7, (Clarke *et al.*, 2006)) assemblages have been reported, generating membrane bundles  
63 predicted to contain up to eighteen alpha helical domains (e.g. hexameric human papillomavirus type 16 (HPV-  
64 16) E5 protein, containing three predicted TMDs, (Wetherill *et al.*, 2012)). Combined with its early  
65 identification, it is therefore perhaps unsurprising that the majority of high resolution structural information  
66 relates to the most simple of viroporin assemblages, namely peptides representing M2 single-TMD tetramers  
67 (reviewed in (Cross *et al.*, 2012)). However, recent progress has extended to the structural characterisation of  
68 hexameric two-TMD HCV p7 channels (OuYang *et al.*, 2013). The number and orientation of TMDs has been  
69 proposed as a means of classifying viroporins, where class I/II refers to the number of TMDs, and a/b  
70 subclasses nominate proteins with either luminal or cytosolic N-termini respectively (Nieva *et al.*, 2012). Whilst  
71 useful in many respects, viroporins predicted to possess three TMDs need to be included and this system does  
72 not account for the fact that structurally related viroporins rarely perform the same function within the  
73 infected cell. Furthermore, examples of 2-TMD viroporins have been shown to flip their C-terminal domains  
74 across the membrane when expressed under certain conditions (Isherwood & Patel, 2005). Nevertheless, in the  
75 absence of sufficient data in many cases to allow functional classification of viroporins, this currently  
76 represents the best means of cataloguing this diverse group of proteins.

77 In addition to their diverse structures and functions, the primitive nature of these virus-coded channel proteins  
78 leads many of them to exhibit a channel-pore dualism, i.e. lacking the highly regulated gating behaviour of  
79 many cellular ion channels. Thus, conflicting data from separate investigations often makes it difficult to assign  
80 the ion specificity, and by inference the biological function, of many viroporins. Furthermore, functional  
81 redundancy common to small RNA and DNA virus proteins means that many viroporins perform additional  
82 roles distinct from their channel forming activity, which may be equally important during the virus life cycle.  
83 Consequently, mutagenesis studies are often confounded by ambiguity concerning which biological functions

84 are disrupted, particularly where viroporins are produced in the context of viral polyproteins. Combined with a  
85 limited chemical toolbox of specific viroporin small molecule inhibitors (Table 2) and examples of strain-specific  
86 functional differences, the challenges associated with the study of viroporins are manifold. This review  
87 attempts to summarise the wide-ranging and often contradictory nature of the viroporin literature, with the  
88 overarching aim of highlighting channel-specific viroporin functions and their current and future potential as  
89 targets for antiviral therapy.

90

## 91 **General Viroporin characteristics**

### 92 **“Simplistic” ion channels exhibiting channel-pore dualism**

93 Viroporins rarely behave as classical voltage- or ligand-gated channels and lack the highly exclusive ion  
94 specificity displayed by cellular proteins. This is likely due to their inherent simplicity and the limited coding  
95 capacity of viruses, but has also led to scepticism concerning whether viroporins form true channels or merely  
96 non-specific pores across membranes. Often, weak ion selectivity and/or indeterminate gating behaviour are  
97 evident *in vitro* or model cell systems, and ionic preferences are difficult to determine using standard  
98 electrophysiological techniques. Nevertheless, most viroporins do display at least a degree of selectivity, such  
99 as the IAV M2 proton channel where numerous structural and biophysical investigations have defined its gating  
100 mechanism based on the ionisation of a conserved His37 residue (Wang *et al.*, 1995). However, M2 channels  
101 will also conduct potassium ions *in vitro* (Duff & Ashley, 1992) and render liposomes (Atkins *et al.*, 2014) and  
102 bacterial cells (Guinea & Carrasco, 1994) permeable to fluorescent dyes and antibiotics respectively. Other, less  
103 well characterised viroporins often reliably display preferences for e.g. cations over anions (or *vice versa*) in  
104 artificial bilayers (e.g. HIV-1 Vpu, HCV p7), although defining the functionally relevant ionic species usually  
105 requires additional cell-based corroboration.

106 As such, viroporins can generally be thought of as membrane ionophores possessing selectivity filters with a  
107 spectrum of both efficiency and selectivity, which allow the passage of ions/solutes through their lumen along  
108 pre-existing electrochemical gradients until equilibrium is reached. At one end of the spectrum, more “channel-  
109 like” viroporins such as M2 display discrete single channel events in artificial bilayers, reminiscent of cellular ion  
110 channels (Duff & Ashley, 1992). Further along the spectrum towards a pore, HCV p7 has been shown to adopt  
111 both single channel and “burst-activity” behaviour in bilayers, with single channel activity also comprising more  
112 than one conductance state (Chew *et al.*, 2009; Clarke *et al.*, 2006; Griffin *et al.*, 2003; Pavlovic *et al.*, 2003;  
113 Premkumar *et al.*, 2004; Whitfield *et al.*, 2011). This may reflect p7 behaviour whilst conducting a non-  
114 preferred ionic substrate, although p7 activity also differentially modulated by several factors, including virus  
115 genotype (Atkins *et al.*, 2014), the formation of different oligomers (Clarke *et al.*, 2006; Luik *et al.*, 2009) and  
116 membrane composition (Whitfield *et al.*, 2011), all of which may influence the meta-stable nature of channel  
117 complexes (Chandler *et al.*, 2012).

118 However, the simplicity of viroporins and their channel-pore dualism can be exploited through the use of  
119 indirect channel formation assays to expedite drug discovery research. This was exemplified by the use of  
120 liposome dye release assays to conduct a high throughput screen of potential HCV p7 small molecule inhibitors  
121 (Gervais *et al.*, 2011). Indeed, many viroporins with variable structures and functions have been shown to  
122 conduct diverse small molecules in addition to ionic species. Such substrates include antibiotics such as  
123 hygromycin B (hygB), fluorescent dyes such as carboxyfluorescein, or other small molecules including 8-  
124 aminonaphthalene-1,3,6 trisulfonic acid (ANTS)/p-xylene-bis-pyridinium bromide (DPX). Conductance of such  
125 molecules may, at first glance, argue against selective channel properties, and is likely indicative of channel-  
126 pore dualism and the plasticity inherent to viroporin channel structures. However, indirect substrates often  
127 possess relatively small Stokes’ radii (e.g. 0.4-0.6 nm for carboxyfluorescein), consistent with their being able to  
128 pass through the luminal apertures of many viroporins, based upon structural data and/or computer models.  
129 Thus, whilst clearly an indirect measure of channel activity, such indirect assays conducted upon multiple

130 channels (e.g. M2, HCV p7, CSFV p7, HPV E5, RSV SH and Picornavirus 2B) have provided important insights into  
131 their activity as well as their inhibition by small molecules, with results generally consistent with those  
132 observed in culture (Agirre *et al.*, 2002; Aldabe *et al.*, 1996; Atkins *et al.*, 2014; Carter *et al.*, 2010; Gladue *et al.*,  
133 2012; Guinea & Carrasco, 1994; Lama & Carrasco, 1992; Perez *et al.*, 1997; Sanz *et al.*, 1994; StGelais *et al.*,  
134 2007; Wetherill *et al.*, 2012; Wozniak *et al.*, 2010).

135

### 136 **Effects of viroporin channel activity on cellular homeostasis**

137 The maintenance of membrane gradients and seclusion of ionic species within defined organelle compartments  
138 is integral to cellular homeostasis. Unsurprisingly, perturbation of these systems through expression of  
139 viroporins can have profound effects on multiple processes, including trafficking, signalling and the induction of  
140 cell death by apoptosis or other mechanisms. Perhaps the most striking example is that of the rotavirus NSp4  
141 protein, which both causes the release of Ca<sup>2+</sup> ions from intracellular stores during infection to promote the  
142 formation of viroplasm and expedite virus release (Browne *et al.*, 2000; Dong *et al.*, 1997; Hyser *et al.*, 2010;  
143 Hyser *et al.*, 2013; Newton *et al.*, 1997; Tian *et al.*, 1996), but which is also secreted via a Golgi-independent,  
144 microtubule-driven mechanism and acts directly as an enterotoxin when applied to the enteric tract, inducing  
145 diarrhoeal symptoms synonymous with rotaviral disease (Einerhand, 1998; Halaihel *et al.*, 2000; Horie *et al.*,  
146 1999; Morris *et al.*, 1999; Tafazoli *et al.*, 2001). Picornavirus 2B channel activity also increases cytosolic Ca<sup>2+</sup> by  
147 releasing it from the Golgi and mitochondria, which is thought to specifically increase viral IRES-mediated  
148 translation at early times during infection and drive membrane instability to expedite the release of viral  
149 progeny at late times (Campanella *et al.*, 2004; de Jong *et al.*, 2006; de Jong *et al.*, 2003; Sandoval & Carrasco,  
150 1997; van Kuppeveld *et al.*, 1997; van Kuppeveld *et al.*, 2002). 2B expression also alters cellular trafficking,  
151 evidenced by effects on the passage of vesicular stomatitis virus G glycoprotein to the cell surface (Doedens &  
152 Kirkegaard, 1995). Expression of both IAV M2 (Ciampor *et al.*, 1992a; Ciampor *et al.*, 1995; Ciampor *et al.*,

153 1992b; Sakaguchi *et al.*, 1996; Takeuchi & Lamb, 1994; Takeuchi *et al.*, 1994) and HCV p7 (Griffin *et al.*, 2004;  
154 Wozniak *et al.*, 2010) has been shown to induce a monensin-like de-acidification of the *trans*-Golgi/endosomal  
155 system, which serves to protect acid-labile proteins/particles during egress. This effect is highly likely to  
156 dysregulate cellular trafficking and the resultant surface expression of various proteins. Expression of M2 and  
157 p7 in isolation has also been shown to induce apoptosis via distinct mechanisms, although the relevance of this  
158 in the context of full infectious virus culture is currently unclear (Aweya *et al.*, 2013).

159 Disruption of cellular ionic gradients through viroporin activity also appears to comprise a novel pathogen  
160 recognition pathway. Several examples of viroporins have been shown to activate the inflammasome via Nodd-  
161 like receptor NLRP3, leading to cytokine production including IL-1 $\beta$  and IL-18. Viroporins showing such activity  
162 include IAV M2 (Ichinohe *et al.*), respiratory syncytial virus SH (Triantafilou *et al.*, 2013), encephalomyocarditis  
163 virus (EMCV) 2B (Ito *et al.*, 2012) and HCV p7 (Shrivastava *et al.*, 2013). Inflammasome activation occurs  
164 primarily following disruption of intracellular K<sup>+</sup> gradients, presumably as an indirect effect of viroporin activity.  
165 However, these effects have primarily been documented within immune cells, which aren't generally infected  
166 by those viruses identified above. Nevertheless, given the number of viruses now recognised to encode  
167 viroporins, it follows that the immune response would evolve to counter such a common viral replication  
168 strategy.

169 Lastly, as discussed above, expression of a variety of viroporins has been shown to induce generalised cellular  
170 permeability to a variety of small molecules, most notably hygB, to which cells are otherwise impermeant  
171 (Gonzalez & Carrasco, 1998; Guinea & Carrasco, 1994; Lama & Carrasco, 1992; Perez *et al.*, 1997; Sanz *et al.*,  
172 1994). Whether or not such permeability has functional relevance to the virus life cycle, again this allows  
173 indirect assessment of viroporin function through hygB effects on translation. Indeed, this phenomenon was  
174 the first indication of viroporin-like function discovered in the 1970s, and was initially targeted as a means of  
175 utilising antibiotics to kill virus-infected cells (Carrasco, 1978).

176

## 177 **Viroporins encoded by RNA viruses**

178 Viroporins were first identified in RNA viruses following the description of channel activity for IAV M2. There  
179 followed a rapid expansion that now sees viroporins identified in multiple virus families, including the  
180 *Flaviviridae*, *Picornaviridae*, *Togaviridae*, *Coronaviridae*, *Paramyxoviridae*, *Orthomyxoviridae*, *Reoviridae* and  
181 *Retroviridae*. M2 remains the best characterised viroporin, but HIV-1 Vpu, HCV p7 and Picornavirus 2B proteins  
182 retain a substantial knowledge base, plus new viroporins are continuously identified. Here, we discuss key  
183 examples of viroporins in detail, as well as selected proteins from other families.

184

### 185 **Influenza A virus M2**

#### 186 *The function of M2 channel activity during the IAV life cycle*

187 Amantadine was licensed for the treatment of IAV in the 60s (Baker *et al.*, 1969; Davies *et al.*, 1964; Sabin,  
188 1967; Togo *et al.*, 1968; Wingfield *et al.*, 1969), yet its target and mode of action remained unknown until the  
189 mid-1980s; selection of resistance to amantadine-mediated inhibition of virus entry identified mutations  
190 clustering within the M2 open reading frame, located on segment seven of the IAV genome (Hay *et al.*, 1985).  
191 In addition, some IAV strains with amantadine sensitivity at a late stage of their life cycle were shown to be  
192 influenced by the origin of the haemagglutinin (HA) envelope glycoprotein (Hay *et al.*, 1985). Thus, amantadine  
193 was initially proposed to disrupt a putative interaction between these two viral proteins. However, M2 was  
194 subsequently shown to form disulphide-linked tetramers (Holsinger & Lamb, 1991; Sugrue & Hay, 1991) and to  
195 raise Golgi/endosomal pH (Ciampor *et al.*, 1992a; Ciampor *et al.*, 1992b; Takeuchi & Lamb, 1994; Takeuchi *et*  
196 *al.*, 1994), providing the first clues to its role as an ion channel. Seminal studies in *Xenopus laevis* oocytes then  
197 confirmed channel activity, where an amantadine-sensitive current was induced in cells in response to reduced  
198 external pH (Pinto *et al.*, 1992). Channel activity was also recapitulated *in vitro* using M2 peptides

199 corresponding to the minimal predicted *trans*-membrane (TM) region of the protein (amino acids 22-46) (Duff  
200 & Ashley, 1992).

201 Several studies then confirmed that M2 displayed selectivity for protons, with activity activated by external  
202 acidic pH and dependent on a highly conserved His37 residue (Shimbo *et al.*, 1996; Wang *et al.*, 1994; 1995;  
203 Wang *et al.*, 1993). The major role of M2 during entry is ubiquitous amongst IAV strains, whereby acidification  
204 of the virion interior destabilises interactions between the ribonucleoproteins and the matrix (M1) protein,  
205 promoting efficient uncoating (Wharton *et al.*, 1994). Strains with late-stage amantadine sensitivity underwent  
206 intracellular cleavage of the HA0 precursor by virtue of a multi-basic furin cleavage site, generating acid-  
207 sensitive mature glycoproteins; M2 channels exerted a monensin-like activity on the TGN/endosomes in such  
208 strains, thereby preserving HA in a functional state as it trafficked to the cell surface (Ciampor *et al.*, 1992a;  
209 Ciampor *et al.*, 1992b; Takeuchi & Lamb, 1994; Takeuchi *et al.*, 1994).

210

#### 211 *Structure and Gating of M2 proton channels*

212 M2 is a 97 amino acid protein with a single TMD which forms disulphide-linked tetramers in membranes  
213 (Holsinger & Lamb, 1991; Sugrue & Hay, 1991). The N-terminal 25 residues are located on the surface of the  
214 plasma/virion membrane and are highly conserved; considerable efforts have been focused on this region as a  
215 pan-influenza vaccine strategy (Neiryneck *et al.*, 1999; Shim *et al.*, 2011). The TMD (aa 25-46) is followed by an  
216 amphipathic helix (aa 47-62) and the remaining cytosolic domain. Channel activity can be recapitulated by a  
217 minimal “TM” domain including the TMD (aa 22-46), although a longer “conductance domain” (CD), including  
218 the amphipathic helices (aa 18-60 or 22-62, depending on the study) displays enhanced channel properties in  
219 oocytes (Ma *et al.*, 2009). Finally, the C-terminus of the protein interacts with the M1 matrix protein during the  
220 formation of the virus particle (Chen *et al.*, 2008).

221 M2 TM domains in mammalian cell membranes showed a 10-fold preference for protons over monovalent  
222 cations (Chizhnikov *et al.*, 1996). Slow conductance ( $\sim 200$  H<sup>+</sup>/sec) and a lack of alkali metal ion conductivity  
223 pointed to the presence of a selectivity filter, which was highly likely to involve protonation of ionisable  
224 residues based upon the induction of activity by reduced external pH (Lin & Schroeder, 2001; Mould *et al.*,  
225 2000). The highly conserved His37 residue within the TMD was shown by mutagenesis to govern M2 selectivity,  
226 although His37 mutants retained amantadine sensitivity (Chizhnikov *et al.*, 1996; Wang *et al.*, 1995). Another  
227 highly conserved Trp41 “gate” residue combines with His37 to form a now well-accepted functional HxxxW  
228 tetrad in all M2 proteins, supported by numerous structural and functional studies. However, the precise  
229 mechanism by which protonation induces channel opening remains a matter of debate. His37 protonation  
230 stabilises M2 tetramers and also occurs at much higher pH compared with His in free solution (Hu *et al.*, 2006),  
231 supporting a “dimer of dimers” model for the His37 tetrad where each pair shares a single proton (Sharma *et*  
232 *al.*, 2010). This allows one His of each pair to interact with adjacent Trp41, whereupon a third protonation  
233 event induces channel opening via alteration of the helical bundle and opening the Trp41 gate (Chizhnikov *et*  
234 *al.*, 1996; Pielak & Chou, 2010). However, alternative models for M2 gating are also proposed including a  
235 “shuttle” mechanism of proton conductance, whereby exchange of protons between His37 and water residues  
236 are facilitated by imidazole ring reorientations (Hong & DeGrado, 2012; Hu *et al.*, 2010; Khurana *et al.*, 2009;  
237 Phongphanphane *et al.*, 2010). Thus, despite its apparent simplicity compared with cellular ion channels and a  
238 wealth of structural information, the fundamental properties of this viroporin paradigm remain a topic of  
239 considerable debate.

240 Whilst a structure for the complete M2 protein remains elusive, numerous atomic structures have been solved  
241 for peptide tetramers representing the TM region, and more recently the CD, in membrane-mimetic  
242 environments (Figure 1). In all cases, M2 forms a left-handed four-helix bundle with a defined lumen containing  
243 both His37 and Trp41 tetrads. M2 structures from multiple influenza A strains have been solved using X-ray  
244 crystallography, ~~solid-state~~ and solution NMR (ssNMR, sNMR). Structures encompass a range of pH

245 conditions in the presence or absence of adamantane inhibitors (amantadine, rimantadine and other  
246 derivatives). Indeed, conflicting structures of drug-bound M2 have generated considerable controversy over  
247 the nature of M2 drug inhibition over recent years (see below). Perhaps the most biologically relevant M2  
248 structure comprises CD peptides in a DOPC/DOPE bilayer at pH 7.5 (pdb: 2LOJ) (Sharma *et al.*, 2010), although  
249 no drug molecule was bound. Recent drug-bound studies include a ssNMR structure in DMPC bilayers with  
250 amantadine bound to the channel lumen (pdb: 2KQT) (Cady *et al.*, 2009; Cady *et al.*, 2010), as well as solution  
251 structures of CD peptides in detergent micelles with four rimantadine molecules bound to a peripheral,  
252 membrane-exposed binding site (pdb: 2RLF) (Schnell & Chou, 2008). Generally, solution structures show more  
253 compacted luminal domains and a varied orientation of the C-terminal basic helices compared with solid state  
254 structures. Thus, consensus over the precise conformation of the M2 channel region has not yet been  
255 achieved, despite many years of intense activity, and this may not be resolved until solutions for the complete  
256 97 amino acid protein in bilayers are available.

257

### 258 *M2 inhibition and drug resistance*

259 The use of adamantane M2 inhibitors for the treatment of influenza A virus has now effectively halted due to  
260 the majority of circulating strains possessing resistance polymorphisms. Whilst direct evidence implicating  
261 adamantane monotherapy in selecting these variants is limited, resistance certainly emerged concomitant with  
262 their use, both in humans and through unsolicited dosing of domestic chicken feed supplements in some  
263 countries. The most common resistance mutations comprise L26F, L28F, V27A, A30T, S31N and G34E, with N31  
264 being most prevalent. This polymorphism occurs in human pandemic H1N1 “swine” influenza as well as highly  
265 pathogenic avian strains such as H5N1 and H7N9, which infect humans with often lethal consequences.

266 Several adamantane-resistant variants occur within the channel lumen, consistent with the majority of  
267 structural studies that place a single adamantane moiety at this position, physically occluding the channel

268 (Cady *et al.*, 2010; Duff *et al.*, 1994; Hu *et al.*, 2007; Stouffer *et al.*, 2008; Wang *et al.*, 2001). However, in 2008 a  
269 drug-bound M2 CD solution structure identified rimantadine molecules bound at four membrane-exposed sites  
270 defined by Asp44 on the channel periphery (Schnell & Chou, 2008). Binding at this site was proposed to  
271 allosterically stabilise the closed form of the channel and correlated with the non-luminal positioning of  
272 mutations such as L26F, L28F and S31N. Consistently, S31N was shown to destabilise the M2 complex *in vitro*,  
273 reducing potential drug binding to the allosteric site (Pielak *et al.*, 2009). Multiple functional, structural and  
274 biophysical studies have followed in an attempt to resolve this controversy, with the luminal site emerging as  
275 the consensus in the majority of cases. Nevertheless, binding to the peripheral site has been modelled, and  
276 documented *in vitro* following saturation of the lipid phase with drug molecules, albeit with reduced efficiency  
277 compared with the lumen (Cady *et al.*, 2010; Du *et al.*, 2009; Rosenberg & Casarotto, 2010). Interestingly, *in*  
278 *vivo* partitioning of adamantanes into membranes is poorly characterised, yet presumably must occur in order  
279 for the drug to reach the surface of respiratory epithelia. Furthermore, many biophysical studies comprise TM,  
280 rather than CD peptides, the former lacking the majority of the predicted peripheral site. However, recent  
281 functional and structural studies lend further support to luminal adamantane binding, including those on  
282 chimeric influenza A/influenza B M2, where the luminal domain originates from the drug-sensitive AM2, and  
283 the peripheral domain from the resistant BM2 (Ohigashi *et al.*, 2009; Pielak *et al.*, 2011). Adamantanes bound  
284 to the lumen in all cases where inhibition occurred, and luminal binding has also been documented for novel  
285 adamantane derivatives shown to inhibit amantadine-resistant S31N mutant M2 channels (Wang *et al.*, 2013a;  
286 Wang *et al.*, 2013c; Williams *et al.*, 2013; Wu *et al.*, 2014).

287 The 2009 H1N1 pandemic combined with the potential for avian viruses to traverse the species barrier and  
288 cause sustainable human infection has prompted renewed interest in discovering M2 inhibitors capable of  
289 blocking amantadine-resistant strains. The majority of novel inhibitors identified to date involve either  
290 derivatisation of amantadine, or another M2-inhibitory compound “BL-1743”, which was identified from a  
291 yeast-based M2 screen (Duque *et al.*, 2011; Kurtz *et al.*, 1995; Rey-Carrizo *et al.*, 2014; Rey-Carrizo *et al.*, 2013;

292 Tu *et al.*, 1996; Wang *et al.*, 2009; Wang *et al.*, 2011a; Wang *et al.*, 2011b; Wang *et al.*, 2013a; Wang *et al.*,  
293 2013b; Wu *et al.*, 2014). Effective inhibitors of several drug-resistant variants have been identified by this  
294 approach, although far fewer hits capable of blocking N31 channels have arisen. Recent efforts have included  
295 extended structural modification of these prototypes, as well as the expansion of the aforementioned yeast  
296 screen to include more substantive compound libraries incorporating additional chemotypes (Balgi *et al.*,  
297 2013). Exciting preliminary hits support the notion that M2 could be revisited as a viable influenza target in  
298 coming years.

299

### 300 **HIV-1 Vpu**

#### 301 *The uncertain role of Vpu channel activity during the HIV-1 life cycle*

302 HIV-1 and related simian viruses (chimpanzee lineage) encode the Vpu accessory protein (Cohen *et al.*, 1988;  
303 Strebel *et al.*, 1988). This small, multifunctional protein is not a virion component, yet plays a pivotal role in the  
304 release of infectious virions. This comprises well understood roles for Vpu in promoting the degradation of CD4  
305 (Willey *et al.*, 1992a; b) and antagonising the restriction factor, Tetherin (Neil *et al.*, 2008). However, Vpu  
306 induces channel activity in oocytes (Schubert *et al.*, 1996b), plus N-terminal Vpu peptides displayed channel  
307 activity *in vitro* with selectivity for Na<sup>+</sup> and K<sup>+</sup> compared with Cl<sup>-</sup> (Ewart *et al.*, 1996). Furthermore, a bacterial  
308 cross-feeding assay linking nutritional requirements to ionic gradients supported a preference for Na<sup>+</sup> (Ewart *et*  
309 *al.*, 1996), although oocyte experiments also showed partial permeability to divalent cations (Schubert *et al.*,  
310 1996b). Vpu peptides displayed sensitivity to amiloride derivatives, but not amiloride itself or amantadine, and  
311 these same compounds inhibited the release of HIV-1 virus-like particles from HeLa cells, implying a role for  
312 Vpu channel activity during egress (Ewart *et al.*, 2002). In addition to its ability to conduct ions, inducible Vpu  
313 expression has been attributed to increasing membrane permeability to a variety of molecules, including

314 [nucleotides and ONPG in prokaryotic cells and hygromycin B and neurobotin in mammalian cells](#) (Gonzalez &  
315 Carrasco, 1998) [\(ref Gonzalez\)](#).

316 Studies showing that membrane depolarisation enhances HIV-1 particle release provided a potential  
317 mechanism by which Vpu channel activity might act during the HIV-1 life-cycle (Hsu *et al.*, 2010). Scrambling  
318 the Vpu TMD also reduces pathogenicity *in vivo* (Hout *et al.*, 2005) and in culture (Schubert *et al.*, 1996a), plus  
319 introduction of a His residue into the Vpu TMD (A18H) generated an adamantane-sensitive HIV-1, supporting a  
320 role for Vpu channel activity (Hout *et al.*, 2006a; Hout *et al.*, 2006b). Introduction of His at this position  
321 generates an HxxxW tetrad in the Vpu sequence, reminiscent of AM2 (Sharma *et al.*, 2011). Both the A18H  
322 variant and the wild type Vpu protein have recently been shown to behave as channels in bacterial growth-  
323 based assays, most likely effecting the conductance of potassium ions (Taube *et al.*, 2014). Alternatively, Vpu  
324 has been proposed to act by interfering with cellular channels rather than exerting its own effects (Coady *et al.*,  
325 1998). Specifically, the Vpu TMD was shown to interact with Twik-related Acid Sensitive K<sup>+</sup> (TASK) channel  
326 TMDs, causing their degradation and so preventing the flow of K<sup>+</sup> ions (Hsu *et al.*, 2004). Thus, it remains to be  
327 seen whether a defined role for Vpu channels can be elucidated and potentially targeted for antiviral therapy.

328

### 329 *Structure and activity of HIV-1 Vpu*

330 Vpu is a class 1 viroporin (i.e. single TMD) comprising 81 amino acids with a mass of ~9 kD. It is separated into a  
331 ~9 residue N-terminal ectodomain, a single TMD and a cytosolic domain containing two (or more) alpha helices  
332 (33-49 and 57-70) (Lemaitre *et al.*, 2006). Peptides corresponding to the first thirty or so residues recapitulate  
333 channel activity *in vitro* and both the TMD and the cytosolic domain interact with CD4 and tetherin,  
334 independent of channel activity (Bolduan *et al.*, 2011; Kuhl *et al.*, 2011; Skasko *et al.*, 2012). NMR structures for  
335 both the cytosolic (PDB: 1VPU, 2K7Y) and TMD (PDB: 2JPX, 2GOF, 2GOH, 1PJE) are available, which have been  
336 assembled into computational models of the full length protein (Lemaitre *et al.*, 2006); [a more recent version](#)

337 of this model is shown in [figure 2](#), courtesy of Prof Wolfgang Fischer, Tapei-. The majority of studies favour the  
338 formation of a pentameric TMD helical bundle, with a lumen lined by both ionisable (e.g. Ser23) and  
339 hydrophobic aromatic side-chains, including Trp22, which could act as a molecular gate (Cordes *et al.*, 2001;  
340 Kukol & Arkin, 1999; Lu *et al.*, 2010; Park *et al.*, 2006; Park *et al.*, 2003; Sharpe *et al.*, 2006). *In vitro*, Vpu TM  
341 peptides display relatively weak channel-like properties, adopting more of a pore-like character with Michaelis-  
342 Menten characteristics in the presence of increasing salt concentration (Mehnert *et al.*, 2008). However,  
343 preferential cation conductance and a critical role for Ser23 in the TM domain for channel activity imply that a  
344 selective, defined gating mechanism exists (Ewart *et al.*, 2002; Ewart *et al.*, 1996; Grice *et al.*, 1997; Mehnert *et*  
345 *al.*, 2007; Mehnert *et al.*, 2008; Romer *et al.*, 2004). Recent studies in yeast and bacteria support that full  
346 length Vpu preferentially conducts potassium ions, notwithstanding earlier studies showing less selective  
347 channel behaviour (Taube *et al.*, 2014).

348

#### 349 *Targeting Vpu channel activity*

350 Hexamethylene amiloride (HMA) and other amiloride derivatives block both Vpu channel activity *in vitro* as  
351 well as HIV-1 virus-like particle production in culture (Ewart *et al.*, 2002; Kim *et al.*, 2006; Lemaitre *et al.*, 2004;  
352 Romer *et al.*, 2004), although the ambiguity concerning Vpu channel function and a lack of resistance  
353 mutations makes it difficult to firmly ascribe Vpu-specific effects. Whilst no direct information concerning the  
354 inhibitory action of HMA is available, docking studies predict it to bind within the Vpu lumen adjacent to Ser23  
355 (Kim *et al.*, 2006). Rimantadine is also able to block engineered A18H Vpu proteins (Hout *et al.*, 2006a; Park &  
356 Opella, 2007), although this has little relevance in developing Vpu-targeted therapies. Various bacterial screens  
357 may provide a means to increase the repertoire of Vpu-selective channel blockers (Taube *et al.*, 2014), and  
358 have already been used to generate a viroporin-targeted small molecule, BIT225 (Khoury *et al.*, 2010), which  
359 has been advanced to human trials.

360 BIT225 is an amiloride derivative, originally selected in an HCV p7 bacterial screen (see below), that was also  
361 found to display activity against Vpu (Khoury *et al.*, 2010; Luscombe *et al.*, 2010). BIT225 is inactive against HIV-  
362 2, which lacks Vpu, and displays a cell culture EC<sub>50</sub> of ~2 μM against HIV-1, with improved efficacy against  
363 macrophage-tropic compared with T-cell tropic strains. Like HMA, the binding mode and inhibitory mechanism  
364 of this small molecule are unknown and resistant polymorphisms in Vpu have not been reported. Nevertheless,  
365 first-in-man studies show BIT225 to have a reasonable safety profile, and phase I/II trials are proceeding in  
366 South East Asia for HIV-1-, HCV- and co-infected individuals.

367

### 368 **HCV p7**

#### 369 *Channel-specific and independent roles for p7 during the HCV life cycle*

370 HCV p7 was the tenth product of the viral polyprotein to be discovered as a result of its inefficient cleavage  
371 from E2-p7 and E2-p7-NS2 precursors by signal peptidase (Lin *et al.*, 1994; Mizushima *et al.*, 1994). p7 is a  
372 highly hydrophobic, 63 amino acid protein predicted to contain two TMDs, separated by a short cytosolic loop  
373 containing two highly conserved basic residues (K/R33 and R35 in most isolates) (Carrere-Kremer *et al.*, 2002).  
374 Double membrane spanning topology was supported by cellular expression studies (Carrere-Kremer *et al.*,  
375 2002), although evidence exists that the C-terminus may also flip across membranes (Isherwood & Patel, 2005).  
376 The protein is therefore considered to be a class 2 viroporin with its termini being oriented towards the ER  
377 lumen. p7 has been shown by over-expression studies and in full length HCV to predominantly localise to ER  
378 membranes (Carrere-Kremer *et al.*, 2002; Haqshenas *et al.*, 2007; Wozniak *et al.*, 2010), including those  
379 associated with mitochondria (Griffin *et al.*, 2005). Cell surface expression has also been noted (Carrere-Kremer  
380 *et al.*, 2002) and recent studies of HA-tagged or native proteins in full length virus have observed associations  
381 with HCV core, E2 and NS5A proteins (Bentham *et al.*, 2013; Vieyres *et al.*, 2013).

382 In 2003 our laboratory showed that p7 (genotype 1b, J4 strain) oligomerised and displayed amantadine-  
383 sensitive channel activity in artificial bilayers (Griffin *et al.*, 2003). Further studies confirmed activity for another  
384 genotype (1a, H77 strain) and identified nonylated imino-sugars and HMA as further inhibitor classes (Pavlovic  
385 *et al.*, 2003; Premkumar *et al.*, 2004). p7 channels displayed both single channel and burst-like behaviour,  
386 consistent with channel-pore dualism. Interest in p7 as a potential ion channel therapeutic target was  
387 stimulated by chimpanzee studies that showed it to be essential for HCV propagation *in vivo* (Sakai *et al.*,  
388 2003).

389 The advent of HCV infectious culture based on the genotype 2a “JFH-1” (Japanese Fulminant Hepatitis)  
390 infectious isolate (Wakita *et al.*, 2005) led to the identification of an essential role for p7 during the production  
391 of infectious HCV particles (Jones *et al.*, 2007; Steinmann *et al.*, 2007a). Viable full length HCV containing IRES  
392 elements inserted between E2 and p7, or p7 and NS2 argued against a functional role for p7 precursors (Jones  
393 *et al.*, 2007). Both early and late-acting defects in virion production have been described where p7 was  
394 (partially) deleted, mutated at specific residues or treated with inhibitors (Bentham *et al.*, 2013; Foster *et al.*,  
395 2014; Foster *et al.*, 2011; Jones *et al.*, 2007; Steinmann *et al.*, 2007a; Vieyres *et al.*, 2013; Wozniak *et al.*, 2010).  
396 This is now known to result from p7 performing multiple functions within infected cells, comprising distinct  
397 protein-protein interactions as well as its channel forming activity. Whilst channel activity clearly depends upon  
398 oligomerisation, the conformation of the protein as it interacts with viral, and possibly cellular factors is  
399 unknown.

400 One well characterised ion channel-independent p7 function is its interaction with NS2, targeting the latter to  
401 defined loci within infected cells where it is thought to act as a “particle assembly scaffold” (Boson *et al.*, 2011;  
402 Jirasko *et al.*, 2008; Jirasko *et al.*, 2010; Ma *et al.*, 2011; Popescu *et al.*, 2011; Stapleford & Lindenbach, 2011;  
403 Tedbury *et al.*, 2011). p7 and NS2 in concert control sub/genotype-dependent compartmentalisation of HCV  
404 core protein between the ER and lipid droplets, with more efficient particle production resulting from ER-  
405 associated core (Boson *et al.*, 2011). Moreover, p7 was recently shown to interact with core, both envelope

406 glycoproteins and NS2 (Hagen *et al.*, 2014), with additional genetic evidence supporting an interaction with  
407 NS5A (Scheel *et al.*, 2012). Such interactions likely underpin the recently described role for p7 during capsid  
408 assembly and the envelopment of HCV particles (Gentzsch *et al.*, 2013).

409 p7 channel activity appears to influence a late-acting phase of the HCV life-cycle, distinct from that concerning  
410 protein-protein interactions; whereas p7 deletions and deleterious point mutations abrogate infectivity in all  
411 compartments (Atoom *et al.*, 2013; Bentham *et al.*, 2013; Brohm *et al.*, 2009; Jones *et al.*, 2007; Steinmann *et al.*,  
412 2007a; Wozniak *et al.*, 2010), small molecule p7 inhibitors (p7i) prevent the accumulation of secreted, but  
413 not intracellular infectivity (Foster *et al.*, 2014; Foster *et al.*, 2011). Point mutations recapitulating the p7i-  
414 induced phenotype have not been identified, yet unlike (partial) deletion mutants (Brohm *et al.*, 2009),  
415 infectivity of HCV carrying mutations to the basic loop region (to either alanine, or the less hydrophobic  
416 glutamine) can be partially restored by *trans*-complementation with influenza A M2 (AM2), or by treating cells  
417 with the vATPase inhibitor Bafilomycin A (BafA) (Bentham *et al.*, 2013; Wozniak *et al.*, 2010). As AM2 does not  
418 interact with HCV proteins, a requirement for proton channel activity exists during the latter stages of HCV  
419 particle release. Consistently, early studies found p7 and M2 to be functionally interchangeable in surrogate  
420 cellular assays for M2-mediated HA surface transport (Griffin *et al.*, 2004), and more recent work found p7 to  
421 raise vesicular pH both of extracted HEK293T microsomes and within HCV-infected Huh7 cells; p7i prevented  
422 both vesicle alkalinisation and virion secretion concomitantly, in a dose-dependent fashion (Wozniak *et al.*,  
423 2010).

424 The functional requirement for p7 proton channel activity is explained by the enhanced acid-sensitivity of  
425 intracellular HCV particles compared with the more stable secreted mature virion (Wozniak *et al.*, 2010), which  
426 may be linked to the stability of E2 (Atoom *et al.*, 2013). This “pH maturation” occurs at a late stage of particle  
427 production, either just prior to or during release, and appears to be directly influenced by p7 (Atkins *et al.*,  
428 2014). As the majority of intracellular HCV infectivity is known to reside in the pH-neutral ER (Gastaminza *et al.*,  
429 2008), p7 likely controls a secretory “bottleneck” with relatively few virions passing through acidic

430 compartments at a particular time. Hence, secreted rather than the bulk of cell-associated infectivity is  
431 sensitive to p7i (Foster *et al.*, 2014; Foster *et al.*, 2011). However, HCV cell-to-cell spread appears less sensitive  
432 to the effects of p7i (Meredith *et al.*, 2013), suggesting that this pathway may be less dependent on channel  
433 activity, albeit with genotype-variability.

434 A controversial role for p7 channel activity during virus entry has been proposed, based upon enhanced  
435 hepatocyte uptake of HCV-LP containing p7 (Saunier *et al.*, 2003), as well as inhibitory effects of p7i added  
436 during the infection process (Griffin *et al.*, 2008). However, despite immuno-gold detection of E2-p7 complexes  
437 in HCV-like particles (HCV-LP) (Isherwood & Patel, 2005), recent studies have failed to demonstrate the  
438 presence of HA-tagged p7 within infectious virions (Vieyres *et al.*, 2013). Whilst this clearly depends upon  
439 antibody detection limits, with potential interference from HCV glycoproteins, a similar outcome resulted from  
440 studies of the related *Pestivirus*, bovine viral diarrhoea virus (BVDV) (Elbers *et al.*, 1996). Furthermore, high  
441 efficiency particle-producing chimaeric HCV strains yield measurable infectivity despite carrying p7 basic loop  
442 mutations (albeit with ~1000-fold reduction in titre); mutant-derived virions possessed equivalent specific  
443 infectivity to that of wild type chimaeric HCV (Steinmann *et al.*, 2007a). However, loop mutations likely disrupt  
444 p7 channel activity indirectly rather than by the formation of inactive channel complexes, via effects upon  
445 protein processing/stability and membrane insertion (Bentham *et al.*, 2013; Perez-Berna *et al.*, 2008; StGelais  
446 *et al.*, 2009). Thus, it is possible that the low level of infectious virions produced in this scenario in fact retain  
447 intact channel complexes. In support of this notion, p7 influences the acid stability of secreted particles (Atkins  
448 *et al.*, 2014) and non-infectious intracellular particles are present within cells harbouring loop mutant JFH-1  
449 (Bentham *et al.*, 2013), although these may also retain envelopment defects (Gentzsch *et al.*, 2013). However,  
450 a conclusive answer to this question should be achievable in the near future, given recent advances in the  
451 purification of infectious HCV particles (Catanese *et al.*, 2013) and the identification of p7i resistant mutants  
452 (see below) (Foster *et al.*, 2011).

453

454 *p7 structure and gating*

455 The stoichiometry of p7 channel complexes has been reported as both hexameric and heptameric in  
456 membrane-mimetic detergents and lipid bilayers, with some studies reporting mixtures of both forms (Clarke  
457 *et al.*, 2006; Griffin *et al.*, 2003; Luik *et al.*, 2009; OuYang *et al.*, 2013; StGelais *et al.*, 2009; Whitfield *et al.*,  
458 2011). Molecular dynamics confirms that both species are theoretically viable, although both display a degree  
459 of metastability (Chandler *et al.*, 2012). The membrane environment appears to exert significant influence over  
460 p7 structure and channel activity, with potential fluctuations in both the monomeric and oligomeric form  
461 proposed to regulate its behaviour (Whitfield *et al.*, 2011). Furthermore, there seems to be genotype-  
462 dependent predominance of heptameric (e.g. genotype 1b) or hexameric (e.g. genotype 2a) channels, although  
463 these have not been directly compared in the same lipid environment. Based primarily upon computer  
464 predictions, the majority of computer-generated p7 channel models have comprised arrangements of  
465 monomeric hairpins made up of two TMDs, with the N-terminal lining the lumen (Chandler *et al.*, 2012; Clarke  
466 *et al.*, 2006; Foster *et al.*, 2011; Patargias *et al.*, 2006; StGelais *et al.*, 2009). In support of such models,  
467 genotype 1a p7 activity was susceptible to blockade using Cu<sup>2+</sup> ions, indicative that a conserved His17 (in  
468 genotype 1 and some others) present on the N-terminal TMD was solvent-exposed (Chew *et al.*, 2009).

469 Elegant transmission electron microscopy (TEM) reconstruction studies of hexameric genotype 2a p7 channel  
470 complexes in detergent micelles revealed a flower-shaped channel complex with both N/C termini membrane-  
471 exposed and oriented to the broad “petals” of the channels by immunogold labelling, consistent with a hairpin  
472 monomeric conformation (Luik *et al.*, 2009). However, the 16 Å resolution of this structure was not sufficient to  
473 discern the precise arrangement of protomers within the channel complex, making further atomic structural  
474 information highly desirable. Early solution NMR studies yielded the structure of the genotype 1b p7 carboxyl  
475 terminus (PDB: 2K8J) (Saint *et al.*, 2009), as well as an NMR-guided molecular dynamics model of the complete  
476 monomer in a hairpin conformation (Montserret *et al.*, 2010). Subsequent solid-state NMR investigations also  
477 supported a monomeric hairpin, albeit with altered helical positioning (Cook & Opella, 2010; 2011).

478 2013 saw three complete p7 solution structures reported (Figure 23): two genotype 1b monomeric structures  
479 (PDB: 3ZD0, 2MTS) (Cook *et al.*, 2013; Foster *et al.*, 2014), and a complete hexameric genotype 5a channel  
480 complex (PDB: 2M6X) (OuYang *et al.*, 2013). Whilst both monomeric structures formed hairpins, protomers  
481 within the 5a structure adopted an unusual *i*+3 “staple-like” conformation, comprising three helical domains  
482 that interacted with three adjacent neighbours. Whilst the two monomeric structures differed slightly in  
483 conformation, likely due to the pH at which they were solved (3ZD0: pH 7.0, 2MTS: pH 4.0), the stark difference  
484 in protomer arrangements within the hexameric 2M6X structure could not have been predicted from previous  
485 bioinformatic analysis. 5a protomers lacked a “basic loop” and their carboxyl-terminus was membrane-  
486 embedded. The resultant channel structure was larger than helical bundles predicted for hairpin protomers,  
487 with a luminal aperture ranging from 6.8 (Ile6) to 10.5 Å (R35), lined predominantly by residues from the first  
488 two helices. Whilst the structure fitted to the genotype 2a EM density (Luik *et al.*, 2009) with a correlation of  
489 0.94, differences were apparent within the “petals” of the 2a structure. Furthermore, the orientation of the 5a  
490 N and C termini within the density is the opposite to that revealed by immunogold labelling of 2a complexes  
491 (Luik *et al.*, 2009), and the embedded 5a carboxyl-terminus would presumably not be detectable by such  
492 methods. Nevertheless, null mutations predicted by the 5a structure (2a: His9Ala, Arg35Asp; 5a: Asn9, Arg35)  
493 reduced activity of 2a channels in two-electrode voltage clamp experiments in *Xenopus* oocytes; functionality  
494 could not be demonstrated for the modified 5a protein (OuYang *et al.*, 2013). It is currently unclear how  
495 genotype 1b monomeric hairpin structures relate to the genotype 5a channel structure, although the  
496 significant genetic distance between the two (~52%) could potentially result in structurally distinct molecules. It  
497 is also possible that “hairpin” monomers undergo conversion to the “staple-like” form upon assembly into an  
498 oligomer. These possibilities will be difficult to reconcile until further oligomeric structures become available  
499 for p7 from other HCV genotypes.

500 p7 has been shown to conduct a variety of ionic species and small molecules *in vitro* and in cells. *In vitro*,  
501 genotype 1a/b p7 displays preferential cation conductance compared with anions, and has been shown to

502 conduct Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> ions in suspended bilayers (Clarke *et al.*, 2006; Griffin *et al.*, 2003; Pavlovic *et al.*,  
503 2003; Premkumar *et al.*, 2004). Genotype 2a channels were also shown to be sensitive to K<sup>+</sup> concentration in  
504 *Xenopus* oocytes (OuYang *et al.*, 2013). p7 channels also adopt multiple conductance states and exhibit “burst  
505 activity”, with a strong influence afforded by the membrane environment, potentially via effects on the overall  
506 channel structure. p7 from a variety of genotypes has also been shown to conduct small molecules, such as the  
507 pH-sensitive fluorophore HPTS (8-Hydroxypyrene-1,3,6-Trisulfonic Acid) (Wozniak *et al.*, 2010), and  
508 carboxyfluorescein (StGelais *et al.*, 2007), indicative of channel-pore dualism; one study recently questioned  
509 the relevance of such behaviour (Gan *et al.*, 2014), yet indirect systems are widely utilised in viroporin studies,  
510 including by these same authors (Li *et al.*, 2014), and results faithfully and consistently reproduced those  
511 obtained for infectious HCV culture (Foster *et al.*, 2014; Foster *et al.*, 2011; Griffin *et al.*, 2008; Wozniak *et al.*,  
512 2010). In this regard, the ability of p7 to mediate proton conductance within infected Huh7 cells remains the  
513 only activity for which a biologically relevant function has been assigned within the HCV life cycle (Wozniak *et*  
514 *al.*, 2010), although roles for other observed conductances cannot be ruled out. Interestingly, p7 from the  
515 related *Pestivirus*, classical swine fever virus (CSFV) was recently shown to behave as an amlodipine-sensitive  
516 Ca<sup>2+</sup> channel (Gladue *et al.*, 2012; Guo *et al.*, 2013), illustrating that not all “p7” sequences necessarily behave  
517 similarly and that genetic divergence, such as that observed between some HCV genotypes, may significantly  
518 affect channel functions.

519 In accordance with its potential role as a proton channel, reduced pH has been shown to activate p7 from some  
520 HCV genotypes (1b, 2a) both *in vitro* and in cell membranes, reminiscent of M2 (StGelais *et al.*, 2007; Wozniak  
521 *et al.*, 2010). However, this was not the case for genotype 1a p7 (H77 strain), which instead adopted more  
522 pore-like behaviour, responding to electrochemical gradients in both directions (Atkins *et al.*, 2014; Li *et al.*,  
523 2012). However, patient-derived variants within the 1a p7 sequence restored an M2-like, pH-activated  
524 phenotype, suggesting that p7 channel gating varies at the quasispecies level as well as between genotypes;

525 caution must therefore be applied when proposing observations based upon one or a few sequences as  
526 general p7 characteristics.

527 Residues controlling the gating of p7 channels have been proposed by functional/mutagenic analysis in the  
528 context of hairpin-monomer models of the channel structure. These include a role for positions 17 and 21,  
529 occupied by His and Tyr/Trp in many, but certainly not all HCV isolates, as an M2-like HxxxW proton  
530 sensor/gate motif (Meshkat *et al.*, 2009). However, genotype 1a (H77) channels retain His17 and are not pH-  
531 activated (Atkins *et al.*, 2014; Chew *et al.*, 2009; Li *et al.*, 2012). Ser/Tyr21, Trp30 and Tyr/His31 have also been  
532 shown to modulate channel activity and/or infectious virion production in various studies (Brohm *et al.*, 2009;  
533 Steinmann *et al.*, 2007a; StGelais *et al.*, 2009). A Phe25ala mutation generates hyper-conductive genotype 1b  
534 and 2a channels *in vitro* (Foster *et al.*, 2011), consistent with channel models based upon the 3DZ0 1b  
535 monomer structure where it forms a hydrophobic “gate-like” constriction (Foster *et al.*, 2014). More recently,  
536 the 5a channel structure points to p7 channels acting as “funnels”, with hydrophobic constrictions at Ile6 (Val  
537 in most isolates) and Asn9 (often substituted by an ionisable His) at one end, and a ring of basic Lys35 residues  
538 at the broader neck of the channel acting as a cation selectivity filter (OuYang *et al.*, 2013).

539 Taken together, whilst a clearer picture of the structure and gating of p7 channels has recently emerged, the  
540 broad genetic diversity between HCV sub/genotypes seemingly precludes a universally applicable model, at the  
541 current time. Broadening both structural and functional analysis to multiple sub/genotypes will likely be  
542 required to obtain a firm grasp upon this enigmatic channel, encoded by perhaps the most diverse of human  
543 viruses.

544

#### 545 *Inhibition of p7 channels*

546 Sensitivity of p7 to the three classes of prototypic p7i: adamantanes, alkyl imino-sugars and HMA was first  
547 identified *in vitro*, using either recombinant protein or peptides (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003;

548 Premkumar *et al.*, 2004). Subsequent studies, including those in the then newly-available JFH-1 infectious  
549 culture system provided conflicting results, yet it later became clear that sub/genotype differences accounted  
550 for variable sensitivity profiles (Griffin *et al.*, 2008; Steinmann *et al.*, 2007b). Whilst commonly accepted for  
551 other HCV targets (e.g. 1<sup>st</sup> generation protease inhibitors), genotype dependence has commonly been cited as  
552 a reason not to pursue p7 as a viable drug target. This was fuelled by both the spectre of amantadine's failings  
553 in the treatment of influenza, combined with a lack of efficacy when prototypes such as amantadine were  
554 combined with interferon/ribavirin (IFN/Rib) in clinical studies (Deltenre *et al.*, 2004; Mangia *et al.*, 2004;  
555 Maynard *et al.*, 2006). Nevertheless, both rimantadine and the imino-sugar NN-DNJ displayed broad genotype  
556 activity (Gottwein *et al.*, 2011; Griffin *et al.*, 2008; Steinmann *et al.*, 2007b).

557 Despite the relatively poor potency of prototype p7i, they did at least point to the presence of at least one  
558 druggable site in the p7 channel complex; prolonged treatment could effectively cure HCV in culture  
559 (Steinmann *et al.*, 2007b). With atomic structures only recently available, early insight into the mode of action  
560 for these molecules arose through correlating candidate p7 resistance polymorphisms with molecular  
561 modelling of p7 channel complexes (Foster *et al.*, 2011). For nonyl imino-sugars, transfer of an F25A  
562 polymorphism from resistant genotype 3a into susceptible genotype 1b and 2a strains conferred resistance.  
563 This correlated with docking studies that predicted NN-DNJ to interact with Phe25 whilst intercalating between  
564 p7 protomers. Accordingly, its mode of action was demonstrated *in vitro* to be through the inhibition of  
565 channel oligomerisation. Encouragingly, adamantane resistance was shown to be entirely separate to that of  
566 imino-sugars, providing the tantalising prospect of drug combinations targeting p7 (Foster *et al.*, 2011).  
567 Adamantanes were predicted to bind to a peripheral, membrane exposed site on the p7 channel surface,  
568 reminiscent of M2 NMR studies (Schnell & Chou, 2008). This site contained both conserved leucine residues  
569 shown to influence amantadine sensitivity *in vitro* (StGelais *et al.*, 2009), as well as Leu20, which had previously  
570 been shown to change to Phe in genotype 1b HCV patients unresponsive to amantadine combined with IFN/Rib  
571 (Mihm *et al.*, 2006). Introducing L20F into susceptible 1b and 2a strains again conferred resistance (Foster *et*

572 *al.*, 2011). Interestingly, peripheral adamantane binding sites are supported by both the 2M6X 5a complete  
573 channel structure (OuYang *et al.*, 2013) as well as structure-guided channel models based upon the 3ZD0 1b  
574 monomer (Foster *et al.*, 2014), with both studies showing interaction data confirming an interaction with  
575 rimantadine. Furthermore, despite the clear structural diversity, position 20 and several of the conserved Leu  
576 residues are present within the peripheral site in both cases. Accordingly, for genotype 1b, an L20F mutation  
577 abrogated NMR interactions with rimantadine (Foster *et al.*, 2014), and *vice versa* for 5a, which naturally  
578 retains Phe20, and was shown to form stronger interactions with rimantadine following introduction of a Leu  
579 residue (OuYang *et al.*, 2013). Thus, p7 joins M2 as the only viroporins for which specific small molecule  
580 resistance polymorphisms have been demonstrated.

581 The third class of prototype p7i, typified by HMA (Premkumar *et al.*, 2004), have not been as extensively  
582 studied and no data is available regarding their activity against HCV in culture, potentially due to cytotoxic  
583 effects (Griffin *et al.*, 2008). However, the BIT225 amiloride derivative has been advanced into clinical trials by  
584 Biotron Ltd. As described above, BIT225 was derived from a bacterial screen vs genotype 1a p7 and has been  
585 shown to exert an antiviral effect against the *Pestivirus*, BVDV (Luscombe *et al.*, 2010). However, the mode of  
586 action for this inhibitor is unknown and activity against HCV in culture has not been published; this may be of  
587 concern given recently reported differences in *Pestivirus* p7 function (Gladue *et al.*, 2012; Guo *et al.*, 2013).  
588 Nevertheless, BIT225 appears to have a reasonable safety profile and preliminary findings in small patient  
589 studies appear encouraging, with larger studies planned (see [www.biotron.com.au](http://www.biotron.com.au)).

590 Ongoing research efforts into the development of p7i with potency suited to drug development programmes  
591 has comprised both high throughput and rational approaches. Screening based upon liposome dye release  
592 assays conducted by Boehringer Ingelheim was found to be robust, generating few false-positives and a  
593 sensible percentage hit rate, although this has not been followed up to date in the literature (Gervais *et al.*,  
594 2011). Moreover, rational compound design based upon the adamantane binding site in 3ZD0 structure-guided  
595 channel models yielded compounds with much improved potency, with nanomolar IC<sub>50</sub> values against HCV in

596 culture (Foster *et al.*, 2014). These structurally novel compounds displayed cross-genotype activity and  
597 effectively suppressed the L20F adamantane resistance polymorphism at sub-micromolar concentrations. Thus,  
598 potential for drug development targeting p7 appears feasible, yet whether this will ultimately prove relevant in  
599 the rapidly evolving landscape of HCV treatment remains to be seen (Griffin, 2014).

600

## 601 **Other RNA virus viroporins**

### 602 *Picornavirus 2B and VP4 proteins*

603 Modulation of membrane permeability is essential for two key stages of the life cycle amongst the  
604 *Picornaviridae*, namely the entry of non-enveloped particles into the host cell and the late phase of infection,  
605 where cell lysis culminates in the release of infectious virions. The *Enterovirus* genus has been most intensively  
606 studied, comprising many significant human pathogens such as poliovirus, Coxsackie viruses, enterovirus 71  
607 (EV71) and human rhinovirus. The non-structural 2B protein is considered to be the principle mediator of host  
608 cell membrane permeability during the replicative phase of the life cycle, whereas VP4 represents a burgeoning  
609 class of viroporins comprising essential components of non-enveloped virus particles.

610 Multiple *Enterovirus* proteins (e.g. 2BC, 2B, 2C) were initially shown to modulate both membrane permeability  
611 (Aldabe *et al.*, 1996; Barco & Carrasco, 1995) and membrane trafficking (Doedens & Kirkegaard, 1995), yet 2B is  
612 now commonly accepted as the principle mediator of such behaviour. 2B is a class 2 viroporin with two helical  
613 TMDs separated by a stretch of highly polar residues. 2B fused to maltose binding protein forms tetramers with  
614 a pore radius of ~6 Å (Agirre *et al.*, 2002), consistent with modelling studies that predict tetrameric pores of 5-  
615 7Å radius with a lumen lined by a stretch of three lysines followed by a serine (Patargias *et al.*, 2009). 2B  
616 multimerisation has been observed in mammalian cells (de Jong *et al.*, 2004; de Jong *et al.*, 2002; van  
617 Kuppeveld *et al.*, 2002) and the protein readily permeabilises vesicles *in vitro* (Agirre *et al.*, 2008; Sanchez-  
618 Martinez *et al.*, 2008). 2B expression gives rise to elevated cytosolic Ca<sup>2+</sup>, which alters vesicle trafficking,

619 induces apoptosis and directly lyses cells as protein levels accumulate, reminiscent of a membrane-active toxin  
620 (Campanella *et al.*, 2004; de Jong *et al.*, 2004; de Jong *et al.*, 2006; de Jong *et al.*, 2003; Sandoval & Carrasco,  
621 1997; van Kuppeveld *et al.*, 1997). Localisation to the Golgi is essential for these functions as the ER-localised  
622 *Hepatitis B* protein does not affect cytosolic  $\text{Ca}^{2+}$  levels. Interestingly, 2B proteins appear to cause  
623 inflammasome activation, adding to the growing number of viroporins associated with phenomenon (Ito *et al.*,  
624 2012). However, it appears that 2B proteins from diverse *Enteroviruses* may, much like p7, display altered  
625 channel activity, as EV71 2B mediates  $\text{Cl}^-$ , rather than  $\text{Ca}^{2+}$  conductance (Xie *et al.*, 2011). This has led to the  
626 only description of a small molecule inhibitor for 2B proteins, namely the generic chloride channel inhibitor  
627 DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid), which blocked both channel activity in *Xenopus* oocytes  
628 as well as EV71 growth *in vitro*. This serves as proof-of-principle that 2B might represent a therapeutic target.

629 The second viroporin encoded by *Enteroviruses*, VP4, is retained on the inside of the virion particle until  
630 internalisation and endosomal acidification begin the process of uncoating (Tuthill *et al.*, 2010). [Interestingly,](#)  
631 [the potential for the formation of channels at the 5-fold vertices of a variety of non-enveloped viruses was](#)  
632 [previously predicted from analysis of crystallographic studies, suggesting functional conservation](#) (Kalko *et al.*,  
633 1992). In co-operation with VP1, VP4 is thought to enable the passage of viral RNA into the cytosol, thus  
634 representing an extreme of the channel-pore dualism observed in viroporins. However, VP4 activity is not  
635 membrane-disruptive and induces discrete channel events in artificial bilayers (Danthi *et al.*, 2003). VP4  
636 channels can be reconstituted *in vitro* using recombinant protein and their activity is amenable to liposome dye  
637 release assays (Davis *et al.*, 2008). Recent studies also support the formation of discrete multimeric complexes  
638 (pentameric and hexameric) of defined pore size, with activity enhanced by myristoylation and reduced pH,  
639 consistent with the scenario within the early endosome (Panjwani *et al.*, 2014). The tantalising prospect of a  
640 small molecule inhibitor of *Enterovirus* entry targeting VP4 is therefore a realistic possibility, which could have  
641 profound impact ranging from polio eradication to treating the common cold.

642

643 *Coronavirus (CoV) E, 3a and other channel forming proteins*

644 Multiple proteins have been assigned viroporin activity in CoV, with studies comprising animal viruses as well  
645 as the severe acute respiratory syndrome CoV (SARS CoV) and other human CoV. The first proteins shown to  
646 display channel forming activity ~~was-were~~ the small envelope membrane [E proteins, from SARS CoV \(Wilson et](#)  
647 [al., 2004\)](#) ~~and murine hepatitis virus-protein, E~~ (Madan *et al.*, 2005; Wilson *et al.*, 2004). E peptides display  
648 cation activity in planar bilayers, with sensitivity to HMA; HMA also blocked the spread of mouse hepatitis virus  
649 (MHV) in culture, yet does not affect attenuated E-deleted viruses (Wilson *et al.*, 2006). SARS CoV lacking E  
650 activity is also attenuated, and shows promise as a vaccine candidate due to its reduced inflammatory stimulus;  
651 E may therefore play a key role during SARS pathology (Netland *et al.*, 2010; Regla-Nava *et al.*, 2015). E is  
652 thought to comprise a type 1 viroporin and forms pentameric bundles (Torres *et al.*, 2006), although its  
653 topology is a matter of some debate (Ruch & Machamer, 2012). Solution NMR structures of the pentameric  
654 TMD have been reported showing an interaction with HMA at both the N-terminal and C-terminal neck,  
655 although these have not been entered onto the PDB (Torres *et al.*, 2006). HMA also blocks E activity in whole  
656 293T cell patch-clamp experiments (Torres *et al.*, 2006) and high concentrations (millimolar range) of  
657 amantadine can also inhibit activity (Torres *et al.*, 2007), although the relevance of such concentrations is  
658 questionable. Asn15Ala and Val25Phe mutations located in the TM domain abrogate channel activity and  
659 attenuate SARS CoV in mice, and both the activity and cation selectivity of E channels are modulated by  
660 membrane composition (Verdia-Baguena *et al.*, 2012).

661 CoV 3a protein forms potassium-selective channels in oocytes, with tetrameric complexes formed by  
662 recombinant protein in membranes stabilised by disulphide linkages (Lu *et al.*, 2006). 3a mediates the  
663 production of infectious viral progeny, potentially linked to cellular trafficking of the spike glycoprotein (Tan,  
664 2005), but has also been proposed to comprise a structural component of the infectious virion (Shen *et al.*,  
665 2005). 3a is pro-apoptotic in a number of cell lines, which appears directly dependent upon channel function,  
666 and may be linked to the induction of an ER stress response (Chan *et al.*, 2009; Freundt *et al.*, 2010; Law *et al.*,

667 2005; M *et al.*, 2005; Minakshi *et al.*, 2009; Padhan *et al.*, 2008). Two studies have reported small molecule  
668 inhibitors targeting 3a. Emodin, a constituent of plant extracts (including Japanese Knotweed), inhibited 3a  
669 channels in *Xenopus* oocytes with an EC<sub>50</sub> of ~20 micromolar and also reduced infectious virion production  
670 (Schwarz *et al.*, 2011). However, Emodin is known to display off-target effects against multiple kinases,  
671 including p56<sup>lck</sup>. Another report describes an inhibitory effect for kaempferol glycosides derived from Chinese  
672 medicinal herbs (Schwarz *et al.*, 2014). Finally, other CoV proteins including ORF8a (Chen *et al.*, 2011; Hsu *et*  
673 *al.*, 2015) and ORF4a (Zhang *et al.*, 2014) have also recently been demonstrated to exhibit channel forming  
674 activity.

675

#### 676 *The small hydrophobic (SH) proteins of Paramyxoviridae*

677 Three genera of the *Paramyxoviridae* encode small hydrophobic (SH) proteins, namely the *Pneumoviruses* (e.g.  
678 respiratory syncytial virus (RSV)), *Metapneumoviruses* (e.g. human metapneumovirus (HMPV)) and  
679 *Rubulaviruses* (e.g. mumps virus (MuV)). Whilst dispensable for growth of MuV or RSV in the majority of culture  
680 systems (He *et al.*, 1998; Takeuchi *et al.*, 1996), SH appears to act as a significant virulence factor; for example,  
681 SH-deleted RSV shows 10-fold and 40-fold reductions in replication in small animal and chimpanzee models,  
682 respectively (Bukreyev *et al.*, 1997; Whitehead *et al.*, 1999). SH has been proposed to antagonise TNF $\alpha$   
683 mediated apoptosis (Fuentes *et al.*, 2007; Lin *et al.*, 2003), but recent reports also point to a role during HMPV  
684 entry, where it modulates both virion membrane permeability and the activity of the viral fusion (F) protein  
685 (Masante *et al.*, 2014).

686 SH is predicted to contain a single TM domain and so comprise a class 1 viroporin of 64 or 65 amino acids, with  
687 an unmodified 7.5 kDa species and carbohydrate-modified forms observed within infected cells. SH is  
688 commonly thought to form pentameric oligomers (Collins & Mottet, 1993; Gan *et al.*, 2008; Gan *et al.*, 2012),  
689 although hexamers have also been reported (Carter *et al.*, 2010). Solution NMR structures have been reported

690 for the pentameric bundles, yet have not been added to the PDB (Gan *et al.*, 2008; Gan *et al.*, 2012). Both SH  
691 TMD peptides and full length protein form cation selective channels *in vitro* (Gan *et al.*, 2008), as well as  
692 promoting bacterial membrane permeability (Perez *et al.*, 1997) and mediating dye release from liposomes  
693 (Carter *et al.*, 2010). The effect of low pH upon channel opening appears to be context dependent, with  
694 conserved His22, His51 and Trp15 residues implicated in channel gating/opening. However, deletion of both  
695 His residues is required to generate non-functional channels and it remains unclear as to the precise effect of  
696 pH upon channel opening (Gan *et al.*, 2008; Gan *et al.*, 2012). Recently, pyronin B was identified as an inhibitor  
697 of SH activity in liposome dye release assays, suspended bilayers and RSV spread in culture (Li *et al.*, 2014).  
698 Binding of this compound was shown by NMR to occur at a peripheral, membrane-exposed region at the  
699 carboxy-terminal end of the TMD, reminiscent of those proposed for both M2 and p7. Pyronin B thus  
700 represents a start-point from which to build inhibitor series, which could have profound impact in the  
701 treatment of RSV and other *Paramyxoviridae*.

702

### 703 *Alphavirus 6K*

704 The *Alphavirus* genus of the *Togaviridae* are insect-borne arboviruses, usually transmitted by mosquitoes, and  
705 include significant human pathogens such as Chikungunya virus (CHIKV). 6K is cleaved from the structural  
706 polyprotein by signal peptidase, following its expression from a viral subgenomic RNA. 6K is an acylated 61  
707 amino acid protein (Gaedigk-Nitschko *et al.*, 1990; Gaedigk-Nitschko & Schlesinger, 1990), predicted to  
708 comprise two TMDs, although a single TMD has also been proposed (Antoine *et al.*, 2007; Melton *et al.*, 2002).  
709 6K appears to function during membrane trafficking and is also a minor virion component; 6K-deleted/mutated  
710 viruses form aberrant particles with altered thermal stability (Gaedigk-Nitschko *et al.*, 1990; Gaedigk-Nitschko  
711 & Schlesinger, 1990; 1991; Ivanova *et al.*, 1995; Lusa *et al.*, 1991; McInerney *et al.*, 2004; Sanz & Carrasco,  
712 2001; Schlesinger *et al.*, 1993; Yao *et al.*, 1996).

713 6K induces bacterial membrane permeability (Sanz *et al.*, 1994) and recombinant protein displays channel  
714 activity in suspended bilayers with preference for Na<sup>+</sup> and over Ca<sup>2+</sup>, and a 15-fold preference for Na<sup>+</sup> over Cl<sup>-</sup>  
715 (Melton *et al.*, 2002). However, experiments in oocytes could not recapitulate channel activity and found that  
716 6K instead induced endogenous Cl<sup>-</sup> (and associated K<sup>+</sup>) efflux (Antoine *et al.*, 2007). Inhibitory small molecules  
717 targeting 6K have not been described and it has been difficult to link its channel activity with a defined role in  
718 the virus life cycle. Interestingly, recent studies have identified a frame-shifted (-1 open reading frame) C-  
719 terminal extension of 6K, termed TF. TF was identified within both purified Sindbis virus (SINV) and CHIKV  
720 virions, and, although not essential, its deletion led to significant decreases in particle release in cultured  
721 mammalian and insect cells without affecting genome replication, particle infectivity, or envelope protein  
722 trafficking (Snyder *et al.*, 2013). SINV TF mutants are attenuated *in vivo*, and the protein induces bacterial  
723 membrane permeability to a similar degree as 6K. Thus, either 6K and/or TF may mediate important stages in  
724 the *Alphavirus* life cycle dependent upon channel activity, which could be exploited as targets for therapy in  
725 this group of emerging viral pathogens.

726

#### 727 *Flavivirus M protein*

728 The 75 amino acid small membrane (M) protein is cleaved from the viral envelope (E) protein by signal  
729 peptidase as a prM precursor, which is then processed in the Golgi and acidifying secretory compartments by  
730 furin-like proteases into M and the pr peptide (Junjhon *et al.*, 2008; Keelapang *et al.*, 2004; Kuhn *et al.*, 2002;  
731 Wong *et al.*, 2012; Yu *et al.*, 2008). The release of virions from the cell surface results in the loss of pr and  
732 resultant formation of a mature, infectious virion (Junjhon *et al.*, 2010; Junjhon *et al.*, 2008; Yu *et al.*, 2009; Yu  
733 *et al.*, 2008). prM is required for efficient trafficking of E to the cell surface and accelerated cleavage of prM is  
734 detrimental to virion production (Junjhon *et al.*, 2010; Junjhon *et al.*, 2008; Keelapang *et al.*, 2004).

735 M protein forms a dual membrane topology within virions and this form of the protein has been shown to lack  
736 channel activity in oocytes (Wong *et al.*, 2011). However, peptides corresponding to a proposed TMD in the  
737 carboxyl-terminus of the protein displayed cation-selective channel activity in suspended bilayers, with  
738 sensitivity to both HMA and amantadine (Premkumar *et al.*, 2005). Similar peptides also induce mitochondrial  
739 membrane permeability, although the relevance of this to natural infection is unclear (Catteau *et al.*, 2003).  
740 Single TMD topology has also been predicted for M, implying that two membrane-associated forms may exist,  
741 potentially as a result of the tight turn (three amino acids) between the two TMDs found within particles (Kuhn  
742 *et al.*, 2002; Yu *et al.*, 2008; Zhang *et al.*, 2003). Much like Vpu and 6K, investigators are yet to assign a  
743 functional role to potential M-mediated channel activity, although mutation of a highly conserved His39 in the  
744 first TMD reduced Dengue virus spread without affecting polyprotein processing or the formation of prM-E  
745 heterodimers, yet this did prevent glycoprotein secretion, which may conceivably relate to channel forming  
746 activity (Pryor *et al.*, 2004).

747

#### 748 *Rotavirus NSP4*

749 *Rotaviruses* are non-enveloped segmented dsRNA viruses from the *Reoviridae* and are the leading cause of life-  
750 threatening viral gastroenteritis among children worldwide. Elevated cytosolic calcium levels are a hallmark of  
751 rotavirus replication and underpin many facets of intestinal disease. A single viral non-structural protein, NSP4,  
752 is sufficient to recapitulate all effects on calcium homeostasis and is present as both an intracellular form and a  
753 secreted endotoxin (Browne *et al.*, 2000; Dong *et al.*, 1997; Einerhand, 1998; Halaihel *et al.*, 2000; Horie *et al.*,  
754 1999; Newton *et al.*, 1997; Tafazoli *et al.*, 2001; Tian *et al.*, 1996; Tian *et al.*, 1995). NSP4 (175 amino acids) is  
755 sub-divided into an amino-terminal helical domain, a coiled-coil region (aa 95-146) for which both tetrameric  
756 and pentameric crystal structures have been solved (Bowman *et al.*, 2000; Chacko *et al.*, 2011; Chacko *et al.*,  
757 2012a; Chacko *et al.*, 2012b; Deepa *et al.*, 2007; Sastri *et al.*, 2014), and a C terminal double-layered particle

758 receptor domain, which is essential for the assembly and egress of rotavirus capsids (O'Brien *et al.*, 2000).  
759 Viroporin activity has recently been shown to exist for the amino-terminal portion of the protein, which  
760 contains multiple predicted helical domains (Hyser *et al.*, 2010; Hyser *et al.*, 2012). Such activity is conserved  
761 across *Rotavirus* sub-types and is dependent upon a conserved region (aa 47-92) containing a penta-lysine  
762 motif and an amphipathic helix.

763 NSP4 viroporin activity enhances bacterial membrane permeability and leads to elevated cytosolic Ca<sup>2+</sup> in  
764 mammalian cells. Associated depletion of ER calcium stores results in the activation of ER calcium sensor  
765 stromal interaction molecule 1 (STIM1), and its subsequent co-localisation with plasma-membrane ORAI-1  
766 calcium channels, increasing Ca<sup>2+</sup> uptake from the extracellular milieu (Hyser *et al.*, 2013). NSP4 viroporin  
767 activity and cytosolic Ca<sup>2+</sup> elevation are essential for *Rotavirus* replication. Thus, inhibitors of NSP4 channels  
768 could act to both suppress virus replication as well as its endotoxin effects on bowel epithelia, dramatically  
769 reducing disease pathology.

770

#### 771 **DNA virus viroporins**

772 Whilst the majority of viroporins identified to date originate from RNA viruses, proteins encoded by small DNA  
773 viruses have recently been shown to exhibit viroporin-like characteristics. Such proteins encoded by some  
774 *Polyomaviruses* and members of the *Papillomaviridae* display diverse functions and may indicate the existence  
775 of other, as yet uncharacterised viroporins in other families and/or genera.

776

#### 777 *Proteins with viroporin activity encoded by Polyomaviruses*

778 Two members of the *Polyomavirus* genus have been shown to encode proteins with viroporin activity. Three  
779 prototypic Simian Virus 40 (SV40) proteins: VP2, VP3 and VP4, have been reported to contribute virion egress  
780 via channel formation. VP4 is a late-acting protein encoded by the same transcript as VP2 and VP3 by internal

781 initiation, although unlike VP2/3 it is not thought to comprise a minor component of the virus capsid. VP4 is  
782 125 amino acids in length and contains a single hydrophobic TMD. VP4 was observed to form channels with an  
783 inner diameter of ~3 nm that promote membrane destabilisation, with a preference for nuclear and plasma  
784 membranes (Raghava *et al.*, 2011). VP2/3 are also thought to form membrane-destabilising channels in ER  
785 membranes (Giorda *et al.*, 2013), with activity regulated by their interaction with the VP1 major capsid  
786 protein (Daniels *et al.*, 2006). Mutations in all three proteins that disrupt membrane association and/or channel  
787 formation severely disrupt the propagation of SV40 in culture.

788 A fourth viroporin identified in the human JC *Polyomavirus* is the agnoprotein (Suzuki *et al.*, 2010). Agnoprotein  
789 is a 71 amino acid multi-functional protein with numerous reported protein-protein interactions (Darbinyan *et*  
790 *al.*, 2004; Endo *et al.*, 2003; Johannessen *et al.*, 2008; Safak *et al.*, 2002; Suzuki *et al.*, 2005). It also retains a  
791 central hydrophobic TM domain which, along with an N-terminal region, is required for ER/plasma membrane  
792 localisation and membrane integration (Suzuki *et al.*, 2010), and forms stable oligomers within infected cells  
793 (Coric *et al.*, 2014; Sami Saribas *et al.*, 2013; Saribas *et al.*, 2011; Suzuki *et al.*, 2010). Agnoprotein expression  
794 both increases plasma membrane permeability and elevates cytosolic calcium, resulting in enhanced virion  
795 release (Suzuki *et al.*, 2010). Both the related human BK *Polyomavirus* and SV40 encode agnoproteins, yet  
796 viroporin activity has not been reported.

797

#### 798 *High risk human Papillomavirus (HPV) E5*

799 The E5 protein is the least-well characterised of the three oncoproteins encoded by high risk HPV16 (Halbert &  
800 Galloway, 1988; Leechanachai *et al.*, 1992; Leptak *et al.*, 1991; Maufort *et al.*, 2007; Pim *et al.*, 1992; Straight *et*  
801 *al.*, 1993; Valle & Banks, 1995). Unlike E6 and E7, HPV16 E5 is highly hydrophobic and is predicted to comprise  
802 three TMDs within its 83 amino acid sequence (Krawczyk *et al.*, 2010; Wetherill *et al.*, 2012). E5 induces  
803 anchorage-independent growth in culture (Leechanachai *et al.*, 1992) and tumour formation in transgenic

804 mouse models (Maufort *et al.*, 2007), with expression detectable in human malignancies (Cavuslu *et al.*, 1996;  
805 Hsieh *et al.*, 2000; Sahab *et al.*, 2012). E5 impairs endosomal maturation, thereby stabilising epidermal growth  
806 factor receptor (EGFR) signalling, and leading to increased extracellular signal-regulated kinase (ERK) mitogen-  
807 activated protein kinase (MAPK) activity (Disbrow *et al.*, 2005; Genther Williams *et al.*, 2005; Leechanachai *et*  
808 *al.*, 1992; Pedroza-Saavedra *et al.*, 2010; Pim *et al.*, 1992; Rodriguez *et al.*, 2000; Straight *et al.*, 1993;  
809 Suprynowicz *et al.*, 2010; Tomakidi *et al.*, 2000). However, understanding of the precise mechanism by which  
810 E5 mediates this is incomplete.

811 A recent study showed that both cell-expressed and recombinant E5 protein formed hexameric oligomers,  
812 forming integral membrane complexes with discernible pores (Wetherill *et al.*, 2012). E5 complexes displayed  
813 channel forming activity with defined pore-size, which was increased by reduced pH. Activity was sensitive to  
814 relatively high concentrations of rimantadine, as well as to a novel small molecule inhibitor generated via *in*  
815 *silico* modelling of E5 complexes and subsequent docking analysis. Importantly, E5-mediated stabilisation of  
816 phosphorylated ERK was prevented by channel-specific small molecules, suggesting that E5 channel activity is  
817 directly linked to its oncogenic function. Thus, E5 represents the first example of an oncogenic viroporin and  
818 illustrates the potential for diverse consequences resulting from viral manipulation of cellular ion homeostasis.

819

## 820 **Conclusions: current and future potential of viroporins as antiviral targets**

821 The identification of viroporins in an increasingly diverse and broad range of viruses, many of which represent  
822 significant human pathogens, represents an important opportunity for the development of novel therapies.  
823 Furthermore, understanding how viruses manipulate cellular ion homeostasis can provide important insight  
824 into both virus- and host-specific processes, including membrane trafficking, apoptosis and growth factor  
825 signalling as just a few examples. Thus, viroporins represent an important, yet relatively unexplored area of  
826 virology, deserving of significant research focus.

827 Ion channel targeted therapeutics have had significant impact in areas such as cardiac medicine, yet viroporins  
828 lag significantly behind as drug targets. Amantadine and rimantadine remain the only licensed antivirals  
829 targeting a viroporin, and, hailing from the 1960s, were not derived using modern drug discovery methods.  
830 Indeed, despite setting a clinical precedent, their failings as effective drugs have perhaps done more to impair,  
831 than to encourage the exploration of viroporins as targets. An extremely limited chemical toolbox of viroporin  
832 inhibitors has led to the “gold standard” of such molecules falling short of the criteria required to pursue drug  
833 discovery projects, with prototype compounds displaying promiscuous, yet only moderate activity. Studies,  
834 particularly in a clinical setting, involving such compounds are therefore highly likely to fail, and in so doing  
835 further add to the scepticism concerning viroporins as targets.

836 However, some encouraging progress has been made in recent times, particularly regarding the accumulation  
837 of atomic structural information and the development of screening assays for several viroporin targets, most  
838 notably M2 and p7, but with SH and CoV E protein not far behind. This is starting to yield improvements in our  
839 ability to e.g. target amantadine-resistant influenza, and to select compounds with cell culture potencies  
840 approaching those suited to drug discovery. However, early progress must be continued if viroporin targets are  
841 to be taken up by pharmaceutical companies and a large amount of laboratory research must be undertaken to  
842 determine more and better atomic structures, expand screening technologies and the apply meticulous  
843 medicinal chemistry. In addition, elucidation of the precise role of viroporin channel activity within virus life  
844 cycles will be necessary to both better define inhibitor effects, as well as to provide appropriate biomarkers  
845 should compounds ever be advanced to human trials. Taken together, viroporins represent an essentially  
846 untapped reservoir of antiviral targets spanning multiple virus families, although their exploitation will require  
847 cohesive, improved and combined efforts in structure-guided and screen-led drug development.

848

849

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1685 **Figure Legends**

1686 **Figure 1. Selected atomic structures for Influenza A M2 proteins.** **A.** Structures solved for M2 “TM” peptides  
 1687 from the DeGrado group with PDB identifiers. Ribbons and transparent electron density are shown, in addition  
 1688 to a single monomer as sphere space-fill. Lumen-bound inhibitors are shown in yellow: amantadine for 3C9J  
 1689 and 2KQT, M2WJ332 adamantane derivative (see table 2) for the 2LY0 structure of an amantadine-resistant  
 1690 N31 channel. **B.** Structures solved for “CD” peptides from the Cross (2LOJ) and the Chou laboratories (2RLF).  
 1691 2RLF shows four peripherally-bound rimantadine molecules (yellow).

1692 **Figure 2. Full length HIV Vpu ~~strucruta~~ structural model.** *In silico* monomeric model, building upon that  
1693 previously reported (Lemaitre *et al.*, 2006), constructed from independent NMR data of the cytoplasmic  
1694 domain (2K7Y) and molecular dynamics predictions for the transmembrane domain (unpublished, Fischer lab).  
1695 Potentially important luminal polar (Ser23) and hydrophobic gate (Trp22) residues illustrated as stick  
1696 sidechains. PDB generously provided by Prof Wolfgang Fischer, Taipei.

1697 **Figure 23. Atomic structures for HCV p7 proteins.** **A.** Full monomeric structures from the Griffin (3ZD0) and  
1698 Opella (2MTS) laboratories, solved at neutral and acidic pH, respectively. Structures are displayed as ribbons,  
1699 showing the side chains of His17, Lys33 and Arg35 for orientation. **B.** Oligomeric p7 channel complexes based  
1700 upon either “hairpin” or “staple-like” protomer conformations, represented by a 3ZD0-based molecular model  
1701 and the 2M6X solution NMR structure from the Chou laboratory. Again, His17, Lys33 and Arg35 side chains are  
1702 shown for orientation, with N and C termini oriented towards the top of each image.

1703

Class	Family	Virus	Name	AA	TM	Ion?	Role of Channel Function	
<b>ssRNA (+)</b>	<i>Picornaviridae</i>	Poliovirus	2B	97	2	Ca <sup>2+</sup>	Particle Production, cell lysis	
			VP4	68	1	-	Entry	
		Coxsackievirus B3	2B	99	2	Ca <sup>2+</sup>	Particle Production, cell lysis	
		EV71	2B	99	2	Cl <sup>-</sup>	Virus Spread	
		Human Rhinovirus	VP4	68	1	-	Entry	
	<i>Flaviviridae</i>	Hepatitis C virus	p7	63	2	H <sup>+</sup>	Particle Production, Entry?	
			BVDV	p7	63	2	?H <sup>+</sup>	Particle Production
			CSFV	p7	63	2	Ca <sup>2+</sup>	Particle Production
			Dengue Virus	M	75	2	K <sup>+</sup> /Na <sup>+</sup>	Particle Production
	<i>Togaviridae</i>	Semliki Forest Virus	6K	60	2	K <sup>+</sup> /Na <sup>+</sup>	Particle Production	
			Sindbis Virus	6K	55	1*	K <sup>+</sup> /Na <sup>+</sup>	Particle Production
			Ross River Virus	6K	62	1*	K <sup>+</sup> /Na <sup>+</sup>	Particle Production
	<i>Coronaviridae</i>	SARS CoV	E	76	1	K <sup>+</sup> /Na <sup>+</sup>	Particle Production	
			3a	27 4	3	K <sup>+</sup>	Virus Spread	
			8a	39	1	K <sup>+</sup> /Na <sup>+</sup>	-	
MHV			E	83	1	K <sup>+</sup> /Na <sup>+</sup>	Particle Production	
<b>ssRNA(-)</b>	<i>Paramyxoviridae</i>	hRSV	SH	64	1	K <sup>+</sup> /Na <sup>+</sup>	TNF antagonist, Pathogenesis	
	<i>Orthomyxoviridae</i>	Influenza A virus	M2	97	1	H <sup>+</sup>	Entry, Particle Production (some)	
			BM2	11 5	1	H <sup>+</sup>	Entry	
		Influenza B virus	NB	10 0	1	H <sup>+</sup>	-	
			CM2	11 5	1	H <sup>+</sup>	Entry	
<b>dsRNA</b>	<i>Reoviridae</i>	Rotavirus	NSP4	17 5	1/3	Ca <sup>2+</sup>	Particle Production, Endotoxin	
<b>RT (RNA)</b>	<i>Retroviridae</i>	HIV-1	Vpu	81	1	K <sup>+</sup> /Na <sup>+</sup>	Particle Production	
		HTLV-1	P13ii	87	2	?K <sup>+</sup>	Mitochondrial Permeability	
<b>dsDNA</b>	<i>Polyomaviridae</i>	SV40	VP4	12 5	1	Ca <sup>2+</sup>	Particle Production	

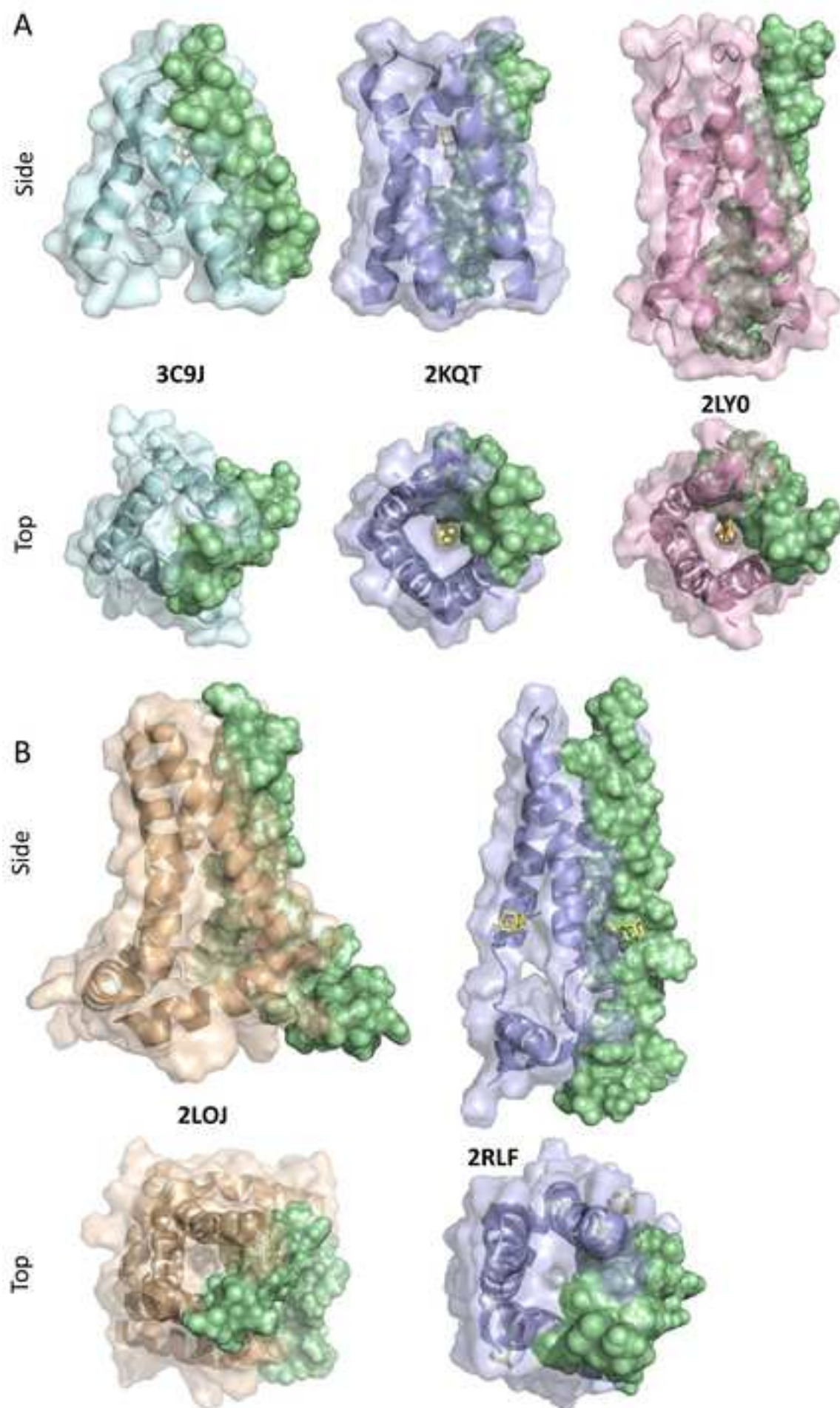
	JC	Agno	71	1	Ca <sup>2+</sup>	Particle Production
<i>Papillomaviridae</i>	HPV-16	E5	83	3	? H <sup>+</sup>	Oncogene, Signalling/Trafficking

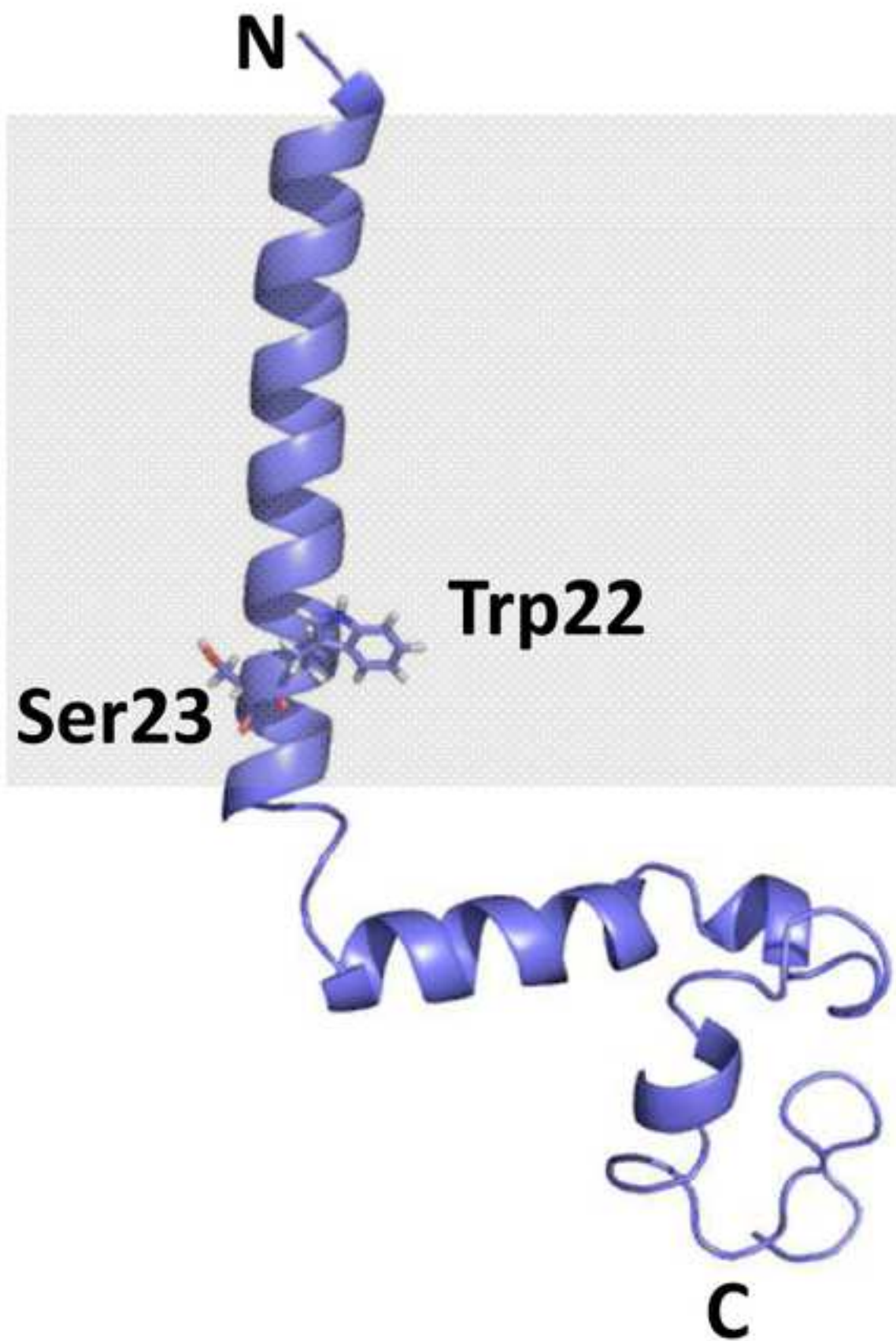
**Table 1: Summary of viroporin characteristics.** Current consensus from the literature regarding viroporin function, size (AA, amino acids) ion specificity (Ion?) and the number of *trans*-membrane domains (TM), including several proteins not discussed herein. \* computer prediction; ? Indirect assays; - unknown/uncertain. Abbreviations: AA, number of amino acids; TM, number of *trans*-membrane domains; Ion, consensus ion specificity; EV71, Enterovirus 71; BVDV, bovine viral diarrhoea virus; CSFV, classical swine fever virus; SARS CoV, severe acute respiratory distress syndrome associated coronavirus; MHV, murine hepatitis virus; hRSV, human respiratory syncytial virus; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-lymphotropic virus type 1; SV40, simian vacuolating virus 40; JC, John Cunningham polyomavirus; HPV-16, human papillomavirus type 16.

Class	Compound	Structure	Target	Resistance
Adamantane	<p>“Amantadine” (1-adamantylamine)</p> <p>Hay <i>et al.</i>, EMBO 1985; Griffin <i>et al.</i>, FEBS Lett 2003; Premkumar <i>et al.</i>, J Membr Biol. 2005</p>		Influenza M2	L26F, L28F, V27A, A30T, S31N, G34E
	<p>“Rimantadine” 1-(1-adamantyl)ethanamine</p> <p>Hay <i>et al.</i>, EMBO 1985; Griffin <i>et al.</i>, Hepatology 2008; Gottwein <i>et al.</i>, J Virol 2012</p>		Influenza A M2	L26F, L28F, V27A, A30T, S31N, G34E
	<p>“H” 5-(1-adamantyl)-2-methyl-1H-imidazole</p> <p>Foster <i>et al.</i>, Hepatology 2011</p>		HCV p7	L20F, genotype 1a (H77), 2a (JFH-1)
	<p>“Spiro[piperidine-2,2'-adamantane] 3</p> <p>Kolocouris <i>et al.</i>, Bioorg Med Chem Letts 2008</p>		Influenza A M2	S31N
	<p>“Spiroadamantane”</p> <p>Wang <i>et al.</i>, JACS 2011</p>		Influenza A M2 (V27A, L26F)	S31N
	<p>“M2WJ332” (3S,5S,7S)-N-([5-(thiophen-2-yl)-1,2-oxazol-3-yl]methyl)tricyclo[3.3.1.1~3,7~]decan-1-aminium</p> <p>Wang <i>et al.</i>, PNAS 2013</p>		Influenza A M2 (S31N)	
Spirane-amine	<p>“BL-1743” (2-[3-azaspiro (5,5)undecanol]-2-imidazoline),</p> <p>Kurtz <i>et al.</i>, Antimicrob. Agents Chemother 1995</p>		Influenza A M2	I35T
Alkyl Imino-Sugar	<p>“MN-NDNJ”: N-nonyl deoxyojirimycin</p> <p>Pavlovic <i>et al.</i>, PNAS 2003</p>		HCV p7	F25A, Genotype 3a (452)
	<p>“NN-DGJ”: N-Nonyl deoxygalactonojirimycin</p> <p>Pavlovic <i>et al.</i>, PNAS 2003</p>		HCV p7	F25A, Genotype 3a (452)
	<p>UT-231b</p>	?	HCV p7	
Amiloride	<p>“HMA”: 5-(N,N-hexamethylene)amiloride</p> <p>Premkumar <i>et al.</i>, FEBS Lett 2004; Wilson <i>et al.</i>, Virology 2006; Premkumar <i>et al.</i>, J Membr Biol. 2005; Ewart <i>et al.</i>, Eur Biophys J. 2002</p>		HCV p7	
			SARS CoV E	
			Dengue M (C-	

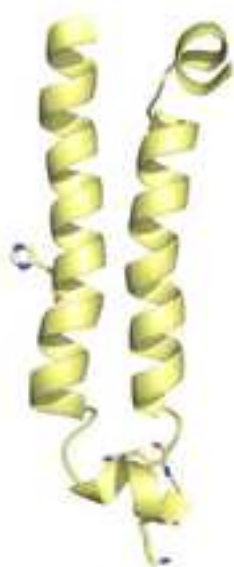
				terminus)	
				HIV-1 Vpu	
	<p>“BIT-225”: (N-[5-(1-methyl-1H-pyrazol-4-yl)-naphthalene-2-carbonyl]-guanidine</p> <p>Luscombe <i>et al.</i>, Antiviral Res. 2010; Khoury <i>et al.</i>, Antimicrob Agents Chemother 2010</p>	<p>BIT225</p>		HCV p7	
				BVDV p7	
				HIV-1 Vpu	
<b>Other</b>	<p>“CD”: 1,3dibenzyl 5(2H1,2,3,4tetraazol5yl) hexahydropyrimidine</p> <p>Foster <i>et al.</i>, Hepatology 2011</p>			HCV p7	L20F
	<p>“LDS25” N-(1-phenylethyl)-2-[4-(phenylsulfonyl)-1-piperazinyl]-4-quinazolinamine</p> <p>Foster <i>et al.</i>, Hepatology 2014</p>			HCV p7	
	<p>“Emodin”: 6-Methyl-1,3,8-trihydroxyanthraquinone</p> <p>Schwarz <i>et al.</i>, Antiviral Research 2011</p>			SARS CoV 3a	
	<p>Verapamil</p> <p>Gladue <i>et al.</i>, J Virol 2012</p>			CSFV p7	
	<p>“DIDS”: 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid</p> <p>Xie <i>et al.</i>, Cell Res 2011</p>			EV71 2B	
	<p>MV006</p> <p>Wetherill <i>et al.</i>, J Virol 2012</p>		?	HPV-16 E5	
	<p>Pyronin B</p> <p>Li <i>et al.</i>, J Virol 2014</p>			RSV SH	

**Table 2: Viroprolin inhibitor toolbox.** Summary of prototypic and derivative viroprolin inhibitors reported in the literature. Virus abbreviations as in table 1.





A



B

