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Viroporins: structure, function and potential as antiviral targets

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Abstract:	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.

Viroporins: structure, function and potential as antiviral targets

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Summary

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.

(200 words)

Introduction

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

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

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

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

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

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

General Viroporin characteristics

"Simplistic" ion channels exhibiting channel-pore dualism

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

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

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

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

Effects of viroporin channel activity on cellular homeostasis

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

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

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

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

Viroporins encoded by RNA viruses

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

Influenza A virus M2

The function of M2 channel activity during the IAV life cycle

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

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

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

Structure and Gating of M2 proton channels

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

M2 TM domains in mammalian cell membranes showed a 10-fold preference for protons over monovalent cations (Chizhmakov *et al.*, 1996). Slow conductance (~200 H^+/sec) and a lack of alkali metal ion conductivity pointed to the presence of a selectivity filter, which was highly likely to involve protonation of ionisable residues based upon the induction of activity by reduced external pH (Lin & Schroeder, 2001; Mould *et al.*, 2000). The highly conserved His37 residue within the TMD was shown by mutagenesis to govern M2 selectivity, although His37 mutants retained amantadine sensitivity (Chizhmakov *et al.*, 1996; Wang *et al.*, 1995). Another highly conserved Trp41 "gate" residue combines with His37 to form a now well-accepted functional HxxxW tetrad in all M2 proteins, supported by numerous structural and functional studies. However, the precise mechanism by which protonation induces channel opening remains a matter of debate. His37 protonation stabilises M2 tetramers and also occurs at much higher pH compared with His in free solution (Hu *et al.*, 2006), supporting a "dimer of dimers" model for the His37 tetrad where each pair shares a single proton (Sharma *et al.*, 2010). This allows one His of each pair to interact with adjacent Trp41, whereupon a third protonation event induces channel opening via alteration of the helical bundle and opening the Tryp41 gate (Chizhmakov *et al.*, 1996; Pielak & Chou, 2010). However, alternative models for M2 gating are also proposed including a "shuttle" mechanism of proton conductance, whereby exchange of protons between His37 and water residues are facilitated by imidazole ring reorientations (Hong & DeGrado, 2012; Hu *et al.*, 2010; Khurana *et al.*, 2009; Phongphanphanee *et al.*, 2010). Thus, despite its apparent simplicity compared with cellular ion channels and a wealth of structural information, the fundamental properties of this viroporin paradigm remain a topic of considerable debate.

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

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

M2 inhibition and drug resistance

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

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

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

The 2009 H1N1 pandemic combined with the potential for avian viruses to traverse the species barrier and cause sustainable human infection has prompted renewed interest in discovering M2 inhibitors capable of blocking amantadine-resistant strains. The majority of novel inhibitors identified to date involve either derivatisation of amantadine, or another M2-inhibitory compound “BL-1743”, which was identified from a 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^+ and K^+ compared with Cl^- (Ewart *et al.*, 1996). Furthermore, a bacterial
308 cross-feeding assay linking nutritional requirements to ionic gradients supported a preference for Na^+ (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

nucleotides and ONPG in prokaryotic cells and hygromycin B and neurobotin in mammalian cells (Gonzalez & Carrasco, 1998) ~~(ref Gonzalez)~~.

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

Structure and activity of HIV-1 Vpu

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 ~9 residue N-terminal ectodomain, a single TMD and a cytosolic domain containing two (or more) alpha helices (33-49 and 57-70) (Lemaitre *et al.*, 2006). Peptides corresponding to the first thirty or so residues recapitulate channel activity *in vitro* and both the TMD and the cytosolic domain interact with CD4 and tetherin, independent of channel activity (Bolduan *et al.*, 2011; Kuhl *et al.*, 2011; Skasko *et al.*, 2012). NMR structures for both the cytosolic (PDB: 1VPU, 2K7Y) and TMD (PDB: 2JPX, 2GOF, 2GOH, 1PJE) are available, which have been assembled into computational models of the full length protein (Lemaitre *et al.*, 2006); a more recent version

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

Targeting Vpu channel activity

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

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

HCV p7

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

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

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

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

p7 structure and gating

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

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

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

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

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

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

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_{50} 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^{2+}, which alters vesicle trafficking,

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

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

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

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

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

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

The small hydrophobic (SH) proteins of Paramyxoviridae

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

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

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

Alphavirus 6K

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

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

Flavivirus M protein

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

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

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

DNA virus viroporins

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

Proteins with viroporin activity encoded by Polyomaviruses

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

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

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

High risk human Papillomavirus (HPV) E5

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

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

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

Conclusions: current and future potential of viroporins as antiviral targets

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

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Figure Legends

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

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

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

Table
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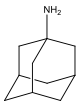
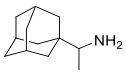
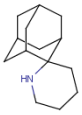
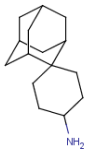
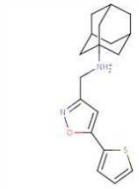
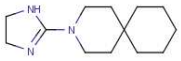
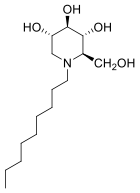
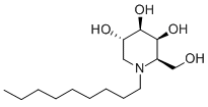
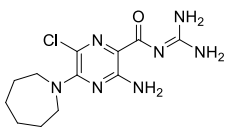
Class	Family	Virus	Name	AA	TM	Ion?	Role of Channel Function
ssRNA (+)	*Picornaviridae*	Poliovirus	2B	97	2	Ca^{2+}	Particle Production, cell lysis
			VP4	68	1	-	Entry
		Coxsackievirus B3	2B	99	2	Ca^{2+}	Particle Production, cell lysis
		EV71	2B	99	2	Cl^{-}	Virus Spread
		Human Rhinovirus	VP4	68	1	-	Entry
	Flaviviridae	Hepatitis C virus	p7	63	2	H^{+}	Particle Production, Entry?
		BVDV	p7	63	2	$?H^{+}$	Particle Production
		CSFV	p7	63	2	Ca^{2+}	Particle Production
		Dengue Virus	M	75	2	K^{+}/Na^{+}	Particle Production
	Togaviridae	Semliki Forest Virus	6K	60	2	K^{+}/Na^{+}	Particle Production
		Sindbis Virus	6K	55	1*	K^{+}/Na^{+}	Particle Production
		Ross River Virus	6K	62	1*	K^{+}/Na^{+}	Particle Production
	Coronaviridae	SARS CoV	E	76	1	K^{+}/Na^{+}	Particle Production
			3a	274	3	K^{+}	Virus Spread
			8a	39	1	K^{+}/Na^{+}	-
		MHV	E	83	1	K^{+}/Na^{+}	Particle Production
ssRNA(-)	*Paramyxoviridae*	hRSV	SH	64	1	K^{+}/Na^{+}	TNF antagonist, Pathogenesis
	Orthomyxoviridae	Influenza A virus	M2	97	1	H^{+}	Entry, Particle Production (some)
		Influenza B virus	BM2	115	1	H^{+}	Entry
			NB	100	1	H^{+}	-
		Influenza C virus	CM2	115	1	H^{+}	Entry
dsRNA	*Reoviridae*	Rotavirus	NSP4	175	1/3	Ca^{2+}	Particle Production, Endotoxin
RT (RNA)	*Retroviridae*	HIV-1	Vpu	81	1	K^{+}/Na^{+}	Particle Production
		HTLV-1	P13ii	87	2	$?K^{+}$	Mitochondrial Permeability
dsDNA	*Polyomaviridae*	SV40	VP4	125	1	Ca^{2+}	Particle Production

		JC	Agno	71	1	Ca^{2+}	Particle Production
	Papillomaviridae	HPV-16	E5	83	3	? H^{+}	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 syncitial 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	"Amantadine" (1-adamantylamine) Hay *et al.*, EMBO 1985; Griffin *et al.*, FEBS Lett 2003; Premkumar *et al.*, J Membr Biol. 2005		Influenza M2	L26F, L28F, V27A, A30T, S31N, G34E
			HCV p7	L20F, genotype 1a (H77), 2a (JFH-1)
			Dengue M (C-terminus)	
	"Rimantadine" 1-(1-adamantyl)ethanamine Hay *et al.*, EMBO 1985; Griffin *et al.*, Hepatology 2008; Gottwein *et al.*, J Virol 2012		Influenza A M2	L26F, L28F, V27A, A30T, S31N, G34E
			HCV p7	L20F, genotype 1a (H77)
	"H" 5-(1-adamantyl)-2-methyl-1H-imidazole Foster *et al.*, Hepatology 2011		HCV p7 (L20F)	
	Spiro[piperidine-2,2'-adamantane] 3 Kolocouris *et al.*, Bioorg Med Chem Letts 2008		Influenza A M2	S31N
	"Spiroadamantane" Wang *et al.*, JACS 2011		Influenza A M2 (V27A, L26F)	S31N
	"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 Wang et al., PNAS 2013		Influenza A M2 (S31N)	
Spirane-amine	"BL-1743"(2-[3-azaspiro (5,5)undecanol]-2-imidazoline), Kurtz *et al.*, Antimicrob. Agents Chemother 1995		Influenza A M2	I35T
Alkyl Imino-Sugar	"*N*N-NDNJ":N-nonyl deoxynojirimycin Pavlovic *et al.*, PNAS 2003		HCV p7	F25A, Genotype 3a (452)
	"NN-DGJ": N-Nonyl deoxygalactonojirimycin Pavlovic *et al.*, PNAS 2003		HCV p7	F25A, Genotype 3a (452)
	UT-231b	?	HCV p7	
Amiloride	"HMA": 5-(N,N-hexamethylene)amiloride Premkumar et al., FEBS Lett 2004; Wilson *et al.*, Virology 2006; Premkumar *et al.*, J Membr Biol. 2005; Ewart *et al.*, Eur Biophys J. 2002		HCV p7	
			SARS CoV E	
			Dengue M (C-	

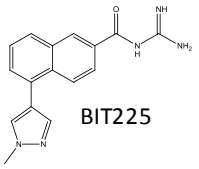
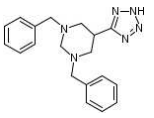
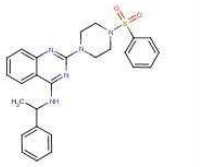
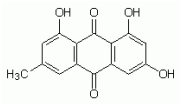
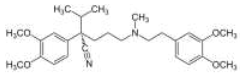
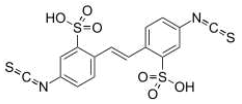
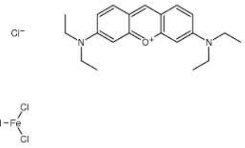
			terminus)	
			HIV-1 Vpu	
	"BIT-225": (N-[5-(1-methyl-1H-pyrazol-4-yl)-napthalene-2-carbonyl]-guanidine Luscombe *et al.*, Antiviral Res. 2010; Khoury *et al.*, Antimicrob Agents Chemother 2010	 BIT225	HCV p7	
			BVDV p7	
			HIV-1 Vpu	
Other	"CD": 1,3dibenzyl 5(2H1,2,3,4tetraazol5yl) hexahydropyrimidine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

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

Figure
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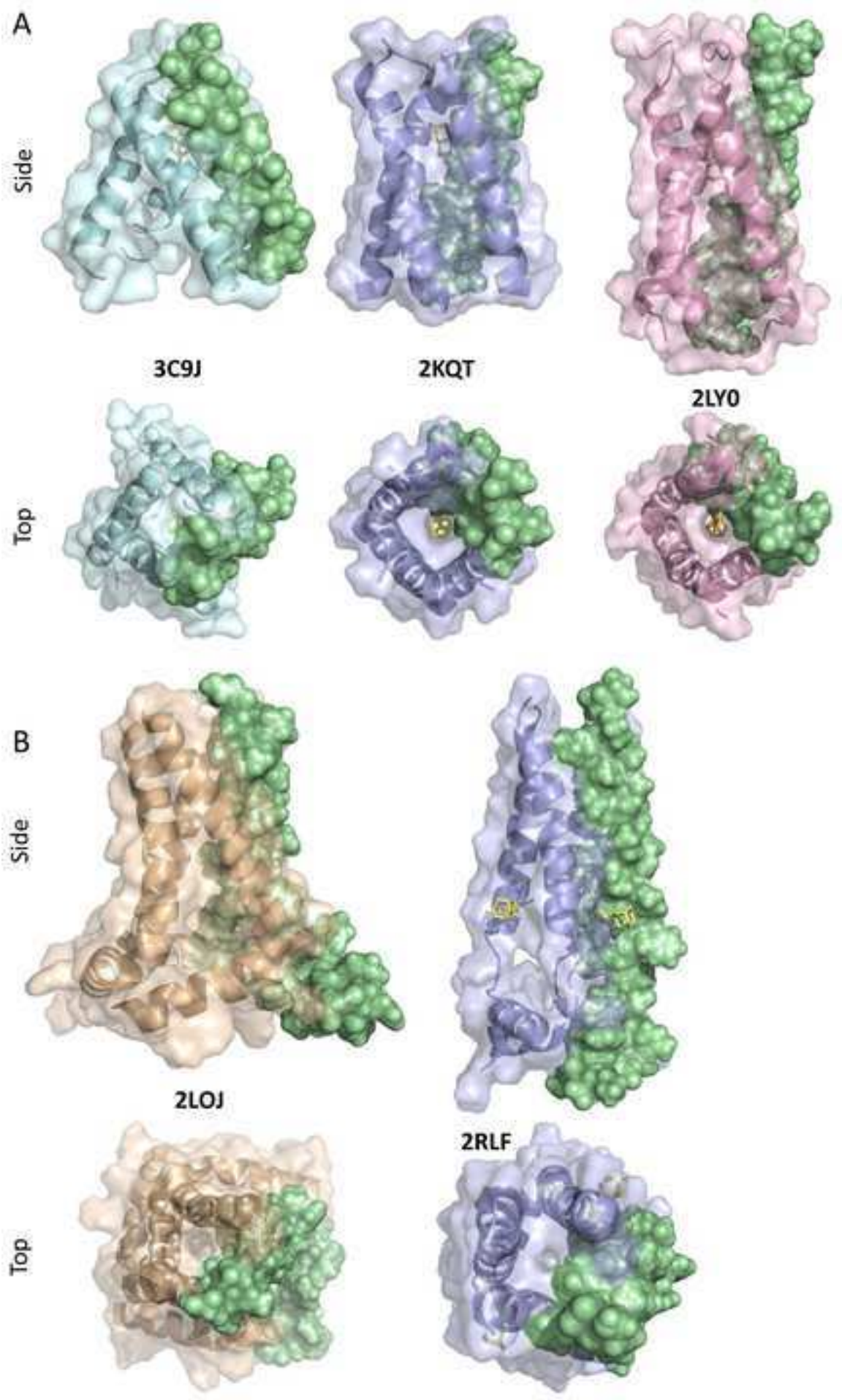

Figure
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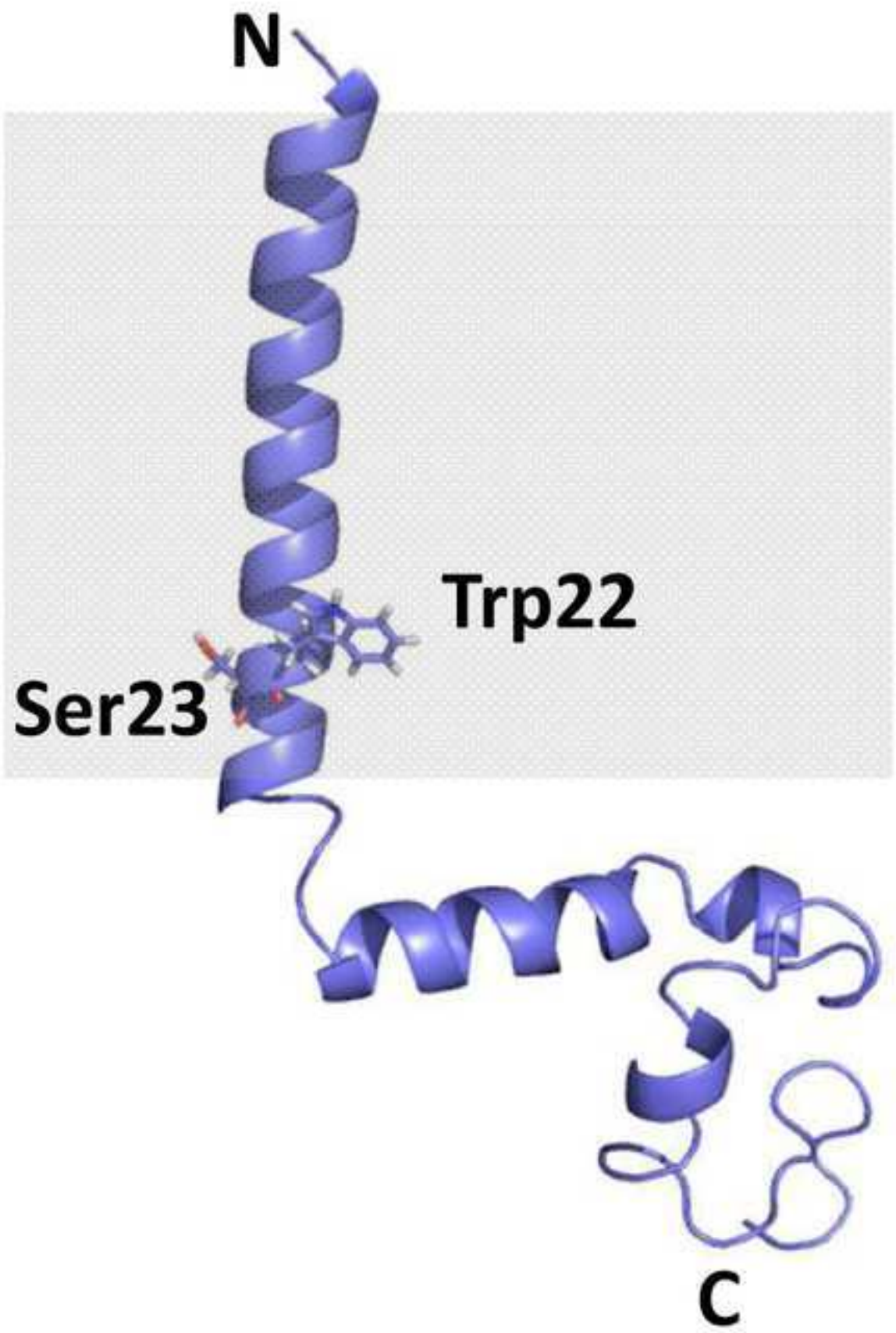

Figure
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