

# Defining Drug Interactions with the Viral Membrane Protein Vpu from HIV-1

V. Lemaitre, C.G. Kim, D. Fischer, Y.H. Lam, A. Watts, and W.B. Fischer

The replication of HIV-1 is strongly enhanced by a small membrane protein called virus protein U (Vpu). Vpu achieves its task by (a) interacting with CD4, the HIV-1 receptor, and (b) by amplifying particle release at the site of the plasma membrane. While the first role is due to interactions of the cytoplasmic site of Vpu with CD4, the second role may be due to ion channel activity caused by the self-assembly of the protein. Recently, a blocker has been proposed which abolishes channel activity. In this chapter, the mechanism of blocking is described using computational methods, including a brief overview of other viral ion channel blockers.

## 1. Introduction

The discovery of the structure and function of a protein goes mostly in parallel with the wish and also the need to find potential modulators inhibiting the discovered function, especially in the case of viral proteins. Several textbook discoveries are described in the literature (for a general review, see Blundell *et al.*, 2002) in a solely rational approach, and potential drugs have been design for the HIV-1 protease (Lam *et al.*, 1994; for a review see Wlodawer and Erickson, 1993; Hodge *et al.*, 1997) and influenza neuraminidase (von Itzstein *et al.*, 1993) based on their respective crystal structures (Colman *et al.*, 1983; Varghese *et al.*, 1983; Baumeister *et al.*, 1991).

Even more recent example comprises the discovery of an inhibitor of the main protease M<sup>Pro</sup> of the SARS virus from an application of a structure-based computational approach (Yang *et al.*, 2003). This protein is involved in the proteolytic processing of transcribed

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proteins essential for the viral life cycle. High-resolution data (around 2 Å) obtained in the presence of the hexapeptidyl inhibitor CbZ-Val-Asn-Ser-Thr-Leu-Gln-CMK revealed the binding conformation of the drug. In addition, structures recorded at different pHs reveal large structural arrangements of this protein upon changes in ionization states.

Out of these examples, one protein, neuraminidase, is a membrane protein (see Chapter 17 by Garman and Laver, this book). Up to date, antiviral drugs have been found against two other membrane proteins, including virus adsorption inhibitors (e.g., gp120 from HIV-1) and virus-cell fusion inhibitors like the 5-helix peptide (Root *et al.*, 2001; for a review, see De Clercq, 2002). However, resistance development against these drugs (e.g., in the case of HIV-1 protease, Condra *et al.*, 1995), necessitates the hunt for other targets such as the smaller, so-called accessory proteins, which include also membrane proteins (for HIV-1, see Miller and Sarver, 1997).

### 1.1. Short Viral Membrane Proteins

Compared to the larger soluble proteins, which can be seen as highly structured candidates with large surface areas on which putative binding motifs can be discovered, membrane proteins comprise a more difficult target. This is due to the difficulties in obtaining enough structural information for accessible regions (loops), which is a desirable prerequisite for fast drug discovery. Recent attempts to derive relatively large quantities of the full length of short viral membrane proteins, with up to 100 amino acids by applying standard solid phase peptide synthesis (SPPS), have been successfully achieved for M2 from influenza A (Kochendörfer *et al.*, 1999). The two 50mer ends of the protein have been synthesized and chemically ligated to obtain the full-length protein. It is claimed that with this method, even noncoded amino acids can be incorporated, which would mean in case of Vpu, two phosphorylated serines (Ser-52 and Ser-56) would need to be added. Despite the still missing experimental verification the method will play an important role in the future in obtaining sufficient quantities of small proteins in general (Kochendoerfer *et al.*, 2004).

However, so far structural information has emerged gradually by investigating parts of the whole, for which Vpu from HIV-1 may serve as an example. CD, FTIR, and NMR spectroscopy (see Chapters 11 and 13 by Opella *et al.* and Bechinger and Henklein, respectively, this book) have been carried out on parts of Vpu, for example, the transmembrane (TM) part in various lengths and solely the cytoplasmic part. Thereby, the fragments have been produced either by SPPS or expression methodology. The structural information is sufficient to enable the generation of computational models. These models, especially the models representing the TM segment of Vpu, have been embedded in hydrophobic slabs, surrounded by octane to mimic a lipid bilayer, or in fully hydrated lipid bilayers on an all atom basis (reviewed in Fischer, 2003). Recently, even a full-length model based on the structural information of all these fragments has been produced and embedded in a lipid monolayer (Sun, 2003). The models can be used for docking approaches and molecular dynamics (MD) simulations enabling detailed structural analysis, until high-resolution data are available. Once a high-resolution structure is available, these methods may furthermore be used to refine the static structure, predict protein mechanics and putative drug-protein interactions.

In this chapter, the efforts to obtain information about drug-protein interactions of Vpu are summarized and compared with investigations on other short viral membrane proteins. The emphasis is to outline the computational methods used for this enterprise and to demonstrate their potential for drug discovery especially in the case of Vpu, for which a high-resolution structure is not yet available.

## 1.2. The Vpu Protein

Vpu is an 81 amino acid protein encoded by HIV-1 with a high degree of sequence conservation (Willbold *et al.*, 1997). Its role and structure are reported in detail in other chapters of this book. In brief, its function in the life cycle of HIV-1 is 2 fold (for reviews, see Fischer and Sansom, 2002; Bour and Strelbel, 2003; Fischer, 2003; Montal, 2003): (a) to interact with CD4 in the endoplasmic reticulum to initiate the ubiquitin mediated degradation of the CD4–Vpu complex, and (b) to enhance particle release at the site of the plasma membrane altering the electrochemical gradient via ion channel formation by homo oligomerization in the lipid membrane or interacting with other ion channels (Hsu *et al.*, 2004). While the first function is fairly established, the second is still open to debate (Lamb and Pinto, 1997). For example, it is not known whether ion channel activity is an intrinsic part of Vpu's function. However, Vpu, either with its TM domain synthesized using SPPS or as full-length protein from expression and reconstituted into lipid bilayers, shows channel activity (Ewart *et al.*, 1996; Schubert *et al.*, 1996; Marassi *et al.*, 1999; Cordes *et al.*, 2002; Park *et al.*, 2003). Recently, a drug has been described which blocks channel activity of Vpu *in vitro* (Ewart *et al.*, 2002) and also of p7 from hepatitis C virus (Premkumar *et al.*, 2004). The molecule causing this effect is a derivative of amiloride, cyclohexamethylene amiloride.

Spectroscopic studies of Vpu incorporated into membranes (or mimic membranes) using CD (Wray *et al.*, 1995, 1999), FTIR (Kukul and Arkin, 1999), and NMR spectroscopy (Federau *et al.*, 1996; Willbold *et al.*, 1997; Ma *et al.*, 2002) have identified structural elements allowing the following description of the Vpu structure: a helical TM segment is followed by a larger cytoplasmic domain with a helix–loop helix–helix/turn motif. The cytoplasmic domain seems to be in contact with the membrane (Marassi *et al.*, 1999; Henklein *et al.*, 2000; Zheng *et al.*, 2003). The tilt angle of the TM segment with respect to the membrane normal using different techniques and Vpu constructs has been found to range from approximately 6° to 30° (Kukul and Arkin, 1999; Marassi *et al.*, 1999; Wray *et al.*, 1999; Henklein *et al.*, 2000). The most recent NMR spectroscopic study indicates a kink of the TM segment of about 12–15° around residue Ile-17 (Park *et al.*, 2003). Based on these experimental results, several models of Vpu have been proposed from computer simulations (Grice *et al.*, 1997; Cordes *et al.*, 2002; Lopez *et al.*, 2002; Sramala *et al.*, 2003; Sun, 2003).

For further details of Vpu, the reader may refer to Chapters 11, 12, 13, and 15 by S.J. Opella *et al.*, Wray and Schubert, B. Bechinger and P. Henklein, and P. Gage *et al.*, respectively, in this book.

## 2. The Methods

### 2.1. Docking Approach

Docking approaches have been proven to be valuable tools in identifying not only the drug-binding sites on proteins but also to screen large databases for other potential drugs, if the binding site is known (Blundell *et al.*, 2002; Glick *et al.*, 2002). In docking methods, the protein and the ligand are transferred to a point on a grid. Smaller spacing of the grid increases the accuracy of the method, which proceeds in parallel with an increase in computer time for the calculations. Pioneering work in this field has been achieved by Peter Goodford (Goodford, 1985; Boobyer *et al.*, 1989; Wade *et al.*, 1993). In this method, putative docking sites and the potency of different conformations of a drug have been assessed based on

calculating electrostatic interactions. To date, several other docking software are available differing in the function implemented to evaluate the fit (scoring) of a drug to the binding site (scoring function). In principle, both the protein and the drug are held rigid during the calculation to save computer time. More advanced methods allow for the ligand to be flexible (Wang *et al.*, 1999; Kua *et al.*, 2002) and even the protein (Carlson and McCammon, 2000). Another improvement of the docking approach is the introduction of the energy of solvation of the ligand for evaluation of binding affinities and consequently the scoring (Shoichet *et al.*, 1999). In the present study, AUTODOCK (Morris *et al.*, 1998) was used, in which the Lamarckian genetic algorithm (GA) is applied. The ligand explores randomly translational, orientational, and conformational space with respect to a rigid protein. To avoid extreme CPU time due to the endless number of possible combinations in the search, GAs are combined with a local search (LS) protocol, which allows for energy minimization at low temperatures. However, a general drawback for the docking software is the inability to include the electrostatic contributions of a lipid bilayer environment.

## 2.2. Molecular Dynamics (MD) Simulations

MD simulations *per se* describe the changes of positions, velocities, and orientations of a system with time, based on Newtonian principles. From the trajectories, time averages of macroscopic properties can be deduced. Any screening of a protein surface by a drug or ligand would cost almost endless MD simulation time, which would impose software failures and consequently inaccurate results. Moving a ligand around a protein surface might be envisaged by the use of “artificial” force on the ligand. Steered MD simulation is a step in this direction used till date for protein folding (Lu *et al.*, 1998; Gao *et al.*, 2002) and to propose pathways of molecules into or out of known binding sites (Kosztin *et al.*, 1999; Isralewitz *et al.*, 2001; Shen *et al.*, 2003). A workaround is the combined use of a docking approach followed by a MD simulation or molecular mechanics calculations (Wang *et al.*, 2001; Beierlein *et al.*, 2003). However, using solely MD simulations for the exploration of the drug and the protein is becoming an increasingly valuable tool. Recent investigations on the effect of general anesthetics on the ion channel gramicidin A have been investigated (Tang and Xu, 2002). In this study, the missing local changes on the protein caused by the drugs have been correlated with a low affinity of the general anesthetics to their target. In another approach, effects of mutations on the structure of the HIV-1 integrase have been analyzed using MD simulations (Barreca *et al.*, 2003). Simulations have also been done in the presence of an inhibitor (5CITEP) to address drug–protein interactions. In the present chapter, the putative binding site of amiloride (Am) and cyclohexamethylene amiloride (Hma) with the TM domain of Vpu has been derived from the docking approach. In the docking approach, a pentameric bundle was used to discover the binding site. Simulations were then run with the protein bundle–drug complex for 12 ns. The topology of the drug and its partial atomic charges have been determined by PRODRUG (van Aalten *et al.*, 1996) and adapted for the force field GROMOS43a2.

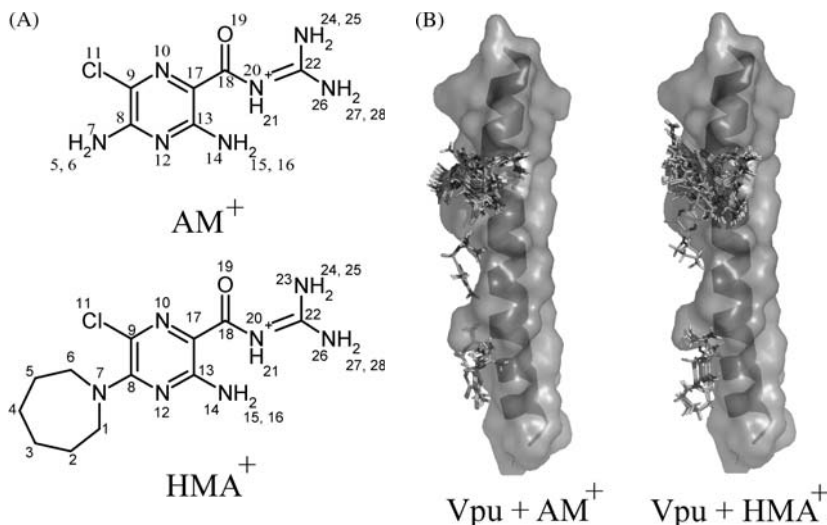
## 3. Analysis of Drug–Protein Interactions of Vpu with a Potential Blocker

According to the results shown experimentally (Ewart *et al.*, 2002), Hma and, to a lesser extent, dimethyl amiloride block channel activity of full-length Vpu, reconstituted into

a lipid membrane, and a peptide representing the TM domain of the protein. Am itself has almost negligible effect. With these data at hand, and the large amount of structural data available (see Fischer, 2003, and references therein; Park *et al.*, 2003), computational methods can be approached to address the following questions: (a) the location of a putative binding site and the affinity of the blocker; (b) the dynamics of the blocker at the binding site and the blocking mechanism; and (c) the entry to the binding site.

### 3.1. Using the Docking Approach

The question of where the blocker binds can be addressed using docking programs, even though they are not designed to search within pore-like structures. In the present study, the program AUTODOCK is used for Am and the most potent blocker Hma, both in protonated ( $\text{AM}^+$ ,  $\text{HMA}^+$ )—the most likely form under physiological conditions—and unprotonated (AM, HMA) forms (C. Kim, V. Lemaitre, A. Watts, W.B. Fischer, in preparation). The Vpu models used are single-stranded Vpu, corresponding to the TM helix of Vpu,  $\text{Vpu}_{1-32}$  (Figure 14.1) and the extended kinked model  $\text{Vpu}_{1-52}$ . Models with assembled helices forming pentameric and hexameric bundles are generated and also used in the docking approach. The single peptides are rationalized by the idea of a possible binding mechanism in which the blocker binds prior to the formation of ion conducting bundles. The results can be summarized in such a way that the putative binding site is toward the C terminal end of the peptides and, in case of the bundles, within the pore. The specific residue to which the blockers establish contact is Ser-23. The type of interaction is via hydrogen bonding. Calculated binding constants are found to be of the same range as those derived from experiments

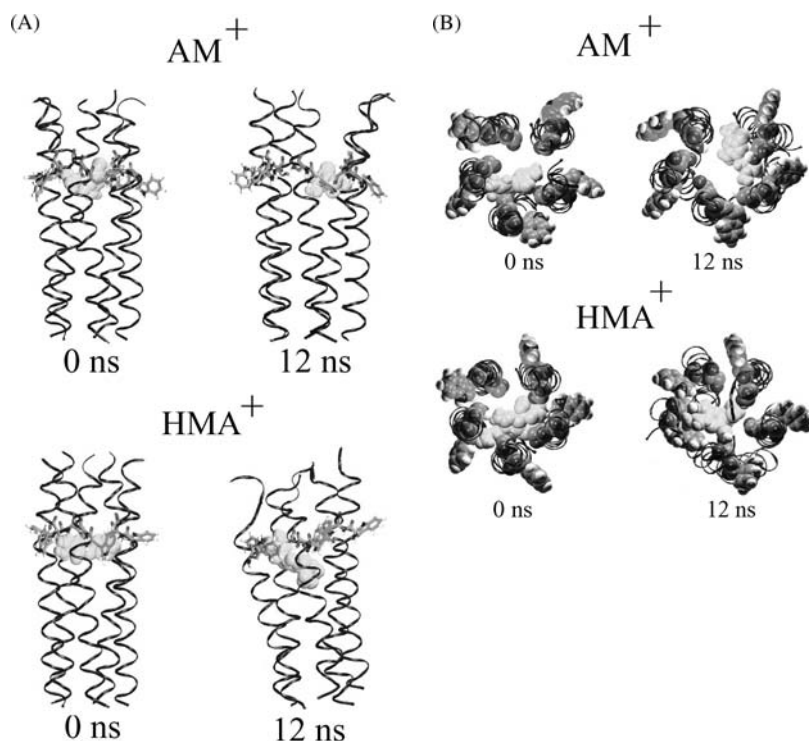


**Figure 14.1.** (A) Structures and atom number used for  $\text{AM}^+$  (top) and  $\text{HMA}^+$  (bottom) for the docking experiments. (B) Docking result of both blockers on a single strand of Vpu (QPIPIVAIVA<sup>10</sup> LVVAIHAIIV<sup>20</sup> VWSVIIIEYR<sup>30</sup> KI, HV1H2, shown in gray with the helix highlighted) corresponding to the TM segment. The TM segment is shown with the results for  $\text{AM}^+$  (left) and  $\text{HMA}^+$  (right).

(Fischer, Lam, Watts, Fischer, unpublished results). Based on these findings MD simulations may follow to address the dynamics of the blocker and the protein in an almost realistic environment of a hydrated lipid bilayer (Lemaitre *et al.*, 2004). Studies of membrane proteins embedded in a hydrated lipid bilayer have been highly successful in describing the mechanism of function of ion channels on an atomic level (Tieleman *et al.*, 1997; Sansom *et al.*, 1998; Shrivastava and Sansom, 2000; Berneche and Roux, 2001; de Groot and Grubmüller, 2001; Im and Roux, 2002; Zhu *et al.*, 2002; Böckmann and Grubmüller, 2002).

### 3.2. Applying MD Simulations

Based on the findings from the docking approach, cyclohexamethylene amiloride and amiloride, both in their protonated and deprotonated states, have been placed within a pentameric bundle (Figure 14.2). The whole system has then been placed into a hydrated lipid bilayer. The analysis gives an insight into the effect of the blockers on the protein structure.



**Figure 14.2.** (A) Side view of the blockers (in light gray) within a pentameric bundle consisting of the circular assembly of the helical TM segments.  $AM^+$  is shown in the top bundles at 0 ns (left) and 12 ns (right),  $HMA^+$  in the lower bundles. The lipid bilayer and the water molecules present during the simulations are omitted for clarity. Tryptophans and serines are indicated by sticks. The C-terminal end is pointing up, while the N-terminal end is pointing down. The same structures as in (A) are shown with a view down into the pore from the C-terminal end (B).

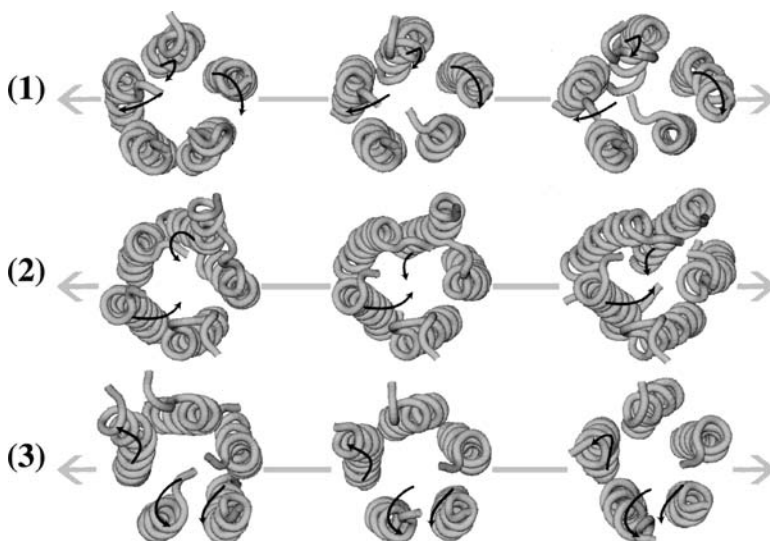
The analysis of the data reveals no major structural rearrangements of the helices. For a more detailed analysis of the pore, the bundle is subdivided into three regions: an N-terminal (residues 1–11), a middle (residues 12–22), and a C-terminal section (residues 23–32). The data uncover small structural sorting which allows the following interpretation: while amiloride induces widening of the pore, cyclohexamethylene induces a more funnel-like shape, with the narrow part at the N-terminal end.

Principal Component Analysis (PCA) has been applied to determine concerted motion taking place in the bundles during the simulation (Figure 14.3). PCA (Amadei *et al.*, 1993), sometimes called “Essential Dynamics,” allows finding correlated motions within an object, for example, a protein (Garcia, 1992; Wong *et al.*, 1993; van Aalten *et al.*, 1995; Yang *et al.*, 2001; Grottesi and Sansom, 2003). These correlated motions or principal components have been shown to describe motions which are relevant for the function of proteins. The technique involves the removal of the overall rotation and translation to isolate the internal motion only. This is achieved by a least square fitting to a reference structure and computation of the covariance matrix  $C$  of the atomic coordinates. The principal components are then obtained by diagonalizing the matrix  $C$  with an orthonormal transformation (Amadei *et al.*, 1993).

The only atoms that have been considered for the analysis of the Vpu bundles are the  $C\alpha$  atoms. This enables noise arising from the random motion of the side chains to be removed and to reduce the size of the matrices which needs to be diagonalized. Furthermore, the analysis is performed on the equilibrated part of the trajectory, discarding the first nanosecond. Figure 14.3 shows the first principal motions which have the largest amplitude in each of the bundles indicated by arrows. The motions describe a correlated change in the twist or in the kink of individual helices forming the pore for the Vpu bundle without blocker (Figure 14.3, (1)) and the Vpu bundle in the presence of  $AM^+$  (Figure 14.3, (3)). The main correlated motion in the presence of  $HMA^+$  (Figure 14.3, (2)) describes a concerted change in the tilt angle of two helices, indicating a closure of the pore which finally explains the funnel-like shape.

The mode of blocking also includes occlusion. AM and  $AM^+$  are within the pore but are not occluding it completely, while HMA and  $HMA^+$  almost completely occupy the space within the pore (Figure 14.3). When in the vicinity of at least one of the serines, the apparent minimum pore radius left within the middle section is about  $1.42 \pm 0.46 \text{ \AA}$  for  $AM^+$  and  $0.63 \pm 0.40 \text{ \AA}$  for  $HMA^+$ . The latter value is too small for even allowing a single filed water (Roux and Karplus, 1991; Woolley and Wallace, 1992; Chiu *et al.*, 1999; de Groot *et al.*, 2002) to pass the blocker. Water molecules in the peptide antibiotic gramicidin are assumed to pass the pore in a single file way, which means that one water molecule has only neighbors in front and in the back.

The root-mean-square deviation (RMSD) values for all blockers remain below 0.2 nm with slightly higher values for HMA and  $HMA^+$ , and the largest fluctuations for the latter (data not shown). The larger values and spread is indicative of the amiloride derivative for the flexible cyclohexamethylene ring. The root mean square fluctuations (RMSF) of the individual atoms of the blockers indicate the central body of Am (a 3,4,6-substituted pyrazine ring) and Hma, remain fairly rigid (RMSF < 0.1 nm) independent of the protonation state of the blockers. Only the hydrogen atoms of the amino groups of the pyrazine ring and the guanidinium group show the largest fluctuations ( $\geq 0.1$  nm). The curves albeit very similar for both blockers, when compared with similar atoms, adopt slightly lower values for all atoms in the protonated blocker. The cyclohexamethylene ring in Hma fluctuates around 0.1 nm, independent of the protonation state. Thus, the hexamethylene ring adds a mobile part to the rigid pyrazine ring.

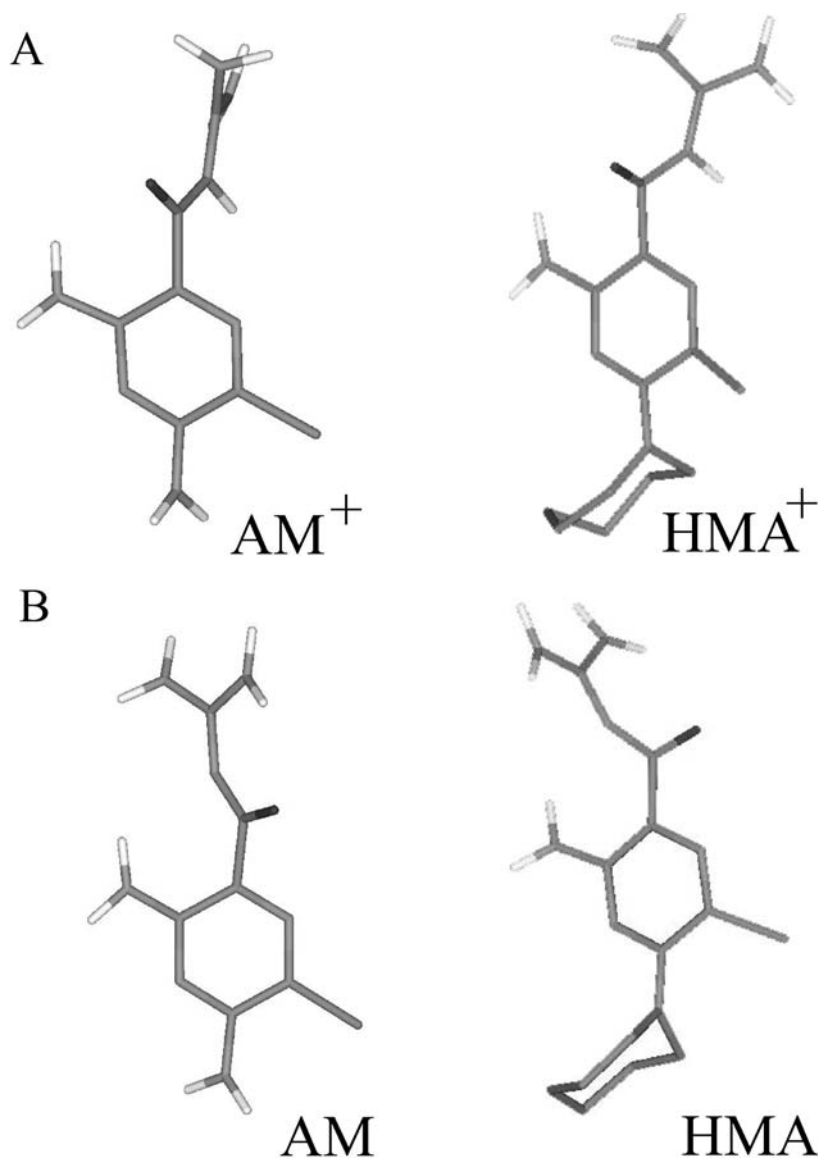


**Figure 14.3.** First principal components, indicating the correlated motion with the largest amplitude, on the C $\alpha$  atoms of the Vpu bundle without any blocker (1), Vpu bundle in the presence of HMA $^+$  (2), and AM $^+$  (3). The small arrows indicate the motion calculated for the C $\alpha$  atoms of the helices. The large horizontal arrows symbolize the protein oscillation between the two conformations on either side. The small arrows highlight the section of the bundle where the motion takes place.

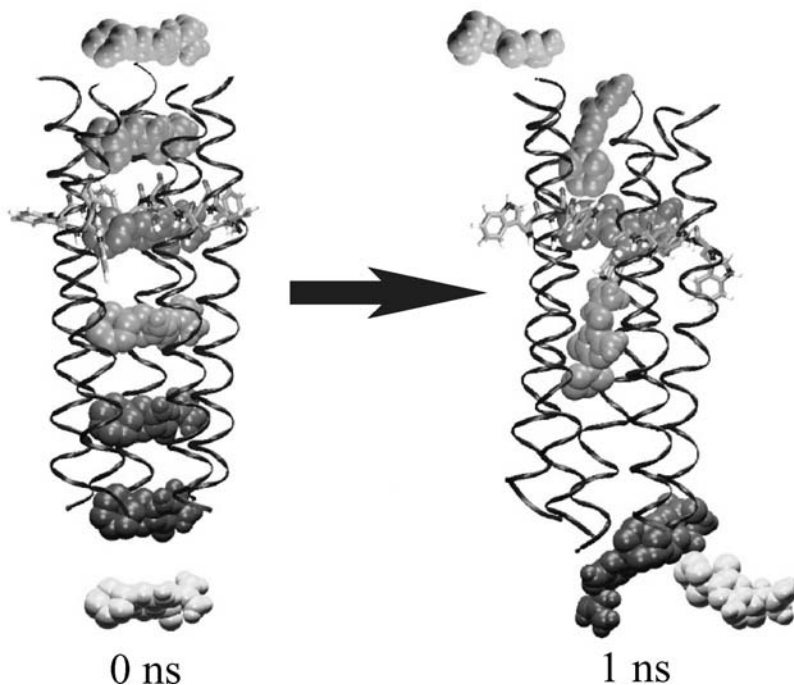
The different conformations generated by the MD simulations for the blockers tested were clustered using a full linkage algorithm, using a cut-off of 0.03 nm for Am and 0.04 nm for Hma. In the case of Am, this means that molecules with an RMSD smaller than 0.03 nm relative to all the existing members of a cluster will belong to this cluster. The mostly populated conformation for AM $^+$  with 96.0% is shown in Figure 14.4A, the most frequently adopted conformation for HMA $^+$  with 95.1%, in Figure 14.4B. The carbonyl group linked to the guanidinium group in the protonated blocker is pointing toward the primary amine group of the pyrazine ring. Deprotonation of the blockers reveals a conformational change in the blocker so that the amine part of the guanidinium group is pointing to the primary amine group of the pyrazine ring (data not shown).

The blockers, independent of their protonation state, interact with the protein via hydrogen bonding. The hydrogen bond partners are the serine side chains (Ser-23) which point into the pore. Therefore, the most prominent difference between AM $^+$  and HMA $^+$  is that, in addition to these hydrogen bonds, HMA $^+$  also interacts with one of the tryptophans (Trp-22) which comes to reside at the helix–helix and helix–lipid interface. The cause of this interaction might be due to the lower average velocity of HMA $^+$  within the site. In addition to this dynamic affect, HMA $^+$  orients with its hydrophobic cyclomethylene ring stronger to the hydrophobic part of the pore toward the N-terminal end. This is reflected by an average angle of  $43.0 \pm 11.8^\circ$  for HMA $^+$  and  $11.3 \pm 12.7^\circ$  for HMA with respect to the membrane plane. AM $^+$  and AM adopt angles of  $5.3 \pm 23.3^\circ$  and  $8.6 \pm 51.8^\circ$ , respectively. The larger standard deviation reflects the higher flexibility of the Am within the pore.





**Figure 14.4.** Most populated structures during the MD simulations for the (A) protonated and the (B) deprotonated blockers.



**Figure 14.5.** A pentameric bundle of the TM segment of Vpu with the individual HMA<sup>+</sup> superimposed at different positions along the pore axis at the beginning (left structure) of the simulation and after 1 ns (right structure). The tryptophans and the serines are highlighted by sticks.

The MD simulations have been based on results obtained from a docking approach performed with the AUTODOCK software. The blocker was placed at a site suggested by AUTODOCK. The question arises, would MD simulations be able to deliver the same result? In order to address this question, a series of 1 ns simulation have been performed with a pentameric bundle and HMA<sup>+</sup> at different position along the *z*-axis within the pore of a pentameric bundle (Figure 14.5). The results in terms of the stability of the bundle and the overall structure remain similar to the results of the long simulations here (data not shown). The integrity of the bundle is not destroyed, independent of the position of the blocker. It is interesting to note that in all of the positions, other than the one used for the longer simulations (position at the Ser-23 site), does the blocker leaves its starting position after 1 ns. To put the individual pictures in context, any position of the HMA<sup>+</sup> toward the C- or N-terminal end of the bundle may force the blocker to orientate its long axis parallel to the pore axis, and beyond a certain threshold position even to escape from the pore.

#### 4. How Realistic is the Protein Model?

Computational approaches are always judged by the relevance of their results which stand and fall with the model assumed. In this case, the question is allowed: how realistic are

the bundles? Experimental studies propose that Vpu exists as an oligomer (Maldarelli *et al.*, 1993). Computational experiments on solely the TM segments have been undertaken to relate the experimentally derived cation selectivity of Vpu to the number of segments forming the bundle (Grice *et al.*, 1997). In these studies, the low energy for a  $K^+$  to pass the pore compared to the higher value obtained for  $Cl^-$  for the pentameric bundle supports the idea of a pentameric model. In a combined experimental and computational approach, the tetrameric bundle was excluded as a putative pore (Cordes *et al.*, 2002). However, direct structural evidence is still lacking. Thus, Vpu could also be a tetramer unless proven by, for example, X-ray, spectroscopy or otherwise, and thus, investigations are carried out with the pentameric and hexameric bundles. The overall orientation of the helices, having the serines facing the lumen of the pore, is based on findings for other ion channels (Leonard *et al.*, 1988; Galzi *et al.*, 1991; Pebay-Peyroula *et al.*, 1997; Pautsch and Schulz, 2000; Sass *et al.*, 2000). Most recent solid-state NMR spectroscopic investigations propose a kink around Ile-17 (Park *et al.*, 2003). The computer models are generated without an explicit kink; however, the analysis of the bundles using residues Ile-16 to Ala-18 as center point also reveal on average a kink of the helices. Differences in the absolute values may derive from the different lipids used and the length of the peptides, including additional residues at the C-terminal end to enable facilitated purification.

## 5. The Putative Binding Site

The site of binding is at the moment pure computationally based speculation. However, we may bring the site into context with experimental evidence.

Am (3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide) is a moderately strong base with a  $pK_a$  of 8.7 (Schellenberg *et al.*, 1985; Kleyman and Cragoe, 1988). At physiological pH at least 95%, Am exists in a positively charged form due to the protonated guanidinium group (Cragoe *et al.*, 1967). In the case of epithelial  $Na^+$  channels, this protonated form is essential for blocking, indicative of a pH-dependent activity (Kleyman and Cragoe, 1988). Am is soluble in water up to 16 mM (Benos, 1982). The deprotonated form is lipid soluble and can easily cross the cell membrane with the consequence of accumulating within the cell and altering a number of cellular processes (Benos, 1982; Kleyman and Cragoe, 1988; Grinstein *et al.*, 1989). Am cannot be metabolized in the body, so it is eliminated intact in urine and has a biological half-life of  $9.6 \pm 1.8$  hr (Smith and Smith, 1973). Am and its derivatives are a class of potent blockers of  $Na^+$  transporters such as the epithelial  $Na^+$  channels (ENaC),  $Na^+/H^+$  exchanger and, to a lesser extent, the  $Na^+/Ca^{2+}$  exchanger (Kleyman and Cragoe, 1988, 1990). Cyclohexamethylene amiloride was reported to be especially selective for the  $Na^+/H^+$  exchanger (Kleyman and Cragoe, 1988). Hydrophobic interactions with parts of the channel adjacent to the Am binding site increases the binding affinity of the blocker (Garty and Palmer, 1997). For Am, a putative binding site in the  $Na^+$  channel has been proposed to be approximately 20% within the TM electrical field (Li *et al.*, 1987; Fyfe and Canessa, 1998) on the extracellular side (Benos, 1982). The orientation is proposed to allow the guanidinium group to penetrate into the pore (reviewed in Alvarez de la Rosa *et al.*, 2000). Using anti-Am antibodies, a six amino acid sequence of WYRFHY (extracellular loop of  $\alpha$ -rENaC), WYKLHY ( $\beta$ - and  $\gamma$ -ENaC subunit) and WYHFHY ( $\delta$ -ENaC subunit) (Kieber-Emmos *et al.*, 1995; Waldmann *et al.*, 1995; Ismailov *et al.*, 1997; Schild *et al.*, 1997) has been identified as a putative binding site. Replacement of the second His-282 in the

$\delta$ -ENaC subunit by glutamate abolishes the blocking while replacement by arginine increases the blocking. Another point mutation within the TM region replacing a crucial serine residue also abolishes affinity for Am (Waldmann *et al.*, 1995). This indicates that the sequence, including an aromatic residue, a positively charged residue, and a serine in the vicinity of a hydrophobic site, is a key feature for binding Am and its derivatives. Consequently, the binding site proposed, with the guanidinium group in the vicinity of the serines of the putative pore of Vpu in the present study, is in agreement with findings on the ENaC.

Also, local anesthetics such as QX-222 and QX-314, which reflect amiloride in their overall structure, are penetrating the pore of the nicotinic acetylcholine receptor and induce blocking via occlusion (Neher and Steinbach, 1978). The binding site for these blockers, and also procaine (Adams, 1977), is proposed to be at a site within the pore which should be near to the ring of serines and threonines found in the pore of the receptor (Leonard *et al.*, 1988). Based on experimental findings, computational experiments, using the Monte Carlo minimization method, have been performed on a model of the nAChR based on its TM helices of M2 (Tikhonov and Zhorov, 1998). The data analyzed the binding geometry of, for example, QX-222 and Chlorpromazin (CPZ). It is found that the charged groups interact with the side chains of the serines and threonines (Thr-4 or Ser-8), and the uncharged groups interact with the ring of hydrophobic residues (Leu-11, Ala-12). The blockers orient their long axis along the axis of the bundle due to the amphiphatic character of the drugs.

These results are indicative of a generalized pattern of binding behavior and underline the relevance of the results obtained for the Am and its derivative in the present study.

One of the important factors for the discovery of potential blocker and drugs is also the knowledge of how the blocker reaches its binding site. The diffusion of the drug to (on rate) the site and away (off rate) from it is involved in defining the binding constant (Lüdemann *et al.*, 2000). Also, it is essential to know the correct description of the energetics within the narrow geometry of the pore. Indirect methods like Brownian dynamics or steered MD, and calculation of free energies and reaction path methods may give a reasonable picture of the diffusion of the blocker into the pore. These methods need to be taken into account to improve the understanding of the mechanism of blocking and the success of virtual drug screening for pore blockers.

## 6. Water in the Pore

During the generation of the bundles and finally the hydration of the bilayer system, water molecules are found within the pore. However, during the 300 ps equilibration step, they totally escape and do not re-enter the pore during the simulation. During the entire duration of the simulations, the hydrophilic C-terminal end is engulfed by water reaching the serines and the blocker. Further toward the N-terminal end, no water molecule remains within the pore.

Simulations on bundles of Vpu consisting of peptides representing the TM sequence IAIVA<sup>10</sup>LVVAIIIAIV<sup>20</sup>VWSIVII indicate a reduced number of water molecules toward the C-terminal end during the early stage of the simulation (Lopez *et al.*, 2002). The pentameric and hexameric bundles are embedded in an octane slab which mimics the lipid bilayer. The long-range electrostatic interactions have been treated with Ewald sums. These findings are indicative for a fluctuating number of water molecules within the pore. Recent investigations

on theoretical hydrophobic pore models show that the presence of water in these pores is strongly dependent on the pore radius (Beckstein and Sansom, 2003). Below 0.4 nm, the water density found decreases to almost zero. Water molecules, if bigger than 0.4 nm, traverse the pore in an “avalanche-like fashion” (Beckstein and Sansom, 2003). For very small radii, simulations on a very long timescale (>50 ns) are needed to assess the proper behavior of the water molecules in confined geometry. Thus, the Vpu bundle model presented here shows a proper bundle with a temporary state of low water content.

## 7. MD Simulations for Drug Screening?

The use of MD simulations for drug screening of membrane proteins still needs further testing. Up to now simulations are still time consuming, which imposes a major drawback when compared to docking approaches. However, the possibility of implementing more realistic conditions upon the models, such as for membrane proteins within a bilayer in an all-atom representation including an explicit representation of solvent, will, in the future, overcome the existing disadvantages through the advancement of both software and hardware (Mangoni *et al.*, 1999). In the meantime, the combination of MD simulations with docking approaches has been proven to be a powerful tool for lead discovery (Wang *et al.*, 2001; Kua *et al.*, 2002). MD simulations with the drug will increasingly play an important role in the exploration of the dynamics of the ligand and its impact on the protein structure (Tang and Xu, 2002). Here, if Vpu is identified as a potential drug target, then identification of residues involved in blocking action will help in further rational drug design.

## 8. Other Viral Ion Channels and Blockers

One of the earliest antiviral drugs, amantadine (amantadine-1), and also remantadine ( $\alpha$ -methyl-1-adamantanemethanamine) (Davies *et al.*, 1964; Hoffmann, 1973) are known to target the viral proton channel M2 from influenza A (Wang *et al.*, 1993), with the latter especially used in chemoprophylaxis and therapy of influenza A. Its site of blocking is assumed to be within the lumen of the pore with residues such as Val-27, Ala-30, and Ser-31 involved (Duff and Ashley, 1992; Pinto *et al.*, 1992; Duff *et al.*, 1993). For a detailed review, see Chapter 8 by Y. Tang *et al.*, this book. Also NB, an influenza B membrane protein forming ion channels, can be blocked by amantadine (Sunstrom *et al.*, 1996; Fischer *et al.*, 2001). However, the binding constants proposed for blocking are too high to propose NB as a potential target. The short viral membrane protein p7 from Hepatitis C virus also exhibits ion channel activity (Griffin *et al.*, 2003; Pavlovic *et al.*, 2003). For this channel, amantadine seems to block channel activity (Griffin *et al.*, 2003). However, it could be shown that this channel can also be blocked by long-alkyl-chain iminosugar derivatives (Pavlovic *et al.*, 2003). Structural and, consequently, computational data, still need to be produced to get more insight about the putative binding site of these blockers. With Vpu as another viral ion channel forming protein, the number of HIV protein targets has been enlarged by the discovery of Am derivatives as potential channel blockers (Ewart *et al.*, 2002; see also Chapter 15 by P. Gage *et al.*, this book). Derivatives of amantadine, spiro 5- and 6-membered analogs, have been identified for weak HIV-1 activity (Kolocouris *et al.*, 1996). Since they have not been

active against HIV-2, which does not encode Vpu, it is tempting to assume blocking of either one or both of the two roles of Vpu in the infected cell. Possibly other viral ion channel proteins and viroporins may become highly important future drug targets and we might be just at the beginning of a fruitful time in this respect.

## 9. Speculation of Binding Sites in the Cytoplasmic Site

It remains speculative of that the potential ligand-binding site of the AM derivatives might be in the cytoplasmic part of Vpu. The beauty of this approach would be to also inhibit Vpu's function of initiating CD4 degradation. Also, in this particular case of Vpu and in general, future drug design has to take into consideration the immediate proximity of the lipid membrane.

## 10. Conclusions

Small viral membrane proteins which are forming ion channels are increasingly in the line of fire as drug targets. Some of these proteins, like Vpu, are highly conserved. However, for some of the viruses, antiviral drugs against other proteins are already available. This is mostly because these other proteins are globular, relatively easily accessible and, with moderate effort, crystallizable. Membrane proteins still remain a difficult target in this respect. On the other hand, these proteins, like Vpu, need to assemble to fulfill their task which comprises another challenge in finding the proper target structure. It seems likely that, especially if functioning as ion channels, these small proteins will show their Achilles' heel and may become potential targets.

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## References

- Adams, P.R. (1977). Voltage jump analysis of procaine action at frog end-plate. *J. Physiol. (London)* **268**, 291–318.
- Alvarez de la Rosa, D., Canessa, C.M., Fyfe, G.K., and Zhang, P. (2000). Structure and regulation of amiloride-sensitive sodium channels. *Annu. Rev. Physiol.* **62**, 573–594.
- Amadei, A., Linssen, A.B., and Berendsen, H.J.C. (1993). Essential dynamics of proteins. *Protein Struct. Func. Genet.* **17**, 412–425.
- Barreca, M.L., Lee, K.W., Chimirri, A., and Briggs, J.M. (2003). Molecular dynamics studies of the wild-type and double mutant HIV-1 integrase complexed with the SCITEP inhibitor: Mechanism for inhibition and drug resistance. *J. Biophys.* **84**, 1450–1463.
- Baumeister, W.P., Ruigrok, R.W. and Cusack, S. (1991). The 2.2Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. *EMBO J.* **11**, 49–56.
- Beckstein, O. and Sansom, M.S.P. (2003). Liquid-vapor oscillations of water in hydrophobic nanopores. *Proc. Natl. Acad. Sci. USA* **100**, 7063–7068.

- Beierlein, F., Lanig, H., Schuerer, G., Horn, A.H.C., and Clark, T. (2003). Quantum mechanical/molecular mechanical (QM/MM) docking: An evaluation for know test systems. *Mol. Phys.* **101**, 2469–2480.
- Benos, D.J. (1982). Amiloride: A molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* **242**, C131–C145.
- Berneche, S. and Roux, B. (2001). Energetics of ion conductance through the K<sup>+</sup> channel. *Nature* **414**, 73–77.
- Blundell, T.L., Jhoti, H., and Abell, C. (2002). High-throughput crystallography for lead discovery in drug design. *Nat. Rev. Drug Discov.* **1**, 45–54.
- Böckmann, R. and Grubmüller, H. (2002). Nanosecond molecular dynamics simulation of primary mechanical steps in F1-ATP synthase. *Nat. Struct. Biol.* **9**, 198–202.
- Boobyer, D.N.A., Goodford, P.J., McWhinnie, P.M., and Wade, R.C. (1989). New hydrogen-bond potentials for use in determining energetically favourable binding sites on molecules of known structure. *J. Med. Chem.* **32**, 1083–1094.
- Bour, S. and Strebel, K. (2003). The HIV-1 Vpu protein: A multifunctional enhancer of viral particle release. *Microb. Infect.* **5**, 1029–1039.
- Carlson, H.A. and McCammon, J.A. (2000). Accommodating protein flexibility in computational drug design. *Mol. Pharm.* **57**, 213–218.
- Chiu, S.W., Subramanian, S., and Jakobsson, E. (1999). Simulation study of a gramicidin/lipid bilayer system in excess water and lipid. I. Structure of the molecular complex. *J. Biophys.* **76**, 1929–1938.
- Colman, P.M., Varghese, J.N., and Laver, W.G. (1983). Structure of the catalytic and antigenic sites in influenza virus neuraminidase. *Nature* **303**, 41–44.
- Condra, J.H., Schleif, W.A., Blahy, O.M., Gabryelski, L.J., Graham, D.J., Quintero, J.C. *et al.* (1995). In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. *Nature* **374**, 569–571.
- Cordes, F.S., Tustian, A., Sansom, M.S.P., Watts, A., and Fischer, W.B. (2002). Bundles consisting of extended transmembrane segments of Vpu from HIV-1: Computer simulations and conductance measurements. *Biochemistry* **41**, 7359–7365.
- Cragoe, E.J., Jr., Woltersdorf, O.W., Jr., Bicking, J.B., Kwong, S.F., and Jones, J.H. (1967). Pyrazine diuretics. II. N-amidino-3-amino-5-substituted 6-halopyrazinecarboxamides. *J. Med. Chem.* **10**, 66–75.
- Davies, W.L., Grunert, R.R., Haff, R.F., McGahen, J.W., Neumayer, E.M., Paulshock, M. *et al.* (1964). Antiviral activity of 1-adamantanamine (amantadine). *Science* **144**, 862–863.
- De Clercq, E. (2002). Strategies in the design of antiviral drugs. *Nat. Rev. Drug Discov.* **1**, 13–25.
- de Groot, B.L. and Grubmüller, H. (2001). Water permeation across biological membranes: Mechanism and dynamics of aquaporin-1 and GlpF. *Science* **294**, 2353–2357.
- de Groot, B.L., Tieleman, D.P., Pohl, P., and Grubmüller, H. (2002). Water permeation through gramicidin A: Desformylation and the double helix: A molecular dynamics study. *J. Biophys.* **82**, 2934–2942.
- Duff, K.C. and Ashley, R.H. (1992). The transmembrane domain of influenza A M2 protein forms amantadine-sensitive proton channels in planar lipid bilayers. *Virology* **190**, 485–489.
- Duff, K.C., Gilchrist, P.J., Saxena, A.M., and Bradshaw, J.P. (1993). The location of amantadine hydrochloride and free base within phospholipid multilayers: A neutron and X-ray diffraction study. *Biochim. Biophys. Acta* **1145**, 149–156.
- Ewart, G.D., Mills, K., Cox, G.B., and Gage, P.W. (2002). Amiloride derivatives block ion channel activity and enhancement of virus-like particle budding caused by HIV-1 protein Vpu. *Eur. J. Biophys.* **31**, 26–35.
- Ewart, G.D., Sutherland, T., Gage, P.W., and Cox, G.B. (1996). The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. *J. Virol.* **70**, 7108–7115.
- Federau, T., Schubert, U., Floßdorf, J., Henklein, P., Schomburg, D., and Wray, V. (1996). Solution structure of the cytoplasmic domain of the human immunodeficiency virus type 1 encoded virus protein U (Vpu). *Int. J. Pept. Protein Res.* **47**, 297–310.
- Fischer, W.B. (2003). Vpu from HIV-1 on an atomic scale: Experiments and computer simulations. *FEBS Lett.* **552**, 39–46.
- Fischer, W.B., Pitkeathly, M., and Sansom, M.S. (2001). Amantadine blocks channel activity of the transmembrane segment of the NB protein from influenza B. *Eur. J. Biophys.* **30**, 416–420.
- Fischer, W.B. and Sansom, M.S. (2002). Viral ion channels: Structure and function. *Biochim. Biophys. Acta* **1561**, 27–45.
- Fyfe, G.K. and Canessa, C.M. (1998). Subunit composition determines the single channel kinetics of the epithelial sodium channel. *J. Gen. Physiol.* **112**, 423–432.
- Galzi, J.L., Revah, F., Bessis, A., and Changeux, J.P. (1991). Functional architecture of the nicotinic acetylcholine receptor: From electric organ to brain. *Annu Rev Pharmacol. Toxicol.* **31**, 37–72.

- Gao, M., Wilmanns, M., and Schulten, K. (2002). Steered molecular dynamics studies of titin i1 domain unfolding. *J. Biophys.* **83**, 3435–3445.
- Garcia, A.E. (1992). Large-amplitude nonlinear motions in proteins. *Phys. Rev. Lett.* **68**, 2696–2699.
- Garty, H. and Palmer, L.G. (1997). Epithelial sodium channels: Function, structure, and regulation. *Physiol. Rev.* **77**, 359–396.
- Glick, M., Robinson, D.D., Grant, G.H., and Richards, W.G. (2002). Identification of ligand binding sites on proteins using a multi-scale approach. *J. Am. Chem. Soc.* **124**, 2337–2344.
- Goodford, P.J. (1985). A computational procedure for determining energetically favourable binding sites on biologically important macromolecules. *J. Med. Chem.* **28**, 849–857.
- Grice, A.L., Kerr, I.D., and Sansom, M.S.P. (1997). Ion channels formed by HIV-1 Vpu: A modelling and simulation study. *FEBS Lett.* **405**, 299–304.
- Griffin, S.D.C., Beales, L.P., Clarke, D.S., Worsfold, O., Evans S.D., Jäger J. et al. (2003). The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, amantadine. *FEBS Lett.* **535**, 34–38.
- Grinstein, S., Rotin, D., and Mason, M.J. (1989). Na<sup>+</sup>/H<sup>+</sup> exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta* **988**, 73–97.
- Grottesi, A. and Sansom, M.S.P. (2003). Molecular dynamics simulations of a K<sup>+</sup> channel blocker: Tc1 toxin from *Tityus cambridgei*. *FEBS Lett.* **535**, 29–33.
- Henklein, P., Kinder, R., Schubert, U., and Bechinger, B. (2000). Membrane interactions and alignment of structures within the HIV-1 Vpu cytoplasmic domain: Effect of phosphorylation of serines 52 and 56. *FEBS Lett.* **482**, 220–224.
- Hodge, C.N., Straatsma, T.P., McCammon, J.A., and Wlodawer, A. (1997). Rational design of HIV protease inhibitors. In S. Chiu, R.M. Burnett, and R.L. Garcea, (eds), *Structural Biology of Viruses*, Oxford University Press, Oxford, pp. 451–473.
- Hoffmann, C.E. (1973). Amantadine, OH and related compounds. In W.A. Carter (ed.), *Selective Inhibitors of Viral Functions*, CRC Press, Cleveland, OH, pp 199–211.
- Hsu, K., Seharaseyon, J., Dong, P., Bour, S., Marbon E. (2004). Mutual functional destruction of HIV-1 Vpu and host TASK-1 channel. *Mol. cell* **14**, 259–267.
- Im, W. and Roux, B. (2002). Ions and counterions in a biological channel: A molecular dynamics simulation of OmpF porin from *Escherichia coli* in an explicit membrane with 1 M KCl aqueous salt solution. *J. Mol. Biol.* **319**, 1177–1197.
- Ismailov, I.I., Kieber-Emmos, T., Lin, C., Berdiev, B.K., Shlyonsky, V.G., Patton, H.K. et al. (1997). Identification of an amiloride binding domain within the alpha-subunit of the epithelial Na<sup>+</sup> channel. *J. Biol. Chem.* **272**(34), 21075–21083.
- Israelowitz, B., Gao, M., and Schulten, K. (2001). Steered molecular dynamics and mechanical functions of proteins. *Curr. Opin. Struct. Biol.* **11**, 224–230.
- Kieber-Emmos, T., Lin, C., Prammer, K.V., Villalobos, A., Kosari, F., and Kleyman, T.R. (1995). Defining topological similarities among ion transport proteins with anti-amiloride antibodies. *Kidney Int.* **48**, 956–964.
- Kleyman, T.R. and Cragoe, E.J., Jr. (1988). Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* **105**, 1–21.
- Kleyman, T.R. and Cragoe, E.J., Jr. (1990). Cation transport probes: amiloride series. *Meth. Enzymol.* **191**, 739–755.
- Kochendörfer, G.G., Salom, D., Lear, J.D., Wilk-Orescan, R., Kent, S.B.H., and DeGrado, W.F. (1999). Total chemical synthesis of the integral membrane protein influenza A virus M2: Role of its C-terminal domain in tetramer assembly. *Biochemistry* **38**, 11905–11913.
- Kochendörfer, G.G., Jones, D. H., Lee, S., Oblatt-Montal, M., Opella, S.J., Montal, M. (2004). Functional characterization and NMR spectroscopy on full-length Vpu from HIV-1 prepared by total chemical synthesis. *J. Am. Chem. Soc.* **126**, 2439–2446.
- Kolocouris, N., Kolocouris, A., Foscolos, G.B., Fytas, G., Neyts J., Padalko, E. et al. (1996). Synthesis and antiviral activity evaluation of some new aminoadamantane derivatives. 2. *J. Med. Chem.* **39**, 3307–3318.
- Kosztin, D., Izrailev, S., and Schulten, K. (1999). Unbinding of retinoic acid from its receptor studied by steered molecular dynamics. *Biophys. J.* **76**(1 Pt 1), 188–197.
- Kua, J., Zhang, Y., and McCammon, J.A. (2002). Studying enzyme binding specificity in acetylcholinesterase using a combined molecular dynamics and multiple docking approach. *J. Am. Chem. Soc.* **124**, 8260–8267.
- Kukul, A. and Arkin, I.T. (1999). Vpu transmembrane peptide structure obtained by site-specific fourier transform infrared dichroism and global molecular dynamics searching. *Biophys. J.* **77**, 1594–1601.
- Lam, P.Y.S., Jadhav, P.K., Eyermann, C. J., Hodge, C.N., Ru, Y., Bacheler, L.T. et al. (1994). Rational design of potent, bioavailable, nonpeptide cyclic ureas as HIV protease inhibitors. *Science* **263**, 380–384.



- Lamb, R.A. and Pinto, L.H. (1997). Do Vpu and Vpr of human immunodeficiency virus type 1 and NB of influenza B virus have ion channel activities in the viral life cycles? *Virology* **229**, 1–11.
- Lemaitre, V., Ali, R., Kim, C.G., Watts, A., Fischer, W.B. (2004). Interaction of amiloride and one of its derivation with Vpu from HIV-1: a molecular dynamics simulation. *FEBS Lett.* **563**, 75–81.
- Leonard, R.J., Labarca, C.G., Charnet, P., Davidson, N., and Lester, H.A. (1988). Evidence that the M2 membrane-spanning region lines the ion channel pore of the nicotinic receptor. *Science* **242**, 1578–1581.
- Li, J.H., Cragoe, E.J., Jr., and Lindemann, B. (1987). Structure-activity relationship of amiloride analogs as blockers of epithelial Na channels: II. Side-chain modifications. *J. Membr. Biol.* **95**, 171–185.
- Lopez, C.F., Montal, M., Blasie, J.K., Klein, M.L., and Moore, P.B. (2002). Molecular dynamics investigation of membrane-bound bundles of the channel-forming transmembrane domain of viral protein U from the human immunodeficiency virus HIV-1. *Biophys. J.* **83**, 1259–1267.
- Lu, H., Isralewitz, B., Krammer, A., Vogel, V., and Schulten, K. (1998). Unfolding of titin immunoglobulin domains by steered molecular dynamics simulation. *Biophys. J.* **75**, 662–671.
- Lüdemann, S.K., Lounnas, V., and Wade, R.C. (2000). How do substrates enter and products exit the buried active site of cytochrome P450? 1. Random expulsion molecular dynamics investigation of ligand access channels and mechanisms. *J. Mol. Biol.* **303**, 797–811.
- Ma, C., Marassi, F.M., Jones, D.H., Straus, S.K., Bour, S., Strebler, K. *et al.* (2002). Expression, purification, and activities of full-length and truncated versions of the integral membrane protein Vpu from HIV-1. *Protein Sci.* **11**, 546–557.
- Maldarelli, F., Chen, M.Y., Willey, R.L., and Strebler, K. (1993). Human immunodeficiency virus type 1 Vpu protein is an oligomeric type I integral membrane protein. *J. Virol.* **67**, 5056–5061.
- Mangoni, M., Roccatano, D., and Di Nola, A. (1999). Docking of flexible ligands to flexible receptors in solution by molecular dynamics simulation. *Protein Struct. Func. Genet.* **35**, 153–162.
- Marassi, F.M., Ma, C., Gratkowski, H., Straus, S.K., Strebler, K., Oblatt-Montal, M. *et al.* (1999). Correlation of the structural and functional domains in the membrane protein Vpu from HIV-1. *Proc. Natl. Acad. Sci. USA* **96**, 14336–14341.
- Miller, R.H. and Sarver, N. (1997). HIV accessory proteins as therapeutic targets. *Nat. Med.* **3**, 389–394.
- Montal, M. (2003). Structure–function correlates of Vpu, a membrane protein of HIV-1. *FEBS Lett.* **552**, 47–53.
- Morris, G.M., Goodsell, D.S., Huey, R., Hart, W.E., Halliday, S., Belew, R. *et al.* (1998). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comp. Chem.* **19**, 1639–1662.
- Neher, E. and Steinbach, J.H. (1978). Local anesthetics transiently block current through single acetylcholine-receptor channels. *J. Physiol.* **277**, 153–176.
- Park, S.H., Mrse, A.A., Nevzorov, A.A., Mesleh, M.F., Oblatt-Montal, M., Montal, M. *et al.* (2003). Three-dimensional structure of the channel-forming transmembrane domain of virus protein “u” (Vpu) from HIV-1. *J. Mol. Biol.* **333**, 409–424.
- Pautsch, A. and Schulz, G.E. (2000). High-resolution structure of the OmpA membrane domain. *J. Mol. Biol.* **298**, 273–282.
- Pavlovic, D., Neville, D.C.A., Argaud, O., Blumberg, B., Dwek, R.A., Fischer, W.B. *et al.* (2003). The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc. Natl. Acad. Sci. USA* **100**, 6104–6108.
- Pebay-Peyroula, E., Rummel, G., Rosenbusch, J.P., and Landau, E.M. (1997). X-ray structure of bacteriorhodopsin at 2.5 Å from microcrystals grown in lipid cubic phases. *Science* **277**, 1676–1681.
- Pinto, L.H., Holsinger, L.J., and Lamb, R.A. (1992). Influenza virus M2 protein has ion channel activity. *Cell* **69**, 517–528.
- Premkumar, A., Wilson, L., Ewart, G.D., and Gage, P.W. (2004). Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. *FEBS Lett.* **557**, 99–103.
- Root, M.J., Kay, M.S., and Kim, P.S. (2001). Protein design of an HIV-1 entry inhibitor. *Science* **291**, 884–888.
- Roux, B. and Karplus, M. (1991). Ion transport in a model gramicidin channel: Structure and thermodynamics. *Biophys. J.* **59**, 961–981.
- Sansom, M.S., Tieleman, D.P., Forrest, L.R., and Berendsen, H.J. (1998). Molecular dynamics simulations of membranes with embedded proteins and peptides: Porin, alamethicin and influenza virus M2. *Biochem. Soc. Trans.* **26**, 438–443.
- Sass, H., Buldt, G., Gessenich, R., Hehn, D., Neff, D., Schlesinger, J. *et al.* (2000). Structural alterations for proton translocation in the M state of wild-type bacteriorhodopsin. *Nature* **40**, 649–653.

- Schellenberg, G.D., Anderson, L., Cragoe, E.J., Jr., and Swanson, P.D. (1985). Inhibition of synaptosomal membrane  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange transport by amiloride and amiloride analogues. *Mol. Pharmacol.* **27**, 537–543.
- Schild, L., Schneeberger, E., Gautschi, I., and Firsov, D. (1997). Identification of amino acid residues in the alpha, beta, and gamma subunits of the epithelial sodium channel (ENaC) involved in amiloride block and ion permeation. *J. Gen. Physiol.* **109**, 15–26.
- Schubert, U., Ferrer-Montiel, A.V., Oblatt-Montal, M., Henklein, P., Strebel, K., and Montal, M. (1996). Identification of an ion channel activity of the Vpu transmembrane domain and its involvement in the regulation of virus release from HIV-1-infected cells. *FEBS Lett.* **398**, 12–18.
- Shen, L., Shen, J., Luo, X., Cheng, F., Xu, Y., Chen, K. et al. (2003). Steered molecular dynamics simulation on the binding of NNRTI to HIV-1 RT. *Biophys. J.* **84**, 3547–3563.
- Shoichet, B.K., Leach, A.R., and Kuntz, I.D. (1999). Ligand solvation in molecular docking. *Protein. Struct. Func. Genet.* **34**, 4–16.
- Shrivastava, I.H. and Sansom, M.S.P. (2000). Simulations of ion permeation through a potassium channel: Molecular dynamics of KcsA in a phospholipid bilayer. *Biophys. J.* **78**, 557–570.
- Smith, A.J. and Smith, R.N. (1973). Kinetics and bioavailability of two formulations of amiloride in man. *Br. J. Pharmacol.* **48**, 646–649.
- Sramala, I., Lemaitre, V., Faraldo-Gomez, J.D., Vincent, S., Watts, A., and Fischer, W.B. (2003). Molecular dynamics simulations on the first two helices of Vpu from HIV-1. *Biophys. J.* **84**, 3276–3284.
- Sun, F. (2003). Molecular dynamics simulation of human immunodeficiency virus protein U (Vpu) in lipid/water Langmuir monolayer. *J. Mol. Mod. (Online)* **9**, 114–123.
- Sunstrom, N.A., Prekumar, L.S., Prekumar, A., Ewart, G., Cox, G.B., and Gage, P.W. (1996). Ion channels formed by NB, an influenza B virus protein. *J. Membr. Biol.* **150**, 127–132.
- Tang, P. and Xu, Y. (2002). Large-scale molecular dynamics simulations of general anesthetic effects on the ion channel in the fully hydrated membrane: The implication of molecular mechanisms of general anesthesia. *Proc. Natl. Acad. Sci. USA* **99**, 16035–16040.
- Tieleman, D.P., Marrink, S.J., and Berendsen, H.J. (1997). A computer perspective of membranes: Molecular dynamics studies of lipid bilayer systems. *Biochim. Biophys. Acta* **1331**, 235–270.
- Tikhonov, D.B. and Zhorov, B.S. (1998). Kinked-helices model of the nicotinic acetylcholine receptor ion channel and its complexes with blockers: Simulation by Monte Carlo minimization method. *Biophys. J.* **74**, 242–255.
- van Aalten, D.M.F., Amadei, A., Linssen, A.B., Eijssink, V., Vriend, G., and Berendsen, H.J.C. (1995). The essential dynamics of thermolysin: Confirmation of the hinge-bending motions and comparison of simulations in vacuum and water. *Proteins* **22**, 45–54.
- van Aalten, D.M.F., Bywater, R., Findlay, J.B., Hendlich, M., Hooft, R.W., and Vriend, G. (1996). PRODRG, a program for generating molecular topologies and unique molecular descriptors from coordinates of small molecules. *J. Comp. Aid. Mol. Des.* **10**, 255–262.
- Varghese, J.N., Laver, W.G., and Colman, P.M. (1983). Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. *Nature* **303**, 35–40.
- von Itzstein, M., Wu, W.-Y., Kok, G.B., Pegg, M.S., Dyason, J.C., Jin, B. et al. (1993). Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* **363**, 418–423.
- Wade, R.C., Clark, K.J., and Goodford, P.J. (1993). Further developments of hydrogen bond formations for use in determining energetically favourable binding sites on molecules of known structure. 1. Ligand probe groups with the ability to form two hydrogen bonds. *J. Med. Chem.* **36**, 140–146.
- Waldmann, R., Champigny, G., and Lazdunski, M. (1995). Functional degenerin-containing chimeras identify residues essential for amiloride-sensitive  $\text{Na}^+$  channel function. *J. Biol. Chem.* **270**, 11735–11737.
- Wang, C., Takeuchi, K., Pinto, L.H., and Lamb, R.A. (1993). Ion channel activity of influenza A virus  $\text{M}_2$  protein: Characterization of the amantadine block. *J. Virol.* **67**, 5585–5594.
- Wang, J., Kollman, P.A., and Kuntz, I.D. (1999). Flexible ligand docking: a multistep strategy approach. *Protein Struct. Func. Genet.* **36**, 1–19.
- Wang, J., Morin, P., Wang, W., and Kollman, P.A. (2001). Use of MM-PBSA in reproducing the binding free energies to HIV-1 RT of TIBO derivatives and predicting the binding mode to HIV-1 RT of efavirenz by docking and MM-PBSA. *J. Am. Chem. Soc.* **123**, 5221–5230.
- Willbold, D., Hoffmann, S., and Rösch, P. (1997). Secondary structure and tertiary fold of the human immunodeficiency virus protein U (Vpu) cytoplasmic domain in solution. *Eur. J. Biochem.* **245**, 581–588.
- Wlodawer, A. and Erickson, J.W. (1993). Structure-based inhibitors of HIV-1 protease. *Annu. Rev. Biochem.* **62**, 543–585.
- Wong, C.F., Zheng, C., Shen, J., McCammon, J.A., and Wolynes, P.G. (1993). Cytochrome c: a molecular proving ground for computer simulations. *J. Phys. Chem.* **97**, 3100–3110.

- Woolley, G.A. and Wallace, B.A. (1992). Model ion channels: Gramicidin and alamethicin. *J. Membr. Biol.* **129**, 109–136.
- Wray, V., Federau, T., Henklein, P., Klabunde, S., Kunert, O., Schomburg, D. *et al.* (1995). Solution structure of the hydrophilic region of HIV-1 encoded virus protein U (Vpu) by CD and <sup>1</sup>H NMR-spectroscopy. *Int. J. Pept. Protein Res.* **45**, 35–43.
- Wray, V., Kinder, R., Federau, T., Henklein, P., Bechinger, B., and Schubert, U. (1999). Solution structure and orientation of the transmembrane anchor domain of the HIV-1-encoded virus protein U by high resolution and solid-state NMR spectroscopy. *Biochemistry* **38**, 5272–5282.
- Yang, C., Jas, G.S., and Kuczera, K. (2001). Structure and dynamics of calcium-activated calmodulin in solution. *J. Biomol. Struct. Dyn.* **19**, 247–271.
- Yang, H., Yang, M., Ding, Y., Liu, Y., Lou, Z., Zhou, Z. *et al.* (2003). The crystal structure of severe acute respiratory syndrome virus main protease and its complex with an inhibitor. *Proc. Natl. Acad. Sci. USA* **100**, 13190–13195.
- Zheng, S., Strzalka, J., Jones, D.H., Opella, S.J., and Blasie, J.K. (2003). Comparative structural studies of Vpu peptides in phospholipid monolayers by X-ray scattering. *Biophys. J.* **84**, 2393–2415.
- Zhu, F., Tajkhorshid, E., and Schulten, K. (2002). Pressure-induced water transport in membrane channels studied by molecular dynamics. *Biophys. J.* **83**, 154–160.