Beyond Membrane Protein Structure: Drug Discovery, Dynamics and Difficulties

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Abstract

Most of the previous content of this book has focused on obtaining the structures of membrane proteins. In this chapter we explore how those structures can be further used in two key ways. The first is their use in structure based drug design (SBDD) and the second is how they can be used to extend our understanding of their functional activity via the use of molecular dynamics. Both aspects now heavily rely on computations. This area is vast, and alas, too large to consider in depth in a single book chapter. Thus where appropriate we have referred the reader to recent reviews for deeper assessment of the field. We discuss progress via the use of examples from two main drug target areas; G-protein coupled receptors (GPCRs) and ion channels. We end with a discussion of some of the main challenges in the area.

Keywords

Structure based drug design • Molecular dynamics • Simulation • Ion channels • Glutamate receptor • G-protein coupled receptor • Docking • Virtual screening

12.1 Structures in Drug Design

One of the most active areas where recent crystal structures have begun to inform drug design in a significant way has been the GPCR renaissance (Jazayeri et al. 2015). In the past, structure based drug design (SBDD) has been the preserve of soluble proteins that readily crystallise. Membrane proteins have remained problematic because of the difficulties associated with generating high-resolution data. However, as described elsewhere in this book, various advances have

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begun to make SBDD a real possibility for many membrane protein targets including GPCRs (Tate 2012). Structures of ion channels, many of which are key therapeutic targets, have also started to appear.

One structure on its own can be tremendously informative, but the appearance of many related structures allows comparative analysis to be done, which can help to define conserved motifs and highlight functionally relevant positions. This is exactly what has been done for GPCRs and has shown conserved interaction networks and characteristic features of ligand binding (Venkatakrishnan et al. 2013). It can also lead to unifying theories of activation (Tehan et al. 2014). Moreover, such analysis is tremendously helpful in developing tools that can be used to assess the quality of models derived from simulation and modelling (Heifetz et al. 2013a). This kind of approach, in conjunction with the huge wealth of mutagenesis data (Isberg et al. 2014), is part of the reason for the renewed interest in GPCRs as drug targets. Today, crystal structures exist for almost every representative sub-branch of the GPCR genome.

A similar approach has been adopted for the analysis of the cys-loop family of ion channels (Spurny et al. 2015) although the variation in the structures, and the fact that many are bacterial in origin, makes cross-comparative work like this more difficult to interpret. The cys-loop family of receptors contains a large number of therapeutic targets, particularly for the treatment of central nervous system (CNS) related disorders. For example, the homomeric α7 nicotinic acetylcholine receptor has been considered a target for the treatment of many disease states associated with cognitive impairment (Uteshev 2014) and there have been several reports of allosteric modulators which may offer therapeutic potential in the future (Young and Geyer 2013).

Ionotropic glutamate receptors on the other hand have the distinct advantage that the ligand-binding domain can be expressed as a soluble construct (that maintains almost wild type binding affinity (Armstrong and Gouaux 2000)) and is readily crystallisable. The AMPA subtype has been recognised as a therapeutic target for neurological disorders including schizophrenia (Ward et al. 2010, 2015; Partin 2015), and structural information has been an important factor in drug design considerations (Harms et al. 2013). Indeed, crystallography has already been integrated with lead optimization methods for the development of new positive allosteric modulators of AMPA receptors (Ward et al. 2010, 2015). A number of patents in this area have been filed (Pirotte et al. 2013) and it also appears to be an area in which academic laboratories are also starting to contribute directly to (Partin 2015; Harms et al. 2013; Caldwell et al. 2015; Chen et al. 2013; Jamieson et al. 2011; Timm et al. 2011; Weeks et al. 2014). Another type of glutamate receptor, the NMDA receptor, can also be modulated in an allosteric fashion at different sites as recently reviewed by Strong et al. (2014). Indeed there is renewed interest in NMDA receptors as therapeutic targets and structural information is starting to guide this in a rational way (Khatri et al. 2014).

Rational approaches to designing compounds acting at the orthosteric binding site of glutamate receptors have also been employed (Demmer et al. 2015; Venskutonytė et al. 2014; Assaf et al 2013; Juknaitė et al 2012; Bunch and Krogsgaard-Larsen 2009; Sivaprakasam et al. 2009). An important goal at this point is to develop sub-type specific compounds, not necessarily as drugs, but as chemical probes. One of the main issues with targeting glutamate receptors is targeting the correct receptor subunit in right part of the brain, but at the present time this is hindered by a lack sub-type specific chemical probes.

In the next sections we briefly highlight some of the principles employed before going on to describe some case studies where these approaches have been used in on going drug-design projects.

12.1.1 In-sillico SBDD

Once one has the structure of a particular target, it can be used as the basis for SBDD and/or virtual screening (Forli and Olson 2015). This is often considered the most promising approach for the discovery of new ligands (Lounnas et al. 2013).

The presence of structural information in a drug-discovery program can lead to a step change in progress; Structure activity relationship (SAR) results can be rationalised, new avenues can be rationally explored and improved hypotheses generated.

In the absence of a structure, drug design typically precedes using ligand-based techniques, but these have well known limitations. Aside from the obvious limitation of the lack of information on how the ligand interacts with its target, it is often the case that any newly discovered molecules will have a similar chemotype to the parent molecule(s) and that can lead to intellectual property (IP) protection problems which render the process non-viable. Additional problems are that activity cliffs cannot be easily rationalized and it is not always possible to isolate effects of chemical modifications. If structural information is available with an important drug or lead compound, then new interactions between ligands and the target can be devised on simple chemical intuition and molecular mechanics principles. It is also easier to design molecules that are completely novel from an IP sense.

Structure-based virtual screening (SBVS) (Lionta et al. 2014) is a useful tool in terms of providing initial filtering of huge chemical libraries and to provide plausible suggestions (Cerqueira et al. 2015) that can be taken forward in a drug-discovery program (Shoichet 2004). However, the detailed prediction of precise modes of action and prediction of affinity, or even more difficult, efficacy (see below in Sect. 12.3.2), is still incredibly challenging and indeed over the years this aspect was arguably overhyped (Seddon et al. 2012). Despite these limitations, the low cost of virtual screening means that it is something employed in most drug design strategies to provide some initial assessment of potential binding modes.

SBVS has been around for a number of years and its development and use have been reviewed substantially (Lounnas et al. 2013; Lionta et al. 2014; Cheng et al. 2012). SBVS is based on high throughput docking where one has the structure of a target (protein usually but not necessarily) and a large (thousands to millions) compounds library. Compounds from this library are then docked to this target structure and ranked according to some kind of scoring function. SBVS relies on these functions, but whilst the ability of these methods to predict accurate poses (typically defined as within 2 Å RMSD of the crystallographic model) is quite reasonable, expert knowledge is often required to obtain that level of performance (Cross et al. 2009). Care must also be taken to ensure that due consideration is given to factors like explicit solvent and the flexibility of the binding pocket (Elokely and Doerksen 2013).

12.1.2 Homology Modelling of Related Targets

Although there has been a steady increase in the number of membrane protein structures being solved, there are still a large number of important targets for which no high resolution structure is available and thus if one wants to pursue any kind of structure-based approach, homology modelling is the only viable solution (Schmidt et al. 2014). Furthermore, even if structural information is available for one complex, if the timescale of obtaining that data is too slow, then homology modelling is often used instead. It is generally considered that in order for homology models to be useful for screening, there needs to be a high quality template with high sequence similarity, although it has been argued that for some approaches, such as off-target prediction, low-to moderate resolution structures can still be of value (Skolnick et al. 2013). Indeed it has also been argued for GPCRs that theoretical models can perform equally as well for some aspects of the drug discovery process (Tang et al. 2012). Similarly, a retrospective comparison for the Dopamine D3 receptor also concluded that well-built homology models can perform as well as crystal structures in terms of *in silico* docking (Levoin et al. 2011). Even in cases where the sequence identity to the target is very low, careful model building in conjunction with site-directed mutagenesis and binding assays can be very useful in aiding the future direction of a

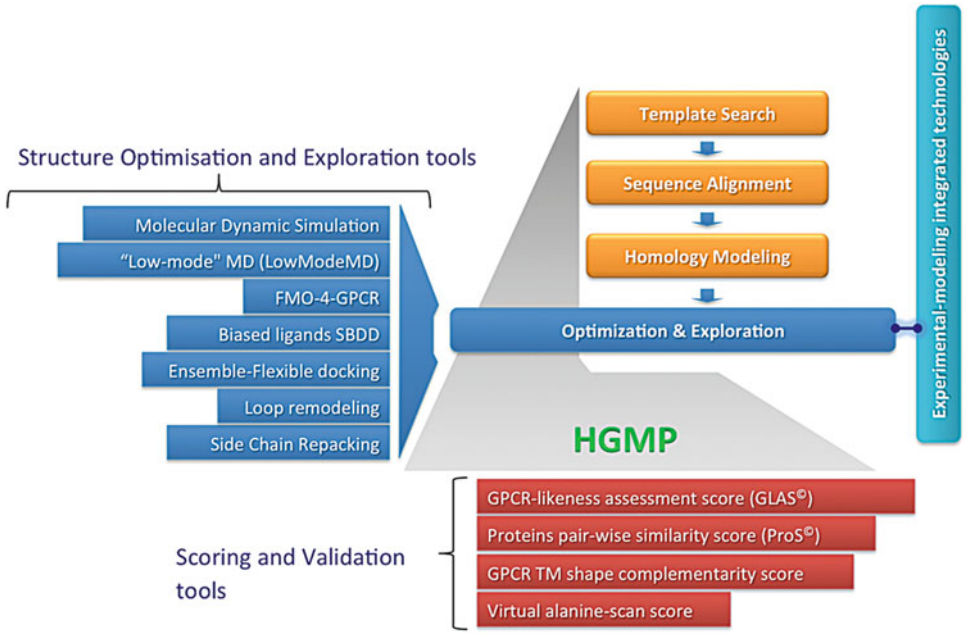

Fig. 12.1 A summary schematic of the Hierarchical GPCR Modelling Protocol (HGMP) developed at Evotec in collaboration with the University of Oxford, illustrating the various components that contribute to the modelling procedure

drug discovery program or indeed rationalizing existing SAR data (Lee et al. 2015).

These approaches can be incorporated into workflows. An example such workflow is the hierarchical GPCR modelling protocol (HGMP), which was developed to support SBDD programs (see Fig. 12.1). The HGMP generates a GPCR model and its potential complexes with small molecules by applying a series of computational methods in a workflow. The protocol makes use of homology modelling following by Molecular dynamics (MD) simulations and docking to predict binding poses and functions of ligands bound to GPCRs. The HGMP is equipped with GPCR-specific "plugins" including for example the GPCR-likeness assessment score (GLAS) to evaluate model quality (Heifetz et al. 2013a). The HGMP also includes a pairwise protein comparison method (ProS) used to cluster structural data and can distinguish between different activation sub-states (Heifetz et al. 2013a). Recently, the capabilities of HGMP have been extended by the addition of GPCR biased ligand tools. The HGMP has been used within real drug-discovery projects at the biotech company Evotec, some of which we briefly outline as case studies below.

12.1.3 SBDD Case Studies

12.1.3.1 Discovery of Selective 5-HT$_{2C}$ Agonists for the Treatment of Metabolic Disorders

In this project, which was performed prior to the crystal structures of 5-HT$_{2B}$ and 5-HT$_{1B}$ being solved, the challenge was to find novel 5-HT$_{2c}$ agonists that were selective in that they did not activate 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors. The HGMP was applied to model both active and inactive receptor conformations, referred to as 5-HT$_{2C}^{active}$ and 5-HT$_{2C}^{inactive}$ respectively. Models were also built of the off targets, 5-HT$_{2A}$ and 5-HT$_{2B}$. Flexible docking was then applied to predict the binding modes of compounds with 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$. The binding site of 5-HT$_{2C}^{inactive}$ was proposed to be shallower compared to the binding site of 5-HT$_{2C}^{active}$ due to residues from TM3 and TM6 forming

stabilizing inter-helical interactions in the 5-$HT_{2C}^{inactive}$ binding site. It was proposed that these inter-helical interactions are broken in the active conformation of the 5-HT_{2C} receptor, which is stabilized by agonist molecules entering deeper into the binding site and compensating *via* interactions with various other residues, including $W324^{6.48}$, a key residue previously identified as a "transmission switch" residue (Han et al. 1997; Holst et al. 2010; Schwartz et al. 2006) and which may form part of a larger "hydrophobic hindering mechanism" recently suggested (Tehan et al. 2014). Agonists were suggested to interact with $W324^{6.48}$, thereby pushing the intracellular half of TM6 in to the active conformation. Agonists were proposed to interact simultaneously with both TM3 and TM6 in 5-HT_{2C}^{active} thus increasing the overall stability of 5-HT_{2C}^{active} and promoting activation. Furthermore, these modeling observations (which are directly supported by the published SDM data) were incorporated into the design of novel 5-HT_{2C} agonists (Tye et al. 2011). Hits were also assessed for hERG liability via docking to a model of the hERG channel. The result was the discovery of a novel compound (EC_{50} = 8.4, 762, 73 nM for 5-$HT_{2C, 2A, 2B}$ and hERG inhibition of 11 % @ 10 CM). The whole design cycle for this project can be summarized in Fig. 12.2.

12.1.3.2 Fighting Obesity with a Sugar-Based Library

Obesity is an increasingly common disease. Although antagonism of the melanin-concentrating hormone-1 receptor (MCH-1R) has been widely reported as a promising therapeutic avenue for obesity treatment, no MCH-1R antagonists have reached the market. Discovery and optimization of new compounds targeting MCH-1R was hindered by low high throughput screening (HTS) success rates and a lack of structural information about the MCH-1R binding site. In this project, a conceptually pioneering approach that integrated GPCR modelling with design, synthesis and screening of a diverse library of

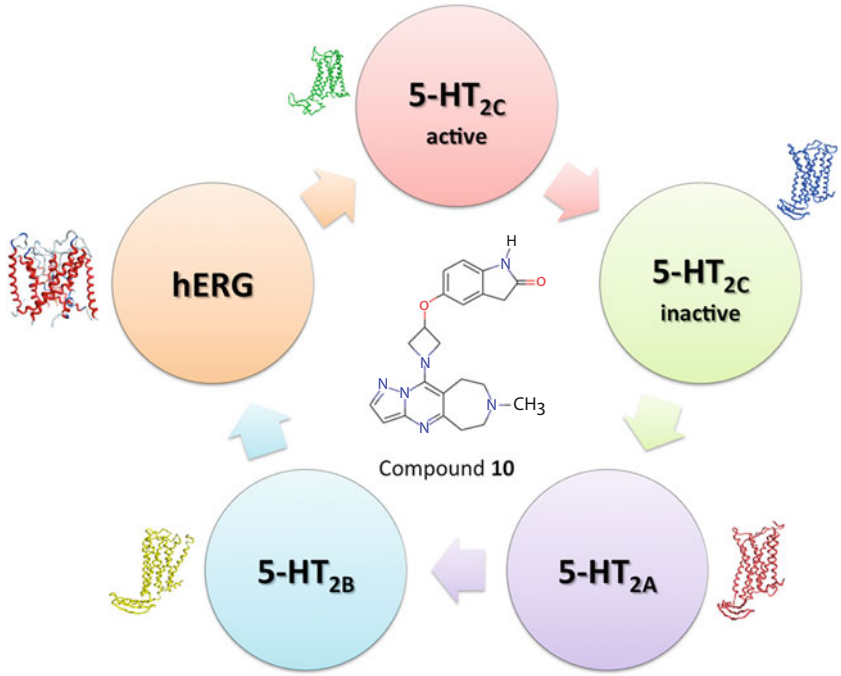

Fig. 12.2 Design cycle for potent, selective and hERG 'clean' 5HT_{2C} antagonists

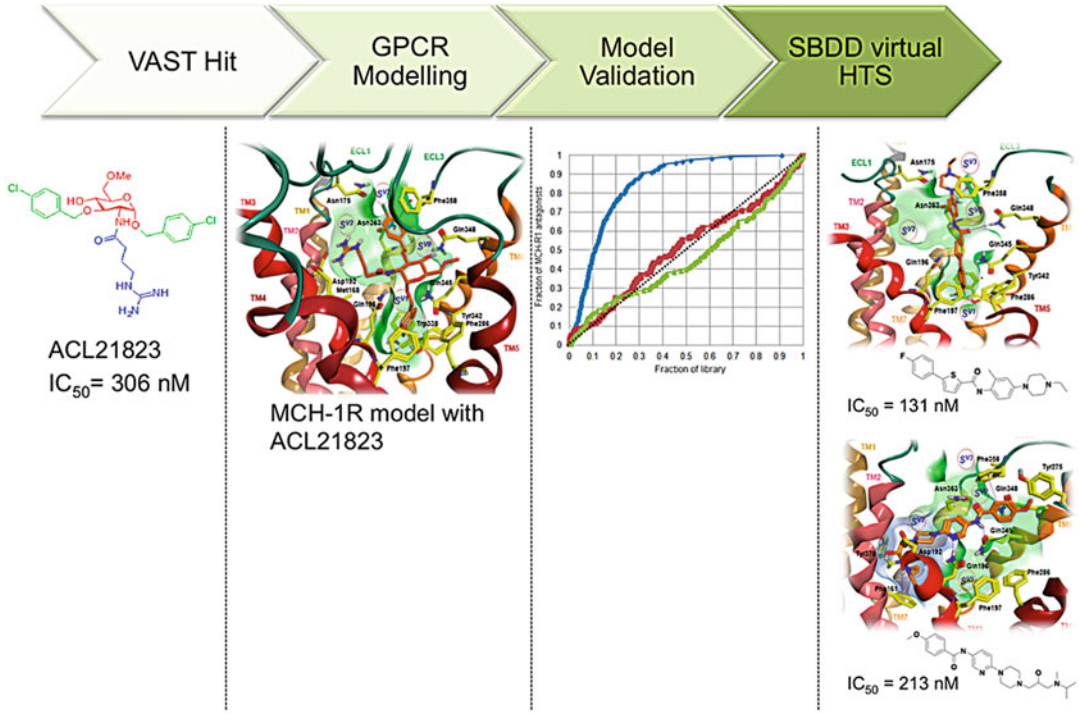

Fig. 12.3 Summary schematic of the VAST-GPCR modelling workflow which led to the discovery of new MCH-1R antagonists

sugar-based compounds from the VAST technology (Versatile Assembly on Stable Templates) was used, to provide structural insights on the MCH-1R binding site (Heifetz et al. 2013a, b, c). The 490 VAST compounds obtained from this library design were screened against MCH-1R, resulting in the discovery of a potent MCH-1R antagonist, ACL21823 (radioligand binding to MCH-1R gave an $IC_{50} = 306$ nM, see Fig. 12.3). The discovery of ACL21823 was utilized in the construction of a high quality MCH-1R model and the refinement of its antagonist binding site. The quality of the MCH-1R model was demonstrated by a virtual enrichment experiment and the model-driven structure-based expansion of ACL21823, which allowed the generation of a list of key MCH-1R residues potentially involved in antagonist binding. The GPCR-VAST method demonstrates how ligand SAR data, when combined with modelling, can provide a useful source of structural information on GPCR binding sites (Heifetz et al. 2013a, b, c). The usefulness of the GPCR-VAST method to drug discovery was demonstrated by a structure-based virtual screen, which achieved a hit rate of 14 % and yielded 10 new chemotypes of MCH-1R antagonists including EOAI3367472 ($IC_{50} = 131$ nM) and EOAI3367474 ($IC_{50} = 213$ nM). This approach creates a cost-efficient new avenue for structure-based drug discovery (SBDD) against GPCR targets.

12.1.3.3 Discovery of Potent and Selective OX_2 Receptor Antagonists

The orexin receptors (OX_1 and OX_2) are linked to a range of different physiological functions including the control of feeding, energy metabolism, modulation of neuro-endocrine function and regulation of the sleep-wake cycle. The key challenges of this project were to increase the OX_2 activity and selectivity over OX_1. This was particularly difficult as OX_1 and OX_2 receptors have over 80 % sequence similarity. This project was completed before the

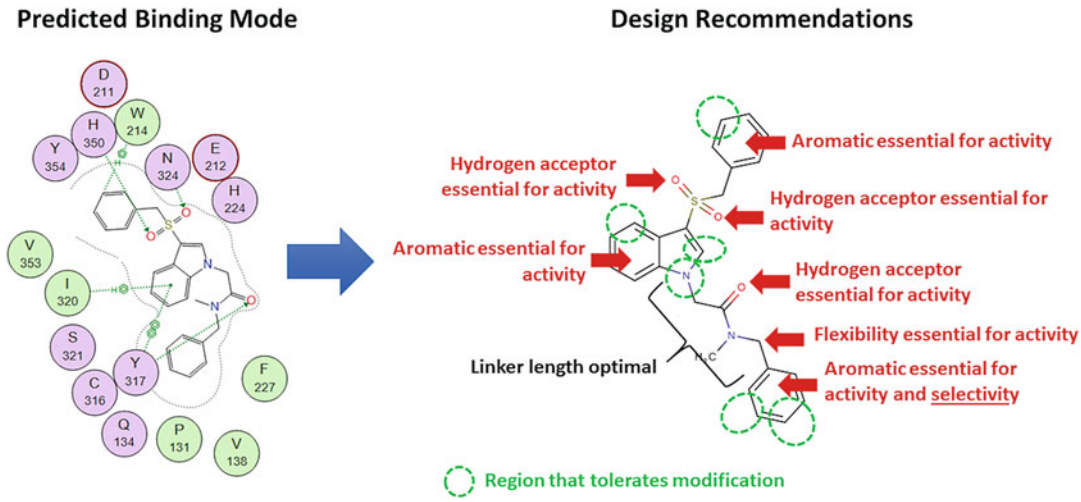

Fig. 12.4 Schematic summarizing how interaction maps derived from structural models and MD data can be used to provide synthesis recommendations

crystal structure of OX_2 bound to Suvorexant was solved. We used Molecular Dynamics (MD) simulations to study the OX_1 vs OX_2 selectivity (Heifetz et al. 2012). The MD process allowed refinement of the models that was not possible with static crystal structures or homology models alone. This study suggested that differences in intra-helical interactions resulted in differences in conformations of transmembranes (TMs) and differences in topology of the binding pocket. These are small differences but sufficient enough to design molecules with OX_2 selectivity. This rational design significantly decreased the amount of synthesis by focusing the effort to the relevant portion of the ligand structure as demonstrated in Fig. 12.4. The final compound, EP-009-0513, had inhibition constants, K_i, of 4363 and 5.7 nM for OX_1 and OX_2 respectively.

12.1.3.4 Discovery of Selective Dual Antagonists of H_3 and H_4 Receptors

An approach that integrates the HGMP with fragment based drug discovery (FBDD) had been applied for the discovery of selective and dual H_3 and H_4 histamine receptor antagonists (see Fig. 12.5). FBDD has emerged as a new tool for drug discovery in recent years and is typically aimed at a target for which a crystal structure can be determined in order to rationally guide fragment-hit expansion. While the majority of historical fragment screens have been focused towards biochemical targets, only a few examples have been published in which this method has been used to identify ligands for GPCRs. This is due to the current infeasibility of regularly crystallizing the GPCR-fragment complexes that are essential for further fragment expansion.

The HGMP was used to generate initial models of these receptors. Primary fragment screens yielded 44 H_3 selective, 21 H_4 selective and 20 dual selective fragment hits. These fragments were used to construct new high-quality H_3 and H_4 models followed by binding site exploration and a structure based virtual screen. Overall, 172 compounds were purchased for testing based on these virtual screening results. Of the 74 compounds predicted to have dual activity, 33 had activity against one or other of the two receptors (44 %), of which 17 had activity against both. Of the 19 compounds predicted to be H_3 selective, 13 were active against H_3 (68 %) and 10 of these also had selectivity over H_4. Of the 79 compounds predicted to be H_4 selective, 36 were active against H_4 (45 %) and 2 of these also had selectivity over H_3. This approach highlights a cost-efficient avenue for structure-based drug

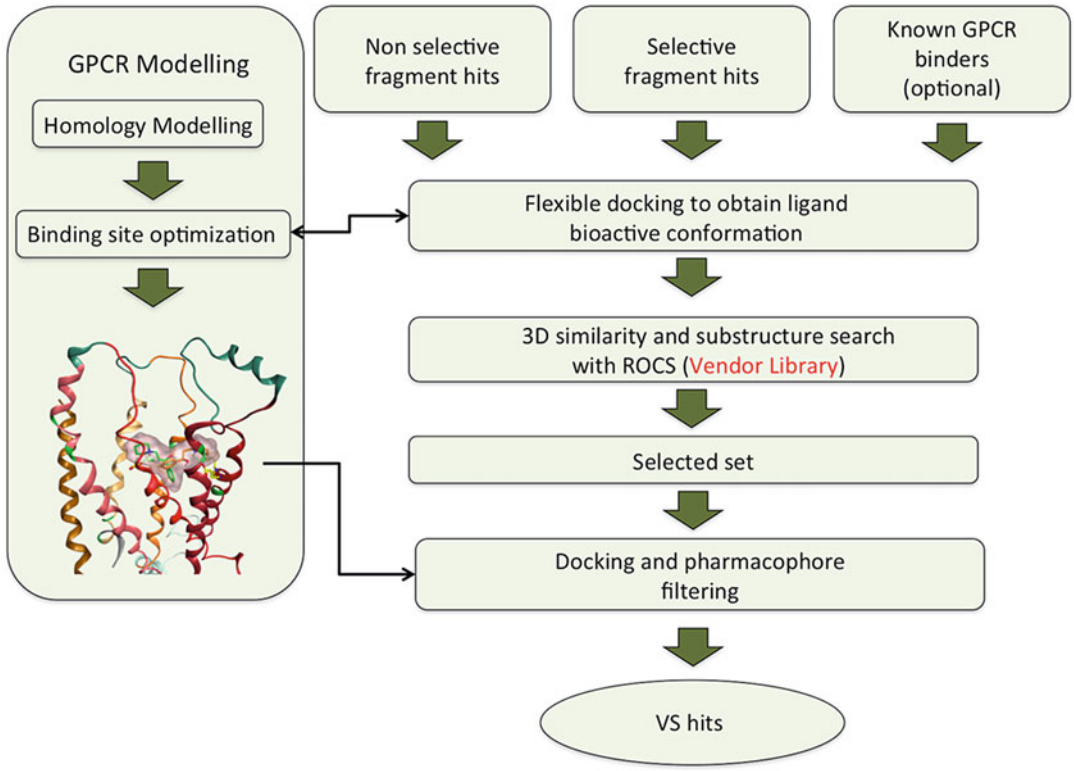

Fig. 12.5 Schematic of the HGMP-FBDD workflow to perform virtual screening

design (SBDD) against GPCR targets (Heifetz et al. 2013b).

12.2 Exploration of the Dynamic Nature of Membrane Proteins

Membrane proteins, by functional necessity, are often very dynamic entities. Indeed, this is one of the reasons why they have been recalcitrant to crystallization efforts. Ion channels have gating machinery for example, whilst GPCRs need to enable a wide variety of signalling pathways through changes in their response to different ligands. Crystal structures and modelling can provide a step-change in our understanding of membrane proteins and in the processes outlined above with SBDD. However, the structure is also the start point for one to be able to examine these dynamic responses and consequently address more complex questions about receptor flexibility and function. Molecular dynamics simulations can be used in a variety of ways including refinement of the structure in a more realistic membrane environment, the analysis of solvent and the discovery of so-called "cryptic sites" which may offer alternative pathways for therapy. We outline some of these aspects below.

12.2.1 Exploration of Membrane Protein Dynamics

Crystal structures are often obtained under conditions that are somewhat artificial and thus, care must be taken to interpret the data in a meaningful way (Wlodawer et al. 2008, 2013). MD simulations can provide refinement of homology models (as alluded to above) and can give information on lipid and solvent interactions for example. The dynamic and heterogeneous nature

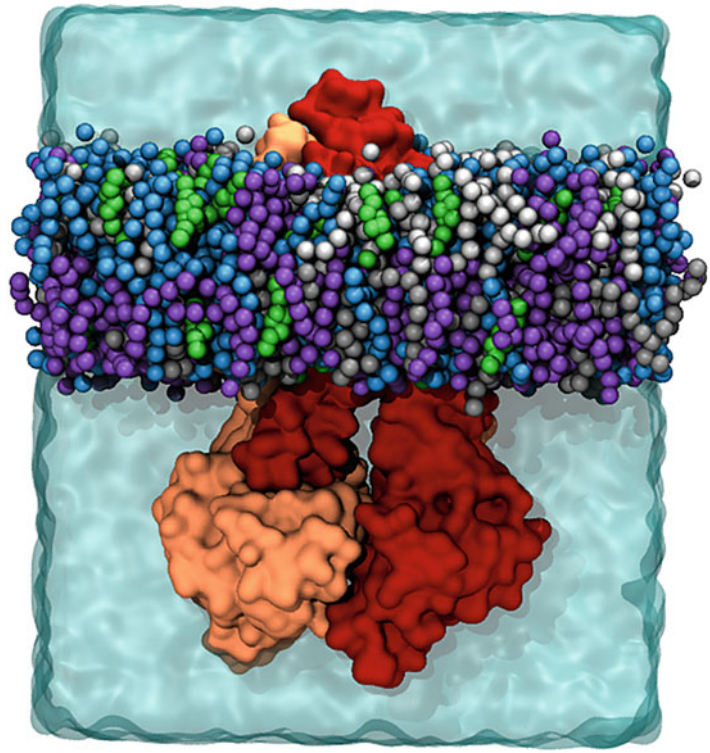

Fig. 12.6 Example simulation box. In case the protein is a homology model of human P-glycoprotein based on the refined mouse structure 4M1M (transmembrane helices 1–6 and 7–12 are *red* and *orange* respectively) embedded within a bilayer system comprised of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; *blue*), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE; *purple*) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS; *dark grey*), sphingomeylin (*light grey*) and cholesterol (*green*). The lipids in front of the protein have been removed for clarity. It is usual for researchers to sodium and chloride ions in the solvent to a concentration that is representative of in vivo or in vitro studies (150 mM NaCl for example) (Figure courtesy of Laura Domicevica)

of lipid bilayers can make the analysis of interactions with membrane proteins difficult. In a typical approach the coordinates are extracted form the PDB and inserted into a lipid bilayer (see Fig. 12.6). Many methods have been published over the years to achieve this and the reader is referred to a specific review on this aspect (Biggin and Bond 2015).

Simulations can provide a unique and molecular view of the interaction of lipids with membrane proteins. Due to its abundance in mammalian membranes, cholesterol has been investigated in great depth (Grouleff et al. 2015) particularly for GPCRs (Sengupta and Chattopadhyay 2015), but as parameters for other lipids become available we can expect analysis of other important lipids and the interactions of more complex membrane systems (Goose and Sansom 2013) including negatively charged ones (Kalli et al. 2013). There is increasing evidence that the energetics of protein-lipid interactions can directly impact the functional properties of the protein (Mondal et al. 2014a, b). Simulations can also provide atomic level information on systems where it would be at best, very challenging if not impossible, to obtain information experimentally. For example, the response of the voltage sensor of potassium channels to a transmembrane voltage can be studied with MD simulations (Jensen et al. 2012) providing unique insight into these functionally important features at an atomistic level of detail.

MD simulations also allow one to explore the possibility of allosteric and cryptic binding pockets (Frembgen-Kesner and Elcock 2006). The latter are not exposed to bulk solvent all of the time and so may be hidden in certain crystallographic structures. MD allows these sites to manifest themselves (Lukman et al. 2014) and so permit docking and similar protocols to be followed in the usual manner. Simulations are also essential for understanding the mechanisms of allosteric modulation. This aspect of GPCRs has been investigated in terms of searching for "hidden" allosteric sites, which may be potential binding pockets (Miao et al. 2014; Ivetac and McCammon 2010). In ion channels, the effect of anesthetic on members of the cys-loop receptor has been postulated to be an allosteric effect, acting at the transmembrane region of the receptor (Murail et al. 2012; Murail et al. 2011 and Salari et al. 2014).

At a more fundamental level, there are questions of trying to understand the dynamics of protein targets as fully as possible (Micheletti 2013) with a view to comparing not just the structural similarity but also the dynamic similarity (Münz et al. 2010, 2012). The power of MD simulations to improve our understanding of function has meant growing interest in its potential in drug discovery (Durrant and McCammon 2011). It can be used in conjunction with many other tools including virtual screening to improve the prospects of finding a new compound (Nichols et al. 2011). The use of MD trajectories to generate an ensemble of possible receptor conformations is best highlighted by the so-called "relaxed complex scheme" (Amaro et al. 2008; Lin et al. 2002), where there has been some success in for soluble targets like HIV integrase (Schames et al. 2004) and the tumor suppressor, p53 (Wassman et al. 2013). The increasing viability of MD simulations with an ever-growing appreciation of the role of protein flexibility and solvent has meant that simulations are starting to attract the attention of the industrial community (Moroni et al. 2015).

12.2.2 The Role of Water Molecules in Receptor-Ligand Binding

Only high-resolution crystal structures will give any reliable indication as to the presence of waters molecules, yet it is known that water-mediated interactions between ligands and protein targets are extremely common (Lu et al. 2007). As it has been shown that the displacement of ordered water molecules can directly affect a ligand's binding affinity (Clarke et al. 2001; Lam et al. 1994), this has been the focus of many drug design strategies with the aim of designing compounds that can displace these waters (Lam et al. 1994; Chen et al. 1998; Wissner et al. 2000).

As one often does not have the presence of water molecules in key positions confirmed by experiment, the first task is the prediction of these sites. In recent years, many methods have been developed to tackle this problem and in general the results are good. Knowledge of the presence of water in binding sites can be useful in its own right and indeed several reports over the years have demonstrated how this is useful for membrane proteins including ligand-gated receptors (Sahai and Biggin 2011; Vijayan et al. 2010; Yu et al. 2014) and GPCRs (Mason et al. 2012).

The issue then is to compute whether it is likely that the water will be displaceable and indeed whether that displacement will give a favourable contribution to the overall free energy of binding. This latter aspect has proven surprisingly difficult to achieve reliable results for, though there have been some reported success mainly for soluble proteins (Mondal et al. 2014a, b; Pearlstein et al. 2013). The prediction of water molecule networks and their perturbation has also been examined in terms of the relationship to kinetics (and residence time – see Sect. 11.3.3 for a series of adenosine A2A receptor antagonists (Bortolato et al. 2013).

Running MD or Monte Carlo (MC) simulations and observing the peaks in water density (Henchman and McCammon 2002; Alvarez-Garcia and Barril 2014) can provide the location

of water binding sites. However, these can be time-consuming to run, especially with buried cavities due to the long time it takes for water to permeate within the protein. Grand Canonical Monte Carlo methods can significantly reduce the length of the simulation, though even that can be quite demanding on resource. Thus, there have been several attempts to develop fast methods. JAWS for example is a grid-based MC method that estimates the free energy of displacing a water molecule into bulk (Michel and Essex 2010; Michel et al. 2009). An integral theory approach (3D-RISM) has also reported success in predicting solvation structure within ligand-binding sites (Imai et al. 2009) and protein cavities (Imai et al. 2007). Short molecular simulations can be used as the data for inhomogeneous fluid solvation theory (IFST), as reported by Lazaridis (1998a, b). This method has the distinct advantage that the free energy can be broken down into enthalpic and entropic components (Li and Lazaridis 2003, 2005a, b). IFST also forms the framework for WaterMap and has been used in number of cases (Abel et al. 2008; Robinson et al. 2010; Young et al. 2006) including glutamate receptors (Frydenvang et al. 2010), the ompC channel (Tran et al. 2013) and GPCRs (Higgs et al. 2010; Newman et al. 2012)

An even faster method, that exploits the docking program AutoDock Vina (Trott and Olson 2010) was found to reproduce water positions to a high degree of accuracy and could also predict whether a water molecule was displaced or conserved to an accuracy of 75 % (Ross et al. 2012). Figure 12.7 shows an example prediction for AMPA bound to ligand-binding domain of the GluA2 ionotropic glutamate receptor. This compromise between speed and accuracy may be desirable at the high-throughput stage of virtual screening.

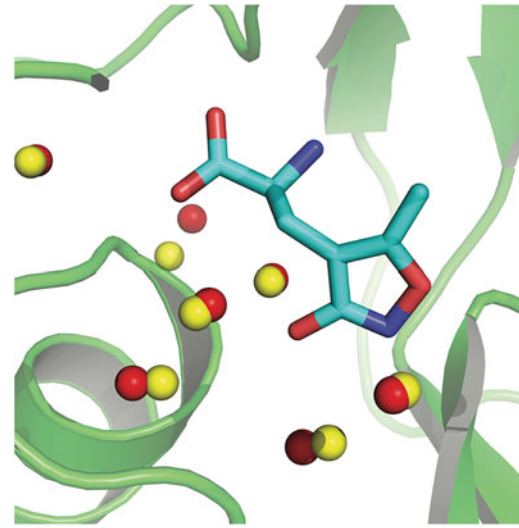

Fig. 12.7 An example of water position prediction from the WaterDock program performed on the ligand-binding domain of the GluA2 ionotropic glutamate receptor in complex with AMPA (shown in liquorice sticks). *Red spheres*: water molecules observed in at least two crystal structures. *Yellow spheres*: predicated water sites. WaterDock is able to predict all of the crystallographically observed water molecules (Figure taken from Ross et al. (2012))

12.3 Challenges for the Future

Experience has shown that the deepest insight can only be achieved when there is good interdisciplinary collaboration between experimental and theoretical groups. Challenges that will require this approach include understanding the conformational changes that are ligand dependent in GPCRs and how those are conveyed to the intracellular signalling cascades (see Bermudez and Wolber 2015 for a recent review). Properties such as accurate prediction of affinity, kinetics, the ever-increasing size and amount of data and the integration of structure into higher-order models are also areas of increasing interest. In this final section, we outline some of these challenges.

12.3.1 Deeper Understanding of Receptor-Ligand Interactions

As we have discussed, structural information of the target plays a central role in the rationalization, efficiency and cost-effectiveness of the drug design process. However, even with the crystal structure in hand, the simple molecule mechanics based approaches to rationalising affinity cannot always explain the full complexity of the

chemical interactions between ligand and target. Quantum mechanics (QM) can provide the most complete description of the interactions including otherwise neglected components such as charge fluctuations and dynamic polarization that can make significant contributions to affinity. However, traditional QM methods are simply not feasible for large biological systems because of their huge computational cost. In recent years though, new QM based methods, such as the fragment molecular orbital (FMO) method developed by Fedorov and Kitaura offers a way forward (Fedorov and Kitaura 2007). The FMO method gives considerable computational speed up over other traditional QM methods and can be applied to membrane proteins and their ligand-complexes. Furthermore, the FMO method has the potential to contribute to the refinement process in terms of X-ray crystallographic data with drug complexes. This better understanding of the enthalpic contributions can help chemists in an intuitive way. However, the omission of entropic effects must be kept in mind and the prediction of the overall free energy of binding, ΔG, is still a major challenge as we discuss in the next section.

12.3.2 Affinity and Efficacy

Although docking programs generally do a reasonable job of pose-prediction, the correct prediction of binding affinity or even predicting the order of binding for a series of compounds, is much more error prone. The development of a generic scoring function that can successfully rank ligands across diverse targets is unlikely to be forthcoming in the near future and indeed it has been mathematically proven that specialized functions will always out-perform any generic scoring function (Ross et al. 2013). At the molecular level, a drug must associate with the receptor in order to cause a response, and the strength of such association is described by its affinity. The availability of structural data was thought to directly provide the information needed to interpret ligand-protein interactions and estimate the affinity of small molecules for any binding pocket (Beddell et al. 1976; Cohen 1977). It was soon realised however that while structural data is necessary, it is not sufficient on its own to describe drug-receptor association as it is in fact a complex process, with significant entropic and solvent effects in most instances that can hardly be explained by structure observation alone (Marshall 2012; Mobley and Dill 2009; Gilson and Zhou 2007). For these reasons, despite decades of efforts in computational studies on the effects of ligand binding to a receptor, the ability to predict affinity is still challenging. Nonetheless, in the last decade there have been continuous improvements in theory and computation that are improving binding affinity prediction methodologies (Chodera et al. 2011; Chipot 2014).

Currently, the most rigorous statistical mechanics approaches to estimate affinities rely upon Molecular Dynamics or Monte Carlo simulations for the sampling of the receptor, ligand and solvent conformations and their associated energies (Chipot 2014; Michel et al. 2010; Gohlke and Klebe 2002). The so called "alchemical" methods are based on a non-physical thermodynamic cycle, where binding free energy is computed as the sum of multiple steps during which the ligand is removed from the solution and inserted into the binding pocket. Steered or pulling methods follow instead a physical pathway, by applying a force that pulls the ligand away from the protein and calculating the work involved in this process (Chipot 2014). The advantage over scoring functions or implicit solvent approaches is that the full flexibility of protein and ligand is taken into account, as well as the discrete nature of the solvent.

While the prediction of absolute binding affinities still faces many challenges, the estimation of relative binding free energies, i.e. the difference in binding affinities between two ligands, appears to be more mature and ready to be applied to a wide range of biological targets (Shirts et al. 2007; Mobley and Klimovich 2012). Recent studies have shown how the prediction of relative affinities can guide medicinal chemistry efforts in lead optimisation (Jorgensen 2009). For instance, the Jorgensen lab has combined computational and medicinal chemistry in the development of potent HIV reverse transcriptase inhibitors (Bollini et al. 2011; Jorgensen et al. 2011). In one study, Bollini et al. used

relative affinity calculations to identify the most promising modifications for an initial 5 μM affinity, which was later turned into a sub-nanomolar ligand. The authors demonstrated how the use of computational methods was pivotal for the identification of optimal substitution patters (Bollini et al. 2011). Similarly, Jorgensen et al. reported the evolution of three low affinity hits into potent inhibitor (<10 nM) of both the wild type and Y181C variant of HIV-1 reverse transcriptase. The use of free energy calculations allowed the identification of these potent and dual-target inhibitors while synthesizing only about 30 compounds (Jorgensen et al. 2011).

Wang et al. (2015) reported instead a large retrospective study with over 200 ligands and 10 protein targets, and using an improved force field. The authors showed how the predictions correlated extremely well (weighted average R-value of 0.75) and were mostly within 1.0 kcal/mol of the experimental values. Additionally, it was shown how the use of the calculations on two prospective studies involving IRAK4 and TYK2 allowed deprioritizing a large number of compounds and enriching the synthesis of tight-binding ligands.

Predicting absolute free energies prospectively, on the other hand, is still a challenge as shown by the latest SAMPL exercise, where participants were asked to predict affinities of HIV integrase inhibitors in the catalytic core domain (Mobley 2014). Whilst in relative affinity calculations sampling issues might have minor effects (Mobley and Klimovich 2012), these are more likely to have large repercussions when calculating absolute binding free energies. Mobley et al. (2007a) showed how undersampling even a valine side-chain reorientation upon binding can lead to an error of several kcal/mol in the binding affinity prediction. For these reasons, prospective applications of absolute binding free energy are still rare and most efforts have focused on retrospective validation of the methodology. One of the most studied macromolecular systems has been the binding pocket of engineered T4 lysozymes. The binding of small fragments to such system has been calculated by Mobley et al. and Boyce et al., achieving root mean square errors just below 2 kcal/mol (Boyce et al. 2009; Mobley et al. 2007b). Another popular test system has been the FK506-binding protein (FKBP12). For this system the binding of drug-like molecules was tested computationally and RMS errors were still around 2 kcal/mol (Wang et al. 2006; Fujitani et al. 2005). More recently, Aldeghi et al. (2016) also achieved good success with absolute free energy calculations performed on a diverse set of drugs against a bromodomain. The RMS errors reported were of the order of 1 kca/mol.

While such level of accuracy would in fact be useful in a drug design context when screening compound libraries (Mobley and Klimovich 2012; Shirts et al. 2010), the performance of the approach still needs to be validated against more complex targets and small molecules. In fact, application to more complex proteins such as ion channels and GPCR where conformational changes play an important role in the function of the receptor (Jensen et al. 2012; Dror et al. 2015; Burg et al. 2015) amplify the challenges that need to be overcome in order to obtain reliable predictions. Similarly to the sampling issue of a side-chain rotation, slow degrees of freedoms like conformational changes upon binding cause significant sampling issues for the timescales currently accessible computationally.

Lin et al. (Lin and Roux 2013; Lin et al. 2013) have however, provided an elegant study that took a large conformational change into account when calculating binding affinity by dividing the calculation in two steps, showing how such calculations are still feasible given sufficient knowledge of the system at hand. Investigating the molecular reasons of Gleevec selectivity, the free energy change from DFG-in to –out conformations of Abl and c-Src kinases was first calculated, followed by the affinity calculation of Gleevec for both DFG-out conformations. The selectivity of Gleevec for Abl over c-Src was found to be a combination of conformational selection, due to the larger work needed to move the loop in c-Src, and differences in binding affinities to the DFG-out conformation (Lin and Roux 2013; Lin et al. 2013).

Considering the challenges that binding affinity predictions face, it is clear that predicting efficacy will be even more challenging at least in

terms of separating the effects out from affinity. Additionally, efficacy is likely not to depend only on the thermodynamic quantity of affinity, but also the kinetics of the binding event, with residence times in the binding site playing a role in the biological response of the receptor to the bound drug. Experimentally, dissecting these various contributions out is also extremely difficult. However, for some ligand-gated ion channels such as the glycine receptor (Yu et al. 2014; Lape et al. 2008), where single channel behavior can be observed, there is the genuine prospect that progress can be made in this area, although there is no doubt that it will be extremely challenging.

Overall, predicting absolute ligand binding affinities is still a challenge for most systems and in particular large, complex membrane proteins. However, it is possible to foresee a point in the near future where binding affinity predictions will be routine and part of the valuable set of computational tools available to accelerate drug discovery. On the other hand, while prediction of efficacy is the natural next step, it will need a deeper mechanistic understanding of membrane protein systems before a solid foundation for such calculations can be laid.

12.3.3 Kinetics and Its Relationship to Structure

In recent years there has been growing interest in trying to relate structure to kinetics (Swinney 2009). The kinetics of drug-binding (Keserü and Swinney 2015) are increasingly being recognized as being important for the clinical effectiveness of drugs (Cusack et al. 2015). Indeed it has been shown experimentally that there is a positive correlation between functional efficacy and its so-called "residence time" at the receptor (Guo et al. 2012, 2014). A long residence time is thought to be important in many cases as it can extend the duration of pharmacodynamic activity during the body. It can thus not only increase the *in-vivo* efficacy but also reduce the potential of off-target effects (Cusack et al. 2015). For a recent review of how residence time has been considered in the development of some compounds the reader is referred to (Hoffmann et al. 2015).

Can we extract relationships between structures (of ligands and/or proteins) and their kinetics (so called Structure-kinetics-relationships, SKRs)? This area of research is still young, but there is tremendous interest in it, because the human body is anything but in equilibrium, and thus kinetics is presumably very important.

As with initial use of affinity, there was some naivety concerning assumptions (for example, that similar ligands would have similar k_{on} rates, etc). The situation has been shown, at least for soluble proteins (like kinases), to be more complex (Schneider et al. 2013) than first hoped. It is also hindered by the fact that there is not only a multitude of ways of performing the experiments but also the manner in which they are reported (Klebe 2015). Nevertheless, efforts have been made to systematically bring together observations across discrete families (Miller et al. 2012) and to analyse properties most likely to influence dissociation rates, with molecular weight apparently contributing the most (Miller et al. 2012). There have also been studies that have successfully developed new compounds against K_v 11.1 potassium channel targets with different off-rates (Yu et al. 2015). Such a consideration may be critical in the consideration of cardiotoxicity.

How do structural features of proteins dictate kinetics? To address that is going to be extremely challenging indeed and certainly much more difficult than the relationship to affinity. Nevertheless, some early attempts to examine this have been impressive in their ambition and the potential insight they can reveal; see (Cavalli et al. 2015). One of the biggest hurdles is simply ensuring that there are enough observations of both "on" and "off" rates and this ultimately comes down to a sampling problem. Various lines of approach have been devised ranging from brute force (i.e. simulate for a very long time using specialized hardware and simply count the on/off rates and residence times) as for example reported for ligand-GPCR binding.

A study of benzamine-trypsin (Buch et al. 2011) showed that 495 × 100 ns simulations could produce 187 binding events. By reconstructing the binding pathway, they were able to show that two intermediate binding states could

be found between the solvent and the final bound state. Another study examined the binding of ligands to GPCRs and highlighted the specific role that dehydration can play in the ligand-binding process (Dror et al. 2011). The use of a large number of simulations for example via "the cloud" (Harvey and De Fabritiis 2015), together with Markov Modelling (Pande et al. 2010) and sophisticated sampling techniques (Doerr and De Fabritiis 2014) is a maturing field that is starting to show exciting results for ligand-binding events and indeed how they can be applied in terms of protein modulation (Shukla et al. 2015).

In addition to the approaches highlighted above, the use of metadynamics has also proven useful in the analysis of ligand-binding events both in term of binding (Limongelli et al. 2013) and unbinding (Tiwary et al. 2015). Metadynamics employs a sampling (in a non-systematic way) along a set of collective variables. A bias is added in a history-dependent fashion that adds Gaussian contributions to the potential energy surface to prevent the system visiting regions of the conformational space that have already been visited. The free energy surface can then be reconstructed as a function of the collective variables. The technique has the advantage that it can provide these insights on fewer computational resources than the Markovian reconstruction approach outlined above. However, the results tend to be sensitive to the choice of collective variables (Barducci et al. 2011).

12.3.4 Data, Pipelining and Unified Models

The previous section alluded to the fact that kinetics can be assessed via the use of a large number of trajectories. In fact this poses part of a more generic problem – the sheer volume of data and how to deal it. This is not a problem specific to simulation or computational methods per se, but is particularly acute for this field. Furthermore, the problem is more than just a data storage issue, managing data across several different compute systems, and in the cloud, requires some considerable strategic thought (Pronk et al. 2011).

However, once a strategy has been devised for simulation and data management more advanced protocols can be developed that can bring added-value to existing data. One such example is MemProtMD (Stansfeld et al. 2015), which is a simulation pipeline for predicting the location of membrane proteins within a lipid bilayer (Fig. 12.8). It will be interesting in the future to

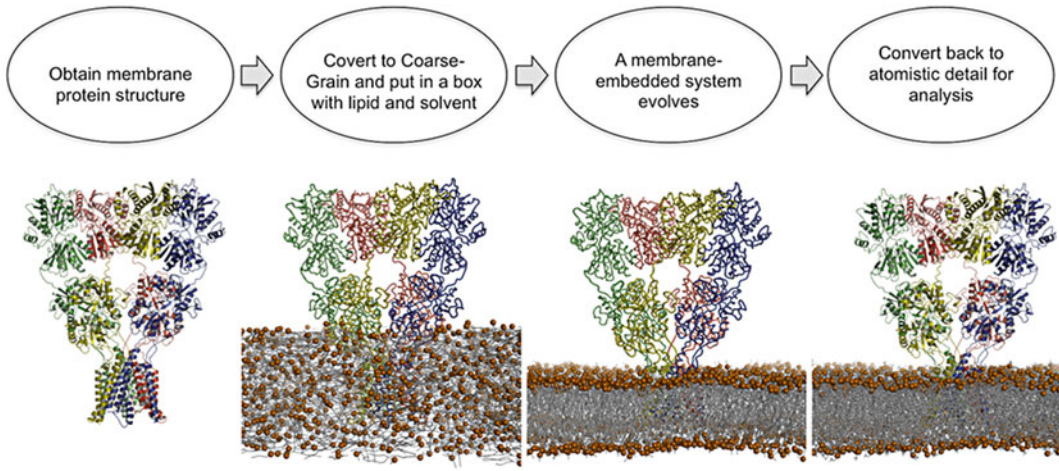

Fig. 12.8 The MemProtMD pipeline for inserting membrane proteins into bilayers. The first step is to detect the protein structures from the PDB, here shown for the GluA2 ionotropic glutamate receptor (PDB: 3KG2). The second is to set up a lipid, water, and protein simulation system. Coarse-grained (CG) simulations are then run (1 μs duration) to assemble and equilibrate a bilayer around each membrane protein structure. The CG simulation system is then converted to atomic resolution, allowing detailed analysis of lipid bilayer/protein interactions (Figure courtesy of Dr Phillip Stansfeld)

see how well computational pipelines can be integrated with high-throughput structural biology pipelines. Another challenge for the future will be how to stitch together the different levels of treatment both in terms of physical models (e.g. coarse-graining (Ayton et al. 2010)) and higher level models that are often called systems biology (Pei et al. 2014).

There are many complex challenges to be solved and even small steps of progress can give important insight for drug design. The changing landscape of the pharmaceutical sector also means that collaborations between academia and industry are likely to play central roles in moving the field forward in the coming decades (Heifetz et al. 2015).

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