

## ***Homology Modelling of Human P-glycoprotein***

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Abbreviations: ATP, adenosine triphosphate; MDR1, multidrug resistance protein 1; NBD, nucleotide binding domain; P-gp, P-glycoprotein; PMF, potential of mean force; POPC, 1-palmitoyl-2-oleoyl-phosphatidyl-choline; RMSD, root mean square deviation; TMD, transmembrane domain; TMH, transmembrane helix.

## **Abstract**

P-glycoprotein is an ATP-binding cassette transporter that exports a huge range of compounds out of cells and is thus one of the key proteins in conferring multidrug resistance in cancer. Understanding how it achieves such a broad specificity and the series of conformational changes that allow export to occur form major, on-going, research objectives around the world. Much of our knowledge to date has been derived from mutagenesis and assay data. However, in recent years, there has also been great progress in structural biology and although the structure of human P-glycoprotein has not yet been solved, there are now a handful of related structures on which homology models can be built to aid in the interpretation of the vast amount of experimental data that currently exists. Many models for P-gp have been built with this aim, but the situation is complicated by the apparent flexibility of the system and by the fact that although many potential templates exist, there is large variation in the conformational state in which they have been crystallized. In this review, we summarize how this approach has been used in the past, how models are typically selected and finally illustrate how molecular dynamics simulations can be used as a means to give more confidence about models that have been generated via this approach.

## Introduction

In recent years there has been substantial progress in using structural and computational methods to improve our understanding of multidrug efflux mechanisms [1] [2]. However, by far the most studied is P-glycoprotein. P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1) is an ATP-dependent efflux pump with broad substrate specificity. It is thought to be one of the main reasons why tumor cells exhibit resistance to drugs and also plays a role in maintaining the blood brain barrier. It is able to export an extremely wide range of compounds [3] and that poses particular problems for the design of anti-cancer compounds, which may otherwise be extremely effective at their target. Current strategies to deal with this problem typically utilize knowledge-based methods on the back of structure-activity relationship data [3]. An understanding of the mechanism by which P-gp not only recognizes compounds, but also how it exports them should improve our prospects for designing compounds that are not susceptible to export by P-gp.

Although there is a vast amount of functional data, the lack of a crystal structure of human P-gp has hindered rational drug design strategies. However, in recent years, structures have started to appear for related proteins that can be used as templates for homology modelling to generate structure-based hypotheses, which in turn, can be tested experimentally.

There are currently a number crystal structures that can and have been used as templates for modelling human P-gp (Figure 1). One the first structures that was used as a template, was the Sav1866 transporter from *S. aureus*, which was crystallized in an outward-open state with both ADP [4] and non-hydrolysable ATP analogue bound [5]. Another outward-open structure that was solved subsequently was that of MsbA [6]. All the other structures solved so far are in some kind of inward-open state and come from a variety of different species (see Table 1 and Figure 1).

The publication of the *C. elegans* P-gp structure by Jin *et al.* [7] highlighted some differences with respect to the first mouse structures. The *C. elegans* structure was supported by arginine mutagenesis data and revealed that the helix register of transmembrane helix (TMH) 5 in the original mouse structure is likely not correct [8]. Several other crystal structures of mouse P-gp have been published since [9, 10], including a refined structure with additional experimental electron density maps that allowed the correct helical register to be modeled [11]. A human homology model from mouse crystal structure was used to dock several compounds in a flexible receptor showing different binding sites for large and small compounds [10, 12].

Of course, as well as serving as templates for modelling human P-gp, these structures are of interest in their own right. The behaviour of mouse P-gp with substrate or inhibitor bound was investigated with molecular dynamics simulations showing that inhibitors keep nucleotide binding domains (NBD)s from approaching each other, while a bound substrate on the other hand would allow closure of the NBDs in a conformational more compatible with

ATP hydrolysis [13]. O'Mara et al. assessed the impact of environment on protein stability [14], noticing that in a cholesterol-enriched 1-palmitoyl-2-oleoyl-phosphatidyl-choline (POPC) bilayer, magnesium ions were required for a stable simulation of the mouse P-gp. However, this study was completed without the flexible linker present in the P-gp structure, a feature important in substrate specificity and drug transport [15, 16]. In another molecular dynamics study with the same structure, the linker was built in to assess its importance in stabilization of the NBDs where it acts as a “damper” reducing the movements of the cytoplasmic regions of P-gp [17]. The transition pathway of MsbA was investigated with nonequilibrium-driven MD simulations revealing the highly cooperative nature of transmembrane domain (TMD) and NBD movement [18].

Presumably owing to it being one of the first structures solved and indeed is still considered an excellent template for the outward-facing state, the Sav1866 structure has been subjected to many MD studies [19-24]. Much can be gleaned from these studies in terms of general exporter function, but in this review, we focus specifically on models of P-gp and then illustrate how such models are typically made and evaluated.

## Modelling Studies

One of the main goals or aims of homology modelling studies has been to build a model that can then be used to rationalize some existing structure-activity relationship (SAR) data [3, 25]. For example, dual inhibitors of P-gp and MRP1 were investigated via homology modelling of human P-gp based on the first mouse structure. Despite the sequence similarity between MRP1 and human P-gp it appears that there is enough sequence variation to give different geometric and physicochemical properties [26]. Palmeira *et al.* used homology models of P-gp in combination with docking to rationalize the P-gp sensitivity of aminated thioxanthone derivatives that have antitumor properties. They were able to show that derivatives could be designed that inhibited P-gp and also led to an improved *in vitro* efficacy in a sensitized drug-resistant (to doxorubicin) cell line [27]. Other studies have used docking to explore explored the binding of potential novel inhibitors such as 6-(methylsulfinyl)hexyl isothiocyanate [28] and guanidine alkaloid [29]. They found in each case that these drugs bind in similar locations to known P-gp inhibitors such as QZ59-RRR [28] and verapamil [29].

Klepsch et al. explored the binding mode of propafenone derivatives in homology models derived from the Sav1866 and mouse crystal structures to capture the differences between the conformational states [30]. The binding modes were similar in both models – propafenone binding occurred on the TMH 5-8 interface overlapping part of the known cyclic inhibitor and verapamil-binding site. This work was followed by docking of benzopyrano[3,4-b][1,4]oxazines, which showed clustering of compounds close to TMHs 4,5 and 6 [31]. Interestingly, inhibitors whose structure-affinity pattern is affected by their ability to permeate the bilayer were distributed close to the entrance gate, while the other molecules could also be found

deeper in the binding site. In a later study [32] they also found that benzophenones bind similarly to the previously investigated propafenone inhibitor.

A study investigating the structure-activity relationship of analogues of the third generation inhibitors, tariquidar and elacridar, in homology models based on mouse P-gp and Sav1866 crystal structures showed that their binding sites might overlap with the Hoechst 33342 and rhodamine binding sites and possibly interacts with both [33]. In another study involving tariquidar and propafenone derivatives, Jara et al. used molecular dynamics simulations and docking in a human homology model from the mouse crystal structure to estimate the binding energy of the modulators [34]. They concluded that hydrophobic interactions and molecular flexibility of the compounds are the main factors for inhibition. Although most studies have focused on the transmembrane cavity, other studies have examined the mode of action of compounds like piperine, which may act via the NBD [35].

### **Studies that Incorporated Dynamics**

The interactions of C<sub>60</sub> fullerene, a potential drug carrier, with P-gp were investigated by a combination of *in vitro* methods and molecular dynamics simulations using a human homology model from the mouse structure inserted into a POPC bilayer [36]. The same model was used in a later study of paclitaxel and doxorubicin interactions with P-gp [37] where free diffusion of both drugs towards the binding site was observed in 100 ns of unbiased molecular dynamics simulations.

The crystal structure of *C. elegans* P-gp has been used to make homology models used in docking of anticancer compounds investigated by molecular dynamics simulations and free energy calculations [38]. One of the compounds, NSC745689, did not show any stable interactions with the binding site, suggesting that it is not a substrate of P-gp and this was confirmed with *in vitro* experiments.

A much more ambitious goal for P-gp modelling is to try to predict the transitions between conformational states. O'Mara and Tieleman [39] built a model of human P-gp based on the Sav1866 structure and, in combination with simulation data from MalK and BtuCD, proposed models of P-gp in the apo state. The transport mechanism of P-gp was also investigated using targeted MD on a homology model based on Sav1866 to obtain structures of intermediate states between the outward-open and inward-open conformations [40]. Docking of 21 substrates and inhibitors on 26 non-redundant transitional structures mostly agreed with previous biochemical evidence of the drug-binding site. Further work on these structures consisted of screening for selective compounds that would inhibit ATP hydrolysis by binding to NBDs and not by targeting the transmembrane binding-site [41].

The communication interface between the NBDs and the TMDs was investigated by homology modelling and molecular dynamics revealing hydrogen bond networks of conserved motifs in the NBDs and intracellular coupling helices [42]. Zeino et al. showed that even though it might be possible to discriminate between substrates, modulators and inhibitors of P-gp by molecular docking alone, care must be taken when interpreting the possible binding site position of a drug [43]. Pan and Aller [44] compared inward and outward models of P-gp with particular emphasis on the allosteric effect of ATP on the conformational dynamics. They also concluded that mouse P-gp and Sav1866 might employ slightly different transport mechanisms, especially with respect to the role of water. More recently, O'Mara and colleagues investigated potential of mean force (PMF) profiles for morphine and nifedipine and showed that they bind at different but overlapping sites within central transmembrane cavity [45].

## **Using Modelling and Molecular Dynamics**

Though more X-ray data is now starting to appear, it is unlikely to provide a complete picture of the mechanism of export for P-gp, even if structures were solved in multiple states. To that end, it is clear that modelling can provide a useful way of generating new hypotheses that can be tested by other experimental methods. As currently, there is no structure for human P-gp in any state, one has to use homology modelling as the starting point. However, the situation is complicated now by the appearance of crystal structures from several different species in similar but not identical states. A key question is how we establish the quality of a model. In this part of the review we demonstrate a typical approach that can be used.

The resolution and sequence identity to the template crystal structure affect the resulting homology model. The crystal structures of membrane proteins are often obtained in the presence of detergents or micelles. This could be a particular issue for P-gp, due to its functional dependence on lipid species and localization [46]. Homology models inherit these flaws and must be further refined, for example, by molecular dynamics to ensure their stability in a membrane bound environment (see for example the work of O'Mara [14]). Although MD has been used in this way, one must remember that a complete sampling of all the conformational states is unlikely (typical current simulation times are of the order of hundreds of nanoseconds) and even with extensive sampling, one still needs an appropriate method to define the quality of the model. Typically, the DOPE (Discrete Optimized Protein Energy) score is evaluated which relies on a statistical potential to evaluate model quality [47]. More simply, the model may be evaluated in terms of the number of residues that sit within the allowed regions of the Ramachandran plot and whilst this provides a good assessment of stereochemical quality, it does not provide any assessment of the likelihood that the structure resembles a native structure. For that, the QMEAN Z-score can be used [48]. When building models that could be derived from several different X-ray templates as is the case here, the Ramachandran plot is often not particularly useful, as the resulting models all tend to have very similar scores.

## P-gp Modeling

In the case of P-gp, a compromise between sequence identity and resolution of the structure often has to be accepted, as the eukaryotic structures generally (with the exception of ABCB10) have lower resolution than those of the bacterial homologues (Table 1). Models of human P-gp can be built on the available crystal structures and assessed in terms of their QMEAN Z-score to gain an idea of the overall quality of the models (Table 2). Since the QMEAN Z-score uses solvent accessibility as one of the scoring components, membrane proteins tend to yield poorer scores, predicting lower quality models than they are in reality [48]. However, this is a known limitation and the scores can provide a useful method to rank and compare very different models such as those from P-gp homologues in various conformational states.

For example, several high quality human homology models based on refined mouse [11], *C. elegans* [7] and *C. merolae* [49] ABCB1 crystal structures can be generated (See Supplementary Information for details). The QMEAN Z-scores of these models ranged from -1.86 (best) to -2.14 (see Table 2). This result is comparable to a medium quality model (mean Z-score -1.75) as previously suggested without taking into account the unfavourable solvent accessibility term [48]. Interestingly, eukaryotic structures of ABCB1 provided better homology models than the structures of bacterial homologues or human ABCB10, suggesting that high sequence identity is preferable for high quality models. However, the cut-off is not exact, as the templates of the best homology models show sequence identity to human P-gp ranging from 39 to 87%.

It should be remembered that the homology model (and indeed the template structure) are single snapshots. In reality the protein is a dynamic object with conformational variability. A way to compare that is to use molecular dynamics. For example, models from the refined mouse, *C. elegans* and *C. merolae* crystal structures can be inserted into lipid bilayers (Figure 2A) and their dynamics monitored (See SI for details). In such simulations the dynamics of P-gp reflect its functional motion. The overall root mean squared deviation (RMSD) of the C $\alpha$  atoms is fairly large for a membrane protein (Figure 2B), but this simply reflects the “breathing motions” of the protein as has also been described by other researchers using MD alongside double electron-electron resonance spectroscopy [50]. The predominant motion is the opening and closing motion that can best be summarized by the distance between the NBD domains (Figure 2C). As can be seen the model based on the *C. elegans* structure exhibits the most dramatic movements over the course of 100 ns, and the model based on the refined mouse crystal structure exhibits the least fluctuation in this regard.

One aspect that modelling and MD simulation in particular could begin to address in the future are the questions surrounding drug access to the central binding cavity. There are two main routes that have been proposed; access

directly from the cytoplasm or the more commonly accepted “hydrophobic vacuum cleaner model” where drugs enter from laterally from within the membrane (Figure 3). The latter route is generally accepted as being highly likely especially for non-polar compounds. In crystal structures and P-gp models, one can visualize distinct entrance portals into the central cavity (Figure 3B); one at the level of the membrane and one situated more towards the cytoplasm. However, during the course of MD simulations, the upper, membrane portals often appear to become occluded (Figure 3B). Thus, how compounds access the central drug-binding cavity remains unclear at this time and thus will require much more detailed investigations.

## **Conclusions**

Consideration of P-gp is essential for many aspects of drug development. Despite excellent progress in recent years particularly in terms of structural biology, there are many key questions that are still open, and are likely to remain open even if we are fortunate enough to obtain a high-resolution structure of human P-gp itself. How is P-gp able to recognize such a diverse range of substrates and what makes a compound a likely substrate? What is the nature of the conformational cycle that pumps compounds out of the cell into the blood stream? How is that coupled to ATP hydrolysis? It is likely that a multidisciplinary approach will provide the best chance of answering these and related questions. There is no doubt that modelling and molecular dynamics simulations can, and will, play a huge role in helping to refine our understanding of this important protein.

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**Table 1.** Overview of P-gp homologue crystal structures.

Protein	PDB	Resolution (Å)	Distance between NBDs (Å)	Sequence identity	Conformation	Reference
<i>S. aureus</i> Sav1866	2HYD	3.0	8.8	34%	Outwards open	[5]
<i>E. coli</i> MsbA	3B5W	5.3			Inwards open	[6]
<i>V. cholera</i> MsbA	3B5X	5.5			Inwards closed	[6]
<i>S. typhimurium</i> MsbA	3B60	3.7			Outwards open	[6]
Mouse ABCB1	3G5U	3.8	22.4	87%	Inwards open	[51]
<i>T. maritima</i> TM287/288	3QF4	2.9	15.6	30%	Inwards open	[52]
<i>C. elegans</i> ABCB1	4F4C	3.4	40.4	46%	Inwards open	[7]
Mouse ABCB1	4KSB	3.8	38.0	87%	Inwards open	[10]
Human ABCB10	3ZDQ	2.85	24.3	37%	Inwards open	[53]
<i>C. merolae</i> CmABCB1	3WME	2.75	32.3	39%	Inwards open	[49]
Mouse ABCB1 (refined)	4M1M	3.8	22.0	87%	Inwards open	[11]
<i>E. coli</i> McjD	4PL0	2.7	8.4	34%	Outwards occluded	[54]

Crystal structures of P-gp homologues. The distance between NBDs is represented by the distance of the N $\zeta$  atom on the Lys of the Walker A motif in NBD1 and the C $\alpha$  atom of the Ser in the Signature Motif in NBD2.

**Table 2.** Overview of human homology models of P-gp

Protein	PDB	QMEAN Z-score
Sav1866	2HYD	-2.34
ABCB1 (mouse)	3G5U	-2.61
ABCB1 ( <i>C. elegans</i> )	4F4C	-1.9
ABCB1 (mouse)	4KSB	-2.46
ABCB10 (Human)	3ZDQ	-2.5
CmABCB1 ( <i>C. merolae</i> )	3WME	-2.14
ABCB1 (Mouse refined)	4M1M	-1.86

A QMEAN Z-score of less than -5 is taken as an indication there could be some problem with the structure [48].

## Figures

### Figure 1 | The structure and conformational states of P-gp homologues

(A) Overall architecture of P-gp and its homologues. The pseudo two-fold symmetry is highlighted by the different colours. (B) Structures of homologues have been solved in different states, which are thought to reflect different stages of the transport cycle. (C) The majority of structures have been solved in the inward-open conformation, but there is huge variation in the separation of the NBDs (see Table 1).

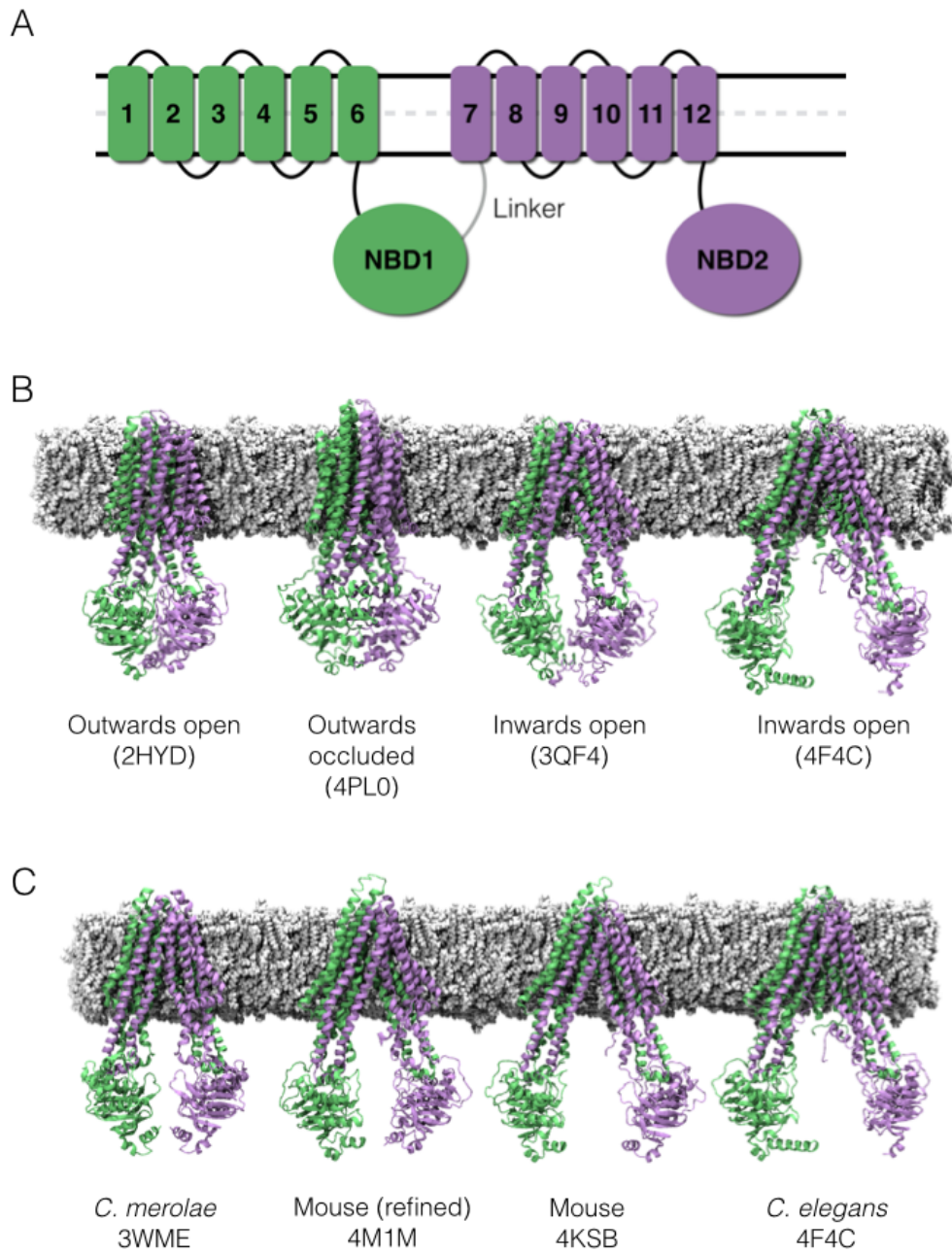
### Figure 2 | The dynamics of P-glycoprotein in a model membrane

(A) Illustration of the simulation box showing the human homology model from the refined mouse crystal structure (4M1M) in a POPC bilayer with counter ions and water and the protein coloured according to the topology diagram in the top panel. (B) RMSD of C $\alpha$  atoms of human homology models from three different templates; 4F4C (*C. elegans*): purple line, 3WME (*C. merolae*): blue line and 4M1M (refined mouse): green line (C) The distance between NBDs is represented by the distance of the N $\zeta$  atom on the Lys of the Walker A motif in NBD1 and the C $\alpha$  atom of the Ser in the Signature Motif in NBD2. Colours as in (B).

### Figure 3 | Substrate entrance pathways

(A) Cartoon depicting how compounds might access the central drug-binding site. The substrates of P-gp have been proposed to reach the central binding site either through the lipid bilayer (the so-called “hydrophobic vacuum cleaner” model) or via the cytoplasm. (B) The homology model suggests that access to the central binding site could occur through an intra-membrane entrance portal (orange box) or a cytoplasmic entrance portal (blue box). In MD simulations (100 ns), the former tends to close, while the latter remains available for substrate passage throughout.

**Figure 1. Domicевичa and Biggin**





**Figure 2. Domicевичa and Biggin**

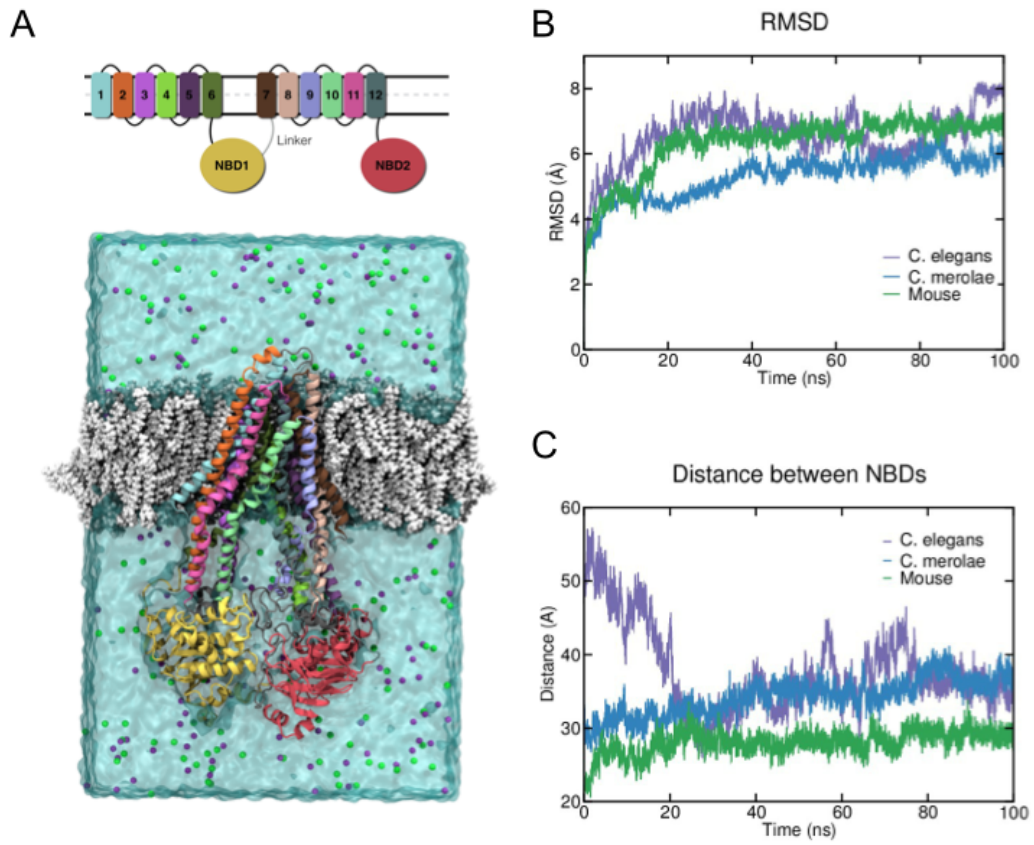


Figure 3. Domicевичa and Biggin

