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Molecular model of the outward facing state of the human multidrug resistance protein 4 (MRP4/ABCC4)

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ABSTRACT

ATP-binding cassette (ABC) transporter multidrug resistance protein 4 (MRP4, ABCC4) is involved in multidrug resistance (MDR), which is an increasing challenge to the treatment of cancer and infections. We have constructed a molecular model of ABCC4 based on the outward facing Sav1866 crystal structure using molecular modeling techniques. Amino acids reported by ICMPocketFinder to take part in substrate translocation were among others Glu103 (TMH1), Ser328 (TMH5), Gly359 (TMH6), Arg362 (TMH6), Val726 (TMH7), and Leu987 (TMH12), and their corresponding amino acids in ABCB1 (P-glycoprotein) have been reported to be involved in drug binding according to site-directed mutagenesis studies. The ABCC4 model may be used as a working tool for experimental studies on ABCC4 and design of more specific membrane transport modulating agents (MTMA).

Multidrug resistance (MDR) is a challenge to the treatment of cancer and infections. Cells exposed to chemotherapeutics and antibiotics can develop resistance by several mechanisms, including increased excretion via drug efflux transporters. Development of membrane transport-modulating agents (MTMA) of drug efflux transporters has been sought for use as a supplement to therapy to overcome multidrug resistance.[1]

ATP-binding cassette (ABC) transporter multidrug resistance protein 4 (MRP4, ABCC4) exports organic anions, including endogenous and exogenous substances, and is involved in MDR.[2–6] ABCC4 is present in most tissues of the human body, including brain,[7] kidney,[8] liver,[9] erythrocytes,[10] platelets,[11] adrenal gland,[12] and pancreas.[13] Experimental studies have demonstrated that ABCC4 is involved in resistance to the anticancer agent topotecan, thus protecting the brain from chemotherapy, suggesting that the therapeutic efficacy of central nervous system-directed drugs that are ABCC4 substrates may be improved by developing ABCC4 MTMA.[3]

There is a limited knowledge of ABCC4's structural, biological and pharmacological features. In lack of an X-ray crystal structure, molecular modeling by homology may be an alternative for gaining structural insight into ABCC4. Information about the molecular properties of the substrate translocation pathway of ABCC4 can be used to design therapeutic agents that may aid to reduce the consequences of MDR.

ABCC4 belongs to the ABC superfamily, which are structurally related membrane proteins featuring intracellular motifs that exhibit ATPase activity.[14–16] ABCC4 has both transmembrane domains (TMD) and nucleotide-binding domains (NBD), and its topology is TMD–NBD–TMD–NBD. Each TMD has six transmembrane helices (TMHs).

The bacterial ABC transporter Sav1866 from *Staphylococcus aureus*, which has a 24% amino acid sequence identity to ABCC4, has been crystallized in an outward-facing ATP-bound state.[17] We have previously used the Sav1866 crystal structure[17] to construct an ABCB1 model[18] and an ABCC5 model.[19] Here, we present a molecular model of ABCC4 based on the Sav1866 crystal structure,[17] together with an identification of ABCC4 amino acid residues that may interact with substrates in the substrate translocation pathway.

The ICM software version 3.4-9b[20] was used to construct the ABCC4 model by homology, and the crystal structure of Sav1866[17] (pdb code 2HYD), which has a 3 Å resolution, was used as a template.

The input alignment was based on (1) a multiple sequence alignment of human ABCC4 (SWISS-PROT Accession No. O15439), human ABCB1 (SWISS-PROT Accession code P08183), human ABCC5 (SWISS-PROT Accession No. O15440), human ABCC11 (SWISS-PROT Accession No. Q9BX80), Sav1866 (SWISS-PROT Accession No. Q99T13), *Vibrio cholerae* MsbA (SWISS-PROT Accession No. Q9KQW9), and *Escherichia coli* MsbA (SWISS-PROT Accession No. P60752), which was obtained using T-COFFEE, Version 4.71 available at the Le Centre national de la recherche scientifique website (http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi), and (2) on secondary structure predictions that were performed to define the boundaries of the TMHs using the PredictProtein server for sequence analysis and structure prediction,[21] and SWISS-PROT.[22]

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ICM constructs the molecular model by homology from core sections defined by the average of Cα atom positions in the conserved regions. Loops are searched for within several thousand high-quality structures in the PDB databank[23] and matched in regard to sequence similarity and sterical interactions with the surroundings of the model. The best-fitting loop is selected based on calculating the maps around the loops and scoring of their relative energies.

The ABCC4 model was subjected to the RefineModel macro of ICM, which globally optimizes side-chain positions and anneals the backbone. The RefineModel macro was performed with (1) a side-chain conformational sampling using 'Montecarlo fast',[24] (2) 5 iterative annealings of the backbone with tethers, which are harmonic restraints pulling an atom in the model to a static point in space represented by a corresponding atom in the template, and (3) a second side-chain conformational sampling using 'Montecarlo fast'. The iterations of 'Montecarlo fast', which samples conformational space of a molecule with the ICM global optimization procedure, consist of a random move followed by a local energy minimization, and the complete energy is then calculated. Based on the energy and the temperature, the iteration is accepted or rejected. Membrane- and water molecules were not included in the calculations.

The Savs Metaserver for analyzing and validating protein structures (http://nihserver.mbi.ucla.edu/SAVS/) was used to check the stereochemical quality of the ABCC4 model. Programs run were Procheck,[25] What_check,[26] and Errat.[27] The pdb file of Sav1866[17] was also checked for comparison with the ABCC4 model.

The ICMPocketFinder was used to identify amino acids lining the substrate translocation pathway of the ABCC4 model, using a tolerance level of 2.0.

Figure 1 shows the refined ABCC4 model. The substrate translocation pathway was formed by a central cavity perpendicular to the cell membrane between TMD1 and TMD2, and TMHs 1, 2, 3, 5, 6, 7, 8, 9, 11, and 12 contributed to the cavity lining. TMH5 and TMH2 of TMD1 were packed against TMH8 and TMH11 of TMD2, respectively. The TMDs were twisted relative to the NBDs, and the substrate translocation chamber was closed toward the intracellular side. Towards the extracellular side, the TMHs diverged into two symmetrical parts, one part consisting of TMHs 1 and 2 of TMD1 and TMHs 9–12 of TMD2, and one part consisting of TMHs 7 and 8 of TMD2 and TMHs 3–6 of TMD1 (Fig. 1). The NBDs, containing the nucleotide-binding sites formed by the motifs Walker A, Walker B, Q-loop, and switch regions, were tightly packed at the intracellular side of the membrane. The loop connecting NBD1 and TMD2 of ABCC4 was α-helical between amino acid residues Lys614-Ser623, Gly628-Lys633, and Pro642-Val682, and the remainder of the loop was in extended conformation.

The Errat option of the Savs Metaserver reported that the overall quality factor of the ABCC4 model was 92.8. A value above 90 indicates a good model. According to the Ramachandran plot provided by the Procheck option, 87.3% of the ABCB1 residues were in the most favored regions, 11.9% were in additional allowed regions, 0.4% were in generously allowed regions, and 0.4% were in disallowed regions. The summary of the Whatcheck option reported that the ABCC4 model was satisfactory. In comparison, the overall quality factor of the crystal structure of Sav1866[17] (pdb code 2HYD) was 93.6, and the Ramachandran plot reported 86.0% (most favored regions), 14.0% (additional allowed regions), 0.0% (generously allowed regions), and 0.0% (disallowed regions). The crystal structure of Sav1866 was also satisfactory, according to the summary of the Whatcheck option.

Detailed structural data for a drug target protein, such as X-ray crystal structures, may provide knowledge about the 3D structure of the drug-target complexation, and the energetic criteria responsible for drug binding, and may aid to map the complementary surface between a drug target and its ligands. Structural information about ABCC4 may be useful in virtual screening experiments searching for possible ABCC4 modulators, and for structure aided drug design. But crystallization of membrane proteins is in general technically difficult, and since no X-ray crystal structure of ABCC4 is available, molecular modeling by homology can be used as a step forward towards structural knowledge of this drug target. Homology between two proteins, such as Sav1866 and ABCC4, is determined by sequence similarity of two proteins having a common ancestor, indicating the presence of similar features such as homologous protein fold. The ABCC4 model presented here may be considered as a working tool for generating hypotheses and designing further experimental studies on ABCC4. Site directed mutagenesis studies and transporter modeling are complementary to each other in the elucidation of drug target structure and function.

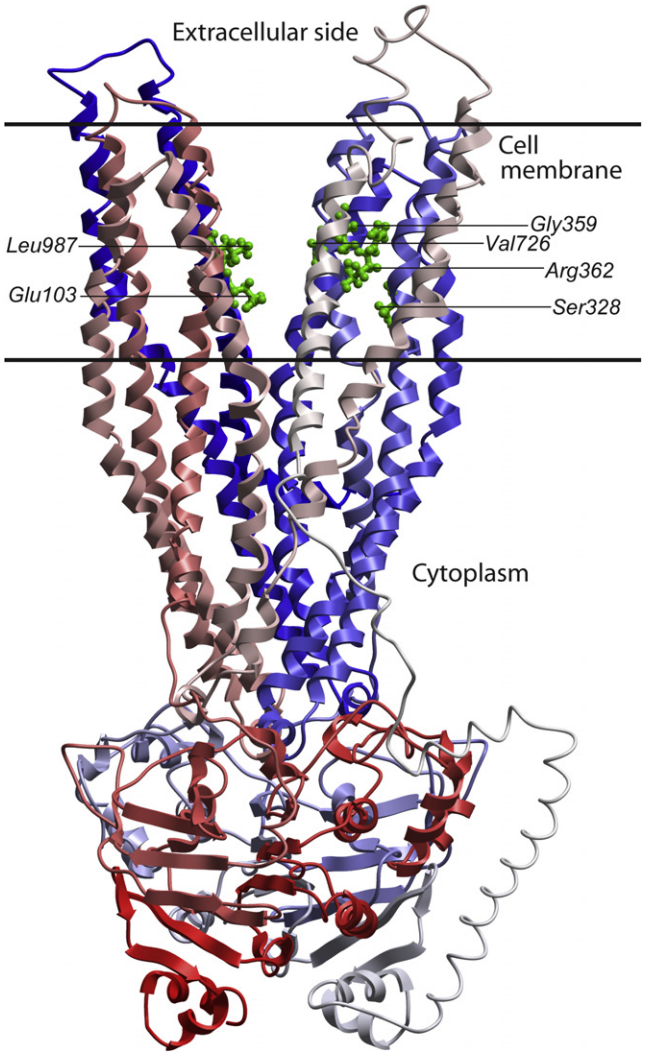

Figure 1. Backbone Cα-trace of the refined ABCC4 viewed in the membrane plane (cytoplasm downwards). Color coding: blue via white to red from N-terminal to C-terminal. Amino acids reported both by ICMPocketFinder and by site directed mutagenesis studies on the homologous transporter ABCB1[29–33] as possible substrate interacting residues are displayed as green balls and sticks.

Table 1 lists a number of amino acids as possible candidates for single-point mutations. These amino acids may interact with substrate during substrate translocation, and some of these may also take part in high-affinity substrate-binding sites accessible from the intracellular side. Experimental studies have shown that ABCC4 may feature multiple allosteric substrate-binding sites.[6] Substrates

Table 1
The amino acids that may interact with substrate during substrate translocation as reported by ICMPocketFinder

TMH	Amino acids
1	Thr99, Glu102, Glu103, Ala105, Lys106, Gln109, Pro110
2	Val140, Cys144, Leu148, His152, Tyr155, Phe156
3	Leu215, Trp216, Gly218, Pro219, Gln221, Val225
5	Leu321, Phe325, Ser328, Lys329, Val332
6	Phe352, Val355, Thr356, Tyr358, Gly359, Ala360, Arg362, Leu363, Thr364, Thr366, Leu367
7	Phe716, Leu719, Leu720, Thr722, Ala723, Val726, Ala727, Leu730
8	Ile780, Leu784, Phe787, Tyr788
9	Asp842, Gln845, Thr846, Gln849
11	Phe948, Ala949, Asp953, Cys956, Ala957, Val960
12	Gly980, Leu983, Ser984, Leu987, Gly991, Met992, Gln994, Trp995, Val997

thus presumably bind to high-affinity binding sites accessible to the intracellular side, and during the translocation process the binding sites change conformation, and the substrates are released to the extracellular side from low-affinity binding sites. The present ABCC4 model is assumed to be in a conformation representing the substrate-releasing conformation. From a pharmacological point of view, the specificity and affinity of ABCC4 substrate binding ias of particular interest, and a conformation open to the intracellular side is more representative for the high-affinity ligand binding and may be more suitable for investigating high-affinity ligand recognition.

According to site-directed mutagenesis studies on ABCB1 (P-glycoprotein), which is a structural homologue of ABCC4,[28] a drug-binding site may include residues Leu65 (TMH1),[29] Ile306 (TMH5),[29] Ile340 (TMH6),[29,30] Phe343 (TMH6),[31] Phe728 (TMH7),[32] and Val982 (TMH12).[33] The corresponding residues in ABCC4 are Glu103 (TMH1), Ser328 (TMH5), Gly359 (TMH6), Arg362 (TMH6), Val726 (TMH7), and Leu987 (TMH12). As shown in Table 1, these amino acids were reported by ICMPocketFinder as possible substrate-interacting residues. The amino acid differences in the binding site areas of ABCB1 and ABCC4 are reflected in their substrate specificity. Thus, despite their homologous fold, ABCB1 transports cationic amphiphilic and lipophilic substrates, while ABCC5 transports organic anions.

The results from ICMPocketFinder were in accordance with site-directed mutagenesis data on the homologous transporter ABCB1,[29–33] which indicates that the Sav1866 X-ray structure[17] may serve as a suitable template for ABCC4 modeling. The putative ABCC4 model presented here may be a step forward toward elucidating ABCC4's structure and drug interaction mechanisms.

Coordinates of the ABCC4 model are available from the authors upon request.

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References and notes

1. Dantzig, A. H.; de Alwis, D. P.; Burgess, M. *Adv. Drug. Deliv. Rev.* **2003**, *55*, 133.
2. Borst, P.; de Wolf, C.; van de Wetering, K. *Pflugers Arch.* **2007**, *453*, 661.
3. Leggas, M.; Adachi, M.; Scheffer, G. L.; Sun, D.; Wielinga, P.; Du, G.; Mercer, K. E.; Zhuang, Y.; Panetta, J. C.; Johnston, B.; Scheper, R. J.; Stewart, C. F.; Schuetz, J. D. *Mol. Cell. Biol.* **2004**, *24*, 7612.
4. Rius, M.; Hummel-Eisenbeiss, J.; Hofmann, A. F.; Keppler, D. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2006**, *290*, G640.
5. Rius, M.; Thon, W. F.; Keppler, D.; Nies, A. T. *J. Urol.* **2005**, *174*, 2409.
6. Van Aubel, R. A.; Smeets, P. H.; van den Heuvel, J. J.; Russel, F. G. *Am. J. Physiol. Renal. Physiol.* **2005**, *288*, F327.
7. Nies, A. T.; Jedlitschky, G.; Konig, J.; Herold-Mende, C.; Steiner, H. H.; Schmitt, H. P.; Keppler, D. *Neuroscience* **2004**, *129*, 349.
8. van Aubel, R. A.; Smeets, P. H.; Peters, J. G.; Bindels, R. J.; Russel, F. G. *J. Am. Soc. Nephrol.* **2002**, *13*, 595.
9. Rius, M.; Nies, A. T.; Hummel-Eisenbeiss, J.; Jedlitschky, G.; Keppler, D. *Hepatology* **2003**, *38*, 374.
10. Klokouzas, A.; Wu, C. P.; van Veen, H. W.; Barrand, M. A.; Hladky, S. B. *Eur. J. Biochem.* **2003**, *270*, 3696.
11. Jedlitschky, G.; Tirschmann, K.; Lubenow, L. E.; Nieuwenhuis, H. K.; Akkerman, J. W.; Greinacher, A.; Kroemer, H. K. *Blood* **2004**, *104*, 3603.
12. Zelcer, N.; Reid, G.; Wielinga, P.; Kuil, A.; van der Heijden, I.; Schuetz, J. D.; Borst, P. *Biochem. J.* **2003**, *371*, 361.
13. Konig, J.; Hartel, M.; Nies, A. T.; Martignoni, M. E.; Guo, J.; Buchler, M. W.; Friess, H.; Keppler, D. *Int. J. Cancer* **2005**, *115*, 359.
14. Higgins, C. F.; Linton, K. J. *Science* **2001**, *293*, 1782.
15. Oswald, C.; Holland, I. B. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2006**, *372*, 385.
16. Saier, M. H., Jr. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 354.
17. Dawson, R. J.; Locher, K. P. *Nature* **2006**, *443*, 180.
18. Ravna, A. W.; Sylte, I.; Sager, G. *Theor. Bio.l Med. Model.* **2007**, *4*, 33.
19. Ravna, A. W.; Sylte, I.; Sager, G. *Eur. J. Med. Chem.* **2008**. doi:10.1016/j.ejmech.2008.01.015.
20. Abagyan, R.; Totrov, M.; Kuznetsov, D. N. *J. Comp. Chem.* **1994**, *15*, 488.
21. Rost, B. *Meth. Enzymol.* **1996**, *266*, 525.
22. Bairoch, A.; Apweiler, R. *Nucleic Acids Res.* **1999**, *27*, 49.
23. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 235.
24. Abagyan, R.; Totrov, M. *J. Mol. Biol.* **1994**, *235*, 983.
25. Laskoswki, R. A.; MacArthur, M. W.; Moss, D. S.; Thorton, J. M. *J. Appl. Cryst.* **1993**, *26*, 283.
26. Hooft, R. W.; Vriend, G.; Sander, C.; Abola, E. E. *Nature* **1996**, *381*, 272.
27. Colovos, C.; Yeates, T. O. *Protein Sci.* **1993**, *2*, 1511.
28. Borst, P.; Evers, R.; Kool, M.; Wijnholds, J. *J. Natl. Cancer Inst.* **2000**, *92*, 1295.
29. Loo, T. W.; Bartlett, M. C.; Clarke, D. M. *Biochem. J.* **2006**, *396*, 537.
30. Loo, T. W.; Clarke, D. M. *J. Biol. Chem.* **2002**, *277*, 44332.
31. Loo, T. W.; Bartlett, M. C.; Clarke, D. M. *J. Biol. Chem.* **2003**, *278*, 50136.
32. Loo, T. W.; Bartlett, M. C.; Clarke, D. M. *Biochem. J.* **2006**, *399*, 351.
33. Loo, T. W.; Clarke, D. M. *J. Membr. Biol.* **2005**, *206*, 173.