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Structural patterns of the human ABCC4/MRP4 exporter in lipid bilayers rationalize clinically observed polymorphisms

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ABSTRACT

The ABCC4/MRP4 exporter has a clinical impact on membrane transport of a broad range of xenobiotics. It is expressed at key locations for drug disposition or effects such as in the liver, the kidney and blood cells. Several polymorphisms and mutations (*e.g.*, p.Gly187Trp) leading to MRP4 dysfunction are associated with an increased risk of toxicity of some drugs. So far, no human MRP4 structure has been elucidated, precluding rationalization of these dysfunctions at a molecular level. We constructed an atomistic model of the wild type (WT) MRP4 and the p.Gly187Trp mutant embedded in different lipid bilayers and relaxed them for hundreds of nanoseconds by molecular dynamics simulations. The WT MRP4 molecular structure confirmed and ameliorated the general knowledge about the transmembrane helices and the two nucleotide binding domains. Moreover, our model elucidated positions of three generally unresolved domains: L_1 (linker between the two halves of the exporter); L_0 (N-terminal domain); and the zipper helices (between the two NBDs). Each domain was thoroughly described in view of its function. The p.Gly187Trp mutation induced a huge structural impact on MRP4, mainly affecting NBD 1 structure and flexibility. The structure of transporter enabled rationalization of known dysfunctions associated with polymorphism of MRP4. This model is available to the pharmacology community to decipher the impact of any other clinically observed polymorphism and mutation on drug transport, giving rise to *in silico* predictive pharmacogenetics.

1. Introduction

Drug crossing through cell and organelle membranes affects drug disposition, activity and toxicity *in vivo*. Membrane crossing can proceed either by (unassisted) passive permeation or through membrane protein transporters. The International Transporter Consortium (ITC) has described a series of human membrane transporters as being of "emerging clinical importance" [1]. This includes ABCB1 (known as P-glycoprotein, P-gp), a highly described member of the superfamily of ATP-binding cassette (ABC) transporters (exporters in mammals). ABC dysfunction is often associated with multidrug resistance and drug-drug interactions in the form of competitive affinity for these transporters, with a clinical impact in many therapeutic domains.

Less attention has been paid to ABCC4/MRP4 (multidrug resistance protein type 4), although it is expressed at key locations for drug disposition/action such as in the liver, the kidney and peripheral blood cells, and it has a broad range of xenobiotics as substrates [2]. Dysfunction of this exporter can modify drug pharmacokinetics or pharmacodynamics. Among the mutations associated with MRP4 dysfunction, the p.Gly187Trp polymorphism [3] is the most frequent in Caucasians and it was described as having the greatest impact on the membrane transport of the

Abbreviations: 6-MP, 6-mercaptopurine; ABC, ATP-binding cassette; ADV, adefovir; AZT, azidothymidine; BLOSUM, blocks substitution matrix; CFTR, cystic fibrosis transmembrane conductance regulator; Chol, cholesterol; cryo-EM, cryo-electronic microscopy; DMA^V, dimethylarsinic acid; $E_2 17\beta G$, β-estradiol-17-(β-D-glucuronide); ECL, extra-cellular loop; GCV, ganciclovir; ICD, intra-cytoplasmic domain; ICL, intra-cellular loop; IF, inward-facing; ITC, international transporter consortium; MD, molecular dynamics; $MMA(GS)_2$, diglutathione conjugate of monomethylarsonous acid; MRP4, multidrug resistance protein 4; NBD, nucleotide-binding domain; OF, outward-facing; PGE2, prostaglandin E2; PMEA, 9-(2-phosphonylmethoxy-ethyl)adenine; POPC, 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphocholine; RMSD, root mean square deviation; TMD, transmembrane domain; TMH, transmembrane helix; WT, wild-type.

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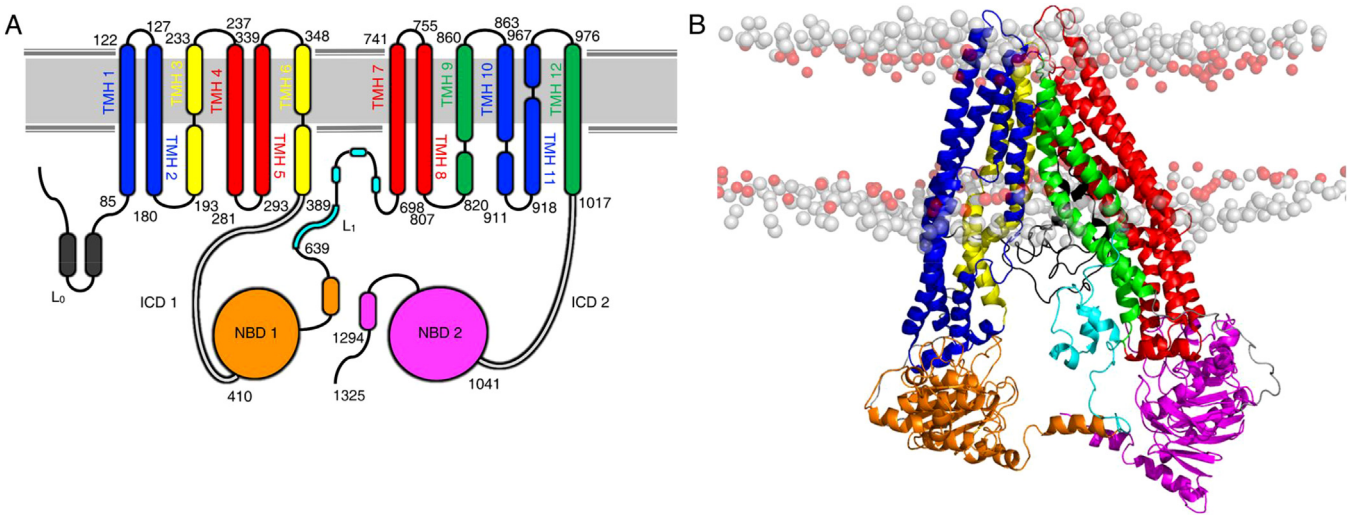

Fig. 1. A) Topology of the human MRP4 exporter, highlighting: the two TMDs made of twelve TMHs (TMH 1–12); the two NBDs (NBD 1 and 2); ICD 1–2; the linker L_1; the zipper helices; the L_0 motif. If any, the irregularities in helices are depicted. B) Fully relaxed 3D-structure of the molecular WT MRP4 model.

two drugs, namely adefovir (ADV, also called PMEA for 9-(2-phosphonylmethoxyethyl)adenine) and azidothymidine (AZT) [4]. This polymorphism was also associated with decreased efflux of ganciclovir (GCV) *in vitro* [5], which is associated with an increased risk of toxicity due to the intracellular accumulation of the drug in neutrophils. The impact of other rare mutations on MRP4 function has been poorly documented so far, which does not allow for a systematic prediction of the overall MRP4 (dys)function and its use in clinical decisions (*e.g.*, in the form of biomarkers or next-generation sequencing data).

Despite highly variable amino acid sequences due to the large number of variants that appeared with phylogeny, all ABC transporters exhibit surprising structural similarities. In their functioning form, they are constituted of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs) with very similar secondary and tertiary structures. Their catalytic sites, where ATP molecules are hydrolyzed, are in two well-conserved and well-defined regions of NBDs [6]. To date, neither the crystallographic nor the cryo-electron microscopy (cryo-EM) structures of any MRP4 protein have been elucidated. Nonetheless, it is known to bear: twelve transmembrane helices (TMH 1–12); two NBDs (NBD 1 and 2); the N-terminal domain, which is constituted of the L_0 motif; and the linker L_1 making a covalent linkage between the two halves of the exporter (Fig. 1A). The supramolecular packing between TMHs is of crucial importance for drug accessibility to the dynamic transmembrane pore, allowing transport from the inner (cytoplasmic) to the outer compartments [7]. The TMHs are arranged in bundles A, B, C and D, which result from an entanglement of the different TMHs of the two halves of MRP4 (Fig. 1). ECLs and ICLs are the extra- and intra-cytoplasmic loops connecting the TMHs with each other, and ICD 1 and 2 are two intra-cytoplasmic domains (covalently) connecting TMDs and NBDs.

Although pure homology molecular models can document on the different domains [8–13], they do not include the surrounding lipid bilayer and a sufficient (dynamic) conformational sampling. In the current study, we used homology modeling to construct the human wild-type (WT) MRP4 protein in its inward-facing (IF) conformer (*i.e.*, drug chamber open towards inner compartment) and in its ligand-free (apo) state. To become predictive and descriptive, this structure was embedded in different lipid bilayers. The models were relaxed by 300 ns molecular dynamic (MD) simulations. This was mandatory to relax any bias associated with homology modeling construction, thus ensuring correction of both secondary and tertiary structures, especially for the most flexible and the less known domains. The topology of the models was thoroughly and critically analyzed with respect to known experimental data. MD simulations also give the considerable advantage to track conformational motions in lipid bilayer. The p.Gly187Trp mutant molecular model was also constructed, which gave clues about how this single nucleotide polymorphism affects the tertiary structure of MRP4, at an atomic resolution, rationalizing its subsequent transport dysfunction.

2. Methods

2.1. Homology modeling

The human MRP4 amino acid sequence was retrieved from the UniProt database [14] with the primary accession number NM_005845.4. Isoform 1 was chosen as being the canonical form. The construction of the IF model of the human wild-type (WT) MRP4 protein was achieved using the I-TASSER webserver [15]. In the first step of this construction, three different relevant templates were identified, namely P-gp from *C. elegans* (PDB ID: 4F4C, X-ray crystallographic structure obtained at 3.4 Å resolution) [16], and from *Mus musculus* (PDB ID: 3G5U and 4M1M, X-ray crystallographic structures, both obtained at 3.8 Å resolution) [17,18]. The robustness of this molecular WT MRP4 model was assessed by a series of 'sequence and structure' similarity scores (see Table S1).

Protonation states of all histidine residues were systematically checked according to the neighboring chemical environment to maximize hydrogen bond networking. Histidines 55, 152, 172, 466, 472, 592, 710, 831, 903, 1037, and 1233 were defined as ε-protonated; Histidines 35, 153, 158, 213, 411, 572, 583, 930, 934, and 1111 were defined as δ-protonated; and Histidines 798, 1060, 1141, and 1225 were defined as double-protonated.

After the construction of this molecular WT MRP4 model in its IF form, the end of the sequence Lys1294 – Leu1325 (PDZ binding domain including the ETAL motif at the end of the sequence) was removed from the structure, as this flexible part (loop plus α-helix) is expected to be far from the NBD domains and likely involved in protein-protein interactions [19]. The molecular p.Gly187Trp mutant model was built from the optimized WT MRP4 model by simply replacing the amino acid residue.

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