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Do Drugs Have Access to the P-Glycoprotein Drug-Binding Pocket through Gates?

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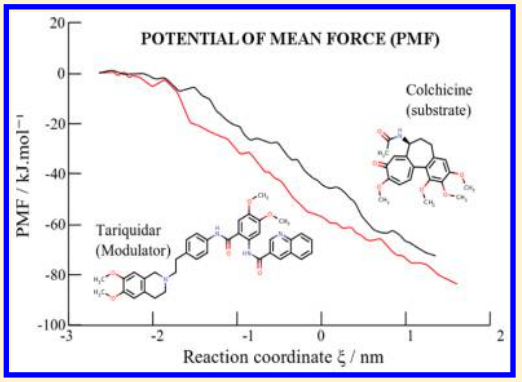
Supporting Information

ABSTRACT: The P-glycoprotein efflux mechanism is being studied since its identification as a leading protagonist in multidrug resistance. Recently, it was suggested that drugs enter the drug-binding pocket (DBP) through gates located between the transmembrane domains. For both a substrate and a modulator, the potential of mean force curves along the reaction coordinate obtained with the WHAM approach were similar, with no activation energy required for crossing the gate. Moreover, drug transit from bulk water into the DBP was characterized as an overall free-energy downhill process.



P-glycoprotein (P-gp, *ABCB1*) is one of the best known members of the ABC transporter superfamily due to its role on multidrug resistance (MDR) in cancer. Together with multidrug resistance-associated protein 1 (MRP1, *ABCC1*) and breast cancer resistance protein (BCRP, *ABCG2*), P-gp is related with drug efflux of structurally unrelated anticancer drugs, lowering their intracellular concentration and impairing their pharmacological action.[1] First identified in Chinese hamster ovary cells,[2] P-gp was correlated with increased resistance to many molecules such as colchicine, vinblastine, and daunorubicin. Nonetheless, studies on P388 leukemia cells showed that P-gp efflux could be modulated by noncytotoxic verapamil dosages, resensitizing MDR sublines to vincristine (a *Vinca* alkaloid with antimitotic properties).[3] Although P-gp efflux was experimentally capable of being modulated, at that time its mechanism was unknown. One of the first efflux models applied to P-gp was based on the ABCB4 lipid translocase mechanism.[4] According to this *flippase* model,[5] drugs and lipids are translocated by P-gp from the cytoplasmic leaflet of the plasma membrane to the extracellular leaflet, overcoming this way the energy barrier associated with molecule flip-flop between leaflets.[6,7] However, since other studies suggested that drugs could be transported directly from the cytoplasmic leaflet to the extracellular medium, the *hydrophobic vacuum cleaner* model was later proposed as the main mechanism for drug efflux.[5,8]

These models show that the presence of a membrane is mandatory for efflux to occur.[9] Although a different model has been proposed in which the membrane would be unnecessary,[10] additional studies proved that only in the presence of a membrane does P-gp retain its ATPase function and the ability to transport drugs.[11] Moreover, it was also proved that the P-gp ATPase function is intimately dependent on membrane composition, with different lipid types and/or membrane components (such as cholesterol, for instance) having a strong influence on the ATP binding and hydrolysis, and drug efflux rates.[12,13] Finally, P-gp seems to transport drugs that mainly build up in the membrane[14] according to their octanol−water partition coefficients.[15,16]

The P-gp polyspecificity of drug transport was also addressed by studies aiming to identify possible drug-binding sites and their correspondent translocation pathways. Shapiro et al. were the first to describe the existence of two cooperative substrate-binding sites, naming them H and R sites based on the ability to bind and transport Hoechst 33342 or Rhodamine-123, respectively.[17,18] Additionally, other studies demonstrated that the efflux only occurs from the cytoplasmic leaflet,[19] also proposing the existence of a third allosteric site where prazosin and progesterone would bind.[20] Following, two distinct translocation pathways were identified for both H and R sites, presumably located in a water-filled internal cavity located within the transmembrane domains.[21]

The publication, in 2009, of the first P-gp crystallographic structure (murine P-gp)[22] was a major turning point in the MDR research. In this structure, an internal drug-binding pocket of approximately 6.000 Å^3 was characterized, and from the location of the co-crystallized QZ59-SSS and QZ59-RRR

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ligands one drug-binding site could be additionally identified.[22] Interestingly, the internal cavity was accessible from both the cytoplasm or lipid bilayer through two "entrance gates" located between transmembrane (TM) helices 4/6 and 10/12,[22] a characteristic that was maintained in all of the P-gp crystallographic structures published so far.[23]

However, since the peptide sequence linking the N-terminal and the C-terminal domains (linker sequence) was missing in all crystallographic structures, the direct accessibility of the internal drug-binding pocket from the cytoplasm was not able to be confirmed experimentally through X-ray crystallography. In addition, as this sequence is known to be related with ATP hydrolysis and drug recognition,[24,25] its overall importance for structure stability, ATP hydrolysis, and drug recognition remained unclear.[23]

Using the crystallographic structure of mouse P-gp reported in 2009, we developed a refined model, comprising both the lipid bilayer and the missing linker sequence.[26] The role of both structures on P-gp stability was clarified, and together with a study of drug interactions and motion pattern analysis, P-gp was characterized as an extremely flexible protein, able to respond to drug binding by changing its native conformation. Moreover, the role of the missing linker in the structure stability was also addressed,[27] three protein−protein contact hot spots being identified next to highly conserved motifs that assist the linker to function as a "damper" between both nucleotide-binding domains (NBDs), stabilizing the cytoplasmic portion of the transporter. By means of molecular docking, three distinct drug-binding sites within the internal drug-binding pocket were also characterized. Two of them were described to be the substrate-binding H and R sites and the third one was ascribed to a modulator site, often referred to as an M site.[28] Interestingly, in MD simulations, only the H site was directly accessible from the lipid bilayer through one "entrance gate" located between TM helices 10/12, and the internal drug-binding cavity was found to be inaccessible from the cytoplasm due to the presence of the linker.[26]

Despite the identification of these drug-binding sites, there is a lack of experimental evidence demonstrating how drugs have access to the internal drug-binding pocket. According to our results, this access is only possible from the cytoplasmic leaflet of the membrane. Therefore, MD simulations were used to study the thermodynamic process of drug permeation from bulk water to the internal drug-binding pocket of P-gp. To that matter, steered MD simulations were performed in which a substrate (colchicine) or a modulator (tariquidar) (Figure 1) was pulled from the entrance gate to the hydrophobic core of the lipid bilayer or into the P-gp drug-binding pocket. As only one entrance gate between TM helices 10 and 12 was found to be open in our simulations, all of the following data are related with drug entry through this specific "gate".

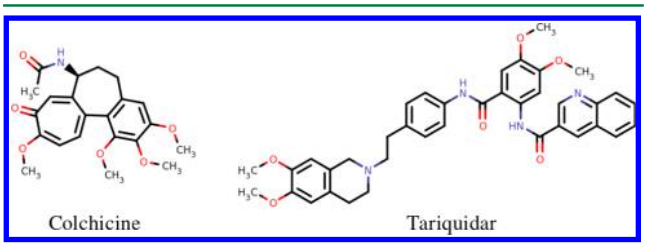

Figure 1. Chemical structures of colchicine (substrate) and tariquidar (modulator).

A system comprising P-gp inserted in a POPC bilayer was used as described elsewhere.[26] Colchicine and tariquidar molecules (neutral forms) were inserted at the entrance gate as previously described for vinblastine.[26] In each case, both molecules were pulled away from the entrance gate along the axes parallel to the bilayer over 10 (pocket) or 15 ns (membrane), using a spring constant of 1000 kJ·mol^{-1}·nm^{-2} and a pull rate of 0.15 nm·ns^{-1}.

These trajectories were the starting points defining a reaction coordinate later used in the umbrella sampling technique with 52 or 58 umbrella windows with a mean width of 0.7 Å (total distance: colchicine, 3.9 nm; tariquidar, 4.2 nm). In each window, 20 ns of MD was performed, for a total umbrella sampling simulation over 2 μs with systems containing ∼200k atoms. Analysis of results was performed through the weighted histogram analysis method (WHAM). The free-energy profiles obtained show that both colchicine and tariquidar enter the internal drug-binding pocket through a spontaneous process (Figure 2). Additional details on the simulation protocol are available as Supporting Information.

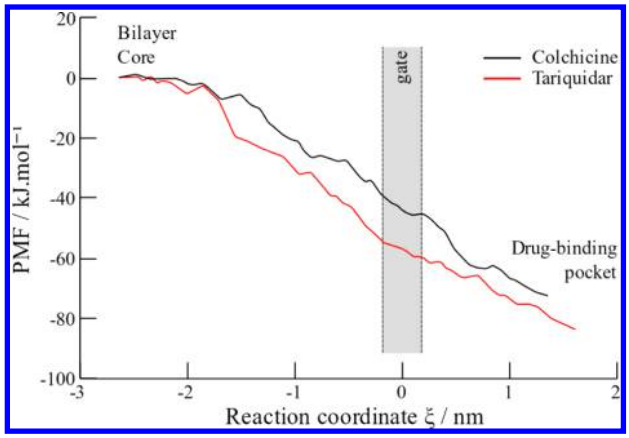

Figure 2. Potential of mean force (PMF) energetic profile for colchicine and tariquidar translocation from the membrane into the drug-binding pocket. The gray shading represents the average radius of the TM helices at the entrance gate (ξ = 0).

When analyzing the reaction coordinate, it is possible to identify that, at distances far from the protein (ξ > 2 nm), there is almost no change in free energy. Thus, it can be considered that at such distances the bulk lipid hydrophobic environment is the main influence on the behavior of the molecules, and the presence of the P-gp is not felt. Then, the free energy decreases steadily with the colchicine slope remaining unchanged until it reaches the entrance gate. However, the slope of the tariquidar PMF is steeper at the beginning (2 < ξ < 1.5 nm), having the same slope as for colchicine afterward. The initial steep decrease on tariquidar slope was observed to be due to a conformational change of tariquidar where the quinoline-3-carboxamide moiety shifted from a parallel to a perpendicular orientation regarding the POPC acyl chains, inducing a more packed conformation and having a positive impact on the motion of tariquidar between the hydrophobic lipid chains (colchicine is unable to make this transition from an elongated to a compact conformation; cf. Figure 1). Interestingly, the stabilization energy due to the conformational change of tariquidar gained at this step is roughly maintained along the reaction coordinate.

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With both molecules already located at the entrance gate, the energetic difference between them is $\Delta\Delta G$ = 12.8 kJ·mol^{-1} (−44.3 vs −57.1 kJ·mol^{-1} for colchicine and tariquidar, respectively). Immediately after crossing the gate, colchicine reorientates, allowing the formation of transient hydrogen bonds with Ser340 and Gln343 (TM 6) favoring a better interaction between colchicine and P-gp.

The crossing of the gate by the molecules could be foreseen as a bottleneck in the pathway to the DBP, in a location that serves as an interface between the lipid environment and the protein/water environment. Interestingly, the data show no activation energy barrier for any of the molecules when crossing the gate. At the end, the calculated free energies for drug permeation from the bilayer core to the drug-binding pocket were ΔG = −72.4 kJ·mol^{-1} for colchicine and ΔG = −79.0 kJ·mol^{-1} for tariquidar, with a $\Delta\Delta G$ = 6.6 kJ·mol^{-1} between both molecules.

It was already determined that P-gp reorganizes the surrounding lipid environment up to a 15−20 nm radius around the protein,[29] inducing a more ordered state that may promote drug efflux, while others showed that P-gp also has a large destabilizing effect on the lipid environment preventing up to 375 ± 197 lipids to undergo phase transition.[30] The lower limit of this lipid estimate is close to the size of our MD box (6 nm radius around the protein). However, the lipids are expected to shield the influence of P-gp on drug-like molecules inside the membrane. From Figure 2, the real impact on the free energy due to the P-gp starts at a distance of about 4.25 nm. This lipid organization will have an impact on the molecules conformations when located at the hydrophobic membrane core. As observed for tariquidar, changes in the orientation of some moieties induce more packed conformations that facilitate drug translocation to the P-gp drug-binding pocket.

These results show a spontaneous route for drug entrance from the hydrophobic membrane core (hydrophobic tail region of the cytoplasmic leaflet) into the internal drug-binding pocket, through the entrance gate located between TM helices 10 and 12. These data are found to be in agreement with both *flippase* and *hydrophobic vacuum cleaner* models.

The herein reported results can be additionally linked to those obtained by Oliveira et al.[31] for Sav1866 in which it was shown that doxorubicin interaction energy profile is downhill inside the Sav1866 drug-binding pocket to the extracellular side along the transmembrane pore. Sav1866 and P-gp are both efflux pumps from the same ABC transporter superfamily; however, Sav1866 crystallographic structure was obtained in an outward-facing conformation.[32] Therefore, there is no reason to think that a drug leaving the DBP of P-gp would behave differently.

Together, these results allow us to conclude that P-glycoprotein can indeed extract drugs from the cytoplasmic leaflet of the membrane through a gate and translocates them to outside of the cell. However, it is worth noticing that, for the last step to occur (molecule leaving the DBP), ATP hydrolysis is required to promote conformational changes that shift the drug-binding sites from high-affinity to low-affinity states[33] promoting drug release from the drug-binding sites into the extracellular medium.

During the translocation of the molecules from the hydrophobic membrane core into the DBP, differences regarding colchicine and tariquidar interaction with P-gp were also observed. Colchicine seems to interact preferentially with polar residues, namely, Asn835 (TM9), Ser988, and Tyr994 (TM12) when already at the entrance gate and with Ser340 and Gln343 (TM6) and Ala991 and Tyr994 (TM12) inside the pocket. However, while still in the hydrophobic membrane core, tariquidar is able to interact with Phe693 and Trp694 (TM7), aromatic residues that are in direct contact with the phospholipids. Contacts with Asn835, Asn838 (TM9), and Tyr994 (TM12) follow as tariquidar approaches the entrance gate, also interacting with Val984, Val987, Ser988, Ala991, and Pro992 (TM12) while entering the pocket. Inside the DBP, tariquidar interacts with all residue types (hydrophobic, polar, and aromatic). Interestingly, tariquidar but not colchicine also interacts with the charged linker residue Arg676, which may also account for the lower ΔG energies registered inside the pocket.

Furthermore, in order to provide additional evidence that would characterize the drug permeation to the DBP for these two molecules, the energetic profile for drug permeation from the bulk water into the hydrophobic membrane core (in the absence of P-gp) was also calculated.

Figure 3 shows that colchicine and tariquidar permeation into the hydrophobic membrane core is energetically favorable.

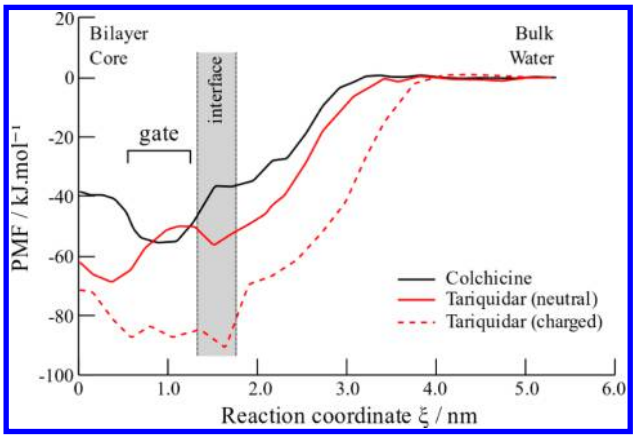

Figure 3. Potential of mean force (PMF) energetic profile for colchicine and tariquidar permeation from bulk water into the hydrophobic membrane core in the absence of P-gp. The gray shading represents an estimation of the headgroup thickness in the POPC membrane.

For tariquidar, the protonation of its tertiary nitrogen (pK_a 8.23, calculated in MarvinSketch[34]), favors a more elongated structure that allows the quinoline-3-carboxamide moiety to enter the membrane while the protonated tetrahydroisoquinoline moiety remains near the phosphate headgroups. Then, phosphate-assisted deprotonation of the tertiary nitrogen occurs[35] to allow neutral tariquidar to enter the membrane.

At the membrane hydrophobic core, the free-energy minima for both colchicine and tariquidar are found to be located below the phosphate headgroups (colchicine) or between the lipid acyl chains of the cytoplasmic leaflet (tariquidar). Since the entrance gate is also found to open to the cytoplasmic leaflet,[29] this allows a more direct access to the drug-binding pocket for both molecules.

At approximately 2 nm from the entrance gate (Figure 2, ξ = −2 nm) both molecules are driven to approach P-gp through a favorable downhill energetic pathway while entering the internal drug-binding pocket. Inside, molecules interact in one (or more) of three identified drug-binding sites,[18,22,28]

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inducing conformational changes that favor ATP binding and hydrolysis.[6,36,37] Thus, while shifting from the inward- to the outward-facing structure, the affinity of the drug-binding sites decreases[4] and drugs are released to the extracellular medium, following an overall downhill energetic pathway as shown by Oliveira et al. for doxorubicin molecule in Sav1866.[31]

The present letter aims to clarify the hypothesis by which drugs can get access to the P-glycoprotein internal drug-binding pocket through entrance gates located between TM helices 4/6 and 10/12. In a previous work, MD simulations on the murine P-gp crystallographic structure were performed to find that only one entrance gate was opened to the membrane core. Following, steered MD simulations were performed to drive drug entry from the membrane into the internal drug-binding pocket and calculating the PMF using the obtained trajectory by means of Umbrella Sampling with the WHAM algorithm. For both colchicine (substrate) and tariquidar (modulator), it was found that the reaction coordinate for drug entrance follows an overall downhill energetic pathway. Although tariquidar slope steeps at the beginning due to a conformational change that stabilizes the molecule, both molecules approach the gate with similar energies, being registered a final $\Delta\Delta G$ = 6.6 kJ·mol^{-1} between colchicine (−72.4 kJ·mol^{-1}) and tariquidar (−79.0 kJ·mol^{-1}).

Finally, these data together with drug permeation profiles from bulk water to the membrane and the data gathered by Oliveira et al.[31] corroborate the fact that P-gp may act accordingly to the *hydrophobic vacuum cleaner* model. Because all but one stage of drug efflux (drug permeation, drug DBP entry, and drug release after ATP hydrolysis) are energetically favorable, P-glycoprotein seems to function as a "facilitator" that bypasses drug flip-flop between membrane leaflets, allowing drugs to pass from the cytoplasmic leaflet directly into the extracellular medium in order to lower its intracellular concentration. However, for drug release to occur, P-gp must shift the binding affinities in drug-binding sites from high to low affinities, with such conformational changes intimately related with ATP binding and hydrolysis at the nucleotide-binding domains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jctc.5b00652.

Detailed description of the MD simulation protocol (Material and Methods) and additional data on drug entry from the lipid bilayer (Figures S1−S10) or drug permeation from bulk water into the membrane (Figures S11−S22) (PDF)

Video of reaction coordinate for colchicine (DBP) as a function of time (AVI)

Video of reaction coordinate for tariquidar (DBP) as a function of time (AVI)

Video of reaction coordinate for colchicine (membrane) as a function of time (AVI)

Video of reaction coordinate for tariquidar (neutral; membrane) as a function of time (AVI)

Video of reaction coordinate for tariquidar (protonated; membrane) as a function of time (AVI)

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Notes

The authors declare no competing financial interest.

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