Cryogenic-electron microscopy (Cryo-EM) structures of a human ABCG2 mutant transporter protein

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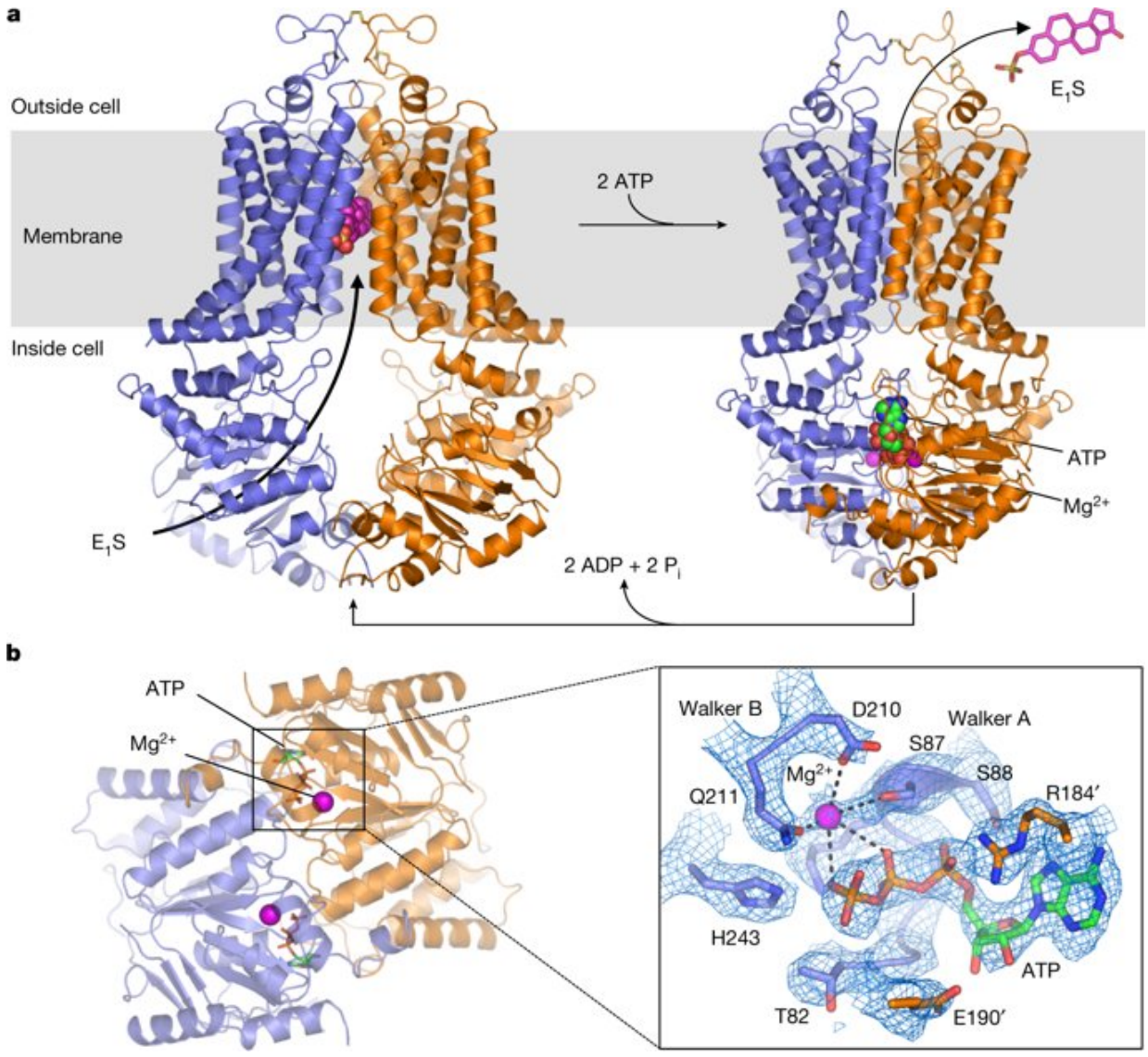


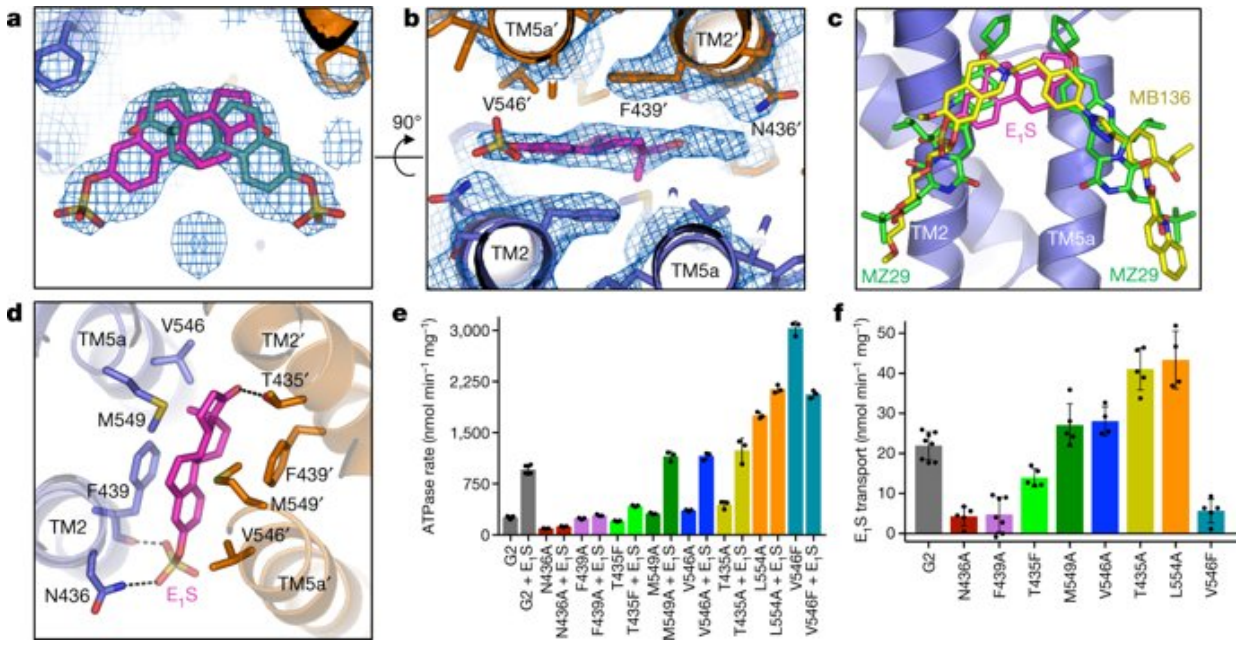
Structure and transport cycle of ABCG2. a) Cartoon representation of ABCG2EQ- E1S (left) and ABCG2EQ-ATP (right). ABCG2 monomers colored blue and orange. Bound E1S, ATP and Mg2 were shown as spheres. In ABCG2EQ the bound 5D3-Fab was omitted for clarity. b) structure of NBD dimer from the ATP-band state viewed from the cytoplasm with bound ATP (sticks) and Mg2+ ions (spheres). Inset rotated ~ 150 degrees to the right, viewed from membrane: electron microscopy density around bound ATP with Walker A and Walker B motif, E190 of the signature motif and the 'switch' histidine (H243) labelled and shown as sticks. The Mg2+ is shown as a purple sphere. Credit: *Nature*, doi: https://doi.org/10.1038/s41586-018-0680-3

The transporter protein ABCG2 belongs to the ATP-binding-cassette (ABC) family. The protein is expressed in the plasma membranes of cells within a variety of tissues and tissue barriers, including the blood-brain, blood-testes and maternal-fetal barrier. The protein can be powered by ATP to translocate endogenous substrates, affect the pharmacokinetics of many drugs and protect against a variety of xenobiotics including anticancer drugs, notably in breast cancer. ABCG2 is often referred to as the breast cancer resistance protein, where previous studies have revealed the ABCG2 architecture and structural basis of ABCG2 inhibition with small molecules and antibodies. The mechanism of substrate recognition by ABCG2 alongside its ATP-driven transport capability remains yet to be determined.

In a new report now published in *Nature*, Ioannis Manolaridis and colleagues presented high-resolution cryoelectron microscopy structures of human ABCG2 in its substrate-bound state of pre-translocation and in the ATP-bound post-translocation state. The scientists used a mutant protein containing a glutamine in place of the catalytic glutamate ($ABCG2_{EQ}$) to observe both states. The mutant $ABCG2_{EQ}$ showed reduced ATPase and transport rates to enable conformational trapping suited for the structural studies. In the substrate-bound state of the study,

about halfway across the membrane in a cytoplasm-facing cavity, a single molecule of estrone-3-sulfate (E_1S) was integrated for functional studies.

Rigid-body shifts of the transmembrane domain were observed as a result of ATP-induced conformational changes, pivoting the nucleotide-binding domains (NBDs) and changes in the relative NBD subdomain orientation. In its mechanism of action, ABCG2 harnessed the energy of ATP binding to extrude E_1S and other endogenous substrates. Based on the size and binding affinity of compounds it was therefore possible to distinguish substrates from inhibitors in the study.



Analyzing the substrate binding cavity and the mutant. a) C2-symmetrized electron microscopy density of ABCG2EQ-EIS; bound EIS molecule (pink or turquoise sticks) shown in two possible orientations rotated by 180 degrees along the y-axis. b) Similar to (a) but rotated 90 degrees and showing one EIS molecule and surrounding residues as labelled. c) Overlay of EIS (pink sticks) and inhibitors M229 (green sticks) and MB136 (yellow sticks) bound in the substrate-

binding cavity after superposition of the three structures. d) Substrate-binding cavity viewed from within the membrane, showing side-chains (sticks) of residues interacting with EIS (pink sticks). e) ATPase activities of liposome-reconstituted wild type and mutant ABCG2 in the presence and absence of EIS (50 μM). f) The initial EIS-transport activities. Credit: *Nature*, doi: https://doi.org/10.1038/s41586-018-0680-3.

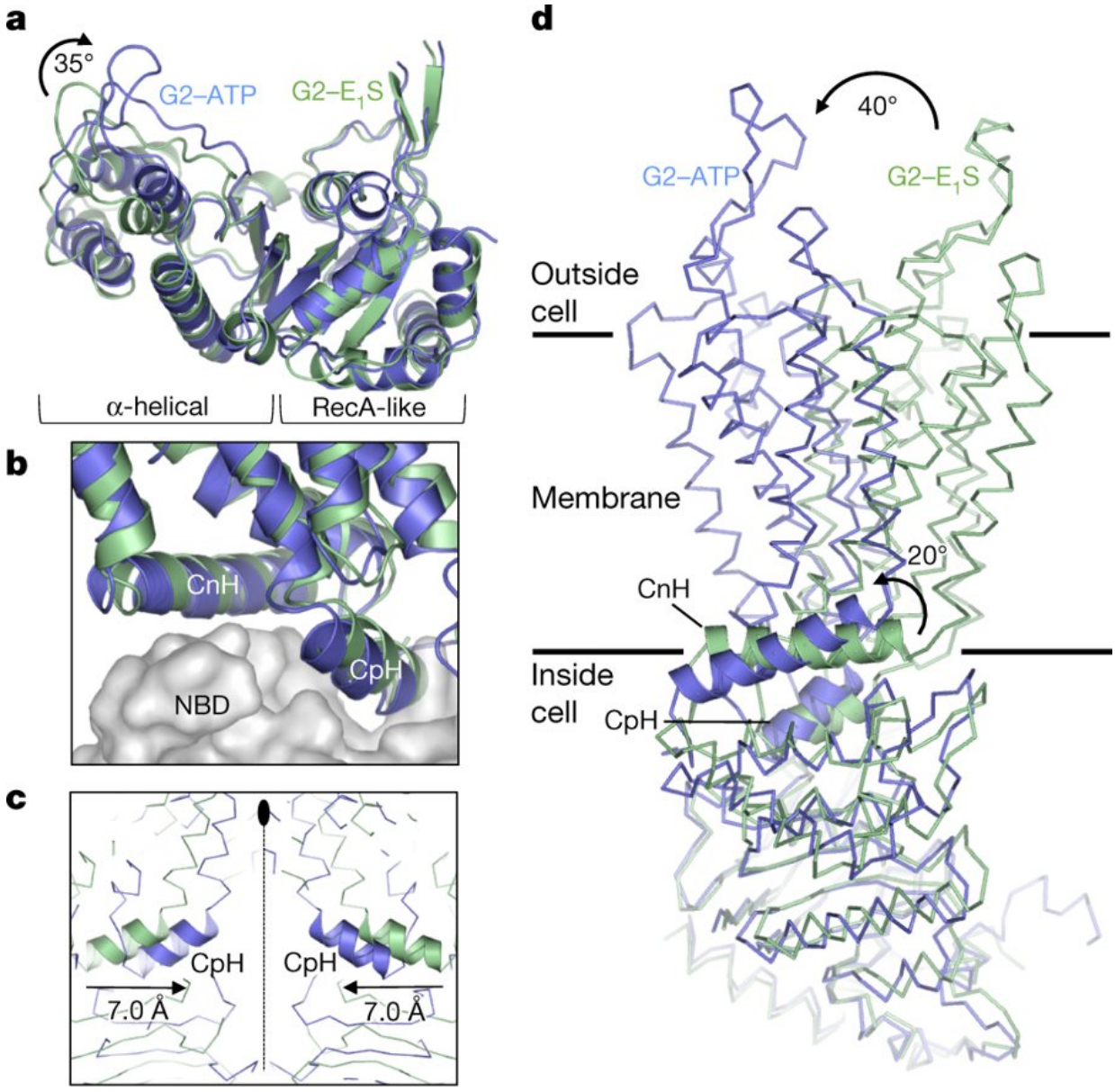
The authors first established that replacing the catalytic glutamate E211 with a glutamine in the Walker B motif (phosphate binding sequence) would result in mutants with greatly diminished (although not abolished), ATP hydrolysis and E_1S transport activity. To determine the E_1S bound structure, an antigen-binding fragment of monoclonal antibody 5D3 (5D3-Fab) was added to the sample – which bound to the external surface of ABCG2, to facilitate high-resolution structure determination. The ATP hydrolysis was slowed down and transport activity of liposome-reconstituted ABCG2 inhibited by the addition of 5D3-Fab, without impacting the interaction between ABCG2 and E_1S substrate. The $ABCG2_{EQ}$ complex revealed an inward-open conformation with an overall resolution of 3.6 Å to clearly visualize the transmembrane domain and the substrate binding cavity.

The authors observed a density feature formed by the transmembrane (TM) helices TM2 and TM5a in the substrate-binding cavity of ABCG2 monomers. The density feature could bind one E_1S substrate in two orientations, although two E_1S molecules could not be bound simultaneously due to steric clashing of the polycyclic ring systems. The substrate binding cavity could also accommodate inhibitors, demonstrating its dual role in substrate and multidrug binding.

In the study, the scientists generated single point mutations for $ABCG2_{EQ}$- E_1S to determine ATPase activity and E_1S transport of the

resulting mutant variants. The stability of all tested mutants showed similarity to the native, wild-type protein allowing direct comparison. The V546F mutant for instance, had impaired transport activity but a 12-fold increase in ATPase activity, although that was inhibited by E_1S in a concentration-dependent manner. This result suggested that the introduction of two phenyl rings into the substrate cavity enabled substrate-binding mimicry for ATPase activity stimulation, but further inclusion of E_1S 'clogged' the transporter. Results for other mutant variants varied in comparison to the wild-type protein in the study, emphasizing the sensitivity of the binding cavity to modifications.

Additional functional studies with cryo-EM microscopy showed that ATP binding caused a 35 degree rotation of the α-helical domains of NBD (nucleotide-binding domains) for dimerization—to enable the 'power stroke' in the transport cycle. In each ABCG2 monomer, individual transmembrane nucleotide binding domain interfaces remained largely unchanged between the nucleotide-free and ATP-bound states. However, ATP-induced conformational changes triggered shifts in NBD causing cytoplasmic components of the transmembrane domain (TMD) to push towards each other. Such ATP-induced conformational changes have important roles in the substrate-translocation pathway.

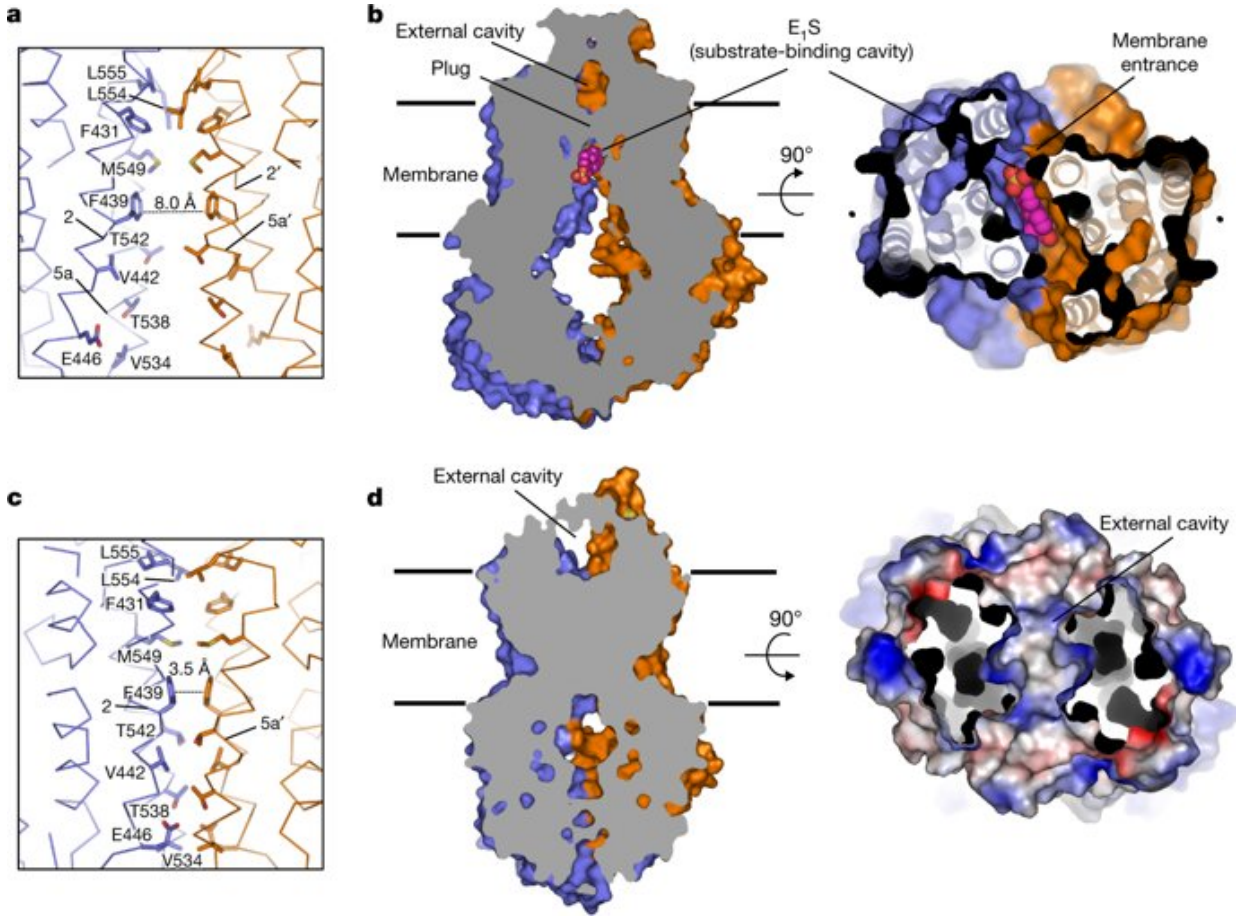


ATP-induced conformational changes. a) Superposition of the RecA-like subdomains of the NBDs (Nucleotide binding domains) of the ABCG2EQ–E1S (green) and ABCG2EQ–ATP (blue) structures. A roughly 35-degree inward rotation of the helical subdomain is observed upon ATP binding. b) Superposition of one ABCG2 monomer of the ABCG2EQ–E1S and ABCG2EQ–ATP structures, with the NBDs shown as a grey surface and the TMDs (transmembrane domains) as ribbons. The interface helices—CpH and CnH—are labelled. c) Superposition of the ABCG2EQ–E1S and ABCG2EQ–ATP structures along the two-fold symmetry axis (dotted line),

showing a 7 Å inward movement of the CpH helices of each ABCG2 monomer. d) Comparison of a single ABCG2 monomer of the ABCG2EQ–E1S and ABCG2EQ–ATP structures. The NBDs have been superimposed, revealing a 20-degree rotation of the CnH and CpH helices (shown as ribbons) as well as a 40-degree rotation of the TMDs relative to one another. Credit: *Nature*, doi: https://doi.org/10.1038/s41586-018-0680-3.

Structurally in the ATP-bound state of $ABCG2_{EQ}$-E_IS, phenyl rings stacked against each other, collapsing the substrate-bending cavity without space for the bound substrate. For transportation across the membrane, the substrates had to move through the translocation pathway at the center of the transporter to reach the external cavity before the pathway closed completely. For this process, transient conformational changes resembling peristaltic motion had to occur to generate space for the substrate.

The authors assumed that the substrate may bind via the cytoplasm or via the "membrane entrance" within the lipid bilayer. Once bound, the NBD dimer could only close when the substrate moved out of the substrate-binding cavity. In a productive transport cycle, the substrate was assumed to move through a translocation pathway at the center of the transporter via a "leucine plug." Once a substrate cleared the plug area, the structure of ATP-bound $ABCG2_{EQ}$ suggested that the plug region closed, and substrate was released to the outside.



Substrate translocation pathway. a) Cα trace of the translocation pathway region of ABCG2EQ–E1S. Residues lining the substrate-binding cavity are shown as sticks; bound E1S omitted for clarity. The dashed line shows the distance between the two F439 residues that stack against bound E1S. b) Vertical slice through a surface representation of ABCG2EQ–E1S, with bound E1S shown as pink spheres and the two cavities and plug region labelled. In the right-hand panel, a 90° rotation of the structure reveals the fit of E1S in the substrate-binding cavity, as viewed from the cytoplasm. The NBDs have been removed for clarity. c) and (d), As for a, b, but with the ABCG2EQ–ATP structure. In the right-hand panel of (d) the molecular surface of the external cavity is viewed from the extracellular space and color-coded by electrostatic potential ranging from blue (most positive) to red (most negative), shown with the extracellular loop EL3 removed for clarity. Credit: *Nature*, doi: 10.1038/s41586-018-0680-3.

One caveat in the study was that the E211Q mutation may have influenced the energetics of the conformational changes involved. The findings suggested that ATP binding may be sufficient for the substrate-extrusion step and the transporter could be reset to an inward-facing conformation by ATP hydrolysis. Unlike other transporters, the ABCG2 did not appear to form a stable, occluded conformation upon substrate binding, but demonstrated a transient conformation with peristalsis-like mechanisms akin to the bacterial BtuCD-F transporter.

A key question that had remained unanswered with polyspecific multidrug transporters such as ABCG2 was why some compounds acted as substrates, while others acted as potent inhibitors. When the authors compared the binding modes of ABCG2 substrate with those of two potent inhibitors, all three molecules bound to the same cavity of the transporter. The study further showed that when the substrate E_1S and ATP bound together to ABCG2, an opening of the plug allowed the substrate to be pushed into the external cavity. In contrast, inhibitors acted as wedges to immobilize the transporters by locking the structure in an inward-facing conformation. The study suggested that the size and binding affinity of the compounds were important for translocation in the transporters, thereby allowing to distinguish substrates from inhibitors. The work provides new insight to further understand dynamics of the transporter protein ABCG2.

More information: Ioannis Manolaridis et al. Cryo-EM structures of a human ABCG2 mutant trapped in ATP-bound and substrate-bound states, *Nature* (2018). DOI: 10.1038/s41586-018-0680-3

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