Structure of a zosuquidar and UIC2-bound human-mouse chimeric ABCB1

Amer Alam†, Raphael Küng‡, Julia Kowal‡, Robert A. McLeod‡, Nina Tremp‡, Eugenia V. Broude*, Igor B. Roninson‡, Henning Stahlberg†, and Kaspar P. Locher†,‡,1

*Institute of Molecular Biology and Biophysics, ETH Zürich, 8093 Zürich, Switzerland; ‡Center for Cellular Imaging and NanoAnalytics, Biozentrum, University of Basel, 4058 Basel, Switzerland; and †Department of Drug Discovery and Biomedical Sciences, College of Pharmacy, University of South Carolina, Columbia, SC 29208

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The multidrug transporter ABCB1 (P-glycoprotein) is an ATP-binding cassette transporter that has a key role in protecting tissues from toxic insult and contributes to multidrug extrusion from cancer cells. Here, we report the near-atomic resolution cryo-EM structure of nucleotide-free ABCB1 trapped by an engineered disulfide cross-link between the nucleotide-binding domains (NBDs) and bound to the antigen-binding fragment of the human-specific inhibitory antibody UIC2 and to the third-generation ABCB1 inhibitor zosuquidar. Our structure reveals the transporter in an occluded conformation with a central, enclosed, inhibitor-binding pocket lined by residues from all transmembrane (TM) helices of ABCB1. The pocket spans almost the entire width of the lipid membrane and is occupied exclusively by two closely interacting zosuquidar molecules. The external, conformational epitope facilitating UIC2 binding is also visualized, providing a basis for its inhibition of substrate efflux. Additional cryo-EM structures suggest concerted movement of TM helices from both halves of the transporters associated with closing the NBD gap, as well as zosuquidar binding. Our results define distinct recognition interfaces of ABCB1 inhibitory agents, which may be exploited for therapeutic purposes.

ABC transporter | structure | small-molecule inhibitors | cryo-EM | mechanism

P-glycoprotein (ABCB1) is an ATP-binding cassette transporter ubiquitously expressed in a wide range of cells and tissues, where it has an important role in cellular detoxification (1, 2). Its wide substrate specificity underlies its physiological relevance in drug transport across multiple blood–organ barriers, resulting in modulation of drug delivery, drug–drug interactions, and drug pharmacokinetics for a host of clinically active agents (1–4). ABCB1-mediated drug efflux has been linked to multi-drug resistance, adversely affecting cancer chemotherapeutic treatment and treatment of various brain disorders (5–7). The ABCB1-specific inhibitory antibody UIC2 was developed with the goal of increasing antitumor drug treatment efficacy (8, 9). UIC2 targets the extracellular moiety of human ABCB1 (ABCB1h) and has been successfully used to investigate the transport cycle of ABCB1 owing to its ability to bind the transporter during various states of the catalytic cycle (9–11). Although the crystal structure of the antigen-binding fragment (Fab) of UIC2 has been determined (12), the conformational epitope on the surface of ABCB1h has remained elusive because direct visualization of the ABCB1–UIC2 interface was limited to low-resolution cryo-EM reconstructions (13). To date, several structures of mouse ABCB1 are available, including those with bound inhibitors (14–17) and nucleotides (18). However, despite added ligands, all these reveal the transporter in an inward-open conformation with varying degrees of nucleotide-binding domain (NBD) separation and a largely unchanged transmembrane domain (TMD) and substrate translocation pathway.

Small-molecule, third-generation inhibitors, including zosuquidar, tariquidar, and elacridar (19–21), have shown potential in model systems for coadministration with various anticancer agents. However, antibody- and small-molecule inhibitor–based strategies have not been successfully utilized clinically (22, 23), emphasizing the need for designing more effective therapeutic and diagnostic strategies targeting ABCB1 expression and inhibition. A detailed understanding of the structure of ABCB1 with bound antibody fragments and drugs/inhibitors in different conformations is central to improving the specificity of inhibitors and to further our understanding of the chemistry governing ABCB1 interaction with high-potency inhibitors. To achieve this, we generated a hybrid construct of human and mouse ABCB1 (ABCB1hm) that contains the extracellular region of the human protein, including the recognition epitope of UIC2, on a background of mouse ABCB1. ABCB1hm shares ~90% sequence identity with the human protein (SI Appendix, Fig. S1) and takes advantage of the higher biochemical stability of mouse ABCB1, which shares high sequence similarity and, except for subtle differences, exhibits highly similar substrate/inhibitor-binding properties with its human homolog, especially with regard to third-generation inhibitors (24). As highlighted in Fig. 1 and SI Appendix, Fig. S1, our results reveal key ABCB1-specific residues involved in the interaction interfaces of both zosuquidar and UIC2 and ABCB1. ABCB1 has been observed to exist in a range of conformations with varying degrees of NBD separation, including a nucleotide-

Significance

The ATP binding cassette transporter ABCB1 (also termed P-glycoprotein) is a physiologically essential multidrug efflux transporter of key relevance to biomedicine. Here, we report the conformational trapping and structural analysis of ABCB1 in complex with the antigen-binding fragment of UIC2, a human ABCB1-specific inhibitory antibody, and zosuquidar, a third-generation ABCB1 inhibitor. The structures outline key features underlining specific ABCB1 inhibition by antibodies and small molecules, including a dual mode of inhibitor binding in a fully occluded ABCB1 cavity. Finally, our analysis sheds light on the conformational transitions underlying by the transporter to reach the inhibitor-bound state.

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†To whom correspondence should be addressed. Email: locher@mol.biol.ethz.ch.

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bound “closed” conformation, where no gap between NBDs exists (13). To understand its interaction with small molecules, we sought to visualize an intermediate conformation of the transporter, where the NBD gap has been partially, but not fully, closed. To visualize and analyze the effect of progressive NBD gap closure on the TMD domains (in the absence of bound nucleotides in this case), we therefore generated a variant that allowed disulfide cross-linking of the NBDs of the transporter (ABCB1<sub>HM-X</sub>). The latter harbors two S→C mutations at positions 559 and 1204 in the dimerization (D)-loops, expected to come within close proximity to each other upon NBD dimerization as seen in the canonical ABC exporter Sav1866, among others, and successfully used for conformational trapping and high-resolution analysis of other ABC transporters (25, 26).

**Results**

**Conformational Trapping and Functional Characterization of ABCB1<sub>HM</sub>.** ABCB1<sub>HM-X</sub> was shown in vivo to confer up to 50-fold higher paclitaxel resistance to mammalian cells when expressed in an inducible HEK293 cell line compared with uninduced cells (SI Appendix, Fig. S2A), showing that the construct retains paclitaxel transport activity. The construct is strongly inhibited by zosuquidar and UIC2, which resensitize ABCB1<sub>HM-X</sub>-expressing cells to paclitaxel (Fig. 1B and C) at nanomolar concentrations. The constructs were expressed in HEK293 cells and purified in a lauryl maltose neopentyl glycol (LMNG) and cholesteryl hemisuccinate (CHS) mixture in contrast to the more commonly used dodecyl maltoside (DDM)-based preparations to avoid interference from nonspecifically bound DDM molecules in the transmembrane (TM) ligand-binding cavity. Disulfide cross-linking of ABCB1<sub>HM-X</sub> was nearly complete in the absence of added nucleotides, as revealed by gel electrophoresis (SI Appendix, Fig. S2B), and ABCB1<sub>H</sub>, ABCB1<sub>HM</sub>, and cross-linked or reduced ABCB1<sub>HM-X</sub> all showed near-identical UIC2-binding affinities (SI Appendix, Fig. S2C).

While previous studies have reported negligible basal ATPase rates for detergent-purified ABCB1 (27), our LMNG/CHS-purified ABCB1<sub>H</sub> and ABCB1<sub>HM</sub> constructs maintain a measurable, albeit low, basal ATPase rate (~20–30 nmol min<sup>−1</sup> mg<sup>−1</sup>), which is stimulated up to 10-fold by addition of zosuquidar or the
anticaner drug paclitaxel (SI Appendix, Fig. S2D), allowing for characterization of drug-mediated ATPase activity in an identi-
cal environment as used for our structural analysis. Although disulfide cross-linking did not alter Kₐₕ values for ATP hydrolysis in ABCB₁₉M and its ATPase stimulation by zosuquidar, we observed a reduction in ATPase stimulation by paclitaxel that could be reversed by addition of DTT (SI Appendix, Fig. S2 D and E). Interestingly, zosuquidar has been shown to stimulate the ATPase rate of detergent (DDM)-solubilized ABCB1 but inhibits the lipid-reconstituted ABCB1 ATPase rate, with the effect in the latter being much stronger (EC₅₀ ~ 200 nM, roughly 50-fold lower than that obtained in DDM micelles) (24). This opposing effect in detergent versus lipid has been speculated to stem from the occlusion of a primary high-affinity zosuquidar-binding site by DDM molecules, leading to zosuquidar binding to a secondary low-affinity site. As seen in SI Appendix, Fig. S2F, while the ATPase activity of both ABCB₁₉ and ABCB₁₉M pur-
rified in LMNG/CHS is stimulated by zosuquidar, the EC₅₀ values for this modulation are ~200 nM, comparable to those reported for native membranes. Combined with the absence of bound detergent molecules in our inhibitor-bound structure, our results suggest that zosuquidar is bound to its primary site in our studies, as explained below.

**Overall Structure of ABCB1–UIC2 in Complex with Zosuquidar.**

The structure of cross-linked, zosuquidar-bound ABCB₁₉M was solved to an overall resolution of 3.58 Å [Fourier shell correlation (FSC) cutoff of 0.143] (Fig. 1D and SI Appendix, Fig. S3). The high disulfide trapping efficiency correlated well with the observation that the overwhelming majority of usable particles corresponded to a single conformation of ABCB1. The local resolution was highest in the TM region, allowing unambiguous de novo building of the TMDs, including the zosuquidar-binding pocket, as well as the ABCB1–UIC2 interface (SI Appendix, Fig. S4). Despite their close association due to the disulfide cross-link, the NBDs did not adopt a fully closed sandwich confor-
mation, which is a hallmark of ATP-bound structures, because no nucleotide was added. They maintained a degree of confor-
mational flexibility reflected in the lower resolution of the electron density, nevertheless allowing atomic interpretation guided by the structures of mouse ABCB1 previously reported with minor manual adjustment where appropriate. Despite the overall lower resolution for the NBDs, density for the introduced disulfide cross-link was visible. The NBD arrangement was somewhat asymmetrical, closely matching that observed in the heterodimeric, bacterial homolog of ABCB1, TM287/288 (28). Asymmetry in ABCB1 hydrolysis has previously been proposed (18, 29) and also observed in a bacterial homolog of asymmet-
rical B family transporters, TmrAB (30). Our structure revealed a single copy of UIC2-Fab bound to the external side of the transporter and tilted by about 70° relative to the membrane plane (Fig. 1D). Whereas the architecture and fold of ABCB₁₉M are similar to those of mouse ABCB1 (15, 18), the trapped conformation is distinct from previous structures because the NBDs are closer together and the TMDs assume a conformation that provides structural insight into an occluded and inhibitor-
bound state of a B-family ABC exporter. As with previously reported ABCB1 structures, no density was observed for the linker region connecting the two halves of the transporter.

**Molecular Details of the ABCB1–UIC2-Binding Interface.** UIC2 rec-
ognizes a conformational epitope of ABCB1 that involves resi-
dues from TM1 and TM2 and the extracellular loops EL1, EL3, and EL4 (Fig. 2A and B), in agreement with biochemical studies that have identified the external part of TM1 (8) and the external loop connecting TM5 and TM6 (EL3) (11) as contributing to the ABCB₁₉–UIC2 interface. The buried surface area amounts to ~1,075 Å², with the heavy chain of UIC2-Fab forming the bulk of the interface (~80%). The observed interactions include polar, electrostatic, and hydrophobic contacts, including methionine-pi stacking. Differences in the amino acid sequences of the external loops of ABCB₁₉M can readily explain the specificity of UIC2 for the human protein over that of rodents (Fig. 2C). Additionally, the observed interaction provides a structural explanation of how UIC2 inhibits transport activity of ABCB1. By clamping the ex-
ternal loops together, UIC2 prevents ABCB1 from converting to an outward-open conformation, which is required to release sub-
strate to the outside (31, 32). This is in line with studies indicating incomplete UIC2 binding in unmodified ABCB1-expressing cells (8), where the transporter is expected to exist in a mixture of con-
formations, including a UIC2-incompatible, outward-open state. It also agrees with the observed modulation of UIC2 affinity for ABCB1 in the presence or absence of various nucleotides and substrates (11). Any alteration of the equilibrium between the outward-open and outward-closed conformations of ABCB1 caused by the binding or release of drugs/nucleotides as part of the transporters’s catalytic cycle could alter its affinity for UIC2.

As ABCB1 has previously been shown to hydrolyze ATP in the presence of UIC2 (33), we conclude that the architecture of ABCB1 allows the NBDs to bind and hydrolyze ATP, while having the TMDs assume an outward-closed conformation. This is in agreement with the expected conformational landscape sampled by ABCB1 based on recent spectroscopic measurements and molecular dynamics studies on mouse ABCB1 (31), where an outward-occluded state with a closed NBD interface and outward closed TMD has been observed. Such a confor-
mation is also compatible with those observed in structures of related prokaryotic ABC exporters (28, 34), as well as the recently determined ATP-bound zebrafish CFTR structure (35), and is expected to be generally shared among various ABC exporter family members (36).

**Details of the Zosuquidar-Binding Pocket.** The zosuquidar-bound structure of ABCB1 reveals a large cavity that spans almost the entire width of the lipid membrane (Figs. 1D and 2 D and E). The cavity is sealed from the external and cytoplasmic sides, and is lined by residues from all 12 TMs of ABCB1 (Fig. 1D and SI Appendix, Fig. S1), comprising many of the residues identified in biochemical studies as contributing to drug interactions of ABCB1 (37–42). Although some of these side chains have been observed to be in contact with ligands in previously reported structures of ABCB1, these structures all described inward-facing conformations, with no enclosed cavities observed and fewer interactions with bound substrates (14, 15, 17, 43). As shown in Fig. 2 D and E, our EM density unambiguously revealed two zosuquidar molecules bound in the enclosed cavity, filling most, but not all of the available space. As a consequence, residues from eight TMs directly interact with bound zosuquidar. The two zosuquidar molecules assume defined orientations, are wrapped around each other, and roughly follow a twofold rota-
tional symmetry. This is in stark contrast to structures of mouse ABCB1 in complex with cyclic inhibitors in the inward-open conformation (43). The distribution of interacting residues, which are fully conserved between our construct and ABCB₁₉ (green dots in Fig. 1B and SI Appendix, Fig. S1), demonstrates that the two halves of ABCB1 contribute in a pseudosymmetrical fashion to zosuquidar binding, as the contacting residues belong to TM1, TM4, TM5, and TM6 (first half of ABCB1) and to TM7, TM10, TM11, and TM12 (second half of ABCB1). In addition to the many aromatic and hydrophobic residues, there are polar or charged side chains in the observed cavity (SI Appendix, Fig. S5). The majority of these have been predicted to be part of the primary drug-binding site of ABCB1 (15, 40, 41, 44). Specifically, Y953 is seen in our structure to hydrogen-bond with one of the zosuquidar molecules (SI Appendix, Fig. S6B) as predicted based on molecular dynamics and mutagenesis studies, although this study predicted
only one zosuquidar bound in the cavity in a different configuration compared with that observed in our structure. The absence of interfering detergent, along with the close association of the bound inhibitors with residues predicted to be primary contributors to specific binding of zosuquidar (37), further validates the relevance of the observed binding site. The observation of two bound zosuquidar molecules agrees with previous biochemical and structural findings suggesting that the ABCB1 drug-binding pocket is capable of accommodating multiple ligands simultaneously (43, 45). The presence of many aromatic residues can offer an adaptive plasticity to the pocket, in line with studies suggesting an induced fit mechanism of ligand recognition (46). We conclude that ABCB1 may bind inhibitors through a combination of specific interactions, leading to defined chemical and structural findings suggesting that the ABCB1 drug-binding pocket is capable of accommodating multiple ligands simultaneously (43, 45). The presence of many aromatic residues can offer an adaptive plasticity to the pocket, in line with studies suggesting an induced fit mechanism of ligand recognition (46). We conclude that ABCB1 may bind inhibitors through a combination of specific interactions, leading to defined
binding modes and a rigid core of bound inhibitor molecules surrounded by TM helices.

**Conformational Changes Associated with NBD Closure.** To understand the contributions of disulfide-mediated NBD trapping and zosuquidar binding to the TMD conformation and the formation of an inhibitor-binding pocket, we determined two additional cryo-EM structures (Fig. 3A). The structure of an amphipol (A8-34) reconstituted, non-cross-linked variant, ABCB1HM-EQ, was determined in the absence of any drugs or nucleotides, but in complex with UIC2-Fab (SI Appendix, Fig. S6). ABCB1HM-EQ harbors an E→Q mutation in the Walker-B motif, which increases the stability and limits the conformational heterogeneity of the sample. The ABCB1HM-EQ structure revealed an inward-open conformation with wide NBD separation, closely matching (rmsd = 1.88 for all aligned Ca atoms) previously reported mouse ABCB1 structures (15, 16, 18, 43) (SI Appendix, Fig. S6E). A detailed structural analysis (Fig. 3B) revealed that the transition from the inward-open state to the disulfide-trapped, zosuquidar-occluded state includes distinct changes in the TMDs, most significantly in the conformations of TM4 and TM10 (Fig. 3C). Despite exhibiting different sequences otherwise, the three residues P223, S238, and G251 in TM4 correspond to identically spaced and located residues P866, S880, and G894 in TM10, allowing for similar helix bending and kinking. Along with the already bent and kinked TM6 and TM12, this amounts to four TM helices of ABCB1 that form significant kinks, forming a cytoplasmic gate to the zosuquidar-binding cavity and closing the lateral membrane opening that exists in the inward-open conformation (Fig. 4A and B). We then determined the structure of disulfide-trapped ABCB1HM-X, also in complex with UIC2-Fab, but in the absence of drugs or nucleotides. We found that the separation of the

![Fig. 3. Conformational changes in ABCB1.](image-url)

(A) Comparison of apo-inward ABCB1HM–UIC2 (blue) and disulfide-trapped, apo ABCB1HM-X–UIC2 (yellow) using UIC2-Fab as an anchor point for the superposition. The gray box represents the approximate location of the plasma membrane. (B) Close-up view of the NBDs of the two structures shown in A, but using NBD1 as the superposition anchor. The coupling helices of ABCB1HM and ABCB1HM-X are colored red and black, respectively. The Ca atoms of V264 and V908 of the coupling helices are represented as spheres, with the distance between them shown as black and red arrows. (C) Superposition of the TM helix pairs TM3–TM4 and TM9–TM10 of the two ABCB1 structures colored as in A. Red and green spheres depict Ca atoms of selected residues, with numbers indicated. A sequence alignment of TM4 and TM10 of human ABCB1 is shown below the structures, and the designated residues are indicated with arrows.
NBDs in the apo ABCB1$_{HM,X}$ structure was identical to that of the zosuquidar-bound state. The conformation of the external loops of ABCB1 and the conformation of the interface with UI2 binding are indistinguishable in the three structures, which is in line with the observation that UI2 binding allows the transporter to cycle through its complete ATP hydrolysis cycle despite the absence of a discrete outward-open conformation (13, 33). Although the structure of disulfide-trapped apo ABCB1$_{HM,X}$ is at slightly lower resolution than the zosuquidar-bound structure, side-chain density is still visible for most TMs, allowing unambiguous determination of the side-chain register. When all particles are used for 3D reconstruction, we observed blurred density in the regions of TM4 (residues 237–244) and TM10 (residues 880–888) owing to local conformational flexibility (SI Appendix, Figs. S7–S9). Upon subclassifying, we could identify distinct and roughly equally populated conformations where TM4 and TM10 either adopt kinked conformations (class 1/map 2) as observed in the zosuquidar-bound state (Fig. 4C) or straight conformations (class 2/map 3) as observed in the apo-inward ABCB1$_{HM,X}$ structure. We conclude that the closing of the NBD gap by disulfide trapping, which is transmitted to the TMDs via the coupling helices, allows TM4 and TM10 to sample both the straight and kinked conformations. Upon binding of zosuquidar, only the kinked conformation is observed. A stabilization of the two TMDs of ABCB1 has been also been reported upon binding of the third-generation ABCB1 inhibitor tarquidar (38), whose proposed binding site overlaps with the zosuquidar-binding site in our structure, pointing to similar modes of interaction with the transporter. In the related multidrug transporter ABCC1/MRP1 (47), binding of substrate was accompanied by a slight reduction in NBD internal cavity volumes are depicted as dark blue and gold-colored surfaces. The da

Conclusions

Our results provide a number of insights into the modulation of ABCB1, as well as the chemistry of the underlying specific ligand interactions. First, we offer visualization of a fully occluded ligand-binding cavity of a type 1 ABC transporter occupied by a small-molecule inhibitor, identifying the details of zosuquidar binding to ABCB1. Such a conformation has been extremely difficult to trap owing to the highly dynamic nature of ABCB1, prompting us to employ covalent linkage of the NBDs. Given that disulfide-trapped ABCB1$_{HM,X}$ hydrolyzes ATP at a similar rate as the native protein and maintains stimulation by zosuquidar, the observed conformation provides physiologically relevant insight. This is further supported by the fact that we observed spontaneous and near-complete cross-linking for the designed cysteine pairs, and our structural analysis revealed the majority of particles to belong to a homogeneous class, consistent with an occluded state of ABCB1. Visualization of an occluded ABCB1 cavity offers a unique tool for in silico studies of drug binding to the transporter, which have so far relied on inward-open structures of ABCB1 and models based on different transporters. The ability of two inhibitor molecules to bind in tandem raises the possibility of cooperativity in binding, which, in the absence of a more sensitive readout for drug binding, we are currently unable to fully analyze. The zosuquidar-inhibited state captured in our structure also raises the question of whether transport substrates can bind similarly in well-defined orientations to the exact same site in ABCB1. Interestingly, based largely on its opposing ATPase

![Fig. 4. Structural changes in the translocation pathway.](Image)

(A) Surface representations of inward-open ABCB1$_{HM}$ and disulfide-trapped ABCB1$_{HM,X}$ structures (without bound UI2-Fab) colored blue and yellow, respectively. Internal cavity volumes are depicted as dark blue and gold-colored surfaces. The dashed oval indicates the location of the occluded TM cavity that binds zosuquidar in the zosuquidar-bound structure. The gray box represents the membrane. (B) Translocation pathways of ABCB1$_{HM}$ and ABCB1$_{HM,X}$ colored as in A, but viewed from the cytoplasmic side of the membrane. TM helices are shown as ribbons and numbered. Arrows indicate the constriction point or cavity gate formed by kinks in TM4 and TM10, closing off the cavity to the cytoplasm in the occluded ABCB1$_{HM,X}$ structure. (C) Side views of ribbon representation of TM4 and TM10 and the corresponding EM density of ABCB1$_{HM,X}$ structures. (Left and Center) Two populations of disulfide-trapped apo ABCB1$_{HM,X}$. Suggesting equilibrium between kinked (closed) and straight (open) conformations. (Right) Zosuquidar-bound structure, where only the kinked conformation exists. Density for the bound zosuquidar molecules is shown as red mesh.
modulatory behavior in lipid- or detergent-embedded ABCB1, zosuquidar, along with elacridar and tariquidar, has recently been proposed to bind to a low-affinity site in the presence of detergent, which is thought to bind to, and hence render, the proposed high-affinity site inaccessible for the inhibitor (24). While we cannot categorically rule out such a different binding mode for zosuquidar, a number of observations help further validate the physiological relevance and significance of our zosuquidar-bound structure. First, we observe no interfering detergent molecules in the binding cavity, arguing against detergent-mediated occlusion of the primary zosuquidar-binding site. Second, our EC50 values for zosuquidar modulation of LMNG-solubilized ABCB1 match those measured in native membranes, with our observed modulation effect being much stronger than that reported for DDM-based preparations (24). Third, the conformational changes in TM4 and TM10, although never before seen to occur in tandem, closely match those observed for TM4 in mouse ABCB1 in complex with select cyclic peptide inhibitors (43). In light of these findings, the explanation for the opposing effects of zosuquidar on ATPase activity of lipid-reconstituted or detergent-solubilized ABCB1 may need to be readdressed.

Second, the ability to narrow the NBD gap by cross-linking rather than addition of nucleotides allowed for the intriguing finding of abrogated ATPase stimulation by paclitaxel. While rather than addition of nucleotides allowed for the intriguing finding of abrogated ATPase stimulation by paclitaxel, which was synthetically generated (Thermo Fisher Scientific) for inducible expression as per manufacturer recommendations. For the variant of the ABCB1–UIC2 complexes (13).

Overall, the work presented herein opens new avenues for the development of more potent antibody and small-molecule inhibitors of ABCB1 that could be used alone or in combination with small-molecule inhibitors, while shedding light on the conformational changes undergone by the transporter upon ligand binding.

Materials and Methods

Protein Expression and Purification. A PCR-free cloning strategy was employed for all ABCB1 constructs, which were synthetically generated (Thermo Fisher Scientific). For the variant of the ABCB1-D-loop cross-link mutant (ABCB1-Dloop X), a 3C protease site (LEVLFQGP) replaced residues 668–675 in the linker connecting the two halves of the transporter, involving conserved, helix-breaking residues. The structural rearrangements in the TMDs may not only occur during inhibition but possibly also during a productive transport cycle.

Finally, we elucidate the conformational epitope of the UIC2 antibody and the mechanism of its inhibitory activity. Most importantly, our structure clearly rules out the existence of an outward-open ABCB1 conformation in the presence of UIC2 as speculated upon based on low-resolution EM analysis of human ABCB1–UIC2 complexes (13). Overall, the work presented herein opens new avenues for the development of more potent antibody and small-molecule inhibitors of ABCB1 that could be used alone or in combination with small-molecule inhibitors, while shedding light on the conformational changes undergone by the transporter upon ligand binding.

Protein Expression and Purification. A PCR-free cloning strategy was employed for all ABCB1 constructs, which were synthetically generated (Thermo Fisher Scientific). For the variant of the ABCB1-Dloop cross-link mutant (ABCB1-Dloop X), a 3C protease site (LEVLFQGP) replaced residues 668–675 in the linker connecting the two halves of the transporter. All genes were cloned into an expression vector harboring the pXLG gene expression cassette in a pUC57 vector (GenScript) (40, 50) between BamH1 and Not1 restriction digestion sites. All genes were cloned with a C-terminal EYFP/rhod-Id4 tag with a C-terminal EYFP/rhod-Id4 tag with a C-terminal EYFP/rhod-Id4 tag with an intervening precision (3C) protease cleavage site between the protein and purification tag. A stable cell line for ABCB1-Dloop X was generated using the Flp-In T-REx Kit (Thermo Fisher Scientific) for inducible expression as per the manufacturer’s guidelines.

Transient expression for ABCB1-Dloop X and ABCB1, (sequence ID: AUA59576.1) constructs was carried out in HEK293T adherent cells. Growth was monitored in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific) at 37 °C with 5% CO2 under humidified conditions. Purified DNA was mixed with branched polyethyleneimine (PEI; Sigma) at a 1.2 (DNA/PEI) ratio and applied to cells after exchanging medium to expression medium (DMEM + 2% FBS). Expression and growth media were supplemented with a penicillin-streptomycin mixture (Thermo Fisher Scientific). For ABCB1, expression was allowed to proceed for 72 h at 37 °C. All synthetic gene constructs were expressed in the presence of 4 mM valproic acid (Sigma) at 30 °C for 96 h. A stable cell line for ABCB1-Dloop X was grown and maintained similarly; induced with 1 μg mL−1 tetracycline and protein expression, it was allowed to proceed for 72 h at 37 °C. Cells were washed with PBS before being harvested and flash-frozen in liquid nitrogen for storage at −80 °C.

For protein purification, frozen cell pellets were thawed and homogenized using a tissue homogenizer in an eightfold (v/v) wt/vol excess of resuspension buffer containing 150 mM NaCl and 25 mM Hepes (pH 7.5), in addition to 10–20% glycerol and a protease inhibitor mix (prestatin A, leupeptin, soy trypsin inhibitor, and phenylmethylsulfonyl fluoride), followed by addition of detergent, except for the ABCB1-Dloop X sample for EM analysis, which was purified in 250 mM NaCl and 50 mM Tris (pH 7.5). For all ABCB1 constructs, protein extraction was allowed to proceed in the presence of a mixture of 0.4% dodecyl maltoside/monoglyceride (DM/MG), 0.1% octaethylene glycol monododecyl ether (C12E8), and 0.1% CHS for 90 min before being centrifuged for 30 min at 37,000 × g in a SA600 fixed-angle rotor. For ABCB1-Dloop X constructs, 0.5/0.05% LMNG/CHS was used for solubilizing protein for 60 min before centrifugation. Clarified supernatant was applied to Sepharose-coupled Rho-ID4 antibody (University of British Columbia) and incubated for 3–18 h. Beads were washed four times with 10 column volumes (CV) of purification buffer containing 150 mM NaCl, 25 mM Hepes (pH 7.5), along with 10–20% glycerol and 0.10,0.01,0.004% DDM/C12E8/CHS (ABCB1) or 0.02,0.004% LMNG/CHS (all ABCB1-Dloop X constructs). For the ABCB1-Dloop X sample for EM analysis, buffer and salt components were adjusted to contain 250 mM NaCl and 20 mM Tris (pH 8.0). For direct comparison of ABCB1-Dloop X and ABCB1-Dloop X constructs in ATPase assays, DDM/C12E8/CHS-solubilized ABCB1 was bound to ID4 columns and exchanged to LMNG/CHS buffer during the wash and subsequent purification steps. Protein was eluted by adding 3 CV of wash buffer containing a 1:10 wt/vol excess of 3C protease or digestion of 0.5 mM 3C protease (GenScript) for 2–18 h. The 3C protease was His-tagged and removed by incubating the cleaved protein with nickel nitritrocetic acid beads (Qiagen). All purification steps were carried out at 4 °C.

Antibody Purification and Fragmentation. UIC2 hybridoma cells were cultured in WheaLen Cell Line Bioreactors as per the manufacturer’s recommendations. Proteins were used for antibody purification and antibody fragmentation, which were carried out using the Fab Preparation Kit protocol (Thermo Fisher Scientific). Fab purity was judged by SDS/PAGE, followed by size exclusion chromatography (SEC), after desalting into storage buffer containing 150 mM NaCl and 25 mM Hepes (pH 7.5) or 250 mM NaCl and 20 mM Tris (pH 8.0) for use with ABCB1-Dloop X for EM analysis.

Cytotoxicity Assays. The ABCB1-Dloop X, stable cell line was grown and maintained as described above. Induced or noninduced cells were plated at a density of 5,000–10,000 cells per well of a 96-well plate and allowed to attach for 1–2 h. Cells were then exchanged to medium containing paclitaxel at various concentrations and incubated for a further 48–72 h. Medium was exchanged, and cell viability was measured using the WST-1 cell proliferation/viability kit (Sigma). The assay was repeated in the presence of 10 μM zosuquidar, added 1–3 h before paclitaxel. To test the effect of UIC2, induced cells were plated as described above and preincubated with varying UIC2 concentrations for 1 h at 37 °C. Paclitaxel was then added directly to the medium (final concentration 0.5 μM), cells were incubated for 48–72 h, and viability was measured described as above. The results shown are for three independent experiments (Fig. 1 B and C and SI Appendix, Fig. 2A). Data were fit to a sigmoidal dose–response curve, plotted in GraphPad Prism 6, and normalized to calculated Bmax values from the fitted curve after subtraction of the calculated B0 values (to adjust for background levels in separate assays) from the respective datasets.

Cysteine Cross-Linking of ABCB1-Dloop X. Detergent-purified protein was incubated with 1 mM dichloro(1,10-phenanthroline)copper(IlI) (Sigma) for 1 h at 4 °C and desalted back into buffer lacking the oxidant using Sephadex G-25 desalting columns (GE Healthcare) to stop the reaction. For ABCB1-Dloop X, cross-linking efficiency was analyzed by SDS/PAGE (SI Appendix, Fig. S2B). The 150 mM NaCl, 25 mM Hepes, and medium were incubated in reducing and nonreducing loading buffer, and the ratio of cross-linked to non–cross-linked transporter was judged by comparing the full transporter band (cross-linked, ~140 kDa) and the cleaved half-transporter bands (non–cross-linked, ~50 kDa). Cross-linked
protein was subsequently used for biochemical assays and EM sample preparation as described below.

ATPase Assays. Measurements of ATP hydrolysis were performed using a
molubdate base colorimetric assay as previously described (51). Protein
concentrations used in the assays in the range of 0.1–0.2 mg/mL zosuquidar
(Medkoo Biosciences) and taxol/paclitaxel (Sigma) were dissolved in DMSO
and added to reaction mixtures at the desired concentrations. The reactions
were started upon addition of 2 nm ATP in the presence of 10 mM MgCl₂
at 37 °C. For kₐ, determination, a range of ATP concentrations was used. Linear
regression and statistical analyses were performed using GraphPad Prism 6.

Antibody-Binding Assay. Purified Avi-tagged proteins were first desalted into
biotinylation buffer [75 mM NaCl, 25 mM Hepes (pH 7.5), 10 mM magnesium
acetate, 10 mM ATP, and 50 μM biotin] containing the detergent/CHS mix
used for protein purification before addition of 5–10 μg biotin ligase BirA
produced in-house), and the reaction was allowed to proceed overnight at
4 °C, followed by desalting into buffer containing 150 mM NaCl, 25 mM
Hepes (pH 7.5), 10–20% glycerol (buffer A), and the respective detergent/
CHS mix. A total of 1–5 pmol of biotinylated proteins was added to each well
of a preblocked 96-well Neutravidin plate (Thermo Fisher Scientific) for up to
1 h at room temperature. All incubations were carried out on a plate shaker
(UNIMAX 1010 Orbital Platform shaker; Heidelberg) at 350 rpm. Unbound
transporters were discarded, and the wells were washed three times with
200 μL of buffer A supplemented with the respective detergent/CHS mix
(buffer AD). A total of 100 μL of serially diluted UIC2 antibody was added to
thes wells and incubated for 30 min at room temperature. Unbound UIC2
was discarded, and the wells were washed three times with buffer AD as before.
Hors eradish peroxidase (100 μL)-conjugated goat anti-IGG (catalog
no. A16072; Thermo Fisher Scientific) diluted to 1 mg/mL (1:1,500) in buffer
A was added to the wells for 30 min. After discarding unbound antibod-
ies, the wells were washed as before, followed by development using the
TM Substrate Kit (Thermo Fisher Scientific). Absorbance (450 nM) was
read using a BioTek Synergy HT plate reader. Readings were plotted against
UIC2 concentrations and fit to a one-site specific binding equation in
GraphPad Prism 6. Data were normalized to calculated Bₘₐₓ values for
comparative purposes.

EM Sample Preparation. LMNG/CHS-purified ABCB1₄m equi constructs were
mixed with a two- to three-fold molar excess of UIC2-Fab. The ABCB1₄m equi–
UIC2 complex was mixed with a 1:10 v/vt v excess of amphipol A8-35
(Anatrace) for 4 h at 4 °C, followed by overnight biobead (BB-SM2)-
mediated removal of detergent. UIC2-Fab complexes of detergent-purified
and amphipol-reconstituted samples were concentrated to 5-8 mg/mL
before being purified on a G4000SW column in buffer containing 250 mM
NaCl and 20 mM Tris (pH 8.0) (amphipol) or 150 mM NaCl and 25 mM Hepes
(pH 7.5) (detergent samples). Peak fractions corresponding to the purified
complexes were pooled and used for cryo-EM grid preparation. For the
zosuquidar complex, the inhibitor was added to a final concentration of
10 μM before grid preparation. Freshly purified samples were applied to
glutaraldehyde-fixed grids (UCSF Chimera, follow ing postprocessing
using automatically determined B-factors as before yielded a
final map with a resolution of 4.14 Å (map 1) that was used for model building.
A second, smaller detergent belt model was similarly generated to create a
second signal-subtracted dataset that served as input for one more round of
3D classification, where a search for three 3D classes yielded two distinct classes
that shared a similar architecture but showed differences in conformations of
TM4 and TM10. Class 1 (153,652 particles) and class 2 (158,827 particles), con-
taining kinked and straight conformations of TM4 and TM10, respectively,
were both refined to a resolution of ~4.5 Å (map 2 and map 3, respectively).
The remaining particles fell into the third class containing blurred density
in the regions of TM4 and TM10 and were not analyzed further.

For the ABCB1₄m equi–UIC2 complex, image stacks comprising 80 frames
each were collected at a nominal magnification of 105,000× in super-
resolution mode with an estimated dose per frame of 1.0 electron per
square angstrom, corresponding to a total dose of 80 electrons per square
angstrom. The software suite Focus (60) was used for online data processing
and prunning, applying motion correction with MotionCor2, including two-
fold binning of the recorded micrographs after motion correction to a
calibrated pixel size of 1.336 Å and CTF estimation with gCTF. Particles were
picked with gAUTOMATCH (61) using a template-based approach, with
the zosuquidar complex solved to near-atomic resolution (discussed above) as a
reference. Of those, two near-identically looking
classifications using a low-pass-filtered map of the cross-linked apo structure
(discussed below) as a reference. Of those, two near-identically looking
classes were unambiguously fit into the corresponding electron density in the ABCB1–
UCSF Chimera, followed by adjustment in Coot. Two molecules of zosuquidar
were both refined to a resolution of 4.33 Å. This map was used to obtain a model map for
the detergent belt, which was generated as described for the zosuquidar
complex dataset and used for signal subtraction using the input particle set
used for the initial refinement. The new signal-subtracted dataset was then
used as input for a single-class AEC method (51) using gEMAN2 (E2PROC2D.py
using e2proc2d.py (EMAN2) (62). The same model was used later as a
starting model in 3D classification. Micrographs were imported for pro-
cessing in Relion, and a total of 347,049 particles were extracted from
2,038 micrographs, followed by rounds of 2D classification, to yield a
particle set of 112,196 particles. Following two rounds of 3D classification,
the remaining 78,282 particles were used for 3D auto-refinement in Relion.
Partial signal subtraction was performed to remove the amphipol belt
for final 3D refinement and postprocessing to yield a final resolution of 6.25 Å.

Model Building and Refinement. Postprocessed maps, as well as non–B-factor
sharpened maps were used for model building in Coot (63) for all datasets.
The quality of electron density in the TMD regions of the apo and zosuquidar
maps allowed for de novo model building. The map quality in the NBD region
was, on average, of lower quality compared with the TMDs, which were re-
solved to near-atomic resolution (SI Appendix, Figs. 54 and 57). Modeling of
the NBD region was therefore guided by published structures of ABCB1
[Protein Data Bank (PDB) ID codes 4M1M and 5KO2], followed by manual
adjustment where required and permitted by map quality. The UIC2 crystal
structure (PDB ID code 5JUE) was manually docked into the Fab density using
UCSF Chimera, followed by adjustment in Coot. Two molecules of zosuquidar
were both refined to a resolution of 4.33 Å. The resulting map was used to
build the NBD using Coot. Missing density was then subsequently added as
phosphatidylethanolamine (PE) in both the apo- and zosuquidar-bound occluded ABCB1₄m equi
structures.
PE and glycan monomers were obtained from the Coot monomer library (monomer codes 3PE and NAG, respectively). Geometric restraints for all ligands were generated in Phenix. The model for ABCB1–UIC2 was validated on the structure of the apo-occluded state. Map quality allowed for rebuilding the TMDs to fit the density and was guided, in part, by the structure of mouse ABCB1 (4M1M).

Real-space refinement of the models was carried out in Phenix (64, 65). For validation of the icosahedral complex model, random coordinate errors up to 0.3 Å were introduced into the refined model, which was subsequently refined against one of the half-maps from the 3D auto-refine run from Relion. Minimal differences in FSCs computed between the refined model and the half-map used for refinement compared with those between the refined model and the half-map excluded from refinement point to a model free from overrefinement (SI Appendix, Fig. S3). All figures were prepared using UCSF Chimera and PyMOL (The PyMOL Molecular Graphics System, Version 1.8; Schrödinger, LLC). Cavity volumes were calculated using HOLLOW (66) as described. Structure alignment and rmsd calculations were done in PyMOL. Ligand interaction diagrams were prepared in LigPict (67). Red numbers in figures are based on equivalent residues in the fully human protein.

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