nature portfolio

Peer Review File

Cryo-EM of human P-glycoprotein reveals an intermediate occluded conformation during active drug transport

Corresponding Author: Dr Maofu Liao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The reviewers comments are available in the uploaded PDF file.

Reviewer #2

(Remarks to the Author)
The manuscript by Culbertson and Liao reports a thorough and novel investigation of the conformational dynamics of Pgp under turnover conditions. These conditions are defined by excess ATP and the presence of transportable substrates. To my knowledge, this is the first study of its kind for Pgp and subject to the disclaimer below reports previously unobserved conformations as well as intriguing results regarding substrate binding sites.
Because I am not an expert on cryoEM, I will take this aspect of the work at face value, assuming other reviewers will weigh in on the rigor of the cryoEM workflow. I find the selection of the two substrate molecules, verapamil and vinblastine, interesting as they have different degree of inhibition at high concentrations. In one of the references cited, it was shown that they stabilize different levels of asymmetric NBSs with a partial inhibitor behavior of Vinblastine.
Of the many findings that arise from the study, one is disappointing. Despite the turnover conditions, the authors were unable to find the OF conformation. As is typical in the field, the lack of such conformation is chalked up to it being transient. May be someone will be more creative one day. However, despite this, the authors were able to capture closed and collapsed conformations, the former with substrate bound providing evidence that its is a state on the transport path.
Multiple IF sates where identified that show distinct degrees of asymmetry in the NBDs. The authors do an exhaustive review and comparison of previous structures to quality this asymmetry. One questions that arises here is whether this asymmetry propagate to the TMD? For the case where the authors got an apo structure does the asymmetry persist?
One troubling aspect for this reviewer and this may be an oversight on my part is what appears to be a missing discussion of whether ATP or ADP were observed in these conformations. How is that reconciled with the fact that the sample was prepared in excess ATP? Or did the authors decide not to discuss the nucleotides.
Beyond the issue with the apparent missing ATP/ADP, the IFs reveal that there are two substrate binding sites. One is well defined in the expected region whereas the other one is novel and appear to be of lower affinity. The binding of substrate introduces asymmetry in the kinking of TM 4 and TM 10 which have been proposed to be integral for substrate binding and transport.
Overall, while I am excited about the work, I find the discussion of its implications somewhat truncated. With the generous page limits here, the authors should consider to expand their comparison with previous Pgp structures and show this in the main manuscript. They should pay attention to what has been proposed in the recent elife paper to be the OF conformation. Do they see evidence of it in their cryoEM ensembles? How does the location of the disulfide linked substrates in that work compare to what is reported here. I am looking forward to read an updated manuscript that more crisply contextualize the work presented.

Reviewer #3

(Remarks to the Author)

This manuscript presents a detailed structural analysis of human P-glycoprotein (Pgp) using cryo-electron microscopy (cryo-EM), capturing multiple conformations during active drug transport, including inward-facing, closed, and intermediate occluded states. A particularly significant finding is the visualization of the drug-bound intermediate occluded conformation, observed in Pgp for the first time. This advances our understanding of the molecular mechanism underlying Pgp function. The study also highlights rearrangements in transmembrane helices (TM4 and TM10) during substrate binding and release, and the identification of a peripheral binding site with potential implications for Pgp inhibition. These findings provide new insights into Pgp's polyspecific drug transport capabilities.

Major Comments:
Resolution and Structural Modeling Concerns: A key limitation of this study is the relatively low resolution of the cryo-EM maps for many of the captured conformational states of Pgp, which affects the reliability of the models presented. This low resolution is likely due to the small number of particles assigned to each conformational state after classification, a common challenge when analyzing dynamic proteins in multiple states. As a result, the density maps are not well-defined in several critical regions, particularly in the nucleotide binding domains (NBDs) and in the side chains of the transmembrane helices. Despite these limitations, the authors have deposited atomic models in the PDB that extend into areas where the density is insufficient for confident interpretation, as indicated in the validation reports. In some regions, particularly in the NBDs, side chains have been modeled even though they are not clearly visible in the density maps. This creates a risk of over-interpretation and misrepresentation of the structural data. To address this, the authors should revise the PDB entries, ensuring that only the regions where the density provides a clear basis for interpretation are modeled, and that areas with insufficient density are omitted. Ambiguous regions should be clearly indicated as such, and speculative regions must be distinguished from well-supported ones.
This revision will require significant updates to both the figures and the text of the manuscript. The figures, especially those showing the cryo-EM maps and models, should accurately reflect the limitations of the data, ensuring that readers can distinguish between confidently resolved regions and areas where interpretation is less certain. In addition, the text should be revised to appropriately temper any conclusions drawn from regions with low resolution. By addressing these issues, the authors will strengthen the robustness of their findings and ensure the data is presented with appropriate caution.

Minor Comments:
1. ATPase Activity Interpretation (Fig. 1a): In Fig. 1a, the ATPase activity measurements show significant basal activity in the absence of drugs. The authors should clarify whether this is due to the intrinsic basal activity of Pgp, possible contamination from other ATPases, or activation by compounds present in the assay buffer. A more thorough discussion is needed to explain this background activity and its potential impact on the results.
2. Comparison with Previous Structures: Several cryo-EM structures of human Pgp bound to inhibitory Fab fragments and small molecules have been published, including a recent high-resolution structure of Pgp bound to three molecules of elacridar (BBRC 2024). It would be valuable to compare the structures obtained in this study with these previously reported structures. This comparison could help contextualize the new findings and highlight any unique features captured in this study.
3. Figure Clarity and Readability: Some of the figures, especially those showing structural models and density maps, use font sizes that are too small, making them difficult to read. Increasing the font size in these figures would improve accessibility, particularly for readers who may have difficulty reading small text.
4. Inconsistent Labeling in Supplementary Figures: The supplementary figures and the legends use inconsistent labeling for the panel identifiers (a, b, c, A, B, C, etc.).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have adequately responded to all the questions by the reviewer.
It is my oppinion now that it is suitable for publication.

Reviewer #2

(Remarks to the Author)
I thank the authors for their constructive response. the work is exciting, the technical elements are solid and the conclusions are important for the field.

Reviewer #3

(Remarks to the Author)
The authors have carefully and thoroughly addressed all of my suggestions and comments. Their responses demonstrate a strong commitment to improving the clarity and rigor of their study. The revisions have significantly enhanced the manuscript, making it more comprehensive and well-supported by the presented data. Given these improvements, I believe this paper deserves to be accepted for publication.

Reviewer 1

GENERAL COMMENTS

The article by Culbertson and Liao aims to shed some light on the elusive mechanism by which efflux pumps – namely Pgp – can transport drugs. The article is, in general, well-written although it is suggested that it should still be thoroughly re-read to correct small misspellings and some sentences that seem out-of-context. The methodology is solid and seemingly reproducible, and the main conclusions are (mostly) supported by the results. In an area in exponential growth since the advent of Cryo-ME, such as membrane proteins, most of the reported results are known, but nonetheless there are several nuances and insights that confers novelty within the field of efflux transporters.

We sincerely thank the reviewer for the support and constructive comments. Below is our point-by-point response to each comment.

MANUSCRIPT COMMENTS

1. *From literature it is known that self-inhibition by vinblastine has an IC50 of ~89μM, while for verapamil the IC50 is ~2.4μM (R-isomer) or ~2.1μM (S-isomer). While verapamil is expected to act as an inhibitor at concentrations above 20μM, it is somewhat surprising that the same would occur with vinblastine at such low concentrations, as claimed in page 2, lines 73-75. The authors must provide a critical comment about this, as it does not correlate with data reported in specialized literature.*

   We thank the reviewer for the feedback. The behavior of Pgp in the presence of verapamil or vinblastine varies widely in literature, and the IC50 values likely depends on experimental conditions. For example, previous studies have shown maximal vinblastine stimulated ATPase at ~10-20 μM and further increasing vinblastine concentration decreased activity (Dastvan et al, 2019, Clouser et al 2022), which is similar to what we observed in our work. In contrast, some studies have shown substrate stimulation up to 250 μM vinblastine (Kim et al, 2018) or even no/little substrate stimulation (Nosol et al, 2020).

   Similar phenomenon can be seen for verapamil, with many studies showing substrate inhibition at low verapamil concentration, but also some showing substrate inhibition only above 400 μM (Dastvan et al, 2019) or no substrate inhibition up to 100 μM (Goda et al, PLOS Gen, 2020).

   These discrepancies for differing activity on substrates between studies likely resulted from different membrane compositions or membrane mimetics. We have added a comment on line 78-80.

2. *The authors aim to identify which residues often interact with either vinblastine or verapamil in the several conformations, which is quite valuable. Nonetheless, the omitted the "empirical site nomenclature" suggested by Shapiro and Ling and based on efflux experiments of Hoechst 33342 and Rhodamine-123 (the H- and R-sites) that, together with initial "modulator" site identified from the first crystallographic ligands in the murine Pgp – QZ diastereoisomers - provides until today a way to adequately "map" the internal binding pocket. The authors are advised to adopt this nomenclature to facilitate the interpretation of the interaction data.*

   We thank the reviewer for the suggestion. At least three previous studies have used different methods for mapping the binding sites of Pgp. We decided to follow the mapping described in

Ferreira et al, 2013, due to its inclusion of all three sites (M-, R-, and H-site). For this we have described the position of our binding sites including how the central cavity is near the bottom of the M-site (line 233-236) and how the peripheral site most closely positioned to the R-site (line 253-254).

3. *In this work (although in line with many other publications), there is no critical assessment that some of the differences reported may simply derive from the different size and volume of vinblastine and verapamil. This should be pointed out.*

   We thank the reviewer for the suggestion. We have added a brief description pointing out that the size of the verapamil molecule, along with it having more rotatable bonds, may play a role in its higher transport efficiency (line 240-242).

4. *On page 5 (lines 215-218) the authors claim that "due to less rigid binding to Pgp, verapamil represents more efficient transport substrates". This was already demonstrated both experimentally (doi: 10.1002/prot.23023) and computationally for Sav1866 (doi: 10.1002/prot.23023) and Pgp (doi: 10.7554/eLife.90174.3). Therefore, references should be added consequently.*

   We thank the reviewer for the suggestion. These references have been added on line 240.

5. *If MD simulations were performed, binding energies should be calculated to support some of the conclusions (e.g., page 6, line 251 or page 7, lines 284-285). Also, for the RMSD calculation the NBDs should be plotted separately from the TMDs, as the authors recognize that "they have higher fluctuations" which could mask the ligand' RMSDs.*

   We thank the reviewer for the insightful suggestions. We have calculated the binding energy for all the simulations and created a separate plot for this (See Fig. S12). In addition, we recalculated the RMSD for the protein molecules, separating the NBD and TMD and plotted them separated. We agree that this makes analysis of these MD simulations more precise. We have added this into the manuscript on line 281-285.

6. *On page 5, lines 260-261, the authors start describing the transport mechanism. However, the very first step – how the ligand may reach the internal cavity – is omitted. The authors must include references (a couple of them can be swiftly found) on this because it will give extra support to the proposed translocation mechanism.*

   We have added a short section to the text on line 324-326, along with references (Raviv 1990, Sharom 2014, and Higgins 1992) for our transport model describing the evidence that Pgp substrates first enter the membrane inner leaflet before being taken up by Pgp. We agree that this addition supports our model, as the peripheral site is positioned at the level of the inner membrane leaflet, consistent with it being the initial substrate binding site.

7. *In the last paragraph of page 7 (lines 285-291), the idea claimed by the authors seems to be inspired by a docking paper in which the enrichment of one of the sites in aromatic rings is responsible for the specific and high-affinity contacts with ligands, with fully stabilized poses suggested to impair conformations transitions from the inward- to the outward-facing conformation (doi: 10.1021/ci400195v). Being an idea already in literature, a suitable reference should be added to the text.*

This section was initially inspired by different features observed in the cryo-EM density maps when comparing various structures. We thank the reviewer for the suggested reference which provides additional support for this idea and have now added it to the text on line 356-358.

8. *Supporting Information, Protein Expression and Purification:*

   a. *Why wasn't cholesterol added to the nanodisc, as several publications identify lipid rafts, higher-order membranes and cholesterol composition as critical for Pgp activity?*

      Our early optimization showed highly stabilized protein in POPG nanodiscs with robust ATPase activity and high enough yield for structural studies. This was the primary goal of this work. Once this was achieved, the lipid composition was kept consistent to allow direct comparison between samples. Moreover, the protein was solubilized in detergents containing cholesterol hemisuccinate (CHS), a cholesterol analog, which was present during nanodisc reconstitution. Indeed, the presence of CHS increased yield during purification, likely through protein stabilization, supporting a similar effect as observed in previous studies. Thus, it is possible that CHS was retained during the detergent removal, allowing any cholesterol-like effects to remain.

9. *Supporting Information, Molecular Dynamics section:*

   1. *A simulation with TM10 in its Cryo-EM determined shape (not straightened) must also be done for comparison purposes. Also, it is not clear how TM10 was "manually straightened": was TM10 replaced by one straight TM10 helix from the Cryo-EM structures? And after 700 (or 1200ns), did the helix kinked again?*

      As suggested by the reviewer, we performed an additional 660ns simulation with TM10 kinked. In this simulation there was slightly higher RMSD and weaker binding energy when TM10 was kinked, but results remained consistent that the central cavity vinblastine was more dynamic compared to peripheral. The results and discussion were added to the manuscript on line 281-285 and added to Fig. S12. The method for manual straightening TM10 was added to the methods section in molecular dynamics on line 223-224 in Sup Info.

      The conformation of TM10 helix after straightening was variable depending on simulation. When vinblastine is bound to both central and peripheral cavity, TM10 remained straight. Whereas when bound to only peripheral, or in the apo state, TM10 kinked slightly inward. This discussion was added to the manuscript (line 274-277)

   2. *Why was IF1 used for vinblastine and IF for verapamil?*

      We assume the reviewer meant "IF2" for verapamil in this comment. The choice of model for MD studies was based on how defined the ligand density was in our cryo-EM maps. For VinATP dataset, IF1 has higher resolution and slightly better-defined vinblastine density. For VerATP dataset, although IF1 has higher overall resolution, the verapamil density was better defined in IF2, increasing our confidence in a more accurate starting position of the ligand. This explanation was added in the methods section (line 225-226).

   3. *It is intriguing why the authors went for POPG for the nanodiscs and POPC:Chol (80:20) for the MD simulations. I would recommend mimicking the composition of the nanodiscs (also*

without cholesterol, optionally with the MSP1D1 protein) unless the authors provide very convincing arguments on why it was done like this (because CHARMM-GUI provides membranes with POPG).

For the MD simulations, we chose POPC over POPG, because although POPG facilitated nanodisc reconstitution and provided highly reproducible samples for structural studies, it is not abundant in human cell membranes. However, for MD simulations, we are not constrained by considerations of protein yield or activity, allowing us to prioritize a lipid composition that is more physiologically relevant.

As the reviewer suggested, we have performed an additional MD simulation of Pgp bound to vinblastine in both central cavity and peripheral site in POPG nanodiscs. The consistency between the simulations of Pgp in POPG nanodiscs and POPC-Chol bilayer was added to the manuscript on line 284-285.

4. *Why was the disordered region between TM1 and TM2 modeled and not the linker between NBD1 and TM7? Also, did the authors see any influence of TM1 on the transport mechanism (from the Cryo-EM data or MD simulations) as suggested in the paper by Zhang et al. (doi: 10.7554/eLife.90174.3)?*

   We thank the reviewer for the suggestion. The loop between TM1 and TM2, which was disordered in our structures, was modeled because this loop is unlikely to influence substrate movement in the central cavity or peripheral site, nor does it affect NBD closure. In contrast, when included in MD simulation, the linker between NBD1 and TM7 frequently positioned itself between the two NBDs and appeared to influence their movement. Depending on whether the linker was included or where it was initially positioned (between or outside the NBDs), we observed highly varied NBD dynamics. Since our primary focus was on the stability and dynamics of ligands within the central cavity and peripheral site, we chose to exclude the linker from the simulations in this study. A comprehensive investigation on exactly how the linker impacts Pgp dynamics is beyond the scope of current work.

   We added a brief discussion comparing our occluded conformation with the NBD closed structures containing cross-linked substrate as described in Gewering et al, 2024. (line 310-317). We propose that our occluded conformation is the step before the collapsed closed state with cross-linked substrates and a dilation in TM1. Further, our cryo-EM analysis showed no similar critical role of TM1, suggesting that such conformations are highly transient and cannot be captured without the chemical crosslinking. This was added into the manuscript (line 310-317).

5. *How many lipids are in the membrane: from its lateral dimensions (96.1 x 96.1) it seems to be very small concerning the maximus distance of the NBDs (from outer edge to outer edge)? The simulation box (.gro), topologies (.itp/.top) and running parameters (.mdp) should ideally be made available to allow reproducibility of the experiments.*

   The lipid membrane contains ~55 cholesterol molecules and ~220 POPC molecules. There are roughly 4-5 lipids between Pgp and the outer edge at the shortest distance and roughly 7-8 lipids at the longest distance between Pgp and the end of the box. We have uploaded all request MD files into supporting data.

- *Figure S4 is identical to Figure S3. Please revise.*

  We thank the reviewer for catching this mistake. The figure was updated and replaced.

- *Figure S5: In the table, specify next to the PDB code if the structure refers to APO or HOLO structures*

  This was added to the table.

- *Figure S6. How did the IF conformations obtained from vanadate correlate with those from Figure S2? Could they be appended to the first ones to increase resolution?*

  IF conformation from ADP-Vi dataset is similar to VinATP IF1 and IF2. The ADP-Vi IF state also showed vinblastine bound to central cavity and additional density at the peripheral site, and the NBD positioning was between IF1 and IF2.

  These datasets are not suitable for combination due to different nature of the samples. The ADP-Vi samples were incubated for extended times to promote completion of trapping the NBD closed state, whereas the continuous turnover VinATP dataset was only incubated for short time to ensure ATP was still saturating and we captured Pgp actively transporting. Thus, combining these datasets will complicate the interpretation of the VinATP IF maps.

- *Figure S11: RMSDs should be plotted only against the TM regions (excluding the highly fluctuating NBDs). This should reduce the noise, e.g. in figure S11C, where is very unlikely that both vinblastine molecules have such a low RMSD when the protein fluctuates immensely. Were the molecules fitted against their initial position or fitted against the protein? Also, it would be interesting to have the area per lipid calculations from the MD runs, to verify that the membrane remained equilibrated and in a liquid- state during the whole MD simulation.*

  We thank the reviewer for the suggestion. We have recalculated the RMSD for all ligands only against the TM regions, rather than whole protein. The RMSD was calculated against their initial position after aligning the TM regions. We have clarified this in the methods section (line 235-236 in Sup Info).

  We thank the reviewer for the suggestion to perform area per lipid (APL) calculations. While we appreciate the value of this analysis for studying lipid bilayer properties, it falls outside the routine methodologies employed in our work, which primarily utilize GROMACS and VMD for simulation and analysis of protein dynamics. In our study, we assessed the membrane dynamics and lipid behavior through visual inspection of the lipid order, ensuring the bilayer maintained a sufficiently dynamic and fluid state throughout the simulation. Although APL analysis could provide additional quantitative detail of the lipid bilayer (in addition to ensuring the membrane was sufficiently dynamic), it is not essential for supporting our key conclusions on the conformational dynamics of Pgp.

14. *Figure S15. Although trusting the data, the reported data do not fit with theoretical concepts. It is assumed that binding of the substrate induces smaller distances between the NBDs that are augmented by the binding of ATP to induce dimerization. The initial part of the data seems to suggest that, as the holo structures has shorter NBD-NBD distances than the apo structures. However, in the presence of ATP the NBDs seem to be more far apart. While for vinblastine this*

is within the margin of error (n=9) for verapamil they are intermediate, suggesting that modulators increase the energetic penalty for changing the conformation from IF > OF. However, the authors bluntly report that "such a conformation is either highly transient of completely absent" in the case of vinblastine. How so, if NBD-NBD distances are smaller in the presence of vinblastine and ATP? May this mean that molecules might be effluxed from the OC conformations sometimes, without need of fully CC? The authors are encouraged to add a short discussion on this.

It is a valuable observation that the 3D reconstructions of Pgp with vinblastine/verapamil and ATP show greater distances than those without substrate transport, i.e., holo or apo. While this seems contradictory to the expectation that addition of substrate should reduce the NBD distance, it can be well explained by the fact that the 3D reconstructions captured during drug transport are snapshots of Pgp undergoing continuous conformational transition. For the actively functioning Pgp, our cryo-EM analysis captures relatively long-lived, but not transient, conformations.

The IF 3D maps from all samples are likely a mix of conformations with a small range of NBD distances, even after extensive 3D classification. This is consistent with lower resolution of the NBDs. During active transport with MgATP, the Pgp proteins with smaller NBD distances, with or without substrate binding, are more likely to close; on the contrary, those with greater NBD distances take longer time to close, thus becoming more long-lived and better represented in the cryo-EM data. Specifically, data in Fig. S16 represents particles classified as IF after initial 3D classification (no NBD-closed particles), where ATP addition likely caused particles with closer NBDs to dimerize and leave the IF pool, leading to increased NBD distances for the continuous turnover datasets, while apo and vin-alone datasets lacked NBD-closed conformations. A summary of this discussion is included in the manuscript on line 176-182.

15. *The analysis of the validation reports also revealed a large number of outliers and poor fitting of the ligands as well. The authors should be absolutely sure that such problems are inherent to the structure and not errors during the fittings.*

    We thank the reviewer for the suggestion. We have updated our models to remove sidechains with insufficient density for accurate model building. This was done by manually running through each model and trimming side chains. This resulted in most side chains on NBDs to be trimmed. In addition, some models (e.g. VerATP-OC, VerATP-CC, Apo), also had many TM side chains trimmed. Our strategy for modifying these models was added to methods sections on line 168-170 in Sup Info. We have a more detailed description of this procedure in response to the first major comment of reviewer #3 below.

Reviewer #2 (Remarks to the Author):

The manuscript by Culbertson and Liao reports a thorough and novel investigation of the conformational dynamics of Pgp under turnover conditions. These conditions are defined by excess ATP and the presence of transportable substrates. To my knowledge, this is the first study of its kind for Pgp and subject to the disclaimer below reports previously unobserved conformations as well as intriguing results regarding substrate binding sites.

Because I am not an expert on cryoEM, I will take this aspect of the work at face value, assuming other reviewers will weigh in on the rigor of the cryoEM workflow. I find the selection of the two substrate

molecules, verapamil and vinblastine, interesting as they have different degree of inhibition at high concentrations. In one of the references cited, it was shown that they stabilize different levels of asymmetric NBSs with a partial inhibitor behavior of Vinblastine.
Of the many findings that arise from the study, one is disappointing. Despite the turnover conditions, the authors were unable to find the OF conformation. As is typical in the field, the lack of such conformation is chalked up to it being transient. May be someone will be more creative one day. However, despite this, the authors were able to capture closed and collapsed conformations, the former with substrate bound providing evidence that its is a state on the transport path.

We thank the reviewer for the positive comments and summary of our key findings.

Multiple IF sates where identified that show distinct degrees of asymmetry in the NBDs. The authors do an exhaustive review and comparison of previous structures to quality this asymmetry. One questions that arises here is whether this asymmetry propagate to the TMD? For the case where the authors got an apo structure does the asymmetry persist?

Measuring the asymmetry in the TM region is challenging because the pseudo-symmetry related TM helices do not overlap well, unlike the NBDs which behave more like rigid bodies. Nonetheless, we split the Apo model into halves and performed a structural alignment between the halves, aligning the region in the membrane. This reveals a minor gradual twist moving down the TMs which correlates with the direction of NBD asymmetry. We have added this into Fig. S16 and the related text on line 171-172.

One troubling aspect for this reviewer and this may be an oversight on my part is what appears to be a missing discussion of whether ATP or ADP were observed in these conformations. How is that reconciled with the fact that the sample was prepared in excess ATP? Or did the authors decide not to discuss the nucleotides.

We thank the reviewer for pointing this out. Comparison of the cryo-EM maps of Apo and Vin-alone IF (nucleotide-free) with the VinATP and VerATP demonstrates additional EM densities, presumably of nucleotides, at both NBSs for VinATP and VerATP maps. However, the resolution of the NBDs is not sufficient for unambiguous assignment of molecule identity. These were modeled as ATP because the sample has an excess of ATP. To clarify this, we have added a description on line 175-177 in the model building section in methods and on line 129-134 of the main text.

Beyond the issue with the apparent missing ATP/ADP, the IFs reveal that there are two substrate binding sites. One is well defined in the expected region whereas the other one is novel and appear to be of lower affinity. The binding of substrate introduces asymmetry in the kinking of TM 4 and TM 10 which have been proposed to be integral for substrate binding and transport.
Overall, while I am excited about the work, I find the discussion of its implications somewhat truncated. With the generous page limits here, the authors should consider to expand their comparison with previous Pgp structures and show this in the main manuscript. They should pay attention to what has been proposed in the recent elife paper to be the OF conformation. Do they see evidence of it in their cryoEM ensembles? How does the location of the disulfide linked substrates in that work compare to what is reported here. I am looking forward to read an updated manuscript that more crisply contextualize the work presented.

We thank the reviewer for the suggestion. We have added an entire section to address this, starting on line 290, entitled “Comparison with previously published Pgp structures”. We have compared our occluded and closed-collapsed conformations with the substrate-crosslinked NBD closed states described in the recent eLife paper (Gewering et al, 2024; line 310-314) and stated that we see no indication of the dilated

TM1 with substrate bound higher in the "exit tunnel", likely because these states are too transient for visualization without chemical cross-linking. (line 312-316). Further, we have compared the central and peripheral site described in our work to previous structures (line 299-307) and the kinking of TM4 and TM10 (line 294-299). We have also added a figure (Fig. S20) with overlays with previous structures, highlighting that vincristine and taxol are in nearly identical positions to our central cavity, while the inhibitor vestibule described in Nosol, et al, 2020 or the three elacridar (Hamaguchi-Suzuki, et al 2024) do not overlap with the peripheral site described in our work, hence demonstrating the novelty of the peripheral site described in this study.

Reviewer #3 (Remarks to the Author):

This manuscript presents a detailed structural analysis of human P-glycoprotein (Pgp) using cryo-electron microscopy (cryo-EM), capturing multiple conformations during active drug transport, including inward-facing, closed, and intermediate occluded states. A particularly significant finding is the visualization of the drug-bound intermediate occluded conformation, observed in Pgp for the first time. This advances our understanding of the molecular mechanism underlying Pgp function. The study also highlights rearrangements in transmembrane helices (TM4 and TM10) during substrate binding and release, and the identification of a peripheral binding site with potential implications for Pgp inhibition. These findings provide new insights into Pgp's polyspecific drug transport capabilities.

We thank this reviewer for the support and summary of our key findings.

Major Comments:
Resolution and Structural Modeling Concerns: A key limitation of this study is the relatively low resolution of the cryo-EM maps for many of the captured conformational states of Pgp, which affects the reliability of the models presented. This low resolution is likely due to the small number of particles assigned to each conformational state after classification, a common challenge when analyzing dynamic proteins in multiple states. As a result, the density maps are not well-defined in several critical regions, particularly in the nucleotide binding domains (NBDs) and in the side chains of the transmembrane helices. Despite these limitations, the authors have deposited atomic models in the PDB that extend into areas where the density is insufficient for confident interpretation, as indicated in the validation reports. In some regions, particularly in the NBDs, side chains have been modeled even though they are not clearly visible in the density maps. This creates a risk of over-interpretation and misrepresentation of the structural data. To address this, the authors should revise the PDB entries, ensuring that only the regions where the density provides a clear basis for interpretation are modeled, and that areas with insufficient density are omitted. Ambiguous regions should be clearly indicated as such, and speculative regions must be distinguished from well-supported ones. This revision will require significant updates to both the figures and the text of the manuscript. The figures, especially those showing the cryo-EM maps and models, should accurately reflect the limitations of the data, ensuring that readers can distinguish between confidently resolved regions and areas where interpretation is less certain. In addition, the text should be revised to appropriately temper any conclusions drawn from regions with low resolution. By addressing these issues, the authors will strengthen the robustness of their findings and ensure the data is presented with appropriate caution.

Thank the reviewer for the suggestion. We have manually gone through the models and trimmed each amino acid with insufficient side-chain density for accurate model building. These updated models were redeposited into the PDB. We kept the backbone model of the NBDs in all the models because these NBD models behave mostly as rigid bodies, and the high-resolution structures of NBDs fit well into the secondary structure features of the cryo-EM maps. Our strategy is now clearly presented in methods

section including the criteria for keeping side chains (line 167-170 in Sup Info), removal of most side chains in NBDs (line 170-171 in Sup Info), modeling of side chains in the TM region (line 171-174 in Sup Info), and modeling of nucleotides (line 174-177 in Sup Info).

These modifications have greatly improved our atom inclusion at the recommended contour described in our validation reports, and these improvements are shown in the table below. We would also like to note that these new atom inclusions values are in line with several previous Pgp cryo-EM structures in various states such as Kim et al, 2018 (PDB 6C0V) with atom inclusion of 0.68, Nosol et al, 2020 (PDB 7A6F) with atom inclusion of 0.43, and Gewering et al, 2024 (PDB 7Zk9) with atom inclusion of 0.41.

Table showing Atom Inclusion

Model	Initial submission	New submission
Apo	0.45	0.65
VerATP-IF1	0.49	0.66
VerATP-IF2	0.41	0.62
VerATP-OC	0.44	0.60
VerATP-CC	0.34	0.52
VinATP-IF1	0.39	0.51
VinATP-IF2	0.39	0.50
ADP-Vi	0.49	0.62

Accordingly, we have added several points in the main text to clarify our conclusions related to limited resolution in certain regions. These included the TMs in the occluded state (line 198-200), the Ver-ATP CC and OC states (line 188-190), nucleotides at the NBS (line 129-132), and the peripheral site (line 249-250).

Minor Comments:
1. ATPase Activity Interpretation (Fig. 1a): In Fig. 1a, the ATPase activity measurements show significant basal activity in the absence of drugs. The authors should clarify whether this is due to the intrinsic basal activity of Pgp, possible contamination from other ATPases, or activation by compounds present in the assay buffer. A more thorough discussion is needed to explain this background activity and its potential impact on the results.

The intrinsic basal ATPase activity without substrate transport has been widely observed for many ABC transporters including Pgp. Such activity can be fully inhibited by specific inhibitors or non-hydrolysable nucleotides. The level of basal ATPase is comparable to previous studies of Pgp in lipid nanodiscs (Dastvan et al, 2019; Alam et al, 2019). We have clarified this in the manuscript on line 73-74 and line 78-80.

2. Comparison with Previous Structures: Several cryo-EM structures of human Pgp bound to inhibitory Fab fragments and small molecules have been published, including a recent high-resolution structure of Pgp bound to three molecules of elacridar (BBRC 2024). It would be valuable to compare the structures obtained in this study with these previously reported structures. This comparison could help contextualize the new findings and highlight any unique features captured in this study.

We thank the reviewer for the suggestion. For comparison with previous structures, we have added a new section (line 290). The content includes comparison of the binding central cavity and peripheral site with

several previous structures, including when bound to three molecules of elacridar (line 299-307), the kinking of TM4 and TM10 (line 294-299), the closed-collapsed states (line 308-310), and the recent eLife paper describing the trapping of substrates through covalent linkage (line 310-317).

3. Figure Clarity and Readability: Some of the figures, especially those showing structural models and density maps, use font sizes that are too small, making them difficult to read. Increasing the font size in these figures would improve accessibility, particularly for readers who may have difficulty reading small text.

We thank the reviewer for the feedback. We have gone through all the figures and increased the font size.

4. Inconsistent Labeling in Supplementary Figures: The supplementary figures and the legends use inconsistent labeling for the panel identifiers (a, b, c, A, B, C, etc.).

Thank the reviewer for the suggestion. We have corrected the labeling in all figures and figure legends.

Journal: Nature Communications

Manuscript ID: NCOMMS-24-65565-T

Article Type: Research Article

GENERAL COMENTS

The article by Culbertson and Liao aims to shed some light on the elusive mechanism by which efflux pumps – namely Pgp – can transport drugs. The article is, in general, well-written although it is suggested that it should still be thoroughly re-read to correct small misspellings and some sentences that seem out-of-context. The methodology is solid and seemingly reproducible, and the main conclusions are (mostly) supported by the results. In an area in exponential growth since the advent of Cryo-ME, such as membrane proteins, most of the reported results are known, but nonetheless there are several nuances and insights that confers novelty within the field of efflux transporters.

MANUSCRIPT COMMENTS

1. From literature it is known that self-inhibition by vinblastine has an IC_{50} of ~89μM, while for verapamil the IC_{50} is ~2.4μM (*R*-isomer) or ~2.1μM (*S*-isomer). While verapamil is expected to act as an inhibitor at concentrations above 20μM, it is somewhat surprising that the same would occur with vinblastine at such low concentrations, as claimed in page 2, lines 73-75. The authors must provide a critical comment about this, as it does not correlate with data reported in specialized literature.
2. The authors aim to identify which residues often interact with either vinblastine or verapamil in the several conformations, which is quite valuable. Nonetheless, the omitted the "empirical site nomenclature" suggested by Shapiro and Ling and based on efflux experiments of Hoechst 33342 and Rhodamine-123 (the H- and R-sites) that, together with initial "modulator" site identified from the first crystallographic ligands in the murine Pgp – QZ diastereoisomers - provides until today a way to adequately "map" the internal binding pocket. The authors are advised to adopt this nomenclature to facilitate the interpretation of the interaction data.
3. In this work (although in line with many other publications), there is no critical assessment that some of the differences reported may simply derive from the different size and volume of vinblastine and verapamil. This should be pointed out.
4. On page 5 (lines 215-218) the authors claim that "due to less rigid binding to Pgp, verapamil represents more efficient transport substrates". This was already demonstrated both experimentally (doi: 10.1002/prot.23023) and computationally for Sav1866 (doi: 10.1002/prot.23023) and Pgp (doi: 10.7554/eLife.90174.3). Therefore, references should be added consequently.
5. If MD simulations were performed, binding energies should be calculated to support some of the conclusions (e.g., page 6, line 251 or page 7, lines 284-285). Also, for the

RMSD calculation the NBDs should be plotted separately from the TMDs, as the authors recognize that "they have higher fluctuations" which could mask the ligand' RMSDs.

6. On page 5, lines 260-261, the authors start describing the transport mechanism. However, the very first step – how the ligand may reach the internal cavity – is omitted. The authors must include references (a couple of them can be swiftly found) on this because it will give extra support to the proposed translocation mechanism.
7. In the last paragraph of page 7 (lines 285-291), the idea claimed by the authors seems to be inspired by a docking paper in which the enrichment of one of the sites in aromatic rings is responsible for the specific and high-affinity contacts with ligands, with fully stabilized poses suggested to impair conformations transitions from the inward- to the outward-facing conformation (doi: 10.1021/ci400195v). Being an idea already in literature, a suitable reference should be added to the text.
8. Supporting Information, Protein Expression and Purification:
    a. Why wasn't cholesterol added to the nanodisc, as several publications identify lipid rafts, higher-order membranes and cholesterol composition as critical for Pgp activity?
9. Supporting Information, Molecular Dynamics section:
    a. A simulation with TM10 in its Cryo-EM determined shape (not straightened) must also be done for comparison purposes. Also, it is not clear how TM10 was "manually straightened": was TM10 replaced by one straight TM10 helix from the Cryo-EM structures? And after 700 (or 1200ns), did the helix kinked again?
    b. Why was IF1 used for vinblastine and IF for verapamil?
    c. It is intriguing why the authors went for POPG for the nanodiscs and POPC:Chol (80:20) for the MD simulations. I would recommend mimicking the composition of the nanodiscs (also without cholesterol, optionally with the MSP1D1 protein) unless the authors provide very convincing arguments on why it was done like this (because CHARMM-GUI provides membranes with POPG).
    d. Why was the disordered region between TM1 and TM2 modeled and not the linker between NBD1 and TM7? Also, did the authors see any influence of TM1 on the transport mechanism (from the Cryo-EM data or MD simulations) as suggested in the paper by Zhang et al. (doi: 10.7554/eLife.90174.3)?
    e. How many lipids are in the membrane: from its lateral dimensions (96.1 x 96.1) it seems to be very small concerning the maximus distance of the NBDs (from outer edge to outer edge)? The simulation box (.gro), topologies (.itp/.top) and running parameters (.mdp) should ideally be made available to allow reproducibility of the experiments.
10. Figure S4 is identical to Figure S3. Please revise.
11. Figure S5: In the table, specify next to the PDB code if the structure refers to APO or HOLO structures
12. Figure S6. How did the IF conformations obtained from vanadate correlate with those from Figure S2? Could they be appended to the first ones to increase resolution?
13. Figure S11: RMSDs should be plotted only against the TM regions (excluding the highly fluctuating NBDs). This should reduce the noise, e.g. in figure S11C, where is very

unlikely that both vinblastine molecules have such a low RMSD when the protein fluctuates immensely. Were the molecules fitted against their initial position or fitted against the protein? Also, it would be interesting to have the area per lipid calculations from the MD runs, to verify that the membrane remained equilibrated and in a liquid-state during the whole MD simulation.

14. Figure S15. Although trusting the data, the reported data do not fit with theoretical concepts. It is assumed that binding of the substrate induces smaller distances between the NBDs that are augmented by the binding of ATP to induce dimerization. The initial part of the data seems to suggest that, as the *holo* structures has shorter NBD-NBD distances than the *apo* structures. However, in the presence of ATP the NBDs seem to be more far apart. While for vinblastine this is within the margin of error (n=9) for verapamil they are intermediate, suggesting that modulators increase the energetic penalty for changing the conformation from IF > OF. However, the authors bluntly report that "such a conformation is either highly transient of completely absent" in the case of vinblastine. How so, if NBD-NBD distances are smaller in the presence of vinblastine and ATP? May this mean that molecules might be effluxed from the OC conformations sometimes, without need of fully CC? The authors are encouraged to add a short discussion on this.
15. The analysis of the validation reports also revealed a large number of outliers and poor fitting of the ligands as well. The authors should be absolutely sure that such problems are inherent to the structure and not errors during the fittings.