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Peer Review File

Inhibition of ABCG2 prevents phototoxicity in mice with erythropoietic protoporphyria

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have generated and characterized new ABCG2 inhibitors derived from Ko143 and followed the effect of one of the inhibitors (K31) in preventing phototoxicity in erythropoietic protoporphyria in a mice model. The inhibitor decreases PPIX concentrations in the skin, thereby attenuating the photosensitivity in these mice. The study appears to be well done, the presentation is clear and the conclusions are based on the presented data. The methodology is sound, but more details are required in the methods section in order to be able to reproduce the results. All in all, I think this is an interesting study with significance to the field and complements the authors' previous study in the abcg2-null mice model.

I have only a few comments:

Does the ABCG2 inhibition affect liver complications due to reduced biliary efflux of PPIX? The follow-up study here was quite short, but could PPIX levels in the liver be measured? It would be interesting data in order to see if liver abcg2 was inhibited by the treatment or if the inhibition was limited to the RBCs.

N-values should be reported in all studies, now it is missing in some, such as in figure 1f, 4c. It is also unclear if the n-value refers to three replicates or three independent experiments for instance in 1b,c and 4 f,g.

More details are required in the methods. For instance:
- regarding the WB, some details on how the membrane proteins were isolated from the RBCs, the primary and secondary antibodies should be indicated more clearly, their dilutions used in the WB as well as details on the detection.
-Regarding the qPCR, the authors are encouraged to familiarize with the MIQE guidelines Bustin SA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22. doi: 10.1373/clinchem.2008.112797.
-For the inhibitory experiments with A549 cells, the culture medium is not mentioned, neither any washing steps before the analysis.
-There is no description on the sex distribution of the mice used in the study.

Reviewer #2

(Remarks to the Author)
The authors investigated whether inhibition of ABCG2 reduces skin phototoxicity in FECH-mutated mice as a model of human erythropoietic protoporphyria. To this aim they synthesized a new ABCG2 inhibitor with improved pharmacokinetic characteristics. They analyzed the effect of this new inhibitor using a number of different techniques. The manuscript is well written and the results are clearly presented and they discuss their findings appropriately. However, a number of questions remain open:
1. The severity of the FECH-mut is influenced by the genetic background (Abibtol M. et al. Am J Physiol 288(6), G1208, 2005). How did the authors confirm that the Fech-mut mice and the Fech-mut/ABCG2 null mice have the same genetic

background?
2. What is the reproducibility of PPIX measurement by UPLC-QTOFMS at the PPIX concentrations measured in the samples? We propose that the PPIX concentrations both in erythrocytes as well as in serum is given as concentrations such as ng/ml which is essential to compare this animal model to human EPP.
3. What kind of light source did the authors use to irradiate the mice? What was the irradiation intensity applied to the mice? Were the mice restrained during light exposure? Why do the authors call it UV light, despite they used light in the visible range (395-410 nm)? Fig 1 Diagram e/f: How many mice were tested for each condition and time point?
4. Why were RBC isolated from blood samples cultured in RPMI 1640 medium? How much of intracellular PPIX diffused out of the cells during this incubation period and what was the PPIX concentration in the red cells at time point zero to correct for variable intracellular concentrations?
5. Histology Figure 2 and 3: The histological findings are different from those described in human EPP-related phototoxicity (e.g. Timonen K et al, J Am Acad Dermatol 43, 489; 2000), as the epidermis is missing and apparently the subepidermal collagen is destroyed. Please describe the observed histological changes by phototoxicity and discuss it with respect to the finding of other authors.
6. Figure 3: The epidermal lesions of the animals of day 6,7 and 8 look surprisingly similar. Please comment.
7. Figure 4a, b: What is the meaning of the units on the abscissa and the ordinate and what do the circles in diagram a) mean? What exactly are the controls? Why was K31 included in the s-plot, as presumably the controls were not treated by K31? A number of metabolites varied much more strongly than PPIX between controls and K31 treated animals. Do they belong to a specific metabolic pattern?
8. Figure 4; Diagram c, f, g: Instead of % of controls use concentrations such as ng/ml in order to enable a comparison to human EPP, but also to other publications with the FECH-m1pas mice.
9. For interpretation of the efflux, the initial concentration of PPIX in the erythrocytes should be given, as it is unlikely that Fech-mut and Fech-mut/ABCG2 null mice have the same intracellular PPIX concentrations at time zero. The efflux should be expressed as percentage of these initial concentrations.
10. Generally, a safety period of 5 days is insufficient. Human EPP and also mouse FECH-mut is a chronic life-long disease and safety of a treatment must therefore be assessed on a prolonged time basis.
a. It is very unlikely that such a short-term exposure can have an effect on the hemoglobin or MCH, as erythrocytes survive on the peripheral blood for several weeks, if there is no acute hemolysis.
b. Liver safety: Five treatment days are unlikely sufficient to reveal any effect on the liver. Taking into account that PPIX after inhibition of ABCG2 accumulates in the erythrocytes and finally this erythrocyte PPIX burden presumably is taken up by the reticuloendothelial system that disposes old erythrocytes. The excess PPIX finally must be eliminated from the body, which likely is by the hepatobiliary route due to the hydrophobicity of PPIX. Indeed, decreased ABCG2 expression has been associated to EPP-related liver damage (Hagiwara S. et al, Hepatology 6: 1638 (2015), although this effect has been disputed by some of the authors of this manuscript (Sachar M et al, Hepatology 64: 2016).
Thus overall, the limitation of the short-term exposure on safety assessment should be emphasized.

Reviewer #3

(Remarks to the Author)
Erythropoietic protoporphyria (EPP) currently has limited options clinically. In this manuscript, Zhu et al. developed a novel oral ABCG2 inhibitor to treat EPP-associated phototoxicity. ABCG2 has been well established as a therapeutic target based on mouse genetics models by the same research group. Overall, this study is well designed with impressive efficacy in mouse models. I highly recommend publishing this work in Nature Communications.

To make this a stronger publication, I have the following recommendations:
1. The authors did not discuss the potential toxicity of targeting ABCG2 in humans. Indeed, based on human genetics, ABCG2 could be a safe drug target as ABCG2 null people appear to be normal (https://pubmed.ncbi.nlm.nih.gov/22246505/), in some populations.
2. Please provide a rationale for why K31 is chosen instead of K3, which has slightly better metabolic stability.
3. What is the unbound fraction of K31 in plasma?
4. What is the selectivity of K31 in a panel of ABC transporters?
5. In all the animal studies, 100 mg/kg PO was used. Do lower doses, such as 30 or 10 mg/kg, still be efficacious? It would be helpful to have the dose-response data, even lower doses may not be as effective. This will help to gauge the dose in future clinical trials.
6. Full gels for WB should be included in supplementary information.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
My concerns with the manuscript have been addressed, and I support its publication.

Reviewer #3

(Remarks to the Author)
Thanks for addressing all my questions. I think this manuscript is ready to be published.

Reviewer #4

(Remarks to the Author)
We'd like to thank the authors for their precise, well-argued answers.
We would still like to clarify a few points.
1/ Concerning the genetic background, 3 FECH m1pas congenic lines have been established with quite different phenotypes. The SJL background has the highest level of erythrocyte PPIX and less severe liver damage than the other two backgrounds, BALB/C and C57BL/6. This supports your results and could be mentioned in the discussion.
You should specify which background you used: BALBC or SJL?
2/ The number of mice in the experiences as a whole is 3 or 4, which makes it impossible to use means and standard deviations and parametric statistical tests. We need to use the median and non-parametric tests.
3/ What's the point of the metabolomics study? The only data that really emerges is the drop in PPIX levels in the serum of K31-treated mice, as demonstrated by direct assay. I would withdraw this study.
4/ With regard to the experiments on PPIX efflux from red blood cells, do the results shown in figures 4f and 4g represent the % of the initial amount of PPIX that leaves red blood cells over time? If so, these results are probably based on the determination of PPIX in RBCs at T0 and at different times.
Why is reference made to a PPIX assay in RPMI? If PPIX assays were indeed performed in RPMI 1640, have you validated that the pink color of RPMI did not interfere with PPIX fluorescence assays, as PPIX emits around 630nm?
Were the RBC culture experiments carried out in the dark? PPIX degrades very rapidly in the presence of light, and light induces the release of PPIX from RBCs, probably independently of ABCG2.
5/ As already mentioned, the question of the fate of erythrocyte PPIX is essential to assess the future of this therapeutic approach. It would have been important to measure PPIX excretion in the stool in a metabolic cage, to ensure that PPIX did not accumulate in the mouse. It would be interesting for the authors to discuss this point.
Safety data over 23 days are already more convincing than over 5, but this is still too short to have a precise idea of the impact of the change in PPIX metabolism in the hepatocyte. The results in the 2019 paper suggesting altered PPIX conjugation in Fech-mut/Abcg2-nul mice would have been very useful in the context of K31 use.

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
The authors' answers are quite convincing. I think this important article is ready for publication.

As the authors are aware, other therapeutic approaches are in advanced phases of clinical research, and initial results on phototoxicity are very promising. The major advantage of ABCG2 inhibitors would be hepatic protection.

RESPONSES TO REVIEWER 1

Reviewer #1 (Remarks to the Author):
The authors have generated and characterized new ABCG2 inhibitors derived from Ko143 and followed the effect of one of the inhibitors (K31) in preventing phototoxicity in erythropoietic protoporphyria in a mice model. The inhibitor decreases PPIX concentrations in the skin, thereby attenuating the photosensitivity in these mice. The study appears to be well done, the presentation is clear and the conclusions are based on the presented data. The methodology is sound, but more details are required in the methods section in order to be able to reproduce the results. All in all, I think this is an interesting study with significance to the field and complements the authors' previous study in the abcg2-null mice model.
Response: We thank the reviewer for these positive remarks.

Does the ABCG2 inhibition affect liver complications due to reduced biliary efflux of PPIX? The follow-up study here was quite short, but could PPIX levels in the liver be measured? It would be interesting data in order to see if liver abcg2 was inhibited by the treatment or if the inhibition was limited to the RBCs.
Response: We thank the reviewer for this insightful comment. We have another ongoing project that focuses on the effects of ABCG2 inhibitors on hepatotoxicity in EPP. In the current work, we focused on phototoxicity, the most common symptom in EPP. We found that ABCG2 inhibitors can effectively prevent PPIX-mediated phototoxicity in an EPP mouse model during a 5-day study (the longest time approved by IACUC because of severe skin damage in control group). However, ABCG2 inhibitors have limited effects on the liver complications of EPP in this short-term study (Supplementary Fig. 7d and 7e). As the reviewer suggested, we measured PPIX levels in the liver, but no significant change was found (Supplementary Fig. 7c). Therefore, we believe that the quick effectiveness of ABCG2 inhibitors in preventing PPIX-mediated phototoxicity is independent of hepatic ABCG2.

N-values should be reported in all studies, now it is missing in some, such as in figure 1f, 4c. It is also unclear if the n-value refers to three replicates or three independent experiments for instance in 1b,c and 4 f,g.
Response: This information has been added to our revised manuscript.

More details are required in the methods. For instance:
- regarding the WB, some details on how the membrane proteins were isolated from the RBCs, the primary and secondary antibodies should be indicated more clearly, their dilutions used in the WB as well as details on the detection.
Response: More details of WB have been added to our revised manuscript in the section of supplementary method.

-Regarding the qPCR, the authors are encouraged to familiarize with the MIQE guidelines Bustin SA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009 Apr;55(4):611-22. doi: 10.1373/clinchem.2008.112797.
Response: More details of qPCR have been added to our revised manuscript in the section of supplementary method.

-For the inhibitory experiments with A549 cells, the culture medium is not mentioned, neither any washing steps before the analysis.
Response: More details of cell culture have been added to our revised manuscript in the method section.

-There is no description on the sex distribution of the mice used in the study.
Response: In the revised manuscript, we have provided the sex distribution of mice used in each study and added the efficacy data from female mice (Supplementary Fig. 4). Throughout our 10 years of investigations in this project, we have been using both male and female mice and no significant gender difference was observed.

RESPONSES TO REVIEWER 2

Reviewer #2 (Remarks to the Author):
The authors investigated whether inhibition of ABCG2 reduces skin phototoxicity in FECH-mutated mice as a model of human erythropoietic protoporphyria. To this aim they synthesized a new ABCG2 inhibitor with improved pharmacokinetic characteristics. They analyzed the effect of this new inhibitor using a number of different techniques. The manuscript is well written and the results are clearly presented and they discuss their findings appropriately.
Response: We thank the reviewer for these positive remarks.

1. The severity of the FECH-mut is influenced by the genetic background (Abibtol M. et al. Am J Physiol 288(6), G1208, 2005). How did the authors confirm that the Fech-mut mice and the Fech-mut/ABCG2 null mice have the same genetic background?
Response: We fully agree with the reviewer that the severity of FECH-mut is influenced by the genetic background. For the studies using Fech-mut/Abcg2-null mice (Fig. 4d-f), they have been backcrossed with Fech-mut mice for 6 generations. Therefore, we believe that the genetic background of Fech-mut/Abcg2-null and FECH-mut mice is highly comparable. In addition, all efficacy studies of ABCG2 inhibitors in the current work (Figs. 1e-f, 2, 3; Supplementary Figs. 1, 4, 5) were conducted using only one strain of FECH-mut mouse, which has the exact same genetic background.

2. What is the reproducibility of PPIX measurement by UPLC-QTOFMS at the PPIX concentrations measured in the samples? We propose that the PPIX concentrations both in erythrocytes as well as in serum is given as concentrations such as ng/ml which is essential to compare this animal model to human EPP.
Response: As the reviewer suggested, we have replaced the relatively quantified data of PPIX with the absolute quantification (ng/ml) in our revised manuscript (Fig. 4c-d; Supplementary Fig. 6). Our detection method for PPIX by UPLC-QTOFMS is highly reproducible with an inter-day difference < 5% (Supplementary Fig. 10a). This information has also been provided in our revised manuscript.

3. What kind of light source did the authors use to irradiate the mice? What was the irradiation intensity applied to the mice? Were the mice restrained during light exposure? Why do the

authors call it UV light, despite they used light in the visible range (395-410 nm)? Fig 1 Diagram e/f: How many mice were tested for each condition and time point?
Response: Same as our previous report (Wang et al., 2019), DULEX Flashlight (395-410 nm, 294 lumens (lm)/m^2) was used as the irradiation source. The mice were restrained in the box during light exposure. We agree with the reviewer that the term "UV light" is not accurate, though it is around the end range of UV-A (400 nm). Corresponding changes have been made in our revised manuscript by adding details of the light source and removing the term "UV". Animal numbers (n = 6) have been added to the legend of Fig 1e/f.

0. Why were RBC isolated from blood samples cultured in RPMI 1640 medium? How much of intracellular PPIX diffused out of the cells during this incubation period and what was the PPIX concentration in the red cells at time point zero to correct for variable intracellular concentrations?

Response: The current work used 1640 medium for culturing RBCs, which is based upon previous reports (Trager and Jensen, 1976; Tiffert et al., 2005). As the reviewer suggested, we determined the intracellular concentration of PPIX in the RBCs of EPP mouse models at the time point zero of incubation. We also measured the percentages of PPIX efflux from RBCs to the medium after 1 and 3 hours of culture. Our data showed that PPIX concentrations were 62.6 and 530.2 ng/10^8 cells in the RBCs of Fech-mut and Fech-mut/Abcg2-null mice, respectively, at the time point zero of incubation (Supplementary Fig. 6). We also found that the percentages (%) of PPIX release from the RBCs of Fech-mut mice were significantly lower than that in the culture groups with ABCG2 inhibitors or with ABCG2 deficiency. These new results have been provided in our revised manuscript (Fig. 4f-g).

1. Histology Figure 2 and 3: The histological findings are different from those described in human EPP-related phototoxicity (e.g. Timonen K et al, J Am Acad Dermatol 43, 489; 2000), as the epidermis is missing and apparently the subepidermal collagen is destroyed. Please describe the observed histological changes by phototoxicity and discuss it with respect to the finding of other authors.

Response: We thank the reviewer for this insightful comment. In our revised manuscript, we have described the histological changes in EPP mice and compared them to the findings in humans "Histological analysis revealed that the epidermis of skin was missing and subepidermal collagen was damaged in the control group of Fech-mut mice after light exposure, but these histological changes were prevented in the groups with K31 treatment (Fig. 2a and 2b, Supplementary Fig. 4). In addition, pretreatment with K31 attenuated oxidative stress and inflammation in the skin of Fech-mut mice exposed to light (Fig. 2c-e). We noted the differences of histological findings in the skin between EPP mouse models and EPP patients, which can be explained by the followings: (1) the EPP mice received a high amount of irradiation without protection, whereas EPP patients usually avoid light with self-protection; and (2) the histological changes in the skin of EPP patients are built up for many years because of the repeated injury and repair, leading to the thickness of vessel walls (Timonen et al., 2000); but the current study on EPP-related phototoxicity in mice only lasted for several days."

2. Figure 3: The epidermal lesions of the animals of day 6,7 and 8 look surprisingly similar. Please comment.

Response: We believe that it is natural/normal for these figures to look similar because they were taken from the same mouse on different days. The skin injury was already very severe on day 6 so that it is hard to see obvious worsening after additional light exposure (days 7 and 8). More pictures of mice from the same experiment have been provided in our revised manuscript, and they all look similar for each individual mouse from days 6 to 8 (Supplementary Fig. 5).

3. Figure 4a, b: What is the meaning of the units on the abscissa and the ordinate and what do the circles in diagram a) mean? What exactly are the controls? Why was K31 included in the s-plot, as presumably the controls were not treated by K31? A number of metabolites varied much more strongly than PPIX between controls and K31 treated animals. Do they belong to a specific metabolic pattern?
Response: Fig. 4a and 4b were generated by the software for metabolomic analysis. In Fig. 4a, the values of x-axis and y-axis represent the score of each sample in orthogonal partial least-squares discriminant analysis. The circles in diagram indicate the group separation of samples from control (vehicle) and K31 treatment. In Fig. 4b, the x-axis is a measure of the relative abundance of ions, and the y-axis is a measure of the correlation of each ion to the model. These details have been added to the figure legend of our revised manuscript.

We conducted metabolomic analysis to explore the potential mechanisms of K31 in preventing PPIX-mediated phototoxicity. As expected, K31 was detected in the sera of mice with K31 treatment, indicating the success of metabolomic analysis. Interestingly, metabolomic analysis also revealed a decrease of PPIX in sera, which provided us with the clue to investigate the connections among K31, ABCG2 in RBCs, and PPIX in sera. As the reviewer pointed out, many other metabolites were also altered after K31 treatment. The current work focused on PPIX because it is the primary cause of EPP-related phototoxicity. Studies are ongoing in our team to identify other metabolites, which might be associated with the secondary effect of K31-mediated protection of PPIX phototoxicity.

4. Figure 4; Diagram c, f, g: Instead of % of controls use concentrations such as ng/ml in order to enable a comparison to human EPP, but also to other publications with the FECH-m1pas mice.
Response: The data in Fig. 4c and 4d have been revised in ng/ml. As the reviewer suggested in next comment, the efflux rate of PPIX from RBCs has also been quantified and expressed as percentage (%) of initial concentrations (Fig. 4f and 4g, Supplementary Fig. 6).

5. For interpretation of the efflux, the initial concentration of PPIX in the erythrocytes should be given, as it is unlikely that Fech-mut and Fech-mut/ABCG2 null mice have the same intracellular PPIX concentrations at time zero. The efflux should be expressed as percentage of these initial concentrations.
Response: We apologize for the confusion. Fech-mut and Fech-mut/ABCG2 null mice have different PPIX concentrations in RBCs and different efflux rates of PPIX from RBCs. In our original Fig 4f, PPIX levels in the medium cultured with the RBCs of Fech-mut mice were set as 1 at 15 min (the 1st time point collected for analysis), and the data in other groups were normalized to the Fech-mut group at 15 min. A similar method was used to present the effect of K31 on the efflux of PPIX from RBCs, in which PPIX levels in the medium cultured with the RBCs of Fech-mut mice but without K31 (Control group) were set as 1 at 15 min, and the data in other groups were normalized to the control group at 15 min.

To avoid the above-mentioned confusion, we have provided the following new results as the reviewer suggested: (1) the initial concentrations of PPIX in the RBCs of Fech-mut and Fech-mut/ABCG2 null mice (Supplementary Fig. 6); (2) the percentage of PPIX efflux from the RBCs of Fech-mut and Fech-mut/ABCG2 null mice (Fig. 4f); and (3) the percentage of PPIX efflux from the RBCs of Fech-mut mice with or without K31 treatment (Fig. 4g).

10. Generally, a safety period of 5 days is insufficient. Human EPP and also mouse FECH-mut is a chronic life-long disease and safety of a treatment must therefore be assessed on a prolonged time basis.
a. It is very unlikely that such a short-term exposure can have an effect on the hemoglobin or MCH, as erythrocytes survive on the peripheral blood for several weeks, if there is no acute hemolysis.
b. Liver safety: Five treatment days are unlikely sufficient to reveal any effect on the liver. Taking into account that PPIX after inhibition of ABCG2 accumulates in the erythrocytes and finally this erythrocyte PPIX burden presumably is taken up by the reticuloendothelial system that disposes old erythrocytes. The excess PPIX finally must be eliminated from the body, which likely is by the hepatobiliary route due to the hydrophobicity of PPIX. Indeed, decreased ABCG2 expression has been associated to EPP-related liver damage (Hagiwara S. et al, Hepatology 6: 1638 (2015), although this effect has been disputed by some of the authors of this manuscript (Sachar M et al, Hepatology 64: 2016).
Thus overall, the limitation of the short-term exposure on safety assessment should be emphasized.
Response: We fully agree with the reviewer on this point. In the current revision, we have provided the safety data of K31 from a sub-chronic study (23 days). We found that 23-day treatment of K31 did not alter growth (body weight gain), the ratios of organ-to-body weight (liver, kidney, and spleen), the biomarkers of liver injury including ALT, AST, and ALP, and liver histology (Supplementary Figs. 8 and 9). In addition, we have added discussions on the safety of ABCG2 inhibitors for EPP therapy "Our short-term and sub-chronic studies showed that treatment with K31 did not alter growth, the ratios of organ to body weight, and the biomarkers of bone marrow, liver, and kidney toxicity, suggesting that ABCG2 inhibitors are unlikely to cause adverse effects on these organs. We also do not expect toxicities from ABCG2 suppression itself because: (i) growth and breeding of Abcg2-deficient mice are normal, and no health issue was observed in these mice (Wang et al., 2019); (ii) the health condition of Fech-mut/Abcg2-null mice is significantly better than that of Fech-mut mice (Wang et al., 2019); and (iii) humans with a genetic deficiency of ABCG2 live normally and show no associations with any particular diseases (Zelinski et al., 2012; Niall et al., 2018; Zhang et al., 2018). Despite the facts mentioned above, long-term studies are needed to further profile the safety of ABCG2 inhibitors for EPP therapy because EPP is a life-long disease."

We did not cite the work from Hagiwara et al regarding the association of ABCG2 expression with EPP-related hepatotoxicity (Hagiwara et al., 2015), which is a case report with only 2 patients, and it did not explore the association of ABCG2 expression with EPP-related phototoxicity. We have argued about their work with a different opinion (Sachar and Ma, 2016). In addition, our follow-up work using Abcg2-null mouse models illustrated that deficiency of

ABCG2 is protective against PPIX-mediated hepatotoxicity in EPP (Wang et al., 2019), which provided solid evidence that the speculation of Hagiwara et al is incorrect.

RESPONSES TO REVIEWER 3

Reviewer #3 (Remarks to the Author):
Erythropoietic protoporphyria (EPP) currently has limited options clinically. In this manuscript, Zhu et al. developed a novel oral ABCG2 inhibitor to treat EPP-associated phototoxicity. ABCG2 has been well established as a therapeutic target based on mouse genetics models by the same research group. Overall, this study is well designed with impressive efficacy in mouse models. I highly recommend publishing this work in Nature Communications.
Response: We thank the reviewer for these positive remarks.

To make this a stronger publication, I have the following recommendations:
1. The authors did not discuss the potential toxicity of targeting ABCG2 in humans. Indeed, based on human genetics, ABCG2 could be a safe drug target as ABCG2 null people appear to be normal (https://pubmed.ncbi.nlm.nih.gov/22246505/),in some populations.

Response: Regarding the safety of ABCG2 deficiency or inhibition, more data and discussions have been added to our revised manuscript: "Our short-term and sub-chronic studies showed that treatment with K31 did not alter growth, the ratios of organ to body weight, and the biomarkers for bone marrow, liver, and kidney toxicity (Supplementary Figs. 7-9), suggesting that ABCG2 inhibitors are unlikely to cause adverse effects on these organs. We also do not expect toxicities from ABCG2 suppression itself because: (i) growth and breeding of Abcg2-deficient mice are normal, and no health issue was observed in these mice (Wang et al., 2019); (ii) the health condition of Fech-mut/Abcg2-null mice is significantly better than that of Fech-mut mice (Wang et al., 2019); and (iii) humans with a genetic deficiency of ABCG2 live normally and show no associations with any particular diseases (Zelinski et al., 2012; Niall et al., 2018; Zhang et al., 2018). Despite the facts mentioned above, long-term studies are needed to further profile the safety of ABCG2 inhibitors for EPP therapy because EPP is a life-long disease."

2. Please provide a rationale for why K31 is chosen instead of K3, which has slightly better metabolic stability.
Response: We thank the reviewer for this insightful comment. Indeed, both K3 and K31 are good candidates with a high ABCG2 inhibitory activity and a high metabolic stability. However, K31 (CC_{50} > 100 μM) is safer than K3 (CC_{50} = 58.9 μM) based upon the evaluation of cytotoxicity (Table 1). Therefore, we chose K31 for further studies.

3. What is the unbound fraction of K31 in plasma?
Response: We have added an experiment to address this question. We found that the unbound fraction of K31 in serum is 5.85%. This result has been provided in our revised manuscript.

4. What is the selectivity of K31 in a panel of ABC transporters?
Response: In our revised manuscript, we have provided the inhibitory data of K31 on ABCB1 (also known as Pgp), one of the most important ABC transporters that pumps many xenobiotics

(including drugs) out of cells (Glaeser, 2011). We found that K31 does not inhibit ABCB1 (Supplementary Fig. 3).

5. In all the animal studies, 100 mg/kg PO was used. Do lower doses, such as 30 or 10 mg/kg, still be efficacious? It would be helpful to have the dose-response data, even lower doses may not be as effective. This will help to gauge the dose in future clinical trials.
Response: In our revised manuscript, we have provided the results from the EPP mice treated with a lower dose of K31 (30 mg/kg), which is also effective (Supplementary Fig. 4).

6. Full gels for WB should be included in supplementary information.
Response: Provided as the reviewer suggested.

REFERENCES

Glaeser H (2011) Importance of P-glycoprotein for drug-drug interactions. *Handb Exp Pharmacol***:**285-297.

Hagiwara S, Nishida N, Park AM, Sakurai T, Kawada A, and Kudo M (2015) Impaired expression of ATP-binding cassette transporter G2 and liver damage in erythropoietic protoporphyria. *Hepatology* **62:**1638-1639.

Niall H, Parth K, and Ian DK (2018) Polymorphisms of the Multidrug Pump ABCG2: A Systematic Review of Their Effect on Protein Expression, Function, and Drug Pharmacokinetics. *Drug Metabolism and Disposition* **46:**1886.

Sachar M and Ma X (2016) Role of ABCG2 in liver injury associated with erythropoietic protoporphyria. *Hepatology* **64:**305.

Tiffert T, Lew VL, Ginsburg H, Krugliak M, Croisille L, and Mohandas N (2005) The hydration state of human red blood cells and their susceptibility to invasion by Plasmodium falciparum. *Blood* **105:**4853-4860.

Trager W and Jensen JB (1976) Human malaria parasites in continuous culture. *Science* **193:**673-675.

Wang P, Sachar M, Lu J, Shehu AI, Zhu J, Chen J, Liu K, Anderson KE, Xie W, Gonzalez FJ, Klaassen CD, and Ma X (2019) The essential role of the transporter ABCG2 in the pathophysiology of erythropoietic protoporphyria. *Science Advances* **5:**eaaw6127.

Zelinski T, Coghlan G, Liu XQ, and Reid ME (2012) ABCG2 null alleles define the Jr(a-) blood group phenotype. *Nature genetics* **44:**131-132.

Zhang W, Sun S, Zhang W, and Shi Z (2018) Polymorphisms of ABCG2 and its impact on clinical relevance. *Biochem Biophys Res Commun* **503:**408-413.

RESPONSES TO REVIEWERS 1 AND 3

Reviewer #1 (Remarks to the Author):
My concerns with the manuscript have been addressed, and I support its publication.

Reviewer #3 (Remarks to the Author):
Thanks for addressing all my questions. I think this manuscript is ready to be published.

Response: Thanks again to reviewers #1, #2, and #3 for their insightful comments and suggestions on our original submission, which really helped us to improve the quality of our revised manuscript.

RESPONSES TO REVIEWER 4

Reviewer #4 (Remarks to the Author):
We'd like to thank the authors for their precise, well-argued answers. We would still like to clarify a few points.
Response: We thank you for these positive remarks. We also thank you for the following comments and suggestions on our manuscript. We have carefully considered each point and revised our manuscript accordingly.

1/ Concerning the genetic background, 3 FECH m1pas congenic lines have been established with quite different phenotypes. The SJL background has the highest level of erythrocyte PPIX and less severe liver damage than the other two backgrounds, BALB/C and C57BL/6. This supports your results and could be mentioned in the discussion.
You should specify which background you used: BALBC or SJL?
Response: The background of Fech-mut mice used in our work is BALB/c. This information together with references have been added to our revised manuscript.

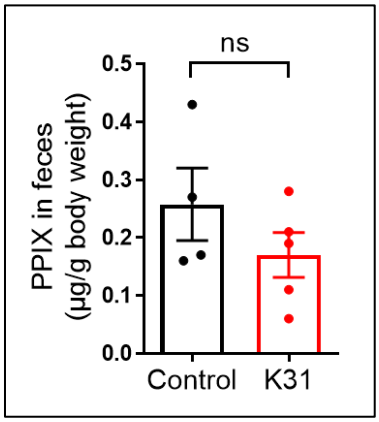
2/ The number of mice in the experiences as a whole is 3 or 4, which makes it impossible to use means and standard deviations and parametric statistical tests. We need to use the median and non-parametric tests.
Response: We thank you for this suggestion. Changes have been made accordingly in our revised manuscript (Fig. 1d, 4c, 4d, and Fig. S3, S8b-d, and S9a-c).

3/ What's the point of the metabolomics study? The only data that really emerges is the drop in PPIX levels in the serum of K31-treated mice, as demonstrated by direct assay. I would withdraw this study.
Response: We agree with the reviewer. The data from the metabolomic study (Fig. 4a-b) have been removed as the reviewer suggested.

4/ With regard to the experiments on PPIX efflux from red blood cells, do the results shown in figures 4f and 4g represent the % of the initial amount of PPIX that leaves red blood cells over time? If so, these results are probably based on the determination of PPIX in RBCs at T0 and at different times.

Why is reference made to a PPIX assay in RPMI? If PPIX assays were indeed performed in RPMI 1640, have you validated that the pink color of RPMI did not interfere with PPIX fluorescence assays, as PPIX emits around 630nm?
Were the RBC culture experiments carried out in the dark? PPIX degrades very rapidly in the presence of light, and light induces the release of PPIX from RBCs, probably independently of ABCG2.
Response: We thank the reviewer for this insightful comment. Yes, the results shown in figures 4f and 4g represent the % of the initial amount of PPIX that leaves RBCs over time, and these results are generated based on the determination of PPIX in RBCs at T0 and at different times. Regarding PPIX detection, we used 1640 medium without phenol red, which avoided its interference with PPIX fluorescence assays. In addition, light protection was considered in each step of PPIX assays. This information has been added to our revised manuscript.

5/ As already mentioned, the question of the fate of erythrocyte PPIX is essential to assess the future of this therapeutic approach. It would have been important to measure PPIX excretion in the stool in a metabolic cage, to ensure that PPIX did not accumulate in the mouse. It would be interesting for the authors to discuss this point.
Safety data over 23 days are already more convincing than over 5, but this is still too short to have a precise idea of the impact of the change in PPIX metabolism in the hepatocyte. The results in the 2019 paper suggesting altered PPIX conjugation in Fech-mut/Abcg2-nul mice would have been very useful in the context of K31 use.
Response: We agree with the reviewer that it is important to explore the fate of erythrocyte PPIX under ABCG2 suppression. As the reviewer suggested, we added an experiment to measure PPIX excretion in the stool of Fech-mut mice after K31 treatment. Briefly, Fech-mut mice were treated with vehicle (n = 4, control) or K31 (100 mg/kg, po, n = 5), and then these mice were held in a metabolic cage individually for 16 hr to collect feces. Afterward, PPIX in feces was extracted and analyzed by UPLC-QTOFMS. As shown in the Figure on the right, K31 has no significant (ns) effect on PPIX excretion. We wanted to share the data with the reviewer, but not for publication because this information is not conclusive due to the short time of K31 exposure (16 hr) vs a long half-life of RBCs (~20 days in mice). In the discussion of our revised manuscript, we highlighted that "Further studies are needed to determine the fate of erythrocyte PPIX under ABCG2 suppression". Indeed, this is an ongoing project in my lab and we are gathering resources for this work including deuterium-labeled ALA, deuterium-labeled PPIX, and conditional Abcg2 KO mice.



We also agree with the reviewer about the need for long-term safety evaluation of K31. However, we cannot provide these data to this manuscript because it is beyond our ability as a small lab to repeat such a long-term safety study when considering the cost of labor, animals, and reagents. Nevertheless, we further discussed the safety concern of ABCG2 suppression in our revised manuscript "We do not expect toxicities from ABCG2 suppression itself because: (i) growth and breeding of Abcg2-deficient mice are normal, and no health issue was observed in these mice; (ii) compared to Fech-mut mice, the health condition of Fech-mut/Abcg2-null mice is significantly better even with a high level of PPIX in RBCs, which is consistent with a

previous report showing that the Fech-mut mice in SJL background have a higher level of erythrocyte PPIX but less severe liver damage than the Fech-mut mice in BALB/c and C57BL/6 backgrounds; and (iii) humans with a genetic deficiency of ABCG2 live normally and show no associations with any particular diseases". We also pointed out in our revised manuscript that "Despite the facts mentioned above, long-term studies are needed to profile the safety of ABCG2 inhibitors for EPP therapy because EPP is a life-long disease".

Overall, we agree with the reviewer regarding the limitations of our manuscript including the fate of erythrocyte PPIX under ABCG2 suppression. We are working on these questions as the reviewer suggested and we believe we can address them in the future. In the end, we hope the reviewer agrees with us that the strengths of this manuscript outweigh its limitations, because this manuscript highlighted the following achievements: (1) development of novel and stable ABCG2 inhibitors; (2) proof-of-concept that ABCG2 inhibitors can effectively prevent PPIX-mediated phototoxicity; and (3) illustration of the mechanism-of-action for ABCG2 inhibitors in preventing PPIX-mediated phototoxicity.