ORIGINAL ARTICLE

# Assessment of porphyrogenicity of drugs and chemicals in selected hepatic cell culture models through a fluorescence-based screening assay

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## Abstract

Compounds that induce 5-aminolevulinic acid [ALA] synthase-1 and/or cytochromes P-450 may induce acute porphyric attacks in patients with the acute hepatic porphyrias [AHPs]. Currently, there is no simple, robust model used to assess and predict the porphyrogenicity of drugs and chemicals. Our aim was to develop a fluorescence-based in vitro assay for this purpose. We studied four different hepatic cell culture models: HepG2 cells, LMH cells, 3D HepG2 organoids, and 3D organoids of primary liver cells from people without known disease [normal human controls]. We took advantage of the fluorescent properties of protoporphyrin IX [PP], the last intermediate of the heme biosynthesis pathway, performing fluorescence spectrometry to measure the intensity of fluorescence emitted by these cells treated with selected compounds of importance to patients with AHPs. Among the four cell culture models, the LMH cells produced the highest fluorescence readings, suggesting that these cells retain more robust heme biosynthesis enzymes or that the other cell models may have lost their inducibility of ALA synthase-1 [ALAS-1]. Allyl isopropyl acetamide [AIA], a known potent porphyrogen and inducer of ALAS-1, was used as a positive control to help predict porphyrogenicity for tested compounds. Among the tested compounds (acetaminophen, acetylsalicylic acid, β-estradiol, hydroxychloroquine sulfate, alpha-methyldopa, D (-) norgestrel, phenobarbital, phenytoin, sulfamethoxazole, sulfisoxazole, sodium valproate, and valsartan), concentrations greater than 0.314 mM for norgestrel, phenobarbital, phenytoin, and sodium valproate produced fluorescence readings higher than the reading produced by the positive AIA control. Porphyrin accumulation was also measured by HPLC to confirm the validity of the assay. We conclude that LMH cell cultures in multi-well plates are an inexpensive, robust, and

simple system to predict the porphyrogenicity of existing or novel compounds that may exacerbate the AHPs.

# 1 | INTRODUCTION

Hepatic 5-aminolevulinic acid synthase-1 [ALAS-1], an enzyme that converts glycine and succinyl coenzyme A into 5-aminolevulinic acid [ALA], is a tightly regulated, rate-determining enzyme in the heme biosynthesis pathway.[1,2] Conditions, including hypoxia and oxidative stress, and compounds, such as alcohol, barbiturates, diverse xenobiotics, progesterone, and hydantoins, may induce ALAS-1 activity and upregulate cytochromes P450, stimulating the production of the end product, heme.[2–6] The size of the regulatory heme pool in hepatocytes plays a major role in ALAS-1 regulation by decreasing transcription of the ALAS-1 gene, reducing the rate of transport of ALAS-1 into the mitochondria at higher concentrations, upregulating ALAS-1 at lower concentrations, and increasing the breakdown of the mature enzyme in mitochondria by the action of LONP1 protease.[7–13]

If a step distal to that of ALAS-1 is defective in the heme biosynthesis pathway, for example, due to an inherited or acquired enzyme deficiency, a relative deficit in a small but critical regulatory heme pool may occur, leading to marked upregulation of ALAS-1 and overproduction of ALA. This overproduction is thought to be the major cause of acute neurovisceral attacks, autonomic abnormalities, and other neuromuscular features, which are hallmarks of acute porphyric attacks.[2,3,14] Patients with acute hepatic porphyrias [AHPs], including ALA dehydratase deficiency porphyria, acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria, may experience acute porphyric attacks with marked induction of ALAS-1.[15] Thus, it is important and clinically relevant to test for porphyrogenicity of common and novel medications to determine whether they have the potential to trigger or exacerbate acute porphyric attacks in patients with the AHPs.

Currently, there is no standard accepted model to assess for porphyrogenicity of drugs or other chemicals. Historically, a standard of cell models in human hepatology has been the utilization of primary human hepatocytes [PHHs]; however, due to many limitations to the use of PHH, such as rapid de-differentiation and slow proliferation in vitro, limited supply, and high costs, alternative cell culture models have been developed and tested with the general goal of providing a physiologically relevant model system.[16] Two notable hepatic cell lines with ALAS-1 inducibility are LMH cells, which are a hepatocellular carcinoma cell line that arose in a male leghorn chicken treated with diethylnitrosamine, and HepG2 cells, which are a human hepatoma cell line originally derived from a hepatocellular carcinoma that

arose in a 15-year-old boy with chronic hepatitis B infection.[10,17–21] Similarly to heme synthesis in humans, ALAS-1 transcription activity in LMH cells is mediated by heme-dependent regulation, which can repress both drug-mediated and basal induction of ALAS-1, supporting the fact that, although LMH cells are not derived from human hepatocytes, they are relevant to human heme biosynthesis.[10] There have been several reports of the use of LMH and HepG2 cells to study porphyrogenicity,however, it is unclear which cell line has retained the capacity for upregulation of ALAS-1 and for porphyrin accumulation to facilitate facile detection and quantification via fluorospectrometry.[10,21,22]

3D hepatic spheroids have recently been emerging as newer models to study drug-induced liver injury-related pathologies and liver toxicology studies purported to exhibit higher sensitivity and specificity in comparison to 2D models.[23–25] They can be derived from primary human hepatocytes or human hepatic-like cells and are phenotypically stable, viable, and can be co-cultured with non-parenchymal cells, allowing this in vitro model, perhaps, to more closely resemble in vivo hepatic tissue.[23,26] Little, if anything, is known of their usefulness in detecting and assessing the porphyrogenicity of drugs and chemicals. Thus, in this study, we studied LMH cells, HepG2 cells, HepG2 spheroids, and human liver organoids to determine which model produced results that are most likely to be pathophysiologically relevant to the AHPs.

We added selected drugs and chemicals to each cell line or spheroid preparation in clinically relevant, escalating doses, measured fluorescence emitted from porphyrin accumulation, mainly protoporphyrin IX [PP], and compared them to the fluorescence produced by known ALAS-1 inducers and porphyrogenic chemicals [AIA, phenobarbital, and phenytoin]. To mimic the effects of the porphyrias, deferoxamine [DFO], a potent iron chelator that blocks the conversion of PP to heme, was added with each drug.[27,28] This also made it possible to have a greater accumulation of fluorescent PP, improving the sensitivity of a high-throughput, microplate-based assay procedure.[28] The addition of ALA, which bypasses the need for upregulation of ALAS-1, provides information regarding the functional status of the biosynthetic pathway from ALA to PP in each cell model. Acetaminophen and acetylsalicylic acid, which have been used without causing acute porphyric attacks or upregulation of ALAS-1 in patients for decades, served as negative controls. β-estradiol, alpha-methyldopa, D (-) norgestrel, sulfamethoxazole, sulfisoxazole, sodium valproate, and valsartan were also tested as they have been hypothesized to induce

acute porphyric attacks. Hydroxychloroquine sulfate was tested as well because, while we were completing this study, this compound gained significant popularity due to its claimed benefits for treating COVID-19 infections and because our patients with AHP asked about its safety.

## 2 | MATERIALS AND METHODS

### 2.1 | Cells and chemicals

The HepG2 cells were supplied by ATCC and maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS and penicillin-streptomycin. The LMH cells were supplied by ATCC and maintained in Waymouth medium (Thermo Fisher Scientific) supplemented with 10% FBS and penicillin-streptomycin. The working solutions for collagen I, rat tail (Thermo Fisher Scientific) and gelatin (Thermo Fisher Scientific), were 50-μg/ml collagen diluted in 20-mM acetic acid and 0.1% gelatin diluted in sterile, distilled water, respectively. The HepG2 organoids were prepared by dispensing 1200 HepG2 cells per well of a 96-well low adhesion plate and allowing them to self-aggregate in complete Hepatocyte Culture Medium (Lonza) for 2–3 days. Multicellular human liver organoids were similarly constructed using a cell mixture of primary human hepatocytes, Kupffer cells, stellate cells, and endothelial cells at 80%, 10%, 5%, and 5%, respectively.[23,26] All cell lines were incubated in a 37°C and 5% $CO_2$ incubator. Mycoplasma was checked in all cell lines using the MycoAlert Mycoplasma Detection Kit (Lonza), and all were confirmed to be Mycoplasma-free.

ALA, DMSO, and most of the compounds assessed for porphyrogenicity were purchased from Sigma-Aldrich. The exception was AIA, which came from Novation Chemicals. All working solutions contained 0.1% DMSO and master stocks were diluted in either DMSO or water. The BioTek Synergy H1 Hybrid Multi-Mode plate reader and the software program, BioTek Gen5 (Software Version: 2.09.2), were used to measure fluorescence and luminescence readings.

### 2.2 | Fluorescence-based in vitro drug screening assay

LMH cells and HepG2 cells were seeded in a black, clear bottom 96-well plate coated with 0.1% gelatin for the LMH cells ($1.5 \times 10^4$ cells per well) and with collagen for the HepG2 cells ($1.5 \times 10^4$ cells per well). The HepG2 spheroids and human liver organoids (100) were seeded in clear bottom 96-well low adhesion plates (Corning Inc.) and cultured overnight at 37°C. Various compounds ranging in concentrations from 0 to 1 mM in half-log increments in the presence and absence of 250-μM DFO were added to the HepG2 and LMH cells and were incubated for 18–24 h. Specific concentrations were selected for the organoids. In addition, each trial had three replicates of 0.314-mM

AIA, as a positive control, as already described. Plates were then covered to prevent exposure to light and were read at an excitation wavelength of 410 nm and an emission wavelength of 625 nm. Background noise was corrected by deducting each fluorescence measurement by the fluorescence produced by DMSO in the absence of DFO. Data were analyzed with the aid of the software, GraphPad Prism 8.

### 2.3 | Cytotoxicity assay

LMH and HepG2 cells were seeded in white, solid bottom 96-well plates ($1.5 \times 10^4$ cells per well) coated with 0.1% gelatin for LMH cells and collagen for HepG2 cells and were prepped concurrently with the fluorescence-based in vitro drug screening assay for each compound. Cells were harvested 18–24 h after the addition of the compounds to measure luminescence, and samples were processed using the ATPLite cytotoxicity assay (PerkinElmer). $CC_{50}$ values were calculated with the aid of the software, GraphPad Prism 8.

### 2.4 | Preparation of samples for HPLC of porphyrins

LMH cells were seeded in 0.1% gelatin-coated, 60-mm dishes ($4 \times 10^6$ cells per plate) and were incubated overnight at 37°C. Afterward, 1-mM ALA and AIA in the presence and absence of 250-μM DFO were added to the cells and were incubated for 18–24 h. Cells were then scraped, transferred into 5 ml of media in a 15-ml Falcon tube, placed on ice, and kept in the dark. Samples were sonicated using the Branson Sonifier 250 with a microtip for 5 s; 750 μl of the cell sonicate was transferred to a 2-ml Eppendorf tube; 750 μl of acetone/conc. HCl (97.5%/2.5%, v/v) was added to each sonicate; samples were vortexed for 30 s and centrifuged at 1000$g$ for 10 m. Then, 900 μl of the supernatant was transferred to a new 2-ml Eppendorf tube and the pH was adjusted to between 3 and 5 using 4-M NaOH and pH paper.[29]

### 2.5 | HPLC assessment of porphyrins extracted from cultured cells

The conditions for HPLC of porphyrins were essential as described in Ref. [29].

### 2.6 | Data handling and statistical analysis

Results are presented as mean values ± SEM ($n = 3$). Data were analyzed with the GraphPad Prism 8.0 software. Statistical analyses were calculated by comparing the fluorescence ratios between the negative and positive controls in the presence of DFO to the ratios between the negative control and the drug concentration that produced the greatest fluorescence reading in the presence of DFO. If

the latter ratio is greater than the former, then porphyrogenicity is indicated at that concentration for the given compound. A two-sided Student's *t*-test was used to assess the statistical significance of the two ratios.

## 3 | RESULTS

### 3.1 | Determination of the lower limit of fluorescence quantification from protoporphyrin IX

Solutions of PP ranging from 0 μM [0 μg] to 325 μM [18.3 μg/ml] were prepared in DMSO and distilled water in black, clear bottom 96-well plates to detect the lower limit of quantification with the BioTek Synergy H1 Hybrid Multi-Mode plate reader. Fluorescence readings below 400 RFU were not high enough to be differentiated from the background. Thus, fluorescence readings above 500 RFU are more likely to produce more accurate and precise results (Figure S1). From this standard curve, an estimated amount of PP can be extrapolated from fluorescence readings produced by different drugs via linear regression models. In these early studies of the BioTek Synergy H1 Hybrid Multi-Mode plate reader, we observed that the standard curve was not strictly linear, so two linear regression equations were established, depending on the fluorescence measurement range. When fluorescence readings are between 680 and 4700 RFU, PP mass can be extrapolated using $y = 701 \times -2701.4$, where $x = $ μg/ml and $y = $ RFU. When readings are between 4700 and 16 420 RFU, PP mass can be extrapolated using $y = 2779.4x - 23\ 034$.

### 3.2 | Fluorescence detection in each cell model after 5-aminolevulinic acid [ALA] administration

In LMH cells, exposure of the cultures for 18–24 h to ALA concentrations above 0.314 mM led to fluorescence readings greater than those produced by exposure to AIA [positive control]. For example, fluorescence readings above 10 000 RFU were observed in cultures exposed to 1-mM ALA and DFO after 18–24 h (Figure 1A). In HepG2 cells, ALA concentrations above 0.0314 mM led to fluorescence readings as high as those produced by the AIA control; fluorescence readings above 5000 RFU were observed in HepG2 cells incubated with 1-mM ALA in the presence of DFO after 18–24 h incubation (Figure 1B). Both LMH and HepG2 cells displayed an increase in fluorescence intensity in a dose-dependent manner. In approximately 200 HepG2 spheroids, 0.5-mM ALA exceeded the reading produced by the AIA control with values above 6000 RFU in the presence of DFO after 18–24 h. These results indicate that the 2D hepatic cell lines and the spheroids have retained the enzymes and cofactors necessary for the stepwise conversion of ALA to PP.

In contrast, phenytoin and phenobarbital failed to produce fluorescence readings above background in the HepG2 organoids (Figure 1C), implying that these spheroids have lost their inducibility of ALAS-1. Similarly, approximately 100 human liver organoids produced a fluorescence reading of above 1600 RFU in the presence of 0.5-mM ALA and DFO after 18–24 h; however, all other compounds did not produce a fluorescence reading above background (Figure S2). Thus, among the four cell models tested, the LMH cells treated with AIA produced the highest fluorescence readings above the background. Thus, LMH cells were selected to test the other compounds studied.
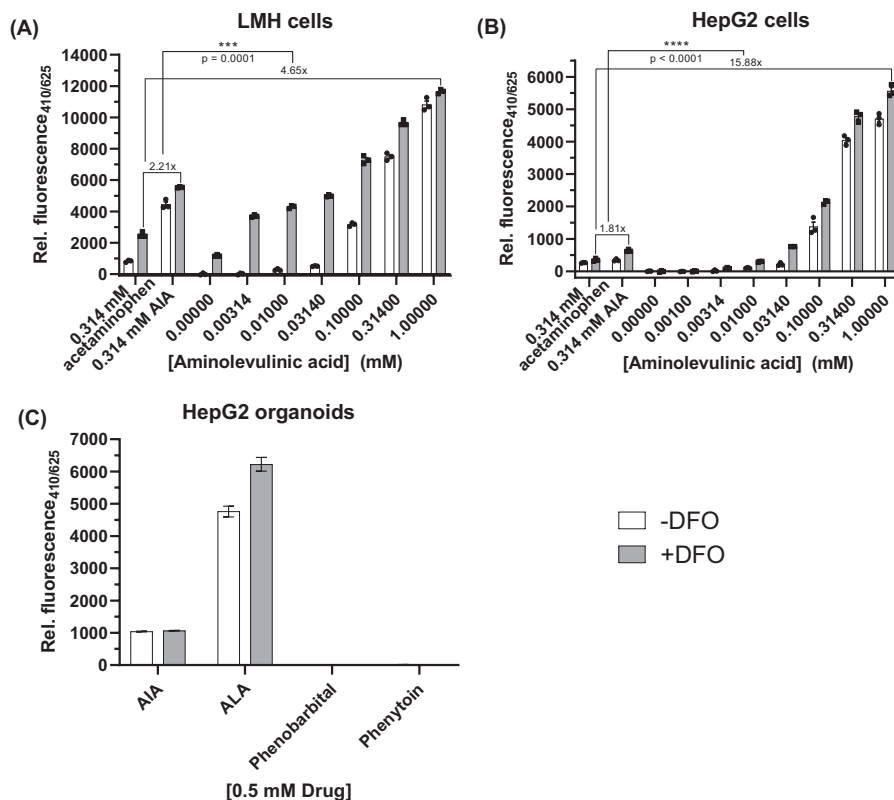


**FIGURE 1** Fluorescence produced in various cell models after 18–24 h AIA or ALA treatment. Porphyrin accumulation produced by increasing concentrations of ALA, ranging from 0 to 1 mM or with 0.314 mM AIA, all in the presence and absence of 250-μM DFO is displayed in (A) LMH cells and (B) HepG2 cells. (C) Fluorescence readings produced in the presence of 0.5-mM AIA, ALA, phenobarbital, and phenytoin with and without DFO are shown in HepG2 spheroids. All fluorescence readings were corrected by deducting background readings. Data are presented as mean values ± SEM of three independent replicates. A two-sided Student's *t*-test was used to assess the statistical significance between the two ratios

## 3.3 | Porphyrogenicity and cytotoxicity of selected compounds

In LMH cells, the positive controls, AIA, ALA, phenobarbital, and phenytoin, produced high fluorescence readings as expected. In the AIA dose-response assay, 0.314 mM produced the highest fluorescence readings, so this concentration was selected as the positive control (Figure 2). Among the selected compounds, only norgestrel, phenobarbital, phenytoin, and sodium valproate produced fluorescence readings above the 0.314-mM AIA control in the presence of DFO. The fluorescence readings produced by 0.314-mM norgestrel, 1-mM phenobarbital, 0.314-mM phenytoin, and 1-mM sodium valproate were $6890 \pm 106$ RFU, $7513 \pm 48$, $5913 \pm 190$, and $4301 \pm 38$ RFU, respectively (Figure 2). None of the other selected compounds produced fluorescence readings above the 0.314-mM AIA control at any concentration in the presence of DFO.

Among the compounds used, β-estradiol, hydroxychloroquine, alpha-methyldopa, and sulfamethoxazole showed clear cytotoxic effects within the concentrations used in each assay with $CC_{50}$ values of 0.071, 0.381, 0.164, and 0.528 mM, respectively, in the presence of DFO (Figure 3). $CC_{50}$ values, which are the drug concentrations that cause 50% cell death, are similar to those selected compounds in the absence of DFO. Although the $CC_{50}$ values of these compounds may not be the same as cytotoxic concentrations in humans, the cytotoxicity data in this in vitro screening assay provide an explanation for the variations in fluorescence and for the decrease in fluorescence, we observed at higher concentrations for certain compounds.

The fluorescence-based in vitro drug screening assay, along with the ATPLite cytotoxicity assay, was also performed with HepG2 cells. Due to the lower amounts of PP accumulation in HepG2 cells (Figure 1B), fewer compounds were tested. AIA and phenobarbital served as positive controls since they are known inducers of ALAS-1 in humans, other mammals, and diverse species. Concentrations above 0.0314 and 0.314 mM of ALA and phenobarbital, respectively, produced fluorescence readings above the 0.314-mM AIA control, while no concentrations of acetaminophen and acetylsalicylic acid produced readings above the AIA positive control (Figure S3). None of these compounds produced significant cytotoxic effects in HepG2 cells at the concentrations tested (Figure S4). Regardless of the expected results, all fluorescence readings, except for the ones produced by ALA, were near the lower detection limit of the fluorospectrometer used, limiting the usefulness of the assay in this cell line.

## 3.4 | HPLC separation and quantification of porphyrins from LMH cells

Porphyrin mixtures were extracted from cultured LMH cells treated with AIA and ALA in the presence and absence of DFO with an acetone/conc. HCl (97.5%/2.5%, v/v) solution. The pH of the supernatant was adjusted to between 3 and 5 with 5-M NaOH and was injected into the HPLC column (Figure S5). The main peaks, from left to right, are 8—[uro], 7-, 6-, 5-, 4- [copro], 3-, and 2- carboxyl-[proto] porphyrins. HPLC confirmed that the most abundant porphyrin in all AIA and ALA treatments was PP, which confirms the use of detecting fluorescence measurements from PP, instead of the other fluorescent porphyrin intermediates.

## 4 | DISCUSSION

In this work, we found that LMH cells have the most robust inducible ALAS-1 from known porphyrogenic drugs and chemicals, in comparison to HepG2 cells, HepG2 organoids, and human liver organoids derived from non-porphyric, healthy human donors. ALA is the intermediate produced by the rate-determining enzyme, ALAS-1, so bypassing this step by adding ALA to the cultures facilitated the assessment of steps in the pathway of PP synthesis distal to ALAS-1. In order to block the conversion of PP to heme, we added DFO to some cultures. Although 1-mM ALA in the presence of DFO showed mild cytotoxic effects, this condition produced approximately 2.1-fold, 1.9-fold, and 7-fold higher readings in LMH cells than in HepG2 cells, HepG2 organoids, and human liver organoids, respectively. Thus, the steps distal to ALAS-1 are also better maintained in LMH cells than in the other model systems that we tested, including human liver cell organoids. Due to a more robust heme synthesis pathway, LMH cells were selected as the primary cell model for testing other drugs and chemicals for porphyrogenicity.

Recently, liver organoids have been shown to have utility in liver toxicology studies due to the organoids' promising properties to retain normal hepatic characteristics, mimicking in vivo environments.[24,25,30] There are still many applications that have not heretofore been reported, such as screening for porphyrogenicity and diseases involving fibrosis and hepatitis, due to multiple limitations, including genomic instability, expression of immature or fetal markers, and limited cell maturation.[31,32] Our results show that HepG2 organoids and human liver organoids do retain the ability to convert ALA to PP, however, under the conditions of our studies, neither organoid system showed any inducibility of ALAS-1, which is the key biochemical hallmark that underlies porphyrogenicity and drug risk in the AHPs. Perhaps, more complicated systems, such as perfused "liver on a chip" may prove to retain such inducibility.[33] Fluorescence readings from the organoid studies might, perhaps, be increased if the cells were treated for a longer period of time, but this would reduce the practicability of the system for use in a rapid, screening assay. The main goal of this assay is to develop a quick and affordable method to detect porphyrogenicity, thus there is no much-added benefit from increasing the length of incubation just to obtain higher fluorescence readings, especially when compared with the results from the LMH cells.

In the heme biosynthesis pathway, uroporphyrin, coproporphyrin, and PP are maximally fluorescent at somewhat different wavelengths in the red part of the visible light spectrum, but in most cell types exposed to ALA or with induction of ALAS-1, PP is the principal
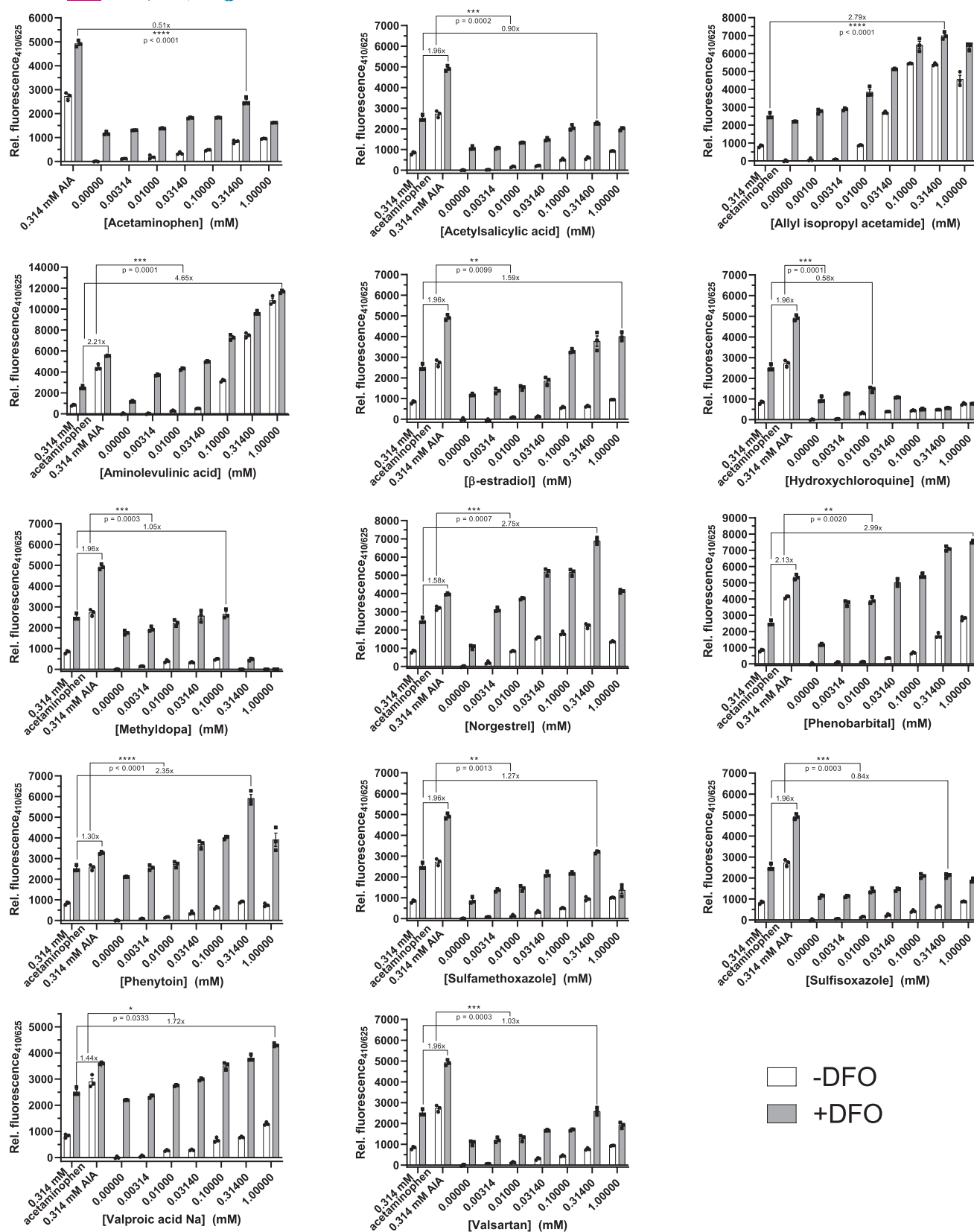
**FIGURE 2** Porphyrogenicity of selected compounds in LMH cells after 18–24 h treatment. Fluorescence readings produced by the positive and negative controls, 0.314-mM AIA and 0.314-mM acetaminophen, respectively, represent clear levels of porphyrogenicity and non-porphyrogenicity, respectively, in LMH cells. Among all the compounds, higher concentrations of ALA, norgestrel, phenobarbital, phenytoin, and sodium valproate produced fluorescence readings greater than the readings produced by AIA. Data are presented as mean values ± SEM of three independent replicates. All results are representative of three independent experiments. A two-sided Student's *t*-test was used to assess the statistical significance between the two ratios
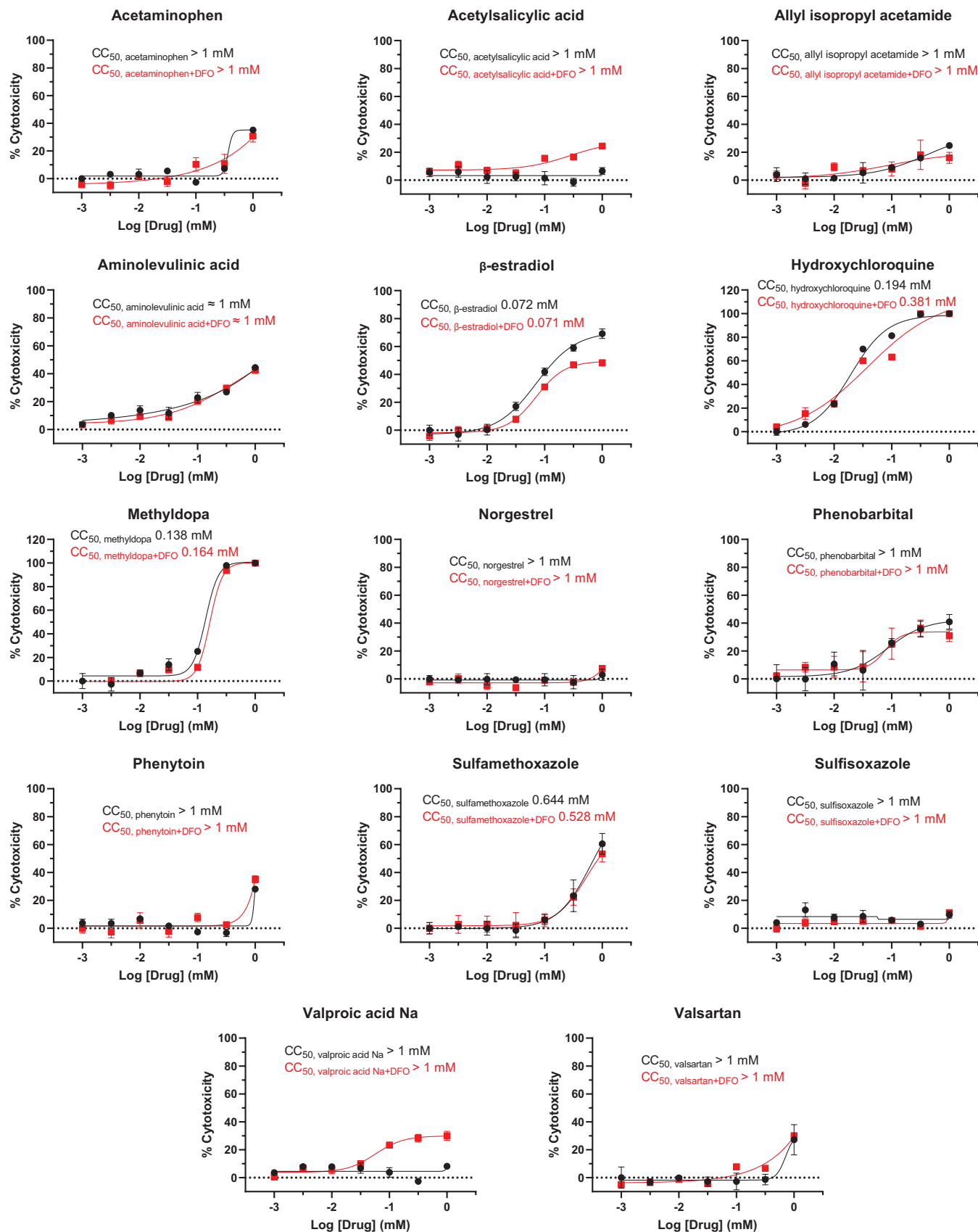
**FIGURE 3** Cytotoxicity of compounds in LMH cells after 18–24 h treatment. A parallel plate with the same treatment as the fluorescence-based in vitro drug screening assay was assayed for cytotoxicity with the ATPLite cytotoxicity assay. $CC_{50}$ values were calculated using the software, GraphPad Prism 8. Data are presented as mean values ± SEM of three independent replicates. All results are representative of three independent experiments

porphyrin that accumulates. This is especially true when DFO is present, which prevents the conversion of PP to heme by chelating iron, which is required for the activity of ferrochelatase.[27,28,34,35] This was confirmed after quantification of these intermediates via HPLC after ALA and AIA treatment in LMH cells (Figure S5). PP was in greater abundance than the other porphyrins [8- to 3-carboxyl-porphyrins].

The complexities of biological in vivo models make it challenging to interpret results from in vitro assays, such as this in vitro porphyrogenic drug screening assay, and to translate them for clinical use. Currently, in the clinical drug development process, pharmacokinetic and pharmacodynamic studies are required for the three phases of drug approval.[36] Since thorough and rigorous studies are needed to be completed for novel drugs before they can enter the market, these required in vivo studies which can help determine maximal drug concentrations in the plasma and various organs, including the liver and brain, and other helpful information that can provide more value to the results of the in vitro drug screening assay. Maximal drug concentrations can be extrapolated from pharmacodynamic/pharmacokinetic studies, which can then be used in assays as herein described to determine if those concentrations lead to high fluorescence values, taken as evidence of porphyrogenicity and potential danger to patients with AHP.

Aspirin and acetaminophen have been used for decades in patients with the AHPs because these compounds have been found to be safe for use and not lead to the accumulation of significant PP,[37,38] http://www.drugs-porphyria.orgpositive). In a study by Nagelschmitz et al., an intravenous and oral 500-mg aspirin dose led to maximal plasma concentrations of 54.25 mg/L [0.30 mM] and 4.84 mg/L [0.027 mM], respectively.[39] From a study by Hong et al., an oral 1000-mg acetaminophen tablet led to a maximum plasma concentration of approximately 8.79 mg/L [0.058 mM].[40] Our data support that even at these maximal plasma concentrations after typical aspirin and acetaminophen doses, these compounds are not porphyrogenic, supporting the validity of the assay. In contrast, phenobarbital is known to be unsafe in patients with the hepatic porphyrias because it upregulates cytochromes P-450 and ALAS-1 activity by decreasing the heme pool and ultimately preventing the negative feedback mechanism in the heme biosynthesis pathway.[41,42] In a study by Nelson et al., a 2.9-mg/kg oral phenobarbital dose led to a maximal serum concentration of approximately 5.5 mg/L [0.024 mM].[43] Our data confirm that concentrations of phenobarbital around 0.024 mM produced fluorescence readings close to the AIA positive control, indicating porphyrogenicity (Figure 2). The positive and negative controls of this experiment support the validity of our LMH model and assay. Thus, it is reasonable to surmise that higher concentrations of norgestrel, phenytoin, and sodium valproate are also porphyrogenic and risky for patients with AHP, as has been observed in clinical practice.[2,3] According to Suthisisang et al., an oral, 300-mg phenytoin sodium dose led to a maximal serum concentration of approximately 1.98 μg/ml [7.22 μM] in humans,[44] which, according to our data, indicates worrisome porphyrogenic potential of phenytoin (Figure 2. Studies of norgestrel and valproic acid reported maximal serum concentrations of approximately 14.1 ng/ml [0.451 mM] and approximately 1271 mg/L [8.81 mM], respectively, in humans[45,46] administered typically used oral doses, which also suggests porphyrogenicity and risk of these drugs (Figure 2).

β-estradiol and norgestrel were used to represent the estrogen and progestogen classes, respectively, and we found that, at higher concentrations, both led to a notable accumulation of PP, especially in the presence of DFO (Figure 2). Norgestrel, however, produced greater fluorescence readings than β-estradiol, which is in keeping with clinical impressions and observations in human subjects.[2,47–52] We also tested alpha-methyldopa because it continues often to be used to control systemic arterial hypertension in pregnant women. Although the drug had a low $CC_{50}$ of 0.164 mM in the presence of DFO, it did not lead to an appreciable accumulation of PP below concentrations that led to appreciable cytotoxicity. We also found that valsartan, another widely used antihypertensive, did not show evidence of porphyrogenicity. This is reassuring for clinicians treating patients with AHP, in whom systemic arterial hypertension is common, especially during acute attacks, and often continuing between attacks. These results extend results from earlier studies in our laboratory, which also showed the safety of losartan, captopril, and lisinopril.[35]

We also tested hydroxychloroquine because, while we were doing this study, this compound gained significant popularity, for a time, due to its claimed benefits for treating COVID-19 infections. Our results indicate that hydroxychloroquine does not upregulate hepatic ALAS-1 and is not risky for patients with AHPs; however, it did show cytotoxicity with a $CC_{50}$ of 0.381 mM in the presence of DFO. We also note that, in recent studies, hydroxychloroquine had no added benefit in treating COVID-19.[53–55]

Sulfonamide-containing antibiotics, such as sulfamethoxazole and sulfisoxazole, have been considered to be risky for use in patients with AHP according to the American Porphyria Foundation Drug Database [https://porphyriafoundation.org/drugdatabase/]; however, in our LMH cell system, neither of these drugs led to excess PP accumulation, even in the presence of DFO (Figure 2). Indeed, our recent [November 3, 2021] PubMed searches on "sulfisoxazole AND acute porphyria" yielded only one citation,[56] which concluded that sulfisoxazole did not affect the activity of hydroxymethylbilane synthase in human erythrocytes or rat livers. A search on" sulfamethoxazole AND acute porphyria" did not yield any citations. The Norwegian Porphyria Centre [https://www.drugs-porphyria.org/] drug database does not list either sulfisoxazole or sulfamethoxazole individually as porphyrogenic or risky for patients with AHP, although it does list the combination of trimethoprim and sulfamethoxazole [Bactrim] as porphyrogenic, suggesting that the agent of concern is more likely trimethoprim. Thus, based on our results and our review of published literature, we are unconvinced that either sulfisoxazole or sulfamethoxazole without trimethoprim is truly risky for use in patients with AHPs.

In summary, among several in vitro model systems tested, the LMH cell model was the best system to predict the porphyrogenicity of various drugs in our fluorescence-based in vitro drug screening

assay. The positive controls, AIA, ALA, phenobarbital, and phenytoin, showed significant accumulation of protoporphyrin, as expected, and the negative controls, acetaminophen and acetylsalicylic acid, showed significantly less effect. Norgestrel, phenobarbital, phenytoin, and sodium valproate all produced fluorescence readings higher than the AIA control at concentrations above 0.314 mM, which indicates that these compounds may likely induce or exacerbate acute porphyric attacks. Pharmacodynamic and pharmacokinetic studies and years of clinical observations support this conclusion; these drugs should best be avoided by patients with AHP. Since such studies are required in the drug approval process and as more novel medications enter the market with the unknown potential to exacerbate the hepatic porphyrias, it would be ideal for the quick and inexpensive assay herein described to be included in the batteries of tests now routinely performed for new candidate drugs. Patients with AHP and their health-care providers would benefit from such knowledge.

## ACKNOWLEDGMENTS

## DISCLOSURE

The authors have no competing conflicts of interest to report.

## AUTHOR CONTRIBUTIONS

H.L.B., C.D.M., C.G.V.H., and C.B. conceptualized and designed the study. C.D.M., C.G.V.H., and M.W. performed, analyzed, and contributed to all the experiments. C.D.M. and H.L.B. wrote the manuscript. All other authors reviewed and contributed to the manuscript. All authors have read and approved the submission of the final manuscript.

## ETHICAL STATEMENT

This study is exempt from ethics approval.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## REFERENCES

1. Bergonia HA, Franklin MR, Kushner JP, Phillips JD. A method for determining δ-aminolevulinic acid synthase activity in homogenized cells and tissues. *Clin Biochem*. 2015;48(12):788-795.
2. Bonkovsky HL, Guo J-T, Hou W, Li T, Narang T, Thapar M. Porphyrin and heme metabolism and the porphyrias. *Compr Physiol*. 2013;3(1):365-401.
3. Bissell DM, Anderson KE, Bonkovsky HL. Porphyria. *N Engl J Med*. 2017;377:862-872.
4. Gerjevic LN, Lu S, Chaky JP, Harrison-Findik DD. Regulation of heme oxygenase expression by alcohol, hypoxia and oxidative stress. *World J Biol Chem*. 2011;2(12):252-260.
5. Held H. Effect of alcohol on the heme and porphyrin synthesis interaction with phenobarbital and pyrazole. *Digestion*. 1977;15(2):136-146.
6. Peters HA. Carbamazepine in seizure management in acute intermittent porphyria. *Neurology*. 1981;31(12):1579-1580.
7. Cable EE, Gildemeister OS, Pepe JA, Donohue SE, Lambrecht RW, Bonkovsky HL. Hepatic 5-aminolevulinic acid synthase mRNA stability is modulated by inhibitors of heme biosynthesis and by metalloporphyrins. *Eur J Biochem*. 1996;240(1):112-117.
8. Correia MA, Sinclair PR, De Matteis F. Cytochrome P450 regulation: the interplay between its heme and apoprotein moieties in synthesis, assembly, repair, and disposal. *Drug Metab Rev*. 2011;43(1):1-26.
9. Hamilton JW, Bement WJ, Sinclair PR, Sinclair JF, Alcedo JA, Wetterhahn KE. Heme regulates hepatic 5-aminolevulinate synthase mRNA expression by decreasing mRNA half-life and not by altering its rate of transcription. *Arch Biochem Biophys*. 1991;289(2):387-392.
10. Kolluri S, Sadlon TJ, May BK, Bonkovsky HL. Haem repression of the housekeeping 5-aminolaevulinic acid synthase gene in the hepatoma cell line LMH. *Biochem J*. 2005;392(Pt 1):173-180.
11. Lathrop JT, Timko MP. Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. *Science*. 1993;259(5094):522-525.
12. Saitoh S, Okano S, Nohara H, et al. 5-aminolevulinic acid (ALA) deficiency causes impaired glucose tolerance and insulin resistance coincident with an attenuation of mitochondrial function in aged mice. *PLoS One*. 2018;13(1):e0189593.
13. Tian Q, Li T, Hou W, Zheng J, Schrum LW, Bonkovsky HL. Lon peptidase 1 (LONP1)-dependent breakdown of mitochondrial 5-aminolevulinic acid synthase protein by heme in human liver cells. *J Biol Chem*. 2011;286(30):26424-26430.
14. Balwani M, Sardh E, Ventura P, et al. Phase 3 Trial of RNAi therapeutic givosiran for acute intermittent porphyria. *N Engl J Med*. 2020;382:2289-2301.
15. Mohan G, Madan A. *Ala Dehydratase Deficiency Porphyria*. StatPearls. 2021.
16. Feng M, Kong R, Pan Y. The breakthrough in primary human hepatocytes in vitro expansion. *Cancer Biol Med*. 2019;16(1):1-3.
17. Donato MT, Tolosa L, Gómez-Lechón MJ. Culture and functional characterization of human hepatoma HepG2 cells. *Methods Mol Biol*. 2015;1250:77-93.
18. Fraser DJ, Podvinec M, Kaufmann MR, Meyer UA. Drugs mediate the transcriptional activation of the 5-aminolevulinic acid synthase (ALAS1) gene via the chicken xenobiotic-sensing nuclear receptor (CXR). *J Biol Chem*. 2002;277(38):34717-34726.
19. Iwasa F, Sassa S, Kappas A. Delta-Aminolaevulinate synthase in human HepG2 hepatoma cells. Repression by haemin and induction by chemicals. *Biochem J*. 1989;262(3):807-813.
20. Kawaguchi T, Nomura K, Hirayama Y, Kitagawa T. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. *Cancer Res*. 1987;47(16):4460-4464.
21. Kolluri S, Elbirt KK, Bonkovsky HL. Heme biosynthesis in a chicken hepatoma cell line (LMH): comparison with primary chick embryo liver cells (CELC). *Biochim Biophys Acta*. 1999;1472(3):658-667.
22. Russo SM, Pepe JA, Cable EE, Lambrecht RW, Bonkovsky HL. Repression of ALA synthase by heme and zinc-mesoporphyrin in a chick embryo liver cell culture model of acute porphyria. *Eur J Clin Invest*. 1994;24(6):406-415.
23. Basharat A, Rollison HE, Williams DP, Ivanov DP. HepG2 (C3A) spheroids show higher sensitivity compared to HepaRG

spheroids for drug-induced liver injury (DILI). *Toxicol Appl Pharmacol.* 2020;408:115279.

24. Ray K. Developing a toolbox for drug-induced liver injury. *Nat Rev Gastroenterol Hepatol.* 2020;17:714.

25. Shinozawa T, Kimura M, Cai Y, et al. High-fidelity drug-induced liver injury screen using human pluripotent stem cell-derived organoids. *Gastroenterology.* 2021;160(3):831-846.e810.

26. Bell CC, Hendriks DFG, Moro SML, et al. Characterization of primary human hepatocyte spheroids as a model system for drug-induced liver injury, liver function and disease. *Sci Rep.* 2016;6:25187.

27. Hahn M, Gildemeister OS, Krauss GL, et al. Effects of new anticonvulsant medications on porphyrin synthesis in cultured liver cells: potential implications for patients with acute porphyria. *Neurology.* 1997;49(1):97-106.

28. Palasuberniam P, Kraus D, Mansi M, et al. Ferrochelatase deficiency abrogated the enhancement of aminolevulinic acid-mediated protoporphyrin IX by iron chelator deferoxamine. *Photochem Photobiol.* 2019;95(4):1052-1059.

29. Bonkovsky HL, Wood SG, Howell SK, et al. High-performance liquid chromatographic separation and quantitation of tetrapyrroles from biological materials. *Anal Biochem.* 1986;155(1):56-64.

30. Lee J-Y, Han H-J, Lee S-J, et al. Use of 3D human liver organoids to predict drug-induced phospholipidosis. *Int J Mol Sci.* 2020;21(8):2982.

31. Nuciforo S, Heim MH. Organoids to model liver disease. *JHEP Rep.* 2021;3(1):100198.

32. Prior N, Inacio P, Huch M. Liver organoids: from basic research to therapeutic applications. *Gut.* 2019;68(12):2228-2237.

33. Jang K-J, Otieno Monicah A, Ronxhi J, et al. Reproducing human and cross-species drug toxicities using a liver-chip. *Sci Transl Med.* 2019;11(517):eaax5516.

34. Lambrecht RW, Gildemeister OS, Pepe JA, Tortorelli KD, Williams A, Bonkovsky HL. Effects of antidepressants and benzodiazepine-type anxiolytic agents on hepatic porphyrin accumulation in primary cultures of chick embryo liver cells. *J Pharmacol Exp Ther.* 1999;291(3):1150-1155.

35. Lambrecht RW, Gildemeister OS, Williams A, Pepe JA, Tortorelli KD, Bonkovsky HL. Effects of selected antihypertensives and analgesics on hepatic porphyrin accumulation: implications for clinical porphyria. *Biochem Pharmacol.* 1999;58(5):887-896.

36. Aronson JK, La Caze A, Kelly MP, Parkkinen V-P, Williamson J. The use of mechanistic evidence in drug approval. *J Eval Clin Pract.* 2018;24(5):1166-1176.

37. Gorchein A. Drug treatment in acute porphyria. *Br J Clin Pharmacol.* 1997;44(5):427-434.

38. James MFM, Hift RJ. Porphyrias. *Br J Anaesth.* 2000;85(1):143-153.

39. Nagelschmitz J, Blunck M, Kraetzschmar J, Ludwig M, Wensing G, Hohlfeld T. Pharmacokinetics and pharmacodynamics of acetylsalicylic acid after intravenous and oral administration to healthy volunteers. *Clin Pharmacol.* 2014;6:51-59.

40. Hong Z, Li Z, Wang Y, et al. Pharmacokinetics and bioavailability study on acetaminophen oral drop in healthy volunteers. *Hua Xi Yi Ke Da Xue Xue Bao.* 1994;25(4):410-413.

41. Klinger W, Müller D. The influence of allyl isopropyl acetamide on d-aminolevulinic acid synthetase and cytochrome P-450. *Acta Biol Med Ger.* 1980;39(1):107-112.

42. Teunissen MWE, Graft MV, Vermeulen NPE, Breimer DD. Influence of allylisopropylacetamide and phenobarbital treatment on in vivo antipyrine metabolite formation in rats. *Xenobiotica.* 1983;13(8):497-502.

43. Nelson E, Powell JR, Conrad K, et al. Phenobarbital pharmacokinetics and bioavailability in adults. *J Clin Pharmacol.* 1982;22(2-3):141-148.

44. Suthisisang CPN, Chulavatnatol S, Towanabut S. Bioavailability of phenytoin sodium capsules available in Thailand. Part II: in vivo study. *J Med Assoc Thai.* 1998;81(1):64-70.

45. Georgoff PE, Nikolian VC, Bonham T, et al. Safety and tolerability of intravenous valproic acid in healthy subjects: a phase I dose-escalation trial. *Clin Pharmacokinet.* 2018;57(2):209-219.

46. Kook K, Gabelnick H, Duncan G. Pharmacokinetics of levonorgestrel 0.75 mg tablets. *Contraception.* 2002;66(1):73-76.

47. Andersson C, Innala E, Bäckström T. Acute intermittent porphyria in women: clinical expression, use and experience of exogenous sex hormones. A population-based study in northern Sweden. *J Intern Med.* 2013;254(2):176-183.

48. Cerovac A, Brigic A, Softic D, Barakovic A, Adzajlic S. Uncontrolled acute intermittent porphyria as a cause of spontaneous abortion. *Med Arch.* 2020;74(2):153-155.

49. Júnior VRS, Lemos VMV, Feitosa IML, et al. Effect of menstrual cycle on acute intermittent porphyria. *Child Neurol Open.* 2017;4:2329048X1773617.

50. Legault N, Sabik H, Cooper SF, Charbonneau M. Effect of estradiol on the induction of porphyria by hexachlorobenzene in the rat. *Biochem Pharmacol.* 1997;54(1):19-25.

51. Sixel-Dietrich F, Doss M. Hereditary uroporphyrinogendecarboxylase deficiency predisposing porphyria cutanea tarda (chronic hepatic porphyria) in females after oral contraceptive medication. *Arch Dermatol Res.* 1985;278(1):13-16.

52. Stein PE, Badminton MN, Rees DC. Update review of the acute porphyrias. *Br J Haematol.* 2017;176(4):527-538.

53. Boulware DR, Pullen MF, Bangdiwala AS, et al. A randomized trial of hydroxychloroquine as postexposure prophylaxis for Covid-19. *N Engl J Med.* 2020;383:517-525.

54. Fiolet T, Guihur A, Rebeaud ME, Mulot M, Peiffer-Smadja N, Mahamat-Saleh Y. Effect of hydroxychloroquine with or without azithromycin on the mortality of coronavirus disease 2019 (COVID-19) patients: a systematic review and meta-analysis. *Clin Microbiol Infect.* 2021;27(1):19-27.

55. Mitjà O, Corbacho-Monné M, Ubals M; Group BC-P-CR. A cluster-randomized trial of hydroxychloroquine for prevention of Covid-19. *N Engl J Med.* 2021;384(5):417-427.

56. Tishler PV. The effect of therapeutic drugs and other pharmacologic agents on activity of porphobilinogen deaminase, the enzyme that is deficient in intermittent acute porphyria. *Life Sci.* 1999;65(2):207-214.

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