NOVEL INSIGHTS INTO 3,5-DIETHOXYCARBONYL-1,4-DIHYDROCOLLIDINE (DDC)-INDUCED PROTOPORPHYRIN IX ACCUMULATION AND LIVER INJURY

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3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) is a porphyrinogenic agent that causes protoporphyrin IX (PPIX) accumulation in the liver and consequently liver damage. DDC-mediated PPIX accumulation is thought to be liver specific by inducing hepatic 5′-aminolevulinate synthase 1, the rating limiting enzyme in the heme biosynthesis pathway, resulting in the increase of PPIX production. In addition, it has been proposed that the hepatic cytochromes P450 (CYPs)-mediated metabolism of DDC is involved in the formation of N-methyl protoporphyrin IX (N-Me PPIX), a potent inhibitor of ferrochelatase that converts PPIX into heme, leading to further PPIX accumulation in the liver. However, the role of hepatic CYPs in DDC-mediated N-Me PPIX production remains unclear. The current work aimed to fill this knowledge gap by using the liver-specific NADPH-cytochrome P450 reductase-null (LCN) mouse model, in which hepatic CYPs are functionally deficient. We found that LCN mice did not attenuate DDC-mediated N-Me PPIX production, suggesting that N-Me PPIX formation is independent on hepatic CYPs. We next used a metabolomic approach to explore the extrahepatic effects of DDC. High levels of PPIX and N-Me PPIX were found in the sera of mice treated with DDC. Our further analyses revealed that the bone marrow is a target of DDC that contributes to the production of PPIX and N-Me PPIX, which can be delivered to the liver and result in liver injury. In summary, DDC-mediated N-Me PPIX formation and PPIX accumulation is not liver specific, and instead the bone marrow seems the major source of N-Me.
PPIX and PPIX after DDC exposure. Moreover, our data suggest that DDC-mediated formation of N-Me PPIX is independent on hepatic CYPs.
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Preface

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1.0 INTRODUCTION

The hepatotoxin 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (Fig. 1) is a porphyrinogenic agent that can cause protoporphyrin IX (PPIX) accumulation in the liver [1-5]. PPIX is an intermediate in the heme biosynthesis pathway and the excessive PPIX is predominantly eliminated from the body through the hepatobiliary system [6]. Because PPIX is highly hydrophobic, it precipitates at high concentrations, leading to bile ducts blockage and liver damage [7-9]. The phenotypes of DDC-mediated liver injury are similar to those observed in patients with erythropoietic protoporphryia (EPP) who also show a phenotype of PPIX accumulation and cholestatic liver injury; thus, DDC is commonly used as a tool agent for studying EPP [10-12]. In addition, DDC has been used as a tool drug to study Mallory-Denk bodies, an hepatic inclusion body commonly found in the cytoplasm of hepatocytes during liver injury like chronic cholestasis. Mallory-Denk bodies can be observed in mice treated with DDC diet at 40 days which is more effective than the first-discovered Mallory-Denk body inducer griseofulvin which takes 60-day of feeding to observe Mallory-Denk bodies[13, 14]. In addition, DDC is also used as model agent to study pericholangitis, periductal fibrosis, ductular reaction, and liver proliferation and repair [10, 15-18].

![Figure 1 Chemical structure of DDC](image)
Two mechanisms have been proposed for DDC-mediated PPIX accumulation in the liver. Firstly, DDC increases the activity of hepatic 5'-aminolevulinate synthase 1 (ALAS1), the first and rating limiting enzyme in the heme biosynthesis pathway, resulting in PPIX over-production [19, 20]. Secondly, DDC induces the formation of N-methyl protoporphyrin IX (N-Me PPIX), a potent inhibitor of ferrochelatase (FECH) that converts PPIX to heme, leading to further PPIX accumulation in the liver [3-5, 21]. Moreover, N-Me PPIX synergizes the effect of DDC on ALAS1 [22]. Together, DDC-mediated induction of ALAS1 and inhibition of FECH result in PPIX accumulation in the liver and liver damage.

It is generally believed that DDC-mediated N-Me PPIX formation relies on cytochromes P450 (CYPs) [3-5]. DDC has been proposed to be activated by hepatic CYPs, and then donate a methyl group to the heme moiety of CYPs to form N-Me PPIX [3, 4, 23-25]. However, the non-selective CYP inhibitor 2-diethylaminoethyl 3,3-diphenylpropylacetate failed to prevent FECH inhibition in mice pretreated with DDC [11], which raised the concern whether CYPs are critical in the DDC-mediated formation of N-Me PPIX. However, if CYPs are needed, it remains unclear which CYP isoenzyme contributes to DDC bioactivation and N-Me PPIX formation.

The current study aimed to determine the role of hepatic CYPs in DDC-mediated N-Me PPIX formation by using the liver-specific NADPH-cytochrome P450 reductase-null (LCN) mouse model. In addition, we incubated DDC with human liver microsomes (HLM) to screen the CYP isoenzymes responsible for N-Me PPIX formation. Our results suggest that DDC-mediated N-Me PPIX formation is independent on hepatic CYPs. Instead, our metabolomic
analyses revealed that the bone marrow is a target of DDC that contributes to the production of PPIX and N-Me PPIX, which can be delivered to the liver and lead to liver injury.
2.0 MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

N-Me PPIX was purchased from Frontier Scientific Inc (Logan, UT). DDC, PPIX, midazolam (MDZ), resorufin (RSF), omeprazole (OMZ) and dextromethorphan (DMP) were purchased from Sigma-Aldrich (St. Louis, MO). Efavirenz (EFR), coumarin (CMR), 7-OH-CMR, and warfarin (WFR) were purchased from TCI American (Portland, OR). Chlorzoxazone (CZX) was purchased from Spectrum Chemical (New Brunswick, NJ). Rosiglitazone (RSG), 7-ethoxy-RSF, 6-OH-CZX, 7-OH-WFR, 5-OH-OMZ, and dextrorphan (DTP) were purchased from Cayman (Ann Arbor, MI). 1-OH-MDZ, 5-OH-RSG, and 8-OH-EFV were purchased from Toronto Research Chemicals (Ontario, Canada). HLM was purchased from Sekisui XenoTech, LLC (Kansas City, KS). All solvents for metabolite analyses were of the highest grade commercially available.

2.2 ANIMALS AND TREATMENTS

Wild-type (WT) and LCN mice [26] were maintained at 22 °C with a 12-h on/12-h off light cycle and were allowed free access to water and diet. Blood, bone marrow, and liver samples were harvested from the mice fed with control (regular chow) or DDC diet (0.1% DDC in chow) for 2 weeks. Liver tissues and bone marrow cells were frozen in liquid nitrogen and
stored at -80 °C until further analysis. The procedures were in accordance with study protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.3 SAMPLE PREPARATION FOR METABOLITE ANALYSIS

The liver tissues were homogenized in 5 volumes of water (100 mg of liver in 500 µl of water). One hundred µl of each homogenate was mixed with 200 µl of acetonitrile:methanol (1:1, v/v). The mixture was vortexed and centrifuged (15,000 g for 10 min). The supernatant was transferred to a new Eppendorf vial for a second centrifugation (15,000 g for 10 min). The serum samples were prepared by adding 80 µl of methanol to 20 µl of serum. The obtained mixture was vortexed and centrifuged at 15,000 g for 10 min. The bone marrow cells were harvested and quantified according to previously described methods [27, 28]. Bone marrow cells (2 × 10⁷ cells) were mixed with 100 µl of methanol:water (4:1) and sonicated for 10 seconds. The mixture was vortexed and centrifuged at 15,000 g for 10 min. The resulting supernatant was transferred to an autosampler vial and analyzed by ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS) (Waters Corporation, Milford, MA).

2.4 METABOLITE ANALYSIS BY UPLC-QTOFMS

Metabolite separation was performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters, Milford, MA). Mobile phase A consisted of 0.1% of formic acid in water,
and mobile phase B consisted of 0.1% of formic acid in acetonitrile. For metabolite quantification, the linear gradient was as follows: 0-1 min, hold at 5% B; 1-12 min, 5% B to 95% B; 12-20 min, hold at 95% B; 20-24 min, re-equilibrated at 5% B. For the characterization of the isomers of N-Me PPIX, the linear gradient was as follows: 0-1 min, hold at 30% B; 1-40 min, 30% B to 50% B. The column temperature was maintained at 50 °C and the flow rate of the mobile phase was 0.5 ml/min. QTOFMS was operated in positive mode with electrospray ionization. MS data were acquired in centroid format, with scanning from 50-1,000 Da. The capillary and cone voltages were set as 0.8 kV and 40 V, respectively. The source temperature was set as 150 °C. The desolvation gas (800 l/hour) was set at 500 °C. Argon was applied as the collision gas. Tandem mass spectrometry fragmentation was conducted with collision energy ramp ranging from 20 to 60 eV. All data were acquired using Masslynx™ V4.1 software and quantified using Quanlynx™ V4.1 (Waters Corp., Milford, MA, USA).

2.5 METABOLOMICS ANALYSIS

Masslynx software (Waters Corporation, Milford, MA) was used to generate mass chromatograms, mass spectra, and centroid and integrated mass chromatographic data. The corresponding data matrices were then imported into SIMCA-P+12 (Umetrics, Kinnelon, NJ) software, transferred to Pareto-scaled data for principal component analysis and orthogonal projection to latent structures discriminant analysis (OPLS-DA).
2.6 ASSESSMENT OF LIVER INJURY

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) levels were analyzed according to the standard procedures provided by the kit manufacture (Point Scientific Inc, Canton, MI). Sections of liver samples were fixed in 10% neutral-buffered formalin overnight, and then dehydrated by passing through a series of ethanol and xylene solutions. The dehydrated liver tissues were then embedded with paraffin and were cut and stained with hematoxylin and eosin (H&E) for histological evaluation.

2.7 WESTERN BLOTTING

Liver homogenates were used for analysis of ALAS1 according to the previously established methods [29]. In brief, 10 µg of total protein from each sample were separated on 10% SDS-polyacrylamide gel. The primary antibodies included a polyclonal rabbit antibody against ALAS1 (ab84962, Abcam, Cambridge, UK). GAPDH was used as a loading control.

2.8 ROLE OF CYP ISOENZYMES IN DDC-MEDIATED N-ME PPIX FORMATION

Incubations were conducted in 1 × PBS (pH 7.4) containing 30 µM DDC, 100 µg of HLM, with or without NADPH (1 mM), in a final volume of 100 µL. Incubations were performed at 37 °C for 3 hrs. The incubations were terminated by adding equal volume of methanol: acetonitrile (1:1), followed by vortex for 1 min and centrifugation at 13,200 rpm for
10 min. One hundred μL of supernatant was collected for UPLC-QTOFMS measurement. To validate the catalytic function of each CYP isoenzyme, classic probes including 7-ER, CMR, EFV, RSG, WFR, OMZ, DMP, CZX, and MDZ were added to indicate CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 activity, respectively. N-Me PPIX and the corresponding metabolites of the probes were measured by UPLC-QTOFMS. All the reactions were conducted in triplicate.

2.9 STATISTICS

All quantified data are expressed as means ± SD. Statistical analysis was performed with two-tailed Student's t tests or two-way ANOVA followed by Tukey test using GraphPad Prism 7 (GraphPad Software, San Diego, CA). A P value less than 0.05 was considered to be statistically significant.
3.0 RESULTS

3.1 METHOD DEVELOPMENT FOR N-ME PPIX ANALYSIS

There are 4 isomers of N-Me PPIX based on the location of methyl group at the pyrrole rings [30]. Two isomers, namely N-Me PPIX 1 and 2, were observed in the liver of WT mice, with a retention time at 28.4 and 37.3 min, respectively, which matched with the retention time of two isomers from authentic standard (Fig. 2A and 2B). The structures of these two N-Me PPIX isomers were further confirmed by exact mass measurement, MS/MS fragmental analysis, as well as by comparing MS/MS with the authentic standard (Fig. 2C). N-Me PPIX 1 and 2 produced the same protonated ion at m/z 577.2827. In addition, these two isomers had the same fragmental ions at m/z 562, 503, 489, 444, and 429 (Fig. 2C). The MS/MS fragmental pattern of N-Me PPIX was highly similar to that of PPIX except one additional methyl group [31].
Figure 2. Identification of N-Me PPIX by UPLC-QTOFMS.

(A) The chromatogram of N-Me PPIX extracted from mouse liver.  (B) The chromatogram of the authentic standard of N-Me PPIX.  (C) MS/MS of N-Me PPIX.
3.2 LIVER INJURY, PPIX ACCUMULATION, AND N-ME PPIX PRODUCTION IN WT MICE TREATED WITH DDC

Consistent with previous reports [1, 15-17, 32], treatment with DDC caused cholestatic liver injury in WT mice (Fig. 3). The serum activities of ALT, AST and ALP in DDC-treated WT mice were significantly increased when compared to the control group (Fig. 3A-C). In addition, histological analysis revealed plugs in bile ducts of DDC-treated mice (Fig. 3D-E). Furthermore, PPIX levels were significantly increased in the liver of WT mice treated with DDC (Fig. 3F). We next explored the mechanisms responsible for DDC-mediated PPIX accumulation and found that ALAS1 expression was significantly upregulated (Fig. 3G), suggesting that DDC activates the heme biosynthesis pathway and increases PPIX production. We also found that the hepatic level of N-Me PPIX 2 was increased after DDC treatment, but not for N-Me PPIX 1 (Fig. 3H, 3I), indicating the involvement of N-Me PPIX 2 in DDC-mediated hepatic PPIX accumulation by suppressing the FECH-mediated conversion of PPIX to heme. However, DDC-mediated ALAS1 induction and N-Me PPIX 2 production (< 4-fold) in the liver seem not to be able to fully explain the magnificence of PPIX accumulation in the liver, whose concentration was increased thousand folds, suggesting that other mechanisms may also contribute to DDC-induced PPIX accumulation in the liver.
Figure 3. DDC-induced PPIX accumulation in the liver of WT mice.

The mice were treated with regular diet or DDC diet (0.1%) for 2 weeks. (A-C) Biochemical analysis of serum ALT (A), AST (B) and ALP (C). (D, E) Histological analysis of liver samples (H&E staining). PV, portal vein. The arrows point to bile plugs. Scale bar = 10 μm.
(F) Abundance of PPIX in the livers. (G) Effect of DDC on ALAS1 expression in the liver. ALAS1 was determined by Western blot. GAPDH was used as a loading control. (H, I) Effect of DDC on the production of N-Me PPIX in the liver. PPIX and N-Me PPIX were detected by UPLC-QTOFMS. All data are expressed as means ± S.D. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001.

3.3 N-ME PPIX IS NOT DETECTED IN THE INCUBATION WITH HLM AND DDC

It has been proposed that DDC is metabolized by hepatic CYPs to transfer a methyl group to the heme moiety of CYPs to form N-Me PPIX [3-5], but it remains unclear which CYP isoenzyme contributes to N-Me PPIX formation. To investigate the role of hepatic CYPs in DDC-mediated N-Me PPIX formation, HLM was used as a source of hepatic CYPs [33] and was incubated with DDC. We firstly verified the activities of major hepatic CYPs in HLM including CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4, and all of them showed expected CYP functions (Fig. 4A-I). However, neither N-Me PPIX 1 nor N-Me PPIX 2 was detected in the incubation with HLM and DDC (Fig. 4J-K), suggesting that hepatic microsomal CYPs may not responsible for DDC-mediated N-Me PPIX formation.
Figure 4. No N-Me PPIX formation was found in the incubations with HLM and DDC.

(A-I) Validation of CYP activities in HLM using probes of CYP isoenzymes. (J, K) N-Me PPIX 1 (J) and N-Me PPIX 2 (K) were not detected in the incubation with HLM and DDC. Metabolites of CYP probes and N-Me PPIX were detected by UPLC-QTOFMS. All data are expressed as means ± S.D. (n = 4). ND, not detected.
3.4 DEFICIENCY OF HEPATIC CYPS DOES NOT PREVENT DDC-MEDIATED PPIX AND N-ME PPIX ACCUMULATION IN THE LIVER OF LCN MICE

We next investigated the role of hepatic CYPs in DDC-mediated N-Me PPIX formation using LCN mice, in which the catalytic activity of hepatic CYPs is profoundly compromised [26]. Compared to the control group, the level of N-Me PPIX 1 was not significantly altered in the liver of LCN mice after DDC treatment (Fig. 5A). However, treatment with DDC significantly increased N-Me PPIX 2 in the liver of LCN mice (Fig. 5B), suggesting that hepatic CYPs are not essential in DDC-mediated N-Me PPIX formation. In addition, DDC also caused a significant accumulation of PPIX in the liver of LCN mice (Fig. 5C), which can be explained by N-Me PPIX 2 accumulation that suppresses PPIX catabolism and by ALAS1 induction that increases PPIX production (Fig. 5D).
Figure 5. DDC-induced N-Me PPIX and PPIX accumulation in the liver of LCN mice.

The mice were treated with DDC diet (0.1%) for 2 weeks. (A, B) Effect of DDC on the production of N-Me PPIX in the liver. (C) Abundance of PPIX in the livers of LCN mice treated with DDC. PPIX and N-Me PPIX were detected by UPLC-QTOFMS. All data are expressed as means ± S.D. (n = 4). ***$P < 0.001$. NS, not significant ($P > 0.05$). (D) Effect of DDC on ALAS1 expression in the liver. ALAS1 was determined by Western blot. GAPDH was used as a loading control.
3.5 DDC INDUCES LIVER INJURY IN LCN MICE

Consistent with DDC-mediated PPIX accumulation in the liver (Fig. 5C), liver injury was observed in LCN mice (Fig. 6). Compared to the control group, high levels of liver injury markers including ALT, AST and ALP were observed in the sera of LCN mice treated with DDC (Fig. 6A-C). Additionally, histological analysis revealed bile plugs and cholestatic liver injury in LCN mice treated with DDC (Fig. 6D-E), which are similar to that observed in WT mice (Fig. 3E). These data indicated that deficiency of hepatic CYPs cannot prevent DDC-induced PPIX accumulation and liver injury.

![Figure 6](image-url)

**Figure 6. DDC-induced liver injury in LCN mice.**

The mice were treated with regular diet or DDC diet (0.1%) for 2 weeks. (A-C) Biochemical analysis of serum ALT (A), AST (B) and ALP (C). All data are expressed as means ± S.D. (n = 4). **P < 0.01, ***P < 0.001. (D, E) Histological analysis of liver samples (H&E staining). PV, portal vein. The arrows point to bile plugs. Scale bar = 10 μm.
3.6 SERUM METABOLOMICS OF WT MICE TREATED WITH DDC

To explore the extrahepatic effects of DDC, we conducted metabolomic analysis of sera from WT mice treated with DDC. The score plot, generated by PCA analysis, revealed two well-separated clusters, corresponding to the control and DDC-treated groups (Fig. 7A). The targeted metabolomic analysis revealed PPIX as one of the top-ranking ions that was significantly increased in the serum of DDC-treated mice when compared to the control group (Fig. 7B and C). The levels of N-Me PPIX 2 were also significantly increased in the serum of DDC-treated mice (Fig. 7D and E). We next investigated the source of the high concentrations of PPIX and N-Me PPIX 2 in sera, which may come from the liver because of DDC-induced cholestatic liver injury and/or the bone marrow because it is the most active organ for the heme biosynthesis pathway [34, 35]. High levels of PPIX were detected in the red blood cells (RBCs) of mice treated with DDC (Fig. 7F), suggesting that the bone marrow is a target of DDC because RBCs are produced from the bone marrow.
Figure 7. Serum metabolomics of WT mice treated with DDC.

The mice were treated with regular diet or DDC diet (0.1%) for 2 weeks. Serum samples were analyzed by UPLC-QTOFMS. (A) Separation of control and DDC groups in a PCA score plot. (B) The variable trend plot for PPIX in control and DDC groups. (C) Relative quantification of
PPIX in the serum of mice treated with DDC. (D) The variable trend plot for N-Me PPIX 2 in control and DDC groups. (E) Relative quantification of N-Me PPIX 2 in the serum of mice treated with DDC. All data are expressed as means ± S.D. (n = 4). ***P < 0.001, ****P < 0.0001.

3.7 DDC INDUCES N-ME PPIX PRODUCTION AND PPIX ACCUMULATION IN THE BONE MARROW

As expected, the level of PPIX in the bone marrow was significantly increased after DDC treatment (Fig. 8A). PPIX accumulation in the bone marrow can be explained by DDC-mediated N-Me PPIX formation that suppresses FECH activity, as DDC significantly increased the production of both N-Me PPIX 1 and N-Me PPIX 2 in the bone marrow (Fig. 8B and 8C). Compared to the control group, N-Me PPIX 1 and 2 levels were increased to 11.2- and 99.8-fold, respectively, in the bone marrow of DDC-treated group. These results confirmed that the bone marrow is the target of DDC.

Figure 8. DDC induces PPIX accumulation and N-Me PPIX production in the bone marrow.
WT mice were treated with regular diet or DDC diet (0.1%) for 2 weeks. (A) Effect of DDC on PPIX accumulation in the bone marrow. (B, C) Effect of DDC on N-Me PPIX production in the bone marrow. PPIX and N-Me PPIX were detected by UPLC-QTOFMS. All data are expressed as means ± S.D. (n = 4). ***$P < 0.001$ vs control group.
4.0 DISCUSSION AND CONCLUSION

N-Me PPIX-mediated FECH inhibition in the liver are thought to be critical in DDC-induced PPIX accumulation and liver injury [3-5]. The isomers N-Me PPIX 1 and 2 are equally potent in FECH inhibition [36-38]. The current study found that DDC caused a dramatic accumulation of PPIX in the liver but no increase of N-Me PPIX 1 and only a mild increase of N-Me PPIX 2, suggesting that other mechanisms may contribute to DDC-induced hepatic PPIX accumulation and liver injury.

The current work identified the bone marrow as a target of DDC that leads to N-Me PPIX formation and PPIX accumulation. We found that DDC significantly increased the formation of both N-Me PPIX 1 and 2 in the bone marrow, which is different from the DDC-mediated regulation of N-Me PPIX production in the liver where DDC only increased the production of N-Me PPIX 2, but not N-Me PPIX 1. Consequently, the level of PPIX was significantly increased in the bone marrow, which can be further transported to the liver through the blood circulatory system [6, 8]. Indeed, high levels of PPIX were detected in the RBCs and sera of mice treated with DDC, which support that the bone marrow is a target of DDC. Additionally, we anticipate that the bone marrow is the major source of DDC-mediated PPIX accumulation rather than the liver because the bone marrow is much more active than the liver in PPIX biosynthesis [6] and DDC caused more N-Me PPIX production in the bone marrow than the liver.

Our data also suggest that DDC-mediated N-Me PPIX production in the liver is CYP-independent. Hepatic CYPs have been proposed to be critical in DDC-mediated N-Me PPIX
formation [3-5, 15, 39, 40]. Using HLM as a source of hepatic CYPs [33], we found that N-Me PPIX cannot be produced in the incubation with HLM and DDC, suggesting that hepatic CYPs are not as essential as described previously for DDC-mediated N-Me PPIX formation. To further determine the role of hepatic CYPs in DDC-mediated N-Me PPIX formation, we used the LCN mouse model, in which the hepatic CYPs are unfunctional [26]. We found that the LCN mice cannot prevent DDC-mediated N-Me PPIX production, PPIX accumulation, and liver injury, indicating that hepatic microsomal CYPs are not needed in DDC-mediated N-Me PPIX formation. This conclusion is also supported by our data showing that DDC induces more N-Me PPIX production in the bone marrow than the liver, but the liver has much more CYPs than most other organs [41, 42]. Our data are consistent with a previous report showing that a non-selective CYP inhibitor could not prevent DDC-induced suppression of FECH activity in mice [11].

In addition to xenobiotic-induced N-Me PPIX formation, endogenous N-Me PPIX has also been found in the liver [23]. The current work identified endogenous N-Me PPIX not only in the liver, but also in the bone marrow. N-Me PPIX is involved in many physiological processes including heme biosynthesis [21, 22], citrulline formation [43, 44], and cholesterol transportation [45, 46]. However, the detailed mechanism remains unclear for the production of endogenous N-Me PPIX. Because DDC strongly induces N-Me PPIX production both in the liver and bone marrow, it can be used as a model drug to further investigate the mechanism of N-Me PPIX biosynthesis.
The current work has limitations and we will address them in our future studies. Firstly, absolute quantification of PPIX and N-Me PPIX are needed for the analysis of samples from control and DDC-treated groups. Secondly, comparisons of PPIX and N-Me PPIX level between WT and LCN mice are needed to further confirm the role of hepatic CYPs in DDC bioactivation and toxicity. In addition to microsomal CYPs, our future work will also investigate the role of mitochondrial CYPs which use different electron transfer pathways rather than CPR [47, 48]. Furthermore, DDC metabolites will be profiled to determine their effects on N-me PPIX formation and PPIX accumulation.

In summary, the current work found that DDC is not liver specific but also targets the bone marrow (Fig. 9). DDC-mediated PPIX accumulation in the bone marrow can be transported to the liver through the blood circulatory system and contribute to DDC-induced liver injury. Our work also suggests that DDC-induced N-Me PPIX formation in the liver is CYP-independent.

Figure 9. Novel insights into the mechanisms of DDC-induced PPIX accumulation and liver injury.
(1) DDC promotes N-Me PPIX formation in the bone marrow; (2) DDC causes PPIX accumulation in the bone marrow; (3) DDC-mediated PPIX accumulation and N-Me PPIX formation in the bone marrow can be transported to the liver through the blood circulatory
system and contribute to DDC-induced liver injury; and (4) DDC-mediated N-Me PPIX formation in the liver is CYP-independent.


36. de Montellano, P.R.O., et al., Inhibition of hepatic ferrochelatase by the four isomers of N-methylprotoporphyrin IX. Biochemical and biophysical research communications, 1980. 97(4): p. 1436-1442.