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# Transgenic mice and metabolomics for study of hepatic xenobiotic metabolism and toxicity

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## Abstract

**Introduction—**The study of xenobiotic metabolism and toxicity has been greatly aided by the use of genetically-modified mouse models and metabolomics.

**Areas covered—**Gene knockout mice can be used to determine the enzymes responsible for the metabolism of xenobiotics *in vivo* and to examine the mechanisms of xenobiotic-induced toxicity. Humanized mouse models are especially important since there exist marked species differences in the xenobiotic-metabolizing enzymes and the nuclear receptors that regulate these enzymes. Humanized mice expressing cytochromes P450 (CYPs) and nuclear receptors including the pregnane X receptor (PXR), the major regulator of xenobiotic metabolism and transport were produced. With genetically-modified mouse models, metabolomics can determine the molecular map of many xenobiotics with a level of sensitivity that allows the discovery of even minor metabolites. This technology can be used for determining the mechanism of xenobiotic toxicity and to find early biomarkers for toxicity.

**Expert opinion—**Metabolomics and genetically-modified mouse models can be used for the study of xenobiotic metabolism and toxicity by: 1) Comparison of the metabolomics profiles between wild-type and genetically-modified mice, and searching for genotype-dependent endogenous metabolites; 2) Searching for and elucidating metabolites derived from xenobiotics; 3) Discovery of specific alteration of endogenous compounds induced by xenobiotics-induced toxicity.

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**Keywords**

genetically-modified mice; CYP; CYP3A4; metabolomics; pregnane X receptor; UPLC-ESI-QTOFMS; xenobiotics

## 1. Introduction

Drug-induced toxicity remains among the top reasons limiting the clinical use of drugs and the development of new chemical entities (NCEs) [1]. For the purpose of this review, drugs will be lumped with other foreign chemicals as xenobiotics since the technologies discussed cab apply to drugs, environmental and industrial chemicals and dietary compounds. Toxicity can be due to metabolism, which while important for the elimination of drugs, can also produce electrophilic metabolites that can cause cell death. For example, acetaminophen at high doses, is metabolized by cytochromes P450 (CYP) to a toxic quinone metabolite that depletes cellular glutathione and can covalently bind macromolecules resulting in elevated oxidative stress, mitochondrial damage and cell death [2, 3]. For decades, acetaminophen has served as the model to explore the mechanisms of drug-induced hepatotoxicities in order to understand idiosyncratic drug reactions involving increased liver enzymes, noted with many clinically-used drugs and drugs under development. In the past decade, these studies have been greatly aided by the use of genetically-modified mouse models and, more recently, by use of LCMS-based metabolomics.

### 1.1. Study of xenobiotic metabolism and toxicity

Many strategies have been used to study drug metabolism and to determine the mechanisms of drug- or xenobiotic-mediated toxicities, particularly liver toxicity, which is first diagnosed by the elevation of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In the pharmaceutical industry, rats have historically been used as the first line to investigate the metabolism and potential toxicity of drugs in whole animals [4]. Later studies can involve the use of dogs. Human hepatocytes have also been found to be of great value in the study of drug metabolism, but their utility in the prediction of drug toxicity is limited as hepatic toxicity, in particular subtle toxicity requires a whole liver context for complete manifestation [5]. Studies with human hepatocytes have been greatly aided by cryopreservation techniques but also limited due to the large interindividual variability in human livers. New engineered human hepatocytes from inducible stem cells could be used to standardize experimental human liver and hepatocytes [6, 7].

*In vitro* evaluation of the metabolic activation of xenobiotics through the use of liver subcellular fractions such as microsomes (fractions from endoplasmic reticulum) and trapping reagents (glutathione, *N*-acetylcysteine, *m*-anisaldehyde) have been employed to determine the production of electrophilic metabolites that result from xenobiotic metabolism [8–10]. As noted above, the use of hepatocyte cutures and liver slices have also been of great value for studying xenobiotic metabolism and the mechanisms of xenobiotic toxicity [11]. However, *in vivo* metabolism is the most relevant means to determine the fate of xenobiotics in mammals and to predict human responses to foreign compounds. *In vivo* animal models have wide utility for the investigation of toxicity mechanisms, but are limited

by their predictive value for humans because mice can metabolize many xenobiotics quite differently from humans, due in part to the marked species differences in the metabolic enzymes, notably the CYPs. For example, the most abundant CYP in humans is CYP3A4, a CYP that metabolizes more than 50% of clinically-used drugs [12–14]. The corresponding mouse homologues are expressed at lower levels than that found for CYP3A4 in liver. In particular, CYP3A4 is expressed in high levels in intestine, incrontrast to low expression of the mouse CYP3As in this tissue [15–17]. There is less species differences in the expression and catalytic activities in the phase 2 conjugating enzymes, while there are differences between humans and rodent models in the expression and specificities of drug transporters [18, 19]. The regulation of xenobiotic-metabolizing enzymes and even drug/xenobiotic transporters can also differ between mice and humans [2, 20].

### 1.2. Use of genetically-modified mice

Genetically modified mouse models (including gene knockout mice and humanized models) have been used to study xenobiotic metabolism and xenobiotic-induced toxicity [21–23]. Through the knockout of specific genes encoding xenobiotic-metabolizing enzymes or xenobiotic receptors involved in the regulation genes encoding xenobiotic-metabolizing enzymes, the contribution of specific enzymes and receptors towards the toxicity of these chemicals can be determined. The creation of humanized mice through the insertion of the human genes into the mouse genome can, to a degree, circumvent the potential species difference for risk assessment and can be faithfully extrapolated to humans [21].

The use of genetically-modified mice models have been reviewed [21–25]. Genes encoding CYPs involved in the metabolism of drugs and other xenobiotics have been individually disrupted and mice lacking these gene have no deleterious phenotypes thus indicating that they are not involved in critical physiological functions or their functions are redundant. For example, mice with a knockout of the hepatic CYP reductase gene, required for the catalytic activity of CYPs, are viable [26, 27], thus indicating that CYPs involved in xenobiotic metabolism are not critical for reproduction and physiological homeostasis with minor alterations in liver function. More recently, in a monumental accomplishment, mice lacking almost all of the known xenobiotic-metabolizing enzyme members of the *Cyp2c*, *Cyp2d*, and *Cyp3a* gene clusters, a total of 30 genes, were created and these mice also were viable with a minor hepatic phenotype of unknown mechanism [28]. This latter study confirms and definitively established that the hepatic, non-mitochondrial CYPs are primarily involved in metabolism of foreign compounds and have no critical physiological role.

The successful application of *Cyp* gene knockout mice in the study of xenobiotic toxicity has been frequently reported. For example, CYP2E1 mediates the oxidative metabolism of a wide range of xenobiotics, that cause toxicity. CYP2E1 is the major rate-limiting enzyme that initiates the cascade of events leading to acetaminophen hepatotoxicity [2]. *Cyp2e1*-null mice were employed to demonstrate the role of CYPs in acetaminophen toxicity. These mice were resistant to the hepatoxic effects of the drug as revealed by LD50 analysis and various biochemical endpoints [29]. In another study, mice lacking expression of CYP1B1 were resistant to bone marrow toxicity when administered the carcinogen 7,12-

dimethylbenz[*a*]anthracene [30]. *Cyp1a1*-null mice were employed to study the toxicity of orally-administered benzo[a]pyrene [31].

### 1.3. Metabolomics

In the last few years, metabolomics has been used to study xenobiotic metabolism and xenobiotic-mediated toxicity [32, 33]. Metabolomics, an area of investigation that measures changes in small molecules downstream of the genome, transcriptome, and proteome, captures the terminal alteration of endogenous chemicals. Metabolomics can also be used to identify and map metabolites derived from xenobiotics [32–34]. Unbiased determination of small molecules in biofluids, or extracts from cells, tissues, or organisms can give a clear description of the alteration of various biological processes. Metabolomics has been successfully introduced into the toxicological studies, as reviewed elsewhere [35–37]. A large number of studies have been performed employing metabolmics to study drug metabolism and toxicity in rats [38–40]. These studies have yielded much information on metabolism and markers for toxicity, including organ-specific toxicity [41]. However, there are not many genetically modified rat models and thus, many recept studies have focused on the study of xenobiotic-metabolism and toxicity using metabolomics and genetically modified mice. In addition, while there is a large literature on the use on $^{1}$H NMR in the study of drug metabolism and toxicity, as reviewed earlier [42], most studies in mice and genetically-modified mice have used liquid chromatography-mass spectrometry. The studies described below largely employ the ultra-performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOFMS) to examine the metabolism of xenobiotics and to find biomarkers for xenobiotic-induced hepatic toxicity [32].

## 2. Acetaminophen-induced hepatotoxicity

One of the most important CYPs for the metabolism of low MW xenobiotics, including industrial solvents and toxicants, is CYP2E1 [2]. It is highly expressed in liver, and to a muuch lower extent in extrahepatic tissues. CYP2E1 is conserved between humans, rats and mice, with no noted differences in catalytic activity between species. There are no known common polymorphisms that alter the expression or catalytic activity of CYP2E1. It is induced by some of its own substrates through a post-translational stabilization mechanism [43]. Despite, its high level of conservation between species and no polymoprhisms in humans, mice lacking expression of CYP2E1 are overtly normal with no notable developmental of physiological defects. It does not appear to influence glucose homeostasis in spite of its role in the metabolism of ketone bodies [44]. This mouse model is thus suitable for the study of the role of CYP2E1 in xenobiotic toxicity.

### 2.1 Metabolite mapping of acetaminophen

The value of gene knockout mice and metabolomics in the study of xenobiotic toxicity can be illustrated by a series of studies using the *Cyp2e1*-null mice and acetaminophen. Mice lacking CYP2E1 were resistance to the hepatotoxic effects of acetaminophen; mice without expression of both CYP2E1 and CYP1A2 were even more resistant to the drug as revealed by LD50 studies [29]. Metabolomics was used with wild-type and *Cyp2e1*-null to determine

the role of CYP2E1-mediated metabolism and down-stream events in mechanism of acetaminophen-induced hepatotoxicity. Metabolite mapping, using the power of metabolomics, was performed and several derivatives of acetaminophen were found only in wild-type mice, with some metabolites diminished in *Cyp2e1*-null mice, indicating that they might be involved in acetaminophen toxicity [45]. Metabolomics revealed the complete metabolic map of nine metabolites of acetaminophen, including three novel metabolites, an acetaminophen dimer formed nonenzymatically in liver, 3-$CH_3$-O-acetaminophen glucuronide and *S*-(5-acetylamino-2-hydroxyphenyl) mercaptopyruvic acid (SAMP) that is likely formed in the kidney through transamination of cysteine-acetaminophen (Figure 1). Metabolites derived from non-acetaminophen related endogenous compounds can also be discovered using metabolomics and these may serve as potential liver toxicity biomarkers [46]. These endogenous compounds might be of value as biomarkers for early toxicity and can offer clues to the mechanism of toxicity as noted below.

### 2.2 Biomarker for acetaminophen-induced hepatotoxicity

Metabolomics was then employed to discover biomarkers for acetaminophen-induced hepatotoxicity, importantly, early biomarkers for the onset of liver damage. Through comparsion of the metabolomics results of serum biosamples between wild-type and *Cyp2e1*-null mice after treatment with a toxic dose of acetaminophen, a differential, time-dependent alteration of serum acylcarnitines was found [47]. Metabolomics revealed mouse strain- and acetaminophen-dependent separation of groups early after exposure to toxic doses of acetaminophen. Notably, the separation was driven by a difference in acylcarnitines that were markedly increased at high acetaminophen doses in wild-type and and not *Cyp2e1*-null mice. While remaining high in wild-type mice, serum acylcarnitine levels gradually returned to normal in *Cyp2e1*-null mice at the end of the 24 h treatment. The early increase in serum acylcarnetines correlated with upregulation of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα) signaling following acetaminophen treatment [47]. PPARα a nuclear receptor that is involved in the control of lipid homeostasis in the liver and kidney and to some extent, in other tissues [48]. It is highly induced by xenobiotic ligands and by endogenous fatty acid metabolites under constions of food restrictions. PPARα controls the expression of genes involved in lipid transport and fatty acid β-oxidation in the peroxisomes and mitochondria. This study suggest that mitochondrial damage was a very early event in the acetaminophen toxicity since acylcarnitine accumulation in the cytosol and leakage into the serum is due in large part to a lack of mitochondrial uptake of these metabolites by mitochondrial transporter carnitine acyltransferases (CPT) [49]. The resultant liver damage leads to acylcarnitine leakage to the blood where they were detected (Figure 2). In addition, the upregulation of PPARα target genes is likely due to an increase in endogenous metabolites, probably derived from fatty acids, produced as a result of the mitochondrial damage, that are agonist for PPARα. In humans, the extent of mitochondrial damage, as revealed by serum biomarkers, correlated with survival after acetaminophen overdose [3].

### 2.3 Mechanism of acetaminophen hepatotoxicity

The transient increase in PPARα signaling may be a defensive mechanism by which fatty acid catabolism is increased along with elevated mitochondrial uncoupling protein 2

(UCP2), encoded buy the PPARα target gene *Ucp2*, as a defense against increased reactive oxygen species produced by acetaminophen overdose (Figure 2) [50]. UCP2 is a membrane protein that transports protons and anions, such as Cl-, Br-, and NO3- across the mitochondrial inner membrane [51]. Activation of PPARα, using the synthetic agonist Wy-14,643, protected mice from acetaminophen-induced hepatotoxicity as a result of indution of *Ucp2*. The mechanism of this protection is likely due to a decrease of acetaminophen-induced reactive oxygen species (ROS) by UCP2, and an explanation by which CYP2E1-mediated metabolic activation of acetaminophen through mitochondrial damage, inhibited mitochondrial fatty acid oxidation [50].

## 3. Alcohol-induced liver disease

Alcohol-induced liver disease (ALD) is a leading cause of nonaccident-related deaths in the United States due in large part to liver injury, notably alcoholic liver disease and liver cirrhosis [52]. Mice lacking PPARα are higly susceptible to ALD, thus making them an excellent model to study the mechanisms of this disease [53]. Metabolomic comparison of alcohol-treated wild-type and *Ppara*-null mice showed that levels of indole-3-lactic acid were markedly elevated in urine, indicating a mechanism where disruption of the PPARα gene impairs conversion of tryptophan to NAD+ through quinolinic acid leading to decreased NAD+-dependent fatty acid β-oxidation [54]. NAD+ is absolutely required for proper cellular functions and thus lower levels of this metabolite would have deleterious consequences. Oxidation of alcohol to acetaldehyde and acetic acid further decreases the NAD+/NADH ratio leading to free fatty acid accumulation in the liver and hepatic steatosis (Figure 3). The buildup of NADH drives the reduction of indole-3-pyruvic acid to indole-3-lactic acid, resulting in an increase in urinary excretion of this compound [54, 55]. Additionally, this metabolite could be a prediction biomarker for alcohol-induced liver disease as revealed in the *Ppara*-null mouse model. The role of PPARα in protection against ALD suggests a potential overlap in the mechanism of toxicity between acetaminophen and ALD that needs to be explored by additonal experimentations.

## 4. Hepatotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

The aryl-hydrocarbon receptor (AhR) is a basic helix-loop-helix trasnsription factor that controls a large number of genes including those encoding CYP1A1, CYP1A2 and CYP1B1 [56]. It is the receptor that medicates the biological activities of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which is among the most potent environmentally toxic compounds. TCDD causes immunotoxicity, chloracne in humans and liver toxicity, but the detailed toxicity mechanism by which it causes liver injury remains unclear. Comparison of serum metabolomics profiles between wild-type mice and aryl-hydrocarbon receptor (*Ahr*)-null mice identified azelaic acid monoesters as significantly increased through downregulation of hepatic carboxylesterase 3 (CES3), an enzyme that hydrolyzes these esters, after treatment with TCDD, in a AhR-dependent manner [57]. Methionine- and choline-deficient (MCD), an experimental model for non-alcoholic steatohepatitis (NASH), was also associated with increased azelaic acid monoesters that result from downregulation of CES3, thus indicating a link between xenobiotic toxicity and NASH. However, no change in CES3 levels and azelaic acid monoesters was found in simple hepatosteatosis. Increased inflammatory

cytokines produced by both TCDD and MCD-induced NASH caused suppression of expression of the *Ces3* gene via TGFβ-SMAD3 and IL6-STAT3 signaling pathways (Figure 4). This study established a role for the AhR signalling pathway in TCDD-induced steatohepatitis and revealed a similarity in mechanism of liver toxicity between TCDD-induced toxicity and NASH.

## 5. Hepatoxicity of isoniazid and rifampicin

*Cyp2e1*-null mice and metabolomics were used to probe the mechanism of toxicity of isoniazid, a first-line medication used in the treatment of tuberculosis [58]. Metabolomic analysis showed that production of metabolites such as *p*-cresol glucuronide and *p*-cresol sulfate were elevated in wild-type mice but not in *Cyp2e1*-null mice. This suggests that the drug altered the gut microbiota population in a CYP2E1-dependent fashion, since these metabolites are derived from the gut flora [59]. The higher abundance of bile acids, bile acid metabolites, carnitine and carnitine derivatives in isoniazid-treated wild-type mice was also not detected in *Cyp2e1*-null mice. These results indicated that CYP2E1 might play a role in isoniazid-induced cholestasis through alteration of gut bacteria, enhancement of bile acid accumulation and mitochondria β-oxidation although a mechanism that remains to be determined.

Rifampicin is usually combined with isoniazid for the treatment of tuberculosis. However, co-therapy with these two drugs results in mild to severe liver toxicity [60]. Since rifampicin is a human PXR specific ligand, *PXR*-humanized mice were used to study the mechanism of hepatotoxicity in mice co-treated with rifampicin and isoniazid. Toxicity, which was characterized by increased serum ALT and alkaline phosphatase, markers for hepatocyte and bile duct injury, respectively, was only observed in *PXR*-humanized mice co-treated with both drugs, and not found in wild-type mice and *Pxr*-null mice [61]. Mild liver toxicity was noted upon treatment with rifampicin alone but not with mono-treatment with isoniazid. In addition, occluded bile ducts were obvious in the livers of treated *PXR*-humanized mice. To investigate the nature of these occlusions, metabolomics was carried out in the bile ducts revealing the presence of high levels of protoporphyrin IX, an intermediate in porphyrin and heme synthesis, in *PXR*-humanized mice; increased protoporphyrin IX was also found in hepatocytes. Rifampicin was found to markedly induce the gene encoding aminolevulinic acid synthase in these mice indicating that the increased in protoporphyrin IX is due in part to its increased synthesis. Treatment with the product of aminolevulinic acid synthase, aminolevulinic acid, was found to potentiate the rifampicin and isoniazid hepatotoxicity. The detailed mechanism by which isoniazid contributes this toxic event remains unknown. However, one possibility is that isoniazid inhibits ferrochelatase activity resulting in the accumulation of protoporphyrin IX as a result of blocking the heme synthesis pathway (Figure 5). This study illustrates the value of *PXR*-humanized in unraveling the mechanism of sporadic hepatic toxicity of a widely used drug therapy. These data may help in the prediction of patients that my exhibit toxicity when treated with rifampicin and isoniazid.

## 6. Bile acid-induced hepatotoxicity

Bile acids are synthesized in the liver from cholesterol by CYP7A1 and CYP8B1. They are conjutated in mice with taruine (and by glycine in humans) and transported through the cannaliculus to the bile duct and into the intestine where they function to help digestion through the solubilization of dietary fats and vitamins [62, 63]. Bile acids are alsom metabolized by gut bacteria to secondary bile acids and >90% are reabsorbed to the blood stream and return to the liver through a process called enteroheptic circulation that is regulated by the farnesoid X receptor (FXR) [64, 65]. When bile acid synthesis and transport are disruped byt liver disease, marked hepatotoxocity can result in part through the hepatic accumulation of certain bile acid metabolites. Lithocholic acid (LCA) is a known hepatotoxin that accumulates in liver diseases such as cholestasis [66]. In another example of the value of gene knockout and humanized mice in exploring the mechanism of hepatotoxicity, mice lacking vitamin D receptor (VDR) expression in intestine and expressing human CYP3A4 in the inestine and liver ($Vdr^{\Delta IEpC}$/3A4) were used to investigate the role of intestine in modulation of bile acid homeostasis and toxicity in liver [67]. CYP3A4 can metabolize and detoxify LCA in liver [68] and earlier studies revealed that LCA can induced CYP3A4 expression in liver through activating VDR [69]. Compared with LCA-treated $Vdr^{\Delta IEpC}$ mice which do not express CYP3A4, LCA-treated $Vdr^{\Delta IEpC}$/3A4 mice that express CYP3A4 showed decreased hepatotoxicity. Metabolomics analysis revealed that this protection was associated with increased LCA metabolism and detoxification, and suppression of bile acid transporter expression in the small intestine [67]. Thus, the small intestine, through VDR signaling, has a role in the protection from the hepatotoxicity of bile acids (Figure 6). This study, using genetically modified mice, shows how the intestine can, under certain circumstances, serve as a defense against bile acid-induced hepatotoxicity

## 7. Ritonavir metabolism

Ritonavir (RTV) is an important component of protease inhibitor (PI) regimens for antiretroviral therapy. RTV inhibits CYP3A-mediated metabolism of PIs, thus increasing plasma concentrations of PIs [66] and making these RTV-boosted PI regimens are highly effective [70]. However, RTV-containing regimens increase the risk of liver injury and the detailed mechanisms of this RTV-induced liver injury remain unknown [71, 72]. Drug metabolism and bioactivation are associated with drug toxicity [73], and previous studies identified multiple metabolic pathways of RTV, including hydroxylation of the isopropyl side chain, *N*-demethylation, and cleavage of the terminal thiazole and isopropylthiazole group [74, 75]. However, it remains unclear whether RTV bioactivation pathways are associated with RTV-induced liver injury.

A recent study explored RTV metabolism using a metabolomic approach [76]. Twenty-six RTV metabolites were identified, including 13 previously reported metabolites and 13 novel metabolites. Among these new RTV metabolites, five were associated with RTV bioactivation [76]. RTV is a potent CYP3A inhibitor, but it is also a CYP3A substrate [74]. Thus, the role of CYP3A in RTV metabolism cannot be fully determined by *in vitro* experiments because RTV inhibits CYP3A. Therefore, the *Cyp3a*-null mouse model was

employed, providing an ideal tool to determine the contribution of CYP3A to RTV metabolism *in vivo*. Compared to wild-type mice, several metabolic pathways of RTV, including the bioactivation, were decreased significantly in *Cyp3a*-null mice, indicating that these metabolic pathways of RTV are CYP3A-dependent [76]. CYP3A is highly expressed in the liver and small intestine [14], and the expression pattern of CYP3A may explain the gastrointestinal adverse effects of RTV, such as diarrhea, nausea, vomiting, and abdominal pain [71, 77]. CYP3A expression is highly inducible, and pre- or co-treatment with a CYP3A inducer may increase RTV bioactivation and potentiate RTV toxicity. Indeed, the hepatotoxicity of RTV-boosted PI regimens was observed in subjects who were pre-treated with rifampicin or efavirenz, a ligand and agonist of PXR that induces CYP3A expression [78–81]. This metabolomic analysis extended the metabolic map of RTV, which identified 13 known and 13 novel RTV metabolites, including 5 bioactivation pathways. Furthermore, CYP3A-dependent metabolic pathways of RTV were determined by using *Cyp3a*-null mice [76]. These data can be used to predict and prevent the potential drug-drug interactions and adverse drug reactions associated with RTV. This study also highlights the power of genetically engineered mouse models and the metabolomic approach in studying of drug metabolism (Figure 7).

## 8. Endogenous biomarkers for xenobiotic toxicity

Xenobiotic-induced toxicity responses can be monitored by the altered levels of endogenous compounds that are produced after toxic insult [41]. For example acyl carnitines are found in the serum after acetaminophen overdose that resulted from disruption of mitochondrial function [47]. Metabolomics analysis can be used to both determine the extent of xenobiotic metabolism and the production of altered endogenous metabolites. However, exposure to high doses of xenobiotics resulted in metabolites derived from the xenobiotics that can interfere with the separation of the endogenous non-xenobiotic metabolites in the control vehicle-treated group and the xenobiotic-treated group. Therefore, discrimination of the contribution of xenobiotic metabolites from xenobiotics-induced endogenous compounds needs to be established in order to find toxicity biomarkers and reveal the detailed toxicity mechanisms. This can be accomplished by filtering out the xenobiotic and its known metabolites during the data analysis step. Stable isotope-based metabolomics is another possible method to achieve this task where it is feasible to synthesize the xenobiotic with a stable isotope such as deuterium or $^{13}C$ [46]. Through comparing urinary ions from ethanol ($C_2H_6O$) and deuterated ethanol ($C_2D_6O$) treatment, a new metabolite of ethanol *N*-acetyltaurine was discovered that can function as a biomarker of hyperlacetatemia [82]. Stable isotope metabolomics can be used to discriminate xenobiotic-derived metabolites from xenobiotic-induced endogenous metabolites. For example, a metabolic map of ten tempol metabolites was uncovered along with other metabolites that resulted from the action of tempol on the intestinal microbiota [83].

## 9. Expert opinion

Genetically-modified mice have distinct advantages in the study of xenobiotic metabolism. Notably, they offer a means to precisely define the roles of specific enzymes in the metabolism of xenobiotics *in vivo*. The CYPs and phase 2 enzymes are ideally amenable to

this technology since they are largely dispensible for normal development and physiological homeostasis. One or multiple enzymes can be disrupted and the mice are otherwise normal and can be used to study xenobiotic metabolism [28]. Similarly, the receptors such as AHR [84, 85], PXR [86, 87] and constitutive androstane receptor (CAR) [88] can be knocked out and the mice are viable, although abnormal immune, hepatic and reproductive phenotypes are present in the *Ahr*-null mice [84, 85]. These mouse models are now widely used and distributed by Jackson Laboratories and other commercial sources. Mice with disrupted phase 2 enzymes such as glutathione S-transferases [89], [90] and UDP-glucuronosyltransferases [91], have also been produced but discussion of these models is beyond the scope of this review.

This review largely focussed on the hepatic toxicity of xenobiotics that is largely driven by metabolism, and the use of metabolomics and genetically-modified mice to detemine the metabolic maps of xenobiotics, to uncover the mechanisms of xenobiotic-induced toxicities, define the genes involved in mediating toxicities and find biomarkers for toxicities. However, xenobiotics can also cause toxicity at other organ sites, such as the central nervous system, intestine, muscle (heart), bladder, kindey, lung and even skin. The mechanism of these toxicities in various organs can be diverse and may not be due to metabolism. While studies using $^{1}$H-NMR metabonomics have been used to find organ-specific endogenous metabolites that can be biomarkers for xenobiotic induced toxicities [41], the use of a more sensitive UPLC-ESI-QTOFMS metabolomics platform has not been exploited to find, using known organ-speficie toxicants, the metabolite biomarkers that reflect xenobiotic toxicities in specific organs.

To produce mouse lines that can be used to predict human xenobiotic metabolism *in vivo*, human CYP and nuclear receptor genes have been introduced into their respective null mouse counterparts. While mouse models humanized for specific CYPs and nuclear receptors have been generated [21], mice in which the complete complement of hepatic regulatory genes and xenobiotic-metabolizing genes have been replaced with their human counterparts have not been developed, although there are ongoing efforts in this area [28, 92, 93]. Ideally, however, it would be of great utility to have a rodent model in which the whole human liver is introduced. It should be noted that the genetically-modified mouse models, in particular the mice expressing human CYPs and xenobiotic receptors, offer a better predictive model for humans than do wild-type mice, they still have many differences from human liver. Thus, studies with human hepatocytes and liver extracts are vital. One possible solution, that has been studies for a number of years, are the chimeric liver replacement mouse models that use hepatocyte transplantation into immunocompromised mice that undergo liver failure [94–96]. These mice are highly predictive of human liver xenobiotic metabolism. However, these mice are expensive since each mouse needs to be custome made and cannot be propagated. They are not viable enough for longer term toxicity studies and they are still dependent on the liver used for the mouse liver replacement. There are other issues from this model, including differences in liver architecture between mice and humans, and influence of the immune system, which shows marked species differences [97], in xenobiotic-mediated toxicity [98].

As noted above, each human liver has a different levels of the various xenobiotic-metabolizing enzymes. One possibel solution to the variability of human livers is to use inducible pluripotent (iPS) cells to make a reliable and reneable source for hepatoctyes to use directly for xenobiotic metabolism studies and for reconstituting mice using the chimeric mouse model. Liver iPS cells is an area of active investigation which will likely lead to cells that are of value for xenobiotic metabolism and toxicity studies [6, 94, 95, 97–99]

As a key component of systems biology, metabolomics plays an increasingly important role in mechanistic elucidation of xenobiotic toxicity. Genetically-modified (including drug-metabolizing enzymes and nuclear receptors) mice can clearly elucidate the role of specific genes in xenobiotics-induced toxicity. The combination of these two key technologies can further promote the understanding of mechanisms of xenobitoic toxicity through analyzing the influence of specific genes towards the xenobiotics-induced alteration of endogenous compounds through the use of metabolic network modeling using a number of commercial and public data bases and programs. However, limitations exist for the application of metabolomics for genetically-modified mouse-based xenobiotic toxicity studies. Therefore, the following steps are recommended for use of metabolomics and genetically-modified mouse models into the study of xenobiotic metabolism and xenobiotic-induced toxicity: 1) Thorough comparison of metabolomics profiles between wild-type and genetically-modified mice, searching for genotype-dependent endogenous metabolites; 2) Based on the structures of a xenobiotic, searching and elucidation of metabolites derived from xenobiotics; 3) Discovery of specific alteration of endogenous compounds induced by xenobiotics-induced toxicity.

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### Article highlights box

- Genetically modified mouse models have served as ideal tools to determine the role of a specific gene in xenobiotic metabolism and xenobiotic-induced toxicity.
- Metabolomics has been used to investigate xenobiotic metabolism and endobiotic metabolism and their associations with xenobiotic-mediated toxicity.
- Transgenic mice and metabolomics provide deep insights for the mechanism of acetaminophen-induced hepatotoxicity.
- Transgenic mice and metabolomics provide novel insights into the toxicities of alcohol; 2,3,7,8-tetrachlorodibenzo-p-dioxin; isoniazid and rifampicin; bile acid; and ritonavir.
- Transgenic mice and metabolomics provide the state-of-art appraoches for research in Pharmacology and Toxicology.

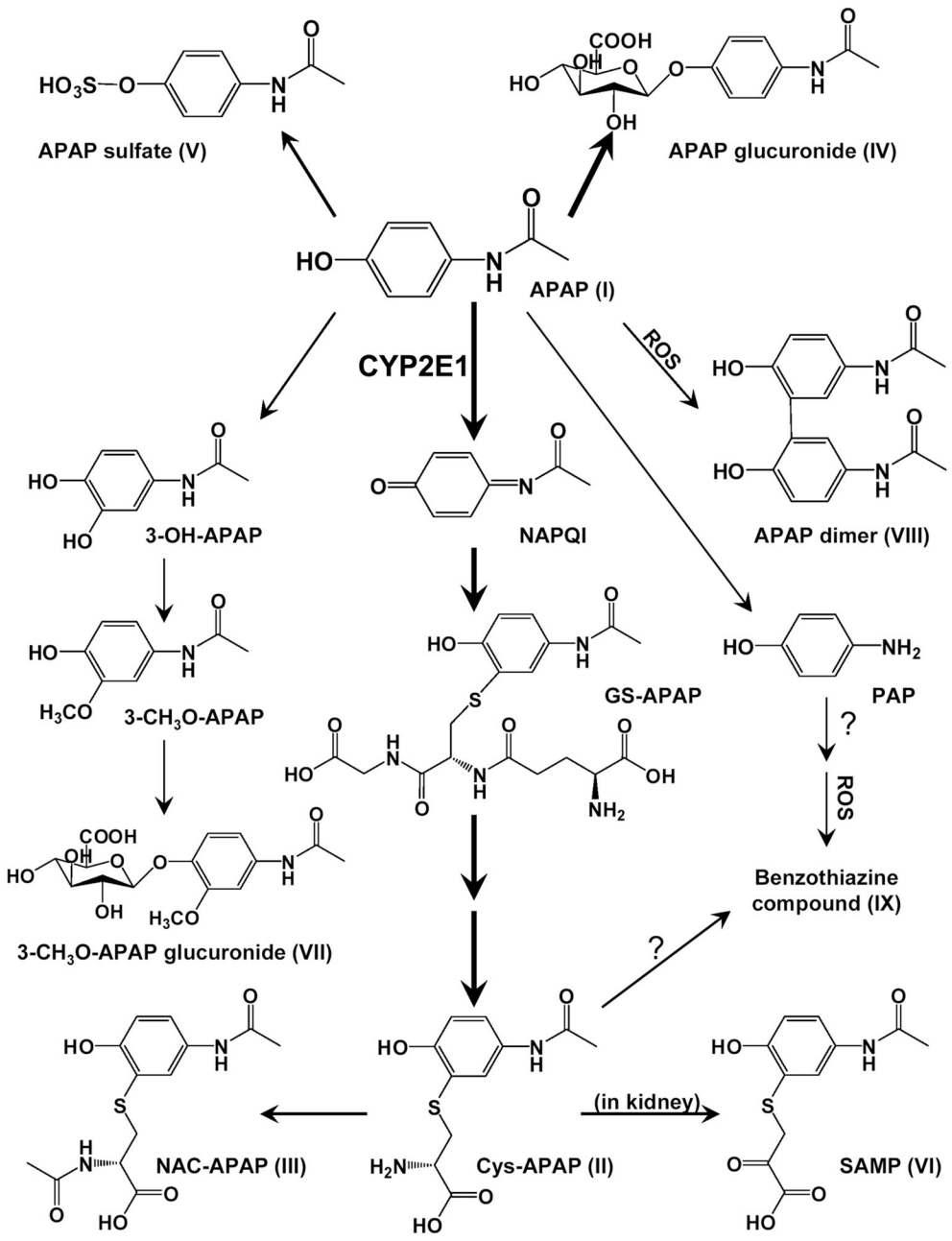


**Figure 1.**

Metabolic map of acetaminophen. Thick lines represent major pathways, and thin lines denote minor pathways. Adapted from [45].

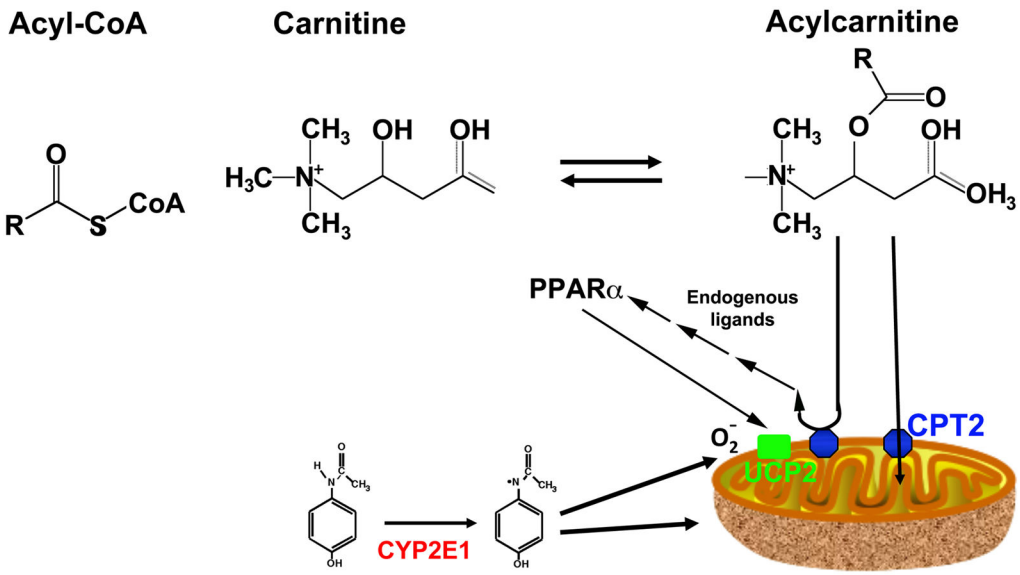


**Figure 2.**

Role of PPARα in acetaminophen-induced hepatotoxicity. Fatty acylcarnitines formed from acyl-CoA and carnitine are required for uptake of fatty acids to the mitochondria by carnitine palmitoyltransferase 2 (CPT2) for fatty acid β-oxidation to occur. Upon mitochondrial damage by an electrophilic metabolite of acetaminophen, NAPQI, the mitochondria is damaged leading to lack of acylcarnitines uptake and leakage to the cytoplasm and serum upon liver cell death. Endogenous ligands from this leakage can activate PPARα that in turn induced fatty acid β-oxidation by peroxisomes and induction of uncoupling protein 2 (UCP2) as a defense against oxidative stress. Data taken from [50].

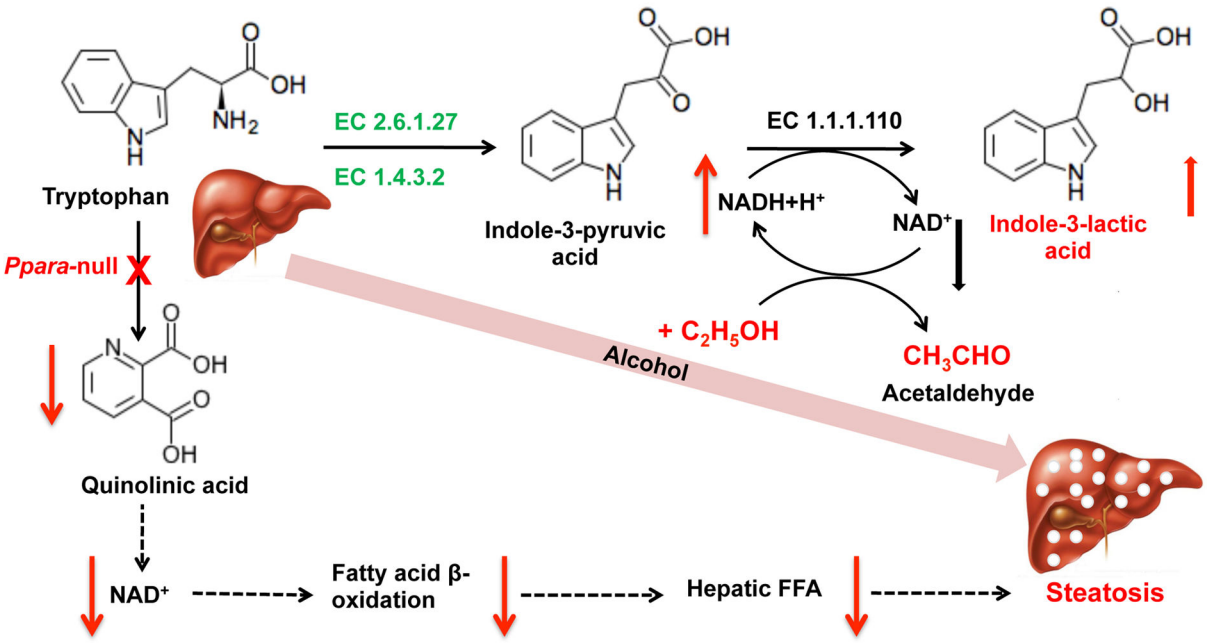


**Figure 3.**

Mechanism of generation of biomarkers that are associated with alcohol and hepatic steatosis. EC 2.6.1.27, tryptophan transaminase; EC 1.4.3.2, L-amino-acid oxidase; EC 1.1.1.110, indolelactate dehydrogenase. Derived from [55].

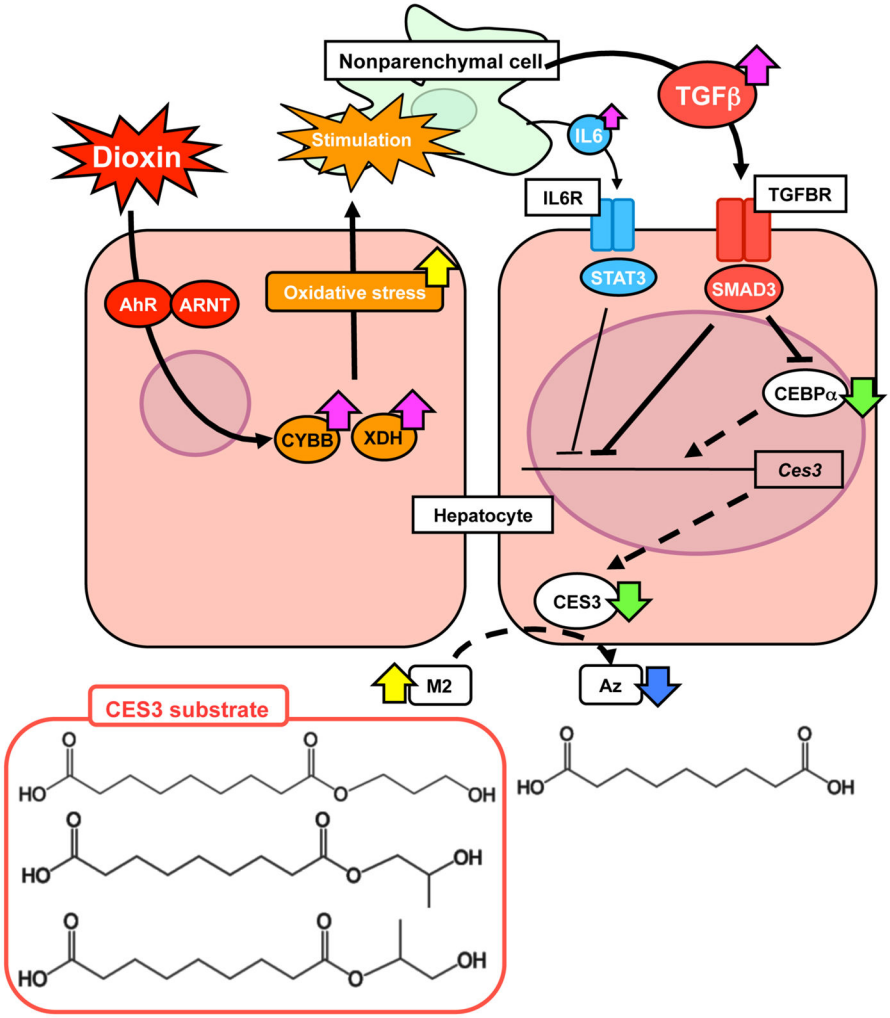


**Figure 4.**

Mechanism by which TCDD increases liver steatosis and azelaic acid esters. TCDD (dioxin) enters hepatocytes and elevates oxidative stress that increases production of proinflammatory cytokines such as IL6 and TGFβ in neighboring Kupffer cells. IL6 and TGFβ act on hepatocytes to suppress STAT3 and SMAD3 signaling resulting in suppression of the Ces3 gene encoding carboxylesterase 3 (CES3) which converts azelaic acid (Az) esters to Az. Lower CES3 expression also results on increase hepatic lipids due to lower hydrolysis of long-chain fatty acid esters and thioesters which may have a role in the elimination of hepatic lipid stores. Derived from [57].

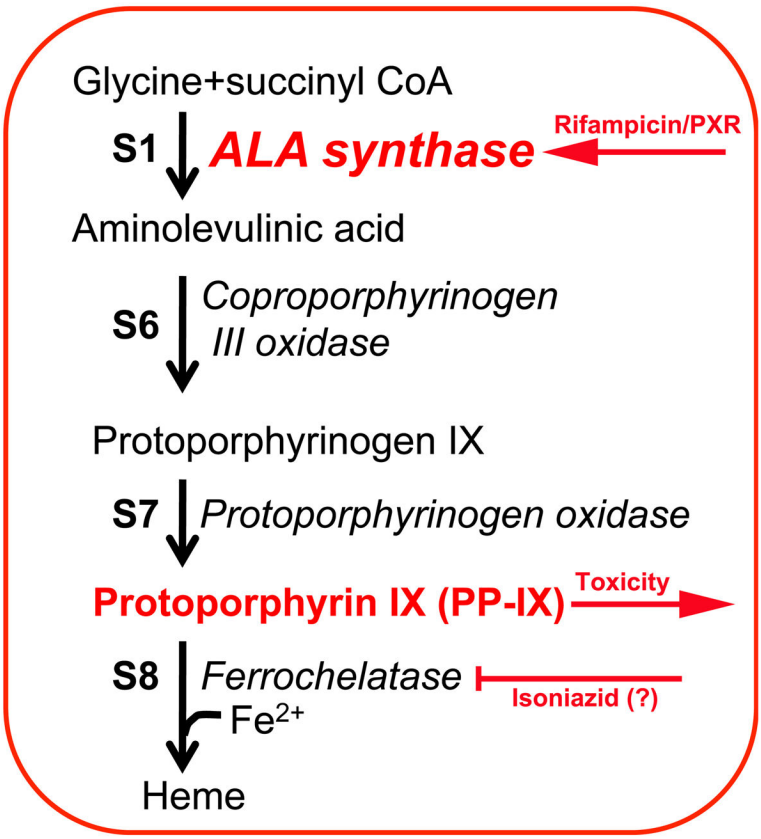


**Figure 5.**

Proposed mechanism for rifampicin and isoniazid co-therapy-induced hepatotoxicity. Rifampicin induces ALA synthase resulting in increased protoporphyrin IX. Isoniazid, through a yet to be determined mechanism, might inhibit ferrochelatase resulting in blockage of further metabolism of this intermediate of heme synthesis. Data taken from [61].

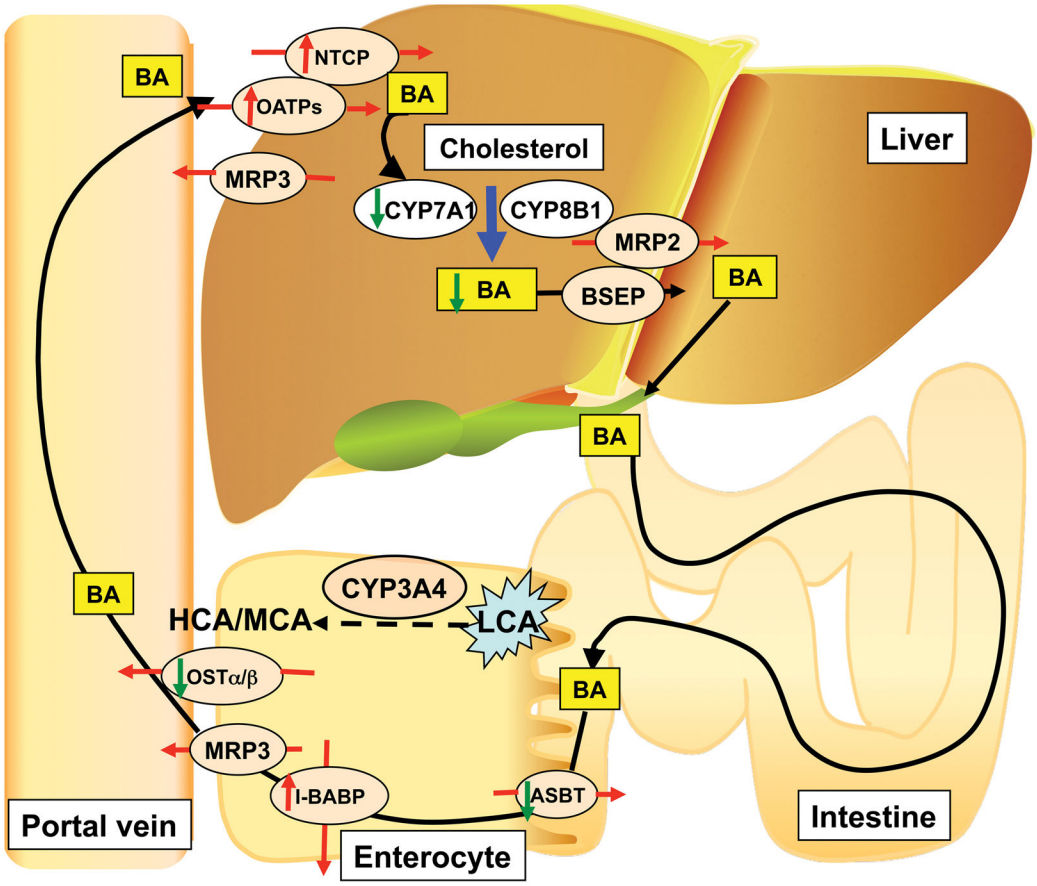


**Figure 6.**

Proposed mechanism for the role of VDR and CYP3A4 in the protection against the hepatotoxicity of lithocholic acid. Abbreviations: ASBT, apical sodium-dependent bile acid transporter; BA, bile acid; BSEP, bile salt export protein; HCA, hydroxycholic acid; I-BABP, intestinal bile acid-binding protein; LCA, lithocholic acid; MCA, muricholic acid; MRP, multidrug-resistance protein; NTCP, $Na^+$-taurocholate cotransporting polypeptide; OATP, organic ion transporting polypeptide; OST, organic solute and steroid transporter. Data taken from [64] and [67].

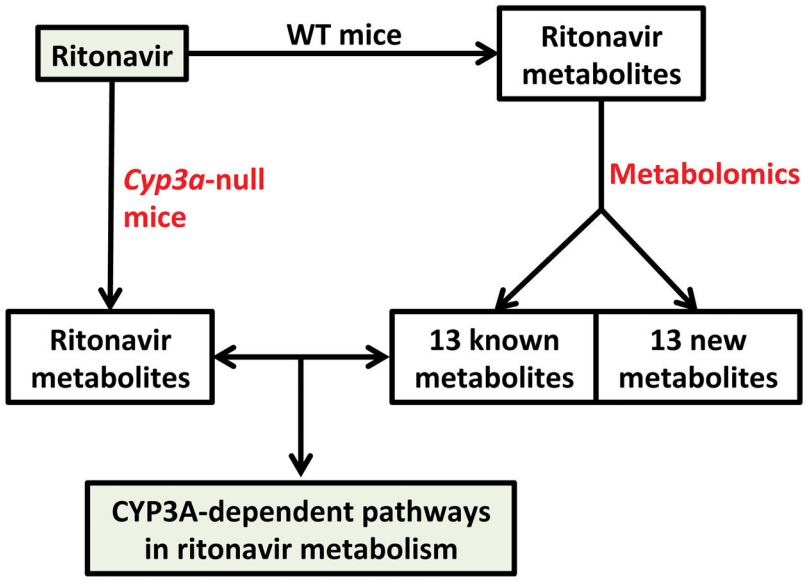


**Figure 7.**

Determine the role of CYP3A in ritonavir metabolism using a metabolomic approach and *Cyp3a*-null mice. The metabolomic analysis extended the metabolic map of ritonavir. The CYP3A-dependent pathways in ritonavir metabolism were confirmed by using *Cyp3a*-null mice. Data taken from [76].