Title: Advances and Challenges in Modeling Cannabidiol Pharmacokinetics and Hepatotoxicity

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Abbreviations: 11-COOH-THC, 11-nor-9-carboxy-tetrahydrocannabinol, or 11-carboxy tetrahydrocannabinol; 7-COOH-CBD, 7-carboxy-cannabidiol; ADH, Alcohol dehydrogenase; ALDH, Aldehyde dehydrogenase; ALT, Alanine aminotransferase; AMPK, 5’ adenosine monophosphate (AMP)-activated protein kinase; APAP, Acetaminophen; BCRP, Breast cancer resistance protein; BSEP, Bile salt export pump; CBD, Cannabidiol; DDI, Drug-drug interaction; DILI, Drug-induced liver injury; FABP, Fatty acid binding protein; FXR, Farnesoid X receptor; JNK, c-Jun N-terminal kinase;
LC-MS/MS, Liquid chromatography-tandem mass spectrometry; MRP1, Multidrug resistance-associated protein 1; MRP2, Multidrug resistance-associated protein 2; NRF2 or NFELE2, Nuclear factor erythroid 2-related factor 2; NTCP, Sodium taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide; OCT, Ornithine carbamyltransferase; OSTα/β, organic solute transporter α and β; P450 or CYP, Cytochrome P450; PBPK, Physiologically based pharmacokinetic (model); P-gp, P-glycoprotein; PPAR-α or PPARA, Peroxisome proliferator activated receptor alpha; QST, quantitative systems toxicology; ROS, reactive oxygen species; THC, Delta-9-tetrahydrocannabinol; TPRA1, Transient receptor potential ankyrin type 1; TRPM8, Transient receptor potential of melastatin type 8 channel; TRPV1, Transient receptor potential vanilloid-type 1; UDPGA, Uridine 5'-diphosphate glucuronic acid; UGT, Uridine 5'-diphospho-glucuronosyltransferase; ULN, Upper limit of normal; VDAC1, Voltage-dependent anion channel 1; VPA, Valproic acid or valproate.
Abstract

Cannabidiol (CBD) is a pharmacologically active metabolite of cannabis that is FDA-approved to treat seizures associated with Lennox-Gastaut syndrome, Dravet syndrome, and tuberous sclerosis complex in children aged one year and older. During clinical trials, CBD caused dose-dependent hepatocellular toxicity at therapeutic doses. The risk for toxicity was increased in patients taking valproate (VPA), another hepatotoxic antiepileptic drug, through an unknown mechanism. With the growing popularity of CBD in the consumer market, an improved understanding of the safety risks associated with CBD is needed to ensure public health. This review details current efforts to describe CBD pharmacokinetics and mechanisms of hepatotoxicity using both pharmacokinetic models and in vitro models of the liver. In addition, current evidence and knowledge gaps related to intracellular mechanisms of CBD-induced hepatotoxicity are described. The authors propose future directions that combine systems-based models with markers of CBD-induced hepatotoxicity to understand how CBD pharmacokinetics may influence the adverse effect profile and risk of liver injury for those taking CBD.
Significance Statement

This review describes current pharmacokinetic modeling approaches to capture the metabolic clearance and safety profile of cannabidiol (CBD). CBD is an increasingly popular natural product and FDA-approved antiepileptic drug known to cause clinically significant enzyme-mediated drug interactions and hepatotoxicity at therapeutic doses. CBD metabolism, pharmacokinetics, and putative mechanisms of CBD-induced liver injury are summarized from available preclinical data to inform future modeling efforts for understanding CBD toxicity.
Introduction

Cannabidiol, or CBD, is a pharmacologically active small molecule derived from Cannabis sativa. CBD is one of over 120 terpenophenolic metabolites in cannabis known as cannabinoids (ElSohly and Gul, 2014; Jazz Pharmaceuticals, Inc, 2023). CBD is FDA-approved to treat seizures associated with Lennox-Gastaut syndrome, Dravet syndrome, or tuberous sclerosis complex in patients one year of age and older under the brand name Epidiolex® (Jazz Pharmaceuticals, Inc, 2023). Though CBD-containing products are readily attainable in the US consumer market, this remains the only FDA-approved indication for CBD. Multiple warning letters have been issued by the FDA to sellers for marketing CBD products in violation of the Food, Drug, and Cosmetic Act (U.S. Food and Drug Administration, 2019). Despite these warnings, products containing CBD are commonly sold in the form of vaping cartridges, edibles, oils, supplements, and cosmetics, among others. US sales of CBD products are anticipated to reach $20 billion by 2024 (PR Newswire, 2020).

In January 2023, the FDA announced that existing regulatory frameworks for foods and dietary supplements were not appropriate for CBD, and additional oversights were needed to minimize potential safety risks associated with CBD (Woodcock, 2023). In making this decision, the FDA cited a lack of research on the long-term safety of cumulative CBD use, including the risk for CBD-induced hepatotoxicity (Woodcock, 2023). In light of these concerns, this review will describe current knowledge of CBD pharmacokinetics (PK) and hepatotoxicity as well as highlight knowledge gaps related to the prediction and prevention of hepatic adverse effects.
CBD Pharmacokinetics in Humans

CBD is highly lipophilic, with a logP of approximately 6 log units (Yeung et al., 2023). It has poor and erratic oral absorption that decreases with increasing dose, with an estimated absolute oral bioavailability of 6% (Perucca and Bialer, 2020). When taken with food, the oral bioavailability increases to 14-25% (Committee for Medicinal Products for Human Use (CHMP), 2019). A recent in vitro bioavailability study by Mozaffari et al. found that CBD micellarization and absorption increases significantly in the presence of lipids and bile salts (Mozaffari et al., 2021). The recommended maintenance dose of Epidiolex® is 20 to 25 mg/kg/day in two divided doses given consistently with regards to meals (Jazz Pharmaceuticals, Inc, 2023). The relatively poor absorption of CBD may be attributed both to incomplete gastrointestinal absorption and extensive first-pass metabolism (Perucca and Bialer, 2020). In phase I studies, the area under the plasma concentration-time curve (AUC) of CBD in 4 males and 8 females increased by up to five-fold when taken with a high-fat meal (Taylor et al., 2018; Jazz Pharmaceuticals, Inc, 2023). Based on its physiochemical properties, CBD is a class II Biopharmaceutical Classification System (BCS) substance, indicating low solubility and high passive permeability.

CBD is highly protein bound, with an estimated unbound fraction of less than 6% in plasma (Jazz Pharmaceuticals, Inc, 2023). CBD is thought to be primarily bound to albumin and lipoproteins in circulation, whereas fatty acid binding proteins (FABPs) function as intracellular carriers of CBD (Elmes et al., 2015; Leboffe et al., 2017; Franco et al., 2020). CBD readily distributes into the brain and adipose tissue upon absorption due to its lipophilicity (Ohlsson et al., 1986). Given the physiochemical properties of
CBD, its estimated apparent volume of distribution is very high, ranging from approximately 21,000 L to more than 42,000 L in healthy volunteers (Taylor et al., 2018). The lipophilicity of CBD suggests that absorption after oral administration may be mediated in part through uptake into the intestinal lymphatic system in addition to the hepatic portal vein via the mesenteric circulation (Taylor et al., 2018).

The distribution, time to effect, and duration of effect of CBD can vary by formulation. Inhaled formulations such as electronic cigarettes and inhaled cannabis, as well as oromucosal sprays, exhibit a significantly lower $T_{\text{max}}$ compared to oral formulations such as Epidiolex® and may avoid hepatic first-pass metabolism (Perucca and Bialer, 2020; Sholler, Spindle, et al., 2022). Orally administered products such as Epidiolex® and edibles display erratic absorption and a lag time that increases with dose, as well as a longer duration of action (Epidiolex®, 2018). A clinical study that evaluated the urinary PK profile of CBD and its metabolites in 11 male and 10 female participants found that the oral formulation significantly impacted the absorption and resultant $C_{\text{max}}$ of CBD: the sesame oil formulation of Epidiolex® exhibited the highest $C_{\text{max}}$, followed by an oral capsule and a syrup formulation (Sholler, Zamarripa, et al., 2022). In contrast, a randomized crossover study by Johnson et al. found no major differences in plasma CBD profiles in eight healthy males following oral administration of CBD-containing capsules versus sublingual drops, suggesting that sublingually delivered CBD may be swallowed prior to mucosal CBD absorption (Johnson et al., 2023). Though topical products containing CBD are popular in the consumer market, there are relatively few studies investigating the topical absorption of CBD. CBD transdermal absorption is thought to be limited by its hydrophobicity, which causes the
molecule to become trapped in the aqueous layer of the skin before reaching the circulation (Varadi et al., 2023). A recently published clinical study including 9 male and 9 female participants claims to be the first to demonstrate topical CBD absorption from a novel transdermal formulation containing equal amounts of CBD and THC (Varadi et al., 2023). The authors reported that the rate and extent of absorption was lower compared to similar doses administered orally or through inhalation, with large interindividual variability in PK for transdermal CBD and THC (Varadi et al., 2023). Recent work by MacNair et al. identified sex differences as a possible source of inter-individual variability in CBD pharmacokinetics (MacNair et al., 2023). In a randomized controlled trial with 17 male and 15 female participants receiving 120-480 mg of CBD orally over 7 days, metabolite-to-parent ratios of 7-OH-CBD and 7-COOH-CBD were found to differ with respect to both time and sex, with female participants showing greater plasma exposure to CBD metabolites over time (MacNair et al., 2023). The authors hypothesized that these findings may be due to sex differences in CBD metabolism or clearance, such as differences in enterohepatic elimination time and volume of distribution, or differences in CYP and P-gp expression (MacNair et al., 2023).

CBD is a high extraction drug with extensive hepatic first-pass metabolism. CBD clearance therefore primarily depends on hepatic blood flow rather than changes in plasma protein binding (Sun and Zhao, 2016). The predominant metabolic clearance pathways for CBD are hydroxylation by the cytochrome P450 (P450) enzyme system at the methyl group located on the 7' position of the substituted cyclohexene ring, or less frequently at one of the carbons in the pentyl side chain (see Fig. 1) (Jiang et al., 2011; Beers et al., 2021), as well as direct glucuronidation by UDP-glucuronosyltransferase
(UGT) enzymes (Havlasek et al., 2023). Of note, the major metabolite 7-hydroxy-CBD (7-OH-CBD) is pharmacologically active and contributes to the anticonvulsant effect of CBD (Whalley et al., 2017). 7-OH-CBD undergoes further oxidation to form the carboxylic acid 7-carboxy-CBD (7-COOH-CBD) (Harvey and Mechoulam, 1990). The most abundant circulating compounds in vivo following oral CBD administration are 7-COOH-CBD > CBD > 7-OH-CBD > 6α-OH-CBD (Taylor et al., 2018). The AUC of 7-COOH-CBD is as much as 40 times higher than that of the parent compound following a single oral dose of CBD, and the majority of CBD is excreted as oxidation products of 7-COOH-CBD and its glucuronide conjugates (Harvey and Mechoulam, 1990; Ujváry and Hanuš, 2016; Taylor et al., 2018).

Formation of the active metabolite 7-OH-CBD is mainly mediated by CYP2C19 and CYP2C9, whereas other monooxygenation products of CBD are generated predominantly by CYP3A4/5 (Jiang et al., 2011; Beers et al., 2021). While prior in vitro studies using human liver microsomes and recombinant P450s cite CYP3A4 as the major enzyme involved in 7-COOH-CBD formation (Epidiolex®, 2018), recent studies with human liver cytosol and S9 fraction have shown that 7-COOH-CBD formation is largely NAD⁺-dependent, with smaller contributions from NADPH-dependent enzymes (i.e., P450 enzymes) (Beers et al., 2023). In addition to P450 metabolism, CBD is glucuronidated by UGT1A3, UGT1A7, UGT1A8, UGT1A9, and UGT2B7 to form CBD-O-glucuronide as well as other minor glucuronidated metabolites (Mazur et al., 2009; Havlasek et al., 2023). Of note, both CBD and its metabolite 7-OH-CBD are known to participate in multiple pharmacokinetic drug-drug interactions (DDIs) mediated by CYPs and UGTs. CBD inhibits CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1,
and CYP3A4/5, as well as UGT1A9 and UGT2B7 *in vitro* (Nasrin *et al.*, 2021a-b). 7-OH-CBD has also been shown to inhibit CPY2C19 and CYP3A4/5 (Bansal *et al.*, 2022).

Though formation of an acyl glucuronide generated from 7-COOH-CBD is chemically feasible, this has yet to be demonstrated in the literature. Of note, the structural isomer THC is known to form an acyl glucuronide from its major metabolite 11-COOH-THC (Williams and Moffat, 1980; Huestis *et al.*, 2020). Another significant but less well-studied pathway of CBD metabolism is beta-oxidation of the pentyl side chain to yield carboxylic acids with fewer carbon atoms in the side chain, as reported by Harvey and Mechoulam, which were isolated from urine collected from an epileptic patient of unknown sex treated with CBD (Harvey and Mechoulam, 1990).

*In vitro* studies have reported that neither CBD nor 7-OH-CBD are substrates for major hepatic or renal transporters, including BCRP, BSEP, MDR1/P-gp, OAT1, OAT3, OCT1, OCT2, MATE1, MATE2-K, OATP1B1, or OATP1B3 (*Epidiolex*®, 2018). Similarly, 7-COOH-CBD was not found to be a substrate for BCRP, OATP1B1, OATP1B3, or OCT1, though it is a P-gp substrate (*Epidiolex*®, 2018). While the O-glucuronide of CBD has been reported as a major metabolite in human urine (Ujváry and Hanuš, 2016), to the author's knowledge, possible transporter interactions with this metabolite have not been studied. Due to their negative charge, glucuronide conjugates such as CBD O-glucuronide commonly require hepatic transporters for basolateral and/or biliary efflux (Smith and Dalvie, 2012; Järvinen *et al.*, 2021). Further work is needed to identify possible transporters involved in the clearance of glucuronide conjugates of CBD.

CBD is subject to prolonged elimination due to its high lipophilicity. The rate-limiting step for CBD elimination is its redistribution into the circulation following
extensive absorption into adipose tissue. The half-lives of CBD, 7-OH-CBD, and 7-COOH-CBD were 61, 32, and 22 h, respectively, after seven days of twice-daily oral dosing in 5 male and 4 female healthy volunteers (Taylor et al., 2018). In a clinical study with 14 male volunteers, the pharmacokinetics of CBD metabolites 7-OH-CBD, 6-OH-CBD, and 7-COOH-CBD were found to vary with CBD formulation and concomitant food intake in a manner similar to CBD (Abbotts et al., 2022). The apparent oral clearance (CL/F) of CBD is approximately 1111 L/h following a single 1500 mg oral dose in 1 male and 5 females (Taylor et al., 2018). CBD and its metabolites primarily undergo hepatobiliary clearance, with minor renal elimination (Jazz Pharmaceuticals, Inc, 2023). In an early mass-balance study, approximately 33% and 16% of the total CBD dose was recovered in feces and urine, respectively, 72 h after intravenous (IV) administration of a single 20 mg dose in 7-10 healthy males (Wall et al., 1976).

**Historical Perspective: Pharmacokinetic Models of CBD**

Model-based approaches, including population PK modeling (Pop PK) and physiologically-based PK modeling (PBPK), have been explored in the literature to understand the PK of CBD. These approaches provide efficient methods for comprehending the CBD PK profile, systemic and tissue availability, and drug exposure. The goal is to ensure optimal efficacy while minimizing undesired effects such as CBD-induced hepatotoxicity.

Some attempts have been made to characterize the PK of CBD using Pop PK (Epidiolex®, 2018; Lim et al., 2020; Liu et al., 2020; Schultz et al., 2022). Such models
offer insights into the interindividual variability of CBD exposure. Pop PK models were
included in the regulatory approval package for CBD (Epidiolex®, 2018). GW
Therapeutics employed data from a phase I ascending dose and food effect study to
develop a Pop PK model of CBD, 7-OH-CBD, and 7-COOH-CBD in healthy volunteers.
Similar models were constructed using phase III clinical data for patients with Dravet
and Lennox-Gastaut syndromes. In addition to characterizing the PK of CBD and its
major metabolites, these models aimed to assess the impact of clinical covariates on
CBD plasma concentrations. A two-compartment model was established for CBD
distribution, incorporating additional compartments for 7-OH-CBD and 7-COOH-CBD.
Using this model, estimates were generated for the food effect on bioavailability and
clearance values for CBD, 7-OH-CBD, and 7-COOH-CBD under fed and fasted
conditions. The model developed for healthy volunteers was also leveraged to evaluate
the effect of diverse CBD titration regimens on the simulated PK profile and extent of
exposure until the target dose was attained. While similar models were developed to
predict CBD exposure in populations with Dravet and Lennox-Gastaut syndromes,
some reviewers have raised concerns regarding the reliability of these models due to
the design of clinical trials. Furthermore, the models only encompass relatively high
doses (1500–6000 mg), potentially omitting the effects of low-dose administration.

Liu et al. developed a Pop PK model after inhaled vaporized CBD administration
in 31 male and 5 female participants (Liu et al., 2020). However, this model was based
on a single randomized, double-blind, crossover placebo-controlled clinical trial, where
many blood samples were collected within 1 hour after the main dose. As a result, the
model primarily addresses the distribution phase rather than the elimination phase.
Lim et al. established the CBD dose-exposure relationship and evaluated the impacts of dosage forms, food intake, and doses on CBD absorption (Lim et al., 2020). They employed a three-compartment model with a Weibull or zero-order absorption model to describe CBD disposition and absorption kinetics. This model provides valuable insights into the PK of various CBD formulations, including oral (capsule, solution) and oromucosal (spray, drops) preparations. However, due to the absence of individual subject data, the model could not account for other sources of variability, such as sex, body weight, food intake type, comorbidities, and between-subject variability.

Schultz et al. developed a three-compartment PK model with a chain of absorption transit compartments and first-order elimination, which adequately described CBD PK for both oral and IV administration (Schultz et al., 2022). However, this model did not encompass the major metabolites, and the clinical trial data were inadequate to fully estimate CBD clearance and volume parameters, given the prolonged terminal half-life of CBD.

Several PBPK models for CBD have also been developed, mainly to predict the potential for P450- and/or UGT-mediated DDIs. GW Therapeutics developed a PBPK model for CBD as a perpetrator for DDIs based on its physiochemical properties and clinical data, as well as in vitro studies with recombinant enzymes; however, the model did not consider the major metabolic enzyme CYP2C19 in CBD metabolism or the formation of 7-OH-CBD or 7-COOH-CBD, thus limiting its predictive power (Epidiolex®, 2018).

Another study by Qian et al. developed a PBPK model for both THC and CBD as perpetrators of drug interaction with methylphenidate, a commonly prescribed attention
deficit hyperactivity disorder (ADHD) medication metabolized by carboxylesterase 1 (CES1) (Qian and Markowitz, 2022). Although in vitro experiments indicated substantial inhibition of CES1 by CBD ($K_{i,u} = 0.091 \, \mu M$ in human liver S9), PBPK model simulations predicted only a mild increase in methylphenidate exposure with repeated oral CBD dosing. However, this study did not evaluate the potential for additional contribution to DDI by the metabolites of CBD and did not account for the contribution of UGTs to CBD metabolism.

Liu et al. outlined a PBPK model to predict the PK profiles of 17 cannabinoids using in vitro and in silico predicted values for physicochemical properties (Liu and Sprando, 2022). In contrast to prior studies, this PBPK model predicted CBD concentrations in both plasma and major organs. Following a single 30-mg oral dose, the highest $C_{\text{max}}$ of CBD was projected in the liver, followed by the brain, adipose tissue, kidneys, reproductive organs, heart, and plasma. Interestingly, concentrations of CBD were predicted to be more than 13 times greater in the liver than in plasma. However, the model lacked validation through additional data and struggled to accurately capture the plasma concentration curves of CBD.

A recent PBPK model by Yeung et al. was developed to assess the in vitro-determined contributions of drug-metabolizing enzymes to CBD metabolism and to simulate drug interaction scenarios for validating estimated contributions (Yeung et al., 2023). This model encompassed both oral and IV CBD administration and integrated existing literature data to predict the plasma PK profiles of CBD and 7-OH-CBD in a simulated drug interaction study. The estimated relative contributions of drug-metabolizing enzymes to CBD metabolic clearance were as follows: CYP3A4 (38%) >
CYP2C19 (21%) > UGT1A9 (16%) > CYP2C9 (11%) > UGT2B7 (10%) > UGT1A7 (4%) (Yeung et al., 2023). The model was able to reasonably predict AUC ratios in CBD measured from clinical drug interaction studies with CBD and itraconazole as a CYP3A4 inhibitor, fluconazole as a CYP2C19 inhibitor, and rifampicin as a CYP3A4 inducer. Nonetheless, it is important to note that a limitation of this model is that the absorption model was fitted to describe the data, thus, extrapolation to a new population will require careful consideration.

Interestingly, Bansal et al. developed an oral CBD PBPK model and got a different conclusion than other published literature (Bansal et al., 2023). They suggested that UGTs (UGT1A9/2B7) play a major role (80%) in CBD metabolism, surpassing P450s (20%) in contribution.

**Historical Perspective: Clinical Evidence and Risk Factors for CBD-Induced Hepatotoxicity**

Given the PK properties of CBD, several factors may influence the risk for CBD-induced liver injury, including interindividual variability in drug-metabolizing enzymes and transporters, possible reactive metabolite formation, interference with endogenous metabolic pathways, or drug metabolizing enzyme-mediated drug interactions. CBD has been shown to cause hepatotoxicity during phase I and II trials for Epidiolex® (Jazz Pharmaceuticals, Inc, 2023). Hepatotoxicity is a major adverse event that often limits drug development and is the leading cause of acute liver failure in the U.S. (Lee, 2003; Kaplowitz, 2005). During pivotal trials for Epidiolex®, alanine aminotransferase (ALT)
elevations greater than 3 times the upper limit of normal (ULN) were reported in 12-13% of clinical trial patients taking CBD (Jazz Pharmaceuticals, Inc, 2023). This clinical threshold is used to identify hepatocellular injury (U.S. Food and Drug Administration, 2009). ALT elevations resulted in treatment discontinuation or dose reduction in two-thirds of all affected patients in clinical trials (Jazz Pharmaceuticals, Inc, 2023).

While transaminase elevations were typically observed within the first two months of treatment initiation, this adverse event has been observed up to 18 months after starting treatment (Jazz Pharmaceuticals, Inc, 2023). The approved labeling for Epidiolex® includes a warning for hepatocellular injury associated with the drug and recommends monitoring serum transaminases and total bilirubin in all patients prior to starting treatment and repeatedly after initiation (Jazz Pharmaceuticals, Inc, 2023). Postmarketing trials are ongoing to investigate the risk for chronic liver injury with long-term CBD use; the estimated completion date for these trials is 2028 (U.S. National Library of Medicine, 2021).

CBD-induced hepatotoxicity is associated both with a higher daily dose of CBD and with concomitant use of valproate (valproic acid, VPA), another antiepileptic drug commonly co-prescribed with CBD (Jazz Pharmaceuticals, Inc, 2023). Transaminase elevations greater than three times the ULN were noted in 8% of patients receiving 10 mg/kg/day of CBD as opposed to 16% of patients receiving 20 mg/kg/day of CBD across controlled trials and the expanded access program for Epidiolex®, indicating dose-dependent toxicity (Epidiolex® Summary Review, 2018).

VPA is known to cause idiosyncratic drug-induced liver injury through a mechanism related to its metabolism. To provide necessary context, VPA metabolism
and toxicity will be discussed here briefly. The major pathway for VPA metabolic clearance is by glucuronidation (accounting for approximately 50% of the total dose), followed by β-oxidation (approximately 40% of dose) and P450 oxidation (approximately 10% of dose) (Dickinson et al., 1989; Argikar and Remmel, 2009; Ghodke-Puranik et al., 2013). VPA is metabolized by multiple UGT enzymes (mainly by UGT1A6, UGT1A9, and UGT2B7, though several other UGTs have been identified (Ethell et al., 2003; Guo et al., 2012; Ghodke-Puranik et al., 2013)) as well as by CYP2C9 and CYP2A6 (Sadeque et al., 1997; Kiang et al., 2006). One prevailing mechanistic hypothesis for VPA toxicity is related to formation of the unsaturated metabolite 4-ene-VPA by CYP2C9 (Kiang et al., 2006), which forms a 4-ene-VPA-CoA ester and is further metabolized to 2,4-diene VPA through mitochondrial β-oxidation (Ghodke-Puranik et al., 2013). The cytotoxic metabolite 2,4-diene VPA may then form a reactive CoA ester, which can be further conjugated with glutathione and form thiol conjugates (Kassahun et al., 1991, 1994; Ghodke-Puranik et al., 2013). VPA and metabolite conjugates with CoA may also contribute to depletion of CoASH and subsequent inhibition of β-oxidation (Li et al., 1991). The reactive metabolites of 4-ene VPA have been found to increase oxidative stress in hepatocytes, leading to mitochondrial permeability transition, cell death, and steatosis (Bjorge and Baillie, 1985; Begriche et al., 2011; Ghodke-Puranik et al., 2013). In addition, VPA has been shown to impair trafficking of the hepatic efflux transporter MRP2 to the cell surface, as well as disrupt hepatocyte polarization mediated by tight junction proteins (Fu et al., 2019).

Interestingly, several 7-COOH-CBD metabolite derivatives contain the structure of the major active and non-hepatotoxic metabolite of VPA, 2-ene-VPA (Kassahun et al.,
Both 7-COOH-CBD and 2-ene-VPA are metabolized by β-oxidation (Kassahun et al., 1994; Ujváry and Hanuš, 2016). This structural similarity has been posited as a possible contributor to the anticonvulsant activity of CBD, though this has not been validated in studies (Ujváry and Hanuš, 2016).

ALT elevations are estimated to occur in 5-10% of patients during VPA therapy (Valproate, 2012). The majority of these cases are asymptomatic and self-limiting (Valproate, 2012). The risk for VPA-mediated hepatotoxicity appears to be increased for children, particularly for those under two years of age treated with multiple medications and with concurrent metabolic disorders (Ghodke-Puranik et al., 2013). While the exact causes for increased hepatotoxicity in children remains uncertain, possible causes include decreased expression of UGTs compared to adults. Since glucuronidation is the predominant pathway for VPA clearance, this may lead to increased VPA exposure in mitochondria, thus contributing to toxic metabolite formation (Ghodke-Puranik et al., 2013). Induction of CYP metabolism by concomitant antiepileptic drugs such as CBD, carbamazepine, phenytoin, topiramate, or phenobarbital may also increase formation of toxic metabolites and contribute to hepatotoxicity (Ghodke-Puranik et al., 2013; Brodie et al., 2013). The estimated risk for fatal hepatotoxicity associated with VPA in this high-risk population is approximately 1 in 600 (Bryant and Dreifuss, 1996; Chateauvieux et al., 2010).

In a recent systematic review and meta-analysis of published clinical trials, CBD use was associated with an increased risk of liver enzyme elevation and drug-induced liver injury, with odds ratios of 5.85 and 4.82, respectively, compared to placebo (Lo et al., 2023). Risk factors included concomitant use of other antiepileptic drugs and CBD.
daily doses greater than or equal to 1000 mg or 20 mg/kg/day (Lo et al., 2023). The risk for liver enzyme elevation was not significantly different between adults and children (Lo et al., 2023). A major increase in the risk for liver injury was observed when both CBD and VPA were taken in pivotal clinical trials, resulting in ALT elevations greater than three times the ULN in 21% of patients versus 3% in patients taking CBD alone (Jazz Pharmaceuticals, Inc, 2023). Despite extensive P450 and UGT-mediated metabolism, the hepatotoxic interaction between CBD and VPA does not appear to be pharmacokinetic in nature; the exact mechanism remains unknown. A phase I DDI study with CBD and the antiepileptic medications VPA, clobazam, and stiripentol found that VPA exposure was not affected by either 7-day or 14-day oral administration of 1500 mg/day CBD in combination with 1000 mg/day VPA (Morrison et al., 2019). Likewise, multiple dose administration of VPA did not significantly affect the steady state plasma concentrations of CBD, 7-OH-CBD, or 7-COOH-CBD (Morrison et al., 2019). The DDI effects of CBD and VPA on their respective hepatic drug (and metabolite) concentrations are unknown.

**CBD-Induced Hepatotoxicity: Key Recent Advances and Proposed Mechanisms**

While the cause of CBD-mediated hepatotoxicity in combination with VPA remains unclear, the findings from several murine and *in vitro* studies may provide some insight into the mechanism(s). The following reports have investigated possible intracellular mechanism(s) of action of CBD for varying indications and identified potential sources of CBD toxicity. As described below, the proposed mechanisms of CBD toxicity are interrelated with overlapping pathways, genes, signaling molecules,
and enzymes involved. Overall, the available data suggest that CBD disrupts intracellular cholesterol and fatty acid metabolism, which appears to be associated with increased oxidative stress and mitochondrial toxicity.

Compared to published work with cells, relatively few studies have investigated CBD toxicity \textit{in vivo}. Recent work by Ewing et al. demonstrated CBD-induced hepatotoxicity in a mouse model (Ewing, McGill, \textit{et al.}, 2019; Ewing, Skinner, \textit{et al.}, 2019). Mice were given a single dose of 246 mg/kg CBD (an allometrically scaled dose equivalent to 20 mg/kg/day in humans), as well as 738 mg/kg and 2460 mg/kg to represent three times and ten times the recommended dose in humans, respectively. To induce sub-acute toxicity, mice were also given repeated doses of 61.5 mg/kg (equivalent to 5 mg/kg in humans), 184.5 mg/kg (equivalent to 15 mg/kg), and 615 mg/kg/day CBD for ten days. For mice treated with the highest acutely toxic dose, CBD was found to increase liver-to-body weight ratios, plasma ALT, AST, and bilirubin (Ewing, Skinner, \textit{et al.}, 2019). In the 615 mg/kg group, mice were terminated after 2-3 doses due to overt CBD toxicity (Ewing, Skinner, \textit{et al.}, 2019). Gene expression analysis also revealed dose-dependent increases in hepatic mRNA for multiple drug-metabolizing enzymes after both acute and chronic CBD administration, including \textit{Cyp1a1}, \textit{Cyp1a2}, \textit{Cyp2b10}, \textit{Cyp2e1}, \textit{Cyp3a4}, \textit{Cyp3a11}, and \textit{Ugt1a1} (Ewing, Skinner, \textit{et al.}, 2019). Across all treatment groups, the authors also noted dose-dependent upregulation of transcripts related to bile acid and drug transport and cholestasis, namely \textit{Abcb1a}, \textit{Abcc2}, and \textit{Abcc3}, accompanied by downregulation of genes related to fatty acid metabolism, including \textit{Car3}, \textit{Fabp1}, and \textit{Ppara} (murine peroxisome proliferator activated receptor alpha, known as PPAR-\(\alpha\) in humans) (Ewing, Skinner, \textit{et al.}, 2019).
A later study by the same group described a drug interaction in which CBD appeared to sensitize mice to acetaminophen (APAP)-induced hepatotoxicity (Ewing, McGill, et al., 2019). Following repeated administration of 116 mg/kg of a CBD-rich extract in sesame oil (equivalent to 10 mg/kg in humans) over three days, a single 400 mg/kg dose of APAP resulted in overt toxicity with 38% mortality, whereas no mortality was observed with APAP alone. This interaction was associated with increased Cyp2e1 expression, glutathione depletion, increased c-Jun N-terminal kinase activation (JNK, an enzyme activated by oxidative stress), and hepatic sinusoidal obstruction identified by histopathology (Ewing, McGill, et al., 2019).

A recent multi-omics-based in vitro study by Guard et al. demonstrated that CBD may cause toxicity through altered cholesterol metabolism (Guard et al., 2022). A fluorescence-based biosensor array was combined with transcriptomics, metabolomics, and proteomics to measure intracellular pathway changes induced following a single treatment with 20 µM CBD in transgenic cancer cell lines derived from keratinocytes and nerve tissue (Guard et al., 2022). The results revealed increases in cytosolic calcium concentrations, a finding that was previously reported by Olivas-Aguirre et al. and may be indicative of mitochondrial toxicity (Olivas-Aguirre et al., 2019). Additional reports have also shown that CBD modulates cytosolic calcium through interaction with TRPM8, TRPA1, TRPV1, and/or VDAC1 ion channels (Bisogno et al., 2001; De Petrocellis et al., 2008, 2011; Rimmerman et al., 2013; Bih et al., 2015).

CBD was also found to incorporate into cell membranes and alter cholesterol synthesis, transport, signaling, and insertion into cell membranes (Guard et al., 2022). While synthesis and storage of cholesterol were increased, membrane insertion of
cholesterol was reduced by CBD (Guard et al., 2022). The authors hypothesized that CBD incorporation into cell membranes altered the orientation of cholesterol by making its hydroxyl moiety more accessible for metabolism by oxidoreductase enzymes (Guard et al., 2022). Apoptosis caused by CBD was rescued by inhibiting cholesterol synthesis by pre-treatment with atorvastatin, a drug that inhibits the rate-limiting enzyme (HMG-CoA reductase) involved in cholesterol synthesis (Guard et al., 2022).

Prior studies also suggested that CBD dysregulates cholesterol metabolism through altered gene expression. CBD has been shown to upregulate murine CYP27a1 transcription (Rimmerman et al., 2011). The CYP27a1 gene encodes sterol 27-hydroxylase, a mitochondrial P450 enzyme involved in cholesterol metabolism and bile acid synthesis in mice and humans (Rimmerman et al., 2011; Jeon, 2016). In an in vivo toxicity study, gene expression of murine lanosterol synthase (Lss), a key enzyme in cholesterol and steroid hormone synthesis, was upregulated following exposure to lower doses of CBD (equivalent to 20 and 60 mg/kg CBD in humans) and downregulated with the highest dose of CBD tested (equivalent to 200 mg/kg in humans) (Ewing, Skinner, et al., 2019). An increase in 5' adenosine monophosphate-activated protein kinase (AMPK) activity was also observed following treatment with CBD, resulting in decreased fatty acid production, increased fatty acid β-oxidation, and upregulation of antioxidant defense genes, including NFE2L2 (nuclear factor erythroid 2-related factor 2, also known as NRF2) (Guard et al., 2022). AMPK is a highly conserved enzyme with multiple signaling functions related to energy homeostasis, including fatty acid oxidation, glucose uptake, sterol synthesis and lipogenesis, and modulation of insulin release (Winder and Hardie, 1999).
In another study using BV-2 murine microglial cells, inhibition of the voltage-dependent anion-selective channel 1 (VDAC1) was identified as a direct cause of CBD-induced cytotoxicity (Rimmerman et al., 2013). VDAC1 is a voltage-dependent ion channel that enables the flux of ions, nucleotides, and metabolites across the outer mitochondrial membrane in its open state and serves as a major mediator for regulating calcium levels between mitochondria and the cytosol (Hodge and Colombini, 1997; Gincel et al., 2001; Shoshan-Barmatz and Mizrachi, 2012). CBD was found to inhibit VDAC1 conductance, which induced a transient increase in intracellular calcium, followed by a larger prolonged increase in cytosolic calcium, leading to loss of mitochondrial membrane potential, generation of reactive oxygen species (ROS), and cell death (Rimmerman et al., 2013). Interestingly, cholesterol has been shown to increase VDAC1 conductance (Popp et al., 1995), and the 3D structure of VDAC1 indicates that cholesterol is necessary for proper VDAC1 protein folding and insertion into the outer mitochondrial membrane (Hiller et al., 2008). It is possible that the toxicity observed by Rimmerman et al. and Guard et al. may have a shared mechanism associated with altered cholesterol metabolism and membrane insertion and subsequent reduced VDAC1 conductance (Rimmerman et al., 2013; Guard et al., 2022).

In addition to disrupting equilibrium calcium concentrations, CBD has demonstrated varying effects on mitochondria across multiple reports in the literature. Most studies have focused on the effect of CBD in cancer cells, and the majority of these have reported activation of mitochondria-led intrinsic apoptosis following CBD treatment in varying cancer cell lines [described in (Massi et al., 2006; McKallip et al., 2006; Shrivastava et al., 2011; Jeong, Jo, et al., 2019; Jeong, Yun, et al., 2019; Chan]
and Duncan, 2021]). Implicated mechanisms of action for apoptosis included activation of pro-apoptotic caspase enzymes (Massi et al., 2006; McKallip et al., 2006; Shrivastava et al., 2011; Jeong, Yun, et al., 2019; Olivas-Aguirre et al., 2019), generation of ROS species (Massi et al., 2006; McKallip et al., 2006; Shrivastava et al., 2011; Jeong, Yun, et al., 2019), and cytochrome C release (Massi et al., 2006; McKallip et al., 2006; Olivas-Aguirre et al., 2019), among others.

In Jurkat cells (an immortal T-cell derived human cell line) treated with 30 µM CBD, a rapid loss in mitochondrial membrane potential was observed and accompanied by increased release of the pro-apoptotic factor cytochrome C (Olivas-Aguirre et al., 2019). A dose-dependent increase in intracellular calcium, followed by mitochondrial calcium overload and mitochondrial permeability transition pore formation was also observed (Olivas-Aguirre et al., 2019). Collectively, these findings support the mechanistic hypothesis of altered VDAC1 conductance in CBD-induced toxicity. It is important to note, however, that the concentrations used in these studies may not reflect in vivo concentrations, and the observed effects of CBD may vary in other cell types.

**Current Challenges and Knowledge Gaps**

While the studies described above have given some important insights into the possible mechanism(s) of CBD-induced liver injury, many knowledge gaps remain, and some limitations have yet to be fully addressed. As mentioned previously, most in vitro studies have been performed in immortal cell lines; thus, results may not be directly translatable to human hepatocytes due to differential gene expression, including but not
limited to varying expression and activity of drug-metabolizing enzymes and transporters. Results may also be difficult to interpret due to differing cell lines, CBD concentrations, and exposure durations tested. Many of the CBD doses tested greatly exceed the estimated total plasma $C_{\text{max}}$ of CBD in vivo (approximately 2 µM fasted and 11 µM in a fed state) (Epidiolex®, 2018); nonetheless, the intracellular hepatic concentrations of CBD may be higher compared to plasma concentrations.

One additional hypothesis for CBD-induced hepatotoxicity that has not been studied is the potential for bile acid-mediated liver injury. This may occur through inhibition of BSEP, a biliary ATP-dependent efflux transporter that is largely responsible for the removal of toxic bile acids from hepatocytes into the bile canaliculi (Cheng et al., 2016). Inhibition of this transporter has been associated with hepatic accumulation of bile acids and subsequent cholestatic drug-induced liver injury. BSEP expression is regulated through the nuclear farnesoid X receptor (FXR) and is inducible through activation of the NRF2 antioxidant response pathway (Ananthanarayanan et al., 2001; Weerachayaphorn et al., 2009). As previously stated, 7-COOH-CBD is a known inhibitor of BSEP, and CBD has been shown to alter NFE2L2 expression (Guard et al., 2022; Jazz Pharmaceuticals, Inc, 2023). Additional work may therefore be warranted to investigate whether inhibited biliary efflux influences the risk for CBD-induced hepatotoxicity.

Finally, despite the fact that patients taking CBD are commonly co-prescribed VPA for seizure management (Devinsky et al., 2017, 2018), very few studies have sought to examine mechanisms of the hepatotoxic DDI observed between CBD and VPA in clinical trials.
Perspective on Future Directions

Future efforts to understand CBD-induced toxicity may benefit by integrating current knowledge with emerging modeling techniques for DILI prediction. Quantitative systems toxicology (QST) uses differential equations to combine known mechanisms of DILI (e.g., mitochondrial dysfunction, reactive metabolite generation, autoimmune activation, ROS production, and bile acid accumulation) with clinical PK and pharmacodynamic data to identify factors contributing to DILI (Watkins, 2019). One recent report combined a PBPK model of CBD developed from clinical trial data with the QST modeling software DILIsym® to predict CBD-induced elevations in ALT with and without concomitant VPA (Lakhani et al., 2023). The resulting simulations suggested that CBD-induced hepatotoxicity is associated with generation of ROS for patients taking VPA who began CBD therapy (Lakhani et al., 2023). These findings could be used to inform future mechanistic studies.

Multi-omics techniques such proteomics, transcriptomics, and metabolomics may also be used not only to discern between different clinical hepatotoxicity phenotypes (i.e., cholestatic, hepatocellular, or mixed), but also to identify intracellular mechanisms of DILI (Quintás et al., 2021). Using principal component analysis, different classes of metabolites may be identified with respect to different mechanisms of liver injury. In a metabolomic analysis of serum from 79 patients with DILI, Quintás et al. identified changes in bile acids, glycerophosphocholines, amino acids, and steroidal glycosides as discriminant of cholestatic DILI versus hepatocellular or mixed DILI (Quintás et al., 2021). Several mitochondrial genes and proteins have also been identified as potential
circulating biomarkers capable of identifying DILI associated with mitochondrial injury (Shi et al., 2015).

Conclusions

In this review, current knowledge of CBD metabolism, pharmacokinetics, modeling efforts, and studies examining CBD-induced hepatotoxicity were described to understand possible factors that contribute to CBD-induced liver injury and inform future research. While multiple pharmacokinetic models for CBD exist for predicting exposure to CBD and metabolites, relatively few models have been developed with special populations, and few have sought to examine risk factors for CBD-induced liver injury. Although current evidence suggests that CBD may cause hepatotoxicity by interfering with lipid metabolism and mitochondrial function, further work is needed to confirm these observations using cells derived from human liver tissue. Ultimately, these findings combined with advances in PBPK and quantitative systems toxicology-based modeling approaches may be used to help predict and prevent hepatotoxicity for the many patients and consumers who use CBD.
Data Availability Statement

The authors declare that all findings described in this minireview are previously published and cited in the References. No new data were generated or analyzed in this minireview.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: J.L. Beers, Z. Zhou, and K.D. Jackson.
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Footnotes

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Figure Legends

**Figure 1.** Simplified pathway of CBD metabolism. Figure adapted with permission from Beers et al. (2021). The active metabolite 7-OH-CBD is generated by CYP2C9 and CYP2C19, and formation of the major circulating metabolite 7-COOH-CBD is catalyzed by CYP3A and ALDH enzymes (Beers *et al.*, 2021; Beers *et al.*, 2023; Jiang *et al.*, 2011). CBD-O-glucuronide is formed by UGT1A7, UGT1A9, and UGT2B7 (Mazur *et al.*, 2009). Minor hydroxylated metabolites are generated primarily by CYP3A4/5 (Jiang *et al.*, 2011).
Figure 1