

# Assessing Liver Effects of Cannabidiol and Valproate Alone and in Combination Using Quantitative Systems Toxicology

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In clinical trials of cannabidiol (CBD) for the treatment of seizures in patients with Dravet syndrome, Lennox–Gastaut syndrome, and tuberous sclerosis complex, elevations in serum alanine aminotransferase (ALT) > 3× the upper limit of normal were observed in some patients, but the incidence was much greater in patients who were receiving treatment with valproate (VPA) before starting CBD. To explore potential mechanisms underlying this interaction, we used DILIsym, a quantitative systems toxicology model, to predict ALT elevations in a simulated human population treated with CBD alone, VPA alone, and when CBD dosing was starting during treatment with VPA. We gathered *in vitro* data assessing the potential for CBD, the two major CBD metabolites, and VPA to cause hepatotoxicity via inhibition of bile acid transporters, mitochondrial dysfunction, and production of reactive oxygen species (ROS). Physiologically-based pharmacokinetic models for CBD and VPA were used to predict liver exposure. DILIsym simulations predicted dose-dependent ALT elevations from CBD treatment and this was predominantly driven by ROS production from the parent molecule. DILIsym also predicted VPA treatment to cause ALT elevations which were transient when mitochondrial biogenesis was incorporated into the model. Contrary to the clinical experience, simulation of 2 weeks treatment with VPA prior to introduction of CBD treatment did not predict an increase of the incidence of ALT elevations relative to CBD treatment alone. We conclude that the marked increased incidence of CBD-associated ALT elevations in patients already receiving VPA is unlikely to involve the three major mechanisms of direct hepatotoxicity.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ A high incidence of serum alanine aminotransferase (ALT) elevations (i.e., potential liver injury) was observed in clinical trials when patients receiving treatment with valproate (VPA) started treatment with cannabidiol (CBD). Because the mechanisms underlying this observation are not known, it is not possible to identify other drug treatments or patient conditions with potential effects like VPA in patients who start treatment with CBD.

### WHAT QUESTION DID THIS STUDY ADDRESS?

✓ This study used quantitative systems toxicology (QST) modeling to explore mechanisms whereby treatment with VPA could increase susceptibility to CBD-induced ALT elevations.

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

✓ Our modeling identified specific mechanisms that could account for ALT elevations produced when patients are treated with either VPA or CBD alone. However, the modeling could not account for the increased ALT elevations in patients treated with both drugs, suggesting the major liver toxicity mechanisms are not involved.

### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

✓ Our study is an example of how QST modeling can identify mechanisms both underlying and not underlying adverse liver effects of drugs.

Plant-derived highly-purified cannabidiol (CBD), marketed as Epidiolex in the United States and as Epidyolex in the European Union, is an approved medicine for the treatment of seizures associated with Dravet Syndrome (DS), Lennox–Gastaut Syndrome (LGS), and tuberous sclerosis complex.<sup>1–8</sup> During clinical trials, some patients receiving CBD experienced elevations in serum alanine aminotransferase (ALT) raising concerns regarding liver

safety.<sup>2–5,9–13</sup> These ALT elevations were accompanied by normal serum alkaline phosphatase and bilirubin values consistent with injury to hepatocytes (i.e., hepatocellular injury).<sup>9</sup> There was a clear dose-dependence, with transaminase elevations observed in 2–7% of patients with DS/LGS treated with ≤ 10 mg/kg/day CBD<sup>1–3</sup> vs. 7–15% of patients with DS/LGS treated with ≥ 20 mg/kg/day.<sup>1,3</sup> In these studies, no clinically serious drug-induced liver injury (DILI)

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cases occurred; that is, there were no potential Hy's Law cases, defined as patients experiencing elevations in serum of both ALT > 3× upper limit of normal (ULN) and total bilirubin > 2× ULN.<sup>1–3,5</sup> However, patients in the clinical trials underwent frequent liver chemistry testing and some patients discontinued treatment due to elevations in serum ALT. For this reason, routine liver chemistry monitoring is recommended for all patients receiving Epidiolex.

An important observation in the pivotal DS/LGS randomized controlled trials was that the incidence and severity of aminotransferase elevations was markedly higher in those patients who were receiving treatment with valproate (VPA) when they started treatment with CBD<sup>1–4</sup>; in one open label extension study, the incidence of ALT elevations > 3× ULN was 31% in patients receiving concomitant treatment with CBD and VPA compared with no elevations > 3× ULN in patients receiving CBD who were not receiving VPA.<sup>2</sup> Two clinical trials have failed to show a significant pharmacokinetic (PK) interaction between VPA and CBD.<sup>11,14</sup>

It is important to identify the mechanisms underlying the liver effects of VPA and CBD in order to identify other potential drugs or patient characteristics that might have similar effects in patients treated with CBD. To this end, we first gathered *in vitro* data assessing the potential for CBD, the two major CBD metabolites (7-COOH-CBD and 7-OH-CBD), and VPA to induce any of the three major mechanisms of hepatotoxicity: inhibition of bile acid (BA) transporters, interference with mitochondrial function, and production of reactive oxygen species (ROS). Next, we built a physiologically-based PK (PBPK) model of CBD and VPA to predict liver exposure during therapeutic dosing. Finally, we integrated the *in vitro* toxicity assay results with the exposure predictions from the PBPK simulations using DILIsym, an *in silico* quantitative systems toxicology model.

## METHODS

### DILIsym overview

DILIsym is a mathematical representation of DILI<sup>15–18</sup> that has been extensively used for evaluating the hepatotoxicity of drugs.<sup>19,20</sup> Briefly, DILIsym integrates multiple submodels (e.g., hepatocyte life cycle, mitochondrial dysfunction and toxicity, BA disposition, and biomarker release) to simulate an organism-level response. The DILIsym software has been developed by the DILI-sim Initiative, which is a public-private partnership among scientists in academia, industry, and the US Food and Drug Administration (FDA).<sup>17,21</sup> In the current study, DILIsym version 8A Patch 2 was used for all mechanistic toxicity simulations.

### PBPK modeling

GastroPlus version 9.7 was used to build PBPK representations for CBD and VPA. The PBPK models were used to describe the dynamics of each compound and its metabolites in humans in both liver and blood. Predicted drug concentrations were exported to DILIsym by utilizing the “DILIsym mode” in GastroPlus to generate “specified data” (e.g., plasma and liver concentration-time profiles) for import into DILIsym.

The PBPK representation of CBD and its metabolites (6-OH-CBD, 7-OH-CBD, and 7-COOH-CBD) was optimized using plasma exposure data from patients receiving 1,500 mg b.i.d. provided by GW Pharmaceuticals (study GWEP1544<sup>22</sup>; Figure S3); the model was then validated against the plasma exposure data from patients receiving 750 mg b.i.d. data (Figure S4).

The PBPK representation of VPA was based on data presented in published studies. Plasma exposure data from 800 mg i.v. bolus and 800 mg p.o.

single dose<sup>23</sup> were used to optimize PBPK model parameters (Figure S2). The constructed PBPK model was validated against data obtained from patients receiving oral dosing of VPA 1,000 mg b.i.d.,<sup>24</sup> which was not used during the optimization process (Figure S6).

### Mitochondrial dysfunction input data

DILIsym represents mitochondrial bioenergetics leading to adenosine triphosphate (ATP) production in hepatocytes. Mitochondrial function in other liver cell populations (e.g., Kupffer cells) is not represented. Compounds may induce mitochondrial dysfunction by inhibiting the electron transport chain (ETC), by inhibiting the mitochondrial F<sub>1</sub>F<sub>0</sub> ATPase, or by uncoupling mitochondrial respiration from mitochondrial ATP synthesis. Compound effects on hepatocyte mitochondrial function can be assessed in laboratory experiments by measuring hepatocyte respiration following culture with compound in a Seahorse XF Analyzer (Agilent Technologies, CA).

To assess the potential mitochondrial liabilities of CBD, 7-COOH-CBD, 7-OH-CBD, and VPA, *in vitro* respiration data were collected in HepG2 cells incubated with various compound concentrations up to 30 μM for 1 or 24 hours. The peak plasma concentrations of CBD in clinical studies are around 3 μM following a 1,500 mg dose of CBD given with a high fat meal<sup>22</sup>; therefore, some of the tested concentrations can be considered supratherapeutic. Importantly, the compound concentration driving an intracellular response (i.e., mitochondrial respiration) may not be equivalent to the nominal media concentration reported in the assay protocol,<sup>25</sup> where the nominal media concentration is defined as the reported (but not measured) concentration of compound in the media. To more closely describe the relationship between concentration at the site of action and effect, the intracellular compound concentrations in parallel HepG2 cultures were assessed via liquid chromatography tandem mass spectrometry (LC-MS/MS). Mitochondrial dysfunction assays were performed by Cyprotex (Macclesfield, UK).

A companion mechanistic mathematical model, MITOSym, which simulates *in vitro* hepatocellular respiration, was designed to reproduce data obtained via the Seahorse assay for the purposes of deriving parameters characterizing compound-induced mitochondrial dysfunction.<sup>26</sup> MITOSym was used to determine parameter values for CBD-, 7-COOH-CBD-, and 7-OH-CBD-mediated-ETC inhibition and to determine parameter values for VPA-mediated uncoupling and VPA-mediated-ETC inhibition. *In vitro* MITOSym parameters were subsequently translated to *in vivo* DILIsym parameters.

### ROS input data

DILIsym represents the generation of ROS in response to compound exposure. Excessive ROS accumulation can ultimately reduce mitochondrial ATP synthesis, leading to hepatocyte death, as well as directly lead to caspase activation and apoptosis. There are several experimental methods that can be used to measure compound-induced oxidative stress, including assays for lipid peroxidation (e.g., thiobarbituric acid reactive substances assay), indirect measurement of superoxide (e.g., dihydroethidium (DHE) fluorescence assay), and indirect measures of multiple ROS (e.g., 2',7'-dichlorofluorescein diacetate fluorescence assay; hydroxyphenyl fluorescein fluorescence assay).

The potential for CBD, 7-COOH-CBD, 7-OH-CBD, and VPA to induce oxidative stress was assessed by high content screening using a fluorescent probe, DHE, in HepG2 cells incubated with various compound concentrations for 6 or 24 hours. Intracellular compound concentrations in parallel HepG2 cultures were assessed via LC-MS/MS. These assays were performed by Cyprotex (Macclesfield, UK).

### BA transporter input data

DILIsym represents BA enterohepatic circulation via BA transporters. More specifically, hepatocyte uptake of BAs from blood occurs by NTCP-mediated transport. BA efflux occurs via BSEP canalicular transport and

multidrug resistance-associated proteins 3 and 4 (MRP3 and MRP4) basolateral transport. Drug-mediated inhibition of efflux transporters may result in hepatocellular BA accumulation, culminating in BA-mediated toxicity, whereas drug-mediated inhibition of NTCP can mitigate this effect. Elevated intracellular BA concentrations can alter mitochondrial function, leading to hepatocyte death.<sup>27</sup> Drug-mediated inhibition of transporters can be assessed in laboratory experiments using cells or membrane vesicles expressing the transporter of interest.

To assess the functional impact of CBD and its major metabolites 7-COOH-CBD, and 7-OH-CBD on human BSEP, human MRP3, and human MRP4, *in vitro* vesicular transport inhibition assays were conducted. To assess the functional impact of CBD, 7-COOH-CBD, and 7-OH-CBD interaction with the human SLC (uptake) transporter NTCP, *in vitro* experiments were performed using CHO cells expressing NTCP. MRP3, MRP4, and NTCP transporter inhibition assays were performed by Solvo Biotechnology (Budaors, Hungary). BSEP inhibition data was provided by GW Pharmaceuticals.

BA transport inhibition data was not collected for VPA because previously published data indicated no transporter inhibition for this compound.<sup>28–30</sup>

### SimPops

SimPops are collections of simulated individuals with parameter variability designed to reflect appropriate biochemical and anthropometric ranges. These collections were used to understand the role of interindividual variability in simulated hepatotoxicity responses. Specifically, this study used two existing SimPops within DILIsym. First, Human\_ROS\_apop\_mito\_BA\_v8A\_1 (v8A\_1) was used to simulate responses in normal healthy volunteers (NHVs); this SimPops includes variability in mitochondrial function, caspase activation (apoptosis), BA transporter expression, and oxidative stress (ROS/reactive nitrogen species) susceptibility. The list and range of DILIsym parameters varied in the v8A\_1 SimPops ( $n = 285$ ) are shown in [Table S9](#). Second, Human\_ROS\_apop\_mito\_BA\_Biogenesis\_v8A\_5 (v8A\_5) was used to simulate responses in NHVs with the “mitochondrial biogenesis” submodel turned on. This additional submodel follows research showing that mitochondria can adapt to bioenergetic stress or other mitochondrial duress, by increasing their number in the cell (i.e., biogenesis). This adaptation has been well-studied in muscle cells,<sup>31</sup> and there is evidence that biogenesis occurs in mouse hepatocytes following exposure to rotenone (a well-known ETC inhibitor).<sup>32</sup> Mitochondrial biogenesis was parameterized in DILIsym based on data obtained in clinical trials for solithromycin where ALT elevations were observed to resolve despite continued treatment with this antibiotic (inhibition of mitochondrial function was the primary mechanism accounting for solithromycin induced elevations in serum ALT).<sup>33</sup> The list and range of DILIsym parameters varied in the v8A\_5 SimPops ( $n = 285$ ) are shown in [Table S10](#).

### SimCohorts

SimCohorts are relatively small populations consisting of a subset of simulated individuals from existing SimPops in DILIsym. This work used 4 SimCohorts, which were created from grouping the 285 individuals in the v8A\_5 SimPops by body weight ([Table S11](#), [Figure S9](#)). These SimCohorts were needed to overcome a technical challenge so that both dose-titration and (approximate) weight-based dosing could be simulated (see [Supplementary Methods](#)).

### Simulation protocols

The simulated dosing protocols for CBD were based on three different maintenance dose levels (5 mg/kg b.i.d., 10 mg/kg b.i.d., and 12.5 mg/kg b.i.d.) and two different meal conditions (entirely fasted or entirely fed). Consistent with clinical CBD dosing protocols, treatment was started at a dose of 2.5 mg/kg b.i.d. for the first week, and the dose was

increased by 2.5 mg/kg b.i.d. each week until the maintenance dose level was reached. VPA dosing was simulated at a maintenance dose of 10 mg/kg b.i.d., which was reached by dosing 5 mg/kg b.i.d. for the first week and increasing to the maintenance dose on the second week. Concomitant dosing of CBD and VPA was simulated in DILIsym by titrating VPA for the first 2 weeks with the above titration schedule to a maintenance dose of 10 mg/kg/day b.i.d.; subsequently, CBD dosing began on day 14 with the above titration schedule to a maintenance dose of 12.5 mg/kg b.i.d.

### Mechanistic investigation simulations

We performed a mechanistic investigation to estimate to what degree each chemical species (CBD, 7-OH-CBD, or 7-COOH-CBD) and each mechanism of toxicity (BA transporter inhibition, mitochondrial dysfunction, or oxidative stress) contributes to the overall simulated liver toxicity. That is, we repeatedly simulated the dosing protocol wherein CBD is titrated to 12.5 mg/kg b.i.d. under fed conditions, and systematically omitted the toxicity impact of one chemical species or omitted one mechanism of toxicity entirely. The simulations were run using the lowest body weight (50–65 kg) SimCohort ( $n = 46$ ; see [Supplementary Methods](#)). ALT elevations were then compared between simulations with an omission and the control simulation (i.e., no omissions, with all molecular entities and all mechanisms present). If a simulation with an omission had a lower frequency of ALT elevation than the control simulation, then this result indicates that the omitted compound or mechanism contributes to simulated toxicity. The degree of contribution is estimated to be proportional to the reduction in frequency of ALT elevations.

## RESULTS

### PBPK modeling

Baseline simulation results from the GastroPlus PBPK representation of CBD following 1,500 mg b.i.d. oral dosing are compared with clinical plasma data in [Figure S3](#). Additionally, calculated PK parameters from this fit are summarized in [Tables S6 and S8](#). Validation simulation results of 750 mg b.i.d. oral dosing are shown in [Figure S4](#); the corresponding PK parameters are summarized in [Tables S7 and S8](#). Population level PBPK simulation results show that, with the typical variation in model parameter values, the model provides adequate coverage of observed clinical data ([Figures S7, S8](#)). These figures and tables demonstrate the accuracy of the PBPK model in predicting the exposure of CBD and its metabolites (7-COOH-CBD and 7-OH-CBD) across b.i.d. dosing regimens. This PBPK model was used to simulate entirely fasted or entirely fed CBD exposure in a population of 285 individuals.

For validation of the GastroPlus PBPK predictions for VPA, the model was assessed against multiple dose clinical data ([Figure S2C](#)). PK parameters for the VPA PBPK model are summarized in [Table S3](#). Population level PBPK simulation results show that, with typical variation in model parameter values, the model has good coverage of observed clinical data ([Figure S6](#)). These figures and tables show the ability of the PBPK model to predict the exposure of VPA across various dosing routes and regimens. This PBPK model was used to simulate VPA exposure in a simulated population of 285 individuals.

### Toxicity parameters

In the mitochondrial respiration assay, CBD, 7-COOH-CBD, and 7-OH-CBD were found to decrease the HepG2 oxygen

consumption rate (OCR; **Figure S12**), suggesting that CBD, 7-COOH-CBD, and 7-OH-CBD are each inhibitors of the ETC. OCR response data, along with measured intracellular concentrations, were used to calculate DILI<sub>sym</sub> ETC inhibition parameters for CBD, 7-COOH-CBD, and 7-OH-CBD (**Table 1**). In the mitochondrial respiration assay, VPA decreased the HepG2 OCR at low concentrations, but increased OCR at higher concentrations (**Figure S10B**). These results suggest that concentrations < 10 mM of VPA inhibit ETC activity; however, at higher concentrations, VPA behave as an uncoupler of oxidative phosphorylation (i.e., inhibition of ATP synthesis without inhibition of the ETC). OCR response data, along with measured intracellular concentrations of VPA, were used to calculate DILI<sub>sym</sub> ETC inhibition and mitochondrial uncoupling parameters for VPA (**Table 1**).

In the oxidative stress assay, CBD, 7-COOH-CBD, and 7-OH-CBD were found to increase ROS in a concentration-dependent manner, suggesting that these molecular species can each elicit oxidative stress (**Figure S13**). In contrast, there was no evidence of VPA-driven generation of oxidative stress (**Figure S10A**). DILI<sub>sym</sub> parameters for CBD-, 7-COOH-CBD-, and 7-OH-CBD-induced ROS production were optimized to recapitulate intracellular concentration vs. cellular ROS data by simulating *in vitro*-like conditions within DILI<sub>sym</sub> (**Figure S13, Table 1**).

Transporter inhibition studies indicated that CBD and 7-COOH-CBD each inhibited 4 BA transporters: BSEP, MRP3, MRP4, and NTCP; whereas 7-OH-CBD inhibited BSEP and MRP3 (**Figures S11, S14, S15**). The BA transporter inhibition constants for CBD, 7-COOH-CBD, and 7-OH-CBD were used directly in DILI<sub>sym</sub> (**Table 1**). To investigate the greatest possible

inhibition of basolateral efflux transport (i.e., the most toxicity), the lower of the two half-maximal inhibitory concentration values for MRP3 and MRP4 was used for the basolateral efflux transport inhibition constants for CBD and 7-COOH-CBD. BA transporter inhibition data was not collected for VPA because previously published data indicated no transporter inhibition by this compound.<sup>28–30</sup>

### Toxicity investigations

Simulations conducted with CBD alone at different dosing levels (maintenance doses of 5 mg/kg b.i.d., 10 mg/kg b.i.d., or 12.5 mg/kg b.i.d.) under the fed condition predicted a dose-dependent increase in the frequency of ALT elevations in the v8A\_1 SimPops (**Figure 1, Table 2**). Simulations (of CBD 12.5 mg/kg b.i.d.) under the fed condition predicted significantly higher frequency of ALT elevations than under the fasted condition (**Figure 1a, Table 2**).

Simulation results with VPA alone, titrated to a maintenance dose of 10 mg/kg b.i.d. in the mitochondrial biogenesis (v8A\_5) SimPops were consistent with clinical observations (5–10% with ALT > 3× ULN<sup>34</sup>): ALT levels resolved upon continued dosing (**Figure 2, Table 2**) due to mitochondrial biogenesis.

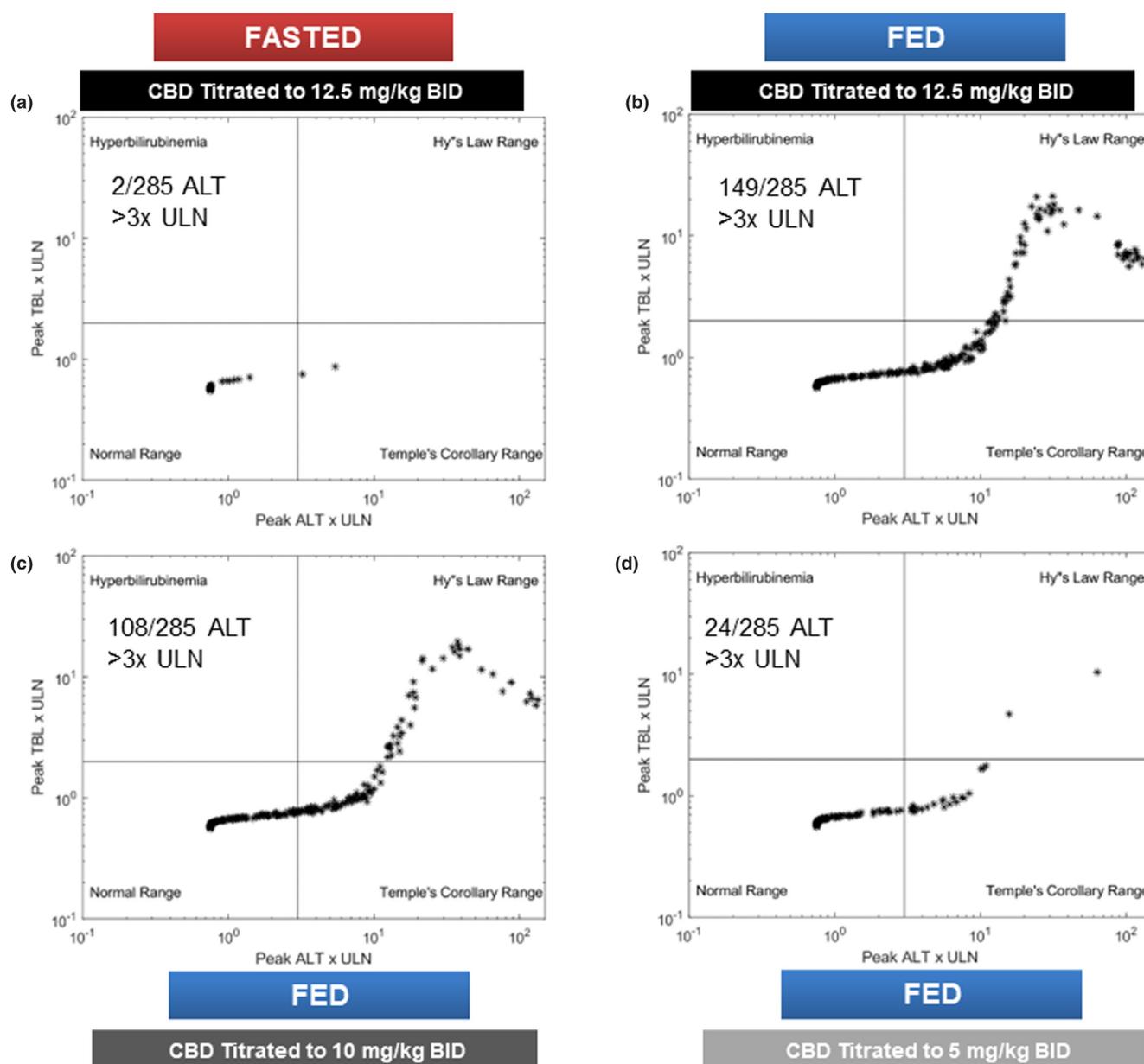
In simulating concomitant dosing of CBD and VPA, the VPA was first dosed alone for 2 weeks (to reach a maintenance dose of 10 mg/kg b.i.d.), followed by the addition of CBD (titrated to a maintenance dose of 12.5 mg/kg b.i.d.) under fasted conditions in the mitochondrial biogenesis (v8A\_5) SimPops. The predicted frequency of ALT elevations > 3× ULN after starting CBD 2 weeks after starting treatment with VPA did not differ from the frequency predicted when CBD dosing was simulated

**Table 1** DILI<sub>sym</sub> toxicity parameter values for CBD and VPA

Mechanism	DILI <sub>sym</sub> parameter	VPA <sup>a</sup>	CBD <sup>a</sup>	7-OH-CBD <sup>a</sup>	7-COOH-CBD <sup>a</sup>	Unit
Bile acid transporter inhibition <sup>b</sup>	BSEP inhibition constant	Published data indicate no transporter inhibition for VPA <sup>28–30</sup>	39.8	14.4	96.1	μM
	NTCP inhibition constant		79.7	—	73.7	μM
	Basolateral inhibition constant <sup>c</sup>		522.5	132.6	21.5	μM
Mitochondrial dysfunction	Coefficient for Uncoupler 1	2.24 × 10 <sup>5</sup>	—	—	—	μM
	Coefficient for ETC inhibition 1	—	—	347	3.47 × 10 <sup>3</sup>	μM
	Coefficient for ETC inhibition 3	3.47	174	10.4	1.74 × 10 <sup>3</sup>	μM
	Max inhibition by ETC inhibition 3	0.56	0.834	0.424	0.924	dimensionless
Oxidative stress	ROS 1 Production Rate	—	—	8 × 10 <sup>-4</sup>	—	mL/nmol/hour
	ROS 4 V <sub>max</sub>	—	0.25	—	0.06	1/hour
	ROS 4 km	—	40	—	250	μM
	ROS 4 Hill	—	2	—	3	Dimensionless

CBD, cannabidiol; DILI, drug-induced liver injury; DSS, DILI<sub>sym</sub> Services Inc; ETC, electron transport chain; IC<sub>50</sub>, half-maximal inhibitory concentration; ROS, reactive oxygen species; V<sub>max</sub>, maximum value; VPA, valproate.

<sup>a</sup>Values shown in the table for DILI<sub>sym</sub> input parameters should not be interpreted in isolation with respect to clinical implications, but rather, should be combined with exposure in DILI<sub>sym</sub> to produce simulations that have predictive and insightful value. <sup>b</sup>IC<sub>50</sub> values; default assumption is mixed inhibition type with α = 5, based on the experience of the DSS team. <sup>c</sup>Basolateral inhibition constant represents the lowest IC<sub>50</sub> of the experimentally derived MRP3 and MRP4 IC<sub>50</sub> values.



**Figure 1** CBD dose-peak serum ALT response relationship in the simulated population. (a) CBD titrated to 12.5 mg/kg b.i.d. under fasted condition. (b) CBD titrated to 12.5 mg/kg b.i.d. under fed condition. (c) CBD titrated to 10 mg/kg b.i.d. under fed condition. (d) CBD titrated to 5 mg/kg b.i.d. under fed condition. Comparing panels (b), (c), and (d), the frequency of ALT elevations decreases as the maintenance dose decreases. Furthermore, because of the effect of food to increase CBD absorption,<sup>10</sup> the frequency of ALT elevations is much higher in the fed state compared with the fasted state. These comparisons support an exposure-response relationship between CBD and ALT elevation. ALT, alanine aminotransferase; CBD, cannabidiol; TBL, total bilirubin; ULN, upper limit of normal.

without prior VPA treatment. This reflected the fact that VPA-induced ALT elevations had resolved before the CBD-induced elevations occurred due to compensatory mitochondrial biogenesis (Figure 3, Figure S16).

### Mechanistic investigations

The relative importance of each molecular species and each hepatotoxicity mechanism in DILISym to the predicted CBD-induced ALT elevations was investigated in a 46 SimCohort receiving a 12.5 mg/kg b.i.d. maintenance dose of CBD administered under fed conditions.

Table 3 shows the frequency of ALT elevations as the toxicity from each chemical species is entirely removed from DILISym; that is, although the molecule is present and simulated in the model, the chemical species that is “turned off” no longer engages any of its toxicity mechanisms. Out of the three chemical species tested, eliminating toxicity from the parent compound reduced the frequency of ALT elevations the most. Thus, the parent compound itself appears to play the largest role in CBD-driven ALT elevations predicted by DILISym; the two CBD metabolites make more minor contributions to predicted CBD-driven ALT elevations. Next, we determined which of the three toxicity mechanisms was

**Table 2 Summary of CBD- and VPA-mediated ALT elevations in SimPops**

Compound	Maintenance dose <sup>a</sup>	ALT > 3× ULN	Bili > 2× ULN
VPA alone	10mg/kg b.i.d.	33/285 (11.6%)	0/285 (0%)
CBD alone	12.5mg/kg b.i.d. fasted	2/285 (0.7%)	0/285 (0%)
	12.5mg/kg b.i.d. fed	149/285 (52.3%)	63/285 (22.1%)
	10mg/kg b.i.d. fed	108/285 (37.9%)	39/285 (13.7%)
	5mg/kg b.i.d. fed	24/285 (8.4%)	2/285 (0.7%)
VPA for 2 weeks then CBD <sup>b</sup>	VPA 10mg/kg b.i.d. CBD 12.5mg/kg b.i.d. fasted	35/285 (12.3%)	0/285 (0%)

All simulations with VPA used the Biogenesis (v8A\_5) SimPops (i.e., “VPA Alone” and “VPA for 2 weeks then CBD”). All other simulations (i.e., “CBD Alone”) used the standard (v8A\_1) SimPops.

ALT, alanine aminotransferase; Bili, bilirubin; CBD, cannabidiol; ULN, upper limit of normal; VPA, valproate.

<sup>a</sup>VPA titration started at 5mg/kg b.i.d. and increased to 10mg/kg b.i.d. on the second week. CBD titration started at 2.5mg/kg b.i.d. and increased by 2.5mg/kg b.i.d. weekly until the indicated maintenance dose was reached. <sup>b</sup>VPA was titrated to the maintenance dose during the first 2 weeks; subsequently, CBD titration started on the third week. The titration schedule is the same as above.

the most significant contributor to the simulated ALT elevations. **Table 3** also shows the frequency of ALT elevations as each toxicity mechanism is systematically turned “off”; that is, that mechanism of toxicity is not simulated. When the ROS mechanism is turned “off” none of the simulated individuals experience elevated ALT levels. Thus, the production of ROS appears to be the dominant mechanism underlying ALT elevations produced by CBD treatment.

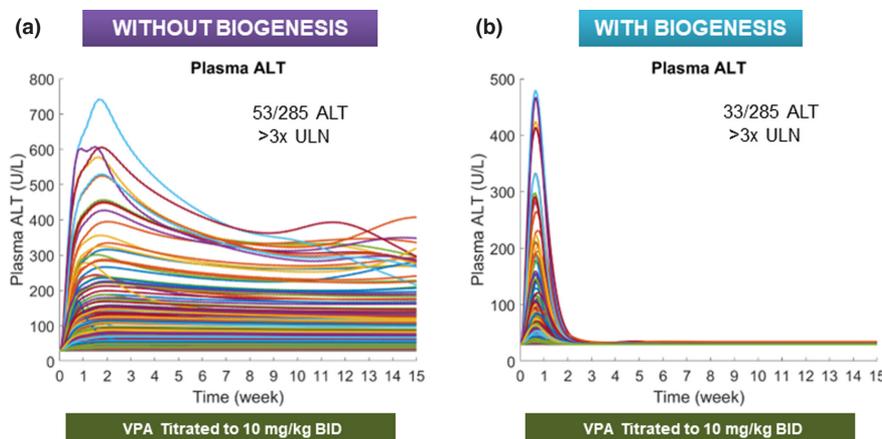
## DISCUSSION

The primary goal of this study was to assess the potential mechanisms underlying the marked increase in incidence of serum ALT elevations when CBD treatment is started in patients receiving treatment with VPA. We built PBPK models in GastroPlus for both CBD and VPA to predict liver exposure. We also gathered data from *in vitro* assays assessing the potential for CBD, CBD-7-COOH, 7-OH-CBD, and VPA, to induce 3 of the most common mechanisms of direct hepatotoxicity: inhibition of BA

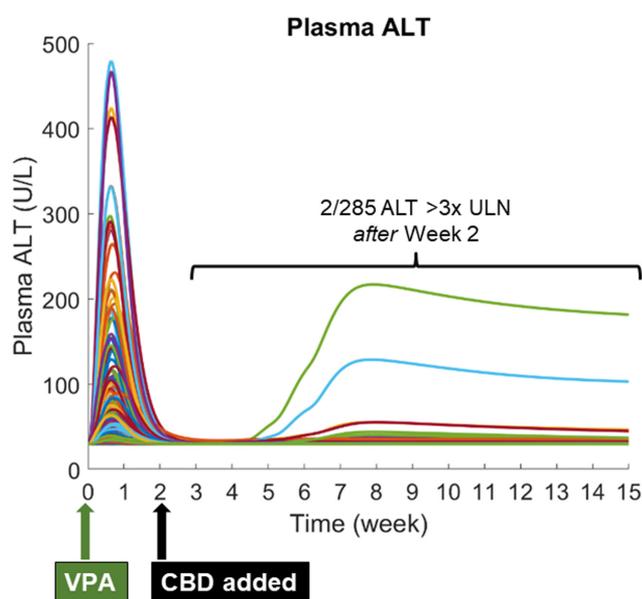
transporters, mitochondrial dysfunction, and oxidative stress. SimPops simulations in DILIsym combined predicted exposure and effects on the hepatocyte toxicity mechanisms to predict population-level frequency and dynamics of ALT elevations.

Simulations of CBD (dosed alone) displayed a dose-dependent increase in predicted ALT elevations, with a much higher frequency of predicted ALT elevations in the fed scenario compared with the fasted scenario, consistent with the exposure differences. Simulated ALT elevations in response to CBD treatment were observed to be largely driven by oxidative stress; whereas, mitochondrial dysfunction played some but a minimal role in the simulated elevations of ALT (**Table 3**). Moreover, the mechanistic investigation showed the parent compound (CBD) made the largest contribution to the simulated ALT elevations, with more minor contributions from the major metabolites (7-OH-CBD and 7-COOH-CBD; **Table 3**).

In DILIsym, about 11.5% of simulated individuals receiving VPA treatment experienced ALT elevations > 3× ULN and this is



**Figure 2** VPA DILIsym toxicity predictions in standard SimPops vs. Biogenesis SimPops. (a) Simulations of VPA using the standard (v8A\_1) SimPops parameters produced predicted ALT elevations in higher the frequency, severity and duration relative to ALT elevations observed in clinical studies in patient populations. (b) Simulation of VPA using the Biogenesis (v8A\_5) SimPops yields predictions consistent with clinical observations: the frequency is around 11.5% and the elevated levels resolve within 2–3 weeks with continued dosing. Hence, DILIsym accurately recapitulates VPA hepatotoxicity when using the Biogenesis (v8A\_5) SimPops. ALT, alanine aminotransferase; ULN, upper limit of normal; VPA, valproate.



**Figure 3** Plasma ALT profile for concomitant VPA and CBD dosing. Concomitant dosing was simulated in the Biogenesis SimPops. The resulting plasma ALT profiles, displayed here, show ALT elevations from VPA dosing are resolved by the end of week 2. These dynamic ALT profiles are nearly identical to the SimPops simulation with VPA dosing alone (Figure 2b). CBD dosing begins to be titrated on the morning of day 15 (1 day after 2 weeks). Two individuals have ALT elevations after CBD was brought on board (after week 2). These two individuals have the same response as without VPA on board (Figure S16). ALT, alanine aminotransferase; CBD, cannabidiol; ULN, upper limit of normal; VPA, valproate.

comparable to the 5–10% incidence reported clinically.<sup>34</sup> Due to inclusion of mitochondrial biogenesis in the model, the VPA induced ALT elevations were transient and this is consistent with the known resolution of ALT elevations usually observed in patients despite continuing treatment with VPA.<sup>34</sup>

Combination simulations, where CBD dosing was started after 2 weeks treatment with VPA, showed the initial VPA-induced ALT elevations had resolved prior to initiation of CBD dosing due to mitochondrial biogenesis. Initiation of CBD treatment at this time then caused a second increase in frequency of simulated ALT

**Table 3** Frequency of simulated ALT elevations in the SimCohort in the mechanistic investigation simulations

Mechanisms off <sup>a</sup>	Chemical species off <sup>b</sup>	ALT > 3× ULN
None	None	24/46
ROS	None	0/46
ETCi	None	24/46
BAi	None	24/46
None	CBD	11/46
None	7-COOH-CBD	16/46
None	7-OH-CBD	23/46

ALT, alanine aminotransferase; BAi, bile acid inhibition; CBD, cannabidiol; ETCi, electron transport chain inhibition; ROS, reactive oxygen species; ULN, upper limit of normal.

<sup>a</sup>“Mechanisms off” refers to mechanisms that were disabled during the simulation. <sup>b</sup>“Chemical species off” refers to drug/metabolite species whose toxicity was disabled during the simulation.

elevations. However, the frequency of predicted ALT elevations > 3× ULN after CBD was added to ongoing VPA treatment was the same as the frequency predicted for CBD alone. In summary, our simulation results did not reproduce the remarkable increase in susceptibility to CBD-induced ALT elevations in patients already receiving treatment with VPA. It is possible that mitochondrial biogenesis might not completely restore mitochondrial function yet eliminate ALT elevations. However, incomplete adaptation would not alone account for the magnitude of co-treatment effect observed clinically.

It should be noted that our predictions for ALT elevations due to CBD treatment clearly overestimate the incidence and severity of ALT elevations that were actually observed in the CBD clinical trials. This likely is because mechanisms that respond to oxidative stress in the liver (e.g., NRF-2 activation) were not incorporated in the simulations. Such tolerance and adaptive mechanisms presumably account for why, in the clinic, CBD induced ALT elevations typically resolve despite continuing CBD treatment.<sup>1</sup> The recommended titration schedule for dosing may also permit adaptation, minimizing the incidence of ALT elevations. Although we overpredicted the incidence and severity of ALT elevations during CBD treatment in the patient populations, it should be noted that in a phase I study involving 16 healthy adult volunteers receiving a similar CBD dose titration protocol and receiving oral doses in the fed state, ALT elevations > 5× ULN were observed in 5 (31%).<sup>9</sup> Because the simulated population was designed to approximate healthy adults, it is possible that patients with DS, LGS, and tuberous sclerosis complex, who are predominantly children and adolescents, are less susceptible to ALT elevations. Our simulations would also not have detected whether disease or pediatric-specific factors contribute to the VPA/CBD toxicity interaction.

Another potential limitation of our study is that we utilized Hep G2 cells for our analysis of oxidative stress and mitochondrial effects and these cells lack the full complement of drug metabolizing enzymes; the effects of all potential metabolites may therefore have been missed. Specifically, we did not investigate the effects of the major metabolite of VPA, 4-ene-VPA. We did not feel this was necessary because we reproduced the clinically observed ALT elevations with parent VPA alone, and in a clinical study of PK interaction between CBD and VPA, CBD treatment did not increase circulating levels of the 4-ene metabolite as well as parent VPA.<sup>14</sup> However, we cannot completely exclude a contribution of this or potentially other metabolites to the serum ALT effects of combined treatment with VPA and CBD.

Had our simulations successfully predicted the ALT effects of concomitant VPA and CBD treatment, it would have been possible to identify other patient conditions or concomitant treatments that could potentially increase susceptibility to CBD liver effects. For example, if combined interference of mitochondrial function accounted for the effect, then concomitant treatment with other drugs capable of this effect (e.g., metformin) or certain disease states associated with reduced mitochondrial function (e.g., non-alcoholic steatohepatitis) could have a similar effect. This could have informed CBD pharmacovigilance and possibly risk management.

Our failure to predict the interaction based on the three common mechanisms for direct hepatotoxicity, suggests that the hepatotoxic interactions between VPA and CBD involve rarer mechanisms that are less likely to be shared by many other drugs or patient conditions. Of note, treatment of mice with VPA greatly increases their susceptibility to liver injury due to acetaminophen. This effect appears to result from increased expression of steroidogenic acute regulatory protein and subsequent signaling involving phosphorylation of JNK1 and JNK2.<sup>35</sup> VPA has also been shown to cause a loss of polarity in cultured hepatocytes, and this has been recently proposed as a mechanism for increasing susceptibility to hepatotoxicity.<sup>36</sup> These and other potential mechanisms should now be explored.

#### SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website ([www.cpt-journal.com](http://www.cpt-journal.com)).

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#### CONFLICT OF INTEREST

V.L., G.G., B.H., and D.L. are employees of DILIsym Services Inc. P.W. chairs the Scientific Advisory Board for the DILI-sim Initiative and is compensated for this. He does not hold equity in DILIsym Services or its parent company Simulations Plus. He served as a paid consultant for GW Pharmaceuticals Ltd. at the time of this study consistent with his university's policies on external activities for pay policy. He did not receive compensation for preparing this manuscript.

#### AUTHOR CONTRIBUTIONS

V.V.L., G.G., B.A.H., D.M.L., and P.B.W. wrote the manuscript. B.A.H., D.M.L., and P.B.W. designed research. V.V.L., G.G., and D.M.L. performed the research. V.V.L., G.G., B.A.H., D.M.L., and P.B.W. analyzed the data. V.V.L., G.G., B.A.H., and D.M.L. contributed analytical tools.

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