

ORIGINAL ARTICLE

Elucidating Differences in the Hepatotoxic Potential of Tolcapone and Entacapone With DILIsym[®], a Mechanistic Model of Drug-Induced Liver Injury

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Tolcapone and entacapone are catechol-O-methyltransferase (COMT) inhibitors developed as adjunct therapies for treating Parkinson's disease. While both drugs have been shown to cause mitochondrial dysfunction and inhibition of the bile salt export protein (BSEP), liver injury has only been associated with the use of tolcapone. Here we used a multiscale, mechanistic model (DILIsym[®]) to simulate the response to tolcapone and entacapone. In a simulated population (SimPops[™]) receiving recommended doses of tolcapone (200 mg t.i.d.), increases in serum alanine transaminase (ALT) >3× the upper limit of normal (ULN) were observed in 2.2% of the population. In contrast, no simulated patients receiving recommended doses of entacapone (200 mg 8× day) experienced serum ALT >3× ULN. Further, DILIsym[®] analyses revealed patient-specific risk factors that may contribute to tolcapone-mediated hepatotoxicity. In summary, the simulations demonstrated that differences in mitochondrial uncoupling potency and hepatic exposure primarily account for the difference in hepatotoxic potential for tolcapone and entacapone.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC? Both tolcapone and entacapone uncouple the mitochondrial proton gradient and display modest inhibition of BA transport. Clinical hepatotoxicity has been observed with tolcapone in human clinical studies. Entacapone is not hepatotoxic in humans. • WHAT QUESTION DOES THIS STUDY ADDRESS? What accounts for the difference in the hepatotoxicity between tolcapone and entacapone? • WHAT THIS STUDY ADDS TO OUR KNOWLEDGE Combining otherwise difficult to interpret *in vitro* mitochondrial toxicity endpoints with exposure through a mechanistic model allowed for the correct prediction of differences in hepatotoxic potential between tolcapone and entacapone. Mitochondrial function and hepatic drug exposure were important contributors to tolcapone-mediated hepatotoxicity and to the lack of observed entacapone toxicity. • HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS This study illustrates the capability of DILIsym[®] to combine clinical data, *in vitro* data, predicted liver compound exposure, and interpatient differences to provide an account of how exposure, biological variability, and multiple hepatotoxicity mechanisms may come together to result in DILI.

Catechol-O-methyltransferase (COMT) inhibitors are drugs that increase the elimination half-life of levodopa, the primary treatment for Parkinson's disease. Tolcapone was the first COMT inhibitor approved for use in Parkinson's disease. Following approval, four instances of acute liver failure were attributed to the use of tolcapone, causing its withdrawal from the European market and requirements for liver enzyme monitoring in the United States.^{1–5} In contrast, no risk of hepatotoxicity has been attributed to entacapone, the second COMT inhibitor approved for Parkinson's disease.^{1,2,5,6}

In vitro assays have shown that both tolcapone and entacapone are capable of inducing mitochondrial dysfunction in a dose-dependent manner.^{7–9} Both compounds cause uncoupling of the mitochondria proton gradient, leading to reduced adenosine triphosphate (ATP) synthesis and increased heat production.^{7–9} In addition, recent work using *in vitro* systems has demonstrated that both drugs have the potential to alter hepatobiliary transport.¹⁰ Tolcapone and

entacapone caused modest inhibition of the bile salt export pump (BSEP), an efflux transporter that secretes bile acids (BAs) from the liver into the bile, and the basolateral efflux transporters (MRP3 and MRP4) that secrete BAs into the blood.¹⁰ Inhibition of efflux transporters can cause hepatocellular accumulation of BAs leading to BA-dependent hepatotoxicity, another underlying mechanism that has been linked to liver injury in humans.^{10–12} Systems pharmacology modeling allows for the integration of data related to multiple physiological processes and biochemical mechanisms that contribute to the development of hepatotoxicity and may enable more accurate predictions of drug-induced liver injury (DILI).

In the current study a mechanistic model of DILI (DILIsym[®]) was used to integrate pharmacokinetic data and *in vitro* toxicity data to simulate the *in vivo* response in humans to tolcapone and entacapone. Responses to tolcapone and entacapone were analyzed in a simulated human population (SimPops[™]), which included variability to

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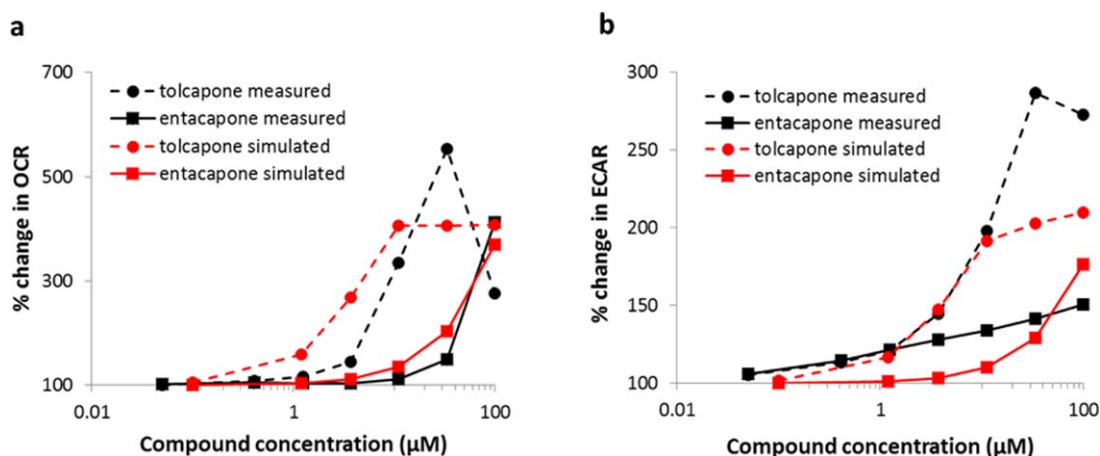


Figure 1 Observed and simulated percentage change in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in response to tolcapone and entacapone. *In vitro* respiration data was obtained from HepG2 cells treated with tolcapone (dashed black lines) or entacapone (solid black lines) and measured using the Seahorse XF96 instrument.⁸ Simulated OCR (a) and ECAR (b) responses for tolcapone (dashed red lines) and entacapone (solid red lines) were generated using MITOSym[®] with the uncoupling parameter (Michaelis–Menten K_m) value for tolcapone and entacapone optimized to the measured data.

account for potential intersubject differences in key biochemical areas related to hepatotoxicity. Potential risk factors for tolcapone-mediated hepatotoxicity were assessed using SimPops[™]. In addition, DILIsym[®] was utilized to test the hypothesis that mitochondrial dysfunction is the primary mechanism underlying tolcapone-mediated toxicity. Further, compound-specific differences responsible for the difference in hepatotoxic potential for tolcapone and entacapone were identified.

METHODS

DILIsym[®] version 4A

A mechanistic, mathematical model of drug-induced liver injury (DILIsym[®], <http://www.dilism.com>), was utilized to explore the divergent toxicological responses for tolcapone and entacapone in human clinical studies. DILIsym[®] consists of smaller submodels that are mathematically integrated to simulate an organism-level response.^{13–19} The current work utilized submodels representing drug distribution, mitochondrial dysfunction and toxicity, BA physiology and pathophysiology, hepatocyte life cycle, and liver injury biomarkers (**Supplementary Figure S1a**). DILIsym[®] is developed and maintained through the DILI-sim Initiative, a public-private partnership involving scientists in academia, industry, and the US Food and Drug Administration (FDA).

MITOSym[®] version 2A

MITOSym[®] is a mechanistic, mathematical model of *in vitro* hepatocellular respiration designed to simulate cellular respiration data obtained via the Seahorse assay (Seahorse Bioscience, North Billerica, MA) for the purposes of deriving parameters characterizing compound-induced mitochondrial dysfunction (**Supplementary Figure S1b**).²⁰ MITOSym[®] parameters characterize the measured *in vitro* mitochondrial dysfunction and can be subsequently translated into DILIsym[®] parameters for simulating the *in vivo* setting.

Determination of mitochondrial dysfunction parameter values for tolcapone and entacapone

MITOSym[®] was used to determine parameter values for tolcapone- and entacapone-mediated mitochondrial uncoupling effects. The uncoupling mechanism is described as a Michaelis–Menten function in MITOSym[®], and drug-specific uncoupling parameters were optimized by fitting the simulated results with published cellular respiration data for HepG2 cells exposed to tolcapone or entacapone.⁸

Figure 1 shows observed and simulated metabolic changes in response to treatment with tolcapone and entacapone. The same mechanistic descriptions of mitochondrial pathways are included in MITOSym[®] and DILIsym[®], while DILIsym[®] includes additional descriptions (such as dietary intake patterns) to account for *in vivo*-only environments.^{18,20} Thus, conversion factors were needed to translate MITOSym[®] parameter to DILIsym[®] (**Supplementary Table S1**). DILIsym[®] parameter values were used in the subsequent simulations.

Determination of BA transport inhibition parameter values for tolcapone and entacapone

The published half-maximal inhibitory concentrations (IC_{50}) of BSEP and MRP4 for tolcapone or entacapone¹⁰ were used to represent inhibition constants (K_i) in DILIsym[®] (**Supplementary Table S1**).

Simulation protocols

DILI responses in humans after administration of tolcapone or entacapone were simulated using physiologically based pharmacokinetic (PBPK) submodel predictions of tolcapone or entacapone disposition (**Supplementary Figure S2**, **Supplementary Table S2**) and previously developed submodels in DILIsym[®] (**Supplementary Figure S1a**). In the simulations, to replicate the clinical dosing of each drug tolcapone was dosed for 1 week at 200 mg t.i.d. (8-hour dosing period), and entacapone was dosed for 1 week at 200 mg 8× per day (3-hour dosing period). Mitochondrial

Table 1 List of parameters varied in the SimPops™ and results of multiple-regression analysis in SimPops™ administered tolcapone (200 mg t.i.d. for 1 week)

Parameter name	Parameter description	Significance
Mitochondrial toxicity submodel		
Basal value of mito ETC flux	The basal value of the standardized ETC flux	$P < 0.001$
Scaling coefficient representing reserve mitochondria function	Scaling coefficient for representing the amount of reserve mitochondria ETC function	$P < 0.001$
Bile acid homeostasis submodel		
LCA-sulfate uptake V_{max}	Maximum velocity of hepatic uptake of LCA-sulfate	NS
LCA-sulfate canalicular efflux V_{max}	Maximum velocity of biliary excretion of LCA-sulfate	NS
CDCA-amide uptake V_{max}	Maximum velocity of hepatic uptake of CDCA-amide	NS
CDCA-amide canalicular efflux V_{max}	Maximum velocity of biliary excretion of CDCA-amide	NS
CDCA-amide basolateral efflux V_{max}	Maximum velocity of hepatic basolateral efflux of CDCA-amide	NS
CDCA amidation V_{max}	Maximum velocity of CDCA amidation in hepatocytes	NS
LCA-amide sulfation V_{max}	Maximum velocity of LCA-amide sulfation in hepatocytes	NS
LCA synthesis V_{max}	Maximum velocity of LCA synthesis by the gut microbiome	NS
Uptake regulation scaling factor	Scaling factor governing the magnitude of feedback regulation of hepatic uptake transporter function by hepatic bile acid accumulation	NS
Canalicular efflux regulation scaling factor	Scaling factor governing the magnitude of FXR-mediated feedback regulation of hepatic canalicular transporter function by hepatic bile acid accumulation	NS
Other system-specific parameters		
Body weight	Body weight	NS ($P = 0.02$)
ATP decrement necrosis V_{max}	Maximum reaction rate in the equation relating the ATP decrement to the necrosis rate	NS
HGF-mediated regeneration V_{max}	Maximum turnover rate in the equation relating HGF to liver regeneration	NS
Tolcapone PBPK submodel		
Tolcapone oral bioavailability	The fraction of an oral dose of tolcapone that reaches the blood	NS
Tolcapone hepatic clearance	The hepatic clearance rate of tolcapone	NS ($P = 0.03$)
Tolcapone oral absorption K_{ab}	First-order rate constant for tolcapone absorption into the blood after an oral dose	NS
Entacapone PBPK submodel		
Entacapone oral bioavailability	The fraction of an oral dose of entacapone that reaches the blood	NA
Entacapone hepatic clearance	The hepatic clearance rate of entacapone	NA
Entacapone oral absorption K_{ab}	First-order rate constant for entacapone absorption into the blood after an oral dose	NA

ATP, adenosine triphosphate; CDCA, chenodeoxycholic acid; ETC, electron transport chain; LCA, lithocholic acid; K_{ab} , first-order rate constant for absorption; NA, not applicable; NS, not significant; PBPK, physiologically based pharmacokinetic; V_{max} , maximum velocity.

The SimPops™ incorporates variability in parameters governing mitochondrial dysfunction and bile acid homeostasis as well as in other system-specific parameters. Parameters in the PBPK submodels for tolcapone and entacapone were also varied. (See **Supplementary Information** online for methods used to construct the SimPops™). In the SimPops™ administered tolcapone (200 mg t.i.d. for 1 week), a multiple regression analysis was performed to identify the most important parameters in tolcapone-mediated hepatotoxicity using 18 varied parameters as independent variables and maximum serum alanine transaminase (ALT) as the dependent variable. Statistical significance was calculated using R software (v. 3.2.0; R Project for Statistical Computing (<http://www.r-project.org/>)).

and bile acid toxicity parameters values used in the present study are listed in **Supplementary Table S1**.

Construction of simulated human populations (SimPops™)

A human population sample ($n = 229$) with variability in parameters in the mitochondrial and BA submodels as well as in system-specific parameters such as body weight was constructed within DILIsym®. PBPK submodel parameters governing tolcapone and entacapone disposition were also varied in the SimPops™. Simulated individuals with compromised mitochondrial function consistent with observations for nonalcoholic steatohepatitis (NASH) patients²¹ were included in the SimPops™ at an incidence approximating the estimated prevalence of NASH in the general

population.²² All parameters varied and data used to construct the SimPops™ are listed in **Table 1** and **Supplementary Table S3**. Details related to the development of the SimPops™ can be found in the **Supplementary Information** and in prior publications.^{13,15,19}

RESULTS

Simulating responses to tolcapone and entacapone in the baseline human

The baseline human in the DILIsym® software represents a typical normal, healthy volunteer. The parameter solution for the baseline human is consistent with data for all mechanisms of toxicity, compounds, protocols, and ancillary tests represented in DILIsym®.

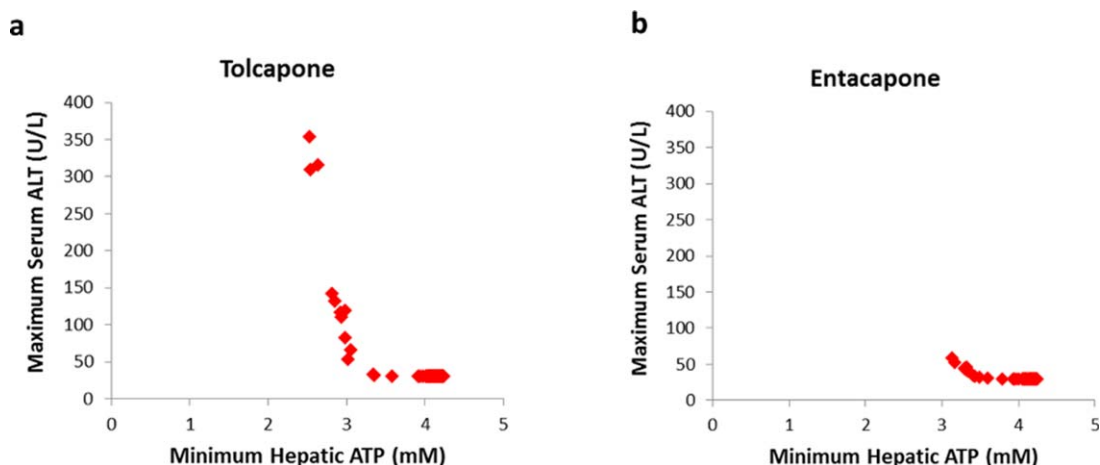


Figure 2 Simulated drug-induced liver injury (DILI) responses in 229 individuals in the SimPopsTM administered tolcapone or entacapone. Predicted DILI responses (i.e., minimum hepatic ATP levels vs. maximum serum alanine transaminase (ALT) levels) postdose in SimPopsTM for (a) oral administration of tolcapone (200 mg t.i.d. for 1 week) and (b) oral administration of entacapone (200 mg 8× per day for 1 week).

No hepatotoxicity (i.e., no increase in alanine transaminase (ALT) or bilirubin and no hepatocellular loss) was predicted in the baseline human either following oral administration of tolcapone (200 mg t.i.d. for 1 week) or entacapone (200 mg 8× per day for 1 week) (data not shown).

Simulating responses to tolcapone and entacapone in simulated populations (SimPopsTM)

A SimPopsTM that included variability in biochemical areas related to hepatotoxicity and drug disposition (Table 1) was utilized to explore tolcapone and entacapone hepatotoxicity at the population level. Figure 2 shows simulated DILI responses in the SimPopsTM following oral administration of either tolcapone (200 mg t.i.d.) or entacapone (200 mg 8× per day). Tolcapone administration resulted in decreased hepatic ATP levels (i.e., minimum ATP levels up to 31% lower than baseline values), and increased serum ALT levels >3× the upper limit of normal (ULN = 40 U/L) in a subset of individuals in the SimPopsTM (Figure 2a). In contrast, entacapone administration caused minimal changes in hepatic ATP levels (i.e., minimum ATP levels up to 14% lower than baseline values), and no increases in ALT >3× ULN were observed (Figure 2b). The simulated incidences of elevated serum ALT for tolcapone and entacapone are summarized in Table 2; the reported incidences of ALT elevations in clinical trials are also listed. In the SimPopsTM,

tolcapone administration induced elevations in serum ALT >3× ULN in 2.2% of the population. This incidence of ALT elevation was similar to observations from clinical trials, in which tolcapone (100–200 mg t.i.d.) induced serum ALT elevations >3× ULN in 1.3–5.0% of patients.^{3,5} Simulated bilirubin levels did not exceed 2× ULN (ULN = 1 mg/dL) for any of the simulated individuals with tolcapone-induced ALT elevations. The lack of simulated Hy’s law cases for tolcapone is consistent with the lack of reported cases of serious liver injury in any of the patients treated with tolcapone in clinical trials.³ Entacapone administration did not elicit any ALT elevations >3× ULN in the SimPopsTM. This lack of hepatotoxicity in response to entacapone is consistent with clinical observations thus far.^{5,6,23}

Multiple linear regression analysis in SimPopsTM administered tolcapone

Simulations revealed a subset of SimPopsTM individuals susceptible to tolcapone-mediated hepatotoxicity. To identify the most important SimPopsTM parameters in the context of tolcapone-mediated DILI, multiple regression analysis was performed with maximum serum ALT as the dependent variable and the SimPopsTM parameters as independent variables. Table 1 lists the statistical significance of the SimPopsTM parameters. Among the 18 parameters varied, two were statistically significant predictors of serum ALT levels. Notably, both of the parameters that reached

Table 2 Summary of tolcapone- and entacapone-mediated hepatotoxicity in SimPopsTM and clinical trials

	Tolcapone		Entacapone	
	Simulations ^a 200 mg t.i.d.	Clinical trials ^{3,5} 100-200 mg t.i.d.	Simulations ^a 200 mg 8x per day	Clinical trials ^{5,6} 200 mg up to 8x per day
ALT >3x ULN ^b	2.2%	1.3–5.0%	0.0%	0–0.9%

ALT, alanine transaminase; t.i.d., 3 × per day; ULN, upper limit of normal.

^aEach dose level was simulated for 1 week.

^bIn the SimPopsTM, ULN was 40 U/L. The majority (223 of 229) of the individuals in the SimPopsTM had approximately the same baseline ALT (30–40 U/L) before drug administration. Six simulated individuals had baseline ALT values >40 U/L (these simulated individuals had compromised mitochondrial function within the observed range for NASH patients, Supplementary Information online). Four of the five simulated individuals with ALT >3x ULN following tolcapone administration had baseline ALT values >40 U/L. Peak ALT levels were greater than 3x baseline for all of the simulated individuals who had ALT >3x ULN following tolcapone administration.

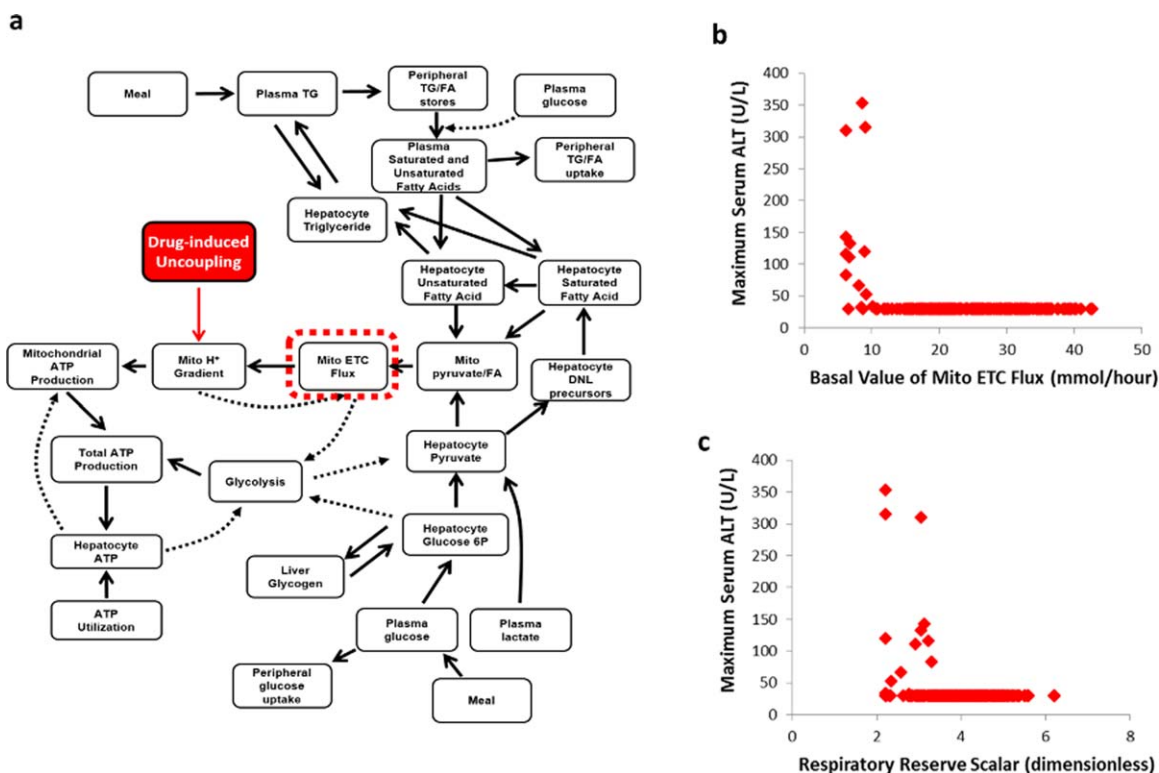


Figure 3 Mitochondrial toxicity parameters associated with susceptibility to tolcapone-mediated hepatotoxicity. The mitochondrial toxicity submodel in DILIsym® is shown in (a). As indicated in the diagram, drug-induced uncoupling affects the mitochondrial proton gradient. The mitochondrial SimPops™ parameters that were significantly associated with tolcapone-mediated hepatotoxicity impact the mitochondrial electron transport chain (ETC) flux (dotted outline). SimPops™ parameter values vs. predicted maximum serum alanine transaminase (ALT) following oral administration of tolcapone (200 mg t.i.d. for 1 week) are shown for (b) the basal value of the standardized ETC flux and (c) the scaling coefficient for representing the amount of reserve mitochondria ETC function. These two SimPops™ parameters reached statistical significance ($P < 0.001$) in a multiple linear regression analysis that was performed to identify the most important parameters in tolcapone-mediated hepatotoxicity (Table 1).

statistical significance (the basal value of the standardized electron transport chain (ETC) flux and the scaling coefficient for representing the amount of reserve mitochondria ETC function) are parameters within the mitochondrial toxicity submodel. Figure 3 shows the relationship between the mitochondrial toxicity parameter values and simulated serum ALT levels following tolcapone administration. The SimPops™ individuals with tolcapone-mediated ALT elevations had relatively low basal ETC flux values and relatively low respiratory reserve values. However, there were simulated individuals with low basal ETC flux values who did not have increased ALT levels (Figure 3b), and there were also individuals with low respiratory reserve values who did not have tolcapone-mediated hepatotoxicity (Figure 3c). As shown in Figure 4a, the simulated individuals with the highest ALT elevations had relatively low values for both the basal ETC flux and the respiratory reserve parameter. These results demonstrate the multifactorial nature of the DILI response to tolcapone.

In the multiple regression analysis, two additional SimPops™ parameters, body weight and tolcapone hepatic clearance, had relatively small P values, although statistical significance was not reached at a $P < 0.001$ threshold (Table 1). Both parameters are involved in determining the hepatic exposure following tolcapone administration and,

consequently, are correlated with peak tolcapone liver concentrations (Supplementary Figure S3). These results suggest that tolcapone liver concentrations and, consequently, factors influencing hepatic exposure may also play an important role in tolcapone-mediated hepatotoxicity. The importance of hepatic exposure in tolcapone-mediated hepatotoxicity is further supported by Figure 4b, which shows that the highest ALT responders also had relatively high peak liver tolcapone concentrations. These results demonstrate that multiple factors, including mitochondrial function and hepatic exposure, are likely involved in tolcapone-mediated hepatotoxicity.

Sensitivity analysis

Results of the multiple regression analysis suggested that compromised mitochondrial function plays an important role in tolcapone-mediated hepatotoxicity. To investigate the sensitivity of DILI responses to the Michaelis–Menten constant (K_m) for the effect of tolcapone-mediated mitochondrial uncoupling, simulations were performed with 10-fold smaller and larger K_m values. Simulated maximum serum ALT levels in SimPops™ treated with tolcapone (200 mg t.i.d.) are presented in Figure 5a. Tolcapone-induced ALT elevations were sensitive to the K_m value; when K_m was decreased by 10-fold, 9.2% of the population exhibited

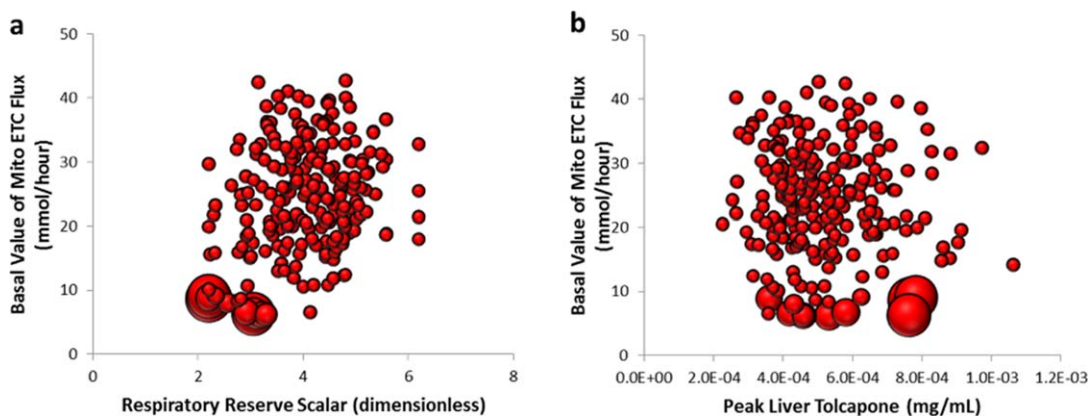


Figure 4 Relationship between simulated maximum serum alanine transaminase (ALT) in the SimPops[™] following tolcapone administration and multiple variables. The magnitude of the simulated maximum serum ALT following oral administration of tolcapone (200 mg t.i.d. for 1 week) is represented by the size of the bubbles, with the largest bubble representing the greatest relative increase. The relationship between simulated ALT, SimPops[™] parameter values for the basal value of the standardized ETC flux, and SimPops[™] parameter values for the scaling coefficient for representing the amount of reserve mitochondria ETC function is shown in (a). The relationship between simulated ALT, SimPops[™] parameter values for the basal value of the standardized ETC flux, and simulated maximum liver tolcapone concentration is shown in (b).

serum ALT $>3\times$ ULN compared to only 2.2% of the population with the optimized value of K_m . None of the individuals showed elevated serum ALT $>3\times$ ULN when K_m was increased 10-fold.

Next, the sensitivity of tolcapone-mediated hepatotoxicity to the value of the BSEP inhibition constant was investigated. The K_i value for tolcapone had very little influence on serum ALT elevations (Figure 5b); 2.6% and 2.2% of the population exhibited a serum ALT $>3\times$ ULN when the K_i was decreased and increased by 10-fold, respectively, compared to an incidence of 2.2% with the measured K_i . These findings are consistent with the results of the multiple regression analysis that suggest that mitochondrial dysfunction is the dominant mechanism underlying tolcapone-mediated DILI responses, while BA-mediated effects are unlikely to contribute substantially to tolcapone-induced liver damage.

A sensitivity analysis for the toxicity parameter values was also performed for entacapone. In the SimPops[™], simulated serum ALT levels did not exceed $3\times$ ULN values when the K_m for entacapone-mediated uncoupling was decreased by 10-fold (Figure 5c), or when the K_i for BSEP inhibition by entacapone was decreased 10-fold (Figure 5d). Notably, the optimized value for the uncoupling K_m for entacapone is ~ 10 -fold higher than the optimized K_m value for tolcapone (Supplementary Table S1). Thus, the difference in the mitochondrial uncoupling strength between entacapone and tolcapone does not completely explain the difference in hepatotoxic potential for the two compounds.

As described above, hepatic exposure also likely plays a role in tolcapone-induced liver injury. Simulated peak liver concentrations for entacapone were, on average, approximately three times lower than peak tolcapone liver concentrations predicted for SimPops[™] individuals. High hepatic clearance for entacapone relative to the hepatic clearance for tolcapone is one of the primary factors responsible for

the lower predicted hepatic exposure for entacapone relative to tolcapone (Supplementary Figure S3). To assess whether the difference in the simulated hepatic exposure was responsible for the difference in the predicted hepatotoxicity for the two drugs, simulations were performed with entacapone dosed at three times the maximum therapeutic dosing regimen. While simulated maximum liver compound concentrations in the SimPops[™] treated with tolcapone at typical therapeutic doses (200 mg t.i.d.) were comparable to simulated maximum liver compound concentrations following treatment with entacapone at three times the maximum therapeutic dose (i.e., 600 mg $8\times$ per day), no ALT elevations $>3\times$ ULN were observed for entacapone, while 2.2% of the individuals treated with tolcapone had ALT elevations $>3\times$ ULN (Supplementary Figure S4). Thus, differences in the simulated hepatic exposure alone do not completely explain differences in the predicted hepatotoxicity between tolcapone and entacapone. Instead, the combined effect of compound-specific differences, including uncoupling strength and hepatic exposure, accounts for the difference in the predicted hepatotoxic potential. Consequently, simulated ALT elevations $>3\times$ ULN could be induced in the SimPops[™] following alterations to *both* the uncoupling parameter value (≥ 10 -fold decrease) and the hepatic exposure (via a threefold increase in dosing) for entacapone (data not shown).

DISCUSSION

DILI is one of the primary reasons for the termination of drug candidates in preclinical or clinical development and is a frequent cause for safety-related drug withdrawals.^{24,25} Late-stage attrition due to liver toxicity leads to substantial costs for drug developers. Improving preclinical screening of new drugs for hepatotoxic effects will increase the efficiency of drug development and will enhance patient care.

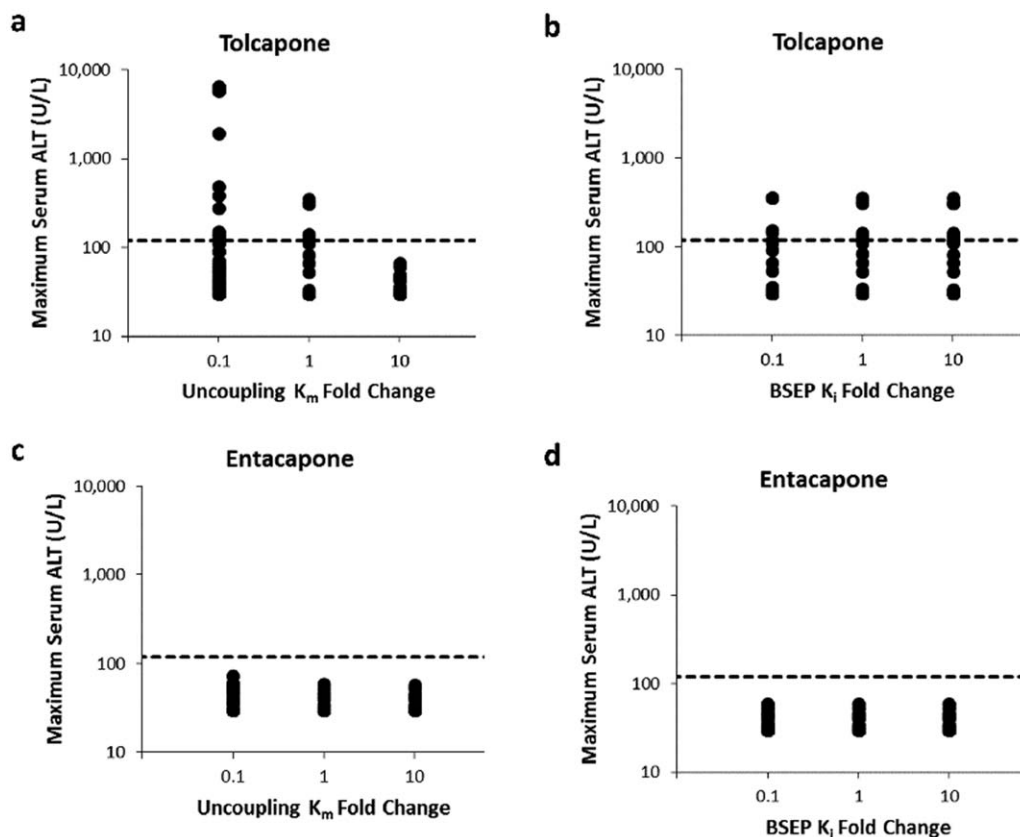


Figure 5 Sensitivity analysis of toxicity parameter values. Values for the Michaelis–Menten constant (K_m) for the effect of mitochondrial uncoupling were altered 10-fold in either direction of the optimized parameter values (Figure 1) for tolcapone (a) and entacapone (c). Values for the BSEP inhibition constant (K_i) were altered 10-fold in either direction of the published values (Supplementary Table S1) for tolcapone (b) and entacapone (d). Predicted maximum serum ALT concentrations in the SimPops™ are presented after (a,b) oral administration of tolcapone (200 mg t.i.d. for 1 week) and (c,d) oral administration of entacapone (200 mg 8× per day for 1 week). Dashed lines represent 3× ULN alanine transaminase (ALT) in the SimPops™.

In silico approaches using computational models can assist in the evaluation of the hepatotoxic liability of therapeutic compounds. For example, DILIsym® is a mechanistic, mathematical model that can be applied to predict toxicity based on preclinical *in vitro* and/or *in vivo* data and to gain insight into the mechanisms responsible for DILI.^{13–19} In the current study, DILIsym® was used to investigate the difference in hepatotoxic potential between tolcapone and entacapone.

Tolcapone, a drug developed for the treatment of Parkinson's disease, was associated with dose-related increases in liver enzymes in clinical trials and four instances of acute hepatotoxicity were attributed to tolcapone in postmarketing surveillance studies.^{1–5} Uncoupling of oxidative phosphorylation and the subsequent reduction in mitochondrial energy production has been postulated to be the main underlying cause of tolcapone-induced hepatotoxicity.^{9,26} In addition, recent work has shown that tolcapone causes modest inhibition of hepatocellular efflux transporters including BSEP, MRP3, and MRP4.¹⁰ Furthermore, genetic polymorphisms in the *UGT1A* gene (which encodes enzymes in the main elimination pathway of tolcapone) have been shown to be

associated with liver enzyme elevations in patients taking tolcapone, indicating that impaired elimination of the drug may contribute to tolcapone-induced liver toxicity.²⁷ Entacapone, a COMT inhibitor with a chemical structure similar to that of tolcapone, has not been associated with hepatotoxicity.¹ *In vitro* assays have shown that entacapone is also capable of causing mitochondrial dysfunction⁸ and is a modest inhibitor of BA transport.¹⁰

The potential for multiple toxicity mechanisms (i.e., mitochondrial dysfunction and BA transport inhibition) and intersubject differences in exposure contribute to tolcapone-mediated hepatotoxicity and the divergent toxicity profiles for tolcapone and entacapone provided a unique opportunity to probe the predictive capabilities of DILIsym® and to utilize DILIsym® to address the following objectives: (1) to gain insight into risk factors that may contribute to patient susceptibility to tolcapone-induced liver injury; (2) to test the hypothesis that mitochondrial dysfunction is the primary mechanism responsible for tolcapone-mediated toxicity; and (3) to explore compound-specific properties underlying the observed difference in hepatotoxic profiles for tolcapone and entacapone. To

accomplish these goals, multiple integrated DILIsym® sub-models representing drug distribution, mitochondrial dysfunction and toxicity, BA physiology and pathophysiology, hepatocyte life cycle, and liver injury biomarkers^{13–19} (**Supplementary Figure S1a**) were used to simulate the response in humans to tolcapone and entacapone. Inter-patient variation was taken into account by simulating treatment protocols in a SimPops™ that included variability in parameters relevant to hepatotoxicity mechanisms and drug disposition (**Table 1**).

Following clinically relevant dosing of tolcapone (200 mg t.i.d.), the simulated incidence of elevated serum ALT $>3\times$ ULN was 2.2%, which is similar to that observed in clinical trials (1.3–5.0%) (**Table 2**). There were no simulated Hy's law cases associated with tolcapone, consistent with the lack of reported cases of serious liver injury in patients treated with tolcapone in clinical trials.³ While four cases of severe liver dysfunction in patients receiving tolcapone have been reported in postmarketing surveillance studies (notably, monitoring recommendations were not followed in these cases), all of these patients were on multiple medications and it is possible that the other medications may have contributed to the development of liver injury.³ The results shown here suggest that the severe injury reported in these cases was not likely solely due to the use of tolcapone.

Compromised mitochondrial function was identified as a potential risk factor that may make certain patients more susceptible to tolcapone-mediated toxicity. Specifically, decreased basal ETC flux and decreased reserve mitochondrial ETC function were significantly associated with tolcapone-mediated ALT elevations in the SimPops™ (**Table 1**). While the basal ETC flux parameter represents the amount of functional ETC activity, the respiratory reserve parameter reflects the additional ETC activity that can contribute when mitochondria are under duress. The higher susceptibility to tolcapone-induced injury in SimPops™ individuals with low ETC activity can be attributed to the inability to effectively compensate for the reduction in ATP production caused by the uncoupling effect of tolcapone. These results suggest that impaired mitochondrial function may be one factor contributing to the ALT elevations observed in a subset of patients treated with tolcapone. While defects in ETC activity have been observed in NASH patients,²¹ there is also evidence for mitochondrial dysfunction in Parkinson's disease patients.^{28,29} Specifically, reduced complex I activity has been observed in regions of the brain in Parkinson's disease patients, and various studies have suggested that abnormalities in ETC activity are present in peripheral tissues of Parkinson's disease patients.^{28,29} Although the SimPops™ utilized for this study was not designed to reflect disease characteristics associated with Parkinson's disease, the SimPops™ included a subset of individuals with abnormal mitochondrial function typical of NASH and thus allowed for an analysis of the impact of ETC dysfunction, which may also occur in Parkinson's disease patients on tolcapone-mediated responses.

The SimPops™ analyses also suggested that hepatic tolcapone exposure plays a role in tolcapone-mediated hepatotoxicity (**Figure 4b**, **Supplementary Figure S4**). Body

weight and tolcapone hepatic clearance (SimPops™ parameters that influence hepatic exposure, **Supplementary Figure S3**) were weakly associated with tolcapone-mediated ALT elevations (statistical significance was not reached at a $P < 0.001$ threshold, **Table 1**). The weak relationship between hepatic exposure and tolcapone-induced ALT elevations in the current study is consistent with recent findings that genetic variation in metabolic enzymes involved in tolcapone elimination were significantly, but weakly, associated with tolcapone-induced ALT elevations.²⁷ The conclusion drawn from the pharmacogenetic study was that risk genotype has limited predictive power and, by itself, does not represent a clinically useful tool for the prediction of susceptibility to tolcapone-induced liver injury.²⁷ The results shown here demonstrate the multifactorial nature of tolcapone-mediated hepatotoxicity and the necessity for tools to incorporate the multiple contributing factors to accurately predict DILI risk.

Sensitivity analyses revealed that tolcapone-induced ALT elevations in the SimPops™ were sensitive to 10-fold changes in the value of the Michaelis–Menten constant (K_m) for the effect of tolcapone-mediated mitochondrial uncoupling (**Figure 5a**); the simulated incidence of elevated serum ALT $>3\times$ ULN increased from 2.2% to 9.2% with a 10-fold decrease in K_m and dropped to 0% with a 10-fold increase in K_m . In contrast, the simulated incidence of tolcapone-mediated hepatotoxicity was relatively insensitive to 10-fold changes in the value of the BSEP inhibition constant (K_i) (**Figure 5b**); 2.6% and 2.2% of the population exhibited a serum ALT $>3\times$ ULN when the K_i was decreased and increased by 10-fold, respectively. These findings support the hypothesis that mitochondrial dysfunction is the primary mechanism responsible for tolcapone-mediated toxicity.

Administration of entacapone (200 mg 8 \times per day) did not induce ALT elevations $>3\times$ ULN in the SimPops™, consistent with the lack of hepatotoxicity reported for patients treated with entacapone (**Table 2**). Even with a 10-fold decrease in the K_m for entacapone-mediated uncoupling, simulated serum ALT levels did not exceed 3 \times ULN values (**Figure 5c**). Because the uncoupling K_m for entacapone (optimized with *in vitro* data) is ~ 10 -fold higher than the uncoupling K_m for tolcapone (also optimized with *in vitro* data), these results demonstrated that the difference in the uncoupling strength between the two compounds does not entirely explain the difference in the hepatotoxic effects of tolcapone and entacapone. Another factor that may contribute to differences in the toxicity profiles for the two compounds is a difference in hepatic exposure. Via the use of PBPK submodels developed to describe the disposition of tolcapone and entacapone, DILIsym® predicted lower hepatic exposure for entacapone than for tolcapone for clinically relevant dosing regimens of each compound. Simulations performed with extremely high entacapone doses (threefold higher than maximum therapeutic doses) resulted in peak liver entacapone concentrations similar to predicted peak liver tolcapone concentrations, yet no ALT elevations $>3\times$ ULN were predicted in the SimPops™ with the increased entacapone dosing levels (**Supplementary Figure S4**). Thus, individually, differences in uncoupling strength and differences in predicted hepatic exposure

could not account for the difference in predicted DILI responses for tolcapone and entacapone. Instead, the simulation results indicated that the combined effect of differences in mitochondrial uncoupling strength and compound exposure leads to the observed differences in DILI liability for the two compounds.

The current study demonstrated that DILIsym®, which integrates physiological information and experimental data, correctly predicted differential hepatotoxicity between tolcapone and entacapone. These results further substantiate the predictive value of DILIsym®.^{13,17,19} In addition, patient-specific risk factors for susceptibility to tolcapone-induced hepatotoxicity were identified. Further, the simulation results support the hypothesis that mitochondrial dysfunction is the primary mechanism underlying tolcapone-mediated toxicity. However, one of the primary limitations of this study is the lack of available data for the full repertoire of potential DILI mechanisms. Because the *in vitro* toxicity data available for tolcapone and entacapone was limited to cellular respiration data and bile acid transport inhibition data, the contributions from alternative hepatotoxicity mechanisms, such as oxidative stress, were not assessed here. Finally, the combined effect of multiple compound-specific properties, including mitochondrial uncoupling strength and hepatic exposure, was responsible for the difference in simulated hepatotoxicity profiles for tolcapone and entacapone.

In conclusion, DILIsym® represents a powerful tool to aid in the evaluation of the hepatotoxic liability of novel drugs.

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Conflict of Interest. D.M., Longo, B.A., Howell, and S.Q., Siler are employees of DILIsym Services, Inc., a company that licenses the DILIsym software for commercial use. D.M., Longo, B.A., Howell, S.Q., Siler, and P.B., Watkins have equity positions in DILIsym Services Inc.

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