An Analysis of N-Acetylcysteine Treatment for Acetaminophen Overdose Using a Systems Model of Drug-Induced Liver Injury


Received March 26, 2012; accepted May 3, 2012

ABSTRACT

N-acetylcysteine (NAC) is the treatment of choice for acetaminophen poisoning; standard 72-h oral or 21-h intravenous protocols are most frequently used. There is controversy regarding which protocol is optimal and whether the full treatment course is always necessary. It would be challenging to address these questions in a clinical trial. We used DILIsym, a mechanistic simulation of drug-induced liver injury, to investigate optimal NAC treatment after a single acetaminophen overdose for an average patient and a sample population (n = 957). For patients presenting within 24 h of ingestion, we found that the oral NAC protocol preserves more hepatocytes than the 21-h intravenous protocol. In various modeled scenarios, we found that the 21-h NAC infusion is often too short, whereas the full 72-h oral course is often unnecessary. We found that there is generally a good correlation between the time taken to reach peak serum alanine aminotransferase (ALT) and the time taken to clear N-acetyl-p-benzoquinone imine (NAPQI) from the liver. We also found that the most frequently used treatment nomograms underestimate the risk for patients presenting within 8 h of overdose ingestion. V_max for acetaminophen bioactivation to NAPQI was the most important variable in the model in determining interpatient differences in susceptibility. In conclusion, DILIsym predicts that the oral NAC treatment protocol, or an intravenous protocol with identical dosing, is superior to the 21-h intravenous protocol and ALT is the optimal available biomarker for discontinuation of the therapy. The modeling also suggests that modification of the current treatment nomograms should be considered.

Introduction

Acetaminophen (APAP) toxicity is a leading cause of liver injury, accounting for 40 to 50% of all cases of acute liver failure in the United States (Ostapowicz et al., 2002; Larson et al., 2005). APAP poisoning is the result of a reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which is formed via CYP450. In therapeutic doses, this reactive metabolite is conjugated by glutathione (GSH). However, in overdose conditions, the reactive metabolite depletes cellular GSH stores, which leads to oxidative stress, mitochondrial dysfunction, and cell death (Bajt et al., 2004).

Treatment with N-acetylcysteine (NAC) has been the standard therapy for APAP overdose since the 1970s (Rumack et al., 1981; Heard, 2008). NAC is actively transported into hepatocytes, where it serves as a precursor to GSH (Yang et al., 2009). Effective treatment with NAC provides sufficient GSH to neutralize NAPQI and avert oxidative stress and cell death. Clinically, there are two main treatment courses used for patients with APAP overdose, as well as an alternate intravenous bolus protocol that has been proposed (Smilkstein et al., 1991) but not widely adopted. These protocols are summarized in Table 1. Historically, the oral treatment has been used in the United States, whereas the intravenous route is typically used elsewhere. Both routes of administration have been demonstrated to be effective for most patients (Whyte et al., 2007). Some clinicians believe the optimal route depends on the gap between drug ingestion and patient presentation; the intravenous route is believed to be better soon after overdose, whereas the oral route has been pro-

ABBREVIATIONS: APAP, acetaminophen; NAPQI, N-acetyl-p-benzoquinone imine; GSH, glutathione; NAC, N-acetylcysteine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; SimPops, simulated sample population; RNS-ROS, reactive nitrogen species-reactive oxygen species; INR, international normalized ratio.
posed to be better for longer delays between dose and presentation (Yarema et al., 2009). Others believe that the oral protocol should always be better, because more NAC is given over the course of the treatment (Gupta et al., 2009). Still others believe there is no difference in outcomes between the two protocols, but prefer one to the other because of practical issues with administration of treatment (Hayes et al., 2008; Buckley et al., 2010).

Modifications of the standard oral and intravenous protocols have also been proposed, including shortening the oral NAC course (Kociancic and Reed, 2003) and tailoring the duration of treatment to biomarkers of liver injury (Betten et al., 2009) or APAP blood level (Woo et al., 2000; Tsai et al., 2005). Shorter treatment protocols may be advantageous, because some research suggests that NAC may impede recovery if taken beyond its period of usefulness. For example, NAC has been found to impede hepatocyte regeneration in mice (Yang et al., 2009), and in vitro data also support this effect (Athuraliya and Jones, 2009). Furthermore, there are potential side effects to NAC treatment, as well as the cost of a prolonged hospital stay (Woo et al., 2000). It is therefore important to investigate when a shorter course of NAC treatment might be sufficient. A direct comparison of the effectiveness of different NAC protocols would be difficult to carry out in a clinical setting, because it probably would require very large numbers of subjects given varied doses of APAP, varied NAC treatment delays, varied NAC treatment lengths, and interindividual differences in susceptibility to APAP liver injury.

In this study we used DILIsym (from The Hamner Institutes, http://www.thehamner.org/dili-sim), a mechanistic computer simulation model of drug-induced liver injury, to predict the optimal NAC treatment for an average patient and also for a more varied in silico population sample. We simulated mild, moderate, and severe overdoses of APAP and intervention with three advocated protocols of NAC treatment to compare the outcomes. We also simulated variations on the duration of the treatment protocols to determine the shortest duration of treatment that preserved efficacy. We then compared treatment protocols across a simulated sample population (SimPops) to determine which treatment protocols produce better results when interpatient variability is taken into account. Finally, we investigated which parameters had the greatest impact on individual susceptibility to APAP liver injury and response to NAC treatment.

### Materials and Methods

Simulations were performed by using DILIsym, an ordinary differential equation-based model of drug-induced liver injury implemented in the MATLAB computing platform (The MathWorks, Inc., Natick, MA). The model contains 282 differential equations and consists of the following submodels: 1) a physiologically based pharmacokinetic model of APAP, NAC, and the major APAP metabolites (glucuronide, sulfate, and NAPQI); 2) a model of GSH depletion and synthesis; 3) a model of mitochondrial dysfunction based on production of reactive nitrogen and oxygen species caused by NAPQI; 4) a model of the hepatocyte life cycle and the effects of ATP depletion and mitochondrial dysfunction on cell death; 5) a model of proinflammatory, anti-inflammatory, and prorregenerative mediator production and effects; 6) a model of injury propagation; and 7) a model of the dynamics of serum biomarkers such as bilirubin and ALT. Further information on the model is included in the supplemental materials.

The human data used to calibrate and validate the model are pulled from several cohort studies in the literature (Gazzard et al., 1975; Portmann et al., 1975; Davidson et al., 1976; Davis et al., 1976; Schüdt et al., 2001). In almost all of those studies, there were more females than males present, and age varied widely within all study groups, although all patients in the studies were adults. No data on ethnicity or other physical characteristics were reported in those studies. A more specific breakdown of the study populations used in this model is in Supplemental Table S5.

In our model, NAC alleviates acetaminophen toxicity by serving as a precursor to GSH. It does not conjugate directly with the reactive metabolite. This is consistent with the findings of Lauterburg et al. (1983), who saw no significant binding of NAC to the reactive metabolite in rats, and with currently accepted theories on the mechanism of NAC protection (Jones, 1998; Yuan and Kaplowitz, 2009; Liang et al., 2010). Other researchers have posited that NAC serves other functions, such as increasing interleukin-6 production (Masubuchi et al., 2011), serving as a scavenger of reactive oxidants (González et al., 2009), and improving liver blood flow (Jones, 1998). There is, however, insufficient data available in the literature to adequately model these hypotheses, and including these hypotheses was not necessary to construct a model that is consistent with published data on the effect of NAC treatment. Data from validation and calibration simulations, as well as a discussion of the possible effect of alternative NAC mechanisms on the model, are included in the supplemental materials.

To understand the population effects of NAC treatment for acute APAP poisoning, we created an in silico population sample (SimPops) of 957 individuals by varying 11 distinct characteristics (model parameters). The list of parameters varied and the ranges over which they were varied are listed in Table 2. The SimPops was generated by the use of a genetic algorithm, which randomly selects values of variables within specified bounds and optimizes these parameters based on the model's fit to a specified fitness function. This method is explained in depth in the supplemental materials. The comparison of our population to the results reported by Davis et al. (1976) is in Supplemental Fig. S1.

To compare our population sample response with the treatment nomograms, we ran simulations on each simulated patient at four different APAP doses (7.5, 10, 15, and 20 g) with no NAC treatment.
to identify individuals with a hepatotoxic response. Clinically, APAP hepatotoxicity is generally characterized by an ALT value of more than 1000 U/liter (Rumack et al., 1981; Sivilotti et al., 2005; Green et al., 2010). In our model after a single overdose of APAP a peak ALT value of 1000 U/liter corresponds to a loss of 18% of hepatocytes, so we defined as hepatotoxic any individual with less than 82% of hepatocytes viable. Furthermore, we classified any simulated individual with less than 45% remaining viable hepatocytes as having a life-threatening (severe) hepatotoxicity, because patients with this fraction of viable hepatocytes have been shown to have high mortality (Portmann et al., 1975; James et al., 2008). We then compared the predicted blood APAP concentration at 1-h intervals with the time since APAP ingestion to determine the location of each hepatotoxic, life-threatening hepatotoxic, and nonhepatotoxic simulated patient on the treatment nomograms.

Death in our model occurs when the simulated individual loses 85% of his/her viable hepatocytes. This is consistent with estimates made from analyses of liver biopsies obtained in patients with fatal and nonfatal liver injuries (Gazzard et al., 1975; Portmann et al., 1975).

Statistical analysis was performed by using JMP 9 from SAS Institute (Cary, NC). Correlation coefficients were measured by using a standard multivariate linear regression model. Statistical significance was determined by using Student’s t test. Because of the high number of data points for each set of simulations, SimPops results all were judged to be statistically significantly different (p < 0.05); statistical significance is therefore not presented with the SimPops data.

## Results

### NAC Treatment in an Average Individual

To address which NAC protocol (listed in Table 1) is most effective for the treatment of acute APAP overdose, we ran simulations of the DILIsym model for an average 70-kg human. Figure 1 shows the results of our simulation for a predicted lethal 60-g acute overdose of APAP followed by treatment with the three different NAC protocols. Each treatment was started after a delay ranging from 4 to 44 h from time of overdose. The nadir in viable hepatocyte fraction always occurred between 36 and 72 h regardless of treatment protocol or latency in starting treatment. The minimal return of hepatocyte fraction at 240 h is consistent with the data on human hepatocyte turnover, which is much slower than that observed in rodents (Portmann et al., 1975). As Fig. 1 shows, we found that the 72-h oral protocol (protocol A in Table 1) and the 48-h intravenous protocol (protocol C in Table 1) produce remarkably similar results. However, treatment with the 21-h standard intravenous protocol (protocol B in Table 1) leads to a lower fraction of viable hepatocytes after short treatment delays compared with protocols A and C. For example, when administered 4 h after overdose in our model, both protocols A and C preserved 70% of hepatocytes compared with 62% for protocol B. However, this difference diminished as the NAC treatment delay increased; if administered 34 h after overdose protocol A preserved 32% of hepatocytes, whereas protocol B preserved 31%. All three protocols failed to prevent death in the simulated average patient if administered more than 34 h after overdose.

In Fig. 1d, we show the results of our simulation for an intravenous infusion protocol similar to the 21-h intravenous protocol but where the third-stage infusion (6.25 mg/h) was extended to 67 h for a total duration of 72 h (protocol D in Table 1). Although this protocol was an improvement over protocol B at short treatment delay lengths, it remained worse than the oral protocol; at 4 h after overdose the 72-h intravenous infusion preserved 66.8% of hepatocytes versus 70% for the oral protocol.

When the APAP dose was increased to 85 g (Fig. 2) the difference between oral and 21-h intravenous NAC became more pronounced. After a 9-h delay in treatment, protocol A preserved 46.7% of hepatocytes, whereas protocol B preserved only 35.2% of hepatocytes. After 24 h protocol A preserved 32.0% of hepatocytes, whereas protocol B preserved 28.8%. Neither NAC course saved the patient if administered more than 24 h after overdose.

When we decreased the APAP dose to 30 g (Fig. 3) the difference between oral and 21-h intravenous NAC was less apparent. After a 9-h delay protocol A preserved 83.0% of hepatocytes, whereas protocol B preserved 80.1%. At 24 h after overdose both the oral and intravenous courses preserved 68.5% of hepatocytes. In addition, both treatment protocols had some positive effect on the patient even after a 44-h delay, although the effect was clearly diminished at that late stage. We see that at this dose protocol B administered with a 4-h delay was slightly less effective than the same treatment offered with a 9-h delay.

With an 85-g overdose the difference between the oral protocol A and the 21-h intravenous protocol B became more

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>S.D. Up</th>
<th>S.D. Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>82</td>
<td>130</td>
<td>50</td>
<td>19.2</td>
<td>12.8</td>
</tr>
<tr>
<td>Baseline GSH, mol/ml</td>
<td>5.50E-06</td>
<td>3.00E-06</td>
<td>1.00E-06</td>
<td>1.00E-06</td>
<td></td>
</tr>
<tr>
<td>APAP uptake rate from gut, 1/h</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucuronidation maximum rate, Vmax, mol/ml/h</td>
<td>3.88E-04</td>
<td>1.76E-04</td>
<td>5.29E-05</td>
<td>5.29E-05</td>
<td></td>
</tr>
<tr>
<td>Sulfation maximum rate, Vmax, mol/ml/h</td>
<td>3.44E-05</td>
<td>1.80E-05</td>
<td>3.2775E-06</td>
<td>3.2775E-06</td>
<td></td>
</tr>
<tr>
<td>NAPQI maximum rate, Vmax, mol/h</td>
<td>1.71E-03</td>
<td>1.88E-04</td>
<td>6.10E-04</td>
<td>6.10E-04</td>
<td></td>
</tr>
<tr>
<td>Reactive metabolite creation of RNS-ROS, Vmax, 1/h</td>
<td>2.91</td>
<td>1.51</td>
<td>0.28</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>RNS-ROS effect on ATP Vmax, 1/h</td>
<td>1.28</td>
<td>1.584</td>
<td>0.25</td>
<td>0.1216</td>
<td>0.412</td>
</tr>
<tr>
<td>Necrotic flux effect on propagation Kmax, cells/h</td>
<td>7.00E-07</td>
<td>1.12E-06</td>
<td>6.88E-07</td>
<td>1.88E-07</td>
<td></td>
</tr>
</tbody>
</table>

NAC Treatment Analysis Using a Systems Model of the Liver 531

Sex, a binary variable, is not included. The influence of sex on the model is limited to the differences in lean body mass between a male and female of the same overall body mass.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>S.D. Up</th>
<th>S.D. Down</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>82</td>
<td>130</td>
<td>50</td>
<td>19.2</td>
<td>12.8</td>
</tr>
<tr>
<td>Baseline GSH, mol/ml</td>
<td>5.50E-06</td>
<td>3.00E-06</td>
<td>1.00E-06</td>
<td>1.00E-06</td>
<td></td>
</tr>
<tr>
<td>APAP uptake rate from gut, 1/h</td>
<td>5</td>
<td>8</td>
<td>2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucuronidation maximum rate, Vmax, mol/ml/h</td>
<td>3.88E-04</td>
<td>1.76E-04</td>
<td>5.29E-05</td>
<td>5.29E-05</td>
<td></td>
</tr>
<tr>
<td>Sulfation maximum rate, Vmax, mol/ml/h</td>
<td>3.44E-05</td>
<td>1.80E-05</td>
<td>3.2775E-06</td>
<td>3.2775E-06</td>
<td></td>
</tr>
<tr>
<td>NAPQI maximum rate, Vmax, mol/h</td>
<td>1.71E-03</td>
<td>1.88E-04</td>
<td>6.10E-04</td>
<td>6.10E-04</td>
<td></td>
</tr>
<tr>
<td>Reactive metabolite creation of RNS-ROS, Vmax, 1/h</td>
<td>2.91</td>
<td>1.51</td>
<td>0.28</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>RNS-ROS effect on ATP Vmax, 1/h</td>
<td>1.28</td>
<td>1.584</td>
<td>0.25</td>
<td>0.1216</td>
<td>0.412</td>
</tr>
<tr>
<td>Necrotic flux effect on propagation Kmax, cells/h</td>
<td>7.00E-07</td>
<td>1.12E-06</td>
<td>6.88E-07</td>
<td>1.88E-07</td>
<td></td>
</tr>
</tbody>
</table>
pronounced (Table 3). Protocol B failed to save the average simulated patient if administered 4 h after the 85-g overdose (less than 15% of hepatocytes preserved; Fig. 2; Table 3). However, an interesting observation is that protocol B saved the patient if offered 9, 14, 19, or even 24 h after overdose (Fig. 3; Table 3). This suggests that the 21-h intravenous infusion course is too short when administered early to effectively treat extreme overdoses, as has been suggested by others (Doyon and Klein-Schwartz, 2009). This idea was supported by the observation that after the 85-g overdose the 72-h intravenous infusion (protocol D) saved the patient if administered 4 h after an 85-g overdose (Fig. 2d; Table 3), although it was still inferior to the oral and intravenous bolus protocols (Table 3). When administered 4 h after over-
dose protocol D preserved 39.3% of hepatocytes, and at 9 h after overdose it preserved 38.4% of hepatocytes. This was superior to protocol B, but still well below the percentage preserved by protocols A and C (Table 3).

We next investigated whether the entire 72-h oral course of NAC is always required for optimal treatment. We ran simulations for a 60-g APAP overdose with oral NAC treatment starting between 4 and 34 h after overdose and varied the total number of NAC doses administered in the treatment. These results are displayed in Fig. 4, which plots the minimum fraction of viable hepatocytes against the number of doses for different treatment delay lengths. When started 4 h after overdose, the effectiveness of NAC treatment in preserving hepatocyte viability was limited to the first 10 doses. When we began oral NAC treatment at 14 h after overdose hepatocyte viability did not increase appreciably for any treatment course longer than eight doses. When treatment was started at 24 h after APAP overdose the number of useful doses was even fewer; only six doses had a beneficial effect. In each of these cases, the final dose with a therapeutic effect was the dose taken between 44 and 48 h after APAP overdose.

**TABLE 3**

Nadir hepatocyte fraction as a function of simulated APAP dose and delay in onset of treatment for each of the four NAC treatment protocols

At 85 g D indicates that the simulated patient died despite treatment (i.e. reached a fraction of viable hepatocytes of ≤0.15).

<table>
<thead>
<tr>
<th>Dose</th>
<th>4 h</th>
<th>14 h</th>
<th>24 h</th>
<th>34 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>30 g</td>
<td>0.855</td>
<td>0.801</td>
<td>0.854</td>
<td>0.809</td>
</tr>
<tr>
<td>60 g</td>
<td>0.702</td>
<td>0.626</td>
<td>0.699</td>
<td>0.668</td>
</tr>
<tr>
<td>85 g</td>
<td>0.534</td>
<td>D</td>
<td>0.527</td>
<td>0.393</td>
</tr>
</tbody>
</table>

**Fig. 3.** Fraction of hepatocytes viable versus time for a simulated 35-g acute overdose of APAP followed by NAC treatment given by the 72-h oral (a), standard 21-h intravenous (b), 48-h intravenous bolus (c), and 72-h intravenous (d) infusion protocols. The treatment was initiated between 4 and 44 h after overdose.

**Fig. 4.** Minimum fraction of hepatocytes viable after oral NAC treatment with a varying number of doses. Simulated APAP doses given were 60 g (a) and 85 g (b). NAC treatment was delayed by 4, 14, and 24 h.
If we increase the APAP dose to 85 g we see a larger number of NAC doses having a therapeutic effect. When oral NAC was started 4 h after an 85-g overdose 14 doses increased the minimum fraction of viable hepatocytes (Fig. 4b). If oral NAC was started 14 h after overdose 12 doses were useful; at 24 h after overdose only 10 doses were useful. In each of these cases, the final dose with a therapeutic effect was taken between 60 and 64 h after overdose.

The intravenous bolus protocol (protocol C) had the same number of effective doses as the oral course for all delay lengths investigated (data not shown). Because the intravenous bolus protocol administers the same amount of NAC as the oral protocol at each dose, this indicates that the route of administration of NAC does not matter. It is noteworthy that with continuous intravenous infusion protocols (protocols B and D) the maximum effective treatment duration was longer than the maximum effective length of the oral NAC or bolus intravenous courses (Fig. 5). If started 4 h after an 85-g overdose intravenous infusion treatment was useful up to 73 h after overdose, and if started 14 h after overdose it was useful up to 71 h after overdose. At 24 h after overdose the treatment was useful until 73 h after overdose.

We also investigated the length of time the reactive metabolite NAPQI was simulated to remain in the liver as well as the amount of time required for GSH levels to return to baseline. In cases where protocol A was administered between 4 and 34 h after an 85-g overdose the predicted NAPQI concentration in the liver fell to zero at 60 h (Supplemental Fig. S2), and the GSH was replenished between 28 h (for the 4-h delay) and 44 h (for the 34-h delay) before rising above baseline levels. For the 60-g overdose NAPQI was eliminated from the liver at 48 h after overdose for all lengths of NAC delay, and the GSH was replenished between 22.5 h (for the 4-h delay) and 40 h (for the 34-h delay). The NAPQI residence time in the liver varied with the infusion length when intravenous NAC was administered; however, for the longest NAC infusions NAPQI was cleared from the liver at 72 h after overdose (Supplemental Fig. S3).

**Population Effects.** Reaction to APAP overdose and NAC treatment is likely to highly depend on variability within the human population. As such, it is important when considering a clinical treatment to look not only at the effects of that treatment on the average human but also on the most and least susceptible members of a large population.

We first sought to compare our results to the treatment nomograms that are often used in the clinic to determine whether a patient is at risk for hepatotoxicity and thus needs NAC treatment. Treatment nomograms involve plotting the plasma acetaminophen concentration of the patient versus the estimated time since overdose and comparing where this point falls on the plot relative to a “treatment line.” The most well known of these nomograms is the Rumack-Matthew nomogram, where the treatment line begins at a blood APAP concentration of 200 μg/ml (at 4 h after APAP ingestion); at the Food and Drug Administration’s request, this line was later lowered to begin at 150 μg/ml (Rumack et al., 1981).

Modifications to the Rumack-Matthew nomogram are fairly common in clinical practice; a survey of Australasian hospitals by Reid and Hazell (2003) showed that the treatment line varied among hospitals, with some choosing a 100 μg/ml line, some the 150 line, and others the 200 line.

We administered four doses of APAP (7.5, 10, 15, and 20 g) to our SimPops virtual population of 957 individuals. At 7.5 g 1.04% of the population developed hepatotoxicity (i.e., a viable hepatocyte fraction of ≈82%), and at 20 g 56.6% of the population developed hepatotoxicity. We plotted the APAP concentration of each patient at 1-h intervals from 4 to 24 h after overdose. Over all four doses investigated, we found that 9.31% of individuals whose blood APAP concentrations fell below the 150 line developed hepatotoxicity, whereas 3.19% of those falling below the 100 line developed hepatotoxicity. However, there is a difference in risk between patients presenting at 4 h and patients presenting at 24 h. At 4 h after overdose 17.16% of individuals falling below the 150 line developed hepatotoxicity, whereas 5.21% of individuals falling below the 150 line at 24 h after overdose developed hepatotoxicity. A similar disparity was present for the 100 line; 6.28% of individuals below the 100 line at 4 h after overdose developed hepatotoxicity, whereas only 2.33% of individuals below the 100 line at 24 h developed hepatotoxicity. The risk of hepatotoxicity below the 150 line for our simulated patients declined to 7.77% at 12 h after overdose.

To explain the increase in hepatotoxic risk for individuals falling below the nomogram line at early presentation, we compared the APAP pharmacokinetics of the minimum hepatotoxic dose to the modeled average human (17 g; peak ALT = 1000 U/liter) to the nomogram lines (Fig. 6). The time course of APAP concentration has a flatter trajectory than the nomogram treatment lines until approximately 12 h after overdose; in fact, the average model human concentration-time curve crosses over the 200 line at approximately 8 h after overdose. This demonstrates that the slope of the current nomogram lines does not reflect the pharmacokinetics of APAP in the blood, and because of this, an early presenting patient whose blood APAP concentration is initially below the nomogram lines and would not receive NAC may actually
have a blood APAP concentration in the at-risk region of the nomogram at a later time. The validation of our model APAP pharmacokinetics is shown in the supplemental materials.

**NAC Treatment on a Sample Population.** We modeled the response of the simulated population to 30- and 60-g acute overdoses of APAP with 1) no NAC treatment, 2) oral NAC treatment using protocol A, 3) intravenous infusion NAC treatment using protocol D, or 4) intravenous bolus NAC treatment using protocol C extended to 72 h. The effects of these treatment courses on the simulated population are described in Supplemental Table S1. As with the average patient, protocols A and C performed better than protocol D when administered to the simulated population soon after overdose. After a 30-g overdose and a 4-h treatment delay 11.1% of the population developed hepatotoxicity after protocol A compared with 11.5% after protocol C and 22.9% after protocol D. However, when administered with a 14-h delay the difference among the treatment protocols shrunk substantially; 37.1% of the simulated population developed hepatotoxicity after protocol A compared with 37.7% of those who received protocol C and 38.7% of those who received protocol D.

After a 60-g overdose, the oral and intravenous bolus courses outperformed the intravenous infusion course at delay lengths of 4 and 14 h. After a 14-h delay 13.8% of the simulated population that received protocol A developed severe hepatotoxicity compared with 14.3% after protocol C and 18.6% after protocol D. For comparison, we also simulated protocol B; after a 60-g overdose and a 14-h treatment delay and as expected from our simulations in the average individual, this protocol was the least efficacious because 22.4% of the simulated population developed severe hepatotoxicity. In addition, we investigated the effect of using a shorter oral NAC course on the population; we found that there was little difference between a 12-dose course and the standard 18-dose course when treatment began 4 or 24 h after either a 30- or 60-g overdose.

**Biomarker Analysis.** Woo et al. (2000) suggested the use of serum APAP concentration as a biomarker to guide the termination of NAC treatment. Others have suggested that treatment may be safely stopped once serum ALT or AST have reached their peak and are decreasing or below a certain value (Betten et al., 2009). Based on the assumptions in the DILIsym model, NAC is no longer effective once the reactive metabolite NAPQI has cleared from the liver; as such, we examined the correlation between liver NAPQI and serum concentrations of APAP and aminotransferases in our virtual patient population as a function of dose (60 and 30 g) and treatment latency (4, 14, 24, 34, and 44 h). We then compared the time required for liver NAPQI levels to reach 1% of its peak value to the time required for blood APAP concentration to fall to 5 µg/ml or when the peak ALT/AST concentration was reached. When the time required for NAPQI to reach 1% of its peak value is less than the time required for the biomarker to reach its critical value guiding therapy by this biomarker should overtreat the patient; when the time required for the biomarker to reach its critical value is shorter the patient should be undertreated. The comparison of the time required for NAPQI to drop below 1% of its peak value with the time required for blood APAP concentration to drop below 5 µg/ml is in Fig. 7a; statistics for the biomarker analysis can be found in Supplemental Table S2. Only 0.16% of patients would be undertreated by stopping NAC treatment when blood APAP is below 5 µg/ml (and none would be undertreated more than 4 h, meaning a missed oral or intravenous bolus dose). The maximum overtreatment is 48 h, and the median overtreatment is 14 h. At a 24-h delay length, however, 4.60% of patients were undertreated (0.21% more than 4 h), and at a 44-h delay length 73.9% of patients were undertreated (55.38% more than 4 h). Because APAP is often undetectable in the bloodstream so long after overdose, it is not surprising that this biomarker is a weak one at long delay times.

Serum ALT performed better than blood APAP concentration at the 30-g APAP dose. Using peak ALT (Fig. 7b) undertreated the fewest patients (maximum of 1.88% at 4 h after overdose), but median overtreatment time using ALT was 15 to 20 h, which is the worst of the four biomarkers. Using peak AST also undertreated 1.88% of patients at 4 h after overdose, but only had a median overtreatment time of 16.6 h at 4 h after overdose and 7.15 h at 44 h after overdose (Fig. 7c; Supplemental Table S2). It is noteworthy that using blood bile acid concentration (Fig. 7d) undertreated 7.84% of patients after a 4-h delay (although only 0.10% for more than 4 h), but the median overtreatment time was between 2 and 3 h for all treatment delays, making it the best of the bio-
markers by that measure. We also tracked peak international normalized ratio (INR), which is a commonly used clinical biomarker for the cessation of NAC treatment (Fig. 7e). We found that using INR only undertreated 0.10% of the population; however, the median overtreatment with peak INR was between 56 and 59 h, which is far greater than any other biomarker examined. Further discussion of INR as it relates to NAC treatment can be found in the supplemental materials.

After a 60-g overdose, however, the results were different. Stopping treatment at serum APAP concentration of 5 μg/ml (Fig. 8a) undertreated only 7.21% of the population after a 44-h treatment delay, and using peak ALT (Fig. 8b) undertreated no one at any delay length. Conversely, using peak AST (Fig. 8c) undertreated between 6 and 19% of the population, whereas using peak blood bile acids (Fig. 8d) undertreated between 5 and 10% of the population (Supplemental Table S2). Like ALT, using peak INR (Fig. 8e) did not undertreat anyone; however, the mean and median overtreatment with INR was approximately twice the mean and median overtreatment for peak ALT (Supplemental Table S2).

We also measured the ideal treatment length for each patient, defined as the length of time between the initiation of NAC treatment and clearance of NAPQI from the liver (data not shown). Even for a massive overdose, the ideal treatment time does not extend far beyond the current clinical recommendations; after a 100-g overdose, the longest ideal treatment time in our simulated population was 81 h for NAC administered after a 4-h delay and 63 h for NAC administered after a 24-h delay. According to our model, the current 72-h clinically recommended oral course would undertreat 1.57% of the population when administered 4 h after a 100-g APAP overdose.

**Parameter Correlation Comparison.** To determine which variables in the population model had the greatest effect on the hepatocyte fraction, we performed a multiple regression analysis both without NAC and with oral NAC treatment with simulated patient characteristics (Table 4). We found that the 10 parameters that varied continuously (excluding sex, which is binary) over the population varied in terms of correlation with hepatocyte fraction. With no NAC treatment the most important variables were those directly related to toxic metabolite production and effects: NAPQI $V_{\text{max}}$ (CYP2E1 activity) $> \text{RNS-ROS generation}$ $V_{\text{max}} > \text{ATP decrement}$ $V_{\text{max}} > \text{injury propagation trigger point}$. Other less important factors were: body mass $> \text{GSH baseline level}$ $> \text{GSH transport} V_{\text{max}}$. Variation in sulfation $V_{\text{max}}$ and APAP uptake rate had no effect on hepatotoxicity susceptibility.

With oral NAC dosing (protocol A) the effects of the variables were similar, but glucuronidation $V_{\text{max}}$, baseline GSH concentration, and GSH transport $V_{\text{max}}$ were generally less important. The length of treatment delay changed the level of correlation between some variables and hepatocyte fraction. The correlation coefficient for the injury propagation trigger point was higher after a 34-h NAC treatment delay ($r = 0.3500$; third) than after a 4-h delay ($r = 0.1243$; fifth). It is also interesting to note that the GSH precursor transport $V_{\text{max}}$ correlation coefficient was essentially not correlated with hepatocyte fraction after a 4-h treatment delay ($p = 0.1261$), but was the fifth most important variable after a 34-h treatment delay ($r = 0.1843; p < 0.0001$). The relative

**Fig. 7.** Plot of the time required for NAPQI to reach 1% of its peak value against the time required for blood APAP concentration to reach 5 μg/ml (a), ALT to reach its peak value (b), AST to reach its peak value (c), blood bile acid concentration to reach its peak value (d), and INR to reach its peak value (e) after a 30-g APAP overdose. Seventy-two-hour oral NAC treatment was modeled with delays of 4, 14, 24, 34, and 44 h. The blue lines denote where the biomarker matches the disappearance of NAPQI perfectly; below and to the right of the blue lines is overtreatment, and above and to the left of the blue lines is undertreatment.
importance of the correlation coefficients and the differences among coefficients at different lengths of treatment delay were similar after a 60-g overdose.

**Discussion**

There is controversy about whether oral or intravenous NAC provides the best protection against APAP toxicity and when completing the full protocol is unnecessary. We addressed these questions by using DILIsym, a computer model based on current knowledge about the mechanisms underlying APAP toxicity. Computer modeling can simulate experiments and measurements that would be impossible on human subjects; as such, potentially important insights can be gained from such a mechanistic computer model.

Our principal conclusion is that the 72-h oral course (protocol A) is superior to the 21-h intravenous course (protocol B) when treatment is started within 24 h of overdose. There is little difference between protocol A and the intravenous bolus protocol C in the outcome of the simulated patient for all APAP doses and treatment latencies. Because these two protocols administer NAC at similar rates, the difference in effectiveness between standard oral and intravenous NAC courses is not method of delivery but rather the duration and structure of the treatment. As Fig. 9 shows, there is a significant difference in the total amount of NAC administered by the standard oral and intravenous infusion protocols beyond 8 h after starting treatment, and this may become more important the earlier NAC is administered.

It is noteworthy that the opposite conclusion concerning the merits of oral versus intravenous NAC was reached based on a prospective analysis of 4048 patients (Yarema et al., 2009). Those investigators found that, for patients treated <12 h postingestion, the incidence of hepatotoxicity was lower in those receiving intravenous treatment.

**TABLE 4**

Correlation coefficients ($R$) between each varied parameter and the fraction of hepatocytes viable

<table>
<thead>
<tr>
<th>APAP Dose</th>
<th>Treatment Delay</th>
<th>Body Mass</th>
<th>GSHo</th>
<th>APAP Uptake Rate</th>
<th>Glucuronidation $V_{max}$</th>
<th>Sulfation $V_{max}$</th>
<th>NAPQI $V_{max}$</th>
<th>RNS-ROS $V_{max}$</th>
<th>ATP Decrement $V_{max}$</th>
<th>Propagation $K_{m}$</th>
<th>GSH Transport $V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 g</td>
<td>4 h</td>
<td>0.2603***</td>
<td>0.0329</td>
<td>-0.0046</td>
<td>0.0040***</td>
<td>0.0117</td>
<td>-0.5427***</td>
<td>-0.4252***</td>
<td>-0.2780***</td>
<td>0.1343***</td>
<td>0.0477</td>
</tr>
<tr>
<td>30 g</td>
<td>34 h</td>
<td>0.1234***</td>
<td>0.1098***</td>
<td>-0.0060</td>
<td>-0.0027</td>
<td>0.0331</td>
<td>-0.4462***</td>
<td>-0.4926***</td>
<td>-0.3099***</td>
<td>0.3506***</td>
<td>0.1843***</td>
</tr>
<tr>
<td>60 g</td>
<td>4 h</td>
<td>0.1618***</td>
<td>0.0555</td>
<td>-0.0159</td>
<td>0.0266</td>
<td>0.0281</td>
<td>-0.5249***</td>
<td>-0.4328***</td>
<td>-0.2789***</td>
<td>0.3137***</td>
<td>0.0285</td>
</tr>
<tr>
<td>60 g</td>
<td>34 h</td>
<td>-0.1000</td>
<td>0.0800</td>
<td>-0.0333</td>
<td>0.0282</td>
<td>0.0004</td>
<td>-0.4095***</td>
<td>-0.5803***</td>
<td>-0.3026***</td>
<td>0.2336***</td>
<td>0.0970***</td>
</tr>
<tr>
<td>15 g</td>
<td>No NAC</td>
<td>0.2269***</td>
<td>0.1167***</td>
<td>-0.0173</td>
<td>0.0239***</td>
<td>0.0538</td>
<td>-0.5321***</td>
<td>-0.4385***</td>
<td>-0.2928***</td>
<td>0.1168***</td>
<td>0.1812***</td>
</tr>
<tr>
<td>20 g</td>
<td>No NAC</td>
<td>0.2070***</td>
<td>0.1316***</td>
<td>-0.0224</td>
<td>0.0009***</td>
<td>0.0591</td>
<td>-0.5087***</td>
<td>-0.4480***</td>
<td>-0.3147***</td>
<td>0.1696***</td>
<td>0.1939***</td>
</tr>
<tr>
<td>30 g</td>
<td>No NAC</td>
<td>0.1504***</td>
<td>0.1138***</td>
<td>-0.0143</td>
<td>0.0097***</td>
<td>0.0428</td>
<td>-0.4710***</td>
<td>-0.4743***</td>
<td>-0.3122***</td>
<td>0.2358***</td>
<td>0.2228***</td>
</tr>
<tr>
<td>60 g</td>
<td>No NAC</td>
<td>-0.0152***</td>
<td>0.0722</td>
<td>-0.0385</td>
<td>0.0414</td>
<td>0.0059</td>
<td>-0.4284***</td>
<td>-0.5223***</td>
<td>-0.2842***</td>
<td>0.1670***</td>
<td>0.1536***</td>
</tr>
</tbody>
</table>

*** denotes a statistically significant correlation ($p < 0.001$).

---

**Fig. 8.** Plot of the time required for NAPQI to reach 1% of its peak value against the time required for blood APAP concentration to reach 5 μg/ml (a), ALT to reach its peak value (b), AST to reach its peak value (c), blood bile acid concentration to reach its peak value (d), and INR to reach its peak value (e) after a 60-g APAP overdose. Seventy-two-hour oral NAC treatment was modeled with delays of 4, 14, 24, 34, and 44 h. The blue lines denote where the biomarker matches the disappearance of NAPQI perfectly; below and to the right of the blue lines is overtreatment, and above and to the left of the blue lines is undertreatment.
ever, the orally and intravenously treated cohorts were different; the former was from the United States, and the latter was in Canada, and the median time from ingestion to NAC initiation differed significantly. Furthermore, the details of the intravenous protocol referenced in this paper were not provided. In preliminary modeling, we explored whether addition to our model of direct antioxidant effects of NAC independent of GSH repletion (González et al., 2009) altered our conclusions, and it did not (Supplemental Figs. S27 and S28). Although we would not at this time recommend a change in clinical practice based solely on our modeling, DILIsym provides a mechanistic, data-based, and rigorous approach to the problem, and our results should prompt reexamination of relevant clinical data.

We also examined biomarkers that might predict when treatment can be safely terminated. Others suggest that when blood APAP drops below detectable levels NAC should be stopped (Woo et al., 2000). We found that blood APAP is a reasonable biomarker of hepatic NAPQI when NAC is administered <24 h after overdose; however, if NAC is administered >24 h after overdose, APAP will often be eliminated from the bloodstream before NAPQI has completely cleared the liver. Following APAP blood levels in this instance would lead to undertreatment of many patients.

We found improved performance with serum ALT. We demonstrated that terminating treatment once peak ALT is observed will prevent undertreatment for most patients; using AST in this context presents a greater risk of undertreatment presumably because of its shorter half-life in serum. However, terminating NAC treatment after peak ALT is observed will lead to ≥20 h of unnecessary treatment for some patients. Because overtreatment is preferable to undertreatment, our results support the use of ALT to guide therapy termination. Because simulations suggest that extending NAC treatment beyond 72 h generally provides no additional benefit, terminating NAC treatment at 72 h regardless of ALT measures would help minimize overtreatment issues.

Peak serum bile acid concentration also seemed to be a promising biomarker for stopping NAC treatment, resulting in the lowest median overtreatment. These observations should be interpreted with caution. Our model of bile acid homeostasis is based on physiological values for bile acid concentrations in the blood and the liver and tracks well with dose-response results from patients with APAP overdose (James et al., 1975). However, we were unable to find reports of a time course of blood bile acids after APAP overdose, and as such we do not know whether the time course of blood bile acids in our model is an accurate representation of what is seen in the clinic. The point is moot from a clinical perspective because serum assays of bile acids are slow and not generally available in the clinic.

Some clinicians suggest using the INR as a biomarker for stopping NAC treatment (Betten et al., 2009). We found that the INR does not undertreat anyone, but is the worst biomarker for overtreatment. Furthermore, NAC acts to prolong the INR (Jepsen and Hansen 1994), and the INR can be increased in patients with APAP overdose without hepatotoxicity after NAC treatment (Schmidt et al., 2002; Lucena et al., 2005).

The DILIsym model predicted that the initiation of NAC therapy is not effective in the average human when administered >34 h after an overdose (however, some members of a sample population benefit from treatment starting as late as 44 h after overdose). This is true despite the fact that the model predicted that NAPQI has not cleared the liver by then. In addition, DILIsym indicated that NAC therapy, once started, can be beneficial up to 72 h after overdose. The explanation for these apparently discordant observations is the critical role of the injury propagation response. Limaye et al. (2003) noted that propagation effects continue in liver injury after the toxic compound has cleared the liver and probably are a contributor to lethality. Mehendale and Limaye (2005) implicate calpain as a contributor to this propagation effect; others point out that inflammation (Tukov et al., 2006) or communication through gap junctions (Patel et al., 2012) may play a role in propagation. In our model, the propagation effect is modeled by a steep Hill function that represents a “trigger” determined by the size and duration of the necrotic flux. In both the simulated fatal overdoses of 60 and 85 g the propagation effect is triggered at 48 h; after this time it is injury propagation that leads to death, not direct toxicity from NAPQI. Our model predicts that NAC treatment must begin before 34 h after overdose in the average patient to provide enough therapeutic effect to avoid the injury propagation that leads to death; however if propagation does not occur, as with nonlethal doses of APAP, less-susceptible individuals, or when NAC has been started in time, the continuation of NAC treatment can be effective until NAPQI is cleared from the liver.

The hypothesis that NAC treatment must be administered early enough to prevent injury propagation is reinforced by the increased importance of the location of the propagation trigger when NAC is administered late. In our model, after a 30-g overdose the correlation coefficient of the variable controlling the location of the trigger point increased almost 3-fold when NAC was administered at 34 h after overdose compared with a 4-h delay. This is likely because early administration of NAC allows the hepatocyte GSH to replenish fast enough to eliminate NAPQI and prevent the level of injury necessary for propagation in all but the most susceptible individuals. However, the later NAC is administered the more likely it is that the necrotic flux will reach the range over which the trigger point location varies within the population. When NAC is delayed 34 h hepatocyte injury is already well underway, and the die is cast on whether the propagation response will occur.

We also found that variation in $V_{\text{max}}$ for NAPQI formation, which should correlate chiefly with hepatic activity of the

---

**Fig. 9.** A diagram of the total NAC dosed over time (in milligrams/kilograms) for the intravenous infusion protocol (red line) and the oral/intravenous bolus protocol (blue line).
enzyme CYP2E1, was the most significant factor underlying susceptibility to APAP toxicity. This is consistent with the observation that chronic alcoholics, who have generally higher CYP2E1 activity (Hu et al., 1995), are at increased risk of APAP hepatotoxicity (Ali et al., 2008).

We also used DILISym to assess the adequacy of nomograms used to determine which patients receive NAC treatment. It has been established that the 150 μg/ml line popularized by Rumack and Matthew is inappropriate for highly susceptible patients (James et al., 2008; Ali et al., 2008). We found that using the 100 line to determine treatment initiation led to only 3.19% of simulated patients developing hepatoxicity. We also noted that patients who presented early (4–8 h after overdose) and seemed on the nomogram below the 100 line (and would likely not receive NAC therapy) had a higher risk of hepatotoxicity than patients who presented below the 100 line later. In our model, this observation can be explained by the slow absorption of very large doses of acetaminophen, which delays the fall in serum APAP concentrations. This suggests that patients with early presenting APAP overdose who fall below but near either the 100 or 150 nomogram lines should be considered for NAC therapy.

In conclusion, DILISym predicts that the standard oral NAC treatment protocol, or an intravenous protocol with identical dosing, is superior to the standard 21-h intravenous protocol in preventing hepatotoxicity after acetaminophen overdose. The modeling also indicates that because of the slow absorption of large APAP overdoses, current treatment nomograms may underestimate the risk of hepatotoxicity for some early presenting patients. Once NAC therapy is initiated, the modeling indicates that serum ALT is the optimal available biomarker to guide safe discontinuation of the therapy.

Acknowledgments
We thank partners of the DILL-sim Initiative (The Hamner Institutes for Health Sciences) for many informative conversations.

Authorship Contributions
Participated in research design: Woodhead, Howell, Yang, Harrill Clewell, Andersen, Siler, and Watkins.
Conducted experiments: Harrill.
Performed data analysis: Woodhead.
Wrote or contributed to the writing of the manuscript: Woodhead, Howell, Andersen, Siler, and Watkins.

References
acute liver failure: recommendations of the U.S. Acute Liver Failure Study Group. 


Address correspondence to: Paul B. Watkins, The Hamner Institutes for Health Sciences, 6 Davis Drive, Box 12137, Research Triangle Park, NC 27709. E-mail: pwatkins@thehamner.org
An Analysis of N-Acetylcysteine Treatment for Acetaminophen Overdose Using A Systems Model of Drug-Induced Liver Injury

Supplementary Materials

Jeffrey L. Woodhead, Brett A. Howell, Yuching Yang, Alison H. Harrill, Harvey J. Clewell III, Melvin E. Andersen, Scott Q. Siler, Paul B. Watkins

Journal of Pharmacology and Experimental Therapeutics

The Hamner-UNC Institute for Drug Safety Sciences
The Hamner Institutes
6 Davis Drive
Research Triangle Park, NC 27709
Figure S1. Fit of our sample population to the data reported by Davis et al.\textsuperscript{1} comparing plasma bilirubin levels to APAP doses for patients who consumed varying doses of APAP.
Figure S2. Average liver NAPQI concentration (a,b) and glutathione concentration (c,d) versus time after overdose with oral NAC treatment beginning at 4, 14, 24, and 34 hours after overdose. APAP doses were 60g (a,c) and 85g (b,d).
Figure S3. Average liver NAPQI concentration (a,b) and glutathione concentration (c,d) versus time after overdose with 21-hour intravenous NAC treatment beginning at 4, 14, 24, and 34 hours after overdose. APAP doses were 60g (a,c) and 85g (b,d).
Supplemental Tables

Table S1. Summary of outcomes for population samples involving different NAC treatment protocols, APAP doses, and treatment delay lengths.

<table>
<thead>
<tr>
<th>Treatment route</th>
<th>APAP dose (g)</th>
<th>Treatment delay (h)</th>
<th>Deaths %</th>
<th>Severe hepatotoxicity %</th>
<th>Hepatotoxicity %</th>
<th>Mean hepatocyte death fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>no NAC</td>
<td>30</td>
<td>0</td>
<td>56</td>
<td>5.85%</td>
<td>141</td>
<td>14.73%</td>
</tr>
<tr>
<td>72h oral</td>
<td>30</td>
<td>4</td>
<td>2</td>
<td>0.21%</td>
<td>2</td>
<td>0.21%</td>
</tr>
<tr>
<td>72h oral</td>
<td>30</td>
<td>14</td>
<td>12</td>
<td>1.25%</td>
<td>12</td>
<td>1.25%</td>
</tr>
<tr>
<td>72h oral</td>
<td>30</td>
<td>24</td>
<td>38</td>
<td>3.97%</td>
<td>56</td>
<td>5.85%</td>
</tr>
<tr>
<td>72h oral</td>
<td>30</td>
<td>34</td>
<td>53</td>
<td>5.54%</td>
<td>93</td>
<td>9.72%</td>
</tr>
<tr>
<td>72h oral</td>
<td>30</td>
<td>44</td>
<td>54</td>
<td>5.64%</td>
<td>120</td>
<td>12.54%</td>
</tr>
<tr>
<td>48h oral</td>
<td>30</td>
<td>4</td>
<td>2</td>
<td>0.21%</td>
<td>2</td>
<td>0.21%</td>
</tr>
<tr>
<td>48h oral</td>
<td>30</td>
<td>24</td>
<td>38</td>
<td>3.97%</td>
<td>56</td>
<td>5.85%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>30</td>
<td>4</td>
<td>2</td>
<td>0.21%</td>
<td>2</td>
<td>0.21%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>30</td>
<td>14</td>
<td>13</td>
<td>1.36%</td>
<td>16</td>
<td>1.67%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>30</td>
<td>24</td>
<td>38</td>
<td>3.97%</td>
<td>59</td>
<td>6.17%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>30</td>
<td>34</td>
<td>53</td>
<td>5.54%</td>
<td>93</td>
<td>9.72%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>30</td>
<td>44</td>
<td>54</td>
<td>5.64%</td>
<td>120</td>
<td>12.54%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>30</td>
<td>4</td>
<td>2</td>
<td>0.21%</td>
<td>2</td>
<td>0.21%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>30</td>
<td>14</td>
<td>12</td>
<td>1.25%</td>
<td>12</td>
<td>1.25%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>30</td>
<td>24</td>
<td>40</td>
<td>4.18%</td>
<td>60</td>
<td>6.27%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>30</td>
<td>34</td>
<td>53</td>
<td>5.54%</td>
<td>94</td>
<td>9.82%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>30</td>
<td>44</td>
<td>54</td>
<td>5.64%</td>
<td>120</td>
<td>12.54%</td>
</tr>
<tr>
<td>no NAC</td>
<td>60</td>
<td>0</td>
<td>481</td>
<td>50.26%</td>
<td>634</td>
<td>66.25%</td>
</tr>
<tr>
<td>72h oral</td>
<td>60</td>
<td>4</td>
<td>26</td>
<td>2.72%</td>
<td>48</td>
<td>5.02%</td>
</tr>
<tr>
<td>72h oral</td>
<td>60</td>
<td>14</td>
<td>61</td>
<td>6.37%</td>
<td>132</td>
<td>13.79%</td>
</tr>
<tr>
<td>72h oral</td>
<td>60</td>
<td>24</td>
<td>176</td>
<td>18.39%</td>
<td>385</td>
<td>40.23%</td>
</tr>
<tr>
<td>72h oral</td>
<td>60</td>
<td>34</td>
<td>327</td>
<td>34.17%</td>
<td>525</td>
<td>54.86%</td>
</tr>
<tr>
<td>72h oral</td>
<td>60</td>
<td>44</td>
<td>437</td>
<td>45.66%</td>
<td>577</td>
<td>60.29%</td>
</tr>
<tr>
<td>48h oral</td>
<td>60</td>
<td>4</td>
<td>26</td>
<td>2.72%</td>
<td>48</td>
<td>5.02%</td>
</tr>
<tr>
<td>48h oral</td>
<td>60</td>
<td>24</td>
<td>176</td>
<td>18.39%</td>
<td>385</td>
<td>40.23%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>60</td>
<td>4</td>
<td>37</td>
<td>3.87%</td>
<td>87</td>
<td>9.09%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>60</td>
<td>14</td>
<td>64</td>
<td>6.69%</td>
<td>178</td>
<td>18.60%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>60</td>
<td>24</td>
<td>177</td>
<td>18.50%</td>
<td>405</td>
<td>42.32%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>60</td>
<td>34</td>
<td>327</td>
<td>34.17%</td>
<td>528</td>
<td>55.17%</td>
</tr>
<tr>
<td>IV infusion (72h)</td>
<td>60</td>
<td>44</td>
<td>438</td>
<td>45.77%</td>
<td>577</td>
<td>60.29%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>60</td>
<td>4</td>
<td>26</td>
<td>2.72%</td>
<td>48</td>
<td>5.02%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>60</td>
<td>14</td>
<td>62</td>
<td>6.48%</td>
<td>137</td>
<td>14.32%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>60</td>
<td>24</td>
<td>179</td>
<td>18.70%</td>
<td>385</td>
<td>40.23%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>60</td>
<td>34</td>
<td>330</td>
<td>34.48%</td>
<td>526</td>
<td>54.96%</td>
</tr>
<tr>
<td>IV 1h bolus (72h)</td>
<td>60</td>
<td>44</td>
<td>438</td>
<td>45.77%</td>
<td>578</td>
<td>60.40%</td>
</tr>
<tr>
<td>IV infusion (21h)</td>
<td>60</td>
<td>4</td>
<td>46</td>
<td>4.81%</td>
<td>107</td>
<td>11.18%</td>
</tr>
<tr>
<td>IV infusion (21h)</td>
<td>60</td>
<td>14</td>
<td>65</td>
<td>6.79%</td>
<td>214</td>
<td>22.36%</td>
</tr>
<tr>
<td>IV infusion (21h)</td>
<td>60</td>
<td>24</td>
<td>177</td>
<td>18.50%</td>
<td>408</td>
<td>42.63%</td>
</tr>
<tr>
<td>IV infusion (21h)</td>
<td>60</td>
<td>34</td>
<td>327</td>
<td>34.17%</td>
<td>529</td>
<td>55.28%</td>
</tr>
<tr>
<td>IV infusion (21h)</td>
<td>60</td>
<td>44</td>
<td>438</td>
<td>45.77%</td>
<td>577</td>
<td>60.29%</td>
</tr>
</tbody>
</table>
Table S2. Summary of biomarker performance at 30g and 60g APAP overdose after 72-hour oral NAC treatment delayed by 4, 14, 24, 34, and 44 hours. Undertreatment is when NAPQI clears the liver after the biomarker would suggest the cessation of treatment; overtreatment is when NAPQI clears the liver before the biomarker suggests the cessation of treatment.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Dose (g)</th>
<th>Treatment delay (h)</th>
<th>Under treated</th>
<th>Undertreatment %</th>
<th>Mean overtreatment (h)</th>
<th>Median overtreatment (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAP conc.</td>
<td>30</td>
<td>4</td>
<td>1</td>
<td>0.1%</td>
<td>48</td>
<td>16.1</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>30</td>
<td>14</td>
<td>2</td>
<td>0.21%</td>
<td>41</td>
<td>13.3</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>30</td>
<td>24</td>
<td>44</td>
<td>4.60%</td>
<td>37</td>
<td>8.88</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>30</td>
<td>34</td>
<td>318</td>
<td>33.23%</td>
<td>35.3</td>
<td>2.98</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>30</td>
<td>44</td>
<td>707</td>
<td>73.88%</td>
<td>27.7</td>
<td>-3.28</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>30</td>
<td>4</td>
<td>18</td>
<td>1.88%</td>
<td>91.7</td>
<td>20</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>30</td>
<td>14</td>
<td>3</td>
<td>0.31%</td>
<td>114.1</td>
<td>21.6</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>30</td>
<td>24</td>
<td>3</td>
<td>0.31%</td>
<td>116.7</td>
<td>21.3</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>30</td>
<td>34</td>
<td>3</td>
<td>0.31%</td>
<td>199</td>
<td>22.4</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>30</td>
<td>44</td>
<td>3</td>
<td>0.31%</td>
<td>190</td>
<td>19.8</td>
</tr>
<tr>
<td>Peak AST</td>
<td>30</td>
<td>4</td>
<td>18</td>
<td>1.88%</td>
<td>47</td>
<td>16.6</td>
</tr>
<tr>
<td>Peak AST</td>
<td>30</td>
<td>14</td>
<td>3</td>
<td>0.31%</td>
<td>62</td>
<td>15.7</td>
</tr>
<tr>
<td>Peak AST</td>
<td>30</td>
<td>24</td>
<td>3</td>
<td>0.31%</td>
<td>54</td>
<td>14</td>
</tr>
<tr>
<td>Peak AST</td>
<td>30</td>
<td>34</td>
<td>3</td>
<td>0.31%</td>
<td>42</td>
<td>11.1</td>
</tr>
<tr>
<td>Peak AST</td>
<td>30</td>
<td>44</td>
<td>21</td>
<td>2.19%</td>
<td>42</td>
<td>7.15</td>
</tr>
<tr>
<td>Peak BA</td>
<td>30</td>
<td>4</td>
<td>0</td>
<td>0.00%</td>
<td>91.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Peak BA</td>
<td>30</td>
<td>14</td>
<td>0</td>
<td>0.00%</td>
<td>114.1</td>
<td>4.31</td>
</tr>
<tr>
<td>Peak BA</td>
<td>30</td>
<td>24</td>
<td>1</td>
<td>0.10%</td>
<td>116.7</td>
<td>5.98</td>
</tr>
<tr>
<td>Peak BA</td>
<td>30</td>
<td>34</td>
<td>3</td>
<td>0.31%</td>
<td>140</td>
<td>6.06</td>
</tr>
<tr>
<td>Peak BA</td>
<td>30</td>
<td>44</td>
<td>75</td>
<td>7.84%</td>
<td>130</td>
<td>4.59</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>60</td>
<td>4</td>
<td>5</td>
<td>0.52%</td>
<td>69</td>
<td>19.1</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>60</td>
<td>14</td>
<td>5</td>
<td>0.52%</td>
<td>66</td>
<td>22.1</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>60</td>
<td>24</td>
<td>8</td>
<td>0.84%</td>
<td>86.7</td>
<td>23.7</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>60</td>
<td>34</td>
<td>30</td>
<td>3.13%</td>
<td>78</td>
<td>21.6</td>
</tr>
<tr>
<td>APAP conc.</td>
<td>60</td>
<td>44</td>
<td>69</td>
<td>7.21%</td>
<td>76</td>
<td>14.1</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>60</td>
<td>4</td>
<td>0</td>
<td>0.00%</td>
<td>107.2</td>
<td>18.4</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>60</td>
<td>14</td>
<td>0</td>
<td>0.00%</td>
<td>100.2</td>
<td>21.4</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>60</td>
<td>24</td>
<td>0</td>
<td>0.00%</td>
<td>86.7</td>
<td>26.8</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>60</td>
<td>34</td>
<td>0</td>
<td>0.00%</td>
<td>89</td>
<td>27.5</td>
</tr>
<tr>
<td>Peak ALT</td>
<td>60</td>
<td>44</td>
<td>0</td>
<td>0.00%</td>
<td>195</td>
<td>21.8</td>
</tr>
<tr>
<td>Peak AST</td>
<td>60</td>
<td>4</td>
<td>57</td>
<td>5.96%</td>
<td>58</td>
<td>9.21</td>
</tr>
<tr>
<td>Peak AST</td>
<td>60</td>
<td>14</td>
<td>101</td>
<td>10.55%</td>
<td>59</td>
<td>6.9</td>
</tr>
<tr>
<td>Peak AST</td>
<td>60</td>
<td>24</td>
<td>128</td>
<td>13.38%</td>
<td>43</td>
<td>5.88</td>
</tr>
<tr>
<td>Peak AST</td>
<td>60</td>
<td>34</td>
<td>165</td>
<td>17.24%</td>
<td>28</td>
<td>4.82</td>
</tr>
<tr>
<td>Peak AST</td>
<td>60</td>
<td>44</td>
<td>185</td>
<td>19.33%</td>
<td>31</td>
<td>2.67</td>
</tr>
<tr>
<td>Peak BA</td>
<td>60</td>
<td>4</td>
<td>95</td>
<td>9.93%</td>
<td>107.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Peak BA</td>
<td>60</td>
<td>14</td>
<td>82</td>
<td>8.57%</td>
<td>100.2</td>
<td>4.79</td>
</tr>
<tr>
<td>Peak BA</td>
<td>60</td>
<td>24</td>
<td>73</td>
<td>7.63%</td>
<td>86.7</td>
<td>9.37</td>
</tr>
<tr>
<td>Peak BA</td>
<td>60</td>
<td>34</td>
<td>57</td>
<td>5.96%</td>
<td>73.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Peak BA</td>
<td>60</td>
<td>44</td>
<td>50</td>
<td>5.22%</td>
<td>131</td>
<td>8.95</td>
</tr>
</tbody>
</table>
DILIsym™ MODEL OVERVIEW

The DILIsym™ model has been developed to help evaluate novel compounds as they are considered for use as drugs in the clinical setting. The DILIsym™ model can be used to predict hepatotoxicity in mice, rats, and humans based on a minimal set of measured laboratory data. These predictions can be used to direct subsequent experiments, efficiently enhancing confidence in the safety level for a given compound.

The current version of the DILIsym™ model (version 1A) represents some of the hepatotoxic mechanisms that have been described to contribute to DILI, with more to be added in future versions. Currently, the DILIsym™ model includes reactive metabolite-induced mitochondrial dysfunction and the resultant necrosis. The necrosis associated with reactive metabolite protein adducts is also included. There are several drugs that have been used as exemplars, providing data for determining the quantitative biochemical and cellular relationships that contribute to the hepatotoxicity. These include acetaminophen (APAP), methapyrilene (MP), furosemide, and Aflatoxin B1. Additionally, the disruption of bile acid homeostasis by glibenclamide is included in the DILIsym™ model; the resultant hepatotoxic effects of cellular bile acid accumulation have not yet been included in the current version of the model, however. A full list of the submodels included in DILIsym™ is available in Table S3; quantitative model metrics such as the number of ODEs in the model are in Table S4.

Table S3. Qualitative Descriptors of the DILIsym™ Model (version 1A)

<table>
<thead>
<tr>
<th>Biological Systems Represented</th>
<th>Mechanistic Model Outputs</th>
<th>Clinical Model Outputs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug ADME Processes</td>
<td>Drug Concentrations (tissue)</td>
<td>Drug Concentrations (blood)</td>
</tr>
<tr>
<td>GSH Dynamics</td>
<td>Reactive Metabolite Flux</td>
<td>GSH Dynamics (blood)</td>
</tr>
<tr>
<td>Cellular Energy Balance</td>
<td>GSH Dynamics (hepatocytes)</td>
<td>Bile Salt Concentrations (blood)</td>
</tr>
<tr>
<td>Mitochondrial Dysfunction</td>
<td>Oxidative Stress Dynamics (hepatocytes)</td>
<td>Plasma ALT</td>
</tr>
<tr>
<td>NRF-2 Adaptation Response</td>
<td>ATP Dynamics (hepatocytes)</td>
<td>Plasma AST</td>
</tr>
<tr>
<td>Bile Salt Homeostasis</td>
<td>Bile Salt Concentrations (hepatocytes)</td>
<td>Plasma bilirubin</td>
</tr>
<tr>
<td>Hepatocyte Life Cycle</td>
<td>Viable Liver Mass Dynamics</td>
<td>INR and Prothrombin Time</td>
</tr>
<tr>
<td>Innate Immune Response</td>
<td></td>
<td>Plasma HMGB1 (acetylated and non-acetylated)</td>
</tr>
<tr>
<td>Clinical Biomarkers</td>
<td></td>
<td>Plasma Keratin 18 (K18)</td>
</tr>
</tbody>
</table>
The current version of the model encompasses liver homeostasis at a high level and includes the responses and adaptations in a number of biochemical, cellular, and immunologic areas as a consequence of xenobiotic perturbations (see Fig. S4). These are represented as distinct and interconnected sub-models of the DILIsym™ model and include: drug pharmacokinetics and metabolism (conjugation and/ biotransformation), glutathione dynamics, mitochondrial dysfunction (i.e., reactive oxygen/nitrogen species [RNS/ROS] balance), cellular energy (adenosine triphosphate--ATP) balance, the NRF-2 adaptation response pathway, bile salt homeostasis, the hepatocyte life cycle, the innate immune response, and the production of associated biomarkers of necrosis, apoptosis, and cellular function.
The exemplar drug acetaminophen provides an example of how these sub-models can interact to produce hepatotoxicity in the DILIsym™ model: APAP biotransformation to the reactive metabolite NAPQI initiates changes in oxidative stress levels, hepatic mitochondrial ATP production, hepatocellular necrosis, apoptosis, and regeneration. The initiation of cell death leads to the release of pro-inflammatory, anti-inflammatory, and pro-regenerative mediators and the subsequent exacerbation of necrosis rates and increases in hepatocellular regeneration. The mechanism for MP toxicity is represented with the same pathways as well. Other exemplar (or novel) drugs can produce hepatotoxicity in the DILIsym™ model via similar or alternative mechanisms.

The DILIsym™ model contains parametric representations of humans, Sprague-Dawley rats, and C57Bl/6 mice. These simulated patients, simulated rats, and simulated mice can be used to facilitate the translation of preclinical data to patients. There is a baseline simulated patient, rat, and mouse that encompasses the average quantitative relationships and responses for each species. The sections below describe some of the sub-models listed above and provide some examples of the data used to optimize the model parameters for each species.
Figure S5. Diagram of the PBPK sub-model within the DILIsym™ model. The PBPK structure includes a blood compartment, muscle compartment, three liver compartments that account for the zonal nature of the liver (centrilobular, midlobular, and periportal), a gut compartment representing the basolateral side of the gut, a gut lumen compartment, and a stomach compartment.

**PBPK Sub-model**

This sub-model consists of a physiologically based pharmacokinetic (PBPK) model used to calculate the tissue distribution and elimination of a xenobiotic. Fig. S5 shows a diagram of the PBPK sub-model. The dosing routes in the DILIsym™ model include oral, intraperitoneal, and intravenous.
Tissues explicitly represented include the liver, gut (small intestine), muscle, and blood. The 'other' tissue is comprised of all other tissues not explicitly shown. The process of oral absorption includes the stomach and lumen compartments to allow for gastric emptying effects. Additional tissues can be added to the PBPK model if compound concentrations are required in organs not specifically represented here. The metabolism of the xenobiotic in the periportal, midlobular, and centrilobular zones of the liver is represented in metabolism sub-models. The size of the liver and the amount of blood are functions of the amount of cellular necrosis and the subsequent viable mass in the liver. For modeling novel compounds not already included in the DILIsym™ model, data are required to estimate chemical-specific kinetic parameters such as the rate of gastric emptying, fraction unbound in plasma, and tissue partition coefficients. In vitro inputs may be estimated based on the physical chemical properties of the drug.
Figure S6. Diagram showing the general metabolism structure within the DILIsym™ model. The centrilobular zone is shown, but the midlobular and periportal zones are similar. Three metabolic pathways are included by default. One pathway labeled "RM" allows for a potential reactive metabolite with protein adducts. Pathway B also includes a substrate depletion structure. The default quantitative representation for all three pathways is Michaelis-Menten reaction kinetics.

**Xenobiotic Metabolism**

This sub-model represents the metabolism of a xenobiotic to metabolite A, metabolite B, and reactive metabolites 1 and 2 (RM) in each zone of the liver (Fig. S6). The primary method of clearance from circulation for many xenobiotics is biotransformation in the liver. The metabolites are eventually eliminated in the urine. The periportal, midlobular, and centrilobular regions are each represented because many enzymes responsible for drug metabolism, such as the cytochrome P450 (CYP450) family of enzymes, are distributed asymmetrically across the liver acinus. The enzymes involved in phase II biotransformation of xenobiotics are generally expressed at similar levels across the zones. The metabolism pathways are represented with Michaelis-Menten kinetics. The primary xenobiotic metabolites can be released into the sinusoid, where they move from the periportal zone into the midlobular zone, then into the centrilobular zone, and then into the blood, following the direction of...
blood flow. Biliary and renal excretion of the metabolites is also accounted for. After the conversion of the xenobiotic to the reactive metabolite (RM), the RM can also bind to proteins or bind to glutathione (GSH). Phase I metabolism, catalyzed by CYP450 and other enzymes such as amidases, converts compounds to stable or reactive metabolites. Reactive metabolites disrupt cellular function by modifying macromolecules or disturbing the oxidative balance of hepatocytes. With a therapeutic dose, reactive metabolites can be detoxified and excreted. Phase II metabolism involves the conjugation of compounds and/or their metabolites to a variety of side groups, including GSH, acetyl groups, glucuronide, and sulfate. This increases the water solubility of the compound and allows for excretion in the urine or feces, thereby minimizing hepatocellular toxicity.
Figure S7. Selected pharmacokinetic data used to optimize the APAP metabolism sub-model for rodents, shown with the corresponding simulation results produced with the DILIsym™ model.

A) Plasma APAP concentration data ( ◆ ), simulated plasma APAP concentration ( - - - ), plasma APAP-sulfate concentration data ( ▲ ), simulated plasma APAP-sulfate concentration ( • • • ), plasma APAP-glucuronide concentration data ( ○ ), simulated plasma APAP-glucuronide concentration ( - - - - ) for C57Bl/6 mice dosed via oral gavage with 300 mg/kg APAP. Data were produced by Dr. Alison Harrill and is available with the corresponding methods from the Jackson Laboratory’s Mouse Phenome Database (http://phenome.jax.org/db/q?rtn=views%2Fsearch&req=harrill; file names "Threadgill1" and "Threadgill2"). Mean and SD shown.

B) Plasma APAP concentration data ( ◆ ), simulated plasma APAP concentration ( - - - ), plasma APAP-sulfate concentration data ( ▲ ), simulated plasma APAP-sulfate concentration ( • • • ), plasma APAP-glucuronide concentration data ( ○ ), simulated plasma APAP-glucuronide concentration ( - - - - ) for Sprague-Dawley rats dosed intravenously with 300 mg/kg APAP².

Fig. S7a shows data generated by Harrill et al. in mice used to optimize the model in addition to corresponding DILIsym™ simulation results for APAP and the two primary metabolites, APAP-glucuronide and APAP-sulfate. Similarly, Fig. S7b shows metabolism data and simulation results for rats where APAP and its sulfate and glucuronide metabolites were reported after a single dose. The
DILIsym™ model parameters for rats were optimized using that data set and several others. Fig. S7 shows how rodent metabolism pathway partitioning can be different across species. In Fig. S8, a dose response relationship for cumulative urinary APAP metabolites over 24 hours as a function of dose is shown for humans. The dose response data was used to optimize the human APAP metabolism parameters.

![Graph showing dose response relationship for APAP metabolites](image)

**Figure S8.** Selected pharmacokinetic data used to optimize the APAP metabolism sub-model for humans, shown with the corresponding simulation results produced with the DILIsym™ model.

A) Measured cumulative APAP-sulfate conjugates excreted in urine over 24 hours (▲), simulated cumulative APAP-sulfate conjugates excreted in urine over 24 hours (● ● ●), measured cumulative APAP-glucuronide conjugates excreted in urine over 24 hours (〇), and simulated cumulative APAP-glucuronide conjugates excreted in urine over 24 hours (——) for humans at various oral doses of APAP³.

B) Measured cumulative conjugates excreted in urine over 24 hours (●) and simulated cumulative NAPQI pathway conjugates excreted in urine over 24 hours (——●) for humans at various oral doses of APAP³.

**GSH Dynamics**

This sub-model, depicted in Fig. S9, captures the synthesis, steady state turnover, and depletion of glutathione (GSH) in each zone of the modeled liver acinus.
Figure S9. Diagram showing the zonal structure for glutathione (GSH) turnover, depletion, and recovery within the DILIsym™ model. GSH synthesis is controlled by enzyme availability and precursor availability, and is effluxed into the plasma. The model includes conjugation with reactive metabolites and GSH precursor transport and depletion. The adaptive response mediated by the NRF-2 pathway is also included.

GSH, an important antioxidant within hepatocytes, is depleted by the reactive metabolites of xenobiotics. GSH synthesis occurs in two steps, both requiring ATP. The first, rate-limiting step is the formation of gamma-glutamyl-cysteine from glutamate and cysteine. In the second step, this glutamate-cysteine product joins to glycine to form GSH. GSH synthesis is regulated in several ways, including competitive feedback inhibition and cysteine availability. GSH is synthesized in the cytosol of the hepatocyte and transferred into the mitochondria. A major function of GSH in cellular homeostasis is as an antioxidant, and a reduction in GSH stores leaves hepatocytes vulnerable to oxidative stress. As reactive metabolites deplete the liver of GSH, the hepatocytes' defenses are saturated and reactive metabolites are more likely to bind to proteins and cause injury. GSH is represented in the periportal, midlobular, and centrilobular hepatocytes of the DILIsym model to allow for localized GSH depletion.
effects. The basic framework for the GSH model originated from a mathematical model reported in D'Souza 1988. This sub-model includes the following components: endogenous GSH loss and synthesis, GSH conjugation with xenobiotic reactive metabolites, delayed GSH synthesis up-regulation and inhibition (to mimic enzyme synthesis up or down-regulation), direct GSH feedback synthesis inhibition, GSH precursor tracking, GSH efflux and influx into plasma, red blood cell GSH efflux into plasma, and the nuclear factor (erythroid-derived 2)-like 2 (NRF-2) response effect on GSH synthesis.

Multiple tissues contribute to the regulation of plasma GSH levels, but they are a good indicator of the hepatic GSH concentration. One of the main non-hepatic contributors to plasma GSH is the red blood cells (RBC). Red blood cells act as a supply source for plasma GSH, taking in GSH precursors and releasing GSH. Minimal GSH is taken up directly by the RBCs. GSH release by RBCs is a zero order process. When hepatic GSH is depleted, additional GSH precursors from elsewhere in the body are transported into the plasma to allow RBCs to continue the synthesis process. Under homeostatic conditions, RBCs contribute a lower proportion of the plasma GSH supply than the liver. One of the major routes for clearance of GSH from the plasma is the kidneys. To represent this source of GSH loss in the DILIsym model, the plasma GSH loss is represented as first order loss where the first order rate constant is calculated so that plasma GSH is maintained at a constant level in the absence of a GSH depletion stimulus. The transport of GSH from hepatocytes into the plasma is controlled by active transport and is saturated at the physiological hepatic concentration of GSH. The liver is a major source of GSH for the body, so GSH efflux is much larger than GSH influx in healthy liver cells. GSH is also transported into the liver from the plasma through a saturable process. However, because the saturation constant governing the process is much larger than the plasma GSH concentration, it is modeled as a first order process.

The GSH formation rate inside hepatocytes is the product of the GSH synthesis rate and the normalized GSH response signal. The combination of these two inputs represents the combined effect of enzyme activity/expression levels and GSH precursor levels on the rate of GSH formation. The GSH sub-model includes a positive term that is up or down-regulated by the 'GSH synthesis response' variable, and a negative term that is controlled by the 'GSH synthesis baseline' variable, so that down-regulation occurs to balance up-regulation. This type of control system mimics the feedback that occurs inside the cell as it senses a loss of GSH and produces more necessary enzymes to synthesize it, and then down-regulates the production of those enzymes as GSH levels return to baseline. If the GSH precursor supply is sufficient, the cell can proceed with up-regulation effectively, but if GSH precursor becomes depleted, the up-regulation response is mitigated.
Direct inhibition of GSH synthesis is also included in the model, and mimics competitive feedback inhibition, where GSH inhibits its own synthesis as a last resort control system for the cell. This depends on the current GSH concentration and does not require a time delay\textsuperscript{17,20,21}. This process can be contrasted with the GSH synthesis response, which inhibits GSH synthesis at GSH levels above baseline, but with a time delay. The direct inhibition expression represents the direct interference of GSH with the GSH synthesis reaction through competitive binding\textsuperscript{17,20,21}. This inhibition prevents GSH from reaching excessively high levels.

The model GSH precursor pool represents the intracellular cysteine or cystine concentration because they are the rate limiting precursors, depending on the species\textsuperscript{17}. The mass balance for GSH precursor depends on two inputs and one output at steady state (without a GSH depletion stimulus). The first precursor input is the recycling of GSH as it is converted to GSSG and other GSH products, which is represented by the GSH loss rate in the DILIsym™ model. The second input is the difference between GSH efflux and influx. This difference is the net loss of GSH to the plasma, and it is replaced by an extracellular precursor source in the model equations to represent the constant replacement of precursor through dietary sources. This source is primarily derived from the methionine pathway for GSH precursor supply, where methionine is converted to cysteine through the transsulfuration pathway\textsuperscript{17,22}. As GSH becomes depleted, cellular ATP available also decreases\textsuperscript{23}. When GSH is depleted, GSH efflux into the plasma is reduced, and the methionine pathway source of GSH precursor is also reduced. The reduced GSH mimics reduced ATP, which leads to a reduced methionine pathway, due to ATP dependence. At this point, the GSH precursor pool becomes depleted, and the GSH precursor transport pathway, representing the cystine/cysteine influx pathway, attempts to replenish GSH.

The transport of GSH precursor into hepatocytes is a key process in the regulation of cellular GSH. This expression quantitatively represents the influx of cysteine for rodents\textsuperscript{17,24} and cysteine for humans\textsuperscript{22} into the cell after the depletion of cystine or cysteine due to the depletion of intracellular GSH\textsuperscript{17,22,24}. The cystine/cysteine pathway, rather than the methionine pathway, is largely responsible for precursor replenishment during GSH depletion due to its lack of ATP dependence\textsuperscript{17}. The influx of cystine/cysteine is saturable\textsuperscript{22,24}. The Km value for this response was optimized based on the observation that GSH recovery is dose, and therefore precursor, limited at high drug concentrations\textsuperscript{22}. Once the transport of GSH precursor into hepatocytes occurs and intracellular hepatocyte GSH precursor concentrations increase to unusually high levels, such as in the case of N-acetyl cysteine (NAC) treatment, a precursor export term in the model acts to return the intracellular precursor levels to baseline\textsuperscript{17}. The efflux of cysteine from cells has been observed experimentally\textsuperscript{25}. The kinetics of the
precursor transport system mimic those of the GSH efflux exporter, which have been characterized\textsuperscript{15}. The parameters used to quantitatively describe the precursor export were chosen based on the effect of NAC on GSH concentrations during APAP injury\textsuperscript{26,27,28,29}. GSH concentrations increase beyond their baseline levels, but usually not significantly more than double\textsuperscript{30}. In addition, the values were chosen so that intracellular GSH levels would return to baseline within 24-48 hours\textsuperscript{26}.

NAC treatment is one case where GSH precursor levels are manipulated therapeutically to improve the outcome in the case of an APAP overdose. NAC influx into hepatocytes is represented with saturable kinetics and depends on the extracellular concentration of NAC. Cysteine and cystine are substrates for the System (Ala-, Ser- Cys-prefering) (ASC) transport system\textsuperscript{31,32}. When extracellular levels of cystine or cysteine increase, ASC is activated and intracellular cysteine concentrations also increase\textsuperscript{17,24}. The parameters associated with NAC influx into hepatocytes were optimized with NAC treatment data where the effect of NAC on hepatic GSH levels was measured.

Finally, when hepatocytes experience oxidative stress, the NRF-2-Kelch-like ECH-associated protein 1 (KEAP) complex (NRF-2-KEAP) uncouples to yield activated NRF-2, which induces physiological responses aimed at mitigating possible injury\textsuperscript{33,34,35}. One effect of NRF-2 is to boost the level of GSH in the cell\textsuperscript{36,37,38,39,40}. This is accomplished in at least two ways. First, NRF-2 causes the enzymes responsible for GSH synthesis to increase in a time-dependent fashion\textsuperscript{34,35,37}, causing the rate of GSH synthesis to increase in a substrate-dependent process. Second, NRF-2 also increases the concentration of the rate-limiting GSH precursors, cysteine/cystine\textsuperscript{38}, which helps to boost the GSH synthesis rate. Both enzyme and precursor effects are included in the model through a time-mediated signal that amplifies these effects following significant increases in oxidative stress. Evidence for a lack of a meaningful NRF-2 response at very low levels of stress has also been shown\textsuperscript{41}, and the model parameter values have been set such that a threshold of oxidative stress is required to induce the NRF-2 response.

Examples of the types of data used to optimize the many model parameters necessary to characterize the GSH response are shown in Fig. S10. Fig. S10A shows a time dependent GSH depletion event by a single, large dose of APAP for rodents, and the characteristic recovery response as evidenced by the measured data and mimicked by the simulation results. Figs. S10B and S10C show additional comparisons between simulation results and measured GSH data for humans; dynamic plasma GSH levels and AUC decrement changes were recorded with and without NAC treatment for a low, single dose of APAP.
A) Liver GSH data relative to baseline (●) used for model parameter optimization and simulated liver GSH relative to baseline (---) for young Sprague Dawley rats given 1250 mg/kg APAP via the intraperitoneal route\textsuperscript{42}. Liver GSH data relative to baseline (◆) used for model parameter optimization and simulated liver GSH relative to baseline (---) for C57Bl/6 mice given 800 mg/kg APAP via the intraperitoneal route\textsuperscript{43}.

B) Plasma GSH data relative to baseline (●) used for model validation and simulated plasma GSH relative to baseline (---) for humans given 2000 mg of APAP orally\textsuperscript{6}.

C) Plasma GSH AUC decrement data (▌) used for model validation and simulated plasma GSH AUC decrement (---) for humans given 2000 mg of APAP orally\textsuperscript{7}.

**N-Acetyl-Cysteine Intervention**

The DILIsym\textsuperscript{™} model includes a sub-model to calculate the pharmacokinetics of N-acetyl cysteine (NAC) from a variety of dosing methods. Fig. S11 shows the scheme used to represent the NAC pharmacokinetics in the DILIsym\textsuperscript{™} model. The mechanistic connection is evident in Fig. S9, where NAC acts as a GSH precursor to speed GSH synthesis. NAC is frequently given as a treatment for acetaminophen overdose, and acts by increasing GSH synthesis and possibly as an antioxidant as well. In this sub-model, a pharmacokinetic model gives the circulating NAC concentration and elimination. The elimination of NAC is represented with renal elimination and non-renal elimination. However, the non-renal elimination is typically not used in simulations, as non-renal elimination is also mechanistically represented in DILIsym\textsuperscript{™} with direct hepatocyte uptake.
The NAC Intervention sub-model includes intravenous and intraperitoneal dosing schemes for bolus doses and infusions. Oral bolus dosing is also included. Renal, non-renewal (empirical), and hepatic uptake (mechanistic) clearance mechanisms account for NAC elimination. 20% to 30% of total NAC clearance is attributable to renal clearance. The circulating NAC concentration is connected to the GSH sub-models so that synthesis can be affected when NAC is given. The baseline volume of distribution for NAC was found from studies in humans in Borgstrom 1986, Jones 1997, Olsson 1988, and Prescott 1989. The value scales directly with body weight for the conversion to mice and rats.

The NAC submodel has been optimized using data from the literature. NAC pharmacokinetics in the human were calibrated using Borgstrom 1986 and De Caro 1989, a comparison of those experimental data and our simulation results is in Fig. S12. Our model is also calibrated with the results of Burgunder 1989, which measured the effect of NAC on GSH decrement in healthy volunteers (Fig.
The pharmacodynamics of NAC in rodents has been well-documented in the literature, and our model utilizes some of that data to calibrate the behavior of NAC in all three species. The mouse NAC data from Whitehouse 198529 and the rat NAC data from Lauterburg 198327 was used to optimize the model (Fig. S14).

Figure S12. Selected pharmacokinetic data used to optimize the NAC sub-model within DILIsym™.

A) Data for a single 600 mg IV bolus dose given to healthy volunteers45 and corresponding simulation results.

B) Data for a single 600 mg oral bolus dose given to healthy volunteers45 and corresponding simulation results.

C) Data for a single 9.47 mg/kg oral bolus dose given to healthy volunteers48 and corresponding simulation results.
Figure S13. Data used to validate the effect of NAC treatment on GSH levels in humans. The figure is a comparison between the AUC decrement in plasma GSH in healthy volunteers who take 2 g APAP and 2 g APAP with 2 g NAC (black), and corresponding simulation results (red).
Figure S14. C57Bl/6 mouse and SD rat data used for model parameter optimization and corresponding simulation results for the effect of NAC treatment.

A) Data for liver GSH concentration expressed as a percentage of baseline for mice given 350 mg/kg APAP intraperitoneally (○) and corresponding simulation results ( ); liver GSH concentration expressed as a percentage of baseline for mice given 350 mg/kg APAP and 500 mg/kg NAC intraperitoneally (○) and corresponding simulation results ( ).

B) Data for liver GSH concentration expressed as a percentage of baseline for rats given 1000 mg/kg APAP orally (○) and corresponding simulation results ( ); liver GSH concentration expressed as a percentage of baseline for mice given 1000 mg/kg APAP and 1200 mg/kg NAC (after an 80 minute delay) orally (○) and corresponding simulation results ( ).

Mitochondrial Dysfunction

The balance of reactive nitrogen/oxygen species (RNS/ROS) was designed based on the idea that reactive metabolites can increase the production of RNS and ROS in hepatocytes (Fig. S15). Numerous reports have indicated increases in hepatic RNS/ROS following exposure to drugs known to generate reactive metabolites (e.g., acetaminophen). Some possible mechanistic explanations for this observation include: reduction of cellular antioxidants, protein binding in various locations inside the cell, and direct reactive metabolite-membrane interactions. The primary assumption in the design rationale of RNS/ROS clearance was that glutathione (GSH) recovery has a time course similar to that of the decrease of elevated RNS/ROS back to baseline levels. This gave unique dynamics for each dose in
each strain. This assumption allowed some information about the human, since GSH recovery could provide a surrogate for the lack of available RNS/ROS data. This relationship has not yet been measured in the lab, and could be measured experimentally to fill a key knowledge gap.

Figure S15. Diagram showing the mitochondrial dysfunction sub-model within the DILIsym™ model. ATP is modeled with a simple production and utilization balance at this point, with reactive nitrogen species (RNS) and reactive oxygen species (ROS) causing a disruption in ATP production. The NRF-2 pathway is activated by RNS and ROS.

Manov et al.\textsuperscript{57} exposed human hepatocytes to various levels of APAP and measured increases in ROS. These data were used to infer a $V_{\text{max}}$ value for the effect of reactive metabolites to increase RNS/ROS production; free cellular NAPQI concentrations were used to determine the $K_{m}$. Simulated exposure to 4 g and 12.7 g single doses in simulated human patients predicted a return to baseline for RNS/ROS at approximately 21 and 31 hours respectively, in accordance with the GSH recovery timing.

Basal ATP turnover in the DILIsym™ model was designed to include zero order production and first order utilization. The steady state ATP turnover rate was calculated based on the measured whole body basal metabolic rate\textsuperscript{58,59,60,61}, the fraction of the basal metabolic rate from the liver\textsuperscript{62}, the mass of
the liver, and the weighted average of the energetic cost of synthesizing ATP from fatty acids, carbohydrates, and amino acids\textsuperscript{63,64}. Baseline hepatic ATP levels in the DILIsym\textsuperscript{™} model were based on data from Ghosh 2009\textsuperscript{65}, Jaeschke 1990\textsuperscript{66}, Knight 2002\textsuperscript{51}, and Saito 2010\textsuperscript{28} for the mouse, Ghosh 2010\textsuperscript{54} and Kaminsky 1984\textsuperscript{67} for the rat, and Noren 2005\textsuperscript{68} and Meyerhoff 1990\textsuperscript{69} for the human.

There is currently a threshold (i.e., a sigmoidal relationship) for the inhibitive effects of RNS/ROS on ATP production. This implementation was based on the experimental dose-response observations between APAP dose and reported hepatocellular injury. In mice, virtually no injury is reported at doses less than 200 mg/kg. Similarly in rats, little injury is reported below 400 mg/kg. The RNS/ROS effects on ATP production in the DILIsym\textsuperscript{™} model currently have no effect at APAP levels commensurate with doses less than 200 mg/kg in the simulated mouse or less than 400 mg/kg in the simulated rat. The primary data used to calibrate the mouse parameters describing the effect of RNS/ROS on ATP production were from Hanawa 2008\textsuperscript{56}. The sigmoidal relationship was assumed from the observed dose response, and the ATP data in Hanawa 2008 were used to determine the parameter values (shown in Fig. S16). The parameter values found from using the mouse data to describe the effect of RNS/ROS on ATP production were also used for the rat. This is based on the general principle that cellular level events should be somewhat conserved across species.
Figure S16. C57Bl/6 mouse data used for model parameter optimization and corresponding simulation results for the mitochondrial dysfunction sub-model within the DILIsym™ model.

A) Normalized data for reactive nitrogen species (RNS) and/or reactive oxygen species (ROS) in the liver\textsuperscript{70} for C57Bl/6 mice given 100 mg/kg APAP via the intraperitoneal route (\(\bullet\)) and corresponding simulation results (\(\bullet\)-\(\bullet\)); normalized data for RNS and/or ROS in the liver\textsuperscript{71} for C57Bl/6 mice given 300 mg/kg APAP via the intraperitoneal route (\(\Delta\)) and corresponding simulation results (\(\Delta\)-\(\Delta\)).

B) Liver ATP data\textsuperscript{56} relative to baseline for C57Bl/6 mice given 600 mg/kg APAP via the intraperitoneal route (\(\bullet\)) and corresponding simulation results (\(\bullet\)-\(\bullet\)).

The primary data used to validate the rat parameter values were Katyare 1989\textsuperscript{72} and Vendemiale 1996\textsuperscript{73}. The key points from these papers are as follows:

1. A dose response: little to no effect on ATP production (300 mg/kg), mild effects on ATP production (500 mg/kg), severe effects on ATP production (750 mg/kg).

2. Suppression of the ATP formation rate in Katyare 1989 at 24 hours: \(~40\%\) across all of their substrates.

3. Although the dynamics of ATP inhibition seen in Vendemiale at 500 mg/kg are somewhat different depending on what value is used (respiratory control index versus ATP production...
rate), their peak inhibition value of ATP production is about 18%, which agrees well with the peak in the simulations. For humans, only in vitro data was available. ATP depletion data in human Chang hepatocytes reported by Shon 2002\textsuperscript{74} were used to adjust the Km for RNS/ROS effects on ATP so that ATP depletion would be around 40% for the 60 g dose.

One key question is how the acute drug effects of RNS/ROS on ATP production interact with the effects of pro-inflammatory mediators. The current working hypothesis in the DILIsym™ model is that the pro-inflammatory mediators elicit necrosis in hepatocytes that already have reduced ATP levels. The pro-inflammatory mediators do not currently reduce ATP levels in hepatocytes that lack a direct, intracellular drug effect for ATP levels. With this implementation, pro-inflammatory mediators enhance acute injury rather than maintaining an injured state.

Another facet of the mitochondrial sub-model is the ability of organisms to adapt to injury. Nuclear factor erythroid derived-like factor 2 (NRF-2) is a cellular transcription factor that provides an adaptive response to increases in ROS/RNS. NRF-2 is bound in the cytoplasm to its chaperone protein, KEAP. When intracellular ROS/RNS levels increase, key cysteines on KEAP-1 appear to react with stressors, leading to dissociation of the NRF-2-KEAP1 dimer. NRF-2 then translocates to the nucleus. This process is rapid, as evidenced by reports that NRF-2 was observed in the nucleus of hepatocytes within one hour after dosing with CCl4, bromobenzene, or APAP\textsuperscript{34}. NRF-2 causes changes in the expression of many genes (GSH synthase, UDP-glucuronosyltransferase, multidrug resistant protein, metallothionen, etc.), primarily increasing the hepatocyte’s ability to mitigate the increased ROS/RNS burden. The generation of the NRF-2 signal in the DILIsym™ model is based on disruptions to the RNS/ROS balance. The timing of the effect was based on data from Suh 2004\textsuperscript{35}, where lipoic acid was administered to rats and glutathione ligase catalytic subunit (GCL) protein levels were measured over the subsequent 48 h. Aleksunes 2008 and Randle 2008 were also used to establish the relationship between RNS/ROS balance disruptions and the generation of the NRF-2 signal\textsuperscript{37,34}.

One particular hypothesis that has been captured within the DILIsym™ model is that reactive metabolites can bind to and alter the function of proteins. In particular, the effect of the reactive metabolite binding to proteins (e.g., Na+/K+ pump) and increasing cellular ATP utilization is represented in this sub-model. This has been employed to represent one of the hypotheses for the hepatotoxicity of large doses of furosemide in mice (in preparation).
Figure S17. Diagram showing the hepatocyte life cycle representation within the DILIsym™ model for the centrilobular zone of the liver. Mature hepatocytes can necrose, apoptose, and move to the mitotic state where they subsequently divide into two young cells.

Hepatocyte Life Cycle

This sub-model, depicted in Fig. S17 (only one zone shown), captures the life cycle of the hepatocytes. Hepatocytes undergo death by apoptosis or necrosis, and both are components of the model. There are a variety of determinants for stressed cells to be pushed toward apoptosis or necrosis; one of the predominant factors being the energy state of the cell. Without sufficient ATP, the cell cannot support energy-requiring reactions (including apoptosis), and necrosis will result. Apoptosis can be initiated by signals originating in mitochondria or elsewhere. Mitochondrial signals arise when the permeability of the outer mitochondrial membrane and/or the number of open inner mitochondrial
pores (due to the mitochondrial permeability transition-mPT) are increased. Extra-mitochondrial apoptotic signals come from the so-called death receptors and/or endoplasmic reticulum (ER) stress. In addition to the cellular energy state, other factors contribute to necrosis. Increased levels of oxidative species, such as reactive metabolites of drugs or endogenous species, can compromise the integrity of the plasma membrane and cause hepatocytes to lyse. Proteases released by Kupffer cells and neutrophils may also damage the plasma membrane and elicit necrosis.

Explicit tracking of individual hepatocyte pools (necrotic, apoptotic and mitotic) enables comparisons with histological data. Mature and young hepatocytes may have different rates of necrosis and apoptosis, and non-viable cells are cleared with specified half-lives. With appropriate internal and external cues, mature hepatocytes can commit to mitosis and divide into young hepatocytes. Cell division can be slowed by crowding signals resulting in cell cycle arrest. Oval cells contribute to hepatocyte populations in the periportal zone and hepatocytes may 'stream' (i.e., migrate over time) from periportal to midlobular and then to centrilobular. The contributing rates of cell turnover are regulated by mediators (described below in the 'Mediator Production and Regulation' and 'Regulation of Hepatocyte Rates' sections).

All zones have the same density of hepatocytes, and hepatocytes have the same volume. In all species, centrilobular (CL) is 1/9, midlobular (ML) is 3/9 and periportal (PP) is 5/9 of total liver volume, based on a 'cake-slice' like three dimensional configuration of the average acinus. The CL zone is perfused by the venous blood and a decreasing oxygen gradient exists from CL to PP. In practice, some of the 'inter-septal' hepatocytes in the PP zone may also be poorly perfused and have slightly different properties. While inter-septal morphology is not explicitly modeled, a fraction of poorly perfused PP hepatocytes is factored in when interpreting histological data.

The baseline homeostasis rates associated with the hepatocyte life cycle are calculated such that the fraction of hepatocytes that should be young, mature, mitotic, and apoptotic at baseline are consistent with the user defined inputs for these values. Hepatocytes have a long life cycle in the baseline, homeostatic state, living 6-12 months. The default values used in the DILIsym™ model for these fractions are from the references listed below.

1. The fraction of mitotic and apoptotic hepatocytes at homeostasis was based on Grisham 1962. Necrosis is assumed not to occur at homeostasis.
2. The rate of clearance of apoptotic cells was based on Bursch 1990 in rats. This rate is not available in the data for other species. In the current version of the model, the cell clearance
rates do not further affect rates of necrosis, apoptosis, and/or proliferation. Future versions may include this type of feedback, however.

3. The rate of clearance of necrotic cells is calibrated in the rat based on time-series data of hepatocytes marked as necrotic. As similar data were unavailable for other species, the same rate is used for other species.

4. The rate of cell cycle progression is based on Chanda 199682.

5. At baseline, the rate of maturation to go from young to mature hepatocytes is rapid compared to other rates. The differences in the model between young and mature hepatocytes are in their rates of necrosis and apoptosis in each zone. This effect can be explored further with the model.

Mediator Production and Regulation

There is an inflammatory response to hepatocyte necrosis in the model, modeled as a response to the rate of hepatocyte necrosis (necrotic flux). The nonparenchymal cells of the liver contributing to the pathophysiology of acute DILI include structural cells (e.g., stellate cells, sinusoidal endothelial cells) and cells of the innate immune system (e.g., Kupffer cells, NK cells, NKT cells, PMNs). Nonparenchymal cells participate in acute DILI through the production of various mediators, cell contact mediated effector activity, and phagocytosis. Individual cytokines and immune cells are not explicitly represented in version 1A of the DILIsym™ model. Rather, broad classes of mediators are modeled to capture their high-level regulation of key behaviors associated with hepatotoxicity (serum biomarkers, histological necrosis, lethality). The classes of immune mediators include pro-inflammatory, anti-inflammatory, and pro-regenerative. See Fig. S18 for an overview of the mediator production sub-model.
A delayed signal from the necrotic flux drives the production of pro-inflammatory mediators, while anti-inflammatory mediators down-regulate their production. The clearance of pro-inflammatory mediators is dictated by a specified half-life. Necrosis rates are increased in each acinar zone with elevated levels of pro-inflammatory mediators. Anti-inflammatory mediators are produced as a result of necrotic flux and have an additional time delay to ensure that they reach peak levels later than the other mediator classes, consistent with data on the relative kinetics of pro-inflammatory versus anti-inflammatory mediator release. The clearance of the anti-inflammatory mediator class is dictated by
the specified half-life. They dampen production of pro-inflammatory and pro-regenerative mediators. Like the pro-inflammatory mediators, the production of pro-regenerative mediators is up-regulated by necrotic flux and down-regulated by anti-inflammatory mediators. These mediators clear with a specified half-life. Pro-regenerative mediators increase hepatocellular proliferation rates.

**Zonal Hepatocyte Life Cycle Rate Regulation**

Rates of necrosis in each zone are dependent upon intracellular and extracellular influences, with the hepatocellular necrosis equations computing the sum of those components. The intracellular influences include the level of hepatocyte ATP and/or reactive metabolite adducts, each of which can have a direct effect on hepatocyte necrosis in a given acinar zone. Additionally, the level of extracellular pro-inflammatory mediators can combine with the intracellular ATP and/or reactive metabolite adducts to exert an additional effect on necrosis. This implementation corresponds with existing hypotheses, which posit that the immune response only affects damaged cells.\(^3\) Regulatory factors for apoptosis and regeneration rates are also described below. A diagram showing the factors regulating cell cycle rates in DILIsym™ version 1A are shown in Fig. S19.
Figure S19. Diagram showing the hepatocyte life cycle rate regulation sub-model within the DILIsym™ model. The damage index in each zone, which is dependent upon the ATP decrement or protein adducts and the pro-inflammatory mediators, causes hepatocyte necrosis. Crowding signals control the proliferation rate so that hepatomegaly does not occur. Pro-regenerative mediators induce an increased rate of proliferation. Cell cycle arrest also helps to avoid hepatomegaly when the liver nears complete regeneration. Only the midlobular zone is shown in this diagram; the model structure is conserved across zones.

The equations for the intracellular direct effects of ATP and reactive metabolite adducts are Hill functions, with parameter \(K_m, V_{max}, \text{Hill coefficient}\) values that have been selected by comparing to a variety of data (e.g., dose-dependent necrosis, ALT) for each species. The equations for the effects of pro-inflammatory mediators are also Hill equations with parameter values selected by a similar approach.
Necrosis is possible in both mature and young hepatocytes. There is evidence indicating that hepatocytes need to reach a certain level of maturity before they can be susceptible to drug-induced necrosis, however. The DILIsym™ model accommodates this hypothesis by including a 'young cell protection effect' parameter, which can be a number from 1 (new cells are equally susceptible) to 0 (new cells do not undergo necrosis). The current DILIsym™ model does not include any effects of drugs on hepatocellular apoptosis, although it is in the plans for future versions. The pro-regenerative mediators initiate the increase of the proliferation rate in all zones, while quorum sensing crowding signals serve to down-regulate the proliferation rate based on the ambient cell population. Crowding signals also prevent some of the cells already in the mitotic state from dividing (cell-cycle arrest). Moreover, a compromised cellular energy state can minimize and/or delay hepatocellular proliferation in response to injury. Mitosis contributes to the simulated regenerative response and is included to provide a comparison with histology-based measurements. Some evidence suggests that it takes less time for a cell to divide under conditions of hyper-proliferation. Our model accommodates this possibility. Conversely, cell cycle arrest can minimize mitosis rates when there is a dramatic increase in crowding signals. This observation is also captured in the model. The rate of maturation defines the rate at which young hepatocytes mature. Adjusting the value of this parameter allows the user to test the hypothesis that young hepatocytes may be less susceptible to injury than mature ones. In the current model, oval cells and the 'streaming' (movement of hepatocytes from one zone to the next) of hepatocytes contribute to basal, untreated hepatocyte fluxes. Neither oval cell proliferation nor streaming participate in re-populating after drug-induced damage, however. The hepatocytes of each zone divide to re-populate the lost hepatocytes.

Post-injury, proliferative responses appear to depend on the degree of injury. Hepatic regeneration may be delayed as hepatotoxicant doses increase, with little mitosis observable in dying animals until very late. This effect influences the model through the proliferation rate. Through those rate changes, we are hypothesizing that a lack of ATP availability minimizes and/or delays post-injury hepatocellular proliferation.

Crowding signals in the model provide an indication to the dividing hepatocytes in a given acinar zone that the number of hepatocytes is near the pre-injury level. Multiple observations in the literature after heptectomy and/or DILI show that the liver completely regenerates to the pre-treatment size but no greater. Certain signals cause hepatocytes to revert from the G1 mitotic phase back to G0. These signals are represented in the model as cell cycle arrest; after that, we assumed that the number of cells that can then proceed all the way to division is reduced by rapid increases in the
crowding signal. Cell cycle arrest thus combines with reduced initiation of cellular division to minimize hepatocellular proliferation as the liver nears full restoration of cell number after injury.

Clinical Biomarkers

Several clinical biomarkers are included in the model, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time, total plasma bilirubin, keratin 18, and high mobility box group 1 protein (HMGB1). Since data from the ALT and bilirubin sub-models is shown in this paper, those two sub-models are discussed in more detail below.

ALT is an intracellular enzyme responsible for amino acid metabolism and gluconeogenesis in the liver and other tissues. In DILI, circulating ALT serves as a marker of hepatocellular injury. An increase in ALT represents compromised hepatocellular membrane integrity due to necrosis. Care must be taken when using ALT as a biomarker, however; ALT rises have been observed in situations unrelated to DILI. For example, ALT increases have been observed due to increased muscle release following high intensity exercise\textsuperscript{92}. 
ALT levels in the model are driven largely by the rate of hepatocyte necrotic flux. The intermediate states of ALT enable the serum ALT to have the appropriate dynamics. The clearance of ALT is driven by the specified half-life. The half-life of ALT is altered by reductions in the number of viable hepatocytes, i.e. less hepatocytes leads to a longer ALT half-life.

ALT levels in the model are driven largely by the rate of hepatocyte necrotic flux (Fig. S20). The intermediate states of ALT enable the serum ALT to capture the dynamics as seen in the data sets. The clearance of ALT has a specified half-life. The half-life of ALT is altered by reductions in the number of viable hepatocytes, i.e. less hepatocytes lead to a longer ALT half-life. Cellular ALT levels were calculated from the literature and used to estimate the amount of ALT released per necrotic cell$^{93,94}$.
The mouse ALT optimization was done using dynamics shown in Aleksunes 2005 and 2008\textsuperscript{95,96}. The dose response was optimized using data from a large number of papers where ALT was measured after APAP at 24 hours. Those papers are listed in the caption for Figure S21. Fig. S21A shows the dose response data and corresponding model prediction for the baseline mouse. The dose response ALT not only allowed for ALT sub-model optimization, but helped to define the 'tipping point' for the C57Bl/6 mouse, where injury propagation occurred and significant necrosis ensued. The rat ALT optimization was done using Gueguen 2007, Chen 2009, Sugimura 1998, Wang 1999, Waters 2001, Zieve 1985, and Chanda 1995\textsuperscript{97,26,99,100,81,84}. The dose response was used from the papers at 24 hours (shown in Fig. S21B). The human ALT optimization was done using Schiodt 2001\textsuperscript{101}. Three time courses were taken from Schiodt at three different doses. NAC protocols were included in the simulations, and were given when specified in the paper. Figs. S22A and S22B display the dynamic ALT response for three APAP doses in humans.
A) Plasma ALT at 24 hours after APAP IP dosing for C57Bl/6 mice shown as a function of dose. Data are from Aleksunes 2005\textsuperscript{95} (●), Aleksunes 2008\textsuperscript{37} (◇), Fujimoto 2009\textsuperscript{102} (□), Campion 2008\textsuperscript{103} (◆), Liu 2004\textsuperscript{104} (○), Liu 2006\textsuperscript{55} (▲), Henderson 2007\textsuperscript{105} (◇), Maddox 2010\textsuperscript{106} (◇), Bourdi 2002\textsuperscript{107} (Δ), Dambach 2006\textsuperscript{108} (△), Gunawan 2006\textsuperscript{109} (◇), James 2003\textsuperscript{110} (□), Nakagawa 2008\textsuperscript{111} (●), Shinohara 2010\textsuperscript{112} (●), and Srinivasan 2001\textsuperscript{43} (●). Simulation results (●) from the DILisym™ model are shown for the baseline C57Bl6 mouse.

B) Plasma ALT at 24 hours after APAP IP dosing for Sprague Dawley rats shown as a function of dose. Data are from Gueguen 2007\textsuperscript{97} (△), Chen 2009\textsuperscript{26} (●), Sugimura 1998\textsuperscript{88} (◇), Wang 1999\textsuperscript{99} (◇), Waters 2001\textsuperscript{100} (△), Zieve 1985\textsuperscript{81} (○), and Chanda 1995\textsuperscript{84} (●). Simulation results (●) from the DILisym™ model are shown for the baseline Sprague Dawley rat.

C) Total plasma bilirubin (mg/dL) as a function of time after doses of various hepatotoxins in Sprague Dawley rats. The data points shown correspond to 2000 mg/kg APAP (◇) given orally\textsuperscript{113}, 2 mL/kg carbon tetrachloride (○) given via the intraperitoneal route\textsuperscript{114}, 2 mL/kg carbon tetrachloride (◇) given via the intraperitoneal route\textsuperscript{115}, and 300 mg/kg thioacetamide (▲) given via the intraperitoneal route\textsuperscript{116}. Simulation results from the DILisym™ model are shown for the baseline Sprague Dawley rat with intraperitoneal APAP at 400 mg/kg (●), 600 mg/kg (●), 800 mg/kg (●), and 960 mg/kg (●). The APAP doses for the simulations were chosen such that the ALT responses in the simulations and data were comparable, since some of the data involved other hepatotoxins besides APAP.
Combined monitoring of ALT and bilirubin levels is often used as a diagnostic test for DILI. Hy’s law, for example, includes the combination of ALT (or AST) increases 3 fold greater than the upper limit of normal with increases of serum total bilirubin 2 fold higher than the upper limit of normal. For this reason and because total plasma bilirubin is an important clinical marker for liver function, a bilirubin sub-model has been included.

Bilirubin is a byproduct of red blood cell lysis and serves as a marker of hepatocellular function. Bilirubin is derived from the heme molecule, which is abundant in red blood cells. Bilirubin is released to the circulation with red blood cell lysis and is cleared by hepatocytes. Once inside the hepatocyte, bilirubin is conjugated (to glucuronide, primarily) and exported to bile for excretion. These are ATP-dependent processes. Bilirubin levels correlate well with the change in hepatic glutathione\textsuperscript{114,115}. Moreover, hepatic glutathione correlates well with hepatic ATP levels\textsuperscript{23,118}. ATP reductions are the likely mechanistic link underlying the correlation between bilirubin and glutathione levels. Increases in unconjugated or total bilirubin are used in DILI as a marker of compromised hepatic function. Caution
must be taken when using bilirubin as a diagnostic test for DILI, as levels may increase due to increases in red blood cell lysis instead of decreased hepatocellular function.

Bilirubin levels in the model are regulated by changes in clearance, as the source is constant and determined by literature values. The primary regulator of hepatocellular bilirubin uptake is the number of viable hepatocytes (current hepatocyte number as a fraction of baseline)\textsuperscript{117}. Glucuronidation and transport of bilirubin out of hepatocytes is regulated by cellular ATP levels; this regulation mechanism correlates well with changes in bilirubin prior to cell loss. Conjugated bilirubin is not currently included in the model, but is likely to be integrated into the bile acid transport model in future versions of DILIsym™. A schematic of the bilirubin model is shown in Figure S23.

![Figure S23. Diagram showing the bilirubin sub-model within the DILIsym™ model. Bilirubin levels in the model are regulated by changes in clearance, as the source is constant and determined by literature values. The primary regulator of hepatocellular bilirubin uptake is the number of viable hepatocytes. Glucuronidation and transport of bilirubin out of hepatocytes is regulated by cellular ATP levels; this regulation mechanism correlates well with changes in bilirubin prior to cell loss.](image-url)
Bilirubin levels in the human after acetaminophen overdose were optimized using Davidson 1976 (shown in Fig. S22C)\textsuperscript{117}. In the rat, we used response to carbon tetrachloride to calibrate the dynamics of bilirubin levels after acetaminophen overdose\textsuperscript{115,116,114,113}. The rat bilirubin dose response from the model and the data used to shape that response are shown in Fig. S21C. No data were available for the bilirubin response in mice.

Prothrombin Time

Prothrombin time is a measure of the clotting activity of the blood. It is commonly used as a biomarker for liver functionality in humans. A prolonged prothrombin time means that the liver is not producing clotting factor proteins effectively\textsuperscript{119}. Clinically, the prothrombin time is generally measured as the international normalized ratio (INR), which is the ratio of the clotting time to a baseline value raised to an exponent determined by the accuracy of the laboratory equipment. The exponent is almost always near 1\textsuperscript{120}.

In our model (Figure S24), the prothrombin time is based on the release of clotting factor, especially clotting factor VII, from the liver. This clotting factor is released into the bloodstream. The prothrombin time is calculated as an empirical function of the concentration of clotting factor. The production of clotting factor by the liver is regulated by the fraction of viable hepatocytes; fewer hepatocytes lead to less clotting factor in the bloodstream, though this relationship is non-linear.
Prothrombin time is regulated by both the concentrations of clotting factor VII and of NAC in the blood. The model contains both prothrombin time and INR as outputs.

Some researchers have also proposed that N-acetylcysteine (NAC) has the side effect of artifactually prolonging a patient’s prothrombin time. Jepsen (1994)\textsuperscript{121} reported that infusions of NAC lowered the clotting activity of the blood in healthy volunteers, and Schmidt (2002)\textsuperscript{122} and Lucena (2005)\textsuperscript{123} both reported prolonged prothrombin times in acetaminophen overdose patients with otherwise normal biomarkers of liver necrosis (e.g. ALT/AST) and liver function (e.g. bilirubin). The effect of NAC on prothrombin time is included in our model as an empirical correlation between NAC concentration and INR.

The human model was calibrated using dose-response data from Portmann 1975\textsuperscript{124}. The NAC effect was calibrated using Jepsen 1994\textsuperscript{121}. The rat and mouse models use the human values. Validation and calibration experiments are shown in Figure S25.
Figure S25. Data and simulation results used to calibrate the prothrombin time portion of the DILIsym™ model. Data are in black, and simulation results are in red.

A) Maximum prothrombin time in patients as a function of fraction of viable hepatocytes after acetaminophen overdose\(^{124}\) and corresponding simulation results.

B) Maximum prothrombin time in patients as a function of approximate acetaminophen ingested\(^{1}\) and corresponding simulation results.

C) Prothrombin (clotting factor VII) activity as a fraction of baseline in healthy volunteers given an initial bolus dose of 10 mg/kg NAC followed by an infusion of 10 mg/kg/h NAC over 32 hours\(^{121}\) and corresponding simulation results.

Generating Population Samples (SimPops™) Using a Genetic Algorithm

This section serves to provide more information on the process of generating SimPops™. SimPops™ were necessary for the application examples in this paper. Model research is best done in the context of considering variability, since all mathematical models are only estimates and approximations of true behavior. In addition, comparisons of DILI responses across species must be made with some idea of the variability present in the organism. Predicted responses are enhanced by knowledge of how the majority of the population will compare to the mean response.
Figure S26. Diagram showing the general methods used to generate SimPops™ to assess variability. Model parameters that exhibit variability are chosen, with ranges and distributions assigned based on the available information from literature and best practices. Data are selected, either one set or multiple sets, and various parameter combinations are picked by the genetic algorithm in the MATLAB computing platform (The MathWorks, Natick, MA). The parameter combinations are used to run the DILIsym™ model and compare the model output to the selected data, while the parameter values are also compared to their probability distributions. An equal weighting between distribution fitting and data fitting is assigned, and some parameter combinations are kept, while others are discarded. The parameter sets can then be used to assess other compounds or NAC treatment protocols as well.

Fig. S26 helps to explain the process of SimPops™ creation in more detail. Start by viewing the left side of the figure. The parameters referred to as 'A', 'B', and 'C' represent any general set of parameters chosen for a given SimPops™. As mentioned in the primary text, Table 1 gives the specific parameters chosen for the treatment population. Parameters are chosen that allow for the most realistic physiological variability, include the full spectrum of events occurring during DILI, and minimize simulation time required for SimPops™ generation and subsequent simulations. In this case, we began with a list of model parameters that were the most plausibly variable within a population, and then selected 11 parameters that either had the most impact on model outcomes in preliminary simulations or were proposed to have significant impact on the outcome of a simulation by the literature. We made
an effort to ensure that the 11 parameters that were selected represented as many sub-models as possible. A distribution is subsequently assigned to each parameter, which is illustrated in Fig. S26. The distributions are based on the best sources of information available for each parameter. Some parameters are well characterized through experimentation, leading to greater confidence in the distribution. Others are less directly applicable to any one experiment and require a model sensitivity analysis. The distributions do not have to be normal. In the current study, the distributions were all considered to be normal, but were sometimes split into two normal distributions, due to unequal upper and lower bounds (Table 3 in primary text).

The next step in the process is to provide the Genetic Algorithm with initial parameter values. In this study, we used a randomly generated normal distribution for each parameter. At this point, focus on the center of Fig. S26. The Genetic Algorithm receives the initial parameter values, runs the DILIsym™ model with the values for Drug 'A' (in this case, acetaminophen), and compares the outputs to data for Drug 'A' (in this case, the Davis 1976 bilirubin dose response data). The algorithm also determines the likelihood of the parameter values existing based on their pre-defined distributions. The parameter distribution score combines with the data comparison score. The result, known as the fitness score, determines how the algorithm picks the next iteration of parameter values. This process is repeated for a pre-determined number of generations. At the conclusion of the run, each individual will be composed of a set of parameter values, as the green blocks (1), red blocks (2), and blue blocks (3) on the left in Fig. S26 show. Once a group of parameter value sets, or SimPops™, is generated, it can then be used to test the effectiveness of NAC treatment across the population.
Discussion of Alternate NAC Mechanisms

In our model, NAC acts solely as a precursor to GSH. However, as outlined in the main manuscript, other researchers have proposed alternative mechanisms for the protection against APAP-induced hepatocellular injury by NAC. We are not able to model any of these alternative mechanisms due to the lack of quantitative data that would allow us to construct an equation relating NAC concentration to the direct result of these modes of action.

We were able to conduct some brief exploratory modeling using extremely basic relationships to explore the qualitative effect that adding these mechanisms to our model would have on the conclusions of this paper. We modeled two of the alternative hypotheses: 1) that NAC directly removes NAPQI from the cell, and 2) that NAC acts as an antioxidant, reducing the amount of oxidative stress (RNS/ROS) in the cell. We assigned each reaction a rate constant and modeled these hypotheses with a 60g APAP overdose and either oral or IV NAC (Protocol A and Protocol D) given after a 4 hour delay.

Figure S27. Effect of including a direct interaction between NAC and NAPQI on a model patient given 60g APAP and either oral or IV NAC after a 4-hour delay.

Figure S27 shows the results from our modeling exercise for the hypothesis that NAC removes NAPQI from the cell. This method results in more viable hepatocytes for both oral and IV NAC; however, oral NAC remains superior to IV NAC. This is the same conclusion reached in Figure S28, where NAC reacts directly with RNS/ROS and removes it from the system; indeed, the difference between oral and IV NAC grows when the effect of NAC on RNS/ROS is considered. The conclusions of this paper,
therefore, are likely to be unaffected by the presence of these proposed alternative mechanisms for NAC hepatoprotection.

![Graph showing HC fraction viable over time for different conditions](image)

**Figure S28.** Effect of including a direct interaction between NAC and reactive nitrogen/oxygen species (RNS/ROS) on a model patient given 60g APAP and either oral or IV NAC after a 4-hour delay.

**Discussion of Human Data Used for Model Optimization and Calibration**

We have reported here on data from five studies that were used to construct and validate the DILIsym™ model. The physical characteristics of the study groups are reported in Table S5.
Table S5. Summary of the physical characteristics of the patients in the study groups used for calibration and validation of the outcomes of the DILIsym™ model in humans. None of the studies reported weight, BMI, or ethnicity.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Figure</th>
<th>NAC given?</th>
<th>Gender composition</th>
<th>Age range</th>
<th>APAP dose range (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schiodt 2001 A</td>
<td>S22A</td>
<td>Yes (6 +/- 5 hour delay)</td>
<td>16% male, 84% female</td>
<td>32 +/- 16</td>
<td>26 +/- 16</td>
</tr>
<tr>
<td>Schiodt 2001 B</td>
<td>S22A</td>
<td>Yes (34 +/- 27 hour delay)</td>
<td>41% male, 59% female</td>
<td>37 +/- 13</td>
<td>39 +/- 25</td>
</tr>
<tr>
<td>Schiodt 2001 C</td>
<td>S22B</td>
<td>Yes (44 +/- 16 hour delay)</td>
<td>33% male, 67% female</td>
<td>38 +/- 11</td>
<td>41 +/- 41</td>
</tr>
<tr>
<td>Davidson 1976</td>
<td>S22C</td>
<td>No</td>
<td>40% male, 60% female</td>
<td>Not given</td>
<td>10-125</td>
</tr>
<tr>
<td>Portmann 1975</td>
<td>S25A</td>
<td>No</td>
<td>38% male, 62% female</td>
<td>15-58</td>
<td>10-100</td>
</tr>
<tr>
<td>Davis 1976</td>
<td>S1, S25B</td>
<td>No</td>
<td>43% male, 57% female</td>
<td>Not given</td>
<td>15-100</td>
</tr>
<tr>
<td>Gazzard 1975</td>
<td>Used in modeling death representation</td>
<td>No</td>
<td>Not given</td>
<td>Not given</td>
<td>Not given</td>
</tr>
</tbody>
</table>

Supplemental Materials References


61. Lazzer, S. *et al.* Relationship between basal metabolic rate, gender, age, and body composition in 8,780 white obese subjects. *Obesity (Silver Spring)* **18**, 71–78 (2010).


