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Use of a systems model of drug-induced liver injury (DILIsym®) to elucidate the mechanistic differences between acetaminophen and its less-toxic isomer, AMAP, in mice

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ABSTRACT

Acetaminophen (APAP) has been used as a probe drug to investigate drug-induced liver injury (DILI). In mice, 3′-hydroxyacetanilide (AMAP), a less-toxic isomer of APAP, has also been studied as a negative control. Various mechanisms for the divergence in toxicological response between the two isomers have been proposed. This work utilized a mechanistic, mathematical model of DILI to test the plausibility of four mechanistic hypotheses. Simulation results were compared to an array of measured endpoints in mice treated with APAP or AMAP. The four hypotheses included: (1) quantitative differences in drug metabolism profiles as a result of different affinities for the relevant enzymes; (2) differences in the amount of reactive metabolites produced due to cytochrome P450 (CYP450) inhibition by the AMAP reactive metabolites; (3) differences in the rate of conjugation between the reactive metabolites and proteins; (4) differences in the downstream effects or potencies of the reactive metabolites on vital components within hepatocytes. The simulations did not support hypotheses 3 or 4 as the most likely hypotheses underlying the difference in hepatoxic potential of APAP and AMAP. Rather, the simulations supported hypotheses 1 and 2 (less reactive metabolite produced per mole of AMAP relative to APAP). Within the simulations, the difference in reactive metabolite formation was equally likely to have occurred from differential affinities for the relevant drug metabolism enzymes or from direct CYP450 inhibition by the AMAP reactive metabolite. The demonstrated method of using simulation tools to probe the importance of possible contributors to toxicological observations is generally applicable across species.

1. Introduction

Drug induced liver injury (DILI) is a significant healthcare problem (Bleibel et al., 2007; Corsini et al., 2012; Hayashi and Watkins, 2009; Kaplowitz and DeLeve, 2013; Lee, 2003; Watkins, 2005). This is true for clinicians and patients, as many cases of liver injury, acute liver failure, liver transplant, and death are caused each year worldwide from DILI (Larson et al., 2005; Lee, 2008, 2003). DILI is equally problematic for drug developers, who commonly abandon promising drugs in the midst of development due to unexpected signs of liver injury, or worse, have drugs removed from the market after a few patients experience, rare, idiosyncratic DILI (Senior, 2007; Watkins, 2011). In an effort to resolve these issues, many researchers have studied DILI with the goal of identifying key attributes of DILI-causing agents. No exemplar hepatotoxicant has been studied more often or more thoroughly than acetaminophen (APAP). As the dominant cause of DILI cases seen clinically, including liver failure (Lee, 2008), APAP is an important drug to study for obvious reasons. APAP is also used as a probe drug to understand mechanistic linch-pins in the DILI process. This is commonly done in mice, which show a greater sensitivity to APAP-induced liver injury than rats (McGill et al., 2012).

In the midst of studying APAP in mice, an analogue of APAP, 3′-hydroxyacetanilide (AMAP), has been utilized as a comparator to APAP due to its apparent lack of liver toxicity in mice. AMAP was originally patented as a possible alternative to APAP (Nelson, 1980a). While it appears to be non-toxic in mice, AMAP has been examined in human and rat liver slices, and shown to potentially be more toxic in these species (Hadi et al., 2012). Over the past 30 years, many hypotheses describing the mechanistic underpinnings of the divergence in liver toxicity seen between APAP and AMAP in mice have been proposed by various researchers. However, one could argue that definitive conclusions have not

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yet been reached. The goal of this work was to employ a mechanistic, mathematical model of DILI (DILIsym® v1A) to test the plausibility of several of these hypotheses. The DILIsym® model allowed for comparisons between simulation results based on different hypotheses and published results on APAP/AMAP responses within a quantitative, mechanistically focused framework. While this investigation focused entirely on APAP and AMAP in mice, it represents a generally applicable approach that can be applied elsewhere in toxicology, including to human DILI events. In addition, DILIsym® can be applied to cross-species hepatotoxicity investigations, where the focus is understanding why different species respond differently to the same drug (Howell et al., 2012).

A wide variety of studies on APAP versus AMAP have been conducted. Many have focused on the in vivo metabolism of one or both of the molecules (Dai et al., 2006; Hamilton and Kissinger, 1986; Lee et al., 2009; McGill et al., 2013; Rashed et al., 1990; Vaccarino et al., 2007). Others have taken an in vitro approach to understanding metabolism and potency differences using microsomes or hepatocytes (Bauman et al., 2009; Holme et al., 1991; Rashed et al., 1989; Streeter et al., 1984). Covalent binding studies have also been a prevailing theme (Rashed et al., 1990; Roberts et al., 1990). In vivo toxicity endpoints, such as glutathione (GSH) and liver injury biomarkers, have commonly been measured (Nelson, 1980b; Priyadarsiny et al., 2008; Salminen et al., 1997; Tirmenstein and Nelson, 1989). Mechanistic investigations have also focused on drug metabolism, where enzyme inhibition studies have shown AMAP to inhibit its own reactive metabolite production (Halmes et al., 1998). More recently, investigations of downstream perturbations of liver homeostasis, such as gene expression and critical pathway analysis, have been done (Priyadarsiny et al., 2008; Salminen et al., 1997; Stamper et al., 2010). The totality of the literature was reviewed for distinct mechanistic hypotheses that could explain the divergence in toxicological response between APAP and AMAP. The various reports were narrowed to four primary hypotheses that offered explanations for the observed differences. Of the various hypotheses listed above, the four below were deemed most supported by published data and most comprehensive:

- Hypothesis 1 – the structure of AMAP lends itself to a quantitatively different drug metabolism profile than APAP (and less reactive metabolite produced as a result);
- Hypothesis 2 – the default metabolism parameters describing the conversion from parent compound to glucuronide, sulfate, and reactive metabolite conjugates are the same for APAP and AMAP, but the AMAP reactive metabolite inhibits its own production through mechanism-based inhibition of CYP2E1;
- Hypothesis 3 – the AMAP reactive metabolite binds to cellular proteins at a higher rate than the APAP reactive metabolite (and thus depletes less GSH);
- Hypothesis 4 – AMAP reactive metabolites cause injury or disrupt cellular processes in different (and less potent) ways on an equimolar basis than APAP reactive metabolites.

Hypotheses 1 and 2 can be viewed as 'upstream' or drug metabolism hypotheses, while Hypotheses 3 and 4 relate more to the action or properties of the reactive metabolites generated by APAP and AMAP.

2. Materials and methods

A mechanistic, mathematical model of drug-induced liver injury was the primary means used to accomplish the goal of this work: elucidating the most likely mechanistic explanation for why APAP and AMAP show divergent toxicological responses in mice. Accordingly, some general information about the model (DILIsym® v1A) is discussed below. The general process of simulating exposure to APAP and AMAP in mice is summarized. Next, the four hypotheses tested with the model in the baseline mouse are described in detail, including which parameters in the model were adjusted to simulate each scenario.

2.1. The DILIsym® model, version 1A

The DILI-sim Initiative is a joint effort between the Hamner Institutes for Health Sciences and the pharmaceutical industry. The goals of the DILI-sim Initiative include developing DILIsym®, a predictive, mechanistic, mathematical model of drug induced liver injury (DILI), and advancing the knowledge of DILI for all parties involved (see www.DILIsym.com for more information on the goals and scope of DILI-sim). The method for model design is best described as 'middle out.' The 'middle out' approach involves starting at the organ level (liver in this case), and working down to the molecular level or up to the organism level when necessary (Michelson et al., 2006; Shoda et al., 2010). As a result, this is a multi-scale approach, where models based on different scales are connected through scaling factors. The model is organized into various smaller sub-models, but all sub-models are mathematically integrated to simulate an organism level response. DILIsym® version 1A primarily focuses on C57Bl6 mice, Sprague Dawley rats, and humans (the mouse representation was used for this analysis). Exemplar hepatotoxic compounds were sequentially used to add mechanistic detail to the model. APAP was the first exemplar represented. DILIsym® v1A is therefore primarily a model of oxidative stress-induced hepatotoxicity.

Since the necessary drug absorption, distribution, metabolism, and excretion (ADME) sub-model frameworks were built within version 1A using APAP, this analysis of APAP versus AMAP was a natural extension of previous work. Additional DILI mechanisms have since been and are currently being added to future versions of the model. More information on DILIsym® version 1A is available through several previously published articles (Bhattacharya et al., 2012; Howell et al., 2012; Shoda et al., 2014; Woodhead et al., 2012). These articles include a more thorough description of the DILIsym® sub-models, data samples used for model optimization, and a comprehensive list of the data used for version 1A. The model is also directly available to industry through membership in the DILI-sim Initiative. Academic and non-profit groups may access the model by contacting the Hamner Institutes for Health Sciences or the corresponding author.

2.1.1. Comparing AMAP to APAP in mice in DILIsym®

The underlying assumption of the present work is that DILIsym® v1A adequately simulates a hepatotoxic event in mice arising from APAP exposure. The methods used to construct the model were discussed in several previous publications (Bhattacharya et al., 2012; Howell et al., 2012; Shoda et al., 2014; Woodhead et al., 2012). To briefly summarize the series of assumptions leading to APAP toxicity simulation in DILIsym® v1A, the inputs include the desired dosing quantity and route. With regard to this study, the simulated quantities of APAP and AMAP dosed, as well as the routes, were taken from the cited publications used for the comparison. Once the simulation is initiated, DILIsym® v1A accounts for the absorption and distribution of the drug. APAP (or AMAP) is then converted via one of three metabolic pathways: sulfation, glucuronidation, or CYP-mediated reactive metabolite production. The sulfate and glucuronide metabolites are represented as inert with regard to liver toxicity. The reactive metabolite is conjugated in two possible ways. GSH conjugation is represented with an extremely high reaction rate, but is only available in limited quantities (substrate limited). Protein adduction is also represented. Protein availability is not assumed to be a constraint, but the rate of reaction for reactive metabolite/protein conjugation is much lower than for GSH conjugation, based on published data used to optimize the rate constant (Howell et al., 2012; Woodhead et al., 2012). Once GSH depletion occurs, the reactive metabolite can build up in the representative liver tissue compartments until GSH recovery occurs or protein adduction occurs. The reactive metabolite concentration in the liver is the actual perpetrator of toxicity in DILIsym® v1A. A function connects the concentration of reactive metabolite in the liver to oxidative stress generation, which is subsequently connected to ATP production. As reactive metabolite accumulates, oxidative stress accumulates, ATP production and concentration decline, and necrosis ensues. Details on the equation structure and parameters dictating these processes are available in previously published articles (Bhattacharya et al., 2012; Howell et al., 2012; Woodhead et al., 2012) or in DILIsym® version 1A, which can be accessed as directed above. The level of mechanistic detail included in v1A is important to understand when considering each of the four hypotheses discussed below. For each hypothesis, any limitations or assumptions that could influence conclusions regarding APAP versus AMAP are pointed out.

To utilize DILIsym® v1A to identify the most likely mechanistic rationale for divergent toxicological responses for APAP versus AMAP in mice, the baseline APAP model for mice was first used. Baseline mice in the model represent 'average' or 'mean' responses when compared to a larger group of mice. For the purposes of this work, the APAP model in mouse was considered to be the starting point. Experimental protocols where APAP was administered to mice were initially simulated (Priyadarsiny et al., 2008; Rashed et al., 1990; Salminen et al., 1997; Tirmenstein and Nelson, 1989). Changes were then made to parameter values within the model in a step-wise fashion to mimic hypothesized mechanistic differences between APAP and AMAP hepatotoxicity (Hypotheses 1–4), and experimental protocols where AMAP was dosed to mice were also simulated (Priyadarsiny et al., 2008; Rashed et al., 1990; Salminen et al., 1997; Tirmenstein and Nelson, 1989). While the entirety

of the steps and complexities involved in the hypotheses were not included (i.e. simplifications were made), we believe the broad hypotheses tested remain consistent with the design and parameterization of the mathematical framework. Underlying the parameter value changes in a step-wise fashion is a key assumption: all other processes and modelling assumptions for APAP, besides the specific changes implemented, were assumed to be exactly the same between the two molecules. The possibility that more than one mechanistic difference exists between the two molecules cannot be ruled out. As such, the reader should consider this approach as a method for determining which process is likely to be the dominant reason for the divergence in response. Note that simulation results for isolated hypothetical mechanisms are capable of suggesting that no single mechanism is sufficient to account for the divergent response. The methods for implementing the four hypotheses simulated herein are outlined below.

2.1.2. Hypothesis 1 – metabolic partitioning is different

Hypothesis 1 basically assumes that both APAP and AMAP produce a reactive metabolite that could lead to oxidative stress in hepatocytes, which results in ATP loss and cell death, but less reactive metabolite is produced from AMAP than APAP. This hypothesis was based on the cumulative APAP and AMAP recovery data in mice reported by Rashed et al. (1990). They observed significantly more glucuronidated AMAP species than APAP, and significantly less reactive metabolite species for AMAP compared to APAP. Thus, the three maximum velocity parameter values that control the amount of APAP converted to sulfate, glucuronide, and N-acetyl-p-benzoquinone imine (NAPQI, which is the APAP reactive metabolite) conjugates were adjusted to match the 24 h cumulative AMAP profile from Rashed et al. (1990). Table 1 shows the parameters that were adjusted.

The non-reactive metabolite pathways were increased and the reactive metabolite pathway was decreased. Under this assumption, the reactive metabolites were assumed to cause the same amount of oxidative stress per mole. Therefore, of critical importance for this assumption was the existence of the model of mouse response to APAP, which had been calibrated to produce the correct amount of simulated oxidative stress per mole of NAPQI produced.

2.1.3. Hypothesis 2 – AMAP reactive metabolite inhibits CYP2E1

Hypothesis 2 is similar to Hypothesis 1 in that it focuses solely on the amount of reactive metabolite produced. Underlying Hypothesis 2 is the assumption that the drug metabolism parameters describing the conversion rates from parent compound to glucuronide, sulfate, and reactive metabolite conjugates are the same for APAP and AMAP, but that the AMAP reactive metabolite inhibits its own production through mechanism-based inhibition (covalent binding) of CYP2E1. CYP inhibition for the AMAP reactive metabolite was initially reported by Halmes et al. (1998). This inhibition was simulated by reducing the maximum velocity parameter for the production of NAPQI from AMAP by 75%, consistent with reported rates (Halmes et al., 1998). Reactive metabolite production rates decreased, although the sulfate and glucuronide pathways were not altered. An important distinction between Hypothesis 1 and 2 involves the methods for arriving at the parameter value alterations. For Hypothesis 1, the three parameters mentioned above were 'tuned' to the 24 h cumulative metabolite profile reported by Rashed et al. For Hypothesis 2, the maximum velocity for reactive metabolite production was multiplied by '0.25' to simulate 75% inhibition. No comparisons to the cumulative metabolite profiles were done to choose this level of inhibition. Rather, it was based solely on the level of inhibition reported in the literature. Halmes et al. reported inhibition levels consistent with a 75% reduction in the maximum velocity parameter. For exploratory purposes, other levels of inhibition were also simulated and the results are discussed briefly in Section 4.

2.1.4. Hypothesis 3 – AMAP reactive metabolite is more reactive than NAPQI

Unlike Hypotheses 1 and 2, Hypothesis 3 hinges on the reactive metabolites from APAP and AMAP interacting with hepatocytes differently. Specifically, Hypothesis 3 suggests that the AMAP reactive metabolite covalently binds to cellular proteins at a higher rate than NAPQI. This has been suggested by several authors previously (Rashed et al., 1990; Roberts et al., 1990; Salminen et al., 1997; Streeter et al., 1984). Despite observations of similar levels of protein adducts for APAP and AMAP in mouse livers (Rashed et al., 1990), the rate of protein binding to the AMAP reactive metabolite was still likely higher due to less total reactive metabolite produced for AMAP (and a resulting lower reactive metabolite to protein adduct ratio). Higher reactive metabolite reactivity could lead to lower toxicity for AMAP in at least two ways. First, more AMAP binding to proteins could result in less AMAP binding to glutathione (GSH), which could prevent GSH from reaching critically low levels. In this scenario, covalent binding is acting as an elimination pathway for the reactive metabolite. To simulate this hypothesis, the rate constant that specifies the first-order reaction rate between reactive metabolite and proteins was increased by over two fold. Thus, the primary question addressed was: could higher AMAP reactive metabolite binding act as a quantitatively significant clearance route for the reactive metabolite? A 1:1 stoichiometry was assumed for the simulations (one molecule of reactive metabolite binds to one protein).

On the other hand, Hypothesis 3 could also be interpreted differently. Many researchers have proposed that higher reactivity with proteins could cause the AMAP reactive metabolite to selectively bind to proteins near the site of reactive metabolite generation, while the APAP reactive metabolite (NAPQI) may be more capable of diffusing to critical sites, such as mitochondria, and binding to more critical proteins (Birge et al., 1989; Holme et al., 1991; Rashed et al., 1990; Roberts et al., 1990; Streeter et al., 1984; Tirmenstein and Nelson, 1989). DILIsym® v1A does not include specific proteins bound by reactive metabolites and the functions associated with each of those proteins. As a result, this component of Hypothesis 3 cannot be directly simulated with the DILIsym® model. An opinion and analysis of selective protein binding and its potential effect on toxicity is addressed in Section 4, despite the inability to directly simulate this hypothesis.

2.1.5. Hypothesis 4 – downstream damage pathway processes drive the divergence

The fourth and final hypothesis assumes that reactive metabolites are causing injury or disrupting cellular processes in different ways on an equimolar basis. Several researchers have examined the downstream differences in toxicity for AMAP versus APAP (Priyadarsiny et al., 2008; Salminen et al., 1997; Stamper et al., 2010). All have shown differences in pathways, proteins, or genes affected by APAP compared to AMAP. Still, one important question regarding these studies remains: are the resulting differences a consequence of upstream metabolic factors driving divergent toxic responses, or are the differences causing the divergence seen (cause or effect)? Hypothesis 4 assumes they are the cause of the divergence, and provides the opportunity to test this theory and compare the available data to simulations under this assumption.

To implement Hypothesis 4, all parameter values within DILIsym® v1A for APAP in mice were assumed to be the same for AMAP except the parameter connecting the concentration of reactive metabolite in the liver to oxidative stress production in the liver. Many details surrounding the events of oxidative stress induction remain unclear (Burke et al., 2010; Hinson et al., 2010), and this process is therefore simplified and empirically modelled in DILIsym® v1A. A change in the parameter value governing this process acts as a surrogate for a whole host of potential injury pathway divergences, including gene regulation. This parameter was reduced by 67% to give a similar level of liver injury for AMAP as observed in published reports (Priyadarsiny et al., 2008; Tirmenstein and Nelson, 1989). This is analogous to how Hypothesis 1 was tested, except that Hypothesis 1 involved 'tuning' the model to the correct cumulative drug metabolism pathway outputs, whereas Hypothesis 4 involved 'tuning' the model to the correct liver injury outputs.

2.1.6. Endpoints for comparison between APAP and AMAP

For each of the four hypotheses described above, a different modification to DILIsym® v1A parameter values was implemented to represent AMAP instead of APAP. The resulting simulations were compared to the AMAP data and APAP simulations and data to determine how well simulated toxicological responses between APAP and AMAP mimicked the differential responses observed in the published studies. Three primary mouse data sets were used: cumulative urinary parent and metabolite accumulation (Rashed et al., 1990), minimum GSH levels observed during in vivo studies (Rashed et al., 1990; Salminen et al., 1997), and maximum ALT values observed (Priyadarsiny et al., 2008; Tirmenstein and Nelson, 1989). ALT was an appropriate primary endpoint for acetaminophen hepatotoxicity due to a previously demonstrated correlation between histologic assessment of liver necrosis and serum ALT (Harrill et al., 2009). For each of these data components, the important consideration for this study was how the reported response differed between APAP and AMAP, and correspondingly, how the response differed from simulated APAP to simulated AMAP. To elucidate the mechanistic differences in toxicity between the compounds, the relative change from one compound to the other was more critical than the absolute response for one compound compared to the data for that compound. The figures and text in Section 3 show the data and simulation results for both compounds for all four hypotheses tested. Note that the APAP/AMAP data and APAP simulation results are the same across all the figures, as only the AMAP parameter representations were adjusted for each hypothesis.

3. Results

3.1. Hypothesis 1 testing in the baseline mouse – metabolic partitioning is different

The simulated and reported cumulative parent and metabolites recovered for AMAP (Hypothesis 1) are shown in Fig. 1A. The simulated APAP values are also shown alongside the AMAP data.

Note that the APAP data shown was not used to optimize DILIsym® v1A and the APAP simulations agree with the APAP data, providing validation that the APAP model is a reasonable predictor of APAP metabolism in mice. The APAP predictions of minimum GSH (Fig. 1B) and maximum ALT (Fig. 2) are also in line with the data, particularly when considering between-study variability. Most importantly, the simulations and data qualitatively agree for APAP: significant liver injury occurred.

Table 1
Parameters describing the metabolism of N-acetyl-meta-aminophenol (AMAP) versus acetaminophen (APAP) in mice used for Hypothesis 1 testing.

Parameter name	Parameter in DILIsym® v1A	APAP	AMAP	Fold change (AMAP relative to APAP)
V_{max} – CYP450 reactive metabolite formation[a] (mol/h/kg$^{0.75}$)	Vmax_NAPQI	2.74e−4[b]	6.85e−5[c]	0.25
V_{max} – glucuronidation (mol/h/kg$^{0.75}$)	Vmax_gluc	8.4e−3[b]	1.09e−2[c]	1.3
V_{max} – sulfation (mol/h/kg$^{0.75}$)	Vmax_sulf	8.53e−5[b]	1.28e−4[c]	1.5

[a] Once APAP or AMAP is converted to their respective reactive metabolites, the DILIsym® model allows for covalent binding to glutathione and/or proteins (to form protein adducts). The first order reaction rates for the reactive metabolite/protein reactions were estimated using covalent binding data from several sources as explained and cited in Howell et al. (2012).
[b] Values first published in Howell et al. (2012).
[c] V_{max} values were adjusted for AMAP based on cumulative APAP and AMAP metabolism studies in Rashed et al. (1990).

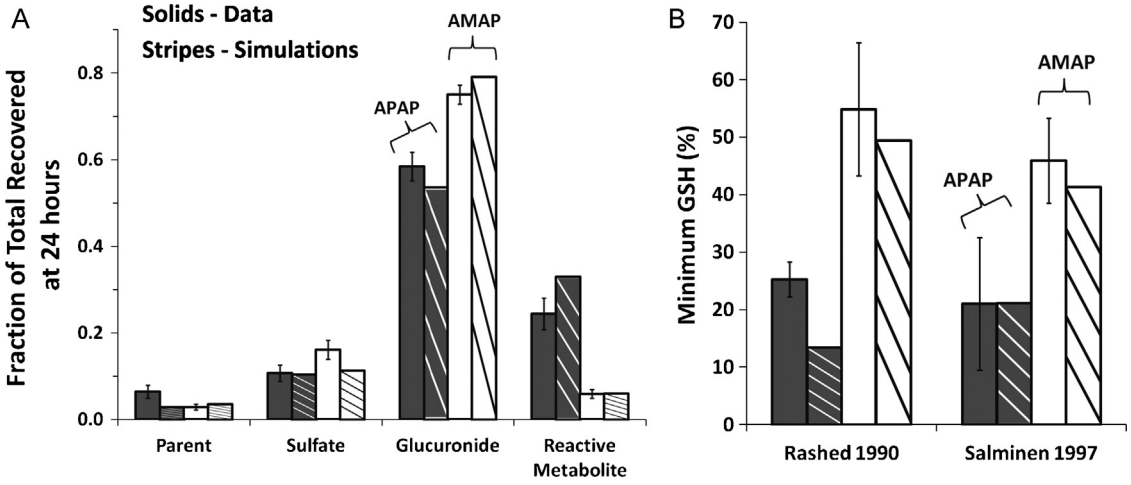

Fig. 1. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of different metabolic partitioning (Hypothesis 1). (A) Cumulative metabolite and parent elimination at 24 h after 250 mg/kg APAP and 600 mg/kg AMAP in mice (normalized to total amount recovered). Solid bars show data measured in Rashed et al. (1990). Dark grey bars represent APAP and white bars represent AMAP. Striped bars show corresponding simulated profiles from DILIsym® v1A. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. (B) Comparison of minimum glutathione (GSH) in the liver as a percentage of baseline for APAP and AMAP, including measurements and simulations. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Rashed et al., 1990), and 200 mg/kg and 1000 mg/kg (Salminen et al., 1997). All simulations and measurements are for mice.

The observed increase in glucuronidation and decrease in reactive metabolite generation (relative to APAP) are captured in the simulated AMAP results. This is no surprise, as Hypothesis 1 involved 'tuning' the model to match the AMAP data shown in Fig. 1A (Table 1). The key figures to review for Hypothesis 1 are Figs. 1B and 2. Fig. 1B demonstrates that the higher minimum GSH observed for AMAP was accurately predicted. Fig. 2 illustrates how the model agreed with the AMAP data with regard to minimal liver injury. Hypothesis 1 led to simulation results that agreed well with measured values for AMAP.

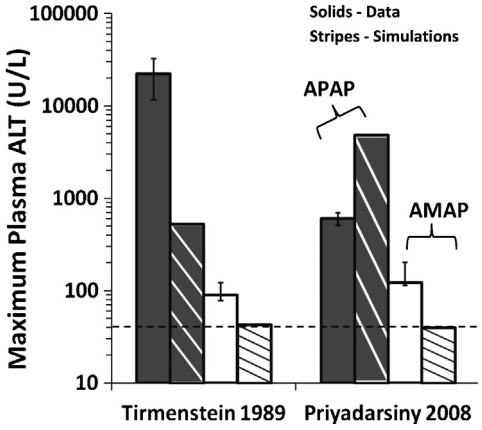

Fig. 2. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of different metabolic partitioning (Hypothesis 1). Comparison of maximum observed or simulated alanine transaminase (ALT) in serum for APAP and AMAP, including measurements and simulations. Solid bars show data while striped bars show corresponding simulated profiles from DILIsym® v1A. Dark grey bars represent APAP and white bars represent AMAP. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Tirmenstein and Nelson, 1989), and 400 mg/kg and 400 mg/kg (Priyadarsiny et al., 2008). All simulations and measurements are for mice.

3.2. *Hypothesis 2 testing in the baseline mouse – AMAP reactive metabolite inhibits CYP2E1*

When implementing a parameter change to mimic 75% CYP2E1 inhibition by the AMAP reactive metabolite (Hypothesis 2), the reduced level of reactive metabolite production for AMAP was similar to levels observed in literature (Fig. 3A).

As a result of the smaller reactive metabolite pathway flux, the glucuronidation flux was also increased, despite the parameter value for glucuronidation being equal to the value for APAP (Fig. 3A). Corresponding predictions of minimum GSH in Fig. 3B show the correct increasing trend for AMAP compared to APAP, although the simulations are not as quantitatively in agreement for AMAP GSH compared to Hypothesis 1. Regardless, the liver injury predicted for AMAP under Hypothesis 2, indicated by ALT in Fig. 4, was minimal compared to APAP. Hypothesis 2 also led to simulation results that generally agreed well with measured values for AMAP.

3.3. *Hypothesis 3 testing in the baseline mouse – AMAP reactive metabolite is more reactive than NAPQI*

Hypothesis 3 tested the idea that an increased reactivity between AMAP reactive metabolites and proteins could account for the observed differences. The simulated increase in the rate of

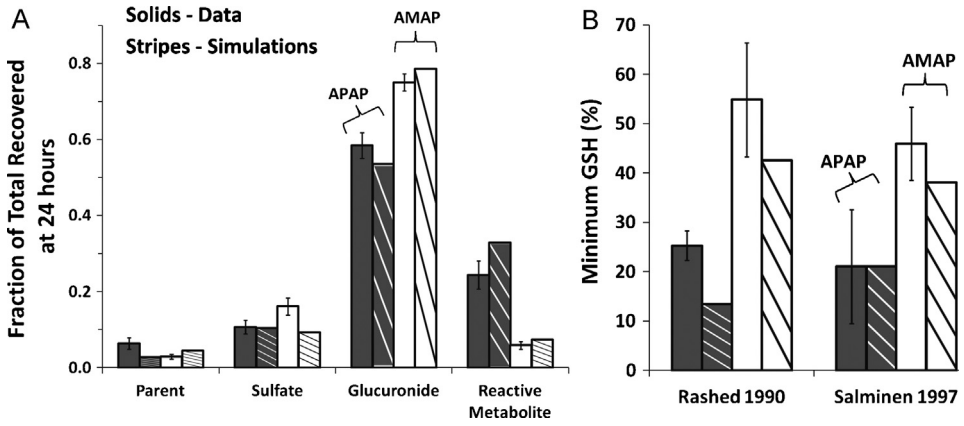

Fig. 3. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of self-inhibition of CYP2E1 (75%) by the AMAP reactive metabolite (Hypothesis 2). (A) Cumulative metabolite and parent elimination at 24 h after 250 mg/kg APAP and 600 mg/kg AMAP in mice (normalized to total amount recovered). Solid bars show data measured in Rashed et al. (1990). Dark grey bars represent APAP and white bars represent AMAP. Striped bars show corresponding simulated profiles from DILIsym® v1A. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. (B) Comparison of minimum glutathione (GSH) in the liver as a percentage of baseline for APAP and AMAP, including measurements and simulations. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Rashed et al., 1990), and 200 mg/kg and 1000 mg/kg (Salminen et al., 1997). All simulations and measurements are for mice.

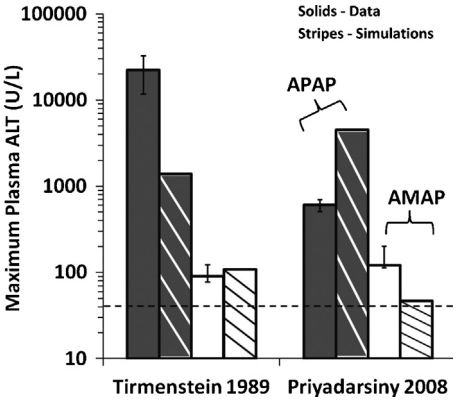

Fig. 4. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of self-inhibition of CYP2E1 (75%) by the AMAP reactive metabolite (Hypothesis 2). Comparison of maximum observed or simulated alanine transaminase (ALT) in serum for APAP and AMAP, including measurements and simulations. Solid bars show data while striped bars show corresponding simulated profiles from DILIsym® v1A. Dark grey bars represent APAP and white bars represent AMAP. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Tirmenstein and Nelson, 1989), and 400 mg/kg and 400 mg/kg (Priyadarsiny et al., 2008). All simulations and measurements are for mice.

protein adduction did not shift the metabolism of AMAP towards glucuronidation as the data suggests (Fig. 5A), nor did it result in less reactive metabolite generation (Fig. 5A).

Even still, the additional elimination of reactive metabolite could theoretically lead to less GSH depletion, less oxidative stress, and therefore less injury. Fig. 5B is evidence to the contrary. GSH stores were completely exhausted under this scenario for AMAP. Furthermore, Fig. 6 provides ALT comparisons that suggest injury from AMAP was predicted to be as significant or worse as compared to APAP under this hypothesis.

To help address the question of why a shift towards protein binding did not lead to a simulated decrease in GSH depletion and less liver injury, Fig. 7 was assembled.

Fig. 7 compares the total reactive metabolite products measured and simulated for both compounds with the total protein adducts measured at 24 h in the liver for both compounds. Note that the data for protein adducts were reported in normalized units, and conversions were done to produce protein adduct estimates per whole liver (Brown et al., 1997). The important aspect of Fig. 7 is the magnitude of protein adducts detected compared to the total reactive metabolite mass. In short, the amount of protein adducts is several orders of magnitude lower than the total reactive metabolite mass, making the magnitude of protein binding insignificant

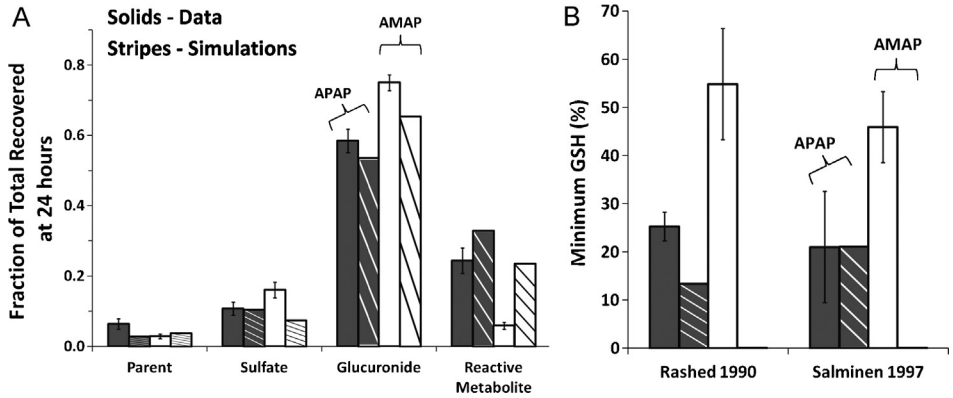

Fig. 5. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of the AMAP reactive metabolite being more reactive than NAPQI (Hypothesis 3). (A) Cumulative metabolite and parent elimination at 24 h after 250 mg/kg APAP and 600 mg/kg AMAP in mice (normalized to total amount recovered). Solid bars show data measured in Rashed et al. (1990). Dark grey bars represent APAP and white bars represent AMAP. Striped bars show corresponding simulated profiles from DILIsym® v1A. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. (B) Comparison of minimum glutathione (GSH) in the liver as a percentage of baseline for APAP and AMAP, including measurements and simulations. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Rashed et al., 1990), and 200 mg/kg and 1000 mg/kg (Salminen et al., 1997). All simulations and measurements are for mice. Note that the minimum simulated GSH bars for AMAP are not visible due to the very low minimum GSH concentration predicted from Hypothesis 3.

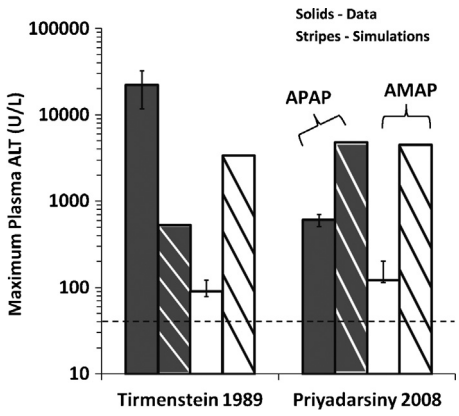

Fig. 6. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of the AMAP reactive metabolite being more reactive than NAPQI (Hypothesis 3). Comparison of maximum observed or simulated alanine transaminase (ALT) in serum for APAP and AMAP, including measurements and simulations. Solid bars show data while striped bars show corresponding simulated profiles from DILIsym® v1A. Dark grey bars represent APAP and white bars represent AMAP. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Tirmenstein and Nelson, 1989), and 400 mg/kg and 400 mg/kg (Priyadarsiny et al., 2008). All simulations and measurements are for mice.

unless levels are simulated beyond the range of the measured data. To further make this point, the reactive metabolite pathway product mass is probably best reported in micromoles, while the protein adduct concentrations are customarily reported in nanomoles.

3.4. Hypothesis 4 testing in the baseline mouse – downstream damage pathway processes drive the divergence

Adjusting the DILIsym® v1A parameter connecting reactive metabolite concentrations in the liver to oxidative stress production for APAP in mice resulted in a reduced level of liver injury for AMAP, which is indicated in Fig. 8.

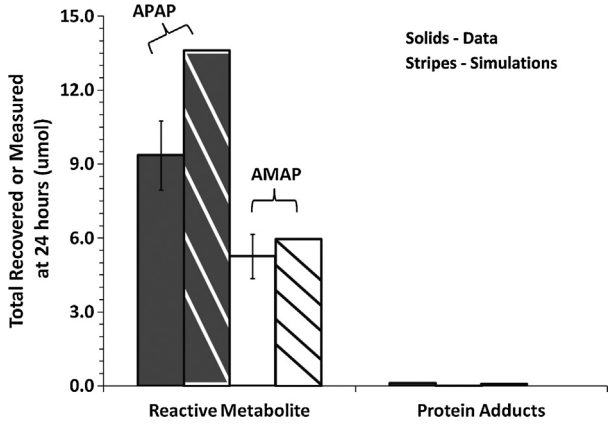

Fig. 7. Comparisons of data and simulations across APAP and AMAP for the amount of reactive metabolite recovered over 24 h and the amount of covalently bound protein adducts in the liver at 24 h. The mechanistic hypothesis of different metabolic partitioning (Hypothesis 1) was used to generate the simulation results. Solid bars show data while striped bars show corresponding simulated profiles from DILIsym® v1A. Dark grey bars represent APAP and white bars represent AMAP. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Rashed et al., 1990). All simulations and measurements are for mice. The data reported in Rashed et al. (1990) were adapted based on an estimated total body weight and liver weight for mice to reach total mass recovered, rather than fractions of the total dose as reported in the paper. Note that the protein adduct bars are not visible due to the magnitude of the values compared to the magnitude of the reactive metabolites recovered.

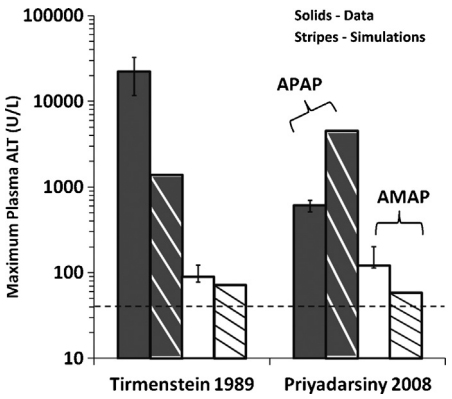

Fig. 8. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of downstream damage processes differing between the AMAP reactive metabolite and NAPQI (Hypothesis 4). Comparison of maximum observed or simulated alanine transaminase (ALT) in serum for APAP and AMAP, including measurements and simulations. Solid bars show data while striped bars show corresponding simulated profiles from DILIsym® v1A. Dark grey bars represent APAP and white bars represent AMAP. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Tirmenstein and Nelson, 1989), and 400 mg/kg and 400 mg/kg (Priyadarsiny et al., 2008). All simulations and measurements are for mice.

This is again no surprise, as Hypothesis 4 involved 'tuning' the model to get a similar level of liver injury as seen in the AMAP studies. Fig. 9 addresses the validity of this hypothesis when considering all the reported metrics simultaneously. Fig. 9A shows clear disagreement between the AMAP data and simulations. Likewise, simulated GSH stores were completely depleted (Fig. 9B). Hypothesis 4 led to reasonable concurrence between the simulations and data for liver injury, but not for drug metabolism or GSH depletion.

4. Discussion

One of the many advantages of utilizing mechanistic, mathematical representations of complex processes is piece-wise hypothesis testing aimed at elucidating important but non-obvious conclusions. This method has been utilized in numerous cases to shed light on difficult problems (Gandelman et al., 2010; Geenen et al., 2012; Howell and Chauhan, 2010; Johnson et al., 2010; Lam and Hunt, 2009; Punt et al., 2009; Woodhead et al., 2012). Simulations of hypotheses on why AMAP toxicity in mice is reduced compared to APAP suggests that two of the four hypotheses tested are more plausible than the other two. Table 2 provides a summary of agreement between simulations and data for each hypothesis. The absolute value of the difference between the measured and simulated endpoints is also shown in Table 2 for each endpoint/hypothesis combination. In cases where the endpoints were measured and simulated for two studies (GSH and ALT), the differences were averaged. The differences quantitatively illustrate that Hypotheses 1 and 2 were suggested as most plausible by the simulations.

4.1. Quantity of reactive metabolite produced appears most plausible

Hypotheses 1 and 2 assume that each mole of reactive metabolite generated from APAP initiates oxidative stress identically to a mole of reactive metabolite generated from AMAP, but less reactive metabolite is produced for the latter case. Both led to simulated cumulative drug metabolism profiles that included three-fold less reactive metabolite from AMAP, which agreed with the published data. Likewise, both Hypotheses 1 and 2 led to GSH depletion and

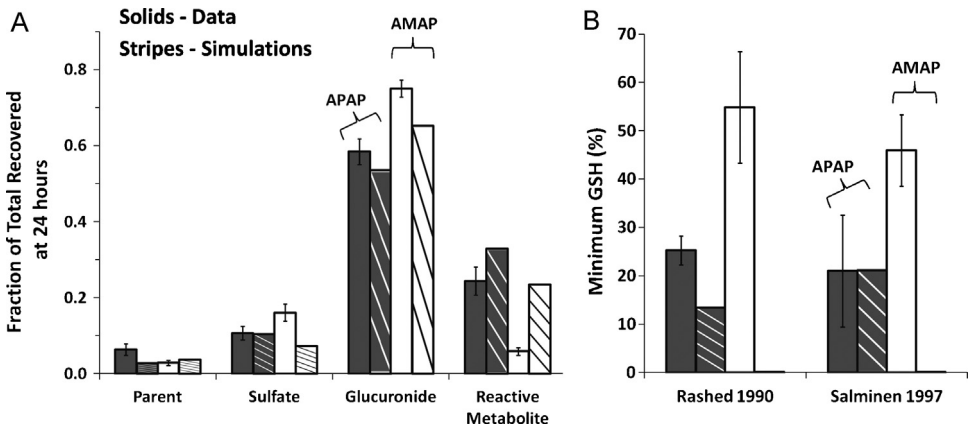

Fig. 9. Comparisons of data and simulations across APAP and AMAP for the mechanistic hypothesis of downstream damage processes differing between the AMAP reactive metabolite and NAPQI (Hypothesis 4). A) Cumulative metabolite and parent elimination at 24 h after 250 mg/kg APAP and 600 mg/kg AMAP in mice (normalized to total amount recovered). Solid bars show data measured in Rashed et al. (1990). Dark grey bars represent APAP and white bars represent AMAP. Striped bars show corresponding simulated profiles from DILIsym® v1A. Dark bars with white stripes represent APAP and white bars with dark stripes represent AMAP. (B) Comparison of minimum glutathione (GSH) in the liver as a percentage of baseline for APAP and AMAP, including measurements and simulations. Doses of APAP and AMAP, respectively, were 250 mg/kg and 600 mg/kg (Rashed et al., 1990), and 200 mg/kg and 1000 mg/kg (Salminen et al., 1997). All simulations and measurements are for mice. Note that the minimum simulated GSH bars for AMAP are not visible due to the very low minimum GSH concentration predicted from Hypothesis 4.

ALT elevation results consistent with published reports. However, the question of whether less AMAP reactive metabolite is produced due to different affinities for the relevant CYP's (Hypothesis 1) versus direct CYP2E1 inhibition (Hypothesis 2) is not directly addressable with DILIsym® v1A. Both are equally supported by the simulations.

Hypothesis 3 focused on the reactivity of the AMAP reactive metabolite. Simulations of increased reactivity between the AMAP reactive metabolite and non-specific proteins did not show protection from GSH depletion and decreased injury in the simulated mouse. Fig. 7 illustrates why: the amount of covalent adducts simulated (and measured) during APAP/AMAP dosing to mice (and other

Table 2
Summary of agreement between measured data and DILIsym® v1A simulation results for N-acetyl-meta-aminophenol (AMAP) after four discrete hypotheses were tested to simulate the differences in observed toxic potencies between AMAP and APAP.

Measured Endpoints for Comparison → / Mechanistic Hypotheses ↓	Cumulative Metabolite Accumulation (at 24 hours) [a]	Maximum GSH Depletion in the Liver [b]	Hepatic Injury Markers (maximum ALT) [c]	Overall Agreement Between Simulations and Data for APAP versus AMAP
Metabolic partitioning is different (Hypothesis 1)	✓ (0.001 fraction)	✓ (5 %)	✓ (64 U/L)	✓
AMAP reactive metabolite inhibits CYP2E1 - 75% inhibition (Hypothesis 2)	✓ (0.016 fraction)	✓ (10 %)	✓ (28 U/L)	✓
AMAP reactive metabolite is more reactive than NAPQI (Hypothesis 3)	⊘ (0.18 fraction)	⊘ (50 %)	⊘ (3850 U/L)	⊘
Downstream damage pathway processes drive the divergence (Hypothesis 4)	⊘ (0.18 fraction)	⊘ (50 %)	✓ (40 U/L)	⊘

[a] Rashed et al. (1990).
[b] Rashed et al. (1990) and Salminen et al. (1997).
[c] Priyadarsiny et al. (2008) and Tirmenstein and Nelson (1989).
✓ Simulation results and measured data agree.
⊘ Simulation results and measured data do not agree.
*The absolute value of the difference between the measured endpoint and the simulated endpoint are shown below the symbols for each endpoint/hypothesis combination. In cases where the endpoints were measured and simulated for two studies (GSH and ALT), the differences were averaged.

species) is several orders of magnitude lower than the amount of total reactive metabolite generated. Thus, the simulations may be pointing to a radical conclusion: covalent binding may simply be a correlative observation associated with reactive metabolites, with little to no mechanistic link to the actual cause of hepatocyte death. Under this scenario, the reactive metabolite itself, when unbound to proteins or GSH (particularly following depletion), is the direct initiator of oxidative stress via interactions with mitochondria. To date, the drug-induced liver injury literature has produced no direct evidence to refute this point, yet many publications point to covalent adducts as a causative feature of reactive metabolite-induced injury (Davern et al., 2006; McGill et al., 2013; Pumford et al., 1990).

Hypothesis 3 contains one important omission: DILIsym® v1A cannot account for which proteins in the hepatocyte are bound by an APAP reactive metabolite compared to an AMAP reactive metabolite. Many researchers have postulated that selective protein binding accounts for the observed differences between the two analogues (Birge et al., 1989; Holme et al., 1991; Rashed et al., 1990; Roberts et al., 1990; Streeter et al., 1984; Tirmenstein and Nelson, 1989). The theory proposes that the AMAP reactive metabolite is more reactive, which is well supported experimentally (Rashed et al., 1990; Streeter et al., 1984), and thus binds to proteins closer to the site of generation than the APAP reactive metabolite (NAPQI), which is able to diffuse to more critically important sites in the cell and initiate changes there. This theory is neither directly supported nor directly refuted by the simulation results shown herein. On the other hand, the modelling in Hypotheses 1 and 2 assumes that APAP and AMAP have equipotent reactive metabolites, accounts for the differences in the magnitude of reactive metabolites produced, and leads to results consistent with the literature. This indirect evidence can be interpreted in the following way: selective binding to less critical proteins by the AMAP reactive metabolite may be occurring, but it is not necessary to explain the differences in observed toxicity. In other words, assuming equipotency and accounting for mass differences are sufficient steps to explain the results, so separating the selective binding theory from the results is difficult unless a mouse study can be done where equal amounts of total reactive metabolites (from APAP and AMAP) are produced (not equal amounts of protein adducts). Beyond the simulations, Holme et al. studied the in vitro potency of the reactive metabolites of APAP and AMAP and concluded that "...the relative toxic potencies of the hydroquinone and quinone metabolites of AMAP were comparable to that of NAPQI..." (Holme et al., 1991). Direct in vitro evidence suggests the two reactive metabolites have similar toxic potencies on a equimolar basis. Lastly, Fig. 7 shows how small the amount of covalent adducts produced is compared to other downstream metabolites recovered. If selective binding were a critical driver of the divergent response, it would have to be extremely selective to have such a drastic effect with such a small amount of proteins affected. Taken as a whole, these simulation results and published data have led us to conclude that increased reactivity of AMAP reactive metabolites has little bearing on the toxicity (or lack thereof) seen in mice.

Hypothesis 4 emphasized a potential difference in the equimolar toxic potency of the APAP reactive metabolite compared to AMAP, with no regard for drug metabolism differences. This hypothesis disagrees with the data from Holme et al. (1991), suggesting equipotency of the reactive metabolites. While this led to inaccurate simulations for cumulative metabolite outputs and GSH depletion, the simulations help to make a key point: focusing on the downstream mechanisms of injury to elucidate a divergent observed response does not make sense unless the exact same amount of reactive metabolite was produced in the mice for both compounds. Thus, this hypothesis was deemed less credible than Hypothesis 1 or 2. While concurrent differences in drug metabolism and reactive metabolite potency were not simulated, the same point made regarding Hypothesis 3 is relevant: the AMAP reactive metabolite may be causing less havoc in the cell on an equimolar basis, but this is not necessary to explain the differences in observed toxicity in the simulations. This points to downstream differences as a minor player at best. Table 2 summarizes the results of comparing simulations of various hypotheses with reported data. Overall, the modelling supports a difference in the net quantity or cumulative flux of reactive metabolite as the most plausible explanation for the differences in response between APAP and AMAP in mice. At first glance, this may seem like an obvious conclusion. Nonetheless, the idea that simple mass-related (flux) issues account for such a well studied divergence in response, and not covalent adducts or mechanistic divergences downstream of drug metabolism, has rarely been suggested in a long history of APAP/AMAP research. This highlights the point that quantitative, mechanistic modelling tools provide the means to test theories that are otherwise difficult to test.

This exercise has supported a hypothesis that may be refuted or accepted in the future as others continue to study APAP and AMAP. At the very least, it will help inform and motivate additional thoughts and studies.

4.2. Limitations

In this example, DILIsym® v1A supported the amount of reactive metabolite produced as the key differentiator between APAP and AMAP in mice. Still, there are several general limitations of the DILIsym® model and the mechanistic modelling approach that must be taken into consideration alongside this conclusion. First, simulation results must always be considered as one additional piece of information rather than as the entirety of evidence for or against a particular hypothesis or proposed mechanism. Mathematical modelling tools are designed to improve understanding of dynamic systems, but they always include approximations that limit their accuracy. Experimental results, experience, and many other factors need to weigh heavily on conclusions regarding the potential for DILI. Another significant challenge for building and using the DILIsym® model lies in data gaps in the DILI literature. Certain aspects of the model, such as autoprotection (adaptation), oxidative stress production from reactive metabolites, and selective protein binding are described as well as possible, but the model presses the edge of the data frontier. This is both a limitation and an exciting opportunity, as the model can be used to identify where data gaps occur and guide new experimentation.

Additional limitations related directly to the assumptions made for APAP and AMAP are also important to highlight. Throughout this work, it was assumed that APAP and AMAP were identical with regard to their effects, except for the specific delineations mentioned for Hypotheses 1–4. The hope is that this over-simplified representation allowed for a better understanding of the critical elements involved in causing DILI without excessive noise that could potentially drown out primary conclusions. On the other hand, such a simplification could lead to omissions that make the supported hypotheses less valid. Also, DILIsym® v1A lacks a representation of the specific proteins conjugated to reactive metabolites. The theory of selective binding cannot be directly addressed with the model for this reason. Despite the contention that such a small absolute amount of reactive metabolites produced (Fig. 7) may make the selective binding theory less plausible, it is nonetheless an important possibility that was not tested.

Furthermore, one additional hypothesis that is missing from this analysis includes selective depletion of GSH from the mitochondrial GSH pool (Tirmenstein and Nelson, 1990, 1989). A thorough review of the literature has led the authors to put forth the following premise: GSH is transported into hepatocellular mitochondria through a one-way, influx transport system (Fernández-Checa

et al., 1997; Lu, 1999; Lu et al., 1992; Shan et al., 1993; Zhong et al., 2008). This avoids depletion of mitochondrial GSH until the cytosolic GSH is sufficiently depleted to prevent further GSH influx into the mitochondria (and the mitochondrial GSH has been exhausted). Under this premise, GSH depletion within mitochondria for APAP (but not AMAP) would simply be the result of more overall cytosolic GSH depletion in the APAP case, as opposed to evidence for a selective mechanism of mitochondrial GSH depletion by the APAP reactive metabolite when compared to the AMAP reactive metabolite. This is not true for cases of ethanol administration, where the mitochondrial GSH influx system is selectively inhibited (Fernández-Checa et al., 1997; Tavoloni and Berk, 1993). Therefore, mitochondrial GSH depletion was not included as an alternative hypothesis. Technically, this hypothesis would fall under Hypotheses 1 and 2, since production of more reactive metabolites would lead to lower cytosolic GSH concentrations. Lower cytosolic GSH would eventually lead to mitochondrial GSH depletion. While the rationale behind omitting selective GSH depletion as a stand-alone hypothesis was based on literature, additional less understood aspects of this mechanism could be important, and were not considered hererin.

Finally, Hypotheses 1–4 were considered in isolation within this work. While it is the authors' opinions that they are probably not synergistic, making this approach valid, such synergies can rarely be completely ruled out within the biological realm. Still, a lack of representation of potential synergies within this work clearly limits the ability for the conclusions to be interpreted as absolutely definitive.

4.3. Summary and conclusions

A mechanistic, mathematical model (DILIsym® v1A) of drug-induced liver injury predicted that the amount of reactive metabolite produced from APAP or AMAP was the key differentiator for the degree of liver injury observed in mice, with significantly less reactive metabolite produced per mole of AMAP leading to less oxidative stress and injury. Within the simulations, this was equally likely to have occurred from different affinities for the relevant enzymes responsible for drug metabolism or from direct CYP inhibition by the AMAP reactive metabolite. The rate of reactive metabolite binding to proteins and differences in downstream (toxicodynamic) processes were not predicted to be as plausible. This proposed explanation for the divergence in response between the two isomers in mice will hopefully spur additional, confirmatory studies in the future.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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