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Assessment of the biochemical pathways for acetaminophen toxicity: Implications for its carcinogenic hazard potential

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ABSTRACT

In 2019 California's Office of Environmental Health Hazard Assessment (OEHHA) initiated a review of the carcinogenic hazard potential of acetaminophen. In parallel with this review, herein we evaluated the mechanistic data related to the steps and timing of cellular events following therapeutic recommended (≤4 g/day) and higher doses of acetaminophen that may cause hepatotoxicity to evaluate whether these changes indicate that acetaminophen is a carcinogenic hazard. At therapeutic recommended doses, acetaminophen forms limited amounts of N-acetyl-p-benzoquinone-imine (NAPQI) without adverse cellular effects. Following overdoses of acetaminophen, there is potential for more extensive formation of NAPQI and depletion of glutathione, which may result in mitochondrial dysfunction and DNA damage, but only at doses that result in cell death – thus making it implausible for acetaminophen to induce the kind of stable, genetic damage in the nucleus indicative of a genotoxic or carcinogenic hazard in humans. The collective data demonstrate a lack of a plausible mechanism related to carcinogenicity and are consistent with rodent cancer bioassays, epidemiological results reviewed in companion manuscripts in this issue, as well as conclusions of multiple international health authorities.

1. Introduction

Acetaminophen, when used as directed, is widely recognized by health authorities, including the U.S. Food and Drug Administration (FDA), and healthcare professionals around the world as a safe and effective analgesic and antipyretic. It is used by tens of millions of people worldwide for a broad array of applications including over-the-counter uses by consumers (e.g., treatment of headaches) as well as prescription-based patient use (e.g., cancer pain management). Currently, the carcinogenic hazard potential of acetaminophen is being reviewed by the California Office of Environmental Health and Hazard Assessment (OEHHA) and the California Carcinogen Identification Committee (CIC) under California's Proposition 65 (OEHHA, 2019). Their review follows numerous safety reviews conducted by key scientific bodies, such as the

Abbreviations: AIF, apoptosis-inducing factor; CIC, Carcinogen Identification Committee; CYP450, cytochrome P450; DILI, drug induced liver injury; GSH, glutathione; JNK, c-Jun N-terminal kinase; KCC, key characteristics of carcinogens; MAPK, mitogen activated protein kinase; MPTP, mitochondria membrane permeability transition pore; mtDNA, mitochondrial DNA; NAPQI, N-acetyl-p-benzoquinone imine; OEHHA, Office of Environmental Health Hazard Assessment; UGT, UDP-glucuronosyltransferases.

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FDA and the World Health Organization's International Agency for Research on Cancer (IARC), who have all previously concluded that the data do not support acetaminophen as a carcinogenic hazard (FDA, 2010; IARC, 1990).

In carcinogenic hazard assessments, weight of evidence conclusions are often based on epidemiological studies, long-term rodent bioassays (referred to as preclinical studies herein), and genotoxicity data. More recently, a greater emphasis has been placed on the assessment of mechanistic data (e.g., WHO, 2019) to help elucidate the potential biological plausibility of a carcinogenic response in humans. Some entities have utilized the proposed key characteristics of carcinogens (KCC) (Smith et al., 2016) to organize mechanistic data. In its review of acetaminophen, OEHHA utilized the key characteristic concept, citing selected studies in the literature, as well as high-throughput screening data (HTS), in the identification of activity related to oxidative stress and electrophilicity (two key characteristics). These characteristics have been extensively studied for acetaminophen in the context of liver toxicity. While acetaminophen is broadly accepted by health authorities and healthcare professionals around the world to be safe at recommended daily adult doses up to 4 g/day, it is also well-recognized that doses above the recommended doses may lead to hepatotoxicity and that acute overdose and repeated supratherapeutic overdose may lead to death (Daly et al., 2004; Davis et al., 1974, 1976; Portmann et al., 1975).

Given the utility of mechanistic data to help elucidate the potential biological plausibility of a carcinogenic response in humans, the objective of this publication is to assemble mechanistic data on acetaminophen hepatocellular effects at therapeutic and overdose exposures and evaluate if they indicate that acetaminophen presents a carcinogenic hazard. The assessment and conclusions regarding its lack of carcinogenic potential are supported by data evaluated in this publication and evidence presented in companion publications in this issue, including a comprehensive assessment of the preclinical carcinogenicity (Murray et al., 2020), genetic toxicology (Kirkland et al., this issue), and epidemiology data (Weinstein et al., this issue), as well as quantitative systems toxicology modeling of mechanistic responses in humans (Eichenbaum et al., 2020).

2. Approach

In order to achieve the overall goal of evaluating mechanistic data relative to carcinogenic hazard, a team of scientists, several of whom have spent substantial portions of their careers evaluating acetaminophen toxicology and pharmacology, was assembled. These subject matter experts include active researchers as well as private-sector scientists involved with the safety and manufacture of acetaminophen. The multidisciplinary team also included experts in hazard and risk assessment, mode of action, and clinical safety.

The general lack of a specific tumor response in preclinical studies (Murray at el., this issue) or humans (Weinstein et al., this issue) precluded the use of standard mode of action techniques to organize and evaluate mechanistic data; i.e., there was not an adverse outcome (i.e., a specific tumor type) for which to develop or assess a mode of action. As such, a more holistic approach to assess the potential for carcinogenicity was pursued. The approach involved assembling information regarding mechanistic events for liver toxicity and evaluating it in the context of the potential for carcinogenicity. Additionally, other mechanistic aspects, including mechanisms related to the KCCs, were considered. The coherence of the mechanistic findings was then considered in regard to the biological plausibility of a carcinogenic response in humans.

Notably, this manuscript assembles mechanistic data from decades of previous research regarding acetaminophen metabolism and drug induced liver injury (DILI) (Athersuch et al., 2018; McGill and Jaeschke, 2013; Raucy et al., 1989). DILI concepts are well-established, and acetaminophen was one of the agents used to develop and validate models of DILI. Further, because of this generally common understanding, the mechanistic data herein were not formally evaluated via a mode of action framework or similar framework; rather, they are assembled in the context of assessing the potential for carcinogenicity as part of, or subsequent to, liver toxicity.

The approach focuses on mechanistic events in the liver as hepatocytes represent a worst-case scenario for N-acetyl-p-benzoquinone imine (NAPQI) formation due to much higher levels of cytochrome P450 isozyme 2E1 (CYP2E1) and the much higher concentrations of acetaminophen and acetaminophen metabolites. Hepatocytes represent a worst-case scenario for acetaminophen exposure, reactive metabolite formation, and potential for DNA damage because of the amount of the drug and drug metabolites that reach the hepatocytes. Acute kidney injury after an acetaminophen overdose occurs mainly in patients who develop acute liver failure (O'Riordan et al., 2011) but this may be caused by a combination of effects that occur during multiple organ failure. Evidence for a direct toxic effect of an acetaminophen overdose on the kidney was reported in animals with a mechanism similar to what is reported for liver injury (Hoivik et al., 1995; Kennon-McGill and McGill, 2018). However, adverse effects of acetaminophen in organs other than the liver or kidney, even following repeated supratherapeutic ingestions or acute overdose, occur very infrequently, and their association with acetaminophen is not well established (Kennon-McGill and McGill, 2018). Thus, the liver is considered the most susceptible and the clinically most relevant target for toxicity and subsequent potential for carcinogenicity herein.

2.1. Evidence identification

The evidence base related to acetaminophen is voluminous, thus requiring a targeted approach to identify relevant data on mechanistic events associated with acetaminophen toxicity and the implications of these pathways on carcinogenic hazard potential. Primary research originally conducted by the authors was considered along with literature identified from a targeted search to identify mechanistic studies that informed and specifically characterized key events associated with liver toxicity. Literature searches specifically targeted studies in primary mouse hepatocytes or mice *in vivo*, which are considered the most relevant model for human pathophysiology as it relates to acetaminophen exposure (McGill et al., 2012a). Critical signaling events in mice have been confirmed in either primary human hepatocytes (Xie et al., 2014), metabolically competent human HepaRG cells (McGill et al., 2011) or in acetaminophen overdose patients (Davern et al., 2006; McGill et al., 2012a, 2014). Targeted searching was also conducted to identify studies that characterized mechanistic events related to oxidative stress and electrophilic conditions that may lead to protein and DNA adducts, two key characteristics that could potentially be related to - though are not exclusively related to - a carcinogenic mode of action. Of note, genotoxicity (a key characteristic) is briefly addressed herein, though it is comprehensively reviewed by Kirkland et al. (this issue).

The preclinical models that were considered in this review have been shown to have systemic exposure to the acetaminophen metabolites that are formed in humans (Murray et al., 2020) and therefore, studies evaluating dosing of the metabolites alone under conditions that may not be physiologically relevant in humans were not considered. Data evaluating phenacetin (a drug withdrawn from the market in the 1970's) were not considered relevant to acetaminophen carcinogenicity; although the primary metabolite of phenacetin is acetaminophen, it is the N-hydroxylated metabolites of phenacetin and not acetaminophen that are considered to be mutagens and carcinogens, and thus responsible for carcinogenic responses for phenacetin (Hinson, 1983; Hinson et al., 1979, 1980). These metabolites are not produced from acetaminophen.

2.2. High-throughput screening (HTS) data

In vitro HTS data for acetaminophen and 4-aminophenol were extracted from the US Environmental Protection Agency (EPA) Summary Files

(invitrodb_v3.2) (EPA, 2019). No data were available for NAPQI, acetaminophen-glutathione (GSH), acetaminophen-hemisuccinate, acetaminophen-cysteine, or paracetamol-mercapturate. Data from individual chemical-assay endpoint pairs are available within these files for thousands of chemicals and a total of 1473 assay endpoints for 20 variables, including: the activity of each chemical in the assay ("hit call"), the concentration at which activity was observed (AC_{50} values, i.e. the concentration eliciting 50% maximal activity), the cytotoxic concentration range, and data flagged for quality issues. Activity calls are indicated by a hit call of 1 ("active") or 0 ("inactive"). Hit calls were based on cytotoxicity criteria previously defined by the US EPA (Judson et al., 2016). Potential assay interference due to cytotoxicity is characterized by Z-scores for these data, indicating the distances between AC_{50} values and concentrations eliciting cytotoxicity, as described by Judson and colleagues (Judson et al., 2016). A Z-score $\geq$3 was deemed by Judson et al. to be a sufficient cut-off indicator for the occurrence of assay activity at a concentration well below the "cytotoxic signal burst" region. Thus, for the purpose of this assessment, assay endpoints in which the test article had a hit call of 1 and also had Z-score $\geq$3 were considered to be active (see Table A1).

HTS data were evaluated in the context of the Key Characteristics of Carcinogens (KCCs) by mapping individual assay endpoints to the KCC. A comprehensive set of assay endpoints from the invitrodb_v3.2 data release was curated using expert judgement and also by consulting several other published mappings: (1) KCC mappings previously employed by IARC Monographs volume 113 to the present, as listed in supplemental materials to the monographs and by Chiu and associates (Chiu et al., 2018), which are not up-to-date with the current ToxCast data release; (2) a recent publication from California EPA scientists (Iyer et al., 2019) which includes only a subset of assays from 3 vendors and also is not up-to-date with the current ToxCast data release; and (3) endocrine pathways as mapped in the ToxCast Endocrine Disruptor Screening Program in the 21st century (EDSP21) program (specific to KCC #8: Receptor-mediated effects). Assay endpoints were mapped to 9 out of the 10 KCC (no assay endpoints were mapped to KCC # 9, Immortalization, as no relevant assays were available in the ToxCast data).

2.3. Evidence assembly and evaluation

As it is commonly accepted that acetaminophen toxicity is dose-dependent, molecular events were organized and described based on two dose categories: (1) therapeutic up to and including the maximum labeled doses ($\leq$4 g/day); (2) supratherapeutic (above the maximum labeled dose but below the defined acute overdose amount) and acute overdose scenarios (defined as $>$ ~15 g). The mechanistic events discussed herein represent a continuous pathway which is influenced by dose, duration of the dose (e.g., if there is repeated supratherapeutic or chronic high-dose exposure), and inter-individual variability or sensitivity. Because these important pharmacological concepts impact the preciseness of the potential occurrence of each molecular event in an individual, and because biologically these events are linked and are in many cases reversible, it is difficult to characterize which specific events occur or do not occur at doses above therapeutic maximum labeled doses. These aspects are comprehensively evaluated and characterized by Eichenbaum et al., (2020) using the DILIsym Quantitative Systems Toxicology model, for which we refer the reader to throughout the subsequent section.

The mechanistic events associated with liver toxicity were further assessed for the potential to be associated with a carcinogenic response. First, the pathway as a whole was considered in determining if observed toxicity could also be associated with carcinogenicity. Second, specific events and data associated with the KCC, as well as other lines of evidence relating to mechanistic activity, are reviewed and assessed relative to liver toxicity and the potential for carcinogenicity.

3. Mode of action for liver toxicity supports lack of carcinogenicity

The biochemical pathways associated with acetaminophen metabolism and disposition have been studied extensively in preclinical models and in humans (Bajt et al., 2006; Cover et al., 2005; Heard et al., 2011; Hu et al., 1993; Kang et al., 2020; McGill and Jaeschke, 2013; McGill et al., 2013; McGill et al., 2012a,b; McGill et al., 2011; Ramachandran and Jaeschke, 2018, 2020; Xie et al., 2015a; Xie et al., 2014). In this section we provide a step-by-step summary of the relevant mechanistic events that occur at therapeutic recommended doses, as well as the mechanistic events observed at doses above the therapeutic recommended dose range (Fig. 1). These events reflect the collective mechanistic evidence that has been generated *in vitro*, in mouse models, and in humans and are discussed and referenced in detail in the sections that follow. This mode of action assessment supports that acetaminophen at therapeutic recommended doses does not result in adverse effects, but at doses above the therapeutic range subsequent mechanistic events can result in toxicity and cell death – events that preclude and are not associated with carcinogenic effects (Fig. 1).

3.1. Glucuronidation and sulfation

Approximately 60% of acetaminophen is conjugated by UDP-glucuronosyltransferases (UGT), UGT1A6 and UGT1A9, and approximately 30% by sulfotransferases, SULT1A1 and SULT1A3, to non-toxic glucuronide and sulfate metabolites (McGill and Jaeschke, 2013). The degree of sulfation is governed by the availability of inorganic sulfate and the rate of metabolism by sulfotransferases. At therapeutic recommended doses, a relatively small fraction of acetaminophen is oxidized by CYP450 isoenzyme 2A6 (CYP2A6) to non-toxic catechols, which can be detected in the urine along with glucuronide and sulfate metabolites of acetaminophen (Chen et al., 1998).

3.2. GSH depletion, NAPQI formation, and cytosolic protein adducts

At therapeutic recommended doses, approximately 5–10% of acetaminophen is oxidized by CYP2E1 to produce NAPQI (Raucy et al., 1989; Thummel et al., 1993), a highly reactive electrophile (Dahlin et al., 1984; Guengerich and Liebler, 1985) that is primarily conjugated to GSH. GSH-NAPQI is subsequently excreted as cysteine, mercapturate, methylthio and methanesulfonyl adducts of acetaminophen, with increased levels of NAPQI formation with higher doses (McGill and Jaeschke, 2013). Human variability in metabolism of acetaminophen as a result of genetic polymorphisms and nongenetic factors (Court et al., 2017; Critchley et al., 1986; van der Marel et al., 2003) is not expected to significantly impact metabolism nor, consequently, safety at therapeutic recommended doses. Glucuronide and sulfate conjugation are the major metabolic pathways (~85–90%) and small changes in oxidation to NAPQI, if there is genetic variability, are not expected to be clinically significant and often fall within the expected range for therapeutic recommended doses (Eichenbaum et al., 2020; de Morais et al., 1992; Forrest et al., 1979; van Rongen et al., 2016; Zapater et al., 2004).

At therapeutic recommended doses, there is limited formation of NAPQI; thus, the amount that is bound to GSH and to cellular proteins is very limited (McGill et al., 2013). It is so limited that only very low levels of acetaminophen-cysteine protein adducts are detectable in both mice and humans with sensitive mass spectrometric methods (Heard et al., 2011; McGill et al., 2013). Although a number of these protein adducts have been identified (Cohen et al., 1997; Qiu et al., 1998), no protein adduct was identified that could cause cell death (Ramachandran and Jaeschke, 2020). As some examples showed, the enzyme activities of covalently adducted proteins were reduced by 25–40%, which was insufficient to directly trigger cell death (Andringa et al., 2008; Pumford et al., 1997).

As GSH becomes depleted and its regeneration lags behind NAPQI

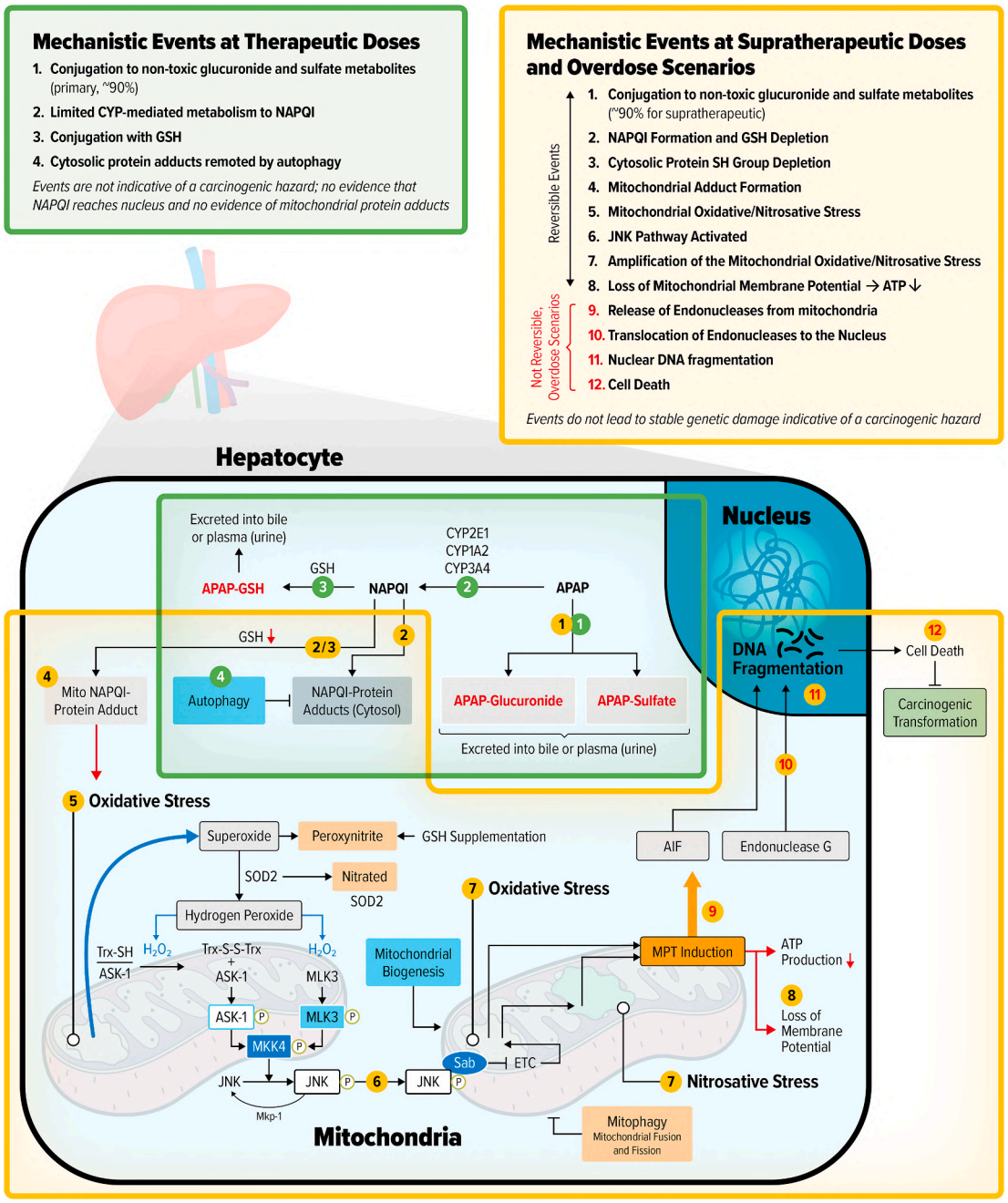

Fig. 1. Schematic diagram showing the mode of action for liver toxicity by dose, recognizing that the pathway represents a continuum of events impacted by dose, duration of exposure, and interindividual variability. At supratherapeutic doses, many events are reversible; only under conditions of repeated, high-dose exposure does the hepatocyte lead to cell death. Such conditions also influence the portion of tissue impacted (e.g., often limited to the centrilobular region following repeated supratherapeutic exposures). Mechanistic events, regardless of dose, do not result in carcinogen transformation.

production, NAPQI binds to hepatic macromolecules initiating a cascade of events outlined in Fig. 1. At supratherapeutic doses, there is some depletion of GSH and cellular proteins that detoxify NAPQI, resulting in mitochondrial adduct formation. In contrast to GSH and general protein binding, adducts formed in mitochondria have negative consequences that can lead to cell death.

With overdose, there can be extensive GSH depletion and a dramatic increase in NAPQI-protein adduct formation (McGill et al., 2013). Following large overdoses, the sulfation pathway becomes saturated, resulting in a greater percentage of acetaminophen being glucuronidated, a non-saturable pathway (Xie et al., 2015a), and oxidized to NAPQI. Simulations of human exposure to various doses were discussed in a companion manuscript (Eichenbaum et al., 2020), which showed the extent of GSH depletion in the supratherapeutic dose range, as well as overdose across a diverse representative patient population. Although CYP1A2 and CYP3A4 show some metabolic activity *in vitro* in human microsomes (Patten et al., 1993; Raucy et al., 1989; Thummel et al., 1993), both enzymes were found to have negligible contribution to hepatotoxicity in human *in vivo* studies compared to other enzymes (Manyike et al., 2000; Sarich et al., 1997).

3.3. *Autophagy or mitochondrial adduct formation*

Because GSH levels in hepatocytes are high (5–10 mM), the very small amount of protein adducts that are formed at therapeutic recommended doses are located predominantly in the cytosol (Hu et al., 2016)

and are subsequently removed by autophagy (Ni et al., 2016). Recent data showed that this mechanism of protein adduct removal is highly effective and prevents adverse effects in hepatocytes even after multiple supratherapeutic doses (Nguyen et al., 2021). Further, the minor adduct formation with therapeutic recommended doses is pathophysiologically irrelevant as neither mitochondrial dysfunction nor DNA damage is detectable (Hu et al., 2016; McGill et al., 2013) and any temporary loss of GSH is rapidly re-replenished (McGill et al., 2013). These data collectively demonstrate that although a small amount of NAPQI is formed, there is no biological consequence related to a carcinogenic response following exposure to acetaminophen at therapeutic recommended doses due to adduct removal by autophagy. Studies with autophagy-deficient mice demonstrated that only long-term, severe cellular stress with extensive cell death and compensatory regeneration will eventually lead to inflammation, fibrosis and liver cancer over the lifetime of the animals (Ni et al., 2014).

Following overdose, mitochondrial protein adduct formation and toxicity can occur. Supratherapeutic doses (in mice) can lead to limited protein adducts in mitochondria (Hu et al., 2016). This is regarded as the principle mode of cellular toxicity, which subsequently drives cell death (Qiu et al., 2001; Tirmenstein and Nelson, 1989; Xie et al., 2015b).

This conclusion is based on mechanistic studies demonstrating no difference in overall protein binding for N-acetyl-p-aminophenol (acetaminophen) compared to its regioisomer N-acetyl-m-aminophenol (AMAP). Only acetaminophen caused protein adducts in mitochondria and induced liver injury (Qui et al., 2001; Tirmenstein and Nelson, 1989). These results were confirmed for mouse hepatocytes but not in human hepatocytes, where AMAP caused mitochondrial adducts and cell death (Xie et al., 2015b). In addition, dose-response studies with acetaminophen *in vivo* showed that mitochondrial dysfunction is correlated with mitochondrial protein adduct formation (Hu et al., 2016).

3.4. Mitochondrial oxidative and nitrosative stress

Following supratherapeutic doses and acute overdose, the mechanistic pathway continues to the generation of oxidative and nitrosative stress in the mitochondria in capacities dependent on dose, duration, and human sensitivity. Peroxynitrite, which is a potent oxidant and nitrating species (Radi, 2004), is the ultimate oxidant responsible for the liver injury following acetaminophen acute overdose (Du et al., 2016; Knight et al., 2002) and potentially occurs also in a limited portion of hepatocytes under some supratherapeutic dosing conditions. Importantly, it is confined to the mitochondria as indicated by selective mitochondrial DNA damage and nitrotyrosine protein adducts inside the mitochondria but not in any other compartment of the cell, including the nucleus (Cover et al., 2005). The limitation of the oxidative/nitrosative processes within the mitochondria is also documented by the selective increase of GSH disulfide (GSSG) concentrations within the mitochondria (Jaeschke, 1990; Knight et al., 2001) and the use of MitoSox, which is a superoxide indicator that accumulates selectively inside the mitochondria (Yan et al., 2010). The pathophysiological importance of the mitochondrial superoxide formation is also demonstrated by the dramatically enhanced peroxynitrite formation and hepatotoxicity in Manganese Superoxide Dismutase (MnSOD)-deficient mice (Ramachandran et al., 2011a) and the protective effect of the selective mitochondria-targeted SOD mimetic Mito-Tempo (Du et al., 2017a). The critical role of MnSOD is to prevent the reaction of nitric oxide with superoxide to form peroxynitrite (Fukai and Ushio-Fukai, 2011). The enhanced dismutation of superoxide to hydrogen peroxide and oxygen allows the detoxification of hydrogen peroxide by glutathione peroxidase. However, it theoretically enhances the risk of a Fenton reaction and lipid peroxidation. The fact that there is only very limited evidence for lipid peroxidation after acetaminophen overdose, and that the lipid-soluble antioxidant vitamin E does not protect against such (Knight et al., 2003), further supports the hypothesis that peroxynitrite, which is limited to the mitochondrial space, is the critical oxidant in the pathophysiology (Du et al., 2016).

3.5. Activation of the JNK pathway

A clear consequence of the mitochondrial NAPQI-protein adducts is a modest oxidant stress, which is insufficient to cause relevant mitochondrial dysfunction but instead activates redox-sensitive mitogen activated protein kinases (MAPK) such as apoptosis signal-regulating kinase 1 (ASK-1) (Nakagawa et al., 2008; Xie et al., 2015c) and mixed lineage kinase 3 (MLK3) (Sharma et al., 2012). The activation of ASK-1 and MLK3 triggers the phosphorylation of mitogen-activated protein kinase 4 (MKK4, Zhang et al., 2017), which results ultimately in the phosphorylation of c-jun N-terminal kinase (JNK) in the cytosol and the translocation of phosphorylated JNK (P-JNK) to the mitochondria (Hanawa et al., 2008). The binding of P-JNK to the anchor protein Sab on the outer mitochondrial membrane and inactivation of intramitochondrial Src kinases results in a further interruption of the mitochondrial electron transport chain and enhanced electron leakage with formation of superoxide in the mitochondrial matrix (Win et al., 2016). The occurrence of these events is limited at supratherapeutic doses relative to the acute overdose scenario (Hu et al., 2016).

3.6. Amplification of mitochondrial oxidative/nitrosative stress

The amplified oxidant stress inside the mitochondria triggered by the JNK pathway leads to the enhanced formation of peroxynitrite (Saito et al., 2010a). Hence, JNK inhibitors effectively prevent acetaminophen-induced oxidant stress and peroxynitrite formation, ultimately preventing liver injury (Akakpo et al., 2019; Saito et al., 2010a). The initial JNK activation, which occurs as early as 1 h after acetaminophen overdose (Xie et al., 2015c), is triggered by the early mitochondrial oxidant stress caused by mitochondrial protein adduct formation (Hanawa et al., 2008). However, continuous JNK activation requires an auto-amplification loop where the amplified oxidant stress in the mitochondria sustains cytosolic JNK activation by release of oxidants such as hydrogen peroxide. Evidence for this mechanism comes from the fact that delayed treatment, i.e. after the initial JNK activation, with inhibitors of ASK1 (Xie et al., 2015c) or JNK (Akakpo et al., 2019) effectively blocks prolonged JNK activation, late oxidant stress and injury. A similar effect is observed when animals are treated with N-acetylcysteine at 1.5 h after acetaminophen (Xie et al., 2015c). N-acetylcysteine treatment promotes the synthesis of hepatic GSH, which directly scavenges peroxynitrite (Knight et al., 2002; Saito et al., 2010b) and supports the detoxification of hydrogen peroxide (Saito et al., 2010b), i.e., interrupts the amplification cycle by removal of the diffusible hydrogen peroxide.

3.7. Loss of mitochondrial membrane potential

Acetaminophen-induced cell death involves the opening of the mitochondrial membrane permeability transition pore (MPTP) resulting in the collapse of the membrane potential and cessation of ATP synthesis *in vitro* (Kon et al., 2004) and *in vivo* (Masubuchi et al., 2005). The MPTP opening can be regulated by cyclophilin D or can be unregulated depending on the severity of the stress (Kim et al., 2003). Consistent with this hypothesis, it was shown that a cyclophilin D-deficient mouse is completely protected against a moderately toxic overdose of acetaminophen ((Ramachandran et al., 2011b) but not against a severely toxic overdose (LoGuidice and Boelsterli, 2011). It is well known that oxidants can trigger the MPTP opening in mitochondria (Kim et al., 2003). The fact that scavengers of reactive oxygen can effectively prevent acetaminophen-induced cell death supports the hypothesis that oxidants are the driving force of the MPTP opening (Saito et al., 2010b). In addition, lysosomal iron translocating to the mitochondria is involved in the MPTP opening (Kon et al., 2010). If enough mitochondria within a hepatocyte are affected, the cell undergoes necrosis (Gujral et al., 2002).

However, damaged mitochondria can also be removed by mitophagy (Ni et al., 2012) and then replaced by mitochondrial biogenesis (Du et al., 2017b) resulting in the survival of cells especially on the periphery of the necrotic area (Ni et al., 2013).

3.8. Release of endonucleases from mitochondria

One of the consequences of the MPTP opening is mitochondrial matrix swelling, which leads to rupture of the outer membrane (Kim et al., 2003). In this case, intermembrane proteins such as cytochrome *c*, Smac/Diablo, endonuclease G, and apoptosis-inducing factor (AIF) are released into the cytosol (Bajt et al., 2006, 2008). An increased permeability of the outer mitochondrial membrane with release of these proteins can also be caused by translocation of the apoptosis regulator BCL-2-associated X (BAX) protein from the cytosol to the mitochondria, where BAX can combine with other proteins such as BCL-2 homologous antagonist/killer (BAK), BCL2-associated agonist of cell death (BAD), and BH3 Interacting Domain Death Agonist (BID) in the outer membrane (Bajt et al., 2008). Mitochondrial BAX translocation occurs within 1–2 h after an acetaminophen overdose and release of these intermembrane proteins can be observed several hours later (Bajt et al., 2008; El-Hassan et al., 2003). Elimination of the BAX pore formation in BAX-deficient mice prevents this early release of the intermembrane proteins and reduces the early injury, however, several hours later there was no longer protection in these animals (Bajt et al., 2008). The reason for the protection only being temporary was that BAX deficiency did not prevent the mitochondrial oxidant stress and thus did not prevent the MPTP formation, matrix swelling, late release of the intermembrane proteins and eventual cell death (Bajt et al., 2008). Thus, release of mitochondrial intermembrane proteins due to the formation of an initial BAX pore and later rupture of the outer mitochondrial membrane caused by MPTP-induced matrix swelling is a critical step in the intracellular signaling events leading to cell death after an acetaminophen overdose.

3.9. Translocation to the nucleus

Some of the mitochondrial intermembrane proteins such as cytochrome *c* and Smac/DIABLO are considered pro-apoptotic proteins that lead to promotion of apoptotic signaling (Jaeschke et al., 2018). Although these proteins are clearly released from mitochondria into the cytosol during acetaminophen hepatotoxicity (Bajt et al., 2006, 2008; El-Hassan et al., 2003), there is no evidence that they promote caspase activation and apoptotic cell death under these conditions (Jaeschke et al., 2018). In contrast, AIF and endonuclease G, when released from the mitochondrial intermembrane space, translocate to the nucleus and cause DNA fragmentation after acetaminophen overdose (Bajt et al., 2006). Both AIF and endonuclease G are known to be involved in nuclear DNA degradation during non-caspase-dependent cell death pathways (Daugas et al., 2000; Low, 2003). Preventing mitochondrial dysfunction after acetaminophen overdose inhibits nuclear translocation of AIF and endonuclease G (Bajt et al., 2006; Cover et al., 2005).

3.10. Nuclear DNA fragmentation

Karyolysis is a hallmark of necrotic cell death (Gujral et al., 2002). Nuclear DNA fragmentation has been documented by DNA ladder (Ray et al., 1990; Cover et al., 2005), the TUNEL assay (Lawson et al., 1999; Cover et al., 2005), anti-histone ELISA (Lawson et al., 1999; McGill et al., 2012b) and expression of phosphorylated H2AX, a marker of DNA double-strand breaks (Borude et al., 2018), during acetaminophen-induced liver injury. Although there is some overlap in DNA fragmentation between apoptosis and acetaminophen-induced necrosis, there are also clear distinctions in terms of the TUNEL staining (Jaeschke et al., 2018) and in the size of the DNA fragments (Jahr et al., 2001). The reason is that nuclear DNA fragmentation during apoptosis is caused by caspase-activated DNases (Nagata et al., 2003), while acetaminophen-induced hepatotoxicity involves mitochondria-derived AIF and endonuclease G (Bajt et al., 2006; Cover et al., 2005).

3.11. Cell death

The release of cytochrome *c* from the mitochondria could theoretically lead to activation of caspase-9 and trigger apoptosis; however, there is no evidence for relevant activation of any caspases (Lawson et al., 1999; Gujral et al., 2002) or morphological characteristics of apoptosis after acetaminophen overdose (Jaeschke et al., 2018). Because the mitochondrial apoptosis pathway involving cytochrome c-mediated apoptosome activation also requires ATP, a potential reason for the lack of apoptosis might be declining ATP levels, which has been observed after acetaminophen overdose (Jaeschke, 1990). Although it is hypothesized that the capacity to maintain cellular ATP levels is necessary for apoptotic cell death (Jaeschke and Lemasters, 2003; Malhi et al., 2006), necrotic cell death in acetaminophen toxicity appears to be independent of hepatic ATP levels (Williams et al., 2011).

Endonuclease G and AIF translocate to the nucleus and cause DNA fragmentation (Bajt et al., 2006). Importantly, the main consequence of this mechanism of DNA fragmentation is karyolysis, which is not repairable. This means that under conditions in which biologically significant DNA fragmentation occurs, the cell passes the “point of no return” and progresses to necrosis, which makes it impossible that such a cell survives or proliferates. That is, following supratherapeutic doses or upon overdose, acetaminophen causes DNA damage only at exposures that result in cell death, making it implausible for acetaminophen to induce the kind of stable genetic damage indicative of a genotoxic or carcinogenic hazard in humans.

3.12. Pathway-based assembly demonstrate lack of carcinogenic potential

There is clear evidence showing that at therapeutic doses NAPQI does not reach the nucleus in the presence of high GSH levels and the presence of cytosolic proteins with free SH groups *in vivo* and that there is no formation of mitochondrial protein adducts, JNK activation, mitochondrial oxidant stress or dysfunction, and no evidence of DNA damage or cell death (Hu et al., 2016; McGill et al., 2013). Review of mechanistic information related to acetaminophen confirms that DNA damage in relevant, well-controlled test systems only occurred at doses/concentrations that result in cell death, which preclude it from having potential to cause any carcinogenic effects via a genotoxic mechanism (Kirkland et al., this issue).

Careful examination of the mode of action in the liver under supratherapeutic and overdose conditions demonstrates that the potential for oxidative stress and DNA effects resulting from formation of a reactive metabolite occurs in a well-characterized sequence that results in cellular toxicity before it can become a carcinogenic hazard. At supratherapeutic, non-toxic doses in the mouse (100–150 mg/kg), there is depletion of GSH due to NAPQI detoxification, mitochondrial protein adduct formation, initial oxidative/nitrosative stress, temporary JNK activation and MPTP opening (Hu et al., 2016; Dunn et al., 2020). Because the MPTP is reversible, mitochondria re-polarize, no DNA damage occurs, and the cell recovers (Hu et al., 2016; Dunn et al., 2020). Under these conditions only few single cells in the centrilobular area may die. In a review of the genotoxic mode of action of acetaminophen, the same mechanism of action (NAPQI-mediated oxidative stress) was identified for genotoxicity following overdose (Bergman et al., 1996).

In acute overdose, in which GSH is depleted, NAPQI binds to mitochondrial proteins resulting in mitochondrial dysfunction; mitochondrial-dependent nuclear DNA fragmentation occurs, leading to cell death to regions beyond the centrilobular area. This acute cell death prevents any effects on nuclear DNA that could drive carcinogenesis. The lack of carcinogenicity potential - at any dose - is consistent with the overall lack of carcinogenic effects in rodent cancer bioassays (Murray et al., 2020) and epidemiological studies (Weinstein et al., this issue).

4. Additional mechanistic evidence demonstrates a lack of carcinogenic potential

4.1. Acetaminophen DNA adducts have not been structurally identified in vivo at any dose level

Nuclear DNA adduct formation in humans or animals *in vivo* have not been identified in studies which use biologically relevant test systems (e.g., reliable specific labeling techniques as discussed by Kirkland et al., this issue). The limited evidence that acetaminophen has the potential to form DNA adducts comes from *in vitro* studies (Dybing et al., 1984; Hongslo et al., 1994; Rogers et al., 1997), and a mouse *in vivo* study (Rogers et al., 1997). These studies show a dose-related increase in the extent of DNA binding of a tritiated label at therapeutic and supratherapeutic concentrations and doses. However, comparisons of the relative binding of the tritiated label to the DNA, chromatin, and nucleus demonstrate that >90% of the label was on the chromatin and nucleus, and not on the DNA; as such, these data demonstrate that the label is binding to histones and other proteins rather than the DNA (Rogers et al., 1997). However, the authors only measured radioactivity in the DNA and assumed this reflected binding of acetaminophen to DNA. In addition, the tritium label can be readily displaced and enter the general cellular pool such that it gets incorporated into normal bases and thence into DNA (metabolic incorporation) and does not represent acetaminophen. There was also no clear observation of adducts in liver DNA using the ^{32}P-postlabeling technique (see Kirkland et al., this issue). No DNA adducts were identified or characterized, and, as indicated above, the presence of radioactivity from the tritium in DNA does not prove that adducts have been formed (Phillips et al., 2000). As stated by Bergman et al. (1996) "Definite proof that the covalent binding of radioactivity from ^{3}H-labeled paracetamol to DNA represents the formation of true DNA adducts would require chemical structural analysis". In contrast to cytosolic and mitochondrial protein adducts, there is no scientifically valid evidence for adduct formation on nuclear DNA after therapeutic or toxic doses of acetaminophen *in vivo*.

4.2. Implications of toxicity pathways for carcinogenicity hazard potential in sub-populations: patients with purported susceptibility to liver injury

A recent critical review of the literature concluded that no patient group is unequivocally at elevated risk of acetaminophen-induced liver toxicity (Caparrotta et al., 2018). This review included clinical studies addressing genetic and nongenetic factors that may alter acetaminophen metabolism, such as enzyme polymorphism, race/ethnicity, Gilbert's syndrome, liver disease, age, obesity, nutritional state, alcohol use, and potential drug interactions. Other reviews of clinical data addressed acetaminophen use by liver-impaired patients (Hayward et al., 2016) and by populations in which low GSH has been observed (Lauterburg, 2002), concluding that there was no evidence for greater risk. Another review highlighted the impact of purported drug, alcohol, and fasting interactions with acetaminophen that were based on data from animal studies, *in vitro* tests, and case reports (Rumack, 2004).

Of note, Eichenbaum, et al. (this issue) modeled mechanistic events in patient sub-populations and in overdose patients using a Quantitative Systems Toxicology Platform called DILIsym that has been developed and validated using acetaminophen. The results of these simulations characterize interindividual variability, including sensitive populations.

4.3. Consideration of potential effects of acetaminophen on DNA repair or genomic stability

Several studies show a potential inhibitory effect of acetaminophen on reparative and replicative DNA synthesis *in vitro* and *in vivo* using a thymidine uptake assay (Hongslo et al., 1994; Lister and McLean, 1997). This is relevant to KCC #3: "alter DNA repair or cause genomic instability." However, the reduced thymidine uptake is transient, reversing *in vivo* within 2–4 h (Hongslo et al., 1994; Lister and McLean, 1997). Additionally, it has been proposed that, by analogy with hydroxyurea, the thymidine uptake assay results may be related to the inhibition of ribonucleotide reductase, which may also be responsible for genotoxic effects seen with acetaminophen at high doses (Bergman et al., 1996; Thybaud et al., 2007).

Another mechanism that has been proposed for acetaminophen effects on DNA is through inhibition of ribonucleotide reductase *in vitro*. There have been several studies that reported inhibition of ribonucleotide reductase in *in vitro* models (Hongslo et al., 1990). However, these models have unclear relevance to humans or animals (e.g., mouse mammary immortalized tumor cell line with mutations introduced and/or in which the conditions tested are implausible in humans; see further discussion in Kirkland et al., this issue). Additionally, a literature search yielded no evidence demonstrating that acetaminophen inhibits ribonucleotide reductase or disrupts the ribonucleotide pool *in vivo*.

There are other potential mechanisms besides direct inhibition of ribonucleotide reductase that could cause the thymidine uptake effects observed in these *in vitro* model systems (Hongslo et al., 1990). One potential alternative mechanism for the effects observed on DNA repair could be acetaminophen-induced mitochondrial permeability transition *in vitro* that occurs in two phases (GSH depletion/covalent binding followed by mitochondrial dysfunction). Mitochondrial dysfunction can inhibit ribonucleotide reductase function in the cytosol (Desler et al., 2007, 2010) and potentially cause the effects on DNA replication that were observed *in vitro*. As discussed herein, the changes to mitochondrial function and GSH depletion only occur at supratherapeutic doses.

Elevation of intracellular Ca^{2+} by high doses of acetaminophen represents another potential cause of genomic instability, as it can result in DNA fragmentation. This has been attributed to formation of acetaminophen protein adducts on the plasma membrane enzyme Ca^{2+} ATPase after hepatotoxic doses of acetaminophen (Tsokos-Kuhn et al., 1988). High concentrations/doses of acetaminophen induce a marked increase in intranuclear Ca^{2+}, resulting in endonuclease activation and DNA fragmentation (Ray et al., 1990; Shen et al., 1991). These effects may be a consequence of cytotoxicity, and, as discussed herein, damaged cells would not survive. Thus, since increased Ca^{2+} levels are associated only with cytotoxicity, the resultant clastogenicity will exhibit a threshold. Human plasma Ca^{2+} concentrations under normal acetaminophen usage are much lower than cytotoxic concentrations, so that under normal usage acetaminophen would not induce genotoxicity associated with increased Ca^{2+} levels. Further, DNA fragmentation that occurs strictly under cytotoxic conditions is likely not related to carcinogenic mechanisms, as would be the case for DNA damage in replicating cells.

4.4. High throughput screening (HTS) data do not support a carcinogenic response to acetaminophen

Acetaminophen was tested for activity in 665 HTS assays, 309 of which measure endpoints relevant to one or more of the KCCs. Acetaminophen was inactive in all but four of the 655 assays in which it was tested, with three of the four active assays mapped to a KCC. These three active KCC-relevant assays were tested in human cell models, as were the majority of the HTS assay data for acetaminophen (cell lines or cell-free). The three active assay endpoints were related to epigenetic alterations, progesterone receptor binding, or androgen receptor antagonism. However, these assays were all flagged for data quality issues according to the ToxCast summary files, bringing their reliability into question (Table A1). Further, while acetaminophen was active in a single assay for androgen receptor antagonism, three other assays for androgen receptor antagonism were inactive, and the overall consensus for androgen receptor antagonism is inactive according to computational modeling of the HTS data (ToxCast pathway model for androgen receptor: https://comptox.epa.gov/dashboard/dsstoxdb/results?search

=DTXSID2020006#bioactivity-toxcast-models)

Thus, acetaminophen at concentrations up to 200 μM was generally considered inactive in HTS assay endpoints related to the KCCs.

4-Aminophenol was active in a total of six ToxCast assay endpoints, five of which were mapped to a KCC. 4-Aminophenol was inactive in another 122 assay endpoints related to one or more KCC. Three assays reporting activity were related to DNA repair. These assays measure cellular ATP content as a determinant of cell viability using luciferase-coupled ATP quantitation. The DT40_100 and DT40_657 are chicken lymphoblast cells that have deletions in genes involved in repairing chromosomal breaks (DT40_100: Ku70 and Rad54 gene deletions (−/−); DT4_657: Rev3 gene deletions (−/−)), which can be compared to response in wild type DT40 cells under the same treatment conditions. 4-Aminophenol was active in all of the DT40 assays regardless of DNA repair gene function, indicating that 4-aminophenol is cytotoxic but does not necessarily indicate the ability of the compound to induce structural DNA damage in these assays. There was also one active assay mapped to KCC #10 Cell proliferation, death and nutrient supply (TOX21_MMP_ratio_down). In such, membrane potential is detected in human liver HepG2 cells with fluorescence intensity signals by homogenous mitochondrial membrane potential assay technology. Because activity in this assay represents loss of signal, this endpoint informs the ability of 4-aminophenol to cause cell death. Thus, 4-aminophenol was active in cell models designed to measure chromosomal damaging potential; however, the activity appears to be related to cytotoxicity as opposed to genotoxicity. Finally, 4-aminophenol was active in an assay for thyroid receptor antagonism in a rat pituitary gland cell line. 4-Aminophenol was also cytotoxic in this assay, but activity for thyroid receptor antagonism occurred at a slightly lower AC_{50} value than significant cytotoxicity (Table A1).

Collectively, these data are consistent with the pathways described. And when viewed in context of the preclinical findings (Murray et al., 2020), which would account for many of the limitations in interpretation of *in vitro* assays, the limited amount of activity observed in the HTS data also considered to have limited biological significance.

4.4.1. Mechanistic data demonstrating potential beneficial (protective) effects

Current evidence regarding acetaminophen's analgesic mechanism of action has been proposed to involve: (1) the inhibition of cellular prostaglandin production (Anderson, 2008; Graham and Scott, 2005); (2) increased cannabinoid receptor activity (Anderson, 2008; Hogestatt et al., 2005; Sharma and Mehta, 2014); (3) the inhibition of nitric oxide production (Sharma and Mehta, 2014); and (4) anti-oxidant/peroxynitrite scavenging properties (Dou et al., 2017; Schildknecht et al., 2008).

Studies conducted *in vitro* have shown that acetaminophen at therapeutically relevant concentrations acts as a cellular peroxynitrite scavenger (Dou et al., 2017; Schildknecht et al., 2008), suggesting it may have a protective effect against oxidative stress, and therefore could be protective against potential carcinogenesis. In tissue and *in vivo* animal studies acetaminophen has also been shown to reduce reactive oxygen species/reactive nitrogen species in multiple tissue types (Blough and Wu, 2011). Acetaminophen has been shown to have antioxidant effects in the rat liver (DuBois et al., 1983) and acetaminophen (20 mg/kg) has also been shown to decrease liver mitochondrial H_2O_2 formation in both control and high fat diet fed mice (Shertzer et al., 2008). Acetaminophen has also been shown to have protective effects at low doses on renal injury in a Zucker rat obesity model for renal injury; the effects appear to be mediated, at least in part, through attenuation of endoplasmic reticulum stress (Wang et al., 2014).

There are also several reports of anti-proliferative and anti-tumor effects of acetaminophen in different nonclinical models. Bush et al. (2016) reported that acetaminophen "exhibited antiproliferative activity against all tested ovarian cancer cell lines" *in vitro* and described potential pathways driving its antiproliferative effects. Takehara et al. (2011) demonstrated that a breast cancer stem cell line treated with acetaminophen *in vitro* resulted in the loss of their tumorigenic ability in nude mice. Furthermore, administration of acetaminophen inhibited the growth of tumor xenografts of MDA-MB-231 cells in both the presence and absence of simultaneous administration of doxorubicin, a typical anti-tumor drug for breast cancer.

4.4.2. Relevance of key characteristics of carcinogens (KCCs)

Acetaminophen has some properties related to what have been described as "Key Characteristics of Carcinogens": i.e., formation of reactive metabolites and the potential to cause oxidative stress under toxic conditions that can lead to DNA damage (OEHHA, 2019). KCCs are not themselves predictive of carcinogenesis (Smith et al., 2016). The KCCs can be useful for identification and prioritization (Bus, 2017; Goodman and Lynch, 2017; Iyer et al., 2019; Wikoff et al., 2019), however, activity or association with "Key Characteristics" does not support a conclusion that a compound has the potential to cause cancer (Becker et al., 2017; Trosko, 2017). Endogenous biomolecules such as NO, $O_2^{\cdot-}$, H_2O_2, and OCl^- are constantly causing biologically significant covalent modifications to DNA in normal cells on a physiologic basis and the body has mechanisms in place to repair the damage (Ames and Gold, 1991). It has been estimated that the total number of all types of oxidative DNA adducts per cell per day formed from endogenous biomolecules due to oxidative stress is on the order of about 10^4 (Ames and Gold, 1991; Marnett and Burcham, 1993). There is also evidence that in cells under normal physiological conditions there is substantial DNA damage from spontaneous depurination, strand breaks, deamination, and endogenous adduction (Marnett, 2000; Marnett and Burcham, 1993; Marnett et al., 2003), which may be increased by the release of endogenous biomolecules from white blood cells as part of responses to bacterial infection or inflammation (Ames and Gold, 1991). These examples highlight the critical nature of going beyond organization of data into characteristics, but to actually examine the data in context of dose, species, specific cancer types, and mode of action considerations – similar to what was done in this manuscript, in which the observed and well-characterized activity which may be related to the key characteristics are associated with toxicity rather than carcinogenicity.

In the case of acetaminophen, the pathways of oxidative stress and resulting DNA effects that could potentially drive a tumorigenic response only occur at doses where there is cell death and no chance for the DNA damage to be propagated to daughter cells. In addition, acetaminophen-induced DNA damage involves a fundamentally different mechanism that is caused by endonuclease-mediated DNA fragmentation that non-reversibly degrades the nucleus of a dying cell and does not involve adduct formation by direct interaction of the chemical with the DNA (Bajt et al., 2006; Cover et al., 2005). This is not repairable and is fundamentally different from a potential DNA modification that could give rise to a cancer cell. These pathways are consistent with oxidative stress effects that have been observed in humans (Jetten et al., 2012; Kozer et al., 2003; Nuttall et al., 2003).

4.5. Clinical evidence support that acetaminophen-induced cellular toxicity does not result in carcinogenicity

The histology of liver injury due to significant overdoses of acetaminophen in rodents and humans is well described (Baeg et al., 1988; Hamlyn et al., 1977). In humans, key histologic features vary from limited centrilobular necrosis to confluent necrosis in more serious cases. In those subjects who recover from this injury, complete recovery characterized by normalization of liver function and restoration of hepatic architecture is the typical pattern (Clark et al., 1973; Hamlyn et al., 1977; Lesna et al., 1976; Portmann et al., 1974). For example, a single case report (Baeg et al., 1988) and case series from the 1970s have evaluated histology of overdose subjects both in the acute phase and three months post overdose (Clark et al., 1973; Hamlyn et al., 1977; Lesna et al., 1976; Portmann et al., 1974). In the case series, in most patients at follow up biopsy, necrotic zones were found to have been

completely reconstituted with restoration of hepatic architecture. In a very small fraction of biopsied patients, minor abnormalities and fibrosis were seen. Fibrosis, if it occurred, was generally mild, and was seen only in very severe cases of injury. In many patients, serial biopsies demonstrated resolution of fibrosis over several months. Of further note, in these cases involving fibrosis, a number of potential causative factors were potentially involved, though there was an absence of pertinent medical information to fully characterize other potential etiologic factors (alcohol, viral, NASH etc.). Collectively, such clinical data generally support the differentiation of toxicity and the potential for carcinogenicity – that clinical case reports on liver injury from acetaminophen overdose when it does not require a liver transplant demonstrate that the injury resolves fully.

5. Conclusion

The sequence of intracellular mechanistic events that occurs following exposure to acetaminophen are well-characterized and are differentiated from mechanistic activity that would result in carcinogenicity at any dose or duration of exposure. When these mechanistic data for liver toxicity are combined with the weight of evidence from the available preclinical carcinogenicity (Murray et al., 2020) and epidemiology studies (Weinstein et al., this issue), the evidence support a lack of carcinogenic hazard.

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Declaration of competing interest

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Appendix A. Supplementary data

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References

Akakpo, J.Y., Ramachandran, A., Duan, L., Schaich, M.A., Jaeschke, M.W., Freudenthal, B.D., Ding, W.X., Rumack, B.H., Jaeschke, H., 2019. Delayed treatment with 4-methylpyrazole protects against acetaminophen hepatotoxicity in mice by inhibition of c-jun n-terminal kinase. Toxicol Sci 1 170 (1), 57–68.

Ames, B.N., Gold, L.S., 1991. Endogenous mutagens and the causes of aging and cancer. Mutat. Res. 250, 3–16.

Anderson, B.J., 2008. Paracetamol (Acetaminophen): mechanisms of action. Paediatr. Anaesth. 18, 915–921.

Andringa, K.K., Bajt, M.L., Jaeschke, H., Bailey, S.M., 2008. Mitochondrial protein thiol modifications in acetaminophen hepatotoxicity: effect on HMG-CoA synthase. Toxicol Lett 1 177 (3), 188–197.

Athersuch, T.J., Antoine, D.J., Boobis, A.R., Coen, M., Daly, A.K., Possamai, L., Nicholson, J.K., Wilson, I.D., 2018. Paracetamol metabolism, hepatotoxicity, biomarkers and therapeutic interventions: a perspective. Toxicol Res (Camb) 7, 347–357.

Baeg, N.J., Bodenheimer Jr., H.C., Burchard, K., 1988. Long-term sequellae of acetaminophen-associated fulminant hepatic failure: relevance of early histology. Am. J. Gastroenterol. 83, 569–571.

Bajt, M.L., Farhood, A., Lemasters, J.J., Jaeschke, H., 2008. Mitochondrial bax translocation accelerates DNA fragmentation and cell necrosis in a murine model of acetaminophen hepatotoxicity. J. Pharmacol. Exp. Therapeut. 324, 8–14.

Bajt, M.L., Cover, C., Lemasters, J.J., Jaeschke, H., 2006. Nuclear translocation of endonuclease G and apoptosis-inducing factor during acetaminophen-induced liver cell injury. Toxicol. Sci. 94, 217–225.

Becker, R.A., Dreier, D.A., Manibusan, M.K., Cox, L.A.T., Simon, T.W., Bus, J.S., 2017. How well can carcinogenicity be predicted by high throughput "characteristics of carcinogens" mechanistic data? Regul. Toxicol. Pharmacol. 90, 185–196.

Bergman, K., Muller, L., Teigen, S.W., 1996. Series: current issues in mutagenesis and carcinogenesis, No. 65. The genotoxicity and carcinogenicity of paracetamol: a regulatory (re)view. Mutat. Res. 349, 263–288.
Blough, E.R., Wu, M., 2011. Acetaminophen: beyond pain and fever-relieving. Front. Pharmacol. 2, 72.
Borude, P., Bhushan, B., Apte, U., 2018. DNA damage response regulates initiation of liver regeneration following acetaminophen overdose. Gene Expr. 18 (2), 115–123.
Bus, J.S., 2017. IARC use of oxidative stress as key mode of action characteristic for facilitating cancer classification: glyphosate case example illustrating a lack of robustness in interpretative implementation. Regul. Toxicol. Pharmacol. 86, 157–166.
Bush, S.H., Tollin, S., Marchion, D.C., Xiong, Y., Abbasi, F., Ramirez, I.J., Zgheib, N.B., Boac, B., Judson, P.L., Chon, H.S., Wenham, R.M., Apte, S.M., Cubitt, C.L., Berglund, A.E., Havrilesky, L.J., Lancaster, J.M., 2016. Sensitivity of ovarian cancer cells to acetaminophen reveals biological pathways that affect patient survival. Mol Clin Oncol 4, 399–404.
Caparrotta, T.M., Antoine, D.J., Dear, J.W., 2018. Are some people at increased risk of paracetamol-induced liver injury? A critical review of the literature. Eur. J. Clin. Pharmacol. 74, 147–160.
Chen, W., Koenigs, L.L., Thompson, S.J., Peter, R.M., Rettie, A.E., Trager, W.F., Nelson, S. D., 1998. Oxidation of acetaminophen to its toxic quinone imine and nontoxic catechol metabolites by baculovirus-expressed and purified human cytochromes P450 2E1 and 2A6. Chem. Res. Toxicol. 11 (4), 295–301.
Chiu, W.A., Guyton, K.Z., Martin, M.T., Reif, D.M., Rusyn, I., 2018. Use of high-throughput in vitro toxicity screening data in cancer hazard evaluations by IARC Monograph Working Groups. ALTEX 35, 51–64.
Clark, R., Borirakchanyavat, V., Davidson, A.R., Thompson, R.P., Widdop, B., Goulding, R., Williams, R., 1973. Hepatic damage and death from overdose of paracetamol. Lancet 1, 66–70.
Cohen, S.D., Pumford, N.R., Khairallah, E.A., Boekelheide, K., Pohl, L.R., Amouzadeh, H. R., Hinson, J.A., 1997. Selective protein covalent binding and target organ toxicity. Toxicol. Appl. Pharmacol. 143, 1–12.
Court, M.H., Zhu, Z., Masse, G., Duan, S.X., James, L.P., Harmatz, J.S., Greenblatt, D.J., 2017. Race, gender, and genetic polymorphism contribute to variability in acetaminophen pharmacokinetics, metabolism, and protein-adduct concentrations in healthy african-American and European-American volunteers. J. Pharmacol. Exp. Therapeut. 362, 431–440.
Cover, C., Mansouri, A., Knight, T.R., Bajt, M.L., Lemasters, J.J., Pessayre, D., Jaeschke, H., 2005. Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J. Pharmacol. Exp. Therapeut. 315, 879–887.
Critchley, J.A., Nimmo, G.R., Gregson, C.A., Woolhouse, N.M., Prescott, L.F., 1986. Inter-subject and ethnic differences in paracetamol metabolism. Br. J. Clin. Pharmacol. 22, 649–657.
Dahlin, D.C., Miwa, G.T., Lu, A.Y., Nelson, S.D., 1984. N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. Proc. Natl. Acad. Sci. U.S.A. 81, 1327–1331.
Daly, F.F., O'Malley, G.F., Heard, K., Bogdan, G.M., Dart, R.C., 2004. Prospective evaluation of repeated supratherapeutic acetaminophen (paracetamol) ingestion. Ann. Emerg. Med. 44, 393–398.
Daugas, E., Susin, S.A., Zamzami, N., Ferri, K.F., Irinopoulou, T., Larochette, N., Prévost, M.C., Leber, B., Andrews, D., Penninger, J., Kroemer, G., 2000. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. Faseb. J. 14 (5), 729–739.
Davern 2nd, T.J., James, L.P., Hinson, J.A., Polson, J., Larson, A.M., Fontana, R.J., Lalani, E., Munoz, S., Shakil, A.O., Lee, W.M., Acute Liver Failure Study Group, 2006. Measurement of serum acetaminophen-protein adducts in patients with acute liver failure. Gastroenterology 130, 687–694.
Davis, D.C., Potter, W.Z., Jollow, D.J., Mitchell, J.R., 1974. Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. Life Sci. 14, 2099–2109.
Davis, M., Labadarios, D., Williams, R.S., 1976. Metabolism of paracetamol after therapeutic and hepatotoxic doses in man. J. Int. Med. Res. 4, 40–45.
de Morais, S.M., Uetrecht, J.P., Wells, P.G., 1992. Decreased glucuronidation and increased bioactivation of acetaminophen in Gilbert's syndrome. Gastroenterology 102, 577–586.
Desler, C., Lykke, A., Rasmussen, L.J., 2010. The effect of mitochondrial dysfunction on cytosolic nucleotide metabolism. J. Nucleic Acids 2010, 701518, 2010.
Desler, C., Munch-Petersen, B., Stevnsner, T., Matsui, S., Kulawiec, M., Singh, K.K., Rasmussen, L.J., 2007. Mitochondria as determinant of nucleotide pools and chromosomal stability. Mutat. Res. 625, 112–124.
Dou, X., Li, J., Danelisen, I., Trush, M., Misra, H., Zhu, H., Jia, Z., Li, Y., 2017. Acetaminophen, the active ingredient of tylenol, protects against peroxynitrite-induced DNA damage: a chemiluminometric and electron paramagnetic resonance spectrometric study. Reactive Oxygen Species 3 (8), 127–134.
Du, K., Farhood, A., Jaeschke, H., 2017a. Mitochondria-targeted antioxidant Mito-Tempo protects against acetaminophen hepatotoxicity. Arch. Toxicol. 91, 761–773.
Du, K., Ramachandran, A., McGill, M.R., Mansouri, A., Asselah, T., Farhood, A., Woolbright, B.L., Ding, W.X., Jaeschke, H., 2017b. Induction of mitochondrial biogenesis protects against acetaminophen hepatotoxicity. Food Chem. Toxicol. 108, 339–350.
Du, K., Ramachandran, A., Jaeschke, H., 2016. Oxidative stress during acetaminophen hepatotoxicity: sources, pathophysiological role and therapeutic potential. Redox Biol 10, 148–156.
DuBois, R.N., Hill, K.E., Burk, R.F., 1983. Antioxidant effect of acetaminophen in rat liver. Biochem. Pharmacol. 32, 2621–2622.
Dunn, K.W., Martinez, M.M., Wang, Z., Mang, H.E., Clendenon, S.G., Sluka, J.P., Glazier, J.A., Klaunig, J.E., 2020. Mitochondrial depolarization and repolarization in the early stages of acetaminophen hepatotoxicity in mice. Toxicology 439, 152464.
Dybing, E., Holme, J.A., Gordon, W.P., Soderlund, E.J., Dahlin, D.C., Nelson, S.D., 1984. Genotoxicity studies with paracetamol. Mutat. Res. 138, 21–32.
Eichenbaum, G., Yang, K., Gebremichael, Y., Howell, B.A., Murray, F.J., Jacobson-Kram, D., Jaeschke, H., Kuffner, E., Gelotte, C.K., Lai, J.C.K., Wikoff, D., Atillasoy, E., 2020. Application of the DILIsym® Quantitative Systems Toxicology drug-induced liver injury model to evaluate the carcinogenic hazard potential of acetaminophen. Regul. Toxicol. Pharmacol. 118, 104788.
El-Hassan, H., Anwar, K., Macanas-Pirard, P., Crabtree, M., Chow, S.C., Johnson, V.L., Lee, P.C., Hinton, R.H., Price, S.C., Kass, G.E., 2003. Involvement of mitochondria in acetaminophen-induced apoptosis and hepatic injury: roles of cytochrome c, Bax, Bid, and caspases. Toxicol. Appl. Pharmacol. 191 (2), 118–129.
EPA, U.S, 2019. ToxCast & Tox21 summary files from invitrodb_v3.2. Retrieved from. https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data_on_11_August_2019. (Accessed 9 August 2019).
FDA, 2010. Supervisor's Secondary Review - Pharmacology/Toxicology Review and Evaluation: Acetaminophen Application. Federal Drug Administration. Available at. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2010/022450Orig1s000PharmR.pdf.
Forrest, J.A., Adriaenssens, P., Finlayson, N.D., Prescott, L.F., 1979. Paracetamol metabolism in chronic liver disease. Eur. J. Clin. Pharmacol. 15, 427–431.
Fukai, T., Ushio-Fukai, M., 2011. Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxidants Redox Signal. 15 (6), 1583–1606.
Goodman, J., Lynch, H., 2017. Improving the international agency for research on cancer's consideration of mechanistic evidence. Toxicol. Appl. Pharmacol. 319, 39–46.
Graham, G.G., Scott, K.F., 2005. Mechanism of action of paracetamol. Am. J. Therapeut. 12, 46–55.
Guengerich, F.P., Liebler, D.C., 1985. Enzymatic activation of chemicals to toxic metabolites. Crit. Rev. Toxicol. 14, 259–307.
Gujral, J.S., Knight, T.R., Farhood, A., Bajt, M.L., Jaeschke, H., 2002. Mode of cell death after acetaminophen overdose in mice: apoptosis or oncotic necrosis? Toxicol. Sci. 67 (2), 322–328.
Hamlyn, A.N., Douglas, A.P., James, O.F., Lesna, M., Watson, A.J., 1977. Liver function and structure in survivors of acetaminophen poisoning. A follow-up study of serum bile acids and liver histology. Am. J. Dig. Dis. 22, 605–610.
Hanawa, N., Shinohara, M., Saberi, B., Gaarde, W.A., Han, D., Kaplowitz, N., 2008. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J. Biol. Chem. 283, 13565–13577.
Hayward, K.L., Powell, E.E., Irvine, K.M., Martin, J.H., 2016. Can paracetamol (acetaminophen) be administered to patients with liver impairment? Br. J. Clin. Pharmacol. 81, 210–222.
Heard, K.J., Green, J.L., James, L.P., Judge, B.S., Zolot, L., Rhyee, S., Dart, R.C., 2011. Acetaminophen-cysteine adducts during therapeutic dosing and following overdose. BMC Gastroenterol. 11, 20.
Hinson, J.A., 1983. Reactive metabolites of phenacetin and acetaminophen: a review. Environ. Health Perspect. 49, 71–79.
Hinson, J.A., Pohl, L.R., Monks, T.J., Gillette, J.R., Guengerich, F.P., 1980. 3-Hydroxyacetaminophen: a microsomal metabolite of acetaminophen. Evidence against an epoxide as the reactive metabolite of acetaminophen. Drug Metab. Dispos. 8, 289–294.
Hinson, J.A., Pohl, L.R., Gillette, J.R., 1979. N-Hydroxyacetaminophen: a microsomal metabolite of N-hydroxyphenacetin but apparently not of acetaminophen. Life Sci. 24, 2133–2138.
Hogestatt, E.D., Jonsson, B.A., Ermund, A., Andersson, D.A., Bjork, H., Alexander, J.P., Cravatt, B.F., Basbaum, A.I., Zygmunt, P.M., 2005. Conversion of acetaminophen to the bioactive N-acylphenolamine AM404 via fatty acid amide hydrolase-dependent arachidonic acid conjugation in the nervous system. J. Biol. Chem. 280, 31405–31412.
Hoivik, D.J., Manatou, J.E., Tviet, A., Hart, S.G., Khairallah, E.A., Cohen, S.D., 1995. Gender-related differences in susceptibility to acetaminophen-induced protein arylation and nephrotoxicity in the CD-1 mouse. Toxicol. Appl. Pharmacol. 130, 257–271.
Hongslo, J.K., Smith, C.V., Brunborg, G., Soderlund, E.J., Holme, J.A., 1994. Genotoxicity of paracetamol in mice and rats. Mutagenesis 9, 93–100.
Hongslo, J.K., Bjorge, C., Schwarze, P.E., Brogger, A., Mann, G., Thelander, L., Holme, J. A., 1990. Paracetamol inhibits replicative DNA synthesis and induces sister chromatid exchange and chromosomal aberrations by inhibition of ribonucleotide reductase. Mutagenesis 5, 475–480.
Hu, J., Ramshesh, V.K., McGill, M.R., Jaeschke, H., Lemasters, J.J., 2016. Low dose acetaminophen induces reversible mitochondrial dysfunction associated with transient c-jun N-terminal kinase activation in mouse liver. Toxicol. Sci. 150, 204–215.
Hu, J.J., Lee, M.J., Vapiwala, M., Reuhl, K., Thomas, P.E., Yang, C.S., 1993. Sex-related differences in mouse renal metabolism and toxicity of acetaminophen. Toxicol. Appl. Pharmacol. 122, 16–26.
IARC, 1990. Monograph for Paracetamol (Acetaminophen), vol. 50. World Health Organization, International Agency for Research on Cancer, Lyon, France, pp. 307–332.
Iyer, S., Pham, N., Marty, M., Sandy, M., Solomon, G., Zeise, L., 2019. An integrated approach using publicly available Resources for identifying and characterizing chemicals of potential toxicity concern: proof-of-concept with chemicals that affect cancer pathways. Toxicol. Sci. 169, 14–24.

Jaeschke, H., 1990. Glutathione disulfide formation and oxidant stress during acetaminophen-induced hepatotoxicity in mice in vivo: the protective effect of allopurinol. J. Pharmacol. Exp. Therapeut. 255, 935–941.
Jaeschke, H., Duan, L., Akakpo, J.Y., Farhood, A., Ramachandran, A., 2018. The role of apoptosis in acetaminophen hepatotoxicity. Food Chem. Toxicol. 118, 709–718.
Jaeschke, H., Lemasters, J.J., 2003. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology 125 (4), 1246–1257.
Jahr, S., Hentze, H., Englisch, S., Hardt, D., Fackelmayer, F.O., Hesch, R.D., Knippers, R., 2001. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Canc. Res. 61, 1659–1665.
Jetten, M.J., Gaj, S., Ruiz-Aracama, A., de Kok, T.M., van Delft, J.H., Lommen, A., van Someren, E.P., Jennen, D.G., Claessen, S.M., Peijnenburg, A.A., Stierum, R.H., Kleinjans, J.C., 2012. 'Omics analysis of low dose acetaminophen intake demonstrates novel response pathways in humans. Toxicol. Appl. Pharmacol. 259, 320–328.
Judson, R., Houck, K., Martin, M., Richard, A.M., Knudsen, T.B., Shah, I., Little, S., Wambaugh, J., Setzer, R.W., Kothiya, P., Phuong, J., Filer, D., Smith, D., Reif, D., Rotroff, D., Kleinstreuer, N., Sipes, N., Xia, M., Huang, R., Crofton, K., Thomas, R.S., 2016. Analysis of the effects of cell stress and cytotoxicity on in vitro assay activity across a diverse chemical and assay space. Toxicol. Sci. 153, 409.
Kang, A.M., Padilla-Jones, A., Fisher, E.S., Akakpo, J.Y., Jaeschke, H., Rumack, B.H., Gerkin, R.D., Curry, S.C., (in press). The effect of 4-methylpyrazole on oxidative metabolism of acetaminophen in human volunteers. . J. Med. Toxicol..
Kennon-McGill, S., McGill, M.R., 2018. Extrahepatic toxicity of acetaminophen: critical evaluation of the evidence and proposed mechanisms. J Clin Transl Res 3 (3), 297–310.
Kim, J.S., He, L., Lemasters, J.J., 2003. Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem. Biophys. Res. Commun. 304 (3), 463–470.
Knight, T.R., Fariss, M.W., Farhood, A., Jaeschke, H., 2003. Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. Toxicol. Sci. 76, 229–236.
Knight, T.R., Ho, Y.S., Farhood, A., Jaeschke, H., 2002. Peroxynitrite is a critical mediator of acetaminophen hepatotoxicity in murine livers: protection by glutathione. J. Pharmacol. Exp. Therapeut. 303, 468–475.
Knight, T.R., Kurtz, A., Bajt, M.L., Hinson, J.A., Jaeschke, H., 2001. Vascular and hepatocellular peroxynitrite formation during acetaminophen toxicity: role of mitochondrial oxidant stress. Toxicol. Sci. 62, 212–220.
Kon, K., Kim, J.S., Uchiyama, A., Jaeschke, H., Lemasters, J.J., 2010. Lysosomal iron mobilization and induction of the mitochondrial permeability transition in acetaminophen-induced toxicity to mouse hepatocytes. Toxicol. Sci. 117 (1), 101–108.
Kon, K., Kim, J.S., Jaeschke, H., Lemasters, J.J., 2004. Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. Hepatology 40, 1170–1179.
Kozer, E., Evans, S., Barr, J., Greenberg, R., Soriano, I., Bulkowstein, M., Petrov, I., Chen-Levi, Z., Barzilay, B., Berkovitch, M., 2003. Glutathione, glutathione-dependent enzymes and antioxidant status in erythrocytes from children treated with high-dose paracetamol. Br. J. Clin. Pharmacol. 55, 234–240.
Lauterburg, B.H., 2002. Analgesics and glutathione. Am. J. Therapeut. 9, 225–233.
Lawson, J.A., Fisher, M.A., Simmons, C.A., Farhood, A., Jaeschke, H., 1999. Inhibition of Fas receptor (CD95)-induced hepatic caspase activation and apoptosis by acetaminophen in mice. Toxicol. Appl. Pharmacol. 156 (3), 179–186.
Lesna, M., Watson, A.J., Douglas, A.P., Hamlyn, A.N., James, O.F., 1976. Evaluation of paracetamol-induced damage in liver biopsies. Acute changes and follow-up findings. Virchows Arch. A Pathol. Anat. Histol. 370, 333–344.
Lister, C.F., McLean, A.E., 1997. Inhibition of DNA synthesis by paracetamol in different tissues of the rat in vivo. Toxicology 116, 49–57.
LoGuidice, A., Boelsterli, U.A., 2011. Acetaminophen overdose-induced liver injury in mice is mediated by peroxynitrite independently of the cyclophilin D-regulated permeability transition. Hepatology 54 (3), 969–978.
Low, R.L., 2003. Mitochondrial Endonuclease G function in apoptosis and mtDNA metabolism: a historical perspective. Mitochondrion 2 (4), 225–236.
Malhi, H., Gores, G.J., Lemasters, J.J., 2006. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 43 (2 Suppl. 1), S31–S44.
Manyike, P.T., Kharasch, E.D., Kalhorn, T.F., Slattery, J.T., 2000. Contribution of CYP2E1 and CYP3A to acetaminophen reactive metabolite formation. Clin. Pharmacol. Ther. 67, 275–282.
Marnett, L.J., Riggins, J.N., West, J.D., 2003. Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. J. Clin. Invest. 111, 583–593.
Marnett, L.J., 2000. Oxyradicals and DNA damage. Carcinogenesis 21, 361–370.
Marnett, L.J., Burcham, P.C., 1993. Endogenous DNA adducts: potential and paradox. Chem. Res. Toxicol. 6, 771–785.
Masubuchi, Y., Suda, C., Horie, T., 2005. Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. J. Hepatol. 42 (1), 110–116.
McGill, M.R., Staggs, V.S., Sharpe, M.R., Lee, W.M., Jaeschke, A.H., Acute Liver Failure Study Group, 2014. Serum mitochondrial biomarkers and damage-associated molecular patterns are higher in acetaminophen overdose patients with poor outcome. Hepatology 60, 1336–1345.
McGill, M.R., Jaeschke, H., 2013. Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis. Pharm. Res. (N. Y.) 30, 2174–2187.
McGill, M.R., Lebofsky, M., Norris, H.R., Slawson, M.H., Bajt, M.L., Xie, Y., Williams, C. D., Wilkins, D.G., Rollins, D.E., Jaeschke, H., 2013. Plasma and liver acetaminophen-protein adduct levels in mice after acetaminophen treatment: dose-response, mechanisms, and clinical implications. Toxicol. Appl. Pharmacol. 269, 240–249.
McGill, M.R., Sharpe, M.R., Williams, C.D., Taha, M., Curry, S.C., Jaeschke, H., 2012a. The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. J. Clin. Invest. 122, 1574–1583.
McGill, M.R., Williams, C.D., Xie, Y., Ramachandran, A., Jaeschke, H., 2012b. Acetaminophen-induced liver injury in rats and mice: comparison of protein adducts, mitochondrial dysfunction, and oxidative stress in the mechanism of toxicity. Toxicol. Appl. Pharmacol. 264, 387–394.
McGill, M.R., Yan, H.M., Ramachandran, A., Murray, G.J., Rollins, D.E., Jaeschke, H., 2011. HepaRG cells: a human model to study mechanisms of acetaminophen hepatotoxicity. Hepatology 53, 974–982.
Murray, F.J., Monnot, A.D., Jacobson-Kram, D., Cohen, S.M., Hardisty, J.F., Bandara, S. B., Kovochich, M., Deore, M., Pitchaiyan, S.K., Gelotte, C.K., Lai, J.C.K., Atillasoy, E., Hermanowski-Vosatka, A., Kuffner, E., Unice, K.M., Yang, K., Gebremichael, Y., Howell, B.A., Eichenbaum, G., 2020. A critical review of the acetaminophen preclinical carcinogenicity and tumor promotion data and their implications for its carcinogenic hazard potential. Regul. Toxicol. Pharmacol. 118, 104801.
Nagata, S., Nagase, H., Kawane, K., Mukae, N., Fukuyama, H., 2003. Degradation of chromosomal DNA during apoptosis. Cell Death Differ. 10, 108–116.
Nakagawa, H., Maeda, S., Hikiba, Y., Ohmae, T., Shibata, W., Yanai, A., Sakamoto, K., Ogura, K., Noguchi, T., Karin, M., Ichijo, H., Omata, M., 2008. Deletion of apoptosis signal-regulating kinase 1 attenuates acetaminophen-induced liver injury by inhibiting c-Jun N-terminal kinase activation. Gastroenterology 135 (4), 1311–1321.
Nguyen, N.T., Akakpo, J.Y., Ramachandran, A., Weemhoff, J.L., Ding, W.X., Jaeschke, H., 2021. Protein adduct removal by autophagy following repeat administration of subtoxic doses of acetaminophen limits liver injury in fed mice. Arch. Toxicol. In press.
Ni, H.M., McGill, M.R., Chao, X., Du, K., Williams, J.A., Xie, Y., Jaeschke, H., Ding, W.X., 2016. Removal of acetaminophen-protein adducts by autophagy protects against acetaminophen-induced liver injury in mice. J. Hepatol. 65 (2), 354–362.
Ni, H.M., Woolbright, B.L., Williams, J., Copple, B., Cui, W., Luyendyk, J.P., Jaeschke, H., Ding, W.X., 2014. Nrf2 promotes the development of fibrosis and tumorigenesis in mice with defective hepatic autophagy. J. Hepatol 61 (3), 617–625.
Ni, H.M., Williams, J.A., Jaeschke, H., Ding, W.X., 2013. Zonated induction of autophagy and mitochondrial spheroids limits acetaminophen-induced necrosis in the liver. Redox Biol 1, 427–432.
Ni, H.M., Bockus, A., Boggess, N., Jaeschke, H., Ding, W.X., 2012. Activation of autophagy protects against acetaminophen-induced hepatotoxicity. Hepatology 55, 222–232.
Nuttall, S.L., Khan, J.N., Thorpe, G.H., Langford, N., Kendall, M.J., 2003. The impact of therapeutic doses of paracetamol on serum total antioxidant capacity. J. Clin. Pharm. Therapeut. 28, 289–294.
OEHHA, 2019. Proposition 65 - Evidence on the Carcinogenicity of Acetaminophen. Reproductive and Cancer Hazard Assessment Branch Office of Environmental Health Hazard Assessment California Environmental Protection Agency, Sacramento, CA, p. 373.
O'Riordan, A., Brummell, Z., Sizer, E., Auzinger, G., Heaton, N., O'Grady, J.G., Bernal, W., Hendry, B.M., Wendon, J.A., 2011. Acute kidney injury in patients admitted to a liver intensive therapy unit with paracetamol-induced hepatotoxicity. Nephrol. Dial. Transplant. 26 (11), 3501–3508.
Patten, C.J., Thomas, P.E., Guy, R.L., Lee, M., Gonzalez, F.J., Guengerich, F.P., Yang, C. S., 1993. Cytochrome P450 enzymes involved in acetaminophen activation by rat and human liver microsomes and their kinetics. Chem. Res. Toxicol. 6, 511–518.
Phillips, D.H., Farmer, P.B., Beland, F.A., Nath, R.G., Poirier, M.C., Reddy, M.V., Turteltaub, K.W., 2000. Methods of DNA adduct determination and their application to testing compounds for genotoxicity. Environ. Mol. Mutagen. 35, 222–233.
Portmann, B., Talbot, I.C., Day, D.W., Davidson, A.R., Murray-Lyon, I.M., Williams, R., 1975. Histopathological changes in the liver following a paracetamol overdose: correlation with clinical and biochemical parameters. J. Pathol. 117, 169–181.
Portmann, B., Talbot, I.C., Day, D.W., Davidson, A.R., Murray-Lyon, I.M., Williams, R., 1974. Histopathological changes in the liver following a paracetamol overdose: correlation with clinical and biochemical parameters. J. Pathol. 117, 169–181.
Pumford, N.R., Halmes, N.C., Martin, B.M., Cook, R.J., Wagner, C., Hinson, J.A., 1997. Covalent binding of acetaminophen to N-10-formyltetrahydrofolate dehydrogenase in mice. J. Pharmacol. Exp. Therapeut. 280 (1), 501–505.
Qiu, Y., Benet, L.Z., Burlingame, A.L., 2001. Identification of hepatic protein targets of the reactive metabolites of the non-hepatotoxic regioisomer of acetaminophen, 3'-hydroxyacetanilide, in the mouse in vivo using two-dimensional gel electrophoresis and mass spectrometry. Adv. Exp. Med. Biol. 500, 663–673.
Qiu, Y., Benet, L.Z., Burlingame, A.L., 1998. Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. J. Biol. Chem. 273, 17940–17953.
Radi, R., 2004. Nitric oxide, oxidants, and protein tyrosine nitration. Proc. Natl. Acad. Sci. U.S.A. 101, 4003–4008.
Ramachandran, A., Jaeschke, H., 2020. A mitochondrial journey through acetaminophen hepatotoxicity. Food Chem. Toxicol. 140, 111282.
Ramachandran, A., Jaeschke, H., 2018. Acetaminophen toxicity: novel insights into mechanisms and future perspectives. Gene Expr. 18, 19–30.
Ramachandran, A., Lebofsky, M., Weinman, S.A., Jaeschke, H., 2011a. The impact of partial manganese superoxide dismutase (SOD2)-deficiency on mitochondrial oxidant stress, DNA fragmentation and liver injury during acetaminophen hepatotoxicity. Toxicol. Appl. Pharmacol. 251, 226–233.

Ramachandran, A., Lebofsky, M., Baines, C.P., Lemasters, J.J., Jaeschke, H., 2011b. Cyclophilin D deficiency protects against acetaminophen-induced oxidant stress and liver injury. Free Radic. Res. 45, 156–164.
Raucy, J.L., Lasker, J.M., Lieber, C.S., Black, M., 1989. Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. Arch. Biochem. Biophys. 271, 270–283.
Ray, S.D., Sorge, C.L., Raucy, J.L., Corcoran, G.B., 1990. Early loss of large genomic DNA in vivo with accumulation of Ca2+ in the nucleus during acetaminophen-induced liver injury. Toxicol. Appl. Pharmacol. 106 (2), 346–351.
Rogers, L.K., Moorthy, B., Smith, C.V., 1997. Acetaminophen binds to mouse hepatic and renal DNA at human therapeutic doses. Chem. Res. Toxicol. 10, 470–476.
Rumack, B.H., 2004. Acetaminophen misconceptions. Hepatology 40, 10–15.
Saito, C., Lemasters, J.J., Jaeschke, H., 2010a. c-Jun N-terminal kinase modulates oxidant stress and peroxynitrite formation independent of inducible nitric oxide synthase in acetaminophen hepatotoxicity. Toxicol. Appl. Pharmacol. 246 (1–2), 8–17.
Saito, C., Zwingmann, C., Jaeschke, H., 2010b. Novel mechanisms of protection against acetaminophen hepatotoxicity in mice by glutathione and N-acetylcysteine. Hepatology 51 (1), 246–254.
Sarich, T., Kalhorn, T., Magee, S., al-Sayegh, F., Adams, S., Slattery, J., Goldstein, J., Nelson, S., Wright, J., 1997. The effect of omeprazole pretreatment on acetaminophen metabolism in rapid and slow metabolizers of S-mephenytoin. Clin. Pharmacol. Ther. 62, 21–28.
Schildknecht, S., Daiber, A., Ghisla, S., Cohen, R.A., Bachschmid, M.M., 2008. Acetaminophen inhibits prostanoid synthesis by scavenging the PGHS-activator peroxynitrite. Faseb. J. 22, 215–224.
Sharma, C.V., Mehta, V., 2014. Paracetamol: mechanisms and updates. Cont. Educ. Anaesth. Crit. Care Pain 14, 153–158.
Sharma, M., Gadang, V., Jaeschke, A., 2012. Critical role for mixed-lineage kinase 3 in acetaminophen-induced hepatotoxicity. Mol. Pharmacol. 82 (5), 1001–1007.
Shen, W., Kamendulis, L.M., Ray, S.D., Corcoran, G.B., 1991. Acetaminophen-induced cytotoxicity in cultured mouse hepatocytes: correlation of nuclear Ca2+ accumulation and early DNA fragmentation with cell death. Toxicol. Appl. Pharmacol. 111 (2), 242–254.
Shertzer, H.G., Schneider, S.N., Kendig, E.L., Clegg, D.J., D'Alessio, D.A., Genter, M.B., 2008. Acetaminophen normalizes glucose homeostasis in mouse models for diabetes. Biochem. Pharmacol. 75, 1402–1410.
Smith, M.T., Guyton, K.Z., Gibbons, C.F., Fritz, J.M., Portier, C.J., Rusyn, I., DeMarini, D. M., Caldwell, J.C., Kavlock, R.J., Paul, F., Lambert, P.F., Hecht, S.S., Bucher, J.R., Stewart, B.W., Baan, R.A., Cogliano, V.J., Straif, K., 2016. Key characteristics of carcinogens as a basis for organizing data on mechanisms of carcinogenesis. Environ. Health Perspect. 124 (6), 713–721.
Takehara, M., Hoshino, T., Namba, T., Yamakawa, N., Mizushima, T., 2011. Acetaminophen-induced differentiation of human breast cancer stem cells and inhibition of tumor xenograft growth in mice. Biochem. Pharmacol. 81, 1124–1135.
Thummel, K.E., Lee, C.A., Kunze, K.L., Nelson, S.D., Slattery, J.T., 1993. Oxidation of acetaminophen to N-acetyl-p-aminobenzoquinone imine by human CYP3A4. Biochem. Pharmacol. 45, 1563–1569.
Thybaud, V., Aardema, M., Clements, J., Dearfield, K., Galloway, S., Hayashi, M., Jacobson-Kram, D., Kirkland, D., MacGregor, J.T., Marzin, D., Ohyama, W., Schuler, M., Suzuki, H., Zeiger, E., Expert Working Group on Hazard Identification and Risk Assessment in Relation to In Vitro Testing, 2007. Strategy for genotoxicity testing: hazard identification and risk assessment in relation to in vitro testing. Mutat. Res. 627, 41–58.
Tirmenstein, M.A., Nelson, S.D., 1989. Subcellular binding and effects on calcium homeostasis produced by acetaminophen and a nonhepatotoxic regioisomer, 3'-hydroxyacetanilide, in mouse liver. J. Biol. Chem. 264, 9814–9819.
Trosko, J.E., 2017. Reflections on the use of 10 IARC carcinogenic characteristics for an objective approach to identifying and organizing results from certain mechanistic studies. Toxicology Research and Application 1, 2397847317710837.
Tsokos-Kuhn, J.O., Hughes, H., Smith, C.V., Mitchell, J.R., 1988. Alkylation of the liver plasma membrane and inhibition of the Ca2+ ATPase by acetaminophen. Biochem. Pharmacol. 37, 2125–2131.
van der Marel, C.D., Anderson, B.J., van Lingen, R.A., Holford, N.H., Pluim, M.A., Jansman, F.G., van den Anker, J.N., Tibboel, D., 2003. Paracetamol and metabolite pharmacokinetics in infants. Eur. J. Clin. Pharmacol. 59, 243–251.
van Rongen, A., Valitalo, P.A.J., Peeters, M.Y.M., Boerma, D., Huisman, F.W., van Ramshorst, B., van Dongen, E.P.A., van den Anker, J.N., Knibbe, C.A.J., 2016. Morbidly obese patients exhibit increased CYP2E1-mediated oxidation of acetaminophen. Clin. Pharmacokinet. 55, 833–847.
Wang, C., Wu, M., Arvapalli, R., Dai, X., Mahmood, M., Driscoll, H., Rice, K.M., Blough, E., 2014. Acetaminophen attenuates obesity-related renal injury through ER-mediated stress mechanisms. Cell. Physiol. Biochem. 33, 1139–1148.
WHO, 2019. Preamble: IARC Monographs on the Evaluation of Carcinogenic Hazards to Humans. World Health Organization International Agency for Research on Cancer, Lyon, France. Available: https://monography.iarc.fr/wp-content/uploads/2019/01/Preamble-2019.pdf.
Wikoff, D.S., Rager, J.E., Chappell, G.A., Fitch, S., Haws, L., Borghoff, S.J., 2019. A framework for systematic evaluation and quantitative integration of mechanistic data in assessments of potential human carcinogens. Toxicol. Sci. 167, 322–335.
Williams, C.D., Koerner, M.R., Lampe, J.N., Farhood, A., Jaeschke, H., 2011. Mouse strain-dependent caspase activation during acetaminophen hepatotoxicity does not result in apoptosis or modulation of inflammation. Toxicol. Appl. Pharmacol. 257 (3), 449–458.
Win, S., Than, T.A., Min, R.W., Aghajan, M., Kaplowitz, N., 2016. c-Jun N-terminal kinase mediates mouse liver injury through a novel Sab (SH3BP5)-dependent pathway leading to inactivation of intramitochondrial Src. Hepatology 63, 1987–2003.
Xie, Y., McGill, M.R., Cook, S.F., Sharpe, M.R., Winefield, R.D., Wilkins, D.G., Rollins, D. E., Jaeschke, H., 2015a. Time course of acetaminophen-protein adducts and acetaminophen metabolites in circulation of overdose patients and in HepaRG cells. Xenobiotica 45, 921–929.
Xie, Y., McGill, M.R., Du, K., Dorko, K., Kumer, S.C., Schmitt, T.M., Ding, W.X., Jaeschke, H., 2015b. Mitochondrial protein adducts formation and mitochondrial dysfunction during N-acetyl-m-aminophenol (AMAP)-induced hepatotoxicity in primary human hepatocytes. Toxicol. Appl. Pharmacol. 289, 213–222.
Xie, Y., Ramachandran, A., Breckenridge, D.G., Liles, J.T., Lebofsky, M., Farhood, A., Jaeschke, H., 2015c. Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury. Toxicol. Appl. Pharmacol. 286 (1), 1–9.
Xie, Y., McGill, M.R., Dorko, K., Kumer, S.C., Schmitt, T.M., Forster, J., Jaeschke, H., 2014. Mechanisms of acetaminophen-induced cell death in primary human hepatocytes. Toxicol. Appl. Pharmacol. 279, 266–274.
Yan, H.M., Ramachandran, A., Bajt, M.L., Lemasters, J.J., Jaeschke, H., 2010. The oxygen tension modulates acetaminophen-induced mitochondrial oxidant stress and cell injury in cultured hepatocytes. Toxicol. Sci. 117, 515–523.
Zapater, P., Lasso de la Vega, M.C., Horga, J.F., Such, J., Frances, R., Esteban, A., Palazon, J.M., Carnicer, F., Pascual, S., Perez-Mateo, M., 2004. Pharmacokinetic variations of acetaminophen according to liver dysfunction and portal hypertension status. Aliment. Pharmacol. Ther. 20, 29–36.
Zhang, J., Min, R.W.M., Le, K., Zhou, S., Aghajan, M., Than, T.A., Win, S., Kaplowitz, N., 2017. The role of MAP2 kinases and p38 kinase in acute murine liver injury models. Cell Death Dis. 8 (6), e2903, 2017 Jun 29.