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Novel strategies for the treatment of acetaminophen hepatotoxicity

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Abstract

Introduction: Acetaminophen (APAP) hepatotoxicity is the leading cause of acute liver failure in the western world. Despite extensive investigations into the mechanisms of cell death, only a single antidote, N-acetylcysteine, is in clinical use. However, there have recently been more efforts made to translate mechanistic insight into identification of therapeutic targets and potential new drugs for this indication.

Areas covered: After a short review of the key events in the pathophysiology of APAP-induced liver injury and recovery, the pros and cons of targeting individual steps in the pathophysiology as therapeutic targets are discussed. While the re-purposed drug fomepizole (4-methylpyrazole) and the new entity calmagafodipir are most advanced based on the understanding of their mechanism of action, several herbal medicine extracts and their individual components are also considered.

Expert opinion: Fomepizole (4-methylpyrazole) is safe and has shown efficacy in preclinical models, human hepatocytes and in volunteers against APAP overdose. The safety of calmagafodipir in APAP overdose patients was shown but it lacks solid preclinical efficacy studies. Both drugs require a controlled phase III trial to achieve regulatory approval. All studies of herbal medicine extracts and components suffer from poor experimental design, which questions their clinical utility at this point.

Keywords

acetaminophen-induced liver injury; Traditional Chinese Medicine; fomepizole; 4-methylpyrazole; calmagafodipir; herbal medicine

1. Introduction

Acetaminophen (APAP, N-acetyl-p-aminophenol, paracetamol) is an FDA approved drug commonly used as an analgesic and antipyretic. The maximal recommended therapeutic dose of 4g per day is generally considered safe and is well tolerated. However, unintentional

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(multiple moderate) and intentional (a single severe) overdose can cause significant hepatotoxicity [1]. In fact, every year in the United States, APAP overdoses are responsible for more than 78,000 emergency department visits and 33,000 hospitalizations [1]. Importantly, APAP overdose is the most common cause of acute liver failure (ALF) in the US, accounting for 46% of all cases which results in about 300-500 deaths annually [2]. This is a persistent health problem because APAP is widely available in the US market. In fact, APAP is found in more than 600 different prescription and over-the-counter (OTC) medicines including pain relievers, fever reducers and sleep aids in addition to cough, cold, as well as anti-allergy medicines [1]. Fortunately, early mechanistic data by Mitchell et al. [3] led to the development of *N*-acetylcysteine (NAC), which is the only FDA approved drug for clinical use against APAP overdose [4]. When patients seek medical attention within 8 hours after an APAP overdose, NAC is highly effective at preventing hepatotoxicity by supporting synthesis of glutathione (GSH), which facilitates the scavenging of the reactive metabolite and of reactive oxygen species [5]. However, most patients seek medical attention much later, when NAC has limited therapeutic efficacy [4], and based on recent data may even have a detrimental effect [6]. Thus, there is clearly a need for novel therapeutic intervention strategies for the treatment of APAP-induced hepatotoxicity to complement NAC in a delayed fashion. Despite extensive mechanistic investigations performed since the 1970's, there is a surprising lack of new treatment options approved against APAP overdose. The key factor is that developing a new drug requires several billions of dollars, which is considered a disproportional investment for the limited number of APAP overdose patients. Interestingly, recently published literature indicates that medicinal plants have become a major source in drug discovery efforts against APAP hepatotoxicity because of their chemical diversity as well as biological and therapeutic properties [7]. Indeed, a high number of studies published by the natural products field are focusing on identifying compounds that show a protective effect against APAP overdose [7,8]. However, the molecular mechanism of action of the specific active chemical compounds in these herbal extracts are usually difficult to identify [9], but a number of common therapeutic targets seem to emerge. On the other hand, repurposing currently FDA approved drugs may be a promising strategy for drug development against APAP overdose because of potentially lower overall development costs and better mechanistic understanding of old drugs [10]. This review discusses these various approaches targeting cellular and molecular mechanisms involved in the APAP pathophysiology to help develop novel rational therapies against APAP overdose.

2. APAP metabolism

The pathophysiological events of APAP hepatotoxicity have been well studied in the murine model of APAP overdose [11]. In fact, drug metabolism and disposition studies demonstrated that the majority of APAP is metabolized in the liver by phase II conjugation reactions [12]. At therapeutic doses, 95% of APAP is metabolized by increasing its hydrophilicity via glucuronidation and sulfation. In adults, glucuronidation is the major conjugation pathway which accounts for 70% of APAP metabolism. The reaction is catalyzed by UDP-glucuronosyltransferases (UGT) 1A6 or 1A7 and requires the high energy cofactor UDP-glucuronic acid (UDPGA). Sulfation is the other pathway responsible for 25%

of APAP metabolism, catalyzed by sulfotransferases (SULT) 1A1, 1A3/4, and 1E1 and the high energy cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) [12]. Because of the significantly lower intracellular concentration of PAPS (80 μ M) compared to UDPGA (400 μ M), sulfation reactions are limited and quickly saturated. In addition to these predominant pathways, 5-10% of APAP is metabolized by cytochrome P450 enzymes (mainly CYP2E1) [12]. This metabolism results in the formation of a highly reactive metabolite, *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is both a strong electrophile and an oxidant in the liver. However, at therapeutic doses, NAPQI is readily detoxified by GSH. It should be mentioned here that the described relative contributions of the metabolic pathways apply to adults. Young children, depending on their age, have a lower glucuronidation capacity, which is compensated by more sulfation (13). In addition, they can have lower CYP2E1 levels (14), which makes infants generally less susceptible to an APAP overdose compared to adults. Together, sulfation, glucuronidation and GSH conjugation represent elimination and detoxification pathways that prevent liver injury after therapeutic doses of APAP.

3. Hepatotoxicity after APAP overdose

3.1 Initiation of APAP-mediated liver injury

After an APAP overdose, a significantly higher amount of APAP is oxidized by CYP2E1 resulting in elevated levels of NAPQI [11]. When the amount of NAPQI generated depletes intrahepatic levels of GSH, NAPQI binds to cellular and mitochondrial proteins to form APAP-cysteine protein adducts (Figure 1) [15]. Formation of adducts on mitochondrial proteins impairs mitochondrial respiration and leads to generation of mitochondrial superoxide [16]. This early APAP-induced mitochondrial oxidative stress triggers activation of redox-sensitive mitogen activated protein kinases ultimately leading to the phosphorylation and activation of c-jun N-terminal kinase (JNK) in the cytosol [17]. Activated P-JNK then translocates to the mitochondria [16] and subsequently amplifies the original mitochondrial oxidant stress, resulting in formation of peroxynitrite and associated nitrosative stress [18]. These events trigger the opening of mitochondrial permeability transition pores (MPTP) and the release of mitochondrial intermembrane proteins like apoptosis-inducing factor (AIF) and endonuclease G, which translocate to the nucleus and cause DNA fragmentation [19]. Collectively, P450-mediated NAPQI formation along with GSH depletion, protein adducts formation, mitochondrial dysfunction, MPTP opening and DNA damage are responsible for necrotic cell death in the centrilobular areas of the liver [11,16].

3.2 Progression of APAP-mediated liver injury

In time course studies it was recognized that the area of necrosis considerably expands between the early (6h) and late (24h) injury phase in mice treated with a moderate overdose of 300mg/kg APAP (Figure 1) [20]. An earlier hypothesis to explain this observation was that a sterile inflammatory response with recruitment of neutrophils caused additional damage [21,22]. This mechanism involved the hepatocellular release of damage-associated molecular patterns (DAMPs) including high mobility group box 1 protein, mtDNA and nuclear DNA fragments, which through binding to pattern recognition receptors on macrophages transcriptionally activate cytokine and chemokine gene expression [22]. These

pro-inflammatory mediators activate and recruit neutrophils into the liver to attack stressed cells and cause additional cell death. Although the sterile inflammatory response after APAP-induced necrosis is undisputed, the controversy involves the question whether the neutrophils and monocyte-derived macrophages actually cause additional damage or have a reparative function after hepatic infiltration. The preponderance of experimental evidence argues against a cytotoxic effect of these inflammatory cells [21,22], which was also confirmed in humans [23, 24]. Nevertheless, inflammatory mediators may promote the progression of the injury independent of leukocytes by affecting intracellular signaling events such as enhancing peroxynitrite formation through inducible nitric oxide synthetase (iNOS) induction [25]. However, pharmacological inhibition of iNOS did not influence NO formation or injury after APAP overdose [18].

Another hypothesis is that necrotic cells release proteolytic enzymes (calpains) or lipases (soluble phospholipase A2), which then damage neighboring cells [26]. An unanswered question is how these “death-inducing” proteins enter healthy cells. Alternatively, neighboring cells transfer cytotoxic mediators through connexin- or pannexin channels [27]. However, functional blockage of these channels *in vivo* had only modest effects on the injury [27].

Another hypothesis is that the zonal expansion of the injury is a time-dependent process related to the severity of the insult, i.e. NAPQI generation with subsequent signaling events, and potential countermeasures such as autophagy to remove damaged mitochondria and delay the injury and mitochondrial biogenesis to replace dysfunctional mitochondria [28,29]. The putative expansion of the necrotic area reflects more the rapid death of cells closest to the centrilobular area and the delayed death and eventual survival of cells due to increasing effectiveness of adaptive mechanisms such as autophagy and biogenesis in cells further away from the central vein [28,29].

3.3 Liver regeneration and recovery

After APAP-mediated hepatocyte necrosis, dead cells must be removed by macrophages and replaced with healthy cells for the liver to recover [30]. This process requires regenerating healthy hepatocytes; the liver is the only organ that can regenerate even when 70% of it has been removed by hepatectomy. Indeed, the robust regenerative capacity of the liver has been demonstrated in the murine APAP model as well [30,31]. After liver injury caused by a moderate APAP overdose, liver regeneration acts as a compensatory response. This process involves proliferating cell nuclear antigen (PCNA), which is essential for DNA replication as well as Ki67 and cyclin D1, which are both required for cellular proliferation in the late phase of APAP-induced liver injury [32]. Moreover, liver regeneration was confirmed by increased α -fetoprotein synthesis, which correlated with better survival rates after APAP overdose in human patients [33]. This supports the idea that timely stimulation of regeneration leads to injury repair. However, in the case of a massive APAP overdose, regeneration is overwhelmed by the injury [30]. In this specific case, liver regeneration fails and acute liver injury has the potential to progress to acute liver failure.

3.4 Mechanisms of liver regeneration

In order to avoid acute liver failure after an APAP overdose, the liver must regenerate to replace dead cells with fully functional hepatocytes around the central vein area. While liver injury induces the expression of a large number of genes that promote liver regeneration [34], exact mechanisms involved in regulation of hepatic division and cell proliferation after APAP-induced liver injury are not well understood. The genes induced after APAP-induced cell death are involved in a cell signalling network that requires the activation of multiple hepatocyte survival pathways which interact with each other to facilitate liver regeneration and recovery after APAP-induced liver injury [35]. These pathways can be classified into 3 major groups - namely cytokines, growth factors, and genes involved in metabolic energy production. The timely onset and interaction between these pathways modulate the initiation, progression and termination phases of liver regeneration [30]. Cytokines are the major players of the initiation phase, which is characterized by the activation of transcription factors required for quiescent hepatocytes to enter the cell cycle and progress from the G₀ to the G₁ phase: a process known as hepatocyte priming [30]. Then, growth factors take over the progression phase required for DNA replication and controlled hepatocyte division. In the late G₁ phase, cyclin D1 expression and activity triggers DNA replication independently of growth factors. Thus, an increase in cyclin D1 expression represents the point at which DNA replication becomes a growth factor-independent process [34]. After APAP-mediated necrotic cell death, liver regeneration depends on the replication of healthy differentiated hepatocytes [30]. Interestingly, it has been proposed that when hepatocytes are unable to proliferate, liver stem cells act as the second line of defense to repopulate necrotic areas and avoid liver failure [35]. Thus, stem cells treatments have the ability to support liver recovery after APAP overdose [30]. In addition to these signaling networks, liver regeneration can also be considered a compensatory response that is coordinated by nutrient availability and organismal nutrient demand [36]. There is a significant increase of the metabolic demand from stressed hepatocytes for survival and regeneration after acute liver injury. In order to ensure optimal function, the liver must metabolize all substrates available to support cellular function [37]. In the healthy liver, metabolism is a complex and dynamic process that supports the basic cellular functions of hepatocytes by providing energy in the form of ATP, building blocks of complex molecules for protein and membrane bilayer lipid synthesis, substrates for protein posttranslational and epigenetic modifications as well as metabolic signals which balance energy production with energy demand. However, to cope with liver injury and survive, stressed hepatocytes must optimize ATP production in order to support cellular function [38]. This requires a metabolic remodeling which reprograms hepatocytes to favor the oxidation of the most abundant substrates. Under a balanced nutrient supply, glycolysis, the citric acid cycle and the electron transport chain are the major ATP production pathways in the healthy liver [37,38]. After short term nutrient deprivation, fatty acid oxidation becomes the major pathway for ATP production. Yet, after prolonged nutrient deprivation, both fatty acid and ketone body oxidation become the major pathways of ATP production [37]. Thus, modulating energy substrate availability is necessary to achieve an energy balance and support liver recovery after APAP overdose. Nonetheless, the molecular and cellular mechanisms of hepatic ATP supply in response to the energy demand after APAP overdose are not fully understood and require further investigations.

4. Novel therapeutic interventions based on the mechanism of APAP overdose

There are many cellular and molecular pathways that can be targeted to manipulate the pathophysiological events of APAP hepatotoxicity specifically during the initiation of the injury, the progression of the injury and during the recovery phase. The following sections will examine novel strategies and potential molecular targets for treatment of APAP hepatotoxicity.

4.1 Disrupting cell death mechanisms

4.1.1 Targeting CYP2E1 activity—The studies discussed in this section highlight the importance of inhibiting CYP2E1 to limit excess production of NAPQI and how that could be a therapeutic approach to prevent APAP-induced liver injury. Generation of the highly reactive metabolite NAPQI from APAP is catalyzed by cytochrome P450 enzymes [12]; this reaction is responsible for GSH depletion and protein adduct formation [3]. More recent work has now established the role of covalent mitochondrial protein adducts as initiating events that lead to necrotic death of hepatocytes after an APAP overdose [11]. Thus, extensive metabolic activation of APAP by cytochrome P450 enzymes is critical for the onset of liver injury. CYP2E1, 1A2, 2D6, and 3A4 are known to be the main enzymes that metabolize APAP to NAPQI [12]. In an *in vitro* study designed to examine formation of toxic metabolites, Hazai et al. characterized APAP metabolism in human liver microsomes [39]. This study showed that at high APAP concentrations, there was a correlation between NAPQI formation and CYP2E1 activity while no correlation was found between the formation of NAPQI and CYP1A2, CYP2A6, and CYP3A4 enzyme activities. These results confirmed clinical studies which suggested that CYP2E1 is the major drug metabolizing enzyme that forms NAPQI in humans [40]. These results are further corroborated by studies showing that mice deficient in CYP2E1 were almost completely protected against APAP hepatotoxicity [41]. Collectively, the data strongly support the hypothesis that CYP2E1 is the primary enzyme essential for the biotransformation of APAP to its reactive metabolites in mice and humans [42]. Hence, targeting CYP2E1 by inhibiting its activity could be a promising strategy to avoid the downstream toxic effects of NAPQI and eliminate APAP induced hepatic necrosis. Indeed, multiple small molecule drugs have been tested for their inhibitory effects on CYP2E1 after APAP overdose. Examples are cimetidine, chlorpromazine, diclofenac, fluphenazine, thioridazine, isoniazid, diallyl sulfide, 4-methylpyrazole (fomepizole), disulfiram and its metabolite, diethyl-dithiocarbamate [39]. However, the only inhibitors of NAPQI formation were found to be disulfiram, its metabolite diethyl-dithiocarbamate, and fomepizole. In addition to these, cimetidine was found to inhibit the activity of CYP2C9, which is a CYP enzyme that does not metabolize APAP. Fomepizole is an FDA approved antidote against ethylene glycol and methanol poisoning. Initial clinical evidence for a potential beneficial effect of fomepizole against APAP hepatotoxicity was from a case report describing the survival of a woman who took a massive APAP overdose [43]. This patient was treated with NAC, the standard of care, and fomepizole for suspected alcohol poisoning. Because of the lethal levels of APAP ingested in this case, 4MP was hypothesized to be the cause for survival in this patient [44]. Consequently, this hypothesis was tested by co-treating C57BL/6J mice with 50 mg/kg

fomepizole, which is less than the dose approved in patients, along with an APAP overdose of 300 mg/kg, which causes severe liver injury [45]. Fomepizole provided almost absolute protection against APAP toxicity, which was mediated by a direct inhibition of CYP2E1 as indicated by inhibition of GSH depletion and oxidative metabolite formation (Figure 1) [45]. The same study confirmed the protective effect of fomepizole in primary human hepatocytes and showed that CYP2E1 inhibition blocked APAP metabolic activation, NAPQI formation and downstream signaling events that typically lead to hepatic necrosis [45]. The efficacy of fomepizole in inhibiting the metabolic activation of APAP was also confirmed in human volunteers [46]. Thus, P450 inhibitors could be potent therapeutics against APAP hepatotoxicity. However, in order to avoid drug-drug interactions, the inhibitor needs to be relatively specific for CYP2E1. Fomepizole seems to be the best candidate as it is specific for CYP2E1. However, the limitation of this therapeutic target is that the drug has to be administered as early as possible as the therapeutic window is limited to the drug metabolism phase.

4.1.2 Targeting hepatic glutathione—Glutathione (GSH) is an essential antioxidant, which plays a major role in preserving hepatocyte function during exposure to reactive metabolites and oxidant stress. GSH can scavenge NAPQI either spontaneously or catalyzed by glutathione-S-transferases [12]. After an overdose of APAP, this reaction depletes the intrahepatic GSH pool while increasing the vulnerability of hepatocytes to subsequent toxic events including protein adduct formation. In addition, mitochondrial GSH levels are critical for scavenging of peroxynitrite and reactive oxygen inside the mitochondria [47]. Based on the reliance of hepatocytes on GSH to scavenge NAPQI, strategies to rapidly re-synthesize GSH were an early focus of therapeutics against APAP hepatotoxicity. This led to the development of NAC, which is still the only clinically approved antidote [4]. Although in use for over 40 years, clinical NAC treatment protocols are still being discussed and refined in terms of dosing and duration of treatment [48]. Mechanistically, NAC does not react directly with NAPQI but primarily provides cysteine for GSH synthesis [49], which depends on the availability of cysteine residues and the activity of the rate-limiting enzyme of GSH synthesis, glutamate-cysteine ligase (GCL). When given within 8h after APAP ingestion, NAC is very effective at replenishing intrahepatic GSH levels and protecting against toxicity in overdose patients [4]. However, early studies considering the therapeutic window of NAC therapy after APAP poisoning in patients show that the efficacy of NAC is declining beyond 8h after APAP ingestion [50]. Hence, additional molecular mechanisms need to be targeted to supplement NAC mediated protection against APAP hepatotoxicity.

4.1.3 Targeting JNK activation and mitochondrial dysfunction—After depleting intrahepatic GSH, NAPQI binds to the cysteine residue of critical mitochondrial proteins leading to mitochondrial dysfunction and superoxide release [51]. Two-dimensional gel electrophoresis and mass spectrometry were used by Qiu and colleagues to successfully identify glutathione peroxidase, aldehyde dehydrogenase, ATP synthase alpha subunit homologous to 2,4-dienoyl-CoA reductase precursor and glutathione S transferase pi as mitochondrial proteins adducted by NAPQI [52]. However, no single adducted mitochondrial protein was identified to be responsible for subsequent mitochondrial dysfunction. More recent data suggest that directional superoxide release towards the cytosol

from adducted respiratory complex components may be involved in activation of cytosolic events ultimately leading to c-jun N-terminal kinase (JNK) activation [16]. JNK phosphorylation (activation) occurs within 1 hour after an APAP dose of 300mg/kg in mice [53] or 6h in human hepatocytes [54] and mitochondrial translocation of P-JNK was observed by 2h in mice and 15h in human hepatocytes [53,54]. Treatment with the JNK inhibitor SP600125 protects against APAP-induced liver injury by preventing mitochondrial oxidant stress [18]. This mechanistic study highlighted the benefit of directly targeting JNK activation to prevent APAP hepatotoxicity. However, SP600125 is an experimental drug only soluble in DMSO and is not in clinical development. Interestingly, a recent study demonstrated that fomepizole (4-methylpyrazole) was still highly effective in mice when treatment was delayed to 1.5h after the metabolism of APAP is completed [55], indicating additional mechanisms of protection (Figure 1). Subsequent biochemical analysis provided evidence that fomepizole bound to the ATP binding site of JNK and competitively inhibited enzyme activity [55]. Direct comparison of 4MP with NAC indicated that fomepizole was superior at severe overdoses in mice [55] and in human hepatocytes. Thus, the clinically approved antidote against methanol poisoning, fomepizole is highly effective in mice against APAP overdose due to its dual mechanism of protection involving both inhibition of CYP2E1 and JNK. Fomepizole is not only effective in preclinical animal experiments [45,55], but also in human hepatocytes [45] and in human volunteers [46] as a CYP2E1 inhibitor. In addition, there is an increasing number of case reports published on the off-label use of fomepizole in patients with severe overdoses of APAP [56,57]. However, these case reports do not prove that fomepizole was the cause of the positive clinical outcome. Together, these data suggest that a randomized phase III clinical trial may be warranted to assess the efficacy of fomepizole in APAP overdose patients.

4.1.4 Targeting mitochondrial oxidant stress—After an APAP overdose, continuous activation and translocation of JNK to mitochondria amplifies the mitochondrial oxidant stress [51]. This further impairs the electron transport chain causing leakage of electrons and production of superoxide [16]. Typically, superoxide generated through the mitochondrial electron transport chain is dismutated by superoxide dismutase 2 (SOD2, MnSOD) located in the mitochondrial matrix to form the less reactive hydrogen peroxide and molecular oxygen. The main protective effect of MnSOD is to limit the reaction of superoxide with nitric oxide to form the highly reactive oxidant and nitrating species peroxynitrite, which has been shown to be the most relevant oxidant in APAP toxicity [47,58]. Thus, GSH synthesis stimulated by NAC treatment can have an additional mechanism of protection, i.e. the direct scavenging of peroxynitrite and the support of glutathione peroxidase to reduce hydrogen peroxide [5]. An important target for modification and inactivation by peroxynitrite is MnSOD [59], which results in a severely compromised capacity to remove superoxide and amplifies peroxynitrite formation during APAP toxicity. The importance of mitochondrial SOD activity is evident in the aggravation of APAP-induced liver injury in mice partially deficient in MnSOD [60] and the effective protection in mice treated with the mitochondria-targeted SOD mimetic Mito-Tempo [61]. Thus, preserving mitochondrial SOD activity could be a therapeutic approach to prevent downstream events and attenuate APAP hepatotoxicity as documented in preclinical studies with Mito-Tempo [61]. Clinical validation of this concept began with the identification that

an MRI contrast agent, mangafodipir, a SOD mimetic, which demonstrated protection against APAP hepatotoxicity in the mouse [62]. Subsequent modification of its structure led to the development of calmagafodipir, which was recently used in a Phase 1 clinical study to demonstrate safety in human APAP overdose patients [63]. Though calmagafodipir showed some minor clinical benefit against APAP hepatotoxicity through measures of liver injury markers such as cytokeratin-18 and miR-122, all patients included in this safety study were those who presented early and were co-treated with NAC and hence unlikely to develop serious liver injury [63]. Thus, based on the beneficial effect of Mito-Tempo, calmagafodipir could be a potentially promising adjunct therapy to NAC (Figure 1). However, detailed preclinical mechanistic studies with this compound are presently lacking. This is relevant since the time of calmagafodipir administration in relation to APAP overdose may be important as it has been shown that delayed treatment with NAC, which presumably scavenges free radicals during regeneration, may be detrimental in APAP-induced recovery [6].

4.1.5 Targeting Nrf2 antioxidant stress response—The nuclear respiratory factor 2 (Nrf2) is an intrahepatic defense mechanism designed to protect hepatocytes from oxidative stress. Under physiological conditions, Nrf2 localizes in the cytoplasm of hepatocytes where it remains bound and inactivated by Kelch-like ECH-associated protein 1 (Keap1) [64]. However, APAP overdose triggers the destabilization and degradation of Keap1 leading to the translocation of Nrf2 into the nucleus where it binds to antioxidant-responsive elements (ARE) [64]. This triggers the transcriptional activation of multiple genes regulating the antioxidant stress response, including glutamate-cysteine ligase (GCL), which catalyzes the first-rate limiting step for GSH synthesis required to scavenge ROS [51]. Nrf2 also induces the expression of UDP-glucuronosyltransferases and glutathione-S-transferases, which are the major pathways of metabolism and detoxification of APAP and NAPQI, respectively. Additionally, Nrf2 mediates the expression of nicotinamide adenine dinucleotide phosphate oxidase, NAD(P)H:quinone oxidoreductase 1 (Nqo1), responsible for the removal of quinones and the scavenging of superoxide [64]. Nrf2 also induces the multidrug resistance-associated protein transporter Mrp2, which transports APAP-glucuronide, APAP-sulfate and APAP-GSH into the bile [65]. Induction of the basolateral membrane transporters Mrp3 and Mrp4 (which transport these metabolites into the bloodstream for kidney excretion [12]), during APAP hepatotoxicity has also been shown to be dependent on Nrf2 [65]. Most importantly, Nrf2-null mice [66] are extremely susceptible to APAP toxicity while Keap-1 null mice [67] are highly resistant. Thus, given the crucial hepatoprotective role of the Nrf2/Keap1/ARE pathway, it is logical to target the antioxidant stress response and investigate potential benefits against APAP toxicity. However, given the time needed for Nrf2-dependent transcriptional activation of genes and protein expression, this approach works best when animals are pretreated and may be of limited therapeutic values for patients who are admitted late to the hospital during the injury phase. Examples of this intervention are predominantly found in the natural products arena and will be discussed in section 5.

4.1.6 Disrupting progression of injury—Amplification of mitochondrial oxidant stress induced by JNK translocation ultimately causes opening of the MPTP and a subsequent collapse of the mitochondrial membrane potential and cessation of ATP

synthesis [11]. Release of mitochondrial intermembrane proteins like endonuclease G and apoptosis inducing factor (AIF) and their translocation to the nucleus then causes nuclear DNA fragmentation [68], which can be considered the point of no-return to cell death. However, these events are dependent on the dose of APAP as lower doses induce reversible mitochondrial changes while higher overdoses result in irreversible MPTP opening and DNA fragmentation [69]. While it could thus be considered that limiting nuclear DNA damage caused by translocation of mitochondrial intermembrane proteins could be a rational therapeutic strategy against APAP toxicity, this is unlikely to succeed in the face of sustained mitochondrial dysfunction and oxidant stress [19]. Hence, preventing mitochondrial dysfunction appears to be a more promising strategy [16]. Alternative hypotheses for the progression of the injury have been discussed. One approach would be to interrupt cell-cell communications [27]. It has been proposed that the liver transmembrane protein channels involving connexins and pannexin 1 help hepatocytes communicate during necrosis to transfer chemical cues that worsen APAP-mediated liver injury [70]. This was based on moderate alleviation of APAP hepatotoxicity by pharmacological inhibitors of these channels [27,70]. However, at least some of the effects were shown to be due to off-target effects such as P450 inhibition [71]. Other studies suggested that proteolytic enzymes like calpain released from dying hepatocytes could be mediating the progression of injury [26]. Although inhibition of these signaling cues could be a therapeutic strategy to limit APAP hepatotoxicity, these approaches are hampered by lack of detailed mechanistic data regarding these signaling events. Interestingly, recent evidence using *in vivo* microscopy indicates that liver injury after an APAP overdose does not spread from individual necrotic cells to their neighbors but occurs in isolated cell islands [72]. Thus, these data suggest that the progression of injury is more a temporary difference in the development of cell death in individual cells caused by the severity of the insult (Cyp2E1 levels) and the adaptive response of autophagy and biogenesis [28,29].

4.1.7 Targeting autophagy and biogenesis—Autophagy is an intracellular process to remove damaged proteins and cell organelles. As such, autophagy has a predominant protective function, but excessively activated autophagy can also lead to cell death [73]. Triggered by adverse cellular conditions during APAP-induced hepatocyte injury, cellular proteins or mitochondria can be enveloped by a membrane to form autophagosomes, which fuse with lysosomes to degrade their content in the autolysosomes [73]. During APAP hepatotoxicity, protein adducts [74] and damaged mitochondria [75] are being removed by autophagy. At the same time, mitochondrial biogenesis is activated to replace the mitochondria removed by autophagy, which also supports cell survival and regeneration [76]. The pathophysiological relevance of these adaptive processes has been demonstrated by chemical inhibitors and activators [74,75,76], which suggests that both activation of autophagy and promotion of mitochondrial biogenesis can limit the extent of the injury by enhancing cell survival as well as promote organ recovery. However, because autophagy and biogenesis can be considered adaptive mechanisms to cellular stress, targeting these processes does not affect the core injury mechanism of APAP-induced cell death (Figure 1). Promoting these adaptive responses will only be effective in cells that do not experience the most severe insults of reactive metabolite formation, oxidant stress and mitochondrial dysfunction, i.e. are located further away from the central vein area [28]. The less severe

insults and the potential delay in the cell death processes allow these adaptive mechanisms to be activated and be able to prevent cell death mainly at the periphery of the necrotic areas [28]. However, because these surviving cells are the ones eventually dividing and replacing the necrotic cells, autophagy and especially mitochondrial biogenesis are critical for the recovery and regeneration [76] and thus could be a relevant drug target.

4.2. Promotion of liver regeneration and recovery

Although the mechanisms of liver regeneration after drug-induced liver injury are ambiguous, several studies highlight the importance of the timely onset of regeneration to facilitate liver recovery after APAP overdose [30]. Timing of DNA replication seems to be an autonomous process [77], while the transition of hepatocytes that have replicated their DNA into mitosis seems to be controlled by the circadian rhythm [78]. Additionally, increase in alpha-fetoprotein (AFP) expression, which is a marker for dedifferentiated hepatocytes, correlates with increased survival of APAP overdose patients [33]. Thus, stimulation of liver regeneration seems to be an evident therapeutic strategy important for recovery after APAP overdose. However, the therapeutic window for intervention targeting liver regeneration must be thoroughly evaluated. Liver regeneration is modulated by multiples survival pathways. After hepatocyte necrosis, DAMPS trigger a sterile inflammation [21,22], which involves generation of cytokines and chemokines causing infiltration of inflammatory cells like neutrophils and monocytes into the liver [21,22]. Although there is still somewhat of a controversy in the literature on whether sterile inflammation aggravates APAP-mediated injury or is only involved in removing necrotic hepatocytes and promoting regeneration, a number of recent reports indicate that the APAP-induced innate immune response is mainly beneficial [21,22]. Both resident Kupffer cells and infiltrating monocyte-derived macrophages are involved clearance of cell debris and promotion of repair [79,80]. The phenotype of the freshly recruited monocytes is generally pro-inflammatory (M1; Ly6C^{high}) but changes rapidly to the pro-regenerative phenotype (M2; Ly6C^{low}) [81]. This conversion of the macrophage phenotype can be induced by cytokines such as IL-6 [82] and neutrophil-derived reactive oxygen species [83]. Thus, the modulation of this sterile inflammation could be a therapeutic target; however, it needs to be kept in mind that an excessive inflammatory response has the potential to aggravate the injury. Cytokines like TNF- α , IL-1 α/β and IL-6 induce many signaling pathways resulting in hepatocyte priming and DNA replication after APAP overdose [30]. At the same time, cell cycle progression to mitosis is driven by important growth factors like hepatocytes growth factor (HGF), epidermal growth factor (EGF) or transforming growth factor- α (TGF- α), which bind to MET and EGF receptors (EGFR) to activate MAPK [34]. However, as the use of an inhibitor of EGFR showed, EGF seems to have a dual role in APAP hepatotoxicity by both promoting liver injury and regeneration [84]. Recent studies have also indicated that delayed activation of the adenosine A2B receptor by the guidance cue netrin-1 modulates the APAP-induced innate immune response to facilitate liver recovery and regeneration [85]. Delayed pharmacological induction of the A2B receptor could thus be a therapeutic approach to induce recovery after APAP hepatotoxicity (Figure 1).

5. Medicinal plant extracts as therapeutics against APAP overdose

Medicinal plant usage has been part of human culture for centuries because they are efficacious against different pathologies. The World Health Organization (WHO) classifies medicinal plants and recommends their safe use as a treatment for many diseases [86]. Consequently, medicinal plants have attracted a lot of interest due to their chemical diversity which could help discover new therapies [9]. In fact, plants are composed of several major bioactive compounds including lignans, saponins, flavonoids, polyphenols, furocoumarins, glycosides, anthraquinone, terpenoids, tannin resins, alkaloids proteins and peptides [87]. Therefore, many countries spend tremendous efforts developing drugs based on the medicinal properties of plants which are thought to be safer than chemically designed drugs. More specifically, the Chinese natural products industry focused on identifying plant extracts with beneficial effects against APAP hepatotoxicity [7,8]. For instance, many extracts tested against APAP toxicity in the herbal product field claim to protect via CYP2E1 inhibition. Indeed, goldenseal extract was recently shown to inhibit CYP enzymes including CYP1A2, CYP2D6, CYP2E1, and CYP3A [88]. Additionally, extracts from Moutan Cortex [89] and *Sasa veitchii* [90] were found to inhibit cytochrome P450 2E1 activity. Yet, the limitation of these studies is the fact that the extracts used are a mixture of bioactive compounds confounding mechanistic analysis, and that they use less relevant APAP toxicity models in a number of cases [15]. Based on these limitations, there is insufficient evidence for advancing any of these natural products towards a potential clinical trial at this time.

Considering the efficacy of NAC in targeting GSH depletion, this has also been a focus from the natural product field and several natural herbal extracts claim to protect against APAP toxicity by modulating intrahepatic GSH levels [91]. Unfortunately, in these cases the experimental design makes it difficult to determine if these extracts maintain the intrahepatic GSH pools or promote GSH synthesis. The pretreatment of Ajoene's herbal extract may actually modulate GSH by blocking the metabolic activation of APAP, thus preventing NAPQI formation. This results in the perceived maintenance of the intrahepatic GSH pool, which is a limitation of the pre-treatment protocols used [91]. Additional issues arise from a mechanistic standpoint, where the interventions target mechanisms which have been proven to be irrelevant to APAP hepatotoxicity. For example, sylibin, which is a flavonoid, has been claimed to protect rats against GSH depletion and lipid peroxidation (LPO) [92]. However, LPO is not a relevant mechanism of APAP hepatotoxicity [93] and rats are not a relevant model as mentioned earlier [15]. Based on the lack of mechanistic understanding in these studies, it appears that targeting GSH synthesis with natural herbal extracts is not a productive endeavor and it is unnecessary to develop a drug to compete with NAC since it is very efficient at replenishing GSH in humans [4]. From the perspective of interrupting oxidative stress and targeting the Nrf2/Keap1 pathway, an increasing number of herbal extracts studies claim to enhance antioxidant properties that protect against APAP overdose by inducing the Nrf2/Keap1/ARE antioxidant response [8,94].

Many investigations of natural products also claim that the herbal extracts have anti-inflammatory properties [8,95]. Again, in almost all cases it is not clear whether the effects observed by these extracts have a direct or only an indirect effect on inflammation. In the latter case the therapeutic effect may be upstream (i.e. CYP2E1 inhibition) and the reduced

injury attenuates the sterile inflammatory response. In either case, based on the current concept of a beneficial role for sterile inflammation [21,22], anti-inflammatory herbal products would be expected to exacerbate APAP hepatotoxicity and delay regeneration. Thus, given the existing complexity of the cellular and molecular signaling pathways for hepatocyte survival after APAP overdose, caution must be exercised when interpreting anti-inflammatory effects by herbal extracts. Other popular protective mechanisms of natural products invoked are stimulation of autophagy [96] and inhibition of apoptosis [97]. During APAP toxicity, autophagy can limit injury by removing damaged mitochondria [75] and adducted proteins [74] and it can be a reasonable mechanism of protection by various compounds [73]. In contrast, the frequently claimed anti-apoptotic properties of natural products is questionable as there is no relevant apoptotic cell death during APAP overdose [98].

Overall, numerous studies published beneficial effects of herbal extracts or their individual components against APAP overdose in animals and suggested the potential for these compounds to be clinically useful. However, most of the discussed mechanisms and the clinical application are questionable. First, most studies used pretreatment of these compounds, which questions the clinical relevance in an overdose scenario. Second, the generally applied high doses of the extracts are unrealistic for human use, especially when taken over a longer period of time. Third, the most frequently applied experimental design of pretreatment with the extract and then administering a single overdose of APAP and assessing numerous parameters at a single time point is flawed as APAP-induced liver injury is a time-dependent process that involves many different signaling events, which need to be evaluated at the appropriate time. Most importantly, the potential effect of an herbal product on drug metabolism and formation of NAPQI needs to be investigated in particular with multi-component extracts. Any interference at this early step of the toxicity will effectively reduce or totally prevent liver injury and normalize all other parameters. If that occurs, only selective drug administration after the metabolism of APAP is complete will allow any conclusions regarding additional protective mechanisms. Fourth, most studies only show correlations between drug treatment and injury but do not test causality. The most prominent example is inflammation, which strictly depends on the initial injury. It is not justified to conclude that a compound has anti-inflammatory properties when there is reduced liver injury and less cytokine production. It requires additional selective interventions against inflammation to support the conclusion that the compound protected because it inhibited inflammation, especially because inflammation is more likely to be beneficial [21,22]. Thus, whereas numerous studies demonstrate beneficial effects of herbal extracts or individual compounds against APAP toxicity, the clinical usefulness is questionable at this time because of pre-treatment and the doses used, and the mechanisms of protection are rarely justified.

6. Expert opinion

Substantial progress has been made over the last decades in elucidating many aspects of the mechanisms of acetaminophen hepatotoxicity. However, despite the identification of numerous potential therapeutic targets, NAC is still the only clinically approved antidote. A major obstacle for the development of new drugs is the cost versus the number of patients

benefiting from the new treatment. One solution to this problem is the repurposing of existing drugs, which may have mechanisms of action that are protecting against APAP toxicity. An example of such a re-purposed drug is fomepizole (4-methylpyrazole), which is an approved antidote against methanol and ethylene glycol poisoning. Fomepizole has been shown to effectively protect against APAP-induced liver injury in mice by inhibiting both Cyp2E1 and JNK [45,55]; the Cyp2E1 inhibition has also been verified in human hepatocytes [44] and in healthy volunteers [46]. In addition, the safety of the compound is well established [99]. Although already being used off-label in high overdose patients presenting late [56,57], a controlled phase III clinical trial is necessary to document the efficacy of fomepizole for this indication. However, the limitation of using re-purposed drugs is that these drugs are generally off patent and thus it can be more challenging to find sponsors for the clinical trials. Another approach is the parallel development of a drug for 2 or more indications including APAP toxicity. This is being attempted currently with calmagafodipir, a SOD mimetic, which is investigated for its potential to reduce nerve damage during chemotherapy [100] and in APAP overdose patients [63]. This splits some of the development cost, but it also requires that the non-APAP indication is successful. Although no relevant side effects were observed in a clinical evaluation [63], at this point, there is limited information about the detailed mechanism of protection by calmagafodipir *in vivo* and there is no evidence for clinical efficacy yet. Again, a controlled phase III clinical trial is needed for this drug to move forward.

Calmagafodipir and fomepizole are the only drugs that are seriously considered as additional clinical antidote against APAP hepatotoxicity (Figure 1). Numerous other compounds, especially in the herbal medicine field, are being evaluated in preclinical models of APAP toxicity and it is mostly concluded that the compounds with their alleged mechanistic targets may be potential drugs against APAP-induced liver injury in patients. However, as discussed in this review, there are many concerns. In order for even one of these numerous compounds investigated in these studies to have a realistic chance of getting close to the clinic, much more rigorous mechanistic investigations are needed. This includes the assessment of the actual mechanisms, which are the cause of the protection, and not just conclusions based on correlations of randomly selected signaling parameters with injury. It also needs to be recognized that some mechanisms, e.g. inflammation, can have both detrimental and beneficial effects. Thus, even if the sterile inflammatory response would cause some aggravation of the injury, the recruited neutrophils and monocytes are certainly involved in regeneration and recovery, which raises the concern that an anti-inflammatory compound may cause more harm than benefit. Also, some of the intervention strategies that protect against the injury, e.g. NAC-induced GSH synthesis involved in scavenging oxidant stress, may impair recovery at later time points [6]. Importantly, all compounds that are considered for potential clinical use as an antidote need to be tested when given after APAP not as a pretreatment. Taken together, if any herbal extract or individual compound will have even a remote chance to be considered clinically, the experimental design of the preclinical studies needs to be dramatically improved to more rigorously investigate the mechanism of protection and potential adverse effects.

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Article highlights

- Increased understanding of acetaminophen hepatotoxicity reveals promising new therapeutic targets.
- Metabolic activation by CYP2E1 and mitochondrial dysfunction with oxidant stress and peroxynitrite formation are key events in acetaminophen-induced cell death.
- Fomepizole (4-methylpyrazole) protects by inhibiting CYP2E1 and attenuating the mitochondrial oxidant stress by inhibiting c-jun N-terminal kinase.
- Mn-SOD mimetics Mito-Tempo and calmagafodipir protect by preventing peroxynitrite formation.
- Both fomepizole and calmagafodipir need a randomized phase III clinical trial to demonstrate efficacy in acetaminophen overdose patients.
- Herbal plant extracts provide a large number of potential antidotes, but the mechanisms of action and the therapeutic potential remains to be investigated.

Figure 1:

Therapeutics can target various steps in the APAP pathophysiology: APAP hepatotoxicity is initiated by formation of the reactive metabolite NAPQI by cytochrome P450 mediated activation in microsomes. Elevated NAPQI depletes hepatic glutathione stores and forms mitochondrial protein adducts resulting in enhanced superoxide release into the cytosol. This activates the MAP kinase JNK, which translocates to mitochondria and amplifies the mitochondrial oxidative and nitrosative stress. This ultimately activates the mitochondrial permeability transition (MPT) resulting in release of endonuclease G (EndoG) and apoptosis

inducing factor (AIF) into the cytosol and their translocation to the nucleus. The resulting nuclear DNA fragmentation induces hepatocyte necrosis and release of damage associated molecular patterns (DAMPs). This initiates a reparative innate immune response by activating resident Kupffer cells, which release cytokines and chemokines to attract circulating neutrophils into the necrotic foci to remove dead cells and activate regeneration. Additional mechanisms of recovery include activation of the Nrf2 pathway resulting in its disengagement from its cytosolic partner Keap 1 followed by nuclear translocation and induction of genes such as those involved in replenishing hepatic GSH content. Stressed cells could also activate mitochondrial biogenesis to compensate for lost mitochondrial function and attempt to recover from the liver injury. Interventions can target various steps of this pathway, with 4-methylpyrazole inhibiting both cytochrome P450 mediated NAPQI formation as well as downstream JNK activation. Calmangafodipir is thought to function as a SOD mimetic to prevent mitochondrial oxidant stress, while activators of the A2B receptors enhance the innate immune response to accelerate recovery. Enhancement of mitochondrial biogenesis by treatments such as SRT1720 also facilitate this process of recovery after APAP-induced acute liver injury. This figure includes templates from Servier Medical Art, which is licensed under a Creative Commons Attribution 3.0 generic license; <https://smart.servier.com>.