Mistaken identity: Paracetamol induces amino acid starvation through mimicry of tyrosine and changes ubiquitin homeostasis
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2019

document version
Publisher's PDF, also known as Version of record

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Chapter 5
Summary, conclusions and future perspectives
Summary

Paracetamol (acetaminophen, APAP) is a widely used analgesic and antipyretic drug and generally considered safe at therapeutic concentrations. However, due to its hepatotoxic potential, availability and presence in many medical formulations, it is worldwide a major cause of acute liver failure (Yoon et al. 2016). The hepatotoxicity is mainly caused by the action of the toxic metabolite NAPQI that is formed in the liver by cytochrome (CYP) P450 enzymes. NAPQI is normally detoxified by glutathione (GSH), but APAP overdose causes GSH depletion, making the liver more vulnerable to covalent binding to proteins and oxidative stress that leads to mitochondrial damage and cell necrosis (Bessems and Vermeulen 2001; Jaeschke et al. 2012). APAP can also cause acute kidney damage (Stollings et al. 2016) and this toxicity is also reported following therapeutic doses (Kato et al. 2014). Side effects at therapeutic doses are very rare but can be life-threatening, such as severe skin damage like Steven-Johnson syndrome and toxic dermal necrolysis (Khawaja et al. 2012; Biswal and Sahoo 2014). In recent years, several large cohort epidemiological studies reported that prolonged maternal APAP use could interfere with normal development of newborns and increase the chance for ADHD, asthma, male infertility and autism symptoms later in life (Jensen et al. 2010; Snijder et al. 2012; Henderson and Shaheen 2013; Brandlistuen et al. 2013; Liew et al. 2014; Thompson et al. 2014; Sordillo et al. 2015; van den Driesche et al. 2015; Avella-Garcia et al. 2016; Ystrom et al. 2017). Although APAP has been used as analgesic and antipyretic agent for many decades, its pharmacological target is not entirely clear. APAP is considered a selective COX-2 inhibitor due to its mild anti-inflammatory effect. However, several studies provided evidence of inhibition of COX-1 as well (Graham et al. 2013). Besides its analgesic and antipyretic properties, APAP has been shown to interfere with the psychological state by reducing feelings of empathy (Durso et al. 2015; Mischkowski et al. 2016). Despite substantial evidence of involvement of CYP-metabolism in APAP toxicity, not all toxic effects can be explained by the action of NAPQI and other toxic metabolites. It has been reported that APAP can cause toxicity before oxidative stress occurs and before glutathione depletion (Prill et al. 2016), and in systems without expressed CYP-enzymes (Jensen et al. 1996; Srikanth et al. 2005; Miyakawa et al. 2015).
The toxicity caused by the parent APAP is a largely unexplored area. Therefore, we designed our research to study genes and pathways involved in APAP toxicity without metabolism to NAPQI. For this purpose, we used yeast S. cerevisiae, a unicellular eukaryotic organism without CYP-enzymes capable of APAP metabolism, but with essential biological processes that are highly conserved within all eukaryotes, with the ultimate goal to translate the findings to humans.

In the first part of Chapter 1, we provide a general introduction on APAP, its pharmacology, metabolism and its toxicity with the special emphasis on NAPQI-dependent and NAPQI-independent toxicity. Furthermore, we provide a detailed overview of using yeast as a model organism to study drug-induced toxicity mechanisms. Yeast is the first eukaryote being entirely sequenced, approximately 80% of its genes being functionally characterized and complete deletion strain libraries of all non-essential genes are commercially available. Over the years, many toxicological studies have been performed in yeast and they provided a wealth of useful information and further understanding of toxicity mechanisms of drugs and other chemicals (Welsch et al. 2003; Wu et al. 2004; Zhou et al. 2009; Dos Santos and Sá-Correia 2011; van Leeuwen et al. 2012; Nijman 2015; Segovia et al. 2017). In the second part, we describe relevant biological processes involved in drug-induced toxicity, with particular emphasis on ubiquitin homeostasis, stress responses regulated by ubiquitination, amino acids sensing, cellular signaling and uptake and nutrient starvation response.

The main aim of this thesis was to study in yeast the link between APAP-induced toxicity and genotype and to get more insight into the mechanism(s) of NAPQI-independent APAP toxicity. For that purpose, we performed a chemogenomic screen using a collection of 1522 deletion and DAmP S. Saccharomyces strains, as presented in Chapter 2. We were able to identify 107 APAP-resistant strains and 126 APAP-sensitive strains when compared to the wild-type. Subsequent Gene Ontology analysis allowed us to find enrichment of biological processes that were involved in APAP toxicity, such as ubiquitin homeostasis, regulation of transcription of RNA polymerase II genes and the retrograde signaling (RTG) pathway were associated with APAP resistance, while histone exchange and modification and vesicular transport were connected to APAP sensitivity. The link between ubiquitin levels and APAP toxicity was also observed and explored.
further by showing that ubiquitin deficiency conferred resistance to APAP toxicity, while ubiquitin overexpression resulted in sensitivity. A small-scale screen using a series of deletion strains with ubiquitin deficiency \((mms2\Delta, doa1\Delta\ ubi4\Delta, doa4\Delta, \text{and } ubp6\Delta)\) was used to compare the toxicity profiles of various drugs and other chemicals to those of APAP and its positional isomer AMAP (Bessems and Vermeulen 2001), showing a unique resistance pattern for APAP. Furthermore, exposure of yeast to APAP increased the level of free ubiquitin and influenced the ubiquitination of proteins. Taken together, these results uncovered a role for ubiquitin homeostasis in APAP-induced toxicity.

Ubiquitination is involved in a range of critical cellular processes like DNA repair, cell cycle regulation, proteasomal protein degradation, gene expression, internalization of membrane proteins and drug sensitivity. In the following parts of this thesis, we focused on determining which of these processes were involved in APAP-induced toxicity. In **Chapter 3**, we describe a small scale chemogenomic screen, which was based on DUB deletion strains to compare toxicity profile of APAP to other drugs/chemicals with known modes of action. The screen comprised 19 non-essential DUB strains, as well as \(ubi4\Delta, mms2, \text{rsp5-DAmp}\) and \(doa1\Delta\). A set of DUB deletion strains was tested for sensitivity and resistance to a diverse series of drugs and other chemicals, including APAP, quinine, ibuprofen, rapamycin, cycloheximide, cadmium, peroxide and amino acids and a cluster analysis was performed. The toxicity profile of APAP was distinct from drugs/chemicals that cause oxidative stress, DNA damage and translational inhibition and similar to drugs and chemicals that cause a nutrient starvation response and degradation of the amino acid permease Tat2. Most DUB deletion strains showed an altered growth pattern when exposed to these compounds by being either more sensitive or more resistant than wild-type, making them suitable for chemogenomic profiling. Toxicity profiling of the DUB strains revealed a remarkable overlap between the amino acid tyrosine and APAP, but not its isomer AMAP. Furthermore, co-exposure of cells to both APAP and tyrosine showed an enhancement of the cellular growth inhibition, suggesting that APAP and tyrosine have a similar mode of action.

In **Chapter 4**, we studied whether APAP can cause a nutrient starvation response. During nutrient starvation, high affinity amino acid permeases are
Summary, conclusions and perspectives

degraded while at the same time the general amino acid permease Gap1 is upregulated, which is a hallmark of the nutrient starvation response (Beck et al. 1999). Therefore, we investigated the effect of APAP exposure on the expression levels of amino acid permeases. We showed that the protein levels of high affinity amino acid permeases Tat2, Tat1, Mup1, and Hip1 were reduced upon APAP exposure, while the expression of the general permease Gap1 was increased, which is consistent with a nutrient starvation response, Chapter 4, Figure 1. We also showed that mainly aromatic amino acids were involved in the induction of this nutrient starvation response, i.e. overexpression of Tat1 and Tat2 (aromatic amino acid transporters), but not Mup1 (methionine transporter), Hip1 (histidine transporter) and Gap1 (general amino acid transporter) conferred resistance to APAP. Furthermore, a tryptophan auxotrophic strain trp1Δ was more sensitive to APAP than wild-type, and addition of tryptophan completely restored the growth restriction of trp1Δ upon APAP exposure, while tyrosine and phenylalanine had an additive effect on APAP toxicity. Addition of other amino acids did not have a significant impact on cell growth upon APAP exposure. We also developed an HPLC-based method to determine intracellular levels of all amino acids upon APAP exposure. APAP exposure caused a decrease in intracellular concentrations of most amino acids, except Glu, Gln, and Gly, which were upregulated during the nutrient starvation response. This effect was less prominent in APAP-resistant ubiquitin-deficient yeast strains that showed a reduced degradation of high affinity amino acid permeases. Finally, we showed a similar decrease in intracellular amino acid concentrations in human hepatoma HepG2 cells. Particularly aromatic and other essential amino acids were reduced indicating an impaired amino acid uptake and potential significance for humans.

In Chapter 5 we provide a summary and conclusions of the research described in this thesis, as well a discussion of our key findings and the perspectives of our work. The key findings are summarized in Figure 1.
Chapter 2: Toxicity screen and GO analysis

- Ub-deficiency confers APAP resistance
- Ub-overexpression confers APAP sensitivity

Chapter 3: DUB chemogenomic screen

- APAP and Tyr identical chemogenomic profiles
- APAP similarity with drugs/chemical that cause nutrient starvation

Chapter 4: APAP causes nutrient starvation response

- Degradation of high affinity AAs and upregulation of general AAP Gap1
- APAP causes Trp starvation
- Additive toxicities of APAP, Tyr and Phe
- APAP causes decrease in essential amino acid levels in yeast and HepG2

Figure 1. A summary of key findings with reference to the chapters in this thesis.
Conclusions and future perspectives

The objective of this study was to study genes and pathways that are involved in APAP-induced toxicity notably due to the parent drug. Until now, APAP-induced toxicity is usually related to its reactive metabolite NAPQI, which causes depletion of GSH, covalent binding to proteins and oxidative stress in the liver during an overdose. However, a limited number of studies observed toxicity and oxidative stress before GSH depletion occurred and also in systems/cells, which did not express metabolic Cyt P450 enzymes (Jensen et al. 1996; Srikanth et al. 2005; Miyakawa et al. 2015; Prill et al. 2016). The underlying mechanisms of this parent APAP toxicity remained unclear. To study toxicities, which are unrelated to APAP metabolism and attributed to the action of the parent APAP, we used baker’s yeast as the model organism because of the absence of oxidative APAP metabolizing enzymes, its simplicity, and high functional conservation with all eukaryotic cells. The final goal was to translate the findings in yeast to humans. The availability of a genome-wide gene deletion library allowed us to screen for genes and pathways involved in APAP-induced toxic in a high-throughput fashion and to perform chemogenomic profiling by comparing the toxicity profile APAP to other drugs, chemicals and amino acids. The genome-wide screen described in Chapter 2 showed a connection between APAP-induced toxicity and ubiquitin levels in yeast. The stress response was mediated by processes regulated by ubiquitination, in particular processes necessary to achieve the nutrient starvation response, such as vacuolar degradation of membrane proteins, the RTG pathway and achievement of growth arrest. Chemogenomic profiling of different drugs, other chemicals and amino acids, described in Chapter 3, revealed a similarity in toxicity profiles between APAP and drugs/chemicals that can cause nutrient starvation response and an almost identical profile similarity with the amino acid tyrosine, which was attributed to their chemical structure similarity. The follow up in Chapter 4, revealed that APAP was causing degradation of high-affinity amino acid permeases and increased the expression of the general amino acid permease Gap1, which are the hallmarks of a nutrient starvation response. This effect was absent in ubiquitin deficient strains, which correlated to APAP resistance. Finally, we showed that APAP caused a decrease in intracellular concentrations of most essential amino acids in yeast and,
importantly, also in the human hepatoma cells HepG2 indicating the relevance of these findings for humans.

In Chapter 2, we studied genes and pathways involved in APAP-induced toxicity and identified ubiquitin homeostasis genes. Protein ubiquitination is an essential process in the regulation of many cellular processes during normal cell growth and stress responses, and it is highly conserved between all eukaryotic cells. Therefore, ubiquitin and ubiquitin homeostasis genes are often identified in chemogenomic deletion screens to be essential for survival during stress such as DNA damage, oxidative stress and heat shock. In the case of APAP, we observed the opposite: ubiquitin deficiency and deletion of a variety of ubiquitin homeostasis genes was correlated with resistance. For example, strains doa1Δ, doa4Δ, ubi4Δ and ubp6Δ, which are involved in the maintenance of free ubiquitin levels and ubiquitin recycling, were uniquely resistant only to APAP, Chapter 2, Figure 5. Furthermore, we identified ubiquitin homeostasis genes to play a role in APAP-induced toxicity, such as subunits of E3 ligase complexes APC/C (APC4 and CDC23) and SCF (CDC34 and UCC1), which are involved in cell cycle progression regulation; HRD1, E3 ligase required for ERAD degradation of misfolded proteins; E2 conjugating enzymes Mms2 and Ubc13 involved in error-free postreplication repair; E3 ligase Upf1 and Upf3, which are components in a nonsense-mediated mRNA decay (NMD) pathway; BMH2 involved in regulation of endocytosis, vesicle transport and Ras/MAPK signaling; Rsp5 E3 ligase, Doa1 and Doa4 required for vacuolar degradation and MVB pathway and deubiquitinase Ubp6 that is involved by proteasomal degradation, Chapter 2, Table 2. These findings suggest that, following changes in the ubiquitin homeostasis, APAP could influence directly and indirectly a variety of cellular pathways.

When studying drug-induced toxicity, polymorphisms of drug metabolizing enzymes and transporters are often considered. Thus, it was shown that different genetic polymorphisms cause altered CYP-activities making some individuals susceptible to drug-induced toxicity through changed metabolic rates (Dong et al. 2018). In our studies, we showed CYP-metabolism independent APAP toxicity with altered ubiquitin homeostasis, suggesting that the parent APAP could influence regulation, expression and activity of a broad variety of genes and, in
combination with genetic polymorphisms, could cause varying responses and outcomes in different individuals. NSAIDs can have a significant impact on ubiquitin-proteasome by causing proteasomal dysfunction and accumulation of abnormal ubiquitinated proteins, and a decrease of free ubiquitin as it was shown for several NSAIDs, such as diclofenac (Ghosh et al. 2016b; Amanullah et al. 2017), ibuprofen (Upadhyay et al. 2016), indomethacin (Amanullah et al. 2018), and meclofenamate and naproxen (Ghosh et al. 2016a). In this regard, APAP seems to be different from the NSAIDs mentioned above, as we do not observe accumulation of ubiquitinated proteins, but rather the opposite with an increase in free ubiquitin, Chapter 2, Figure 4. Interestingly, altered ubiquitination patterns upon APAP exposure have been reported previously. For example, it was shown that both APAP and AMAP inhibited ubiquitin-mediated degradation of rat CYP3A (Santoh et al. 2016) as well as degradation of tumor suppressor p53 by APAP (Lee et al. 2006).

Reliable models for drug-induced toxicity prediction are essential during preclinical drug discovery and development. In recent years, a variety of complex in vitro and in vivo models was developed, such as humanized cell systems and 3D- and tissue culture models (Edmondson et al. 2014; Naritomi et al. 2018). However, these systems are expensive, laborious and require sophisticated equipment, such as imagers and high-throughput analysis. In Chapter 3, we describe a chemogenomic screen based on a collection of yeast DUB deletion strains, which was designed to determine toxicity mode of action of APAP. It was shown that a small-scale screen could also be informative to predict a drug’s adverse mode of action. The DUB deletion strains were tested for sensitivity and resistance with a diverse series of compounds and the toxicity profiles were compared to APAP. We were able to cluster different drugs successfully based on their toxicity profiles and uncovered the similarity in toxicity profiles between APAP and drugs that can cause nutrient starvation response and an almost identical profile similarity with the amino acid tyrosine. Furthermore, we showed different toxicity profiles for various drugs and similar profiles between drugs that have a similar mode of action such as arsenic and cadmium (heavy metals); AMAP, a regioisomeric APAP, quinine and ibuprofen (Trp uptake inhibition); rapamycin and FTY720 (nutrient starvation response); benomyl and peroxide
(oxidative stress and DNA damage), Chapter 4, Figure 5. Interestingly, APAP and DNA damaging agent MMS showed opposite toxicity profiles, Chapter 3, Figure S2, implicating that the genes crucial for survival during MMS treatment, will lead to APAP resistance, indicating their role in the achievement of growth arrest.

The DUB screen in Chapter 3 and the follow-up in Chapter 4 revealed the importance of aromatic amino acids in APAP-induced toxicity. The trp1Δ strain was highly sensitive to APAP toxicity and addition of tryptophan rescued growth, while a surplus of tyrosine or phenylalanine had an additive effect on growth reduction. These findings and the structural similarity between APAP and tyrosine lead to the conclusion that APAP is causing inhibition of tryptophan uptake through mimicry of tyrosine. Furthermore, we showed that APAP caused ubiquitine-dependent degradation of high affinity amino acid permeases and increased expression of the general amino acid permease Gap1, which are the hallmarks of a nutrient starvation response. This effect was absent in ubiquitin-deficient strains, where degradation of Tat2 did not occur, indicating that less ubiquitin correlated with higher Tat2 levels and APAP resistance. Finally, we showed that APAP caused a decrease in intracellular concentrations of almost all amino acids in yeast and also in human hepatoma cells HepG2 indicating the relevance of these findings for humans.

Drug-induced nutrient starvation is not unique for APAP as it has been shown that different drugs can cause amino acid starvation and subsequent nutrient starvation response. For example, the anti-malaria drug quinine inhibits tryptophan uptake causing Tat2 amino acid permease degradation and tryptophan starvation in yeast (Khozoie et al. 2009). Subsequent research revealed similar inhibition of tryptophan uptake in malaria patients (Islahudin et al. 2012). The TOR pathway was first discovered in yeast as a target for the anti-cancer drug and immunosuppressor rapamycin. Rapamycin induced degradation of high affinity permeases and subsequent nutrient starvation response (Beck et al. 1999). These findings led to the discovery of the mTOR pathway in humans (Brown et al. 1994; Sabatini et al. 1994; Sabers et al. 1995) and showed a similar effect of nutrient starvation, whereby tryptophan starvation occurs by TORC inhibition (González and Hall 2017). Two other immunosuppressors, FK506 and FTY720, also induced degradation of amino acid permeases and tryptophan
starvation in yeast (Heitman et al. 1993; Schmidt et al. 1994; Welsch et al. 2003). The mechanisms of drug-induced nutrient starvation response were suggested to be competitive inhibition of tryptophan uptake through structural similarity (quinine, ibuprofen) or inhibition of the TOR pathway (rapamycin). APAP likely shows a similar MoA as quinine and ibuprofen with respect to Trp uptake/depletion.

Malnutrition is often identified as a risk factor for APAP-induced toxicity (Kurtovic and Riordan 2003; Claridge et al. 2010), but the underlying mechanisms are not well understood. A correlation between caloric intake and APAP toxicity and hepatic metabolism in obese patients during food restriction could not be shown (Schenker et al. 2001). However, our studies show a direct link between nutrition and APAP toxicity exemplified by amino acid availability. Imbalances in amino acid availability (deficiency or excess) can cause a nutrient starvation response (Beck et al. 1999; Nikko and Pelham 2009). Our data suggest that mM concentrations of APAP are mimicking an excess amount of tyrosine, thus causing tryptophan deficiency by competitive inhibition and subsequent degradation of high affinity amino acid permeases. Competitive inhibition is possible because amino acid transporters are transporting several amino acids. For example, Tat1 transports Leu, Tyr, Phe, Val, Ile and Trp, while Tat2 transports Trp, Tyr and Phe. An excess amount of an amino acid will consequently cause inhibition of uptake of other amino acids. Amino acids that are present in the cell at low concentrations, such as Trp, Tyr, Phe or Met, are particularly susceptible for competitive inhibition by compounds structurally similar with these amino acids, i.e., tryptophan and quinine (Khozoie et al. 2009). When cells are grown in poor media with APAP, the toxicity is achieved at much lower concentrations (30 mM) than in rich media (70 mM). Of note is that both the rich and poor media contained the same amount of glucose (10g/l). In the case of APAP, it still needs to be determined at which concentration the competitive inhibition takes place, whether the concentration dependent degradation of amino acid transporters takes place in humans, and whether amino acid transporters are responsible for APAP uptake into the cell. A model of competitive inhibition of aromatic amino acids uptake is presented in Figure 2.
The observation that a drug can mimic an amino acid is interesting to be considered in studying and prediction of drug-induced toxicity. Drugs showing structural similarity with aromatic amino acids, such as the phenol moiety in APAP, quinine, and ibuprofen, could cause competitive aromatic amino acid uptake inhibition. This structural feature should rather be recognized and generally applied in drug-induced toxicity prediction of drugs that are used in high dosages. Such drugs are more likely to cause adverse effect, which are often not well understood (Chalasani and Björnsson 2010). APAP, quinine, and ibuprofen belong to this category of drugs with limited daily dosages of 4 x 1000 mg, 9 x 200 mg, and 4 x 800 mg, respectively. Our data suggest that drug-food interactions could have a significant effects on drug-induced toxicity as seen with the opposing roles for tyrosine and tryptophan in APAP toxicity. The effect of amino acids on APAP toxicity remains to be explored in humans. Similarly, the additive effect of amino acids and drugs on toxicity should be tested for drugs that can cause nutrient starvation, such as ibuprofen and quinine.
APAP exposure and subsequent degradation of amino acid permeases resulted in reduced intracellular amino acid levels in yeast but also in human HepG2 cells. In yeast, which lacks metabolic CYP-enzymes, we observed toxicity of APAP that is unrelated to CYP-related metabolism. HepG2 cells (unlike primary human hepatocytes) also have a low CYP-enzyme activity. For comparison, expression levels of CYP2E1 and CYP3A4 (enzymes involved in APAP metabolism) have been reported to be ~ 50- and ~ 200-fold lower, respectively, in HepG2 cells when compared to human primary hepatocytes (Gerets et al. 2012). Moreover, the incubation time in our experiments with HepG2 cells was only 2 hrs, which is insufficient for significant APAP metabolism (Kyriakides et al. 2016). Therefore, the observed amino acid reduction in HepG2 cells is also considered to be due to APAP and not APAP-metabolites. The strongest decrease in amino acid levels in HepG2 cells was measured for Trp, Met, Leu, Val, Phe, Ile, Tyr and Lys, which are all essential amino acids, indicating that the decrease in amino acid levels is caused by uptake inhibition and not by impaired biosynthesis. Of note, the APAP concentrations we used to treat HepG2 cells were physiologically relevant (10 and 20 mM) and often used to study APAP toxicity in human hepatocytes (Utkarsh et al. 2016; Sison-Young et al. 2017).

In yeast, Tat1 and Tat2 transporters are responsible for uptake of aromatic amino acids. In humans, the transporter Tat1 is responsible for the uptake of aromatic amino acids (Palego et al. 2016). Based on the structural similarity between APAP and tyrosine, competitive inhibition of aromatic amino acid uptake could also be the mechanism behind the reduced amino acid levels observed in HepG2 cells. It is not the first time that findings in yeast are translated in human: Trp uptake inhibition by quinine was also first discovered in yeast (Khozoie et al. 2009) and subsequently confirmed in malaria patients (Islahudin et al. 2012).

When measuring intracellular levels of all amino acids upon APAP exposure in yeast and human hepatoma cells HepG2, we observed a decrease in almost all amino acid levels, except for glutamine, glutamate and glycine, which were increased. Interestingly, a metabolomic analysis of mouse liver samples showed an increase in cellular glutamate, while 6h post-treatment GSH and its precursor methionine were depleted (Kyriakides et al. 2016). The increase in glutamate and
glutamine levels occur during nutrient starvation response (Martíez-force and Benítez 1992; Johnson et al. 2014). Considering that APAP causes amino acid depletion, also of methionine, serine and cysteine that are required for GSH biosynthesis, GSH-depletion could be caused not only by binding to NAPQI but also by an impaired biosynthesis due to amino acid starvation. If this is the case, supplementation with amino acids during APAP-induced toxicity could be beneficial during overdose treatment. In rats, a mixture of Cys, Met and Ser was already tested for their protective role during APAP overdose and showed to be equally if not more beneficial than NAC for the treatment of APAP overdose (Di Pierro and Rossoni 2013). NAC and Cys also have a protective thiol group to inactivate NAPQI directly.

During nutrient starvation, yeast cells shift from fermentative to respiratory growth (TCA cycle). A shift to the TCA cycle is regulated by the retrograde signaling pathway and RTG genes, four of which (RTG1, RTG2, RTG3, and MSK1) we identified during the genome-wide screen to confer resistance to APAP upon deletion, Chapter 2, Table 1. Several studies described metabonomic profiles of mammalian cells during APAP treatment showing increased rates of glycolysis and impaired β-oxidation (Coen et al. 2004), a shift taking place during nutrients deprivation (Wu et al. 2013). The shift to glycolysis is regulated by highly conserved AMP kinase (SNF1 complex in yeast). In the genome-wide screen, we identified deletion strains of genes encoding components of the SNF1 complex to confer resistance to APAP, and in mammalian hepatocytes AMPK activation prevented and reversed APAP-induced mitochondrial and hepatocellular injury (Kang et al. 2016). All together, this indicates that nutrient availability may play a significant role in drug-induced toxicity.

It was previously also observed that risk to APAP toxicity is higher in individuals with malnutrition (Michaut et al., 2014), however, the mechanism is still remaining unclear. Additional experiments are necessary to determine whether nutritional status will influence APAP toxicity in vitro and in vivo. Similar experiments as in yeast could be designed for human hepatocytes, in which several toxicity markers can be determined in cells treated with APAP in a medium supplemented with different amounts of amino acids. Similar experiments could also be designed for in vivo studies. A recently established
defective mice tat1−/− model (Mariotta et al. 2012) could be used to determine whether addition of Tyr and Phe would make the mice more susceptible to APAP-induced toxicity. Moreover, it would be interesting to determine whether and to which extent, APAP causes amino acid starvation in humans and whether the addition of Trp would have a beneficial effect upon initiation or treatment of acute APAP overdose.

Amino acids are precursors for the biosynthesis of various bioactive compounds, neurotransmitters and hormones, such as dopamine, serotonin, melatonin, NAD and kynurenine (Trp), thyroid hormones (Tyr) and Glu, a neurotransmitter for the GABA receptor and several others (Osawa et al. 2011; Palego et al. 2016). Therefore, inhibition of tryptophan uptake and other amino acids could potentially impact various cellular processes in humans, Figure 3. Serotonin is an important neurotransmitter that regulates adaptive responses to environmental changes in human CNS, such as mood, behavior, cognition, libido, feeding behavior and body temperature. Peripherally, especially in the gastrointestinal tract, serotonin regulates intestinal movements, the immune and inflammatory responses and blood stem cells differentiation (Richard et al. 2009; Palego et al. 2016). Accordingly, altered serotonin levels have been associated with mood-affective disorders, autism and cognitive deficit, eating disorders and other syndromes presenting peripheral symptoms, such as fibromyalgia, chronic fatigue syndrome and irritable bowel syndrome (IBS) (Palego et al. 2016). Peripheral serotonin is also involved in the hepatic physiological and pathological process and it was shown to have cytoprotective properties during liver injury (Ruddell et al. 2008). A recent in vivo study on APAP-induced hepatotoxicity showed that mice with sufficient serotonin levels (wild-type mice) had a significantly lower mortality rate and a better outcome compared with mice deficient of peripheral serotonin (tph1−/- mice), which was partially attributed to a decreased level of inflammation, oxidative stress response and GSH depletion (Zhang et al. 2015). Since tryptophan is precursor in serotonin biosynthesis, supplementation with tryptophan could potentially influence the outcome during hepatotoxicity.
Several epidemiological studies provided evidence that prolonged use of APAP during pregnancy can result in a higher risk for development of ADHD, autism symptoms and asthma later in life (Källén et al. 2013; Henderson and Shaheen 2013; Liew et al. 2014; Thompson et al. 2014; Sordillo et al. 2015; Avella-Garcia et al. 2016; Ystrom et al. 2017), and recent studies showed that APAP reduces empathy and weakens emotional reactions (Durso et al. 2015; Mischkowski et al. 2016). Tryptophan, tyrosine, serotonin and dopamine brain levels are altered in children with ADHD and autism (Fernell et al. 2007; Johansson et al. 2011), and lack of empathy is linked with serotonin and dopamine deficiency (Siegel and Crockett 2013; Gong et al. 2014), suggesting that the amino acid uptake inhibition by APAP could be the underlying mechanism. In individuals already suffering from certain diseases, such as Parkinson’s patients (dopamine deficiency) or from clinical depression (low serotonin levels), this could also have a significant adverse effect. Tryptophan is particularly important in pregnancy for both mother and fetus, as they need Trp and its kynurenine metabolites for many purposes such as increased protein synthesis, signaling pathways, NAD+ synthesis and to protect the fetus against rejection by immunosuppressive K metabolites (Badawy et al. 2016). The serotonin deficiency during pregnancy is

**Figure 3.** Possible links between amino acid starvation/levels and clinical phenomena and by inference possible relevance of APAP-induced amino acid starvation.
also associated with depression during pregnancy and in the months preceding and following pregnancy (Badawy 2015; Badawy et al. 2016). Recent studies also indicate involvement of amino acids in immune response by regulating the activation and proliferation of T and B lymphocytes, natural killer cells and macrophages. Amino acids such as Cys, Gln, Arg, Phe, Leu and Trp have been shown to be essential for T-lymphocytes proliferation and their ability to recognize and fight cancer (Li et al. 2007; Sikalidis 2015).

In conclusion, in this thesis we have shown for the first time that the parent APAP causes a disturbance of the ubiquitin homeostasis as well as amino acid starvation in yeast and human hepatoma cells. The observed toxicity was CYP metabolism and by inference NAPQI-independent. The parent APAP itself causes depletion of tryptophan and other amino acids through the mimicry of tyrosine, thus confirming the importance of aromatic amino acids in APAP-induced toxicity. This is an important step forward in the understanding of the mechanisms of adverse effects of APAP in humans. In order to fully assess the clinical relevance of the present findings, it remains essential to determine whether APAP indeed causes altered and/or reduced amino acid levels in humans and, furthermore, what the consecutive effects are on short- and long-term toxicities of this widely used analgesic and antipyretic agent.

References


