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

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 (*mms2Δ*, *doa1Δ ubi4Δ*, *doa4Δ*, and *ubp6Δ*) 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 *ubi4Δ*, *mms2*, *rsp5-DAmP* and *doa1Δ*. 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

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**.

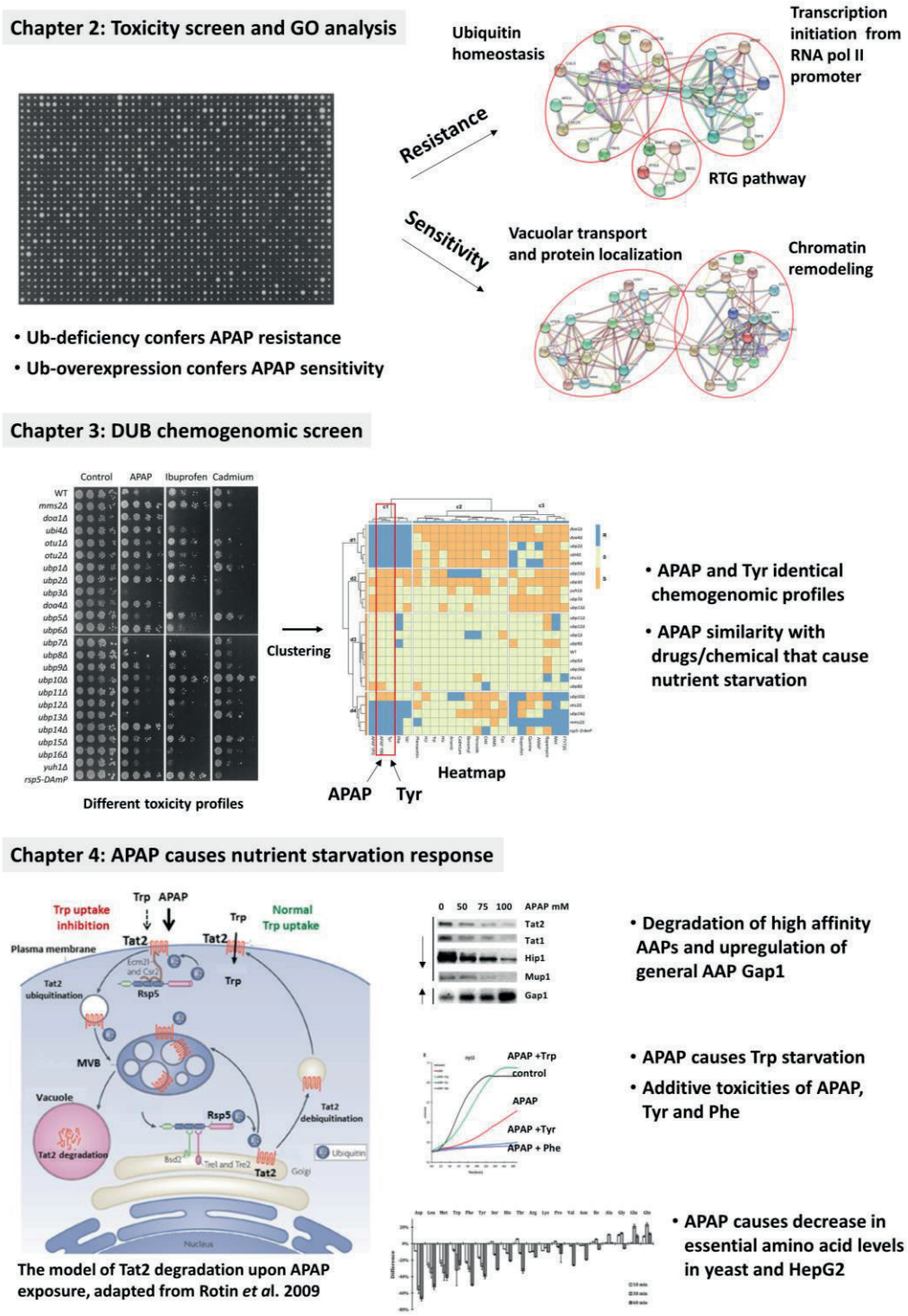

Figure 1 . A summary of key findings with reference to the chapters in this thesis.