Yeast as a model eukaryote in drug safety studies

van Leeuwen, J.S.

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CONCLUSIONS AND PERSPECTIVES

In this thesis, we aimed to investigate the utility of yeast as novel model organism in toxicity studies. Advantages of yeast as model organism are its fast growth, cost-efficiency, eukaryotic background, well-annotated genome and genetic accessibility. When this research started, yeast had mainly been used in toxicity studies to investigate genotoxicity, either in the absence or presence of bioactivation. We focused on yeast as a tool in non-genotoxic drug safety studies. We have chosen diclofenac, a frequently used NSAID that is related to several adverse drug reactions, as model drug in these studies. Another reason for choosing diclofenac is that its toxicity is possibly partly due to both bioactivation as a result of P450 and/or UGT activity and mitochondrial damage. A second aim of this thesis was therefore to investigate the mechanistics underlying diclofenac toxicity.

Summary of the results

We have shown that diclofenac inhibits mitochondrial respiration by interference with subunits Rip1p and Cox9p of the respiratory chain (Fig. 1). Subsequent leakage of electrons from the respiratory chain leads to generation of reactive oxygen species (ROS), causing decreased growth and cell death. Metabolism of diclofenac by model cytochrome P450 BM3 M11 further reduced growth and increased ROS levels. Primary metabolites like 4'- and 5-hydroxydiclofenac, however, were not toxic in yeast either in absence or presence of BM3 M11. Hydroxydiclofenac-derived quinone imines were identified in the BM3 M11-expressing cells by detection of their glutathione conjugates, thus suggesting that the generation of diclofenac quinone imines is not responsible for the increased toxicity. Alternative explanations might be that arene oxides or radical species formed during metabolism are causing toxicity.

Additionally, we studied the mechanisms of adaptation to diclofenac toxicity. Using microarray analysis we found that in particular pleiotropic drug resistance genes and genes under the control of Rlm1p, a transcription factor in the protein kinase C pathway, were upregulated in diclofenac-adapted cells. Genes involved in ribosome biogenesis, rRNA processing and zinc homeostasis were downregulated. We found that diclofenac causes flocculation and thereby probably induces cell wall stress. Furthermore, it possibly lowers intracellular zinc concentrations by zinc chelation. The major pathway of adaptation was found to be active transport of diclofenac out of the cell by Pdr5p. Finally, we investigated if these proteins and processes are also involved in the toxicity of related NSAIDs, all either acetic acid or propionic acid derivatives. We found great variation in the mechanisms underlying the toxicity of these structurally related drugs and divided the NSAIDs into three classes based on the involvement of mitochondrial dysfunction, oxidative metabolism and ABC transporters in their toxicity.

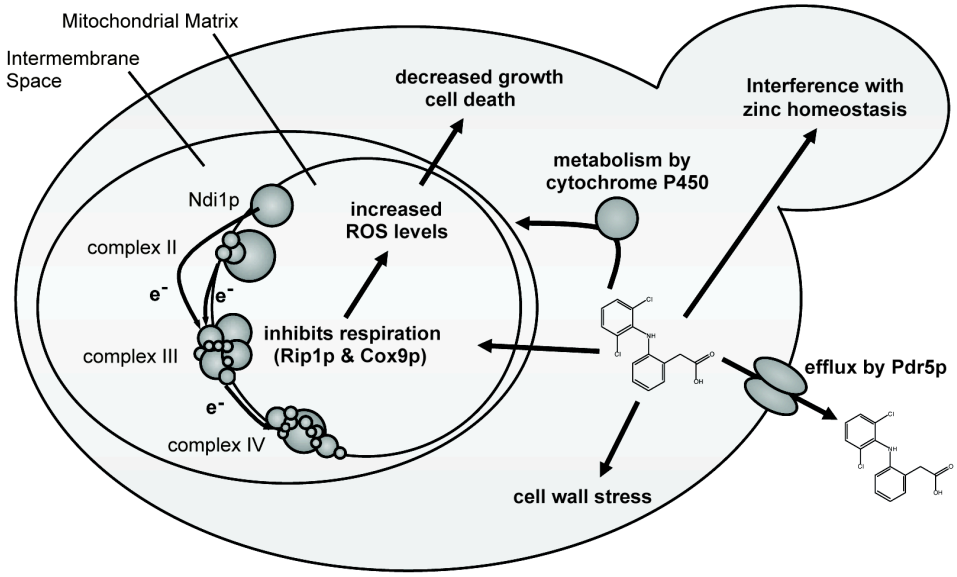

Fig. 1. Mechanisms of diclofenac toxicity described in this thesis. Inside the mitochondria, diclofenac inhibits respiration by interference with subunits Rip1p and Cox9p of the respiratory chain. This leads to increased levels of reactive oxygen species, causing growth inhibition and cell death. Metabolism of diclofenac by cytochrome P450s further enhances ROS levels and increases toxicity. Additionally, diclofenac induces cell wall stress and can possibly lower intracellular zinc concentrations. The major pathway of detoxification is active transport of diclofenac out of the cell by the ABC-transporter Pdr5p.

Mitochondrial toxicity of diclofenac and related NSAIDs

In the past years, increased attention has been drawn to mitochondrial dysfunction as major cause of drug-induced organ failure (Labbe et al., 2008; Nadanaciva and Will, 2009; Tujios and Fontana, 2011; Wallace 2008). Xenobiotics can interfere with mitochondrial respiration by inhibiting electron transport or cause leakage of the electrons, thereby generating reactive oxygen species (ROS) that can induce apoptosis. They can also uncouple oxygen consumption from ATP production, affect fatty acid ß-oxidation or tricarboxylic acid oxidation or interfere with mtDNA replication, transcription or translation. Mitochondrial impairment typically affects the most highly respiring tissues such as the kidney and the heart. Additionally, liver failure is often reported due to the high exposure of the liver to xenobiotics. The multiple organ failures described after NSAID-use fit this profile (Fosbol et al., 2009; Lafrance and Miller, 2009; Laine et al., 2009). Indeed, diclofenac causes decreased ATP formation and ROS production in hepatocytes (Gomez-Lechon et al., 2003; Lim et al., 2006; Masubuchi et al., 2002a). Also other NSAIDs have been related to mitochondrial dysfunction *in vitro* (Moreno-

Sanchez et al., 1999). In our research, we tried to identify the primary cause of diclofenac-induced mitochondrial toxicity in *Saccharomyces cerevisiae*. Importantly, the mitochondrial respiratory complexes II, III and IV are highly conserved among eukaryotes (Lemire and Oyedotun, 2002; Taanman and Capaldi, 1992; Zara et al., 2009). In **Chapter 2** we showed that diclofenac induced mitochondrial dysfunction by interference with subunits Rip1p and Cox9p of the respiratory chain. In **Chapter 5**, we demonstrated that Rip1p and Cox9p also dominate the toxicity of indomethacin and ketoprofen. We speculate that diclofenac and related NSAIDs can bind at one of the complex III ubiquinone binding sites, thereby inhibiting respiration and inducing leakage of electrons. Electron leakage results in ROS production that has a negative impact on growth and viability of the yeast cells. In the absence of Rip1p, the ability of diclofenac to generate ROS is lost, as has also been described for hypoxia-induced ROS in yeast (Guzy et al., 2007). Interestingly, *RIP1* is highly conserved across eukaryotes (Beckmann et al., 1989), and downregulation of the mammalian homologue of Rip1p, RISP (UQCRFS1), also reduced hypoxia-induced ROS production (Guzy et al., 2005). This suggests that Rip1p is also involved in diclofenac-induced mitochondrial dysfunction in mammalian cells. Its relevance in adverse effects in patients has yet to be determined, but elucidation of their RISP sequences would be highly interesting. Cox9p is a small (7 kDa) subunit of complex IV, located at the outside of the complex and is essential for complex IV activity. Although Cox9p is structurally similar to its mammalian counterpart COX6c, sequence homology is limited. Since little is known about the function of Cox9p and it is only partially conserved, the human relevance of this protein in diclofenac toxicity is unclear. Further research is required to confirm our Rip1p hypothesis, to verify the role of RISP and possibly COX6c in diclofenac toxicity in mammalian systems and to understand the role of Cox9p in respiration and diclofenac toxicity. For example, it would be interesting to see whether deletion of *RIP1* or *COX9* also affects toxicity of known complex III or IV inhibitors.

High-throughput screening for mitochondrial toxicity

Incorporation of routine screens for mitochondrial toxicity in the drug-development process is strongly advised (Dykens and Will, 2007). Because the wide occurrence of mitochondrial toxicity only became apparent in the past decade, most assays for mitochondrial toxicity detection are still low-throughput. Several high-throughput assays have been developed in recent years, but many of them use isolated mitochondria (Hynes et al., 2006; Nadanaciva et al., 2007). Only few cell-based assays have been developed. For example, comparing toxicity of a drug on cells that differentially rely on respiration for their energy production provides information on mitochondrial dysfunction (Gohil et al., 2010; Marroquin et al., 2007). Most cell-based high-throughput screens use fluorescent or luminescent markers to measure NADH conversion, ATP levels, mitochondrial membrane potential or ROS formation (for example Schoonen et al., 2005). However, each dye has its disadvantages and changes in fluorescence

can be a result of a variety of processes including pH changes and cell death. A set of yeast deletion strains could provide an efficient high-throughput screening tool for mitochondrial toxicity. An initial screen using WT and rho^0 strains could indicate whether mitochondrial respiratory toxicity occurs, while a set of strains as we used in **Chapter 2 and 5** could be used to identify the position of interference with the respiratory chain. Additional strains could be added to screen for interference with other mitochondrial processes. However, since yeast lacks a complex I, but instead contains single-protein NADH dehydrogenases (Luttik et al, 1998), possible effects on complex I activity are likely missed. Furthermore, upregulation of multidrug resistance genes in certain mitochondrial deficient strains might complicate interpretation of the results, although these genes could be deleted easily as well. Nevertheless, since many mitochondrial disease genes are conserved in yeast (Steinmetz et al., 2002), it may contribute to identification and understanding of mitochondrial toxicities and focus subsequent drug sensitivity studies in mammalian cells.

Oxidative metabolism-related toxicity of diclofenac

Metabolism by cytochrome P450s can yield reactive metabolites that may cause toxicity (Park et al., 2011). In hepatocytes and hepatic cell lines P450 inhibitors can reduce diclofenac-induced release of LDH and ROS-formation (Bort et al., 1999; Kretz-Rommel and Boelsterli, 1993; Lim et al., 2006). However, the metabolites involved remain unknown. In **Chapter 3**, we investigated the metabolism-related toxicity of diclofenac. Strains expressing the model P450 BM3 M11, specifically chosen because of its similar diclofenac metabolite profile compared to human P450s (Damsten et al., 2008), were found to be more sensitive to diclofenac and showed higher ROS levels after diclofenac incubation. Although both 4'- and 5-hydroxydiclofenac and hydroxydiclofenac-derived quinone imines were identified in the yeast cells, either directly or by their glutathione-conjugates, they appeared not to be toxic in yeast. However, this can be different in mammals, since quinone imines can cause protein adducts that may lead to immune reactions (Naisbitt et al., 2007). In yeast, the metabolism-related increase in toxicity is likely coupled to other diclofenac metabolites or other reactive intermediates such as arene oxides or radical species (Blum et al., 1996; Grillo et al., 2008; Masubuchi et al., 2002b). In **Chapter 5** we showed that also indomethacin, ketoprofen and naproxen cause P450-related toxicity. For diclofenac, indomethacin and ketoprofen oxidative decarboxylated metabolites have been identified (Grillo et al., 2008; Komuro et al., 1995). During oxidative decarboxylation reactive carbon radicals are formed that may cause toxicity. In future research, P450 BM3 mutants with an altered diclofenac metabolite profiles could be applied to identify the metabolite responsible for the enhanced toxicity in yeast.

The increased ROS-formation by diclofenac metabolism can be caused by mitochondrial interference of diclofenac metabolites, in a similar way as we found for unmetabolized

diclofenac in **Chapter 2**. Mitochondrial dysfunction specifically induced by metabolites and not by the parent drug has been suggested for acetaminophen and aromatic antiepileptic drugs (Bessems and Vermeulen, 2001; Santos et al., 2008). However, in a preliminary experiment using rho^0 yeast cells that lack mtDNA, diclofenac metabolism still increased ROS formation, suggesting that the mitochondria are not the source of metabolism-related ROS (our unpublished results). Also in hepatocytes, mitochondrial dysfunction is mainly associated with the parent drug instead of diclofenac oxidative metabolites (Lim et al. 2006). Alternative sources of ROS exist in the cell, such as fatty acid ß-oxidation. Growth of BM3 M11 expressing yeast cells on fatty acids in the presence of diclofenac might indicate if ß-oxidation is the source of ROS. ROS can also be formed by uncoupling of BM3 M11, however, in an *in vitro* experiment using purified BM3 M11 diclofenac did not enhance uncoupling or ROS formation (our unpublished results). Finally, the metabolism-related increase in ROS can be a result of cell death, since it is mainly visible at concentrations where cell growth is decreased. A genome-spanning collection of BM3 M11 expressing deletion strains could be generated with techniques like synthetic genetic analysis (SGA) (Tong and Boone, 2006) and applied to investigate the mechanisms of metabolism-related toxicity.

Yeast expressing multiple drug biotransformation enzymes

Additionally, we have successfully combined BM3 M11 expression with expression of human sulfotransferase SULT1A1 in yeast (our unpublished results). Others have reported co-expression of mammalian CYPs with an epoxide hydrolase, glucuronosyltransferase UGT1A6, N,O-acetyltransferase or glutathione-S-transferases (Black et al., 1990; Ikushiro et al., 2004; Kelly et al., 2002; Paladino et al., 1999) showing the potential of combining drug metabolizing enzymes. New techniques for fast deletion of multiple genes in yeast could also be applied for insertion of human metabolic enzymes (Suzuki et al., 2011). Combined with a sophisticated mating and sporulation set-up, this will allow creation of strains expressing all possible combinations of phase I and phase II enzymes. These will provide a valuable tool in future studies in identification of enzymes and metabolites involved in toxicity. Since in the past decade increasing attention has been drawn to studying the interplay of absorption, disposition, metabolism and elimination (ADME), additional combinations with human drug transporters can be made. This can lead to a multifunctional test system to study the interplay between biotransformation enzymes and transporters and provide a human-relevant ADME-related toxicity test system. Similar strategies can yield yeast-based high-throughput screening systems including bioactivation, dedicated to the screening of pharmacological activities.

Adaptation of yeast to diclofenac

In **Chapter 4** we describe the observation that yeast cells can adapt to diclofenac toxicity. Using microarray analysis combined with yeast genetics, we tried to identify pathways and

genes involved in diclofenac resistance or toxicity. Although many studies have applied microarray analysis to examine the response of cells to a compound of interest, transferability of transcriptionally altered genes to genes showing a growth phenotype is often limited (Giaever et al., 2002; Zakrzewska et al., 2010). By using fully adapted strains for transcriptional analysis, we removed initial lethality responses from the results and linked several significantly enriched motif-groups and GO-categories to diclofenac toxicity. This approach may also prove usefull for target identification in studies with other drugs or drug candidates.

We identified multidrug transporter Pdr5p, zinc homeostasis and cell wall stress as major contributors to diclofenac tolerance or toxicity (Fig. 1). Although the human relevance of diclofenac-induced cell wall stress is probably limited, zinc depletion may also occur in patients. Shoji et al. (1993) reported a rapid decrease in serum zinc levels in patients after diclofenac administration. Administration of zinc also markedly reduced gastrointestinal and renal damage caused by diclofenac or indomethacin in rats (Abou-Mohamed et al., 1995; Varghese et al., 2009). Possibly diclofenac can chelate zinc and thereby lower intracellular zinc concentrations. Further studies are needed to identify the precise role of zinc in diclofenac toxicity. A start would be to directly measure the intracellular zinc concentrations directly after diclofenac addition and during adaptation, although this could be complicated since only part of the zinc is freely available in the cytosol.

Adaptation is not something exclusive to yeast. Patients can develop transient elevations of transaminase-levels that subsequently normalize despite continued drug treatment (Stirnimann et al., 2010). Adaptation in mammals may also include upregulation of multidrug transporters, as has been described for adaptation to acetaminophen in mice (Aleksunes et al., 2008). Upregulation of ABC multidrug transporter Pdr5p seems to be the major event in diclofenac adaptation in yeast. Interestingly, a murine homolog of Pdr5p, BCRP1 (ABCG2) can efficiently transport diclofenac *in vitro* (Lagas et al., 2009). In **Chapter 5** we showed that Pdr5p is also involved in resistance to ketoprofen, while multidrug transporter Snq2p plays a role in indomethacin, ketoprofen and sulindac detoxification. Interestingly, all NSAIDs upregulate the pleiotropic drug resistance response in yeast, leading to upregulation of the multidrug resistance transporters, except ibuprofen. Interestingly, ibuprofen also failed to enhance MDR1 expression in human cells (Takara et al., 2009). PDR-regulators in yeast (Pdr1p/Pdr3p) and in mammals (PXR) show high functional analogy and can both directly bind a large variety of xenobiotics, thereby inducing expression of multidrug resistance transporters (Kliewer et al., 2002; Thakur et al., 2008). Although both Pdr1p/Pdr3p and PXR are very promiscuous, ibuprofen is apparently unable to interact in such a way to promote MDR induction. In view of the concerns for human health caused by drug resistant pathogenic

microorganisms and cancer cells, it might be interesting to try to understand why ibuprofen cannot induce MDR while structurally related NSAIDs can. Initial experiments could focus on binding of ibuprofen to Pdr1/3p, either directly by binding assays or by studies on competition with diclofenac for Pdr1/3p binding measured as upregulation of Pdr5p.

Classification of NSAIDs based on their toxicity mechanisms

In **Chapter 5**, we investigated the involvement of mitochondrial toxicity, oxidative metabolism and active transport in the toxicity of a group of structurally related NSAIDs. The general 'order' of NSAID toxicity was similar to that found in rat hepatocytes and human epithelial cell cultures (Allen et al., 1991; Jurima-Romet et al., 1994; Masubuchi et al., 1998). Surprisingly, we found great variation in the mechanisms underlying their toxicity. In a study on structurally related imidazo-pyridines and -pyrimidines also great variation in toxicity mechanisms was found, and the relevance of these findings for mammalian cells was confirmed (Yu et al., 2008). Further research will be required to see if the identified differences in NSAID toxicity mechanisms are also relevant in mammalian cells.

The toxicity of class I drugs diclofenac, indomethacin and ketoprofen was clearly related to mitochondrial dysfunction and oxidative metabolism. However, for class II drugs ibuprofen and naproxen we were unable to identify the main target causing their toxicity. Yeast genetic screens could be applied to further study toxicity of these compounds. Since class III drugs were hardly toxic at all to yeast, their toxicity cannot be easily studied in yeast. Possibly, highly sensitized yeast strains that lack almost all ABC-transporters could be of use here.

Yeast as model organism in toxicology

With the increasing demands for safe drugs and chemicals on one side and for a decrease of laboratory animal use on the other side, alternative models to test toxicity are necessary. Extra pressure is applied by the EU REACH guidelines, requiring toxicity profiles of over 30.000 chemicals on a relatively short time scale. Yeast provides a relatively easy, fast and cost-efficient way to determine various major mechanisms of drug toxicity. Various toxicity screens have been developed in yeast, mainly focusing on genotoxicity or estrogenicity (Routledge and Sumpter, 1996; Westerink et al., 2009). Also metabolism-related genotoxicity using heterologously expressed P450s has been extensively studied in yeast (**Chapter 1**). Our results show that bioactivation-competent yeast can also be used to study non-genotoxic mechanisms of toxicity. Additionally, we used yeast genetics to identify multiple diclofenac off-targets. Although we did not yet confirm relevance of our findings in human cells or animal models ourselves, clear indications exist that similar pathways are involved in mammalian toxicity of diclofenac. Especially the involvement of Rip1p in mitochondrial dysfunction, the finding that diclofenac quinone imines do not cause cell death and the role of

zinc homeostasis in diclofenac toxicity are potentially relevant to mammalian cells. Further support for the suitability of yeast is provided in this thesis by the observation that the general 'order' of NSAID-toxicity in yeast is comparable to that in mammalian cells.

Is yeast the solution to the increasing demand for new toxicity models? Probably not by itself, but the ongoing developments (Suk et al., 2011; Suzuki et al., 2011) in genetic techniques allow straightforward deletion of complete yeast signaling pathways and insertion of complete human pathways in wild type strains or in the deletion strain collection. These genetic tools and "humanized" yeast strains, created by over-expression of human biotransformation and transporter genes, will prove very useful tools in toxicity studies and in combination with mammalian cells they can provide unique and critical information on the mechanisms underlying cellular toxicity.

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