Yeast as a model eukaryote in drug safety studies

New insights on diclofenac-induced toxicity

Jolanda van Leeuwen

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Jolanda Susanne van Leeuwen

The research described in this thesis was carried out in the Leiden Amsterdam Center for Drug Research (LACDR), Division of Molecular Toxicology, Department of Chemistry and Pharmaceutical Sciences, Faculty of Sciences, VU University Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

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Yeast as a model organism for biotransformation-related toxicity

OUTLINE

High drug attrition rates due to toxicity, the controversy of experimental animal usage, and the EU REACH regulation demanding toxicity profiles of a high number of chemicals demonstrate the need for new, *in vitro* toxicity models with high predictivity and throughput. Metabolism by cytochrome P450s is one of the main causes of drug toxicity. In this chapter, we will discuss the use of yeast expressing (mammalian) P450s as powerful, additional model system in drug safety. We will start with a brief introduction to the concepts of toxicology, metabolism by cytochrome P450s, cellular toxicity models and yeast as eukaryotic model. Next, we will more thoroughly discuss the various cellular model systems for bioactivation related toxicity and subsequently describe the properties of yeast as model system. Then we will focus on the endogenous bioactivation enzymes present in yeast, the heterologous expression of cytochrome P450s in yeast and the application of yeasts expressing heterologous P450s or other biotransformation enzymes in toxicity studies. We will conclude this chapter with the aims and outline of this thesis. Unless indicated otherwise "yeast" will refer to baker's yeast *Saccharomyces cerevisiae*.

INTRODUCTION

Although new drug candidates are developed continuously, only few compounds make it through development and get approved by the regulatory authorities (Kola and Landis, 2004). In the past 20 years, pharmaceutical industry has focused on investigating absorption, disposition, metabolism, elimination and toxicity (ADME/Tox) of new drug entities to decrease the high drug attrition rate. One of the main reasons for drug candidates to be terminated from development, next to lack of efficacy, is toxicity and clinical safety (Fig. 1) (Kola and Landis, 2004). Despite years of research, accurately predicting human toxicity with either *in vitro* or animal models remains a challenge (Innovative Medicines Initiative, 2006). Clearly, new toxicity models are needed to increase drug safety and decrease toxicity attrition. Additionally, the EU REACH regulation requires toxicity profiles for over 30.000 chemicals, for which highly predictive and high-throughput toxicity models are needed.

Toxicity can depend on the on-target (mechanism-based), an off-target, biological activation, a hypersensitivity/immunological reaction or can be idiosyncratic (Liebler and Guengerich, 2005). The main causes of attrition due to toxicity are biotransformation-related or target-based (Guengerich and MacDonald, 2007). Around 75% of the top 200 drugs used in the US in 2002 are cleared via metabolism (Williams et al., 2004). Although metabolism is mainly involved in detoxification by improving water-solubility and facilitating excretion from the body, it can also render reactive metabolites that may cause protein- or DNA-adducts leading

to cytotoxicity, mutagenicity or carcinogenicty. Therefore, it is advised to test the toxicity of major metabolites during drug development as well (FDA, 2008).

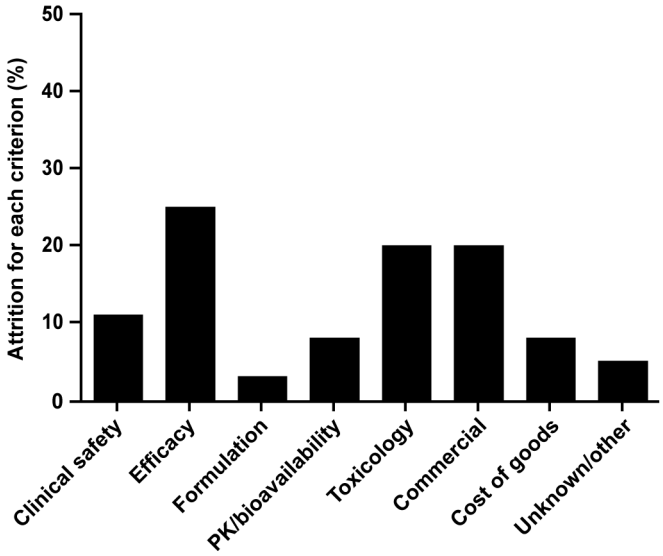

Fig. 1. Reasons for drug attrition in the year 2000. PK: pharmacokinetics. Adapted from Kola et al., 2004.

Around two-thirds of the drugs that are cleared via metabolism are metabolized by cytochrome P450s (Williams et al., 2004). Cytochrome P450s (CYPs, P450s) constitute the main group of phase I metabolic enzymes, consisting of approximately 60 human enzymes that are divided in 18 families. Of these enzymes, only 15 are known to be involved in metabolism of xenobiotics (Guengerich, 2008). CYP3A4 (partly overlapping in activity with CYP3A5), CYP2C9, CYP2C19, CYP2D6 and CYP1A2 (Williams et al., 2004) are together responsible for >95% of P450 drug metabolism. Of these, CYP2C19 and CYP2D6 are highly polymorphic, leading to large differences in pharmacokinetic parameters (Johansson and Ingelman-Sundberg, 2011). The best-studied example of P450 bioactivation-related toxicity is probably the metabolism of acetaminophen by CYP2E1, leading to the reactive quinone imine NAPQI that may cause liver toxicity (Lee et al., 1996). Several P450s are also involved in the activation of pro-carcinogens. Especially CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2E1 and CYP3A4 contribute to carcinogen activation (Guengerich, 2008). A famous example is the bioactivation of aflatoxin B_1 by CYP1A1, CYP1A2 and CYP3A4 into a carcinogenic metabolite (Bedard and Massey, 2006). A second important group of drug metabolizing enzymes consists of the UDP-glucuronosyltransferases (UGTs), belonging to the class of

phase II enzymes and catalyzing conjugation of glucuronic acid to xenobiotics or their metabolites (Williams et al., 2004). Other examples of phase II conjugating enzymes involved in drug metabolism are sulfotransferases and glutathione-S-transferases, catalyzing conjugation of a sulfonate group or glutathione, respectively, to a xenobiotic or metabolite. Conjugation of hydrophilic groups to xenobiotics by phase II enzymes can help in detoxification but can also increase toxicity by creating reactive metabolites, as has been described for glucuronidation of non-steroidal anti-inflammatory drugs (NSAIDs) (Sawamura et al., 2010) or sulfation of benzylic alcohols (Glatt, 2000).

Clearly, it is important to investigate metabolism-related toxicity of drug candidates early in drug development. However, metabolism-dependent toxicity studies are complicated by polymorphisms in drug metabolizing enzymes that cause high variations in metabolic capacity in the population (Johansson and Ingelman-Sundberg, 2011). Additionally, idiosyncratic adverse reactions can occur, that are by definition difficult to predict. Animal models like rats and mice have limited predictability, since species differences are commonly encountered in the expression level, functional activity, and tissue distribution of drug-metabolizing enzymes and drug transporters, leading to altered ADME profiles compared to humans (Tang and Prueksaritanont, 2010). Even if ADME profiles are similar, animal models can at best predict toxicity for an average population. Furthermore, these *in vivo* experiments are time-consuming and expensive to use in early drug development stages. Therefore, cellular model systems to test bioactivation-related toxicity have been developed (Vermeir et al., 2005). Primary hepatocytes obtained from human livers are a popular model since they resemble the liver cells *in vivo*. However, P450 levels decline fast in these cells (see next section). Heterologous expression systems that stably express human P450s have been established in bacteria, yeast and mammalian cells (Friedberg et al., 1999). In these models, the enzymes and metabolites involved in toxicity can be identified and studied in detail.

Yeast expressing (mammalian) P450s is a powerful, additional model system in drug safety. Yeast combines all the advantages of a microorganism in terms of fast growth and straightforward genetics with the characteristics of a eukaryotic cell. Various genetic screens in yeast have led to the identification of drug on- and off-targets (Ho et al., 2011; Smith et al., 2010; Sturgeon et al., 2006). Yeast bioassays such as the yeast estrogen screen and the RadarScreen are widely applied to detect estrogenicity or genotoxicity (Routledge and Sumpter, 1996; Westerink et al., 2009). Also, P450s have been heterologously expressed in yeast to study the enzymology of a particular P450 or for the production of specific metabolites. Also metabolism-related (geno)toxicity has been extensively studied in yeast (Fig. 2). Both the metabolite and the parental drug may affect various cellular processes possibly leading to toxicity. Many bioactivation-dependent toxicity studies in yeast describe the

genotoxicity of the natural toxin aflatoxin B_1 after bioactivation by CYP1A1, CYP1A2 or CYP3A4 (Guo et al., 2005, 2006; Kaplanski et al., 1998; Kelly et al., 2002; Li et al., 2006, 2009b; Sengstag et al., 1996). Yeast cells expressing mammalian CYP1A1, CYP1A2, CYP2B1, CYP2E1 or CYP3A4 were also used in genotoxicity assays for a wide range of other environmental or food contaminants and drugs, including *N*-nitrosodimethylamine, benzo[a]pyrene and the anticancer drug cyclophosphamide (Black et al., 1989, 1992; Del Carratore et al., 2000; Sengstag and Würgler, 1994; Walsh et al., 2005). First, we will focus on the various other cellular bioactivation models before discussing in more detail the properties of yeast as eukaryotic model in biotransformation-related toxicity studies.

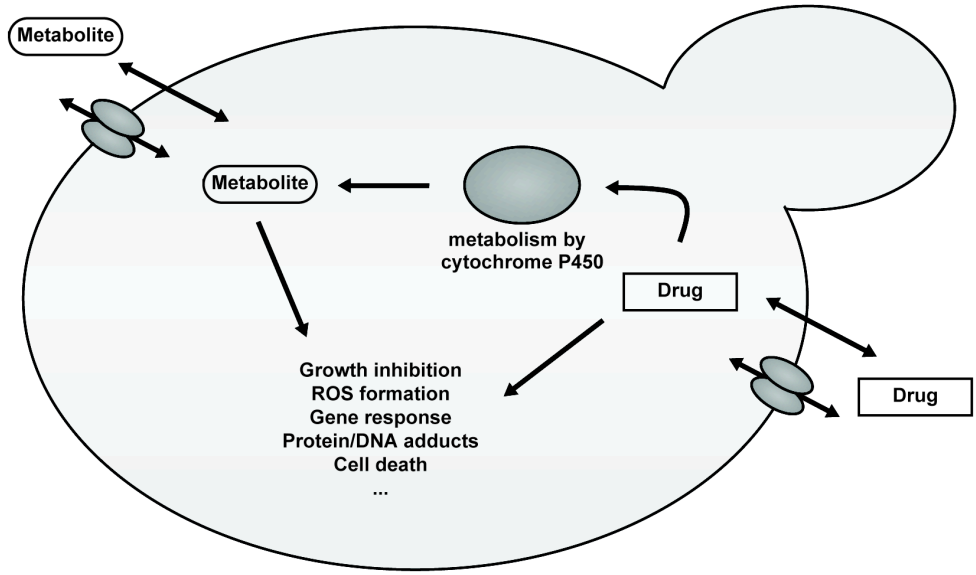

Fig. 2. Yeast expressing mammalian P450s as model in biotransformation-related toxicity studies. Inside the cell, the drug of interest is metabolized by the heterologously expressed P450, yielding a metabolite. Both the drug and the metabolite may affect cellular processes and thereby cause toxicity. Additionally, both the drug and the metabolite may enter or leave the cell either by active transport or via diffusion.

CELLULAR MODEL SYSTEMS FOR BIOTRANSFORMATION STUDIES

Model systems expressing endogenous bioactivation enzymes

As described above, cellular model systems are useful in determination of bioactivation-related toxicity. Friedberg et al. (1999) divided the cellular models for human drug metabolism into “complex systems” that express a complex system of drug-metabolizing enzymes such as hepatocytes and “simple systems” that express only a limited set of enzymes (Table 1).

Model	Remarks
Primary hepatocytes &	+ High resemblance to hepatocytes *in vivo*
Liver slices	+ High endogenous P450 levels
	+ Intact cellular environment of cells in liver slices
	- Limited availability
	- Fast decline P450 levels
	- Use of P450 inhibitors for toxicity studies
	- Cryopreservation decreases GSH and transporter levels
Hepatoma cell lines &	+ Stable P450 expression
Stem-cell derived hepatocytes	+ Human-derived cells
	- Low to moderate P450 expression levels
	- Use of P450 inhibitors for toxicity studies
Cunnighamella	+ Stable P450 expression
	+ Straightforward to use
	- Limited human relevance
	- No isogenic controls
Exogenous activation	+ Stable P450 expression
	+ Straightforward to use
	- Limited uptake and/or stability of formed metabolites
Recombinant *E. coli*	+ Stable P450 expression
	+ Straightforward to use
	+ Low costs
	- Modification of P450s necessary
	- No eukaryotic structures or processes
Recombinant mammalian cells	+ Stable P450 expression possible
	+ Human-derived cells possible
	- Background activity
	- Carcinoma-derived cells can be oversensitive to mutagens
Recombinant *S. cerevisiae*	+ Stable P450 expression
	+ Eukaryotic cellular characteristics
	+ Screens for target-identification
	- High compound concentrations required
	- Not all mammalian cellular targets present

Table 1. Comparison of the various cellular models to study bioactivation related toxicity. Main advantages (+) and disadvantages (-) are indicated. Details are provided in the text.

The advantage of complex models is that they often closely resemble the human liver. However, a limitation of complex cellular models in metabolism-related toxicity studies is the instable and variable expression of cytochrome P450s. The main advantage of simple model systems is the possibility to investigate the role of individual metabolic enzymes in metabolism and toxicity. A general disadvantage of all cellular models is the apparent lack of toxicity of protein adducts that in higher organism may lead to immunological adverse effects.

Of the complex models, primary hepatocytes are currently the model system of choice for metabolism-related toxicity testing, since these cells contain relatively high P450 expression levels and closely resemble hepatocytes in the liver (Gomez-Lechon et al., 2007). However, with hepatocytes either P450 inhibitors or simultaneous experiments with a metabolically incompetent cell line have to be used to study the effect of P450 metabolism on toxicity (Li, 2009a). P450 inhibitors can also affect other cellular processes, such as drug transport and glucuronidation that may also affect toxicity (Raungrut et al., 2010; Wang et al., 2002). Furthermore, P450 expression levels show high variation in primary hepatocytes obtained form different donors (LeCluyse, 2001). Due to problems with availability of primary hepatocytes and the limited time freshly isolated cells can be used, attempts have been made to cryopreserve the cells. Although ongoing developments in cryopreservation of human hepatocytes increased viability in culture from 6h to several days, P450 levels decline around 50% per day in culture, limiting possible exposure times (Li, 2007). An additional disadvantage of cryopreserved hepatocytes is that they contain drastically reduced glutathione (GSH) levels and can internalize transporters (Li, 2007; Sohlenius-Sternbeck and Schmidt, 2005). In a study using human hepatocytes incubated with various CYP-inhibitors and aflatoxin B_1, a CYP1A2 inhibitor failed to show an altered toxicity profile, while in other models such as yeast the role of CYP1A2 in aflatoxin B_1 toxicity has unmistakably been shown and *in vivo* CYP1A2 polymorphisms are clearly associated with aflatoxin B_1 induced hepatocellular carcinoma (Chen et al., 2006; Guo et al., 2005, 2006; Li, 2009a). Besides isolated hepatocytes, also liver slices have been used in bioactivation studies. The main advantage of liver slices is a more integrated cellular tissue architecture mimicking the liver environment *in vivo*. However, also in liver slices P450 levels decline fast, although 24-hour incubations seem feasible (Elferink et al., 2011; Renwick et al., 2000).

Alternative complex model systems are human hepatoma cell lines like HepG2. These cells contain relatively stable levels of many functional phase I and II enzymes, which are lost in most cultured cell lines. However, several main P450s, such as CYP1A2, 2C19 and 2D6, are hardly expressed in HepG2 cells (Ek et al., 2007). New developments using stem cell derived hepatocyte cells or HepaRG cells show improvements in the number of P450s expressed, but expression levels are still low compared to primary hepatocytes (Ek et al., 2007; Guillouzo et

al., 2007). Another disadvantage of these cell lines is that also here P450 inhibitors are needed to test bioactivation related toxicity. Four luciferase-based genotoxicity reporter assays using promoter regions of RAD51C and Cystatin A, and the p53 and Nrf2 responsive elements were developed in HepG2 cells (Westerink et al., 2010). The overall predictivity of the assay was comparable to that of other genotoxicity screens such as the Ames, RadarScreen and Vitotox tests (Westerink et al., 2010). Although the predictivity of genotoxicity for some compounds, such as Ames-negative clastogenic compounds, was higher in the HepG2 assay, for others, especially CYP2B6 and CYP2E1 substrates, the predictivity was lower.

Fungi belonging to the *Cunninghamella* genus express endogenous cytochrome P450s, sulfotransferases and glucuronosyltransferases and can be used as an alternative model for human metabolism of xenobiotics (Amadio and Murphy, 2010). However, although the metabolism in *Cunninghamella* is often roughly comparable to human metabolism of the xenobiotic, only ~25% of the tested compounds yield exactly the same metabolites in both species (Asha and Vidyavathi, 2009). Therefore, most examples in literature on bioactivation studies using *Cunninghamella* are focused on preparation of metabolites or new compounds rather than studying metabolism-related toxicity (Asha and Vidyavathi, 2009).

Models using exogenous activation systems

Other complex models use exogenous activation systems, such as the hepatic S9 fraction of rats, to bioactivate a compound and simultaneously measure cell viability of *E.coli*, yeast or mammalian cells. A sophisticated version of exogenous activation is the MetaChip-DataChip platform (Lee et al., 2008). A single human P450 combined with rabbit NADPH-P450 reductase was spotted together with the test compound and a NADPH regenerating system on the MetaChip. This MetaChip was then stamped on top of the DataChip containing spots of mammalian cells in 3D cultures and after incubating several days cell survival was determined (Lee et al., 2008). Alternatively, a microfluidic device has been applied to simultaneously characterize HLM-generated metabolites by UV detection and test for cytotoxicity on HepG2 cells (Ma et al., 2009). However, metabolites can be too reactive or hydrophilic to penetrate into the cells, as has been described for the epoxide of benzo[a]pyrene (Gautier et al., 1993). Also, in a direct comparison between the two test systems, the number of DNA mutations induced by *N*-nitrosodimethylamine metabolism outside the cell by S9-fractions was about three-fold lower than that obtained by metabolism inside the cell in CYP2E1-expressing yeast cells (Del Carratore et al., 2000).

Models heterologously expressing mammalian bioactivation enzymes

As alternative, many individual CYPs have been expressed in *E. coli*, yeast, and mammalian cells. These simple models can be used as bioreactors to produce high amounts of metabolite

and are useful for characterizing specific steps in the metabolism of a drug, characterizing or identifying inhibitors for the used enzyme, or for identification of the metabolite or enzyme involved in toxicity. Advantages of *E. coli* for heterologous P450 expression are its easy manipulation, lack of endogenous P450s that may interfere, high levels of P450 expression that can be achieved and high yield of P450 protein after purification due to the very high cell densities that can be reached. However, cDNAs of mammalian P450s often have to be modified before they can be expressed in *E. coli*. Especially the hydrophobic membrane anchor is regularly removed, which may affect enzyme properties. Other disadvantages of prokaryotes such as *E. coli* for human P450 expression are the low endogenous heam synthesis, folding problems and the risk of formation of inclusion bodies. Uptake of hydrophobic or bulky xenobiotics in many wild type bacteria is limited due to the relatively impermeable lipopolysaccharide layer found on the outer membrane (Kranendonk et al., 2000). However, several mutations can affect lipopolysaccharide structure and increase permeability (Fralick and Burns-Keliher, 1994). Additionally, since *E. coli* lacks eukaryotic structures and processes, its suitability as model in non-genotoxic bioactivation-related toxicity studies is limited.

The main advantage of P450 expression in transfected mammalian cells is the mammalian cell context, which may be crucial in the evaluation of relevant toxic effects. Sawada and Kamataki (1998) gave an extensive overview of genetically engineered mammalian cells stably expressing P450s, although it is slightly outdated by now. Combined with "omics" techniques, such as transcriptomics, metabolomics or proteomics, mammalian P450-expressing cells can yield valuable information on mechanisms of toxicity (Guengerich and MacDonald, 2007). Furthermore, combining P450 expression with phase II enzyme expression, as has for example been described for CYP2E1 and SULT1A1, allows examination of multiple enzymes acting subsequently in bioactivation or -inactivation (Glatt et al., 2005). However, also here there are some factors to consider. Several standard cell lines are derived from tumor tissue, affecting regular cellular processes such as DNA repair. For example, the V79 and CHO cell lines that are commonly used in metabolism-related genotoxicity assays have a mutated and non-functional p53 protein, causing decreased DNA repair and identification of a high number of false positives in these assays (Chaung et al., 1997; Hu et al., 1999; Knight et al., 2007). Additionally, mammalian cells are slow and rather expensive to grow in large numbers and genetic modification is relatively complicated compared to microbial models. However, mammalian cells are indispensable for verification of the results obtained in other models.

YEAST AS MODEL SYSTEM IN DRUG SAFETY

Yeast as model eukaryote

Saccharomyces cerevisiae is a model eukaryote that is widely used due to its genetic accessibility, cost-effectiveness and rapid growth. The well-annotated genome and various genome-wide screening methods make it a model organism of choice in whole genome assays. At this moment, around 70% of the ORFs contain functionally classified proteins (http://www.yeastgenome.org). Furthermore, ~45% of yeast proteins share at least part of their primary amino-acid sequence with a human protein (Hughes, 2002). Even mammalian genes that lack obvious orthologs in yeast, such as genes involved in angiogenesis, were found to have yeast "phenologs", evolutionary conserved genes that are involved in a different cellular function but show orthologous phenotypes (McGary et al., 2010). Therefore, yeast can for example be used to identify previously unrecognized genes affecting mammalian vasculature formation (McGary et al., 2010).

The determination of drug targets is a very challenging issue (Guengerich and MacDonald, 2007). Yeast has proven to be a convenient and relevant model organism in uncovering drug on- and off-targets. Various reviews have been written on the application of yeast screens and genetic techniques in drug research (Bharucha and Kumar, 2007; Ho et al., 2011; Mager and Winderickx 2005; Menacho-Márquez and Murguía, 2007; Smith et al., 2010; Sturgeon et al., 2006). Valuable tools are the collections of gene deletion strains (Giaever et al., 2002) and overexpression constructs (Magtanong et al., 2011), each covering a large part of the genome. Generally, in these chemogenomic screens, a collection of strains in which expression of a set of genes is altered is incubated with the compound of interest while growth is monitored. Growth can be followed either on plates or in liquid cultures and the set of genes can cover almost the entire genome. Strains showing altered growth profiles indicate the potential involvement of the gene with altered expression in toxicity of or resistance to the compound of interest. As deletion of genes can alter expression of other genes, false positives may be found. Additional techniques to identify drug targets include the yeast two or three hybrid assays, temperature-sensitive mutants, GFP-chimeras, protein chips and microarray analysis. Using chemogenomic assays in yeast, Yu et al. (2008) showed for structurally related imidalo-pyridines and –pyrimidines a differential involvement of mitochondrial dysfunction and DNA damage in their toxicity and confirmed these results in cultured human cells. Other examples for which the relevance of targets identified by yeast chemogenomic screens was shown in human cells are dihydromotuporamine C, a compound that inhibits metastasis, and molsidomine, a drug against angina (Baetz et al., 2004; Lum et al., 2004). Additionally, mitochondrial disease genes are highly conserved among eukaryotes and yeast genetics have been used to study the mechanisms of mitochondrial toxicity of xenobiotics such as paraquat

and diclofenac (Cohemé and Murphy, 2008; Steinmetz et al., 2002; van Leeuwen et al., 2011b).

Yeast is not only useful in off-target identification, but is also a well-established model system for other toxicity studies. Widely used assays for the detection of estrogenic compounds are the yeast estrogen screen (YES) and derivatives thereof (Balsiger et al., 2010; Routledge and Sumpter, 1996; Sanseverino et al., 2005). The assays use yeast strains expressing the human estrogen receptor combined with a reporter containing estrogen response elements fused to a ß-galactosidase or luciferase reporter gene. Upon binding of an activating compound to the estrogen receptor, the receptor will bind to the estrogen response elements thereby inducing transcription of the reporter gene. Yeast is also often used in genotoxicity screens, such as the RadarScreen and GreenScreen in which the DNA damage sensitive *RAD54* promoter is coupled to a reporter like ß-galactosidase or green fluorescent protein (Cahill et al., 2004; Lichtenberg-Fraté et al., 2003; Westerink et al., 2009). Also other DNA damage sensitive promoters such as those of *RNR3* and *HUG1* have been used for the detection of genotoxicity in yeast (Benton et al., 2007; Jia et al., 2002). Johnson & Johnson screened a library of their compounds with the GreenScreen Assay and confirmed that the assay is useful in the identification of genotoxic compounds that are negative in bacterial tests, but positive in mammalian cell tests (van Gompel et al., 2005). Also assays for general cytotoxicity have been developed using the promoter of the housekeeping plasma membrane ATPase gene *PMA1* coupled to a reporter as biomarker (Schmitt et al., 2006). Examples of bioassays where yeast is used as indicator organism are for detection of the food toxin deoxynivalenol (Abolmaali et al., 2008) or to examine the presence of toxicants in wastewater (Keenan et al., 2007).

The main disadvantage of yeast in toxicology studies is the high concentration of compound that is often required to produce a toxic effect, probably due to the barrier presented by the cell wall and the various active efflux pumps. In several of the assays described above, ABC multidrug transporter encoding genes are deleted to increase the sensitivity of yeast to the toxic compound of interest (Abolmaali et al., 2008; Balsiger et al., 2010; Lichtenberg-Fraté et al., 2003; Schmitt et al., 2006). Additionally, since yeast is a unicellular organism it lacks the different cell environments and structures of the various organs in mammals and, on a smaller scale, multicellular properties like gap junctions. Also at the cellular level, yeast does not possess all mammalian features that may affect toxicity. For example, the limited number of biotransformation enzymes in yeast (see below) can affect toxicity of xenobiotics, while missing multi-subunit mitochondrial respiratory complex I might interfere with the detection of mitochondrial toxicants. However, these problems can be partially solved by heterologous expression of the mammalian counterparts in yeast.

Endogenous biotransformation enzymes

Baker's yeast *Saccharomyces cerevisiae* contains only three endogenous P450s, CYP51, CYP56 and CYP61 (Table 2), all involved in housekeeping activities (Cresnar and Petric, 2011; van den Brink et al., 1998). The yeast P450s are associated with high substrate specificity, like most mammalian P450s, but unlike the main mammalian drug metabolizing P450 enzymes. The CYP51 family is found across all kingdoms, whereas CYP61 enzymes are present in fungi and plants and CYP56 enzymes are only found in fungi. The CYP51 enzyme (Erg11p) catalyzes lanosterol 14α–demethylation in the ergosterol biosynthesis pathway in yeast (Aoyama et al., 1989). Specific inhibitors that selectively inhibit fungal CYP51 enzymes over their mammalian counterparts are of considerable importance as antifungal compounds. CYP56 (Dit2p) is a N-formyltyrosine oxidase that catalyzes the production of N,N-bisformyl dityrosine, a component required for spore wall maturation (Briza et al., 1994). The enzyme CYP61 (Erg5p) is a Δ22-desaturase in the membrane ergosterol biosynthesis pathway (Kelly et al., 1997a). Besides having a housekeeping function, the *S. cerevisiae* CYP61 also metabolizes benzo[a]pyrene yielding 3-hydroxybenzo[a]pyrene (Kelly et al., 1997b). To our knowledge, this is the only clear evidence of the evolvement of a cytochrome P450 of *S. cerevisiae* in xenobiotic metabolism. However, several procarcinogens that in mammalian cells require bioactivation to become carcinogenic were found to be genotoxic in yeast (Cahill et al., 2004; Westerink et al., 2009). Since metabolites or involved metabolic enzymes were not characterized, it is unclear if these effects are due to high concentrations of the parent compound or are indeed metabolism-dependent.

Phase II enzymes catalyze the conjugation of a xenobiotic or metabolite to glutathione, glucuronic acid or sulfonate. Yeast does not contain any known genes homologous to glucuronosyltransferases or sulfotransferases. However, yeast contains seven enzymes classified as glutathione transferases (GSTs) (table 2). Grx1p, Grx2p, Gtt1p and Gtt2p show GST activity against the standard GST substrate CDNB (Choi et al., 1998; Collinson and Grant, 2003). All four enzymes protect the cell against oxidative and heat stress and stress induced by xenobiotics (Castro et al., 2007; Choi et al., 1998; Collinson and Grant, 2003; Luikenhuis et al., 1998). Deletion of *GTT2* led to increased toxicity of menadione and decreased levels of the menadione-glutathione conjugate in the medium (Castro et al., 2007). Like the human omega class GSTs, yeast omega GSTs Gto1p, Ecm4p and Gto3p do not show activity towards CDNB but are active against ß-hydroxyethyl disulphide (Garcerá et al., 2006). Several additional proteins, including Ure2p, Mak16p and Yef3p, show some homology to GSTs, although no catalytic GST activity has been observed for these proteins (McGoldrick et al., 2005; Zhang et al., 2008).

Class	Gene	Biological function	Reference
CYP51	ERG11	Biosynthesis of ergosterol	Aoyama et al., 1989
CYP56	DIT2	Biosynthesis of N,N′-bisformyl dityrosine	Briza et al., 1994
CYP61	ERG5	Biosynthesis of ergosterol	Kelly et al., 1997a
-	GTT1	Defense against various stresses	Choi et al., 1998
-	GTT2	Defense against various stresses	Choi et al., 1998
-	GRX1	Defense against various stresses	Collinson and Grant, 2003
-	GRX2	Defense against various stresses	Collinson and Grant, 2003
GST ω	GTO1	Unknown	Garcerá et al., 2006
GST ω	ECM4	Unknown	Garcerá et al., 2006
GST ω	GTO3	Unknown	Garcerá et al., 2006

Table 2. Endogeneous biotransformation enzymes present in *S. cerevisiae*.

Heterologous expression of P450s in yeast

Subsequent to the expression of rat CYP1A1 in baker's yeast in 1985 (Oeda et al., 1985), many other mammalian CYPs have been expressed in *S. cerevisiae*. Since yeast is a eukaryote, it contains an ER membrane and modification processes that resemble those of mammals and expression of full-length P450s is possible without sequence modifications. Also other yeasts, such as *Yarrowia lipolytica* (Fickers et al., 2005) and fission yeast *Schizosaccharomyces pombe* (Peters et al., 2009) have been used for the expression of cytochrome P450s. However, genetic accessibility and annotation of these organisms is not yet as extensive as it is for *S. cerevisiae*. Many P450s have been heterologously expressed in yeast for the purpose of synthesizing a certain metabolite or biological compound. The use of genetically engineered yeast in the synthesis of drug metabolites or other biologically interesting compounds has been extensively reviewed (Dumas et al., 2006; Huang et al., 2008; Lee et al., 2009; Zöllner et al., 2010). Advantages of yeast for this purpose are the possibility of isolated production of key metabolites without interference of other P450s compared to the host organism or mammalian cells and the ease of purification due to an advanced export system of transporters compared to prokaryotes. Disadvantages of yeast for synthetic biology purposes are its lack of subcellular compartmentalization compared to for example the metabolon-vesicles in plants (Winkel, 2004) and the lack of an active import system to increase intracellular substrate concentrations of for example precursor steroids (Zehentgruber et al., 2010). Expression of human CYP2C9 in both fission and baker's yeast yielded gram-scale amounts of the diclofenac metabolite 4'-hydroxydiclofenac (Dragan et al., 2011; Othman et al., 2000). Sophisticated approaches resulted in the heterologous expression of complete biosynthetic pathways, consisting of up to eight enzymes including CYPs, in yeast for the production of complicated biological

molecules such as sesquiterpenes, benzylisoquinoline alkoids or precursors for antimalarial or anticancer drugs (DeJong et al., 2006; Hawkins and Smolke, 2008; Ro et al., 2006; Takahashi et al., 2007).

Yeasts expressing mammalian CYPs have also been used to study bioactivation related toxicity (see next section). Using isogenic yeast strains each expressing a specific P450 or transformed with an "empty" vector as negative control, no P450-inhibitors have to be used and the enzyme responsible for toxicity can be easily identified. Mainly genotoxicity of metabolites has been studied using screens for revertants or point mutations at the locus of a gene required for growth under certain conditions. For example, a standard assay for mutations monitors growth of strains in the presence of the toxic arginine analog canavanine. The uptake of canavanine is solely dependent on the arginine permease Can1p. Therefore, loss-of-function mutations in *CAN1* will lead to increased canavanine resistance and the ability of cells to grow on canavanine is a direct measure for mutagenicity. Furthermore, human mitochondrial CYP11B2 expressing *S. pombe* cells have been used in a whole-cell assay for the screening for CYP11B2 inhibitors for possible treatment of congestive heart failure (Ehmer et al., 2002). Importantly, the inhibitory values found in yeast are consistent with those found in human cells (Ehmer et al., 2002). Also IC_{50} values for inhibition of human CYP17 and CYP21 expressed in fission yeast have been determined (Dragan et al., 2006b). However, in this case the reported IC_{50} values in yeast cells were about one order in magnitude larger than those in human microsomes. Finally, expression of mammalian P450s in yeast has also been used to study CYP degradation (reviewed by Correia and Liao, 2007), localization (Sepuri et al., 2007) and polymorphisms (Hanioka et al., 2010).

With the exception of a few bacterial and fungal fusion-proteins, most cytochrome P450s require a separate reductase partner to provide electrons. ER-bound microsomal P450s primarily receive their electrons from membrane-bound NADPH-cytochrome P450 reductase (CPR) while mitochondrial membrane-bound P450s receive their electrons from a soluble reductase system consisting of adrenodoxin (ADX) and adrenodoxin reductase (ADR) (Omura, 2010). Yeast contains its own CPR; Ncp1p (also known under the alias Cpr1p). Although Ncp1p can donate electrons to mammalian CYPs like 2D6, 2E1, and 3A4, expression of human CPR1 often increases metabolic activity (Cheng et al., 2006; Hawkins and Smolke, 2008; Pompon et al., 1995). Also expression of human cytochrome b_5 may increase human CYP activity in yeast (Hayashi et al., 2000). Additionally, yeast contains an ADR homolog, Ahr1p, that can supply electrons to mammalian mitochondrial CYPs, via mammalian ADX targeted to yeast mitochondria (Dumas et al., 1996; Lacour et al., 1998; Szczebara et al., 2003). Also fission yeast *S. pombe* contains an ADX homolog, Etp1p, that

can function with mammalian mitochondrial P450s and ADR (Bureik et al., 2002; Schiffler et al., 2004) although not as efficiently as mammalian ADX (Hakki et al., 2008).

CYTOCHROME P450-RELATED TOXICITY IN YEAST

Expression of biotransformation enzymes may already lead to toxicity in the absence of a xenobiotic. Although most papers do not report a growth inhibition caused by P450 expression, strong expression of CYP11B2 or CYP21 caused significantly slower growth of fission yeast *S. pombe* (Bureik et al., 2002; Dragan et al., 2006a). In the case of the mitochondrial CYP11B2, this may be due to the formation of structures similar to inclusion bodies between the inner and the outer membrane of the mitochondria (Bureik et al., 2002). Also high expression levels of bacterial P450 BM3 mutant M11 decrease yeast cell growth (our unpublished results). Toxicity related to P450 expression can be dependent on the localization of the enzyme. Mammalian CYP2E1 is located in various organelles, including the ER and mitochondria. Bansal et al. (2010) have altered the targeting of rat CYP2E1 in baker's yeast by mutating the N-terminal signal domain. In the absence of a xenobiotic substrate, mitochondrial-targeted CYP2E1 caused respiratory deficiency, a clear indicator of mitochondrial damage possibly caused by severe ROS formation, while wild type or ER-targeted CYP2E1 did not affect respiratory capacity.

P450 metabolism-related toxicity studies in yeast have mainly focused on the genotoxicity of metabolites. Black et al. (1989) were probably the first to study P450-related toxicity in yeast by expressing rat CYP2B1. When exposed to the anticancer pro-drug cyclophosphamide the mutation frequency, as determined by the development of resistance to the arginine analogue canavanine, increased in a dose-dependent manner over a control strain and was up to 16-fold higher at the highest doses used (Black et al., 1989). Also sterigmatocystin induced mutations in CYP2B1-expressing yeast cells (Black et al., 1992).

Several bioactivation-dependent toxicity studies in yeast use the natural toxin aflatoxin B_1. Biotransformation of aflatoxin B_1 by human CYP1A2 yields a highly unstable epoxide that can form DNA-adducts responsible for the carcinogenic effect of aflatoxin B_1. In wild type yeast cells no aflatoxin B_1 toxicity was observed, while in the strain expressing CYP1A2 dose-dependent reduction of cell survival was seen (Guo et al., 2005, 2006). Also DNA damage was increased in aflatoxin-treated strains expressing CYP1A2, as indicated by an increase in DNA-adducts, chromosomal translocation, minisatellite rearrangements, gene conversion by *trp* revertants, and point mutations by 5-fluoro-orotic acid or canavanine resistance (Guo et al., 2005, 2006; Kaplanski et al., 1998; Kelly et al., 2002; Sengstag et al., 1996). Also human CYP1A1 expression enhanced metabolism-dependent toxicity of aflatoxin B_1 in yeast

(Sengstag et al., 1996). Li and colleagues heterologously expressed carbohydrase genes in yeast cells expressing human CYP3A4 (Li et al., 2006, 2009b). In a fluorimetric microplate bioassay, the carbohydrase activities were quantified as measure of toxicity. Carbohydrase activity is more sensitive to toxicity than cell growth measurements (Engler et al., 1999). The carbohydrase activities were found to be significantly lower, indicating higher toxicity, after aflatoxin B_1 or G_1 incubation in CYP3A4 expressing cells compared to controls transformed with empty vector. Yeast strains expressing CYP1A1 or CYP1A2 were also used to examine the mutagenicity of benzo[a]pyrene-trans-7,8-dihydrodiol, 3-amino-1-methyl-5H-pyrido[4,3-b]indole, benzo[a]pyrene and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (Sengstag and Würgler, 1994). Additionally, homologous mitotic recombination was highly increased in yeast cells expressing rat CYP2E1 incubated with *N*-nitrosodimethylamine, *N*-methylformamide and *N*-ethylformamide compared to cells transformed with an empty plasmid or CYP2E1 expressing cells co-incubated with a CYP2E1-inhibitor (Del Carratore et al., 2000).

A nice example of the advantage of yeast as model system is provided by a study by Guo et al. (2005) who have applied yeast genetics to identify genes involved in metabolism-dependent toxicity. To evaluate the participation of various DNA repair pathways in aflatoxin B_1 toxicity and tolerance, human CYP1A2 was expressed in a series of haploid deletion strains defective in DNA repair or cell cycle checkpoints (Guo et al., 2005). The authors found that nucleotide excision repair, homologous recombination repair, post-replication repair and DNA damage checkpoints are required for the repair of aflatoxin B_1-induced DNA lesions. Several of these pathways are also involved in tolerance to aflatoxin B_1 in mammalian cells (Bedard and Massey, 2006). Additionally, microarray experiments on aflatoxin B_1 treated yeast cells expressing CYP1A2 showed that expression of genes involved in DNA synthesis and repair, cell cycle regulation, or protein degradation and synthesis was significantly altered (Guo et al., 2006; Keller-Seitz et al., 2004).

In the GreenScreen Assay, a plasmid containing the promoter of the DNA damage inducible yeast *RAD54* gene is fused to a gene encoding green fluorescent protein (GFP) (Cahill et al., 2004). Following overnight exposure of the yeast culture to a test substance, the level of GFP fluorescence induction gives a measure of the genotoxicity of the substance. An advantage of this assay is that the whole genome is the target for DNA damage in contrast with reverse mutation assays, which detect DNA damage at a specific locus. Also, reverse mutation assays require more experimental work and time than real-time, high-throughput fluorescence measurements. To improve the applicability of the assay, human cytochrome P450 isoforms were co-expressed in the yeast strain (Walsh et al., 2005). GFP levels were significantly increased in *N*-nitrosodimethylamine, colchicine or cyclophosphamide treated CYP3A4

expressing yeast cells while aflatoxin B_1 increased GFP expression in CYP1A2 expressing yeast cells.

Mammalian CYP expressing yeasts have not solely been used to study genotoxicity. Azole antifungal drugs inhibit fungal CYP51 (Erg11p), resulting in a depletion of ergosterol and an accumulation of 14α-methylated sterols that impair fungal growth. An obvious potential side effect of azoles is the inhibition of human CYP51, which may result in reduced cholesterol synthesis and affect the endocrine system. Parker et al. (2008) replaced native CYP51 by human CYP51 in the yeast genome and thereby created a test system for CYP51-specificity testing of new antifungal drugs. All of the seven azoles tested inhibited cell growth and ergosterol production more severely in yeast CYP51 expressing cells compared to human CYP51 expressing yeast cells.

We have studied the metabolism-dependent toxicity of diclofenac in yeast expressing cytochrome P450 BM3 M11 (van Leeuwen et al., 2011a). BM3 is a cytosolic, bacterial P450 with a coupled reductase domain (Munro et al., 2002). Mutant M11 metabolizes several drugs, including diclofenac, in a similar way as human P450s (Damsten et al., 2008). In the absence of a xenobiotic substrate, yeast cells expressing BM3 M11 had comparable growth and ROS-levels as negative control cells. However, in the presence of diclofenac, expression of BM3 M11 significantly decreased growth and increased ROS levels (van Leeuwen et al., 2011a). The diclofenac oxidative metabolites 4'- and 5-hydroxydiclofenac did not lead to toxicity, indicating that toxicity is either caused by another metabolite or by a reactive intermediate formed during the generation of primary hydroxydiclofenac metabolites. We have confirmed that the metabolism-related toxicity of diclofenac is not caused by genotoxicity (our unpublished results), providing one of the very few examples of non-genotoxic P450-mediated toxicity in yeast. Cellular toxicity is presumably caused by ROS-induced cell death signaling. Interestingly, also the toxicity of the structurally related NSAIDs indomethacin, ketoprofen and naproxen was increased by BM3 M11 metabolism (van Leeuwen et al., 2011c).

(CO-)EXPRESSION OF NON-P450 METABOLIC ENZYMES IN YEAST

Combination of P450s with other bioactivation or detoxification enzymes can provide valuable information on the metabolic pathways involved in toxicity. For example, co-expression of human microsomal epoxide hydrolase reduced the toxicity and mutagenicity of aflatoxin B_1 in both CYP1A1 and CYP1A2 expressing yeast cells (Kelly et al., 2002), thereby providing an elegant example of multistep biotransformation in yeast and strong evidence that epoxide hydrolase is involved in aflatoxin B_1 detoxification.

Glucuronosyltransferases

UDP-glucuronosyltransferases (UGTs) are phase II metabolic enzymes that transfer glucuronic acid to a xenobiotic or its metabolite. This normally increases solubility and thereby facilitates excretion. However, it may also lead to a more reactive metabolite, since glucuronic acid can create an excellent chemical leaving group. Well-known examples of glucuronidation-related toxicity are the carboxylic NSAIDs, where metabolism by UGTs is the main cause of protein-adduct formation leading to immune reactions (Sawamura et al., 2010). Nine human UGTs belonging to the UGT1A, -2A and -2B families have been expressed in fission yeast *S. pombe* for production of glucuronides (Dragan et al., 2010). Expression of the UGTs alone did not produce a growth phenotype, however, co-expression with the cofactor-providing UDP-glucose dehydrogenase decreased biomass yield by 30-50% in the absence of a xenobiotic substrate (Dragan et al., 2010). Mouse and rat UGTs have been expressed in *S. cerevisiae* to study enzyme structure and function (Iwano et al., 1997; Toghrol et al., 1990). Furthermore, human UGT1A6 and B3GAT3 have been expressed in the yeast *Pichia pastoris* for enzyme production and characterization (Lattard et al., 2006; Ouzzine et al., 1999, 2000a, 2000b). Ikushiro et al. (2004) have co-expressed rat CYP1A1 and UGT1A6 in yeast and identified the 7-ethoxycoumarin metabolites 7-hydroxycoumarin and its glucuronide in yeast microsome incubations. However, no toxicity-related studies have yet been performed using UGTs in yeast.

Sulfotransferases

Also sulfotransferases are phase II metabolic enzymes that can both activate and inactivate xenobiotics. For example, sulfation of benzylic alcohols by sulfotransferases leads to toxicity (Glatt, 2000). Human SULT1A3, rat N-deacetylase/N-sulfotransferase-1 (NDST-1) and *Arabidopsis* tyrosylprotein sulfotransferase (TPST) have been expressed in *S. cerevisiae*, all with the purpose of enzyme purification or characterization (Dajani et al., 1999; Komori et al., 2009; Saribas et al., 2004). Additionally, five animal-derived sulfotransferases have been expressed in the yeast *Kluyveromyces lactis* for the biosynthesis of heparin (Zhou et al., 2011). We have successfully expressed human SULT1A1 in combination with cytochrome P450 BM3 M11 in yeast, showing the potential to study a combination of metabolic enzymes (our unpublished results). To our knowledge, so far no toxicity studies using sulfotransferases have been reported in yeast.

Glutathione-S-transferases

Glutathione-S-transferases (GSTs) constitute another group of enzymes that can be involved in detoxification of reactive metabolites by conjugation to glutathione. GSTs also play a regulatory role in cellular signaling by associating with several kinases (Elsby et al., 2003; Sun et al., 2011). Notably, GST function in tumors may decrease the efficacy of anticancer drugs.

This was confirmed by the expression of human GSTA1 or GSTP1 in *S. cerevisiae*, which resulted in a marked decrease in cytotoxicity of anticancer drugs chlorambucil and adriamycin (Black et al., 1990).

Other enzymes

Heterocyclic aromatic amines are potent mutagens found in various food sources. Heterocyclic aromatic amines are mainly metabolically activated by CYP1A2-dependent N-hydroxylation followed by either O-acetylation mediated by N,O-acetyltransferase NAT2 or sulfonation by sulfotransferase. The resulting N-acetoxyesters or N-sulfoxyesters are ultimate carcinogens that readily react with DNA or proteins. In yeast strains expressing both CYP1A2 and NAT2, 2-amino-3-methylimidazo-[4,5-f]quinoline and 2-amino-3,8-dimethylimidazo-[4,5-f]-quinoxaline efficiently induced recombination and mutagenicity, while in absence of one of the enzymes no genotoxicity was observed (Paladino et al., 1999).

CONCLUSIONS

It is apparent that yeast is a useful tool in biotransformation studies. All relevant human P450s involved in metabolism-related toxicity of drugs have been successfully expressed in yeast. These bioactivation-competent strains have been extensively used to study the effect of metabolism on genotoxicity. The few studies on co-expression of P450s with phase II or other human enzymes have shown the potential to study a combination of enzymes in yeast. The power of yeast is that drug metabolism studies can be easily combined with genome-wide screens for on- or off-target identification. The straightforward genetics make it an ideal model for identification and characterization of mechanisms underlying toxicity. Many of the proteins encoded by the yeast genome have human homologs and relevance of the identified targets in yeast for human cells has often been shown. Still, experiments with mammalian cells and animal models are ultimately necessary to determine the relevance of found toxicity mechanisms.

AIMS AND OUTLINE OF THE THESIS

Aims of the thesis

Many new drug candidates never make it to the market. Toxicity is one of the main reasons for drug attrition (Fig. 1) and accurately predicting human toxicity with either *in vitro* or animal models remains challenging (Innovative Medicines Initiative, 2006; Kola and Landis, 2004). Although genotoxicity screens are widely applied during drug development, studies for off-target or biotransformation-related toxicity are less common and these are currently the two main causes of toxicity-related drug attrition (Guengerich and MacDonald, 2007).

Additionally, the EU REACH regulation requires toxicity profiles for over 30.000 chemicals, while restrictions are made on the use of animal models. Clearly, there is a strong need for highly predictive, non-genotoxic toxicity models that can be used in high-throughput set-ups.

As we have described in this chapter, the yeast *S. cerevisiae* is a well-established, powerful model eukaryote suitable for high-throughput analyses. Advantages of yeast as model organism are its fast growth and straightforward genetics combined with the characteristics of a eukaryotic cell (Table 1). The availability of various genetic screens in yeast allows for the identification of drug on- or off- targets (Sturgeon et al., 2006). Many P450s have been heterologously expressed in yeast to study the enzymology of a particular P450 or for the production of specific metabolites. Although yeast has also been used as model in P450-related toxicity studies (Fig. 2), primarily genotoxicity has been studied. The objective of this thesis was to evaluate the use of yeast as model organism in non-genotoxic drug safety studies. We assessed the use of yeast as tool for unraveling cellular toxicity mechanisms and for studying the role of P450s in biotransformation-related toxicity. To achieve bioactivation of model compounds in yeast, cytochrome P450 BM3 mutant M11 was heterologously expressed in the cells. BM3 M11 was selected as it is a highly active, cytosolic P450 that metabolizes various drugs in a similar way as human P450s (Damsten et al., 2008).

The non-steroidal anti-inflammatory drug (NSAID) diclofenac was chosen as model drug in these studies. NSAIDs are drugs that exhibit anti-inflammatory, antipyretic and analgesic properties. Diclofenac is widely used for the treatment of rheumatoid disorders. Presumably, the pharmaceutical efficacy is achieved through inhibition of prostaglandin synthesis by inhibiting cyclooxygenases (Schwartz et al., 2008). Diclofenac use has been connected to rare but severe side effects, including gastrointestinal bleeding and heart, liver and kidney failure (Fosbol et al., 2009; Lafrance and Miller, 2009; Laine et al., 2009; Lewis et al., 2002). Possible causes of the toxicity observed in mammalian cells are mitochondrial dysfunction and oxidative metabolism by cytochrome P450s (Gomez-Lechon et al., 2003; Lim et al., 2006). Also allergic reactions such as anaphylaxis and Stevens-Johnson syndrome have been described after diclofenac use, probably caused by glucuronidation-related protein adducts (Sawamura et al., 2010). In the research described in this thesis, we have used yeast as model system to further investigate the mechanisms underlying diclofenac toxicity.

Outline of the thesis

An overview of the literature regarding models for bioactivation-related toxicity is presented in the current chapter, **Chapter I**, and the suitability of yeast as alternative model system is discussed. Additionally, the application of bioactivation-competent yeasts in toxicity studies is reviewed.

In **Chapter II**, we investigated the mitochondrial toxicity of diclofenac in yeast. We showed the importance of mitochondrial respiration in diclofenac toxicity. Using yeast genetics, the crucial role of respiratory chain subunits Rip1p and Cox9p in diclofenac toxicity was identified and correlated to the formation of reactive oxygen species. A model explaining the role of Rip1p in diclofenac toxicity is presented.

Chapter III describes the metabolism-related toxicity of diclofenac in yeast. By expressing the model cytochrome P450 BM3 M11 in yeast, a bioactivation-competent yeast strain was created. BM3 M11 is a cytosolic, highly active P450 that yields a similar diclofenac metabolite profile as human P450s. Using this BM3 M11-expressing strain in combination with a control strain, the toxicity of diclofenac metabolism and of its hydroxy-metabolites was investigated.

In the research described in **Chapter IV**, microarray analysis was applied to identify additional cellular responses to diclofenac. Diclofenac-adapted yeast strains were used for this purpose to prevent extensive cell-death signaling. Mainly expression of genes involved in the pleiotropic drug resistance response, zinc homeostasis or the protein kinase C signaling pathway was altered. These targets were verified using yeast genetic approaches.

In **Chapter V**, we used yeast to investigate whether the various diclofenac targets and toxicity mechanisms identified in earlier chapters were also involved in the toxicity of other NSAIDs. Therefore, we selected a set of structurally related NSAIDs containing a carboxylic acid group. We divided the NSAIDs in three classes based on the involvement of mitochondrial proteins, a transporter or cytochrome P450 activity in their toxicity.

Finally, in **Chapter VI**, we discuss the research described in this thesis, draw overall conclusions regarding the use of yeast as model system and the possible translation of our results to humans, and provide perspectives for future research. It is concluded that bioactivation-competent yeast can also be used to study non-genotoxic mechanisms of toxicity. 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. We believe that “humanized” yeast strains, created by over-expression of human biotransformation and transporter genes, will prove very useful tools in future studies on the mechanisms underlying cellular toxicity.

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II

Subunits Rip1p and Cox9p of the respiratory chain contribute to diclofenac-induced mitochondrial dysfunction

Jolanda S. van Leeuwen
Rick Orij
Marijke A.H. Luttik
Gertien J. Smits
Nico P.E. Vermeulen
J. Chris Vos

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ABSTRACT

The widely used drug diclofenac can cause serious heart, liver or kidney injury, which may be related to its ability to cause mitochondrial dysfunction. Using *Saccharomyces cerevisiae* as a model system, we studied the mechanisms of diclofenac toxicity and the role of mitochondria therein. We found that diclofenac reduced cell growth and viability and increased levels of reactive oxygen species (ROS). Strains increasingly relying on respiration for their energy production showed enhanced sensitivity to diclofenac. Furthermore, oxygen consumption was inhibited by diclofenac, suggesting that the drug inhibits respiration. To identify the site of respiratory inhibition, we investigated the effects of deletion of respiratory chain subunits on diclofenac toxicity. Whereas deletion of most subunits had no effect, loss of either Rip1p of complex III or Cox9p of complex IV resulted in enhanced resistance to diclofenac. In these deletion strains, diclofenac did not increase ROS formation as severely as in wild type. Our data are consistent with a mechanism of toxicity in which diclofenac inhibits respiration by interfering with Rip1p and Cox9p in the respiratory chain, resulting in ROS production that causes cell death.

INTRODUCTION

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is widely used in the treatment of diseases involving pain and inflammation, such as arthritis. NSAIDs are inhibitors of prostaglandin synthesis and have been tested as anti-fungal agents, because *Candida albicans* and *Cryptococcus neoformans* secrete prostaglandin-like molecules involved in pathogenicity (Alem and Douglas, 2004; Noverr et al., 2001). In humans, diclofenac causes serious adverse drug reactions that target the liver (Laine et al., 2009) heart (Fosbol et al., 2009) and upper gastrointestinal tract (Lewis et al., 2002). Mitochondrial dysfunction is increasingly considered a major cause of drug-induced organ failure (Boelsterli and Lim, 2007; Dykens and Will, 2007). In mitochondria, electron donating compounds like NADH or succinate transfer electrons to the electron transport chain (ETC). The electrons travel through the respiratory chain complexes and are finally donated to oxygen, while protons are pumped across the membrane. This generates a proton motive force consisting of a membrane potential and a pH gradient that is used by ATPase to produce ATP. Various drugs can interfere with the electron transfer, leading to lowering of the mitochondrial membrane potential and decreasing the amount of ATP formed (Labbe et al., 2008). Furthermore, the electrons can leak from the respiratory chain, generating reactive oxygen species (ROS) that can induce apoptosis via the mitochondrial permeability transition (MPT) (Nieminen et al., 1997; Turrens, 2003).

There are several indications for a role of mitochondrial dysfunction in diclofenac toxicity. In rat hepatocytes, diclofenac causes uncoupling of mitochondrial respiration, in which oxygen consumption is no longer linked to proton transport over the inner membrane (Masubuchi et al., 2000). This leads to a decreased ATP production and ATP depletion (Bort et al., 1999; Masubuchi et al., 2002). Furthermore, in several cellular systems, including human hepatocytes, diclofenac induces ROS formation causing successive opening of the MPT pore, cytochrome c release, caspase activation and apoptosis (Gomez-Lechon et al., 2003; Inoue et al., 2004; Lim et al., 2006). However, the primary cause of the mitochondrial dysfunction remains unclear.

We used yeast as a model system to elucidate the mechanisms leading to mitochondrial dysfunction in diclofenac toxicity. Baker's yeast (*Saccharomyces cerevisiae*) is an excellent model organism to study mitochondrial functions, because a detailed knowledge on the composition and assembly of the various respiratory chain complexes can be combined with its genetic tractability. Furthermore, complex II, III and IV are highly conserved among eukaryotes (Lemire and Oyedotun, 2002; Taanman and Capaldi, 1992; Zara et al., 2009). Although yeast lacks complex I, the functional similarity of yeast NADH dehydrogenase Ndi1p allows rescue of NADH oxidase activity in complex I-deficient mammalian systems (Seo et al., 1998).

Here, we clearly link diclofenac toxicity to respiration. We demonstrate the unique role of Rip1p in complex III and Cox9p in complex IV of the respiratory chain in the toxicity of diclofenac and show that there is a strong correlation between diclofenac toxicity and the formation of ROS. These results indicate that diclofenac interferes with respiration at the site of Rip1p and Cox9p, leading to formation of ROS and cell death.

MATERIALS AND METHODS

Chemicals and stock solutions

Diclofenac was purchased as its sodium salt form Sigma Aldrich and dissolved in DMSO (500 mM). 2',7'-Dichlorodihydrofluorescein diacetate was obtained from Alexis Biochemicals and dissolved in ethanol (4 mM). All stock solutions were stored at –20 °C and protected from light. All other chemicals were purchased from Sigma Aldrich at the highest purity.

Strains

The haploid BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) wild type and deletion strains were obtained from EUROSCARF. Strains without mitochondrial DNA (rho^0) were

generated by growth with 10 μg ml^{-1} ethidium bromide for several days in rich medium (YPD: 1% w/v yeast extract, 2% w/v bactopeptone, 2% w/v glucose). Individual colonies were streaked in parallel on YPD and YPEG (1% w/v yeast extract, 2% w/v bactopeptone, 3% v/v ethanol and 3% v/v glycerol). Rho^0 cells grow only on YPD plates (Goldring et al., 1970). Loss of mitochondrial DNA was confirmed by staining the DNA with DAPI (4'-6-diamidino-2-phenylindole).

Construction of rescue constructs

RIP1 and *COX9* genes including 500 bp up- and downstream were amplified by PCR and cloned into YCplac111 (pLEU), a yeast single-copy vector containing the CEN4/ARS1 origin of replication and a *LEU2* marker (Gietz and Sugino, 1988). The plasmids were transformed into the corresponding deletion strains by using the freeze-thaw method (Klebe et al., 1983). Transformed strains were streaked in duplicates on YNB-Leu (0.67% w/w yeast nitrogen base without amino acids, 2% w/w glucose, supplemented amino acids and nucleotides without leucine) and YPEG. The Δcox9 and Δrip1 strains can only grow on YPEG when the deleted gene is complemented by the corresponding rescue construct.

Growth conditions and diclofenac treatment

Strains were grown overnight on minimal medium (YNB) at 30 °C. Overnight cultures were diluted in minimal medium and grown at 30 °C to OD_{600} ~0.2. At this point, 0-100 μM diclofenac was added. Controls were treated with equal amounts of DMSO (max 0.1% v/v). At various time points, the OD_{600} of control and diclofenac treated cultures was measured (Amersham Novaspec II spectrophotometer).

Measurement of cytosolic and mitochondrial pH

Cytosolic and mitochondrial pH were measured as described previously (Orij et al., 2009). Briefly, strains containing a plasmid carrying the pHluorin gene with or without a mitochondrial targeting sequence were grown in black, clear-bottomed 96-well plates in minimal media buffered at pH 5.0 with 25 mM sodium citrate. Fluorescence (λ_{ex} = 390 or 470 nm, λ_{em} = 512 nm) was measured and corrected for the cell density in the wells. The ratio of pHluorin emission intensity resulting from excitation at 390 nm and 470 nm was calculated and compared with a standard curve to determine the pH.

Oxygen consumption measurements

Oxygen consumption rates of whole cells were determined at 30 °C with a Clark-type oxygen electrode. The reaction mixture (4 ml) contained 100 mM potassium phosphate buffer (pH 3.0 or 5.0), 10 mM $MgSO_4$, and ~$6x10^6$ cells. Reactions were started by addition of 0.5% w/v

glucose. After incubating for 2 min, 0-200 µM diclofenac was added. Oxygen uptake rates were calculated based on a dissolved oxygen concentration of 236 M in air-saturated water at 30 °C.

Survival assay

Exponentially growing cultures of OD_{600} ~0.2 were incubated with 0 or 100 µM diclofenac. After 6 hours, the OD_{600} was measured and $1x10^{-5}$ OD units (~300 cells) were plated on YPD plates. After incubation at 30 °C for 3 days, the number of colonies was counted.

Measurement of ROS production

Exponentially growing cultures (2 ml) of OD_{600}~0.2 were treated with 0-50 µM diclofenac in the presence of 10 µM of the fluorescent, ROS-sensitive 2'7'-dichlorodihydrofluorescein diacetate. After 3 hours at 30 °C the cultures were centrifuged (3 min 3000 rpm) and cell pellets were washed and resuspended in 1 ml water. Fluorescence (λ_{ex} = 485 nm, λ_{em} = 535 nm) was measured and corrected for the cell density in the samples.

ß-galactosidase reporter assay

The *PDR3*- and *PDR5*-lacZ reporter constructs in the low copy number vector pSEYC102 were a kind gift from Prof. Scott Moye-Rowley (Katzmann et al., 1994). The plasmids were transformed into yeast by using the freeze-thaw method (Klebe et al., 1983). Yeast cultures were grown at 30 °C to OD_{600} ~0.5. Protein extracts were made in LacZ buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol) by using glass beads. Then, 5-35 µg protein was incubated with 24 µg chlorophenolred-ß-D-galactopyranoside at 30 °C for 10-360 minutes. Absorption at 575 nm was measured and corrected for the protein concentration in the samples and incubation time.

RESULTS

Respiring yeast cells show increased sensitivity to diclofenac

Previous studies in mammalian cells show that mitochondria play a role in diclofenac toxicity (Gómez-Lechón et al., 2003; Inoue et al., 2004; Lim et al., 2006; Masubuchi et al., 2002). To investigate whether diclofenac interferes with mitochondrial respiration in *S. cerevisiae*, we studied the effect of various carbon sources on the sensitivity of wild type cells to diclofenac. Growth of the cultures was monitored on medium containing glucose or galactose as carbon source supplemented with 0, 50 or 100 µM diclofenac (Fig. 1). When yeast is grown on glucose, the cells derive most of their energy from glycolysis. With galactose as carbon source, cells are more reliant on respiration (Fendt and Sauer, 2010). On glucose, cells treated with 50

μM diclofenac grew significantly more slowly than untreated cells (Fig. 1A). However, on galactose, cells grew even more slowly after treatment with 50 μM diclofenac (Fig. 1B). Clearly, when cells are increasingly dependent on respiration, diclofenac toxicity is enhanced. During incubation with 100 μM diclofenac in either glucose or galactose, cells barely grew at all.

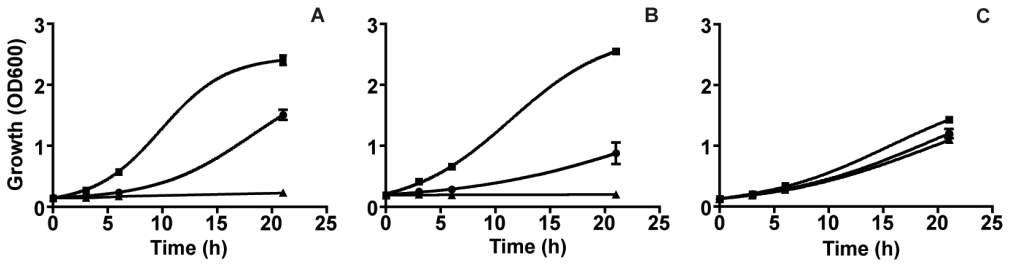

Fig. 1. Respiring cells are more sensitive to diclofenac. The wild type yeast strain grown on glucose (A) or galactose (B) as carbon source and (C) a strain without mitochondrial DNA (rho^0) grown on glucose were incubated with 0 μM (squares), 50 μM (circles) or 100 μM (triangles) diclofenac. Growth is expressed as OD_{600} ± SD. Experiments were performed three times using independently generated rho^0 strains.

To confirm that respiration increases diclofenac toxicity, we tested the diclofenac sensitivity of strains lacking mitochondrial DNA (rho^0). Rho^0 cells are deficient in respiration but can grow on fermentable carbon sources such as glucose, although more slowly than wild type cells. Whereas wild type cells hardly grew on 100 μM diclofenac, this diclofenac concentration had only a minor effect on the growth of rho^0 cells (Fig. 1C), confirming the importance of respiration in diclofenac toxicity.

Diclofenac inhibits respiration but does not dissipate the proton gradient over the mitochondrial membrane

One of the current hypotheses regarding diclofenac toxicity is that it uncouples oxygen consumption from ATP production by dissipating the proton gradient (Masubuchi et al., 2002). We used the pH-sensitive green fluorescent protein pHluorin (Orij et al., 2009) to measure the cytosolic and mitochondrial pH (pH_{cyt} and pH_{mit} respectively). To rule out influences of external pH, cells were grown in media buffered at pH 5. In this buffered medium 200 μM diclofenac has similar effects on cell death and growth as 50 μM diclofenac in non-buffered medium (data not shown). As diclofenac is a weak acid (pKa ~4.0), its uptake is higher in the more acidic non-buffered medium (Wohnsland and Faller, 2001), leading to increased toxicity.

Wild type cells were grown in glucose-containing media and fluorescence was monitored. The exponentially growing cells had a pH_{cyt} of 6.9 and a pH_{mit} of 7.2. After addition of 200 µM diclofenac, both values decreased quickly (Fig. 2A). After 30 minutes, the pH stabilized at 6.4 in the cytosol and 6.6 in the mitochondria and remained constant for at least six hours (data not shown). Previously, the uncoupler CCCP was shown to diminish the difference between pH_{cyt} and pH_{mit} (Orij et al., 2009). As the difference between mitochondrial and cytosolic pH remained intact with diclofenac, we conclude that diclofenac does not uncouple respiration.

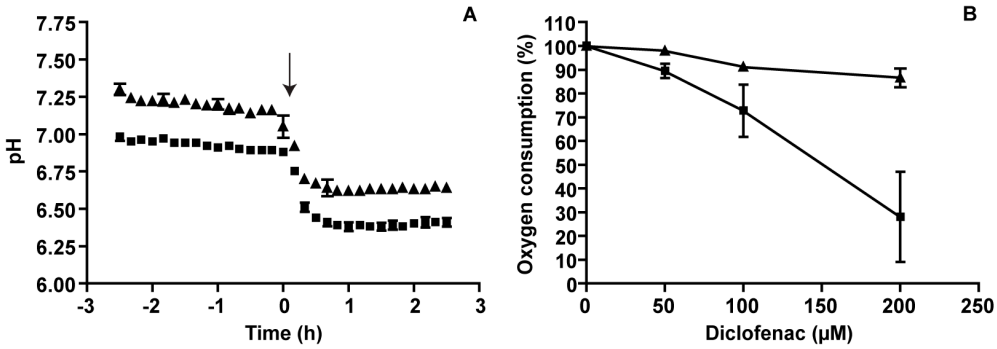

Fig. 2. Diclofenac inhibits oxygen consumption but does not influence the proton gradient. (A) Wild type cells were grown in minimal media buffered at pH 5.0. pH_{cyt} (squares) and pH_{mit} (triangles) were measured by using the pH-dependent fluorescent protein pHluorin. Fluorescence was corrected for cell density. At t=0 (arrow), 200 µM diclofenac was added. Data are expressed as mean ± SD (n=3). (B) Oxygen consumption was measured of wild type cells incubated at pH 3.0 (squares) or pH 5.0 (triangles) with 0-200 µM diclofenac. Data are expressed as % oxygen consumption compared with cells incubated without diclofenac (100 %) ± SD (n=3).

Since diclofenac is more toxic to respiring than to fermenting cells, we examined whether it inhibits respiration in yeast. Wild type cells were incubated in potassium phosphate buffer at pH 3.0 or 5.0 and oxygen consumption was measured in the presence of glucose and 0-200 µM diclofenac (Fig. 2B). Oxygen consumption in the absence of diclofenac was comparable at both pHs (~7.4 fmol min^{-1} $cell^{-1}$) and was set at 100%. Diclofenac dose-dependently inhibited respiration at both pH 3.0 and 5.0. However, at pH 3.0 the inhibition was more severe. At pH 5.0, 200 µM diclofenac inhibited respiration ~13%, which is comparable with the inhibition by 50 µM diclofenac at pH 3.0. Because growth inhibition at these two conditions is comparable, there is a clear correlation between growth inhibition and reduced oxygen consumption at both pHs. Together these results show that diclofenac inhibits, but does not uncouple, respiration.

Diclofenac induces the formation of reactive oxygen species in WT but not in rho^0 strains

As the respiratory chain is the main source of ROS in the mitochondria, we tested the effect of respiration on diclofenac-induced ROS formation. Wild type and rho^0 strains were incubated for 3 hours in the presence of the ROS-sensitive fluorescent compound 2',7'-dichlorodihydrofluorescein diacetate and 0, 10, 30 or 50 µM diclofenac.

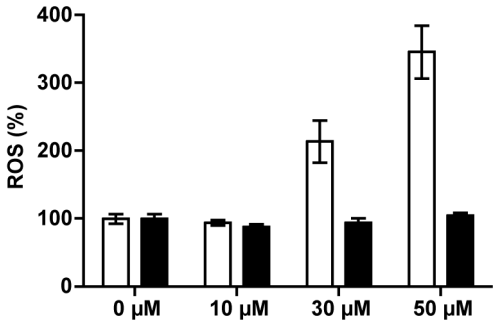

Fig. 3. Diclofenac increases ROS levels in wild type, but not in rho^0 cells. The wild type yeast strain (white bars) and a strain lacking mitochondrial DNA (rho^0, black bars) were grown in the presence of 0, 10, 30 or 50 µM diclofenac. After 3 hours, the ROS levels were measured using the fluorescent ROS marker 2',7'-dichlorodihydrofluorescein diacetate. Measured fluorescence is corrected for cell density. ROS formation is expressed as % fluorescence compared with WT or rho^0 controls incubated without diclofenac (100 %) ± SD. Experiments were performed three times using independently generated rho^0 strains.

ROS levels in the absence of diclofenac were set at 100%. In wild type cells, diclofenac dose-dependently increased the amount of ROS formed (Fig. 3, white bars). Even at diclofenac concentrations of 30 µM the amount of ROS in the cells was doubled compared with wild type cells incubated without diclofenac. At 50 µM diclofenac, the cellular ROS levels were ~3.5-fold higher than in the untreated control cells. In contrast, these diclofenac concentrations did not induce ROS formation in the respiratory deficient rho^0 strain (Fig. 3, black bars) compared with rho^0 controls. These results show a relationship between respiration, ROS formation and diclofenac toxicity.

Crucial role of respiratory chain subunits Rip1p and Cox9p in the toxicity of diclofenac

To investigate the role of mitochondrial respiration in diclofenac toxicity in more detail, we tested the effect of individual respiratory chain proteins on diclofenac toxicity. Single gene deletion strains were used in which non-essential, nuclear encoded subunits of the respiratory chain complexes were deleted. The deletion strains were grown for 6 hours in the presence of 0 or 100 µM diclofenac. For each strain, the growth (increase in OD_{600} in 6 hours) of cultures

incubated without diclofenac was set at 100% and the percentage growth of diclofenac treated cultures was calculated (Fig. 4A).

Growth of wild type cultures incubated with 100 µM diclofenac was only ~5% of that of control cultures. As also seen in Fig. 1, the rho^0 strain was much more resistant to diclofenac, with ~80% growth compared with untreated cells. Deletion of complex I homologue Ndi1p or subunits of complex II did not significantly change the sensitivity to diclofenac compared with wild type cells. However, deletion of subunit Rip1p (Δrip1) of complex III increased the resistance to diclofenac, with ~40% growth compared with control cells. Interestingly, deletion of subunit Cox9p (Δcox9) of complex IV also increased diclofenac resistance.

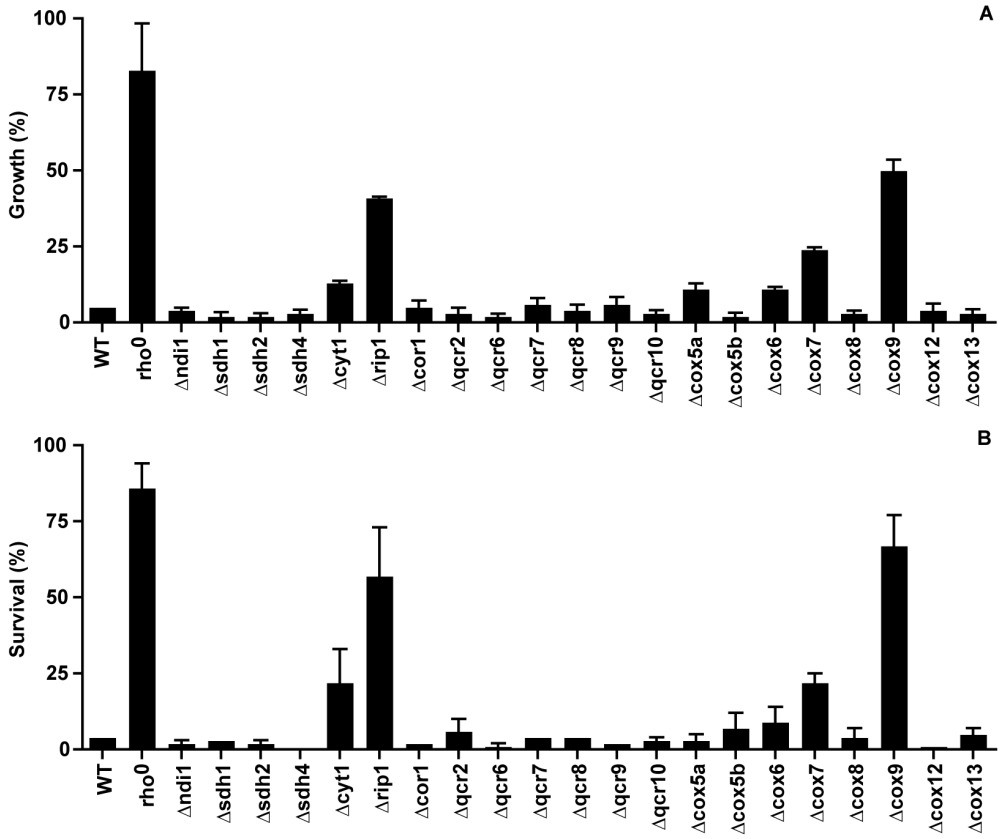

Fig. 4. Diclofenac toxicity is dependent on Rip1p and Cox9p. Growth (A) and survival (B) of the wild type strain (WT), a strain lacking mitochondrial DNA (rho^0) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted after incubation with 100 µM diclofenac for 6 hours. The data are expressed as % growth (OD_{600}) or % surviving cells ± SD compared with cells of the same strain incubated without diclofenac (100 %).

The viability of the deletion strains after incubating 6 hours with 100 µM diclofenac was also analyzed. The results (Fig. 4B) correlate with those of the growth assay. Only ~5% of the WT cells survived the diclofenac treatment. The rho^0, Δrip1 and Δcox9 strains showed strongly increased resistance to diclofenac, with ~85%, ~55% and ~65% of the cells surviving diclofenac treatment, respectively. Deletion of other complex III or IV subunits had some effect on diclofenac toxicity; in particular the Δcyt1 and Δcox7 strains showed slightly increased diclofenac resistance in the growth and survival assays.

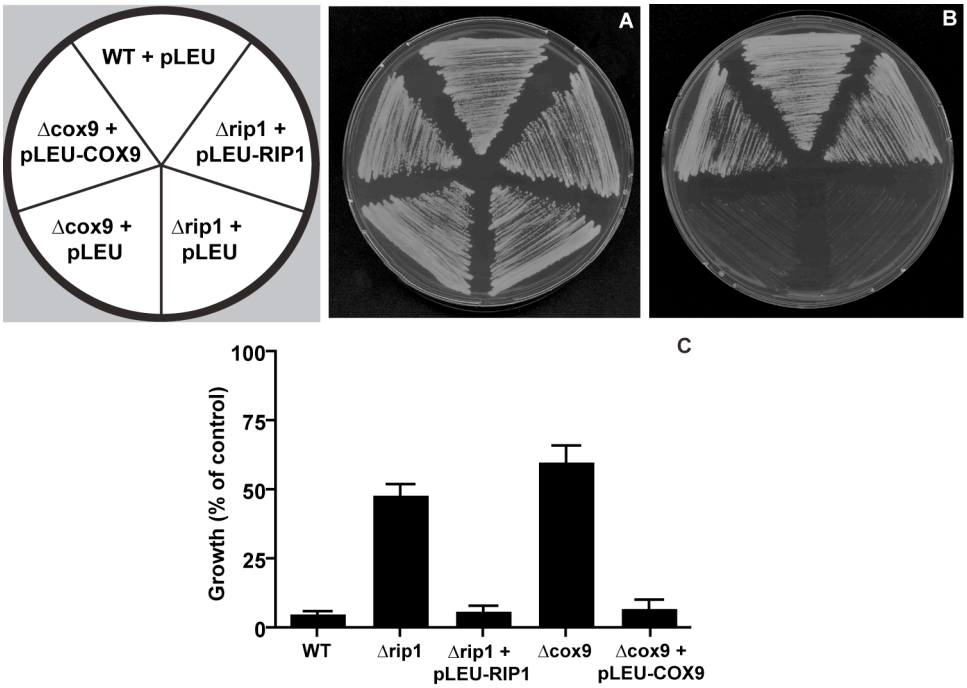

Fig. 5. Complementation of the deleted genes restores respiration and diclofenac sensitivity in Δrip1 and Δcox9 strains. The Δrip1 and Δcox9 deletion strains were transformed with constructs containing the *RIP1* and *COX9* genes under control of their own promoter. Wild type (WT), Δrip1 and Δcox9 strains transformed with an empty vector (pLEU) and the complemented Δrip1 + pLEU-RIP1 and Δcox9 + pLEU-COX9 were grown on plates containing (A) glucose (YPD) or (B) ethanol and glycerol (YPEG) as carbon source. Only strains that are able to respire can grow on YPEG plates. (c) Wild type, Δrip1, Δcox9 and the complemented Δrip1 + pLEU-RIP1 and Δcox9 + pLEU-COX9 strains were grown in the presence of 100 µM diclofenac. The data are expressed as % growth (OD_{600}) ± SD after 6 hours compared with cells of the same strain incubated without diclofenac. Data represent means of two independent experiments performed in duplicate using different transformants.

Complementation of the deleted genes restores diclofenac sensitivity

To confirm that the diclofenac resistance observed in the Δrip1 and Δcox9 deletion strains was solely due to absence of the *RIP1* and *COX9* genes, we introduced a plasmid-based copy of the genes into the corresponding deletion strains. The *RIP1* and *COX9* genes were under control of their own promoter. Successful complementation of the deleted gene should restore the ability to respire and result in a diclofenac-sensitive wild type phenotype.

Wild type, Δrip1 and Δcox9 strains transformed with an empty vector (pLEU) and the complemented strains Δrip1 + pLEU-RIP1 and Δcox9 + pLEU-COX9 were streaked in parallel on plates containing glucose (Fig. 5A) or ethanol and glycerol (Fig. 5B) as carbon source. All strains were able to grow on plates containing glucose. Whereas the Δrip1 and Δcox9 strains transformed with an empty vector were unable to grow on plates containing the non-fermentable carbon sources ethanol and glycerol, the complemented Δrip1 + pLEU-RIP1 and Δcox9 + pLEU-COX9 strains grew on these plates. This shows that the ability to respire was restored in the complemented strains.

We also tested the effect of diclofenac on the growth of the strains. For each strain, growth without diclofenac was set at 100%. Whereas the Δrip1 and Δcox9 strains were resistant to diclofenac, the Δrip1 and Δcox9 strains transformed with the pLEU-RIP1 and pLEU-COX9 constructs, respectively, were sensitive to diclofenac (Fig. 5C), confirming that Rip1p and Cox9p are involved in diclofenac toxicity.

Retrograde upregulation of the PDR response is not involved in diclofenac resistance of complex III and IV deletion strains

In rho^0 cells and in strains lacking certain complex V subunits, several genes involved in pleiotropic drug resistance (PDR), including multidrug transporter *PDR5* and transcription factor *PDR3,* are upregulated compared with wild type cells (Hallstrom and Moye-Rowley, 2000; Zhang and Moye-Rowley, 2001). Deletion of complex III or IV subunits may also cause upregulation of the PDR genes, which can affect diclofenac resistance. Therefore, we examined *PDR3* and *PDR5* expression in the diclofenac resistant Δrip1 and Δcox9 strains. By using a ß-galactosidase reporter assay, we measured *PDR3* and *PDR5* promoter activity. For both genes an increase in *lacZ* expression was observed in the rho^0 strain (Table 1), as reported before by Hallstrom and Moye-Rowley (2000). In the Δrip1 and Δcox9 strains, *lacZ* expression for both genes was similar to that in wild type. This indicates that diclofenac resistance in these deletion strains is not caused by an upregulation of the multidrug resistance genes.

	ß-galactosidase activity (units μg^{-1} min^{-1})	
	PDR3-lacZ	*PDR5*-lacZ
Wild type	1.7 ± 0.3	77 ± 11
Rho^0	3.4 ± 0.4	213 ± 22
Δrip1	2.1 ± 0.1	87 ± 27
Δcox9	1.8 ± 0.2	90 ± 21

Table 1. Multidrug resistance genes *PDR3* and *PDR5* are not upregulated in Δrip1 and Δcox9 strains. *PDR3*-lacZ and *PDR5*-lacZ expression in the wild type strain, a strain lacking mitochondrial DNA (rho^0) and strains in which either subunit Rip1p or Cox9p of the respiratory chain was deleted. Data are expressed as ß-galactosidase activity in units, corrected for the protein concentration and incubation time, ± SD. Data are means of two experiments performed in duplicate using different transformants.

Diclofenac sensitivity correlates with the formation of reactive oxygen species

To further explore why deletion of Rip1p and Cox9p resulted in diclofenac resistance, whereas deletion of other complex III or IV subunits did not, we investigated whether the deletion of these genes had an effect on the amount of ROS in the cell. First, we investigated the ROS levels in the wild type and deletion strains grown in absence of diclofenac to compare the basal ROS levels. The strains were grown for 3 hours in presence of the ROS marker 2',7'-dichlorodihydrofluorescein diacetate. No significant differences in basal ROS levels were observed (Fig. 6A).

Secondly, we tested ROS levels after incubation with 50 µM diclofenac and 2',7'-dichlorodihydrofluorescein diacetate for 3 hours (Fig. 6B). In almost all deletion strains, ROS formation was increased approximately fourfold upon diclofenac treatment compared with ROS levels in the same strain incubated without diclofenac. Apparently, the disruption of stable complex III or IV formation does not decrease the potential of diclofenac to increase ROS formation. However, in the diclofenac resistant Δrip1 and Δcox9 strains, ROS levels were only ~200% and ~150% of untreated controls, respectively. These results show a strong correlation between diclofenac toxicity and ROS formation, and the unique roles of Rip1p and Cox9p within the electron transfer chain.

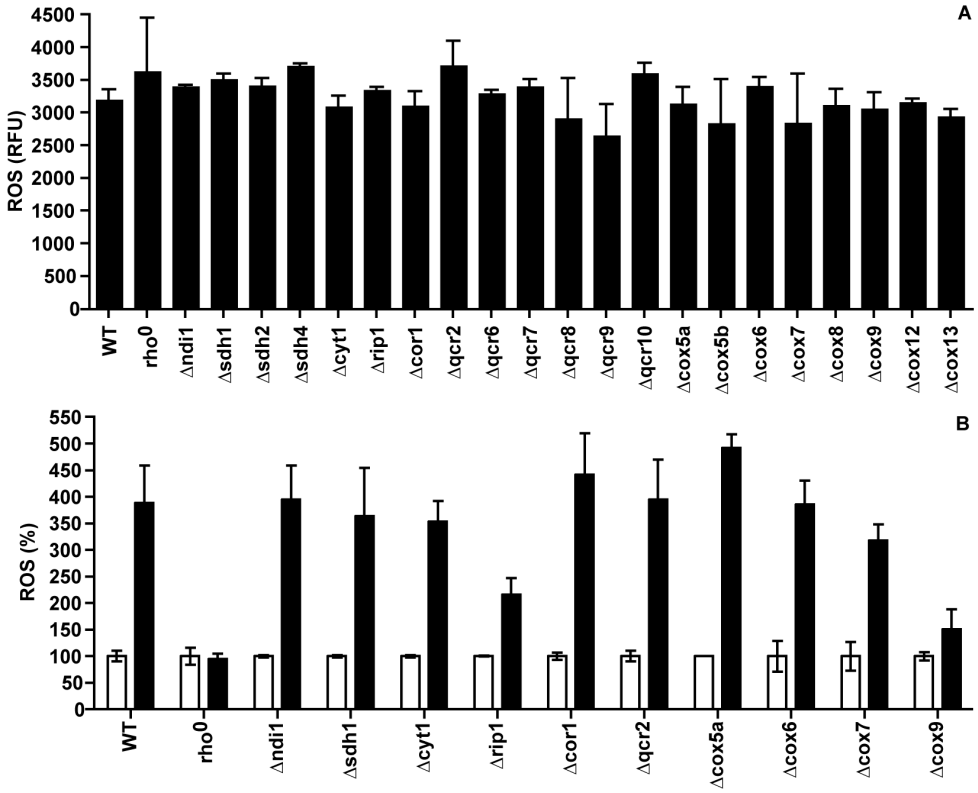

Fig. 6. Diclofenac resistance is correlated with ROS formation. (A) Basal ROS levels in wild type (WT), a strain lacking mitochondrial DNA (rho^0) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted. For this experiment the strains were not incubated with diclofenac. (B) ROS formation in wild type (WT), a strain lacking mitochondrial DNA (rho^0) and strains in which a subunit of respiratory chain complex I, II, III or IV was deleted after 3 hours of incubation without (white bars) or with 50 μM diclofenac (black bars). ROS formation was measured using the fluorescent ROS marker 2',7'-dichlorodihydrofluorescein diacetate. Fluorescence is corrected for cell density and expressed as units (RFU) or % compared with cells of the same strain incubated without diclofenac ± SD (n=4).

DISCUSSION

One of the challenges in examining the toxicity of drugs is identifying the cellular target(s). Yeast cells are useful in the identification of genes important in drug toxicity (Bharucha and Kumar, 2007). An advantage of yeast over mammalian cellular systems is the relatively straightforward way of performing genetic modifications. Here, we used yeast to study the mitochondrial toxicity of diclofenac.

In several cellular systems, including human hepatocytes, diclofenac can induce reactive oxygen species (ROS) formation that causes successive opening of the mitochondrial permeability transition (MPT) pore, cytochrome c release, caspase activation and apoptosis (Gomez-Lechon et al., 2003, Inoue et al., 2004, Lim et al., 2006). Since the respiratory chain is a well-known source of ROS (Kowaltowski et al., 2009; Poyton et al., 2009), we studied the role of respiration in diclofenac-induced ROS formation. We showed that when cells are increasingly dependent on respiration, diclofenac toxicity is enhanced (Fig. 1). Strains that lack mitochondrial DNA (rho^0) are deficient in respiration and show an increased resistance to diclofenac (Fig. 1). Furthermore, loss of mtDNA completely diminished diclofenac-induced ROS formation (Fig. 3). These results indicate a clear link between mitochondrial respiration, ROS formation and diclofenac toxicity. Interestingly, another NSAID, aspirin, has also been linked to mitochondrial dysfunction in yeast, leading to apoptosis (Sapienza et al., 2008).

Most of the oxygen consumed by yeast is used by the respiratory chain (reviewed by Rosenfeld and Beauvoit, 2003). Respiratory inhibitors can almost fully inhibit oxygen consumption. Here, we show that diclofenac can also inhibit oxygen consumption in yeast. Inhibition of oxygen consumption by diclofenac was also found in mammalian cells (Niklas et al., 2009; Krause et al., 2003). Uncoupling of respiration by diclofenac is thought to be responsible for mitochondrial dysfunction. Although a collapse of the mitochondrial membrane potential after diclofenac exposure has been described in mammalian cells (Bort et al., 1998; Inoue et al., 2004; Lim et al., 2006; Masubuchi et al., 2002), this can also be the result of opening of the MPT pore instead of uncoupling. Here, we followed the effect of diclofenac on the cytosolic and mitochondrial pH over time. We observed an immediate decrease in both cytosolic and mitochondrial pH, but the difference between the two values remained intact. Similar results have been described for the complex III inhibitor antimycin A, while the uncoupler CCCP does lead to dissipation of the pH difference (Orij et al., 2009). This indicates that under these experimental conditions, diclofenac does not uncouple respiration or induce MPT pore opening in yeast.

Using single gene deletion mutants, we identified Rip1p and Cox9p of complex III and IV, respectively, as possible targets of diclofenac in the respiratory chain. Deletion of Rip1p or Cox9p markedly increased growth and survival during diclofenac treatment compared with wild type and other deletion strains (Fig. 4). Furthermore, Δrip1 and Δcox9 strains had markedly lower ROS levels during diclofenac incubation than the wild type strain (Fig. 6B). To determine ROS formation we used 2',7'-dichlorodihydrofluorescein diacetate. However, as this probe is slightly pH sensitive we repeated the ROS assay for WT, Δrip1 and Δcox9 strains with another ROS-sensitive probe, dihydroethidium, and obtained similar results (data not shown). This strong correlation between ROS levels and diclofenac sensitivity indicates that

the formation of ROS is the main cause of toxicity. The mitochondrial superoxide dismutase SOD2 has been suggested to be a diclofenac target in neuroblastoma cells as evidenced by reduced levels of SOD2 protein and activity upon diclofenac exposure (Cecere et al., 2010). Therefore, we tested deletion mutants of *SOD1* and *SOD2* in yeast, but did not observe a reduced diclofenac sensitivity compared with wild type over 21 h of incubation.

We confirmed the role of Rip1p and Cox9p in diclofenac toxicity by showing that complementation of the genes in the deletion strains restores the diclofenac sensitive phenotype. Although the diclofenac resistance of Δrip1 and Δcox9 strains was significantly increased compared with wild type cells, they were not as resistant as the rho^0 strain. The higher diclofenac resistance of the rho^0 cells can be explained as an additional effect of lacking both complex III and IV activity. Furthermore, upregulation of the PDR multidrug response in rho^0 cells (Table 1) (Hallstrom and Moye-Rowley, 2000) may contribute to the resistance. For other NSAIDs like acetaminophen (Srikanth et al., 2005) and indomethacin (Mima et al., 2007), a role for ABC transporters on their toxicity towards *S. cerevisiae* has been shown.

Rip1p, a Rieske iron-sulfur protein, is one of the catalytic subunits of complex III. Complex III has two distinct binding sites for ubiquinone; the Qi site and the Qo site. The Qo site consists of the proximal niche close to the heme bL of the mitochondrially-encoded cytochrome B (Cobp) and the distal niche close to the iron-sulfur cluster of Rip1p. In particular, complex III inhibitors that bind in the proximal Qo niche or at the Qi site induce ROS formation by inhibiting electron transport (Muller et al., 2003; Yang et al., 2008). It is tempting to speculate that diclofenac can bind at one of these sites, thereby inhibiting respiration and inducing leakage of electrons. In the absence of Rip1p the ability of diclofenac to generate ROS is lost. Loss of the iron-sulfur protein also abolishes the increase in hypoxia- or methylmercury-induced ROS (Guzy et al., 2007; Lee et al., 2009). Furthermore, inhibition of Rip1p mobility by stigmatellin decreases both basal and induced ROS formation (Armstrong et al., 2004; Muller et al., 2003). This indicates that Rip1p is crucial for ROS formation at complex III. Also, loss of Cyt1p slightly increased diclofenac resistance, which might be explained by the direct interaction of Cyt1p with Rip1p. Interestingly, *RIP1* is highly conserved across eukaryotes (Beckmann et al., 1989), and downregulation of the mammalian homologue of Rip1p, RISP (UQCRFS1), has also been shown to reduce ROS production (Guzy et al., 2005).

Cox9p is a small (7 kDa) protein that is essential for complex IV activity (Wright et al., 1986). In literature, the protein encoded by *COX9* is also referred to as subunit VIIa whereas the gene-product of *COX12* is sometimes named Cox9p. Here, we use the nomenclature of the *Saccharomyces* Genome Database (http://www.yeastgenome.org) and refer to Cox9p as the gene-product of *COX9*. We show that lack of subunit Cox9p lowers diclofenac-induced ROS

formation. Cox9p has been suggested to play a role in complex IV assembly or stability (McEwen et al., 1986). Possibly, interactions of diclofenac with Cox9p interfere with complex IV integrity, leading to ROS formation. Interestingly, complex IV has rarely been associated with ROS formation in literature. As complexes III and IV assemble in a supercomplex, lack of Cox9p might influence electron transfer between the complexes, leading to ROS formation at complex III.

Surprisingly, deletion of several complex III and IV subunits (for example Cor1p and Cox6p) has been reported to cause loss of Rip1p and Cox9p from the complexes (Calder and McEwen, 1991; Crivellone et al., 1988; Dowhan et al., 1985) but does not result in diclofenac resistance or reduced ROS formation (Fig. 4 and 6B). Perhaps in these cases subcomplexes of complex III or IV are formed that are not stable enough for isolation and are therefore not detected.

In conclusion, we demonstrated that subunits Rip1p of respiratory chain complex III and Cox9p of complex IV are crucial to the mitochondrial toxicity of diclofenac. Further research is needed to see whether the mammalian counterparts UQCRFS1 and COX6c (Lenaz and Genova, 2010) are related to diclofenac toxicity in mammalian cells and to understand how these two proteins dictate diclofenac interference at the ETC.

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III

Metabolism related toxicity of diclofenac in yeast as model system

Jolanda S. van Leeuwen
Galvin Vredenburg
Sanja Dragovic
T.F. Jennifer Tjong
J. Chris Vos
Nico P.E. Vermeulen

Adapted from: van Leeuwen et al. (2011) *Toxicol Lett*

ABSTRACT

Diclofenac is a widely used drug that can cause serious hepatotoxicity, which has been linked to metabolism by cytochrome P450s (P450). To investigate the role of oxidative metabolites in diclofenac toxicity, a model for P450-related toxicity was set-up in *Saccharomyces cerevisiae.* We expressed a drug-metabolizing mutant of cytochrome P450 BM3 (BM3 M11) in yeast. Importantly, BM3 M11 yielded similar oxidative metabolite profiles of diclofenac as human P450s. It was found that yeast strains expressing BM3 M11 grew significantly slower when exposed to diclofenac than strains without BM3 M11. Furthermore, the amount of reactive oxygen species (ROS) after incubation with diclofenac was higher in strains expressing BM3 M11 than in strains without this enzyme, confirming that P450 activity increases diclofenac toxicity. Interestingly, 4'- and 5-hydroxydiclofenac had no effect on cell growth or ROS formation in cells expressing BM3 M11, although hydroxydiclofenac-derived quinone imines were identified in these strains by detection of their glutathione conjugates. This suggests that 4'- and 5-hydroxydiclofenac, as well as their quinone imines, are not involved in toxicity in yeast. Rather, the P450-related toxicity of diclofenac is caused by primary metabolites such as arene oxides resulting in hydroxydiclofenac or radical species formed during decarboxylation.

INTRODUCTION

Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), can cause severe liver toxicity (Laine et al., 2009) for which the exact mechanisms remain unclear. In the human liver, diclofenac is mainly metabolized by glucuronosyl-transferase UGT2B7 to its acyl glucuronide and by cytochrome P450 2C9 to the metabolite 4'-hydroxydiclofenac (King et al., 2001; Leemann et al., 1993). Minor metabolites are formed as well, such as CYP3A4-mediated 5-hydroxydiclofenac and other mono- or dihydroxylated, methoxylated or decarboxylated metabolites and conjugates thereof (Blum et al., 1996; Grillo et al., 2008; Yan et al., 2005). In hepatocellular cells, the cytochrome P450 (P450) inhibitors sulfaphenazole and ketoconazole can decrease LDH release and the formation of reactive oxygen species (ROS) caused by diclofenac exposure (Bort et al., 1999; Kretz-Rommel and Boelsterli, 1993; Lim et al., 2006). Although P450 inhibitors clearly decrease diclofenac toxicity, the role of individual diclofenac metabolites and intermediates in the toxicity of diclofenac is still unclear. Both 4'- and 5-hydroxy-derivatives can form, in part spontaneously, reactive quinone imine intermediates (Shen et al., 1999; Tang et al., 1999a) that are suggested to cause the toxicity of diclofenac (Miyamoto et al., 1997; Poon et al., 2001; Shen et al., 1999; Tang et al., 1999b). Furthermore, arene oxides may be involved in the P450-dependent generation of 4'- and 5-hydroxydiclofenac (Masubuchi al., 2002; Yan et al., 2005), leading to toxicity.

Currently, hepatocytes are the standard model for investigating drug metabolism and toxicity (Gomez-Lechon et al., 2007). However, the use of primary hepatocytes has some drawbacks, such as high variation in P450 activities in hepatocytes from different donors and declining P450 expression and de-differentiation during culture (LeCluyse, 2001). P450 inhibitors are often used to differentiate between the toxic effects of drugs and their metabolites. However, these inhibitors can also influence other cellular processes and thereby affect toxicity. For example, CYP3A4-inhibitor ketoconazole that is often used in diclofenac toxicity studies inhibits both glucuronosyl-transferase UGT2B7 (Raungrut et al., 2010; Takeda et al., 2006) and transporter ABCB1/P-gp (Wang et al., 2002).

The yeast *Saccharomyces cerevisiae* is an excellent alternative model organism in pharmacological research (Menacho-Marquez and Murguia, 2007). Advantages of yeast over mammalian cell systems are its easy manipulation, cost-effectiveness, and rapid growth. Because wild type yeast strains lack drug-metabolizing cytochrome P450s and glucuronosyl-transferases, many biotransformation enzymes have been heterologously expressed in yeast (Pompon et al., 1997; Renaud et al., 1993). Expression of mammalian P450s in yeast has been used to investigate the mutagenic effects of oxidative metabolites of xenobiotics, as has been described for N-alkylformamides and aflatoxine B_1 (Del Carratore et al., 2000; Guo et al., 2005).

The cytosolic P450 BM3 from *Bacillus megaterium* is a good alternative candidate for bioactivation studies. Advantages of BM3 over other P450s are its high activity, solubility, stability and coupled reductase domain (Munro et al., 2002). Furthermore, the M11 mutant of BM3 (R47L/E64G/F81I/F87V/E143G/L188Q/E267V) has a high metabolizing activity towards a broad range of drugs and drug-like compounds, including diclofenac (Damsten et al., 2008; van Vugt-Lussenburg et al., 2007).

In this study, we have examined the toxicity of diclofenac and its P450-mediated metabolites in *S. cerevisiae.* For that purpose, a bioactivation-competent yeast strain expressing cytochrome P450 BM3 M11 has been created. Both control and BM3 M11 expressing yeast strains have been used in the detection of P450-related toxicity of diclofenac and its oxidative metabolites. Furthermore, by using 4'- or 5-hydroxydiclofenac as substrate, the toxicity of secondary metabolites was examined.

MATERIALS AND METHODS

Chemicals and stock solutions

Diclofenac was purchased as its sodium salt from Sigma-Aldrich. 4'-hydroxydiclofenac was

obtained from Cypex Ltd, UK at 97.5% purity. 5-hydroxydiclofenac was a kind gift from Prof. Kevin Park, University of Liverpool, UK (Kenny et al., 2004). Diclofenac was dissolved in DMSO or EtOH (100 mM) and 4'- and 5-hydroxydiclofenac were dissolved in EtOH (10 mM). Stock solutions were stored at –20 °C and protected from light. All other chemicals were purchased from Sigma-Aldrich at the highest purity.

Strains and plasmids

The haploid *S. cerevisiae* strain W303 (MATa; ura3-52; trp1Δ2; leu2-3,112; his3-11; ade2-1; can1-100) was used in this research. BM3 M11 was expressed in yeast using pTL26, a galactose inducible yeast expression vector carrying the *HIS3* selection marker and the CEN6/ARSH4 origin of replication (Lafontaine and Tollervey, 1996). The His-tagged BM3 M11 gene was obtained from the previously described pT1-M11 (van-Vugt-Lussenburg et al., 2007) and cloned into the pTL26 vector using standard procedures. The resulting pTL26-M11 and the empty pTL26 were transformed into yeast using the freeze-thaw method (Klebe et al., 1983).

Growth conditions and diclofenac treatment

Strains were grown on selective minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented amino acids and bases without histidine) and switched overnight to minimal media containing galactose to induce BM3 expression. Overnight cultures were diluted and grown at 30 °C to OD_{600} ~0.2. At this point, 0-50 µM diclofenac, 4'-hydroxydiclofenac or 5-hydroxydiclofenac was added. Controls were treated with equal amounts of DMSO or EtOH.

Western blotting of heterologously expressed BM3 M11

Overnight cultures were centrifuged and protein extracts were made by vortexing using glass beads in lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 2 mM PMSF). Protein concentrations were determined using the Advanced Protein Assay (Sigma-Aldrich). 25 µg protein was loaded on a SDS-PAGE gel. After transfer to nitrocellulose, membranes were blocked using 5% milk powder in TBST (10 mM Tris-HCl pH 8.0, 15 mM NaCl, 0.05% Tween 20). Membranes were subsequently incubated for 3 h with an anti-His mouse antibody (1:5000 in TBST, QIAGEN). Unbound antibody was removed by washing 3 x 10 min with TBST. Finally, the membranes were incubated for 1.5 h with anti-mouse IgG HRP linked (1:5000, Cell Signaling Technology) and washed 3 times. The His-tagged proteins were visualized using an ECL detection system (Pierce, USA).

In vitro incubations using purified BM3 M11 and HPLC conditions

The purification of BM3 M11, the *in vitro* incubations, and the HPLC analysis of stable and GSH-conjugated metabolites were performed as described previously (Damsten et al., 2008). For the separation of 4'- and 5-hydroxydiclofenac a C18 column (Luna 5 µm, 4.6 x 150 mm, Phenomenex) was used at isocratic conditions using 40% acetonitrile, 0.2% formic acid for 60 min at 0.5 mL/min and UV detection at 254 nm.

Detection of in vivo generated metabolites in yeast

Diclofenac treated cultures were centrifuged and cellular lysates were made in KPi buffer (100 mM potassium phosphate, pH 7.4) using glass beads. Proteins were precipitated by adding an equal volume of ice-cold MeOH and incubating 10 min on ice. Precipitate was removed by centrifugation (10 min, 14000 rpm) and the supernatant was analyzed by HPLC.

ROS assay

Exponentially growing cultures with OD_{600}~0.2 were treated with 0-50 µM diclofenac, 4'-hydroxydiclofenac or 5-hydroxydiclofenac and 10 µM of the fluorescent, ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate (Alexis Biochemicals). After 3 hours at 30 °C the cultures were centrifuged and cell pellets were washed and resuspended in water. Fluorescence (l_{ex} = 485 nm, l_{em} = 535 nm) was measured and corrected for the cell density in the samples.

Statistical analysis

To compare growth or ROS formation in control and BM3 M11-expressing strains, the two-sided Student's *t*-test for unpaired samples was used. *P* values less than 0.01 were considered statistically significant. When applicable, *P* values were corrected for multiple comparisons.

RESULTS AND DISCUSSION

In this research, we have expressed cytochrome P450 BM3 M11 in yeast to investigate the P450-related toxicity of diclofenac.

Cytochrome P450 BM3 M11 as model enzyme for oxidative diclofenac metabolism

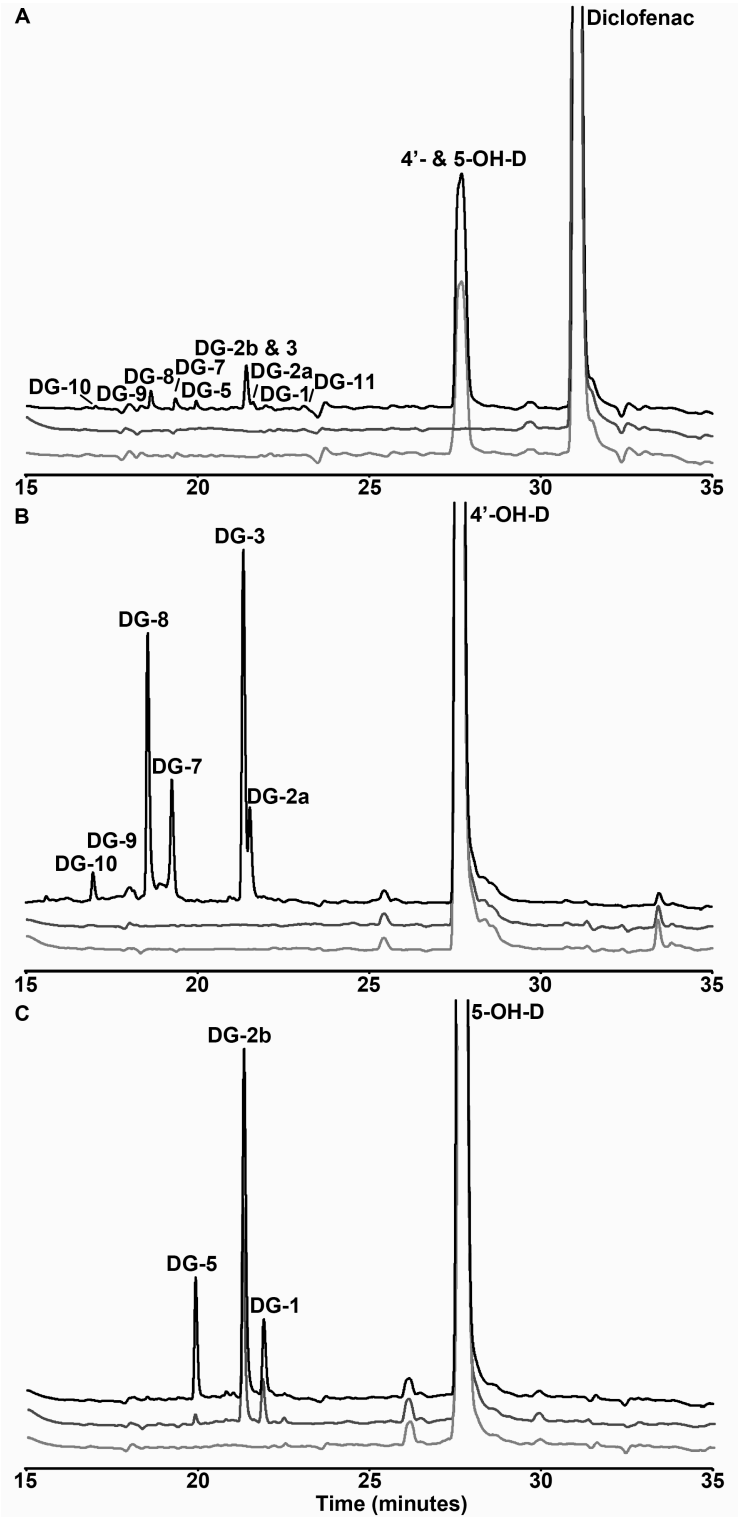
First, the utility of cytochrome P450 BM3 M11 as model P450 for diclofenac metabolism was evaluated. Cytosolic BM3 M11 combines the advantages of a bacterial P450, such as good solubility and high catalytic activity, with drug-metabolizing properties (Damsten et al., 2008). His-tagged BM3 M11 was expressed in *E.coli* using a bacterial expression vector and subsequently purified. The purified enzyme was incubated with diclofenac in the presence of NADPH. By measuring glutathione (GSH) conjugates, we investigated electrophilic reactive metabolites formed from diclofenac.

Damsten et al. (2008) showed that diclofenac was mainly metabolized by BM3 M11 into hydroxydiclofenac and several hydroxydiclofenac GSH-conjugates (DG-1, DG-2a, DG-2b, DG-3, DG-5, DG-7, DG-8, DG-9, DG-10; see Fig. 6 for the biotransformation scheme). Here, we confirmed these results and identified an additional diclofenac metabolite (Table 1, Fig. 1A), namely the GSH-conjugate of the oxidative decarboxylated diclofenac metabolite (2-(2,6-dichloro-phenyl-amino)benzyl-S-thioether glutathione; DG-11). Furthermore, using a different HPLC gradient, we observed that the hydroxydiclofenac peak consisted for ~90% of 4'-hydroxydiclofenac (4'-OH-D) and for ~10% of 5-hydroxydiclofenac (5-OH-D, data not shown). Also in human liver microsome (HLM) incubations, 4'-hydroxydiclofenac is the major metabolite (Shen et al., 1999).

Metabolites			*M11 in vitro*			*M11 in vivo (yeast)*		
	structure	m/z	D	4'-OH-D	5-OH-D	D	4'-OH-D	5-OH-D
4'-OH-D	+O	312.0 [a]	+	+	-	+	+	-
5-OH-D	+O	312.0 [a]	+	-	+	n.d.	-	+
DG-1	+O +SG -H	617.1 [a]	+	-	+	n.d.	-	+
DG-2a	+O +SG -H	617.1 [a]	+	+	-	n.d.	+	-
DG-2b	+O +SG -H	617.1 [a]	+	-	+	n.d.	-	+
DG-3	+O +SG -Cl	583.1 [a]	+	+	-	n.d.	+	-
DG-5	+O +2SG -2H	461.6 [b]	+	-	+	n.d.	-	+
DG-7	+O +2SG -Cl -H	444.6 [b]	+	+	-	n.d.	+	-
DG-8	+O +2SG -2Cl	427.6 [b]	+	+	-	n.d.	+	-
DG-9	+O +3SG -2Cl -H	387.1 [c]	+	+	-	n.d.	n.d.	-
DG-10	+O +3SG -2Cl -H	387.1 [c]	+	+	-	n.d.	n.d.	-
DG-11	-COOH +SG	557.1 [a]	+	-	-	n.d.	-	-

Table 1. Metabolism of diclofenac and 4'- and 5-hydroxydiclofenac by BM3 M11 *in vitro* or *in vivo* in yeast. Metabolites were analyzed by HPLC with UV detection at 254 nm and by LC-MS. Presence (+) or absence (-) of the metabolite is indicated. N.d.: not detected, a: single protonated molecular ion, b: double protonated molecular ion, c: triple protonated molecular ion.

Fig. 1. Diclofenac metabolites formed by BM3 M11 *in vitro*. Shown are HPLC chromatograms of incubations containing 250 nM BM3 M11 purified from *E.coli* with 500 µM diclofenac (A), 500 µM 4'-hydroxydiclofenac (B) or 500 µm 5-hydroxydiclofenac (C) in the presence of NADPH and glutathione. The black, upper traces represent full incubations; the dark grey, middle traces incubations including glutathione but lacking BM3 M11; and the light grey, lower traces incubations including BM3 M11 but lacking glutathione. Identification of all metabolites was confirmed by LC-MS.


A
Diclofenac
4'- & 5-OH-D
DG-2b & 3
DG-10
DG-9
DG-8
DG-7
DG-5
DG-2a
/DG-1/
DG-11
15
20
25
30
35
B
4'-OH-D
DG-3
DG-8
DG-7
DG-2a
DG-9
DG-10
15
20
25
30
35
C
5-OH-D
DG-2b
DG-5
DG-1
15
20
25
30
35
Time (minutes)

Additionally, we used synthetic 4'- and 5-hydroxydiclofenac as substrates in *in vitro* incubations with purified BM3 M11 to differentiate between 4'- or 5-hydroxydiclofenac-derived GSH-conjugates. As expected, most of the GSH-conjugates found in diclofenac incubations were also present in incubations with either 4'- or 5-hydroxydiclofenac (Fig. 1B-C, Table 1). These are most likely GSH-conjugates of the corresponding quinone imines, which are reactive metabolites of 4'- and 5-hydroxydiclofenac. The only exception is DG-11, the GSH-conjugate of the oxidative decarboxylated metabolite of diclofenac, which is only found in diclofenac incubations and is formed directly from diclofenac. Moreover, we observed that GSH-conjugates of 5-hydroxydiclofenac were formed spontaneously, although at lower levels than when BM3 M11 was present (Fig. 1C). This is caused by autooxidation of 5-hydroxydiclofenac to the corresponding quinone imine (Shen et al., 1999). 4'-Hydroxydiclofenac clearly requires activation by BM3 M11 to form GSH-conjugates (Fig. 1B). Similar results were previously reported for 4'- and 5-hydroxydiclofenac in HLM incubations (Madsen et al., 2008).

Importantly, the diclofenac metabolite profile of BM3 M11 (Fig. 1, Table 1) is analogous to that generated by HLMs. In both cases the major metabolite is 4'-hydroxydiclofenac. 5-Hydroxydiclofenac and the GSH-conjugates of 4'- and 5-hydroxydiclofenac (DG-1, DG-2a, DG-2b, DG-3, DG-5, DG-7, DG-8, DG-9, DG-10) and of the decarboxylated metabolite (DG-11) have also been identified in HLM incubations (Damsten et al., 2008; Grillo et al., 2008; Madsen et al., 2008). Damsten et al. (2008) reported a comparison between the ratios of some of the GSH-conjugates in BM3 M11 and HLM incubations. Collectively, these results demonstrate that BM3 M11 generates an oxidative diclofenac metabolite profile comparable to that of HLMs. Therefore, BM3 M11 is a suitable model P450 to study oxidative diclofenac metabolism.

Expression and activity of cytochrome P450 BM3 M11 in yeast

By expressing BM3 M11 in yeast, we aimed to establish a model system for investigating P450-related toxicity. Yeast was transformed with an episomal vector (pTL26-M11) containing the C-terminal His-tagged BM3 mutant M11 (van Vugt-Lussenburg et al., 2007) behind a conditional GAL promoter. Switching the yeast cells from glucose- to galactose-containing medium induced BM3 M11 expression. The presence of BM3 M11 in the strain was determined by Western blotting (Fig. 2). The blot shows that when the yeast strain was grown on medium containing galactose, an antibody against His-tags bound to a protein with a similar size as BM3 M11 purified from *E. coli* (119 kDa).

No protein was recognized in cells transformed with pTL26-M11 and grown on glucose containing medium, showing that P450 expression in the strain is controllable. Also in the

control yeast strain transformed with an empty vector (pTL26), no protein was recognized by the anti-His antibody (data not shown). The expression level was ~5 pmol BM3 M11/mg total protein. This corresponds to ~0.1% (w/w) of all soluble protein, which is comparable to P450 expression levels in mammalian cells (Aoyama et al., 1990; Ding et al., 1997).

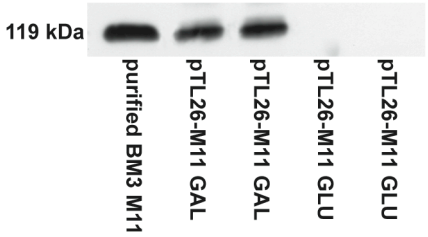


Fig. 2. Westernblot of protein extracts of yeast strains transformed with pTL26-M11. An anti-His antibody was used against the His-tag of M11. Lane 1: purified BM3 M11 from *E. coli*, lane 2 & 3: protein extract (25 µg) of yeast cells transformed with pTL26-M11 grown in medium containing galactose, lane 4 & 5: protein extract (25 µg) of yeast cells transformed with pTL26-M11 grown in medium containing glucose.

Subsequently, the *in vivo* activity of the heterologously expressed BM3 M11 in yeast was examined. The yeast strains were incubated in medium containing galactose and 0 or 50 µM diclofenac. Cellular lysates were analyzed by HPLC for the presence of diclofenac metabolites (Fig. 3). At 27.5 min a clear hydroxydiclofenac peak is visible in the HPLC chromatogram. In yeast strains without BM3 M11 or in strains incubated without diclofenac the hydroxydiclofenac was not detected. The GSH-conjugates were below detection limits in the cellular lysate of the yeast strain expressing BM3 M11. Due to the intrinsic toxicity of diclofenac in yeast, we could only use relatively low diclofenac concentrations for the *in vivo* yeast incubations. Using 200 µM 4'- or 5-hydroxydiclofenac, which are notably not toxic to yeast at these high concentrations, most of the GSH-conjugates (i.e. DG-1, DG-2a, DG-2b, DG-3, DG-5, DG-7 and DG-8) were detected by LC-MS in lysates of BM3 M11 expressing cells (Table 1), demonstrating the generation of quinone imines in yeast cells expressing BM3 M11. DG-11, the GSH-adduct of the decarboxylated metabolite, is formed directly from diclofenac and is therefore not present in incubations with 4'- or 5-hydroxydiclofenac. The triple-GSH-conjugates DG-9 and DG-10 were not detectable in the yeast lysates. To evaluate if heterologously expressed BM3 M11 forms these metabolites, lysates of BM3 M11 expressing yeast cells were incubated with 1 mM diclofenac (for DG-11) or 4'-hydroxydiclofenac (for DG-9 and DG-10). Indeed, these metabolites could be detected in these *in vitro* incubations (data not shown). Since all GSH-conjugates were detected either inside the yeast cells or in incubations with yeast lysates, all reactive intermediates of diclofenac are expected to be generated inside the bioactivation competent yeast cells after incubation with diclofenac.

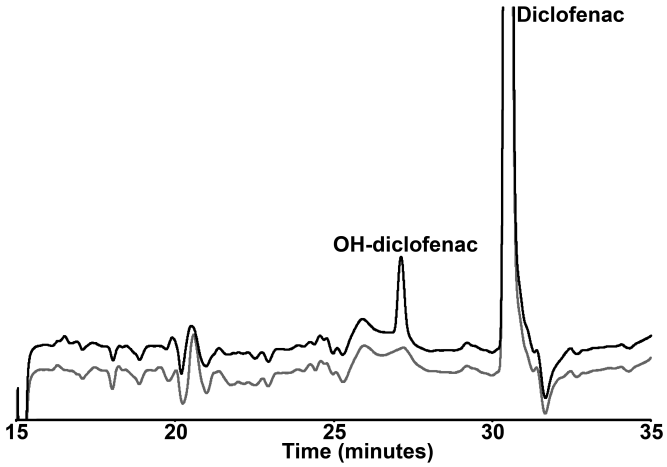


Fig. 3. Diclofenac metabolites formed by BM3 M11 *in vivo*. Shown are HPLC chromatograms of cellular lysates of yeast cells expressing BM3 M11 (black, upper trace) or transformed with an empty vector (grey, lower trace) after *in vivo* incubation with 50 µM diclofenac in selective medium containing galactose.

The effect of BM3 M11 expression on diclofenac toxicity in yeast

To examine the influence of oxidative diclofenac metabolism on yeast growth, W303 strains with or without expressed BM3 M11 were grown for 6 hours with 0-50 µM diclofenac. When grown without diclofenac, the growth of strains expressing BM3 M11 was comparable to that of strains without BM3 M11, showing that BM3 M11 expression in itself is not toxic to yeast. For both strains, growth without diclofenac was set at 100% and the percentage growth of diclofenac-treated cultures was calculated (Fig. 4A). In the presence of 50 µM diclofenac, both strains grew significantly more slowly. This shows that unmetabolized diclofenac is already toxic in yeast. Notably, the strain expressing BM3 M11 grew significantly more slowly in the presence of 50 µM diclofenac (57±1% growth) than the control strain transformed with an empty vector (75±5% growth). This clearly shows that P450-dependent metabolism of diclofenac causes additional toxicity. This P450-dependent growth difference was also visible at 30 µM diclofenac.

Diclofenac can induce the formation of ROS in both mammalian cells (Gomez-Lechon et al., 2003; Inoue et al., 2004; Lim et al., 2006) and in yeast (van Leeuwen et al., 2011). In mammalian cells, it has been shown that sulfaphenazole (CYP2C9 inhibitor) and ketoconazole (CYP3A4 inhibitor) decrease toxic effects caused by diclofenac exposure, such as LDH release (Bort et al., 1999; Kretz-Rommel and Boelsterli, 1993) and ROS formation (Lim et al., 2006). To investigate if ROS levels were changed by the oxidative metabolism of diclofenac in the yeast system, we incubated the strain transformed with an empty vector and the BM3 M11 expressing strain with diclofenac and with the ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate. In the absence of diclofenac, ROS levels in both strains were comparable (Fig. 4B). When incubated with 30 µM or 50 µM diclofenac, the ROS levels were increased in both

strains, but were significantly higher in the BM3 M11 strain than in the control strain. This shows that the increase in diclofenac toxicity observed in BM3 M11 expressing strains is accompanied by increased ROS formation. Similar results were obtained in another yeast strain, BY4741 (data not shown). Overall, these results demonstrate the direct involvement of cytochrome P450 activity in the cellular toxicity of diclofenac.

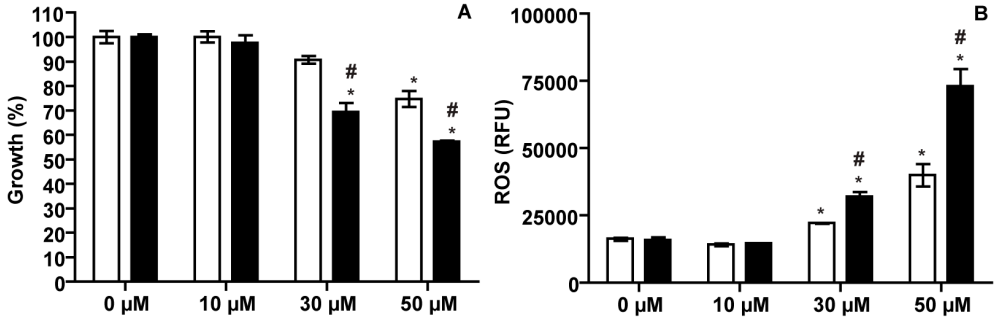


Fig. 4. Growth and ROS formation of yeast strains expressing BM3 M11 (black bars) or without BM3 M11 (white bars) after incubation with different concentrations of diclofenac. (A) Growth of the strains after incubating 6 hours with diclofenac. (B) The strains were incubated for 3 hours with 10 µM of the ROS-sensitive, fluorescent 2',7'-dichlorodihydrofluorescein diacetate and 0-50 µM diclofenac. Strains were grown in selective media containing galactose. The growth is expressed as % growth ± SD compared to untreated cells (100%) and ROS levels are expressed as RFU ± SD. * P<0.01 compared to cells incubated without diclofenac, # P<0.01 compared to cells without BM3 M11.

4'- and 5-hydroxydiclofenac metabolism does not affect growth and ROS formation

The quinone imines formed from 4'- and 5-hydroxydiclofenac (Fig. 6) are considered to be responsible for the toxicity of diclofenac (Miyamoto et al., 1997; Poon et al., 2001; Shen et al., 1999; Tang et al., 1999b). To investigate if the quinone imines could be related to the P450-dependent toxicity of diclofenac in our yeast model, we tested the effect of 4'- and 5-hydroxydiclofenac on growth and ROS levels of yeast cells with or without BM3 M11. Interestingly, when either 4'- or 5-hydroxydiclofenac was added to the yeast strains at concentrations ranging between 0.3 and 50 µM, no negative growth effect could be observed (Fig. 5A, data shown for 30 µM), neither in control strains without BM3 M11, nor in strains expressing BM3 M11. Notably, 4'- and 5-hydroxydiclofenac (30 µM) did not induce ROS formation in control or M11 expressing strains (Fig. 5B) although GSH-conjugates were generated inside the cells. Similar results were obtained in another yeast background (BY4741) or with 4'- and 5-hydroxydiclofenac obtained from another source (Toronto Research Chemicals, Canada; data not shown).

In principle, the lack of toxicity of 4'- and 5-hydroxydiclofenac could be a result of limited uptake by the cells. Using HPLC and LC-MS the uptake of both hydroxylated metabolites was checked (Table 2). When treated with 30 µM 4'- or 5-hydroxydiclofenac for 3 hours, their intracellular concentration was much higher than the hydroxydiclofenac concentration found in BM3 M11 expressing strains incubated 3 hours with 30 µM diclofenac, at which a P450-dependent decrease in growth and increase in ROS was detected.

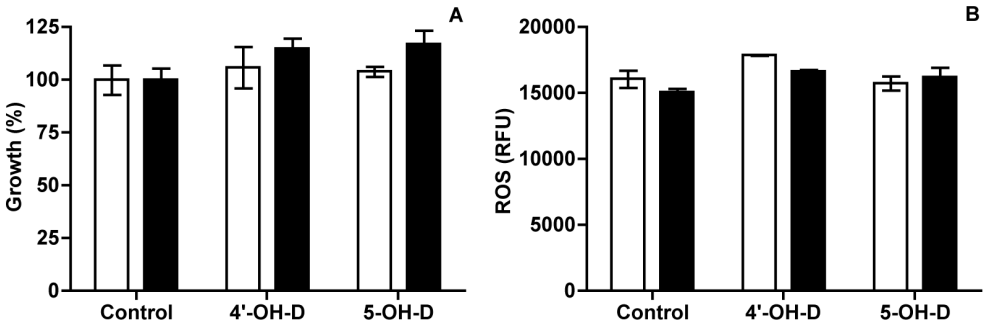


Fig. 5. Growth and ROS formation of yeast strains expressing BM3 M11 (black bars) or without BM3 M11 (white bars) after incubation with 30 µM 4'- or 5-hydroxydiclofenac. (A) Growth of the strains after incubation for 6 hours with 4'- or 5-hydroxydiclofenac. (B) ROS levels after incubating the strains for 3 hours with 4'- or 5-hydroxydiclofenac in the presence of the ROS marker 2',7'-dichlorodihydrofluorescein diacetate. The strains were grown in selective media containing galactose. The growth is expressed as % growth ± SD compared to untreated cells (100%) and the ROS levels are expressed as RFU ± SD. There were no significant differences (P<0.01) between the various conditions.

	Concentration in lysate (pmol/µg)	
	diclofenac	OH-diclofenac
30 µM diclofenac	179 ± 5	0.3 ± 0.2
30 µM 4'-OH-diclofenac	-	15 ± 2
30 µM 5-OH-diclofenac	-	9 ± 3

Table 2. Concentration of diclofenac or hydroxydiclofenac in yeast cells expressing BM3 M11. The cells were incubated for 3 hours with 30 µM (hydroxy)diclofenac. Cellular lysates were analyzed by HPLC and LC-MS. Numbers indicate the amount of (hydroxy)diclofenac in pmol/µg protein.

Collectively these results indicate that 4'- and 5-hydroxydiclofenac and their quinone imines, reflected by the marker GSH-conjugates DG1-10, are not involved in diclofenac toxicity in

yeast. Interestingly, toxic effects such as uncoupling of oxidative phosphorylation, ATP depletion and inactivation of CYP3A4 are also mainly observed with diclofenac and not with isolated diclofenac metabolites (Bort et al., 1999; Masubuchi et al., 2000, 2002). On the other hand, 5-hydroxydiclofenac can bind to cellular protein in HLM incubations in the absence of GSH (Shen et al., 1999). Although the toxicological relevance of protein adducts in isolated cells such as yeast or hepatocytes may be limited (Kretz-Rommel and Boelsterli, 1993), *in vivo* protein adducts might result in an immunological response leading to toxicity (Naisbitt et al., 2007).

As hydroxydiclofenac is not toxic in bioactivation-competent yeast, the observed toxicity and ROS production is likely coupled to another P450-mediated diclofenac metabolite or reactive intermediate. Interestingly, the GSH-conjugate of the decarboxylated metabolite (DG-11) was the only metabolite we could detect in BM3 M11 incubations with diclofenac that was not formed in incubations with 4'- or 5-hydroxydiclofenac. Grillo et al (2008) identified DG-11 previously in incubations with HLMs. Interestingly, also for the other NSAIDs ketoprofen and indomethacin oxidative decarboxylated metabolites have been identified (Komuro et al., 1995). Both ketoprofen and indomethacin caused a P450-dependent increase in ROS formation and toxicity in our yeast model system while other drugs like paracetamol and clozapine did not (data not shown). During oxidative decarboxylation of indomethacin, a carbon radical is formed (Komuro et al., 1995), which is also suggested to be formed in the oxidative decarboxylation of diclofenac (Grillo et al., 2008; Fig. 6). Since free radicals are toxic species, the formation of this decarboxylated metabolite might be responsible for the P450-dependent toxicity of diclofenac. Another possible explanation is that intermediate arene oxides that are involved in the formation of 4'- and 5-hydroxydiclofenac play a role in toxicity (Blum et al., 1996; Masubuchi et al., 2002; Fig. 6). Consistent with this hypothesis, the 2'-hydroxy-3'-GSH-monoclofenac metabolite, identified in HLM incubations by Yan et al. (2005), is considered to result from the diclofenac 2',3'-arene oxide.

Conclusion

A crucial topic in examining the toxicity of drugs is distinguishing between the toxicity of the parent drug and its metabolites. Here, we created yeast strains with inducible expression of the cytosolic, stable and highly active P450 BM3 M11 and isogenic strains transformed with an empty vector as tool to study the P450-dependent toxicity of diclofenac. Advantages of this yeast model over mammalian cellular systems are the controlled P450 expression levels, a defined eukaryotic background and the redundancy of P450 inhibitors. Both stable and GSH-conjugated metabolites of diclofenac were identified inside the BM3 M11 expressing yeast cells.

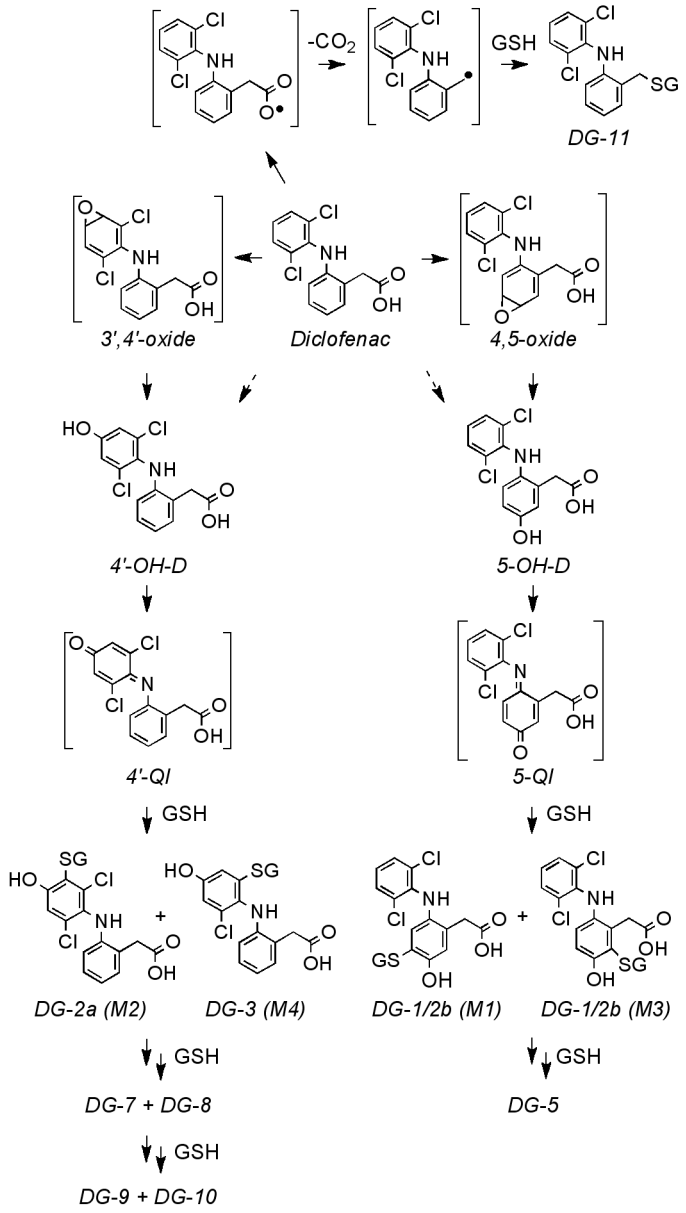


Fig. 6. Biotransformation scheme of diclofenac. Diclofenac metabolism can yield potentially toxic reactive intermediates, including quinone imines, arene oxides and radical species.

We showed a significantly slower growth and higher ROS levels for strains expressing BM3 M11 when exposed to diclofenac than in control strains without BM3. The metabolites 4'- and 5-hydroxydiclofenac had no effect on the cell growth and ROS formation in strains with or

without BM3, showing that these metabolites and their reactive quinone imines or other secondary metabolites are not responsible for P450-mediated diclofenac toxicity in yeast. Alternatively, either reactive arene oxides in the formation of hydroxydiclofenac or radical species in the formation of DG-11 might be responsible for the observed toxicity. Further research is needed to identify which of these two routes causes the toxicity, possibly by using BM3 mutants with an altered metabolite profile. Furthermore, yeast genetics can now be used to determine which genes are involved in the toxicity of diclofenac metabolism.

ACKNOWLEDGMENTS

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IV

Involvement of the pleiotropic drug resistance response, protein kinase C signaling, and altered zinc homeostasis in resistance of *Saccharomyces cerevisiae* to diclofenac

Jolanda S. van Leeuwen
Nico P.E. Vermeulen
J. Chris Vos

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ABSTRACT

Diclofenac is a widely used analgesic drug that can cause serious adverse drug reactions. We used *Saccharomyces cerevisiae* as model eukaryote to elucidate the molecular mechanisms of diclofenac toxicity and resistance. Although most yeast cells died during the initial diclofenac treatment, some survived and started growing again. Microarray analysis of the adapted cells identified three major processes involved in diclofenac detoxification and tolerance. In particular, pleiotropic drug resistance genes and genes under the control of Rlm1p, a transcription factor in the protein kinase C (PKC) pathway, were upregulated in diclofenac-adapted cells. We tested if these processes or pathways were directly involved in diclofenac toxicity or resistance. Of the pleiotropic drug resistance gene products, the multidrug transporter Pdr5p was crucially important for diclofenac tolerance. Furthermore, deletion of components of the cell wall stress-responsive PKC pathway increased diclofenac toxicity, whereas incubation of cells with the cell wall stressor calcofluor white before the addition of diclofenac decreased its toxicity. Also, diclofenac induced flocculation, which might trigger the cell wall alterations. Genes involved in ribosome biogenesis and rRNA processing were downregulated, as were zinc-responsive genes. Paradoxically, deletion of zinc-responsive transcription factor Zap1p or addition of the zinc-chelator 1,10-phenantroline significantly increased diclofenac toxicity, establishing a regulatory role for zinc in diclofenac resistance. In conclusion, we have identified three new pathways involved in diclofenac tolerance in yeast, namely Pdr5p, as main the contributor of the PDR response, cell wall signaling via the PKC pathway, and zinc homeostasis regulated by Zap1p.

INTRODUCTION

Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), is widely used in the treatment of arthritis and related disorders because of its inhibitory effect on prostaglandin synthesis. Additionally, the antifungal potency of diclofenac has been tested against prostaglandin-secreting pathogenic fungi (Alem and Douglas, 2004). Unfortunately, diclofenac may cause serious adverse drug reactions (ADRs) that target the liver (Laine et al., 2009), heart (Fosbol et al., 2009) or upper gastrointestinal tract (Lewis et al., 2002). Furthermore, diclofenac is an environmental hazard to Gyps vultures due to its widespread use as veterinary drug (Oaks et al., 2004). In mammalian hepatocytes, diclofenac toxicity has been linked to mitochondrial dysfunction and oxidative metabolism by cytochrome P450s (Gomez-Lechon et al., 2003; Lim et al., 2006). Gene expression analysis has been performed on murine liver samples (Chung et al., 2006; Deng et al., 2008) and on human and rat hepatocytes (Lauer et al., 2009) treated with diclofenac to further identify the underlying toxicity mechanisms. In particular, genes

associated with oxidative stress, cell death, and cell cycle regulation were identified. However, the specific genes directly involved in diclofenac toxicity remained unclear.

Previously, we have shown in *Saccharomyces cerevisiae* that subunits Rip1p and Cox9p of the mitochondrial respiratory chain are diclofenac targets and that metabolism of diclofenac by cytochrome P450s increases its toxicity (van Leeuwen, 2011a, 2011b). Yeast is an excellent eukaryotic model organism for toxicological research (Hoon et al., 2008; Yaksokawa and Iwahashi, 2010). The advantages of yeast over mammalian cellular systems are its straightforward genetic accessibility, cost-effectiveness, and rapid growth. Furthermore, the pharmacological targets of diclofenac do not exist in yeast, thereby simplifying the test system, whereas many of the mechanisms underlying toxicity and resistance to chemicals and other environmental stresses are conserved (Mager and Winderickx, 2005). For example, both in yeast and in mammalian cells, diclofenac toxicity is related to mitochondrial dysfunction and elevated production of reactive oxygen species (ROS) (Gomez-Lechon et al., 2003; Lim et al., 2006; van Leeuwen et al., 2011a). The availability of a well-annotated genome sequence makes yeast an ideal model system for genome-wide studies. The transcriptional responses of yeast to a wide variety of stress conditions have been studied extensively (Causton et al., 2001; Gasch et al., 2000). Moreover, yeast deletion strain collections and overexpression libraries have been used to identify drug on- and off-targets (Giaever et al., 2004; Luesch et al., 2005).

In this study, we present the mechanisms of diclofenac toxicity and resistance in yeast as eukaryotic model organism. By using microarray analysis of adapted yeast cells and by testing the resistance of relevant haploid deletion strains, we gained insight into the main defense mechanisms circumventing diclofenac toxicity.

MATERIALS AND METHODS

Chemicals and stock solutions

Diclofenac was purchased as its sodium salt from Sigma-Aldrich and was dissolved in DMSO (100 mM). 2',7'-Dichlorodihydrofluorescein diacetate was obtained from Alexis Biochemicals and dissolved in EtOH (4 mM). Stock solutions were stored at –20 °C and were protected from light. All other chemicals were purchased from Sigma-Aldrich at the highest purity.

Strains

The haploid *Saccharomyces cerevisiae* strains W303-1A (MATa; ura3-52; trp1Δ2; leu2-3,112; his3-11,15; ade2-1; can1-100) and BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) were used. BY4741 deletion strains were obtained from EUROSCARF. W303-1A Δpkc1 and Δslt2 were a kind gift from Stefan Hohmann (Tamas et al., 1999).

Growth conditions and diclofenac treatment

Strains were grown overnight at 30°C in selective minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented amino acids and bases). Overnight cultures were diluted in minimal media and grown at 30°C to an OD_{600} ~0.2. At this point, 0-100 µM diclofenac was added. Controls were treated with equal amounts of DMSO (max 0.1%). HPLC analysis of the medium or cellular lysates revealed no degradation products of diclofenac in wild type cells after incubating 24 hours (van Leeuwen et al., 2011a).

RNA extraction and microarray analysis

Five independent yeast cultures were incubated with 100 µM diclofenac for 75 hours. Every 24h, cultures were diluted to an OD_{600} ~0.1 in YNB containing 100 µM diclofenac. Five independent control cultures were grown in the absence of diclofenac. Yeast pellets were frozen at -80°C prior to RNA extraction. mRNA was isolated using the Qiagen RNeasy Mini Kit. Hybridization and array analysis were performed by the MicroArray Department of the University of Amsterdam using GeneChip® Yeast Genome 2.0 Arrays from Affymetrix. Data were normalized using MAS5 and RMA and analyzed for significantly enriched classes or categories of genes in the Gene Ontology (GO) database using T-profiler (Boorsma et al., 2005). T-profiler analysis was also performed to search for significantly enriched groups of genes containing upstream matches to a consensus transcription factor binding motif. The microarray data can be downloaded from the Genome Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE29331.

Overexpression constructs

PDR5 and *SNQ2* overexpression constructs in YEplac181 (2 µm origin, *LEU2* marker) were a kind gift from Karl Kuchler (Mahe et al., 1996). Scott Moye-Rowley kindly provided the *RSB1* overexpression construct pRS426-*RSB1* (2 µm origin, *URA3* marker). The plasmids were transformed into yeast strain BY4741 by using the freeze-thaw method (Klebe et al., 1983).

ß-galactosidase reporter assay

The *TRP5*-, *PDR3*-, *PDR5*-, *RSB1*-, and *SNQ2*-lacZ reporter constructs in the low copy number vector pSEYC102 were a kind gift from Scott Moye-Rowley (Decottignies et al., 1995; Hallstrom and Moye-Rowley, 2000; Katzmann et al., 1994; Panwar and Moye-Rowley, 2006). The plasmids were transformed into yeast strain BY4741 by using the freeze-thaw method (Klebe et al., 1983). Yeast cultures were grown at 30°C to an OD_{600} ~0.2. Diclofenac was added and cultures were incubated for 2 hours. Protein extracts were made in LacZ buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol) by vortexing with glass beads. ~20 µg of protein was incubated with 24 µg chlorophenolred-ß-

D-galactopyranoside at 30°C for ~30 minutes. Absorption at 575 nm was measured and corrected for the incubation time and the protein concentration in the samples.

Measurement of ROS production

Exponentially growing cultures (2 mL) of OD_{600} ~0.2 were treated with 0 or 50 µM diclofenac in the presence of 10 µM of the fluorescent, ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate. After 3 hours at 30 °C the cultures were centrifuged (3 min, 3000 rpm) and cell pellets were washed and resuspended in 1 mL water. Fluorescence (l_{ex} = 485 nm, l_{em} = 535 nm) was measured and corrected for the cell density in the samples.

Figures and statistics

All experiments were performed at least two times in triplo. Standard deviations (SD) were calculated using GraphPad Prism 4 and figures were created with GraphPad Prism 4 and Adobe Illustrator CS5. Contrast of the photographs in Fig. 5D was enhanced using Adobe Photoshop CS5.

RESULTS

Adaptation of yeast to diclofenac

Previously, we reported that yeast cell growth is inhibited at diclofenac concentrations of 50 µM and higher (van Leeuwen et al., 2011a). Upon incubation with 100 µM diclofenac, wild type yeast cells could hardly grow at all and after 3 hours only 10% of the cells were viable. However, when cells after a 24-hours treatment with diclofenac were diluted and treated again with 100 µM diclofenac, no toxicity could be observed anymore (Fig. 1A), showing that the surviving yeast cells had fully adapted to diclofenac.

Diclofenac targets the mitochondria and induces ROS formation during the initial toxicity of diclofenac (van Leeuwen et al., 2011a). In the present study, we investigated the amount of ROS formed in adapted cells compared with freshly treated cells. Yeast cells were either adapted by growing them for 48 hours with 100 µM diclofenac or were grown for 48 hours without diclofenac. Exponentially growing cells were then incubated for 3 hours with 0 or 50 µM diclofenac in the presence of the fluorescent, ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate. Although 50 µM diclofenac caused a growth delay in freshly treated cells, this concentration was not lethal (data not shown). ROS levels in adapted cells that were now incubated without diclofenac were comparable to ROS levels in cells grown continuously in the absence of diclofenac (Fig. 1B). Interestingly, ROS formation in adapted cells incubated with 50 µM diclofenac was significantly lower than in freshly treated

cells. As we have observed previously (van Leeuwen et al., 2011a, 2011b), there is a clear correlation between diclofenac-induced growth inhibition and ROS formation.

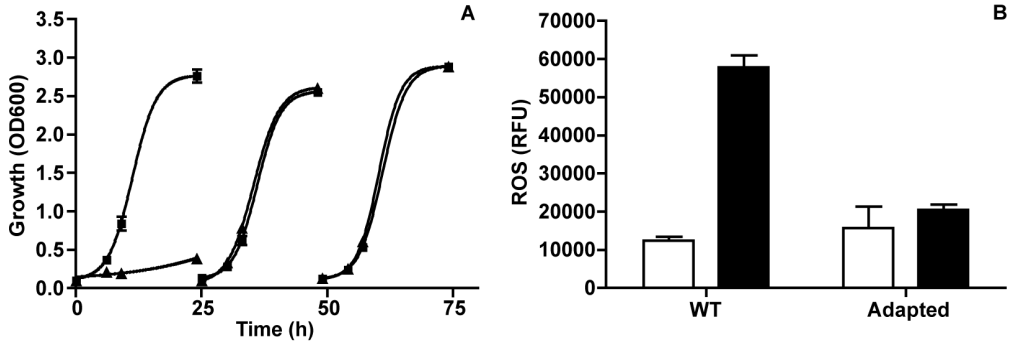

Fig. 1. (A) Yeast cells can adapt to diclofenac. W303 cells were grown in the presence of 0 µM (squares) or 100 µM (triangles) diclofenac in minimal medium. After 24 and 48 hours the cultures were diluted in minimal medium containing 0 or 100 µM diclofenac. Growth is expressed as OD at 600 nm ± SD. (B) Diclofenac-adapted cells have lower ROS levels than wild type cells in the presence of diclofenac. W303 cells pretreated for 48 hours with 0 µM (WT) or 100 µM (adapted) diclofenac were grown for 3 hours with 0 µM (white bars) or 50 µM (black bars) diclofenac in the presence of 10 µM of the ROS-sensitive, fluorescent 2',7'-dichlorodihydrofluorescein diacetate. Data are expressed as fluorescence units corrected for cell density ± SD.

Since loss of mitochondrial DNA (mtDNA) (rho^0 cells) leads to decreased ROS formation and elevated resistance to diclofenac (van Leeuwen et al., 2011a), we investigated whether adapted strains lost their mitochondrial DNA. After several days of incubation with 100 µM diclofenac, cells were plated on plates containing either glucose or glycerol and ethanol as carbon sources. Only cells that are able to respire and therefore contain mtDNA can grow on glycerol/ethanol-plates. Approximately 95% of the adapted cells contained mtDNA, which was comparable to what was found for cells grown without diclofenac. Although rho^0 cells have a higher resistance to diclofenac, no selection for rho^0 cells occurred.

To further investigate the characteristics of adaptation, strains were adapted by exposure to 50 µM diclofenac for 72 hours. When these adapted cells were subsequently treated with 150 µM diclofenac, almost no toxicity was observed (Fig. 2). However, when the adapted strains were grown in the absence of diclofenac for 48 hours and were then treated with 150 µM diclofenac, the resistance was lost (Fig. 2). This reversibility of adaptation is consistent with the observation that adapted strains do not lose their mtDNA, and also excludes selection for a random DNA mutation as has been described for multidrug resistance genes (Carvajal et al., 1997).

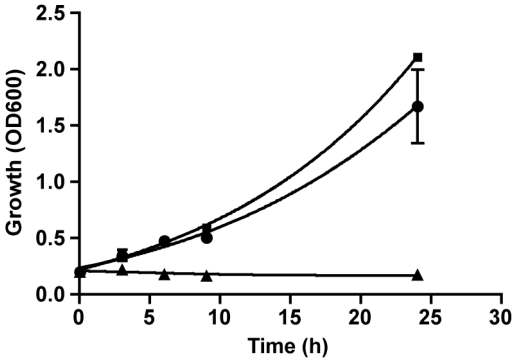

Fig. 2. Adaptation to diclofenac is reversible. W303 strains either were grown in the absence of diclofenac (squares), pretreated for 72h with 50 µM diclofenac and subsequently incubated with 150 µM diclofenac (circles), or first pretreated for 72h with 50 µM diclofenac, then incubated for 48h without diclofenac, and finally incubated with 150 µM diclofenac (triangles). Growth is expressed as OD at 600 nm ± SD.

Motif	T-value[a]	E-value[a]	Genes[b]
PDR	11.3	< 1.0E-15	*AZR1, RSB1, PDR5, GRE2, RTA1, YGR035C, ADY2, PDR15, YPL088W, SNQ2, ICT1, YLR346c, YOR268C, YOR1, YHR140W, YKL071W, HXT1, PDR18, TPO1, YLL066W-B, PRM5*
RLM1	4.5	8.6E-04	*RTA1, YGR035C, YPL088W, YPS3, YCR101C, YGL258W-A, YAL067W-A, YNR066C, ADH2, CSM4, YPL067C, PRM5*
ZAP1	-3.0	3.7E-02	*YOR387C, ADH4, VEL1, ZPS1, YIL169C, ZAP1, CHA1, YLL053C, YGK3, HPF1*
rRPE	-6.7	2.5E-09	*YOR378C, ADH4, VEL1, CYB5, YAP7, BFR2, YDL063C, SUL1, GCV2, YOL029C, BIO2, REX4*
PAC	-8.5	< 1.0E-15	*CHA1, BFR2, GIT1, YGK3*
Other (up)			*ECM12, YJL213W, PFS1, MET2, PRM4, YLR012C, IST3, DAL4, YGR153W, YOL038C-A, RIM15*
Other (down)			*PCL1, PDC6, YGR079W, AQY2, SEO1, DAK2, IRC7, FTR1, GRE1, YNR063W, DAL80, FET3, HLR1*

Table 1. Significantly enriched motif-groups in diclofenac-adapted strains compared with controls. a: T- and E-values were found using T-profiler analysis (Boorsma et al., 2005) on the complete dataset, b: genes, grouped by motif, that are >2 times up- or down-regulated in adapted strains compared with controls.

Genome-wide analysis of cells adapted to diclofenac

Because diclofenac-adaptation was reversible, it was probably related to altered gene expression. Genes whose expression is significantly altered in adapted cells might play an important role in tolerance to diclofenac. Therefore, we performed microarray analysis on

yeast cells adapted to diclofenac and compared the expression profile to that of controls grown without diclofenac. T-profiler analysis (Boorsma et al., 2005) was performed on the complete dataset to search for significantly enriched groups of genes containing upstream matches to a consensus transcription factor binding motif. The search for motifs revealed upregulation of pleiotropic drug resistance (PDR) genes and genes targeted by Rlm1p, a transcription factor in the protein kinase C (PKC) mediated MAP kinase pathway, which is responsive to cell wall stress (Table 1). Also genes containing an Msn2p/4p binding motif and responsive to oxidative or general environmental stress were slightly enriched. However, only Msn2p/4p responsive genes that also contain a PDR responsive element in their promoters were found to be upregulated. *MSN2* and *MSN4* themselves were both downregulated in the adapted cells (-1.1x and –1.4x, respectively) and probably are not involved in adaptation to diclofenac. Genes containing rRPE (ribosomal RNA processing element) or PAC (polymerase A and C box) motifs in their promoters were downregulated. These genes generally encode ribosomal proteins or proteins involved in rRNA and tRNA synthesis and processing and are downregulated in response to a variety of stresses (Gasch et al., 2000). Also, genes containing zinc-responsive promoters (Zap1p binding motif) were downregulated.

	Aspect[a]	T-value[b]	E-value[b]	Mean
(drug) Transporter activity	F	6.1	1.2E-06	0.38
Endoplasmic reticulum	C	6.0	2.7E-06	0.11
Cell wall	C	4.4	1.4E-02	0.13
Transcription	P	-4.7	4.6E-03	-0.11
Mitochondrion	C	-5.7	1.7E-05	-0.06
rRNA processing	P	-11.4	< 1.0E-15	-0.36
Nucleus	C	-11.6	< 1.0E-15	-0.12
Ribosome biogenesis	P	-12.5	< 1.0E-15	-0.36

Table 2. Significantly altered Gene Ontology (GO) categories in cells adapted to diclofenac compared with control cells, redundant or meaningless GO categories were left out. a: F = molecular function, C = cellular component, P = biological process, b: T- and E-values were determined using T-profiler analysis (Boorsma et al., 2005) on the whole dataset.

T-profiler was also used to analyze the data for significantly enriched classes or categories of genes using the Gene Ontology (GO) database (Table 2). Redundant or meaningless GO categories were left out. Interestingly, mRNA levels of proteins located in the ER or cell wall were increased, whereas mRNA levels of nuclear and mitochondrial proteins were lowered. Upregulation of the genes encoding cell wall components might be a consequence of *RLM1*

upregulation. In agreement with the results obtained by motif analysis, genes involved in drug transport (PDR motif) were upregulated and genes involved in rRNA processing and ribosome biogenesis were downregulated (rRPE and PAC motifs). Downregulation of Zap1p responsive genes did not lead to a significantly enriched GO category. In summary, the microarray data point to three pathways involved in diclofenac tolerance: the multidrug resistance response, cell wall stress and zinc homeostasis.

Upregulation of multidrug resistance genes

Our microarray data showed that the pleiotropic drug resistance response is dramatically upregulated in cells adapted to diclofenac. In yeast, multidrug resistance is regulated by transcription factors Pdr1p and Pdr3p (reviewed by Moye-Rowley, 2003). One of their major targets is ABC transporter Pdr5p. To investigate the multidrug resistance response during initial diclofenac toxicity, we followed *PDR5* promoter activity using a ß-galactosidase reporter construct in cells incubated with 30 µM diclofenac. After addition of diclofenac, *PDR5*-lacZ expression increased during the first ~3 hours, after which it remained stable for at least 21 hours (Fig. 3A). *PDR5*-lacZ expression was not changed in cells incubated without diclofenac. Additionally, we measured promoter activity of the other PDR-genes *PDR3*, *RSB1* and *SNQ2* after a 2-hour incubation with 30 µM diclofenac and set the level of activity in strains incubated without diclofenac at 100% (Fig. 3B). Indeed, also for these genes an increase in *lacZ* expression was observed. Promoter activity of a control gene involved in tryptophan biosynthesis (*TRP5*-lacZ) was not significantly altered. These results show that the PDR response is upregulated during initial diclofenac exposure and remains high in adapted cells.

To investigate the roles of the various PDR-transporters in diclofenac resistance, we examined the diclofenac sensitivity of BY4741 strains lacking *AZR1*, *RSB1*, *PDR5*, *ADY2*, *PDR15*, *SNQ2*, *TPO1,* or *PDR12*. *RSB1* and *ADY2* encode long-chain base and acetate transporters, respectively, whereas the other genes encode drug transporters. The wild type BY4741 strain (Fig. 4) showed diclofenac sensitivity and adaptation similar to those of the W303 strain (Fig. 1A) and resumed growth after ~15 h with 100 µM diclofenac (Fig. 4). The strain lacking *PDR5* was much more sensitive to diclofenac and needed ~35 h to adapt (Fig. 4). Deletion of any of the other genes tested had no significant effect on diclofenac toxicity (data not shown).

Furthermore, we tested diclofenac sensitivity of strains overexpressing *RSB1*, *PDR5* or *SNQ2*. Only cells overexpressing *PDR5* were more resistant to diclofenac (Fig. 4), whereas overexpression of the other genes had no effect (data not shown). Additionally, although ~10% of wild type cells survive incubating 3 hours with 100 µM diclofenac, less than 2% of the Δpdr5 cells and all *PDR5* overexpressing cells survive these conditions. However, in a

platereader assay, the minimum concentration of diclofenac that completely inhibited growth for 12 hours ranged from approximately 75 µM (Δpdr5) to 150 µM (WT) and 500 µM (PDR5 overexpressing strain) (data not shown). The relative small differences in these concentrations could indicate that Pdr5p does not actively transport diclofenac but rather indirectly affects diclofenac toxicity via an altered membrane composition (Shahi and Moye-Rowley, 2009). Regardless of the exact role of Pdr5p, the growth and survival data at 100 µM diclofenac clearly show the importance of Pdr5p dosage in diclofenac tolerance.

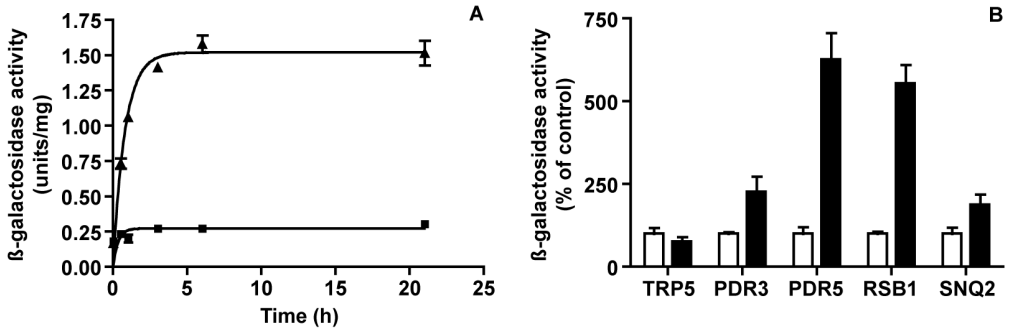

Fig. 3. The pleiotropic drug response (PDR) is dramatically upregulated by diclofenac. (A) Time-dependent expression of *PDR5*-lacZ in a BY4741 strain incubated with 0 µM (squares) or 30 µM (triangles) diclofenac. Data are expressed as ß-galactosidase activity in units, corrected for the protein concentration, ± SD. (B) *TRP5*-, *PDR3*-, *PDR5*-, *RSB1*-, and *SNQ2*-lacZ expression in BY4741 wild type cells incubated 3 hours with 0 µM (white bars) or 30 µM (black bars) diclofenac. LacZ expression is presented as % ß-galactosidase activity compared to untreated controls (100%) ± SD.

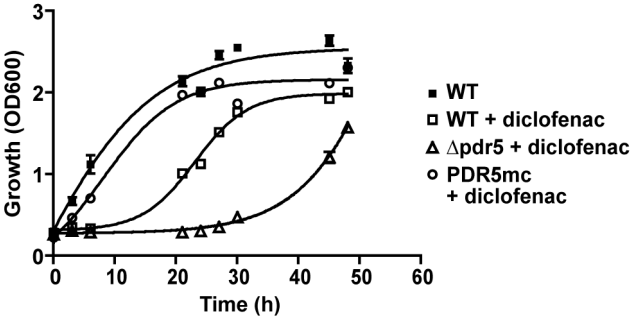

Fig. 4. Pdr5p is important for diclofenac tolerance. BY4741 wild type (open squares), Δpdr5 (open triangles) and PDR5 overexpressing (open circles) cells were grown in the presence 100 µM diclofenac in minimal medium containing glucose. In the absence of diclofenac, wild type (closed squares), Δpdr5 (not shown) and PDR5 overexpressing (not shown) cells grew comparably to each other. Data are expressed as OD at 600 nm ± SD.

Diclofenac resistance and cell wall modification

T-profiler analysis also showed that Rlm1p responsive genes were significantly upregulated in diclofenac-adapted cells (Table 1). The transcription factor Rlm1p is phosphorylated by MAPK Slt2p in the PKC pathway. Therefore, we tested the effect of deleting either *PKC1* or *SLT2* on cell growth in the presence of diclofenac. Both deletion strains showed increased sensitivity to diclofenac (Fig. 5A,B) confirming the important role of the PKC MAPK pathway in diclofenac tolerance.

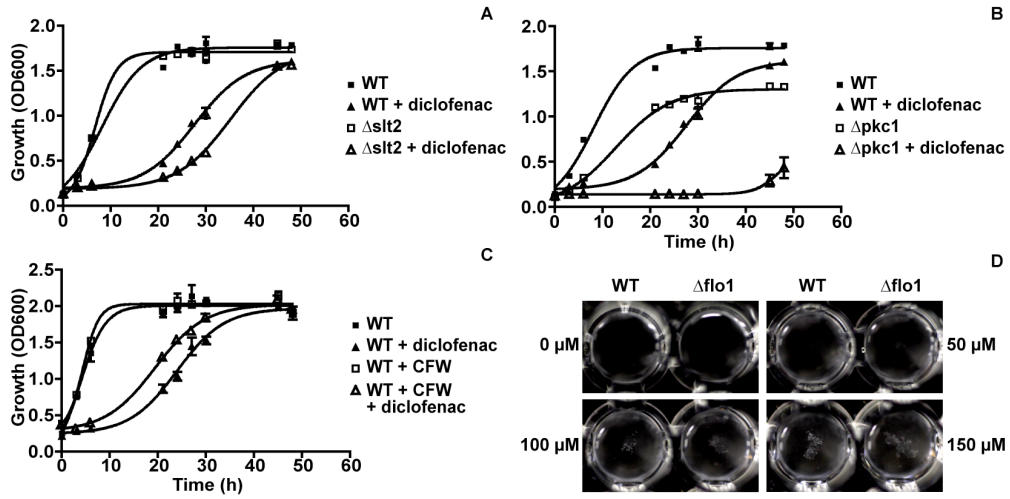

Fig. 5. Diclofenac induces PKC pathway-mediated cell wall stress and flocculation. (A,B) Wild type (closed symbols), Δslt2 (A, open symbols) and Δpkc1 (B, open symbols) W303 cells were incubated with 0 μM (squares) or 100 μM (triangles) diclofenac in minimal medium containing 1 M sorbitol for osmostabilization. (C) Wild type BY4741 cells were grown in the presence of 0 μM (squares) or 100 μM (triangles) diclofenac in minimal medium containing no additives (closed symbols) or 100 μg/ml calcofluor white (open symbols). Growth is expressed as OD600 ± SD. (D) BY4741 WT and Δflo1 cells were grown in minimal medium in a 48-well plate. Photographs were taken 20 minutes after addition of 0, 50, 100 or 150 μM diclofenac.

The PKC pathway is responsive to cell wall integrity, and indeed, many cell wall proteins were upregulated after diclofenac exposure (Table 2). To investigate whether cell wall changes lead to increased diclofenac resistance, cells were incubated with a non-toxic concentration of cell wall stressor calcofluor white, one hour before addition of diclofenac. In the presence of calcofluor white, diclofenac toxicity was decreased, whereas the growth of cultures without diclofenac was not affected (Fig. 5C). When calcofluor white was added one hour after addition of diclofenac, no decrease in toxicity was observed (data not shown). Similar results

were obtained with low concentrations of cell wall stressor zymolyase (data not shown). Apparently, the altered cell wall composition induced by calcofluor white decreases diclofenac toxicity.

During the growth assays we observed that diclofenac caused flocculation (Fig. 5D), which may trigger the cell wall changes. When cells were grown in 48-well plates with continuous shaking, small flocs appeared as early as 10 min after addition of diclofenac, suggesting that flocculation is not transcriptionally regulated but rather is a direct effect of diclofenac. Notably, deletion of *FLO1*, encoding a major lectin-like determinant of cell-cell adhesion, did not prevent flocculation (Fig. 5D). Possibly, diclofenac can bind to the cell wall, thereby causing flocculation, cell wall stress, and activation of the PKC MAPK pathway.

Altered zinc homeostasis during diclofenac exposure

The microarray analysis revealed that Zap1p responsive genes were strongly downregulated (Table 1). Zap1p is a transcription factor that regulates gene expression in response to changes in zinc levels (Herbig et al., 2005). Downregulation of Zap1p and Zap1p-responsive genes suggests that intracellular zinc levels are high in diclofenac-adapted cells. To further explore the role of zinc in diclofenac toxicity, we examined the toxicity of diclofenac in the presence of various zinc concentrations. Addition of 50-500 μM $ZnSO_4$ to the medium one hour before diclofenac addition had no effect on diclofenac toxicity (Fig. 6A, data shown for 50 μm $ZnSO_4$). However, addition of 50 μM of the zinc-chelator 1,10-phenantroline severely increased diclofenac toxicity (Fig. 6B). The increased toxicity was abolished by the addition of $ZnSO_4$, indicating that the chelation of zinc indeed led to the increased toxicity (Fig. 6B). Since the multidrug resistance response regulators Pdr1p and Pdr3p are zinc transcription factors, addition of 1,10-phenantroline might lead to toxicity by preventing the multidrug resistance response. Therefore, we tested the effect of 1,10-phenantroline on *PDR5*-lacZ expression in the presence of 10 μM diclofenac. This diclofenac concentration is not toxic in combination with 50 μM 1,10-phenantroline. Both in the absence or presence of 1,10-phenantroline, *PDR5*-lacZ activity was induced ~6 times by 10 μM diclofenac, showing that 1,10-phenantroline does not interfere with the PDR response (data not shown).

Furthermore, we tested the diclofenac sensitivity of a Δzap1 deletion strain. Surprisingly, deletion of Zap1p increased diclofenac toxicity (Fig. 6C). In presence of 100 μM zinc, the diclofenac resistance of Δzap1 cells was increased to the level of wild type cells, showing that the enhanced diclofenac toxicity of Δzap1 strains was caused by a lack of zinc. Accordingly, the addition of 100 μM $FeSO_4$ had no effect on the diclofenac sensitivity of wild type or Δzap1 strains (data not shown). These results point to a pivotal role for zinc in the ability of yeast cells to adapt to diclofenac exposure.

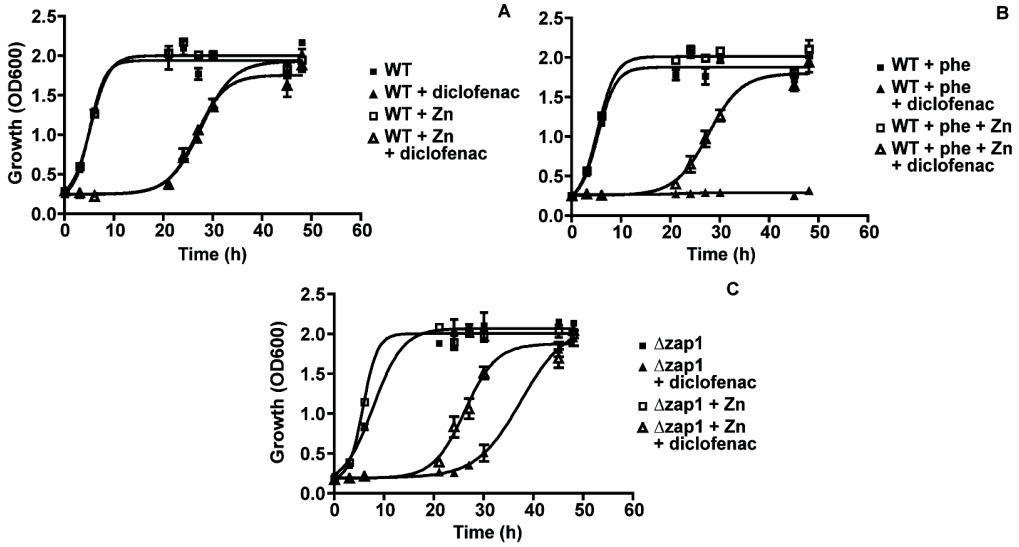

Fig. 6. Diclofenac toxicity is increased under low zinc conditions. Wild type (A,B) or Δzap1 (C) BY4741 cells were incubated with 0 μM (squares) or 100 μM (triangles) diclofenac in minimal medium containing: (A) no additive (closed symbols) or 50 μM $ZnSO_4$ (open symbols); (B) 50 μM 1,10-phenantroline (closed symbols) or 50 μM $ZnSO_4$ and 50 μM 1,10-phenantroline (open symbols); (C) no additive (closed symbols) or 100 μM $ZnSO_4$ (open symbols). Data are expressed as OD at 600 nm ± SD.

DISCUSSION

In the past decades, many studies have applied microarray analysis to examine the response of mammalian cells to a drug of interest (reviewed by Zhou et al., 2009). However, a remaining challenge is to translate these data sets into actual cellular target(s). *S. cerevisiae* can be a valuable tool in toxicology studies, since yeast has a well-annotated genome sequence and is easily genetically modified for validation of potential targets (Yasokawa and Iwahashi, 2010). Here we used microarray analysis of diclofenac-adapted yeast cells to identify the cellular pathways involved in diclofenac toxicity or tolerance, and we subsequently applied yeast genetics for confirmation of the targets we found.

The major group of upregulated genes in diclofenac-adapted cells consisted of pleiotropic drug resistance genes (Tables 1 and 2). Upregulation of multidrug resistance genes is a common response of yeast to xenobiotics. The resistance of yeast to around ~25% of all compounds tested (Hillenmeyer et al., 2008; Kemmer et al., 2009) is dependent on the upregulation of these genes. Although several multidrug resistance transporters are upregulated after diclofenac exposure, a single ABC transporter, Pdr5p, is crucial in diclofenac resistance (Fig. 3

and 4). This may be due either to direct transport of diclofenac by Pdr5p or to secondary effects of Pdr5p deletion or overexpression (Shahi and Moye-Rowley, 2009). Interestingly, a murine homolog of Pdr5p, BCRP1 (ABCG2), can efficiently transport diclofenac *in vitro* (Lagas et al., 2009). Previously, Mima et al. (2007) found that overexpression of the polyamine transporter *TPO1* decreases sensitivity to diclofenac in yeast. However, deletion of *TPO1* did not alter diclofenac toxicity in our assay. Since Pdr5p is the main transporter involved in diclofenac resistance, the continuous upregulation of *PDR5* is likely to be the main mechanism protecting adapted cells from diclofenac-toxicity.

Cells adapted to diclofenac show upregulation of Rlm1p responsive genes (Table 1). Rlm1p is part of the PKC pathway, which is activated in response to various conditions causing cell wall stress (Levin, 2005). Interestingly, cell wall structural alterations have been observed with many weak acids (pKa of diclofenac ~4.0) and possibly reduce the diffusion of the weak acid into the cell (Mira et al., 2010). Deletion of either *PKC1* or *SLT2* increased diclofenac toxicity (Fig. 5A,B), confirming the role of the PKC pathway in diclofenac resistance. Rlm1p responsive genes are also upregulated by zymolyase, which hydrolyzes the ß-1,3-glucan network, and by calcofluor white, which binds to cell wall polysaccharides. Indeed, cells pre-incubated with these agents were more resistant to diclofenac (Fig. 5C). Furthermore, we observed that diclofenac induces *FLO1*-independent flocculation (Fig. 5D), which might trigger the cell wall stress response. Together these results show that diclofenac adaptation involves altered cell wall synthesis and that cell wall alterations protect against diclofenac toxicity.

Although the diclofenac sensitivity of Δpdr5 and Δpkc1 cells is significantly increased, both strains can adapt to 100 µM diclofenac after incubating ~35 and ~45 hours respectively (Fig. 4, 5). Interestingly, when these adapted cells were grown without diclofenac for 48 hours and were subsequently incubated with 100 µM diclofenac again, their adaptation-period was reduced to ~20 hours (data not shown). The reduction in adaptation time indicates an acquired mutation while the remaining ~20 hours required for adaptation reflects the need to make the appropriate gene expression changes that are essential for growth in the presence of diclofenac. Thus, in contrast to our findings for wild type cells (Fig. 2), adaptation of Δpdr5 and Δpkc1 cells involves the selection of a mutation. The growth of these mutant Δpdr5 cells in the absence of diclofenac was comparable to that of the parental strain, but the mutant Δpkc1 cells grew faster without diclofenac than the original Δpkc1 cells, providing further evidence of their altered genetic makeup (data not shown). We have excluded selection for loss of mitochondrial DNA in both adapted Δpdr5 and Δpkc1 cells, but further research is required to identify the nature of the mutation.

Downregulation of the zinc-responsive transcription factor Zap1p in diclofenac-adapted yeast cells indicates that intracellular zinc levels are altered (Herbig et al., 2005). Zinc is essential for the protection of cells against diclofenac, because lowering of the zinc levels by deletion of *ZAP1* or by addition of zinc-chelator 1,10-phenantroline increased diclofenac toxicity, which could be reversed by the addition of extra zinc (Fig. 6). Since diclofenac induces ROS formation (Fig. 1B), the antioxidant properties of zinc (reviewed by Powell, 2000) may protect diclofenac-treated cells against from further oxidative damage, as has been described in rats (Abou-Mohamed et al., 1995). Additionally, diclofenac anions in the cytosol may chelate zinc, thereby lowering the cellular free zinc concentration. A similar process has been described for the chelation of iron by lactic acid (Abbott et al., 2008) or for the chelation of both iron and zinc by hop iso-a-acids (Hazelwood et al., 2010). Interestingly, lowering of zinc levels by diclofenac has also been observed in patients (Shoji et al., 1993).

Downregulation of genes involved in ribosome biogenesis or other aspects of protein synthesis and cellular growth is a common response to stress and part of the "environmental stress response" (ESR) (Causton et al., 2001; Gasch et al., 2000). The typical upregulation of the Msn2p/Msn4p target-genes in the ESR is not clearly observed here. Interestingly, the downregulation of rRPE and PAC genes was reported to be transient (Fardeau et al., 2007; Gasch et al., 2000). However, expression of these genes is still altered after 75h with diclofenac, while no toxicity is observed in terms of the growth rate. Possibly, adapted cells are still stressed, causing downregulation of the rRPE and PAC genes.

Remarkably, adaptation does not mimic typical weak acid adaptation with upregulation of H^+-ATPases and Msn2p/Msn4p-, Haa1p-, and War1p-regulons (Legras et al., 2010; Mira et al., 2010). Also, although in both yeast (van Leeuwen et al., 2011a) and mammalian cells (Gomez-Lechon et al., 2003) the toxicity of diclofenac is directed primarily toward mitochondria, resulting in increased ROS levels, no oxidative stress response with upregulated superoxide dismutases, peroxiredoxins, and catalases, is apparent. The lack of a mitochondrial retrograde response by upregulation of *RTG1-3* indicates that the mitochondria are functional and indeed, we could not find selection for rho^0 strains during adaptation to diclofenac. T-profiler analysis, however, shows a reduced expression of mitochondrial genes, which might contribute to the reduced sensitivity. Apparently oxidative stress, and possibly also weak acid stress, is transient and is involved only in initial diclofenac toxicity. In agreement with this, ROS levels in adapted strains are lower than in freshly treated strains (Fig. 1B). Other cellular changes during adaptation, such as increased Pdr5p expression, may lower diclofenac levels sufficiently to prevent oxidative stress.

In conclusion, we used microarray analysis of diclofenac-adapted strains to identify several processes involved in diclofenac tolerance. By using yeast, we could directly verify the involvement of differentially expressed genes in diclofenac detoxification by applying yeast genetics. Limited transferability of transcriptionally altered genes to genes showing a growth phenotype has been described in many studies (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. Especially, upregulation of multidrug transporter Pdr5p increases resistance to diclofenac. Changes in cell wall composition and zinc homeostasis further contribute to diclofenac tolerance. It remains to be seen whether the discovered importance of zinc for diclofenac tolerance in yeast, discovered here, is relevant for patients with a zinc deficiency (Shoji et al., 1993).

ACKNOWLEDGMENTS

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APPENDIX

Upregulation of the PDR-response by diclofenac is dependent on Pdr1p

As described above, diclofenac severely upregulated the PDR-response in both diclofenac-adapted and freshly treated cells (Table 1, Fig. 3). Especially increased expression of multidrug transporter Pdr5p contributed to resistance to diclofenac (Fig. 4). In yeast, the multidrug resistance response is regulated by transcription factors Pdr1p and Pdr3p (reviewed by Moye-Rowley, 2003). We investigated which of these is involved in upregulation of the PDR-response by diclofenac. WT, Δpdr1, Δpdr3 and Δpdr5 cells were incubated with diclofenac (Fig. S1). Diclofenac-sensitivity of Δpdr3 cells was comparable to that of WT cells. However, Δpdr1 cells showed greatly decreased diclofenac-resistance and were nearly as sensitive to diclofenac as Δpdr5 cells. Additionally, *PDR5* promoter activity was greatly lowered in the absence of Pdr1p, both in the absence and presence of diclofenac (Fig. S2). *PDR5*-lacZ expression was not altered in Δpdr3 cells compared to wild type cells. Other PDR-genes like *RSB1* and *SNQ2* showed a comparable expression pattern as *PDR5*, while expression of the control construct *TRP5*-lacZ was not altered in Δpdr1 or Δpdr3 cells compared to wild type both in presence or absence of diclofenac (not shown). These data strongly suggest that Pdr1p is the main regulator of diclofenac-induced upregulation of *PDR5*. Also for other xenobiotics, Pdr1p is the main transcription factor involved in upregulation of

the PDR-response (Fardeau et al., 2007). The role of Pdr3p seems to be more focused on upregulation of the PDR-genes in response to mitochondrial deficiencies (Devaux et al., 2002; Hallstrom and Moye-Rowley, 2000).

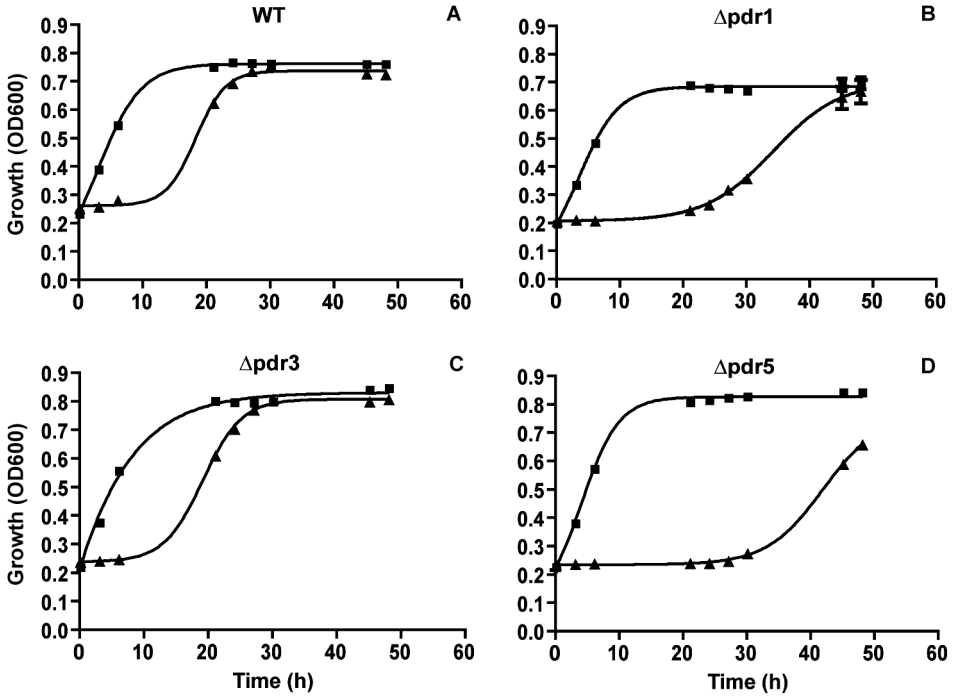

Fig. S1. Diclofenac toxicity is increased in *PDR1*-deficient cells. BY4741 wild type (A), Δpdr1 (B), Δpdr3 (C) or Δpdr5 (D) cells were incubated with 0 µM (squares) or 100 µM (triangles) diclofenac in minimal medium. Data are expressed as OD at 600 nm ± SD.

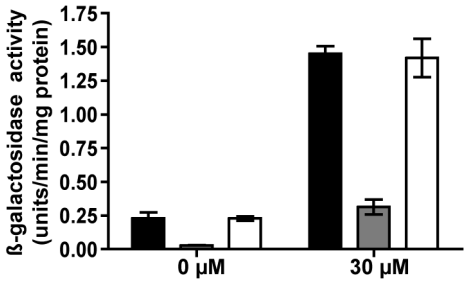

Fig. S2. Upregulation of *PDR5*-lacZ expression is dependent on Pdr1p. *PDR5*-lacZ expression in BY4741 wild type (black bars), Δpdr1 (grey bars) and Δpdr3 (white bars) cells incubated 3 hours with 0 µM or 30 µM diclofenac in minimal medium. LacZ expression is presented as ß-galactosidase activity in units per minute per mg protein ± SD.

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V

Differential involvement of mitochondrial dysfunction, cytochrome P450 activity and active transport in the toxicity of structurally related NSAIDs

Jolanda S. van Leeuwen
Betül Ünlü
Nico P.E. Vermeulen
J. Chris Vos

Adapted from: van Leeuwen et al. (2011) *submitted*

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain and inflammation. However, this group of drugs is associated with serious adverse drug reactions. Previously, we studied the mechanisms underlying toxicity of the NSAID diclofenac using *Saccharomyces cerevisiae* as model system. We identified the involvement of several mitochondrial proteins, a transporter and cytochrome P450 activity in diclofenac toxicity. In this study, we investigated if these processes are also involved in the toxicity of other NSAIDs. We divided the NSAIDs into three classes based on their toxicity mechanisms. Class I consists of diclofenac, indomethacin and ketoprofen. Mitochondrial respiration and reactive oxygen species play a major role in the toxicity of this class. Metabolism by cytochrome P450s further increases their toxicity, while ABC-transporters decrease the toxicity. Mitochondria and oxidative metabolism also contribute to toxicity of class II drugs ibuprofen and naproxen, but another cellular target dominates their toxicity. Interestingly, ibuprofen was the only NSAID that was unable to induce upregulation of the multidrug resistance response. The class III NSAIDs sulindac, ketorolac and zomepirac were relatively non-toxic in yeast. In conclusion, we demonstrate the use of yeast to investigate the mechanisms underlying the toxicity of structurally related drugs.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used class of medication worldwide. They inhibit cyclooxygenases involved in the synthesis of prostaglandins and are widely used in the treatment of pain and inflammation. Unfortunately, NSAIDs can cause serious adverse drug reactions that mainly target the upper gastrointestines (Lewis et al., 2002). Also heart (Fosbol et al., 2009), kidney (Lafrance and Miller, 2009) and possibly liver (Rubenstein and Laine, 2004) toxicity have been reported after human NSAID use. Additionally, glucuronidation of NSAIDs can lead to instable glucuronide-conjugates that, via protein adduct formation, can activate the immune system leading to allergic reactions such as anaphylaxis and Stevens-Johnson syndrome (Sawamura et al., 2010). Gastrointestinal and kidney toxicity have been related to non-selective inhibition of cyclooxygenases, and have decreased after development of selective COX-2 inhibitors (Lafrance and Miller, 2009; Mitchell and Warner, 1999). However, the occurrence of heart and liver failure remains (Fosbol et al., 2009; Rubenstein and Laine, 2004), indicating that additional mechanisms of toxicity exist.

In earlier studies, we have used *Saccharomyces cerevisiae* as model organism to study the toxicity of diclofenac (van Leeuwen et al., 2011a-c). Advantages of yeast as model in toxicity

studies are its well-annotated genome, straightforward genetics, cost-effectiveness and rapid growth (Hoon et al., 2008; Yasokawa and Iwahashi, 2010). Genetic screens using the genome spanning collections of deletion strains or overexpression constructs have led to the identification of on- and off-targets of various drugs (Ho et al., 2011). Many P450s have been heterologously expressed in yeast to study metabolism related (geno)toxicity. Additionally, yeast lacks cyclooxygenases, which simplifies the test system, whereas many of the mechanisms underlying toxicity, such as mitochondrial dysfunction and DNA damage, are conserved (Steinmetz et al., 2002; Yu et al., 2008).

In mammalian cell lines, the toxicity of NSAIDs has been associated with mitochondrial dysfunction (Moreno-Sanchez et al., 1999), metabolism by cytochrome P450s (Jurima-Romet et al., 1994) and efflux by transporters (Mima et al., 2007). However, the underlying mechanisms and relative contribution of these processes to NSAID toxicity remain largely unknown. Previously, we showed that yeast cells lacking mitochondrial DNA (so called rho^0 cells) and thereby missing the mitochondrial electron transport chain (ETC) are resistant to diclofenac. Furthermore, ROS formation induced by diclofenac in yeast is dependent on the presence of the ETC. Within the ETC, we identified subunits Rip1p and Cox9p of the respiratory chain complex III and IV, respectively, as major mitochondrial targets of diclofenac (van Leeuwen et al., 2011a). Additionally, expression of bacterial cytochrome P450 (P450) BM3 mutant M11, which metabolizes diclofenac in a similar way as human P450s, increased the toxicity of diclofenac in yeast (van Leeuwen et al., 2011b). Cytochrome P450 BM3 is extensively genetically modified to mimic drug metabolism by human P450s and has the advantage that it is a soluble protein composed of both an oxidase and reductase domain (Kumar, 2010). Furthermore, we used microarray analysis to study the genetic responses of yeast towards diclofenac exposure and showed that the multidrug resistance (MDR) response and especially ABC-transporter Pdr5p were involved in resistance against diclofenac (van Leeuwen et al., 2011c).

In the current study, we will explore if these proteins and pathways are also involved in toxicity and tolerance of other NSAIDs and compare their relative contribution to toxicity as well as investigate whether yeast cells provide an additional model system to detect toxicity pathways *in vitro*. For this purpose, we selected a set of structurally related NSAIDs, all either acetic acid or propionic acid derivatives (Fig. S1) and studied their toxicity in various yeast strains to identify underlying toxicity mechanisms. In this way, we can subdivide the NSAIDs in several classes based on their mechanisms of toxicity.

MATERIALS AND METHODS

Chemicals and stock solutions

All NSAIDs were purchased from Sigma Aldrich at highest purity. Naproxen was dissolved in ethanol (100 mM) and all other NSAIDs in DMSO (100 mM). 2',7'-dichlorodihydrofluorescein diacetate was obtained from Alexis Biochemicals and dissolved in ethanol (4 mM). All stock solutions were stored at –20 °C and protected from light. All other chemicals were purchased from Sigma Aldrich at the highest purity.

Yeast strains and plasmids

The haploid BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) wild type and deletion strains were obtained from EUROSCARF. Strains without mitochondrial DNA (rho^0) were generated by growth with ethidium bromide (10 µg/ml) as described previously (Goldring et al., 1970). Loss of mitochondrial DNA was confirmed by microscopy after staining the DNA with DAPI (4'-6-diamidino-2-phenylindole). BM3 M11 was expressed in yeast using a previously described, galactose-inducible construct (van Leeuwen et al., 2011b).

Growth conditions and NSAID incubation

Strains were grown overnight on minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented amino acids and bases) at 30 °C. Overnight cultures were diluted in minimal media and grown at 30 °C to an optical density at 600 nm (OD_{600}) of ~0.2. At this point, 25-500 µM of the NSAID was added. Controls were treated with equal amounts of DMSO or ethanol (max 1% v/v). The OD_{600} of control and NSAID-treated cultures was followed for 24-48 hours.

Measurement of reactive oxygen species (ROS) production

Exponentially growing yeast cultures (2 ml) of OD_{600}~0.2 were treated with the NSAID in the presence of 10 µM of the fluorescent, ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate. After 3 hours at 30 °C the cultures were centrifuged (3 min 3000 rpm) and cell pellets were washed and resuspended in 1 ml water. Fluorescence (l_{ex} = 485 nm, l_{em} = 535 nm) was measured as relative fluorescence units (RFU) and corrected for the cell density in the samples.

ß-Galactosidase reporter assay

The *TRP5*- and *PDR5*-lacZ reporter constructs in the low copy number vector pSEYC102 were a kind gift from Prof. Scott Moye-Rowley (Katzmann et al., 1994). The plasmids were transformed into yeast using the freeze-thaw method (Klebe et al., 1983). Yeast cultures (150 µl in 96-well plates) of OD_{600} ~0.2 were treated with the NSAID. After 3 hours, the OD_{600} was

measured and cells were lysed by addition of 50 µl 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in lacZ buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol). After incubating 30 min, 20 µl of chlorophenolred-ß-D-galactopyranoside (1.2 mg/ml in lacZ buffer) was added and the samples were incubated 1-2 hours at 30 °C. Absorption at 575 nm was measured and corrected for the cell density in the samples.

Statistical analysis

To compare yeast cell growth, ROS formation or *lacZ* expression between samples, the two-sided Student's *t*-test for unpaired samples was used. *P* values less than 0.01 were considered statistically significant. When applicable, *P* values were corrected for multiple comparisons.

RESULTS

Measurement of growth inhibition and general NSAID toxicity in yeast

To assess the general toxicity of the NSAIDs in yeast, wild type yeast cells were incubated with 50-500 µM of the drugs and growth of the cultures was followed in time (Fig. 1). The highest concentration used of sulindac (SUL) and zomepirac (ZOM) was 250 µM since both drugs precipitated at higher concentrations. A characteristic growth experiment is shown in Fig. 1A. Most NSAIDs caused a growth delay, but when growth resumed had little effect on the growth rate (Fig. 1A). Likely, adaptation is required for surviving cells to resume growth in the presence of the drug (van Leeuwen et al., 2011c). We used the growth delay (Δt) as measure for toxicity (Fig. 1A,B). Diclofenac (DIC), indomethacin (IND) and ibuprofen (IBU) were most toxic and no growth was observed at concentrations above 100 µM. Ketoprofen (KEP) and naproxen (NAP) showed significant toxicity at 500 µM, while sulindac was only slightly toxic in the range between 50-250 µM (Fig. 1B). Ketorolac (KER) and zomepirac did not cause a significant delay in growth at the concentrations used.

Previously, we described an increase in the formation of reactive oxygen species (ROS) during diclofenac incubation using both 2',7'-dichlorodihydrofluorescein diacetate and dihydroethidium as ROS-sensitive probes (van Leeuwen et al, 2011a). Here, we found that also other NSAIDs induce ROS formation in a concentration-dependent manner (Fig. 2). For most NSAIDs, concentrations that caused a growth delay also induced significant ROS formation. Ketorolac and zomepirac, that did not affect growth, also hardly induced ROS formation. However, there is no obvious correlation between the length of the growth delay and the amount of ROS. Cultures incubated with indomethacin and sulindac produced relatively high amounts of ROS compared to the growth delay, while ketoprofen induced relatively low ROS formation. For instance, at 50 µM indomethacin, we see ~7x increase in

ROS formation, which only corresponds to a Δt of 7.5 hours. In contrast, Δt's longer than 10 hours are seen for 50 µM diclofenac, 500 µM ketoprofen and 100 µM ibuprofen although ROS levels are increased no more than ~2-4x. Also, 250 µM sulindac has a comparable ROS level as 500 µM ketoprofen, but a much shorter Δt (2 and 13 hours, respectively). Interestingly, 50 µM ibuprofen and 250 µM naproxen already caused a significant growth delay (Fig. 1B) without an increase in ROS formation (Fig. 2). The reduced ROS formation at 100 µM versus 50 µM for IND probably reflects the reduced number of viable cells.

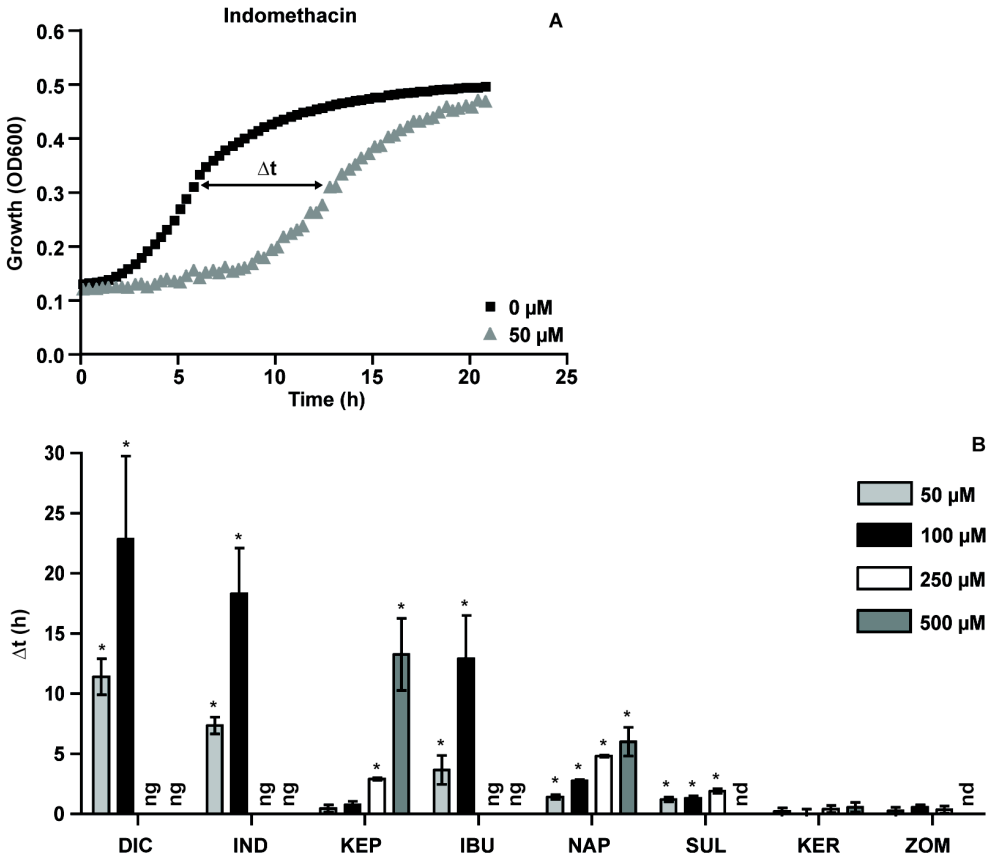

Fig. 1. NSAID toxicity in yeast. (A) Growth of wild type BY4741 yeast cells incubated with 0 µM (black squares) or 50 µM (grey triangles) indomethacin in YNB medium supplemented with glucose. Measurement of the growth delay as Δt is indicated. (B) Delay in growth of BY4741 wild type cells incubated with 50, 100, 250 or 500 µM diclofenac (DIC), indomethacin (IND), ketoprofen (KEP), ibuprofen (IBU), naproxen (NAP), sulindac (SUL), ketorolac (KER) or zomepirac (ZOM). The delay in growth (Δt) is expressed in hours ± SD. * $P<0.01$ compared to cells incubated without drug. N.d. = not determined. N.g. = no growth during 24h.

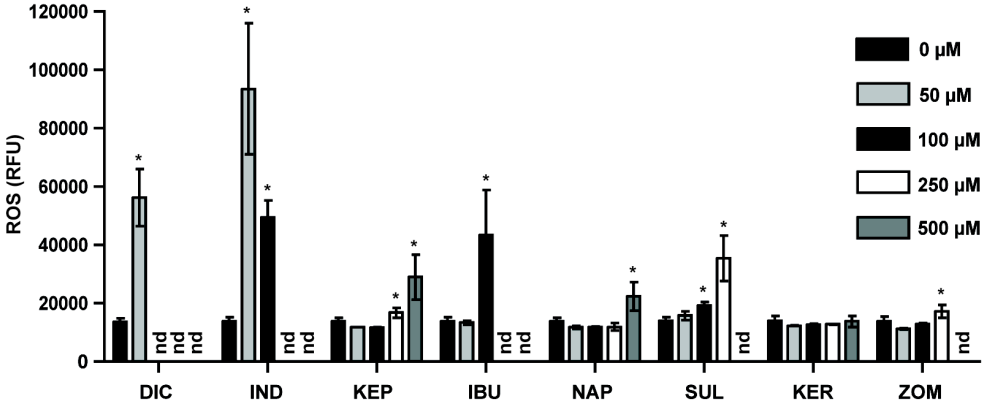

Fig. 2. NSAID-induced ROS formation. ROS levels in BY4741 wild type cells incubated for 3 hours with 0, 50, 100, 250 or 500 µM diclofenac (DIC), indomethacin (IND), ketoprofen (KEP), ibuprofen (IBU), naproxen (NAP), sulindac (SUL), ketorolac (KER) or zomepirac (ZOM) in the presence of the ROS-sensitive, fluorescent 2',7'-dichlorodihydrofluorescein diacetate in YNB medium supplemented with glucose. The ROS levels are expressed as fluorescence units (RFU), corrected for the cell density in the samples, ± SD. * P<0.01 compared to cells incubated without drug. N.d. = not determined.

Mitochondrial toxicity of NSAIDs

The mitochondrial respiratory chain is the main source of ROS in the cell (Kowaltowski et al., 2009). Recently, we found that diclofenac inhibits oxygen consumption in yeast and that toxicity is enhanced when yeast cells rely more on respiration for their energy production (van Leeuwen et al., 2011a). We have shown that rho^0 cells, that lack mitochondrial DNA and are therefore unable to respire, show increased resistance to diclofenac (van Leeuwen et al., 2011a). Mitochondria are possibly also involved in the toxicity of other NSAIDs (Lal et al., 2009; Moreno-Sanchez et al., 1999). We investigated the role of mitochondria in NSAID toxicity by comparing the resistance of a rho^0 strain to wild type cells. Since sulindac, ketorolac and zomepirac were not or only slightly toxic to the wild type strain (Fig. 1B) we did not test the resistance of rho^0 cells to these drugs. We found that, similar to diclofenac, rho^0 cells were also much more resistant to indomethacin and ketoprofen, but lack of mitochondrial DNA did not affect the toxicity of ibuprofen and naproxen to the same extent (Fig. 3) indicative for a different major cellular drug target.

We used yeast deletion strains lacking genes encoding subunits of the mitochondrial electron transfer chain to identify the position of interference of the NSAID with respiration. Especially strains lacking subunit Rip1p or Cox9p of respiratory chain complex III and IV, respectively, were shown to be significantly more resistant to diclofenac (van Leeuwen et al., 2011a). Here,

we tested the NSAID-resistance of strains in which the genes were deleted encoding the yeast equivalent of complex I (Ndi1p) or subunits of complex II (Sdh1p), III (Qcr2p or Rip1p) or IV (Cox6p or Cox9p). Deletion of other complex II, III or IV subunits (Sdh2p, Cor1p or Cox5ap, respectively) revealed similar phenotypes as Δsdh1, Δqcr2 and Δcox6 (data not shown). Resistance to indomethacin and ketoprofen was greatly increased in cells lacking *RIP1* or *COX9* as shown by a reduction in Δt by more than 50% (Fig. 3), indicating an underlying mechanism of toxicity identical to diclofenac. Sensitivity to ibuprofen and naproxen showed only a minor change in Δt of 10-30% in these strains. Apparently, mitochondrial respiration only plays a minor role in the toxicity of these compounds. Notably, although with 100 μM diclofenac the sensitivity of Δndi1, Δsdh1, Δqcr2 and Δcox6 is comparable to that of wild type cells (van Leeuwen et al., 2011a), with 50 μM diclofenac these strains had an increase in resistance compared to wild type (Fig. 3). Also with indomethacin, Δcox6 and Δsdh1 showed increased resistance. However, Rip1p and Cox9p are the main respiratory contributors to diclofenac, indomethacin and ketoprofen toxicity.

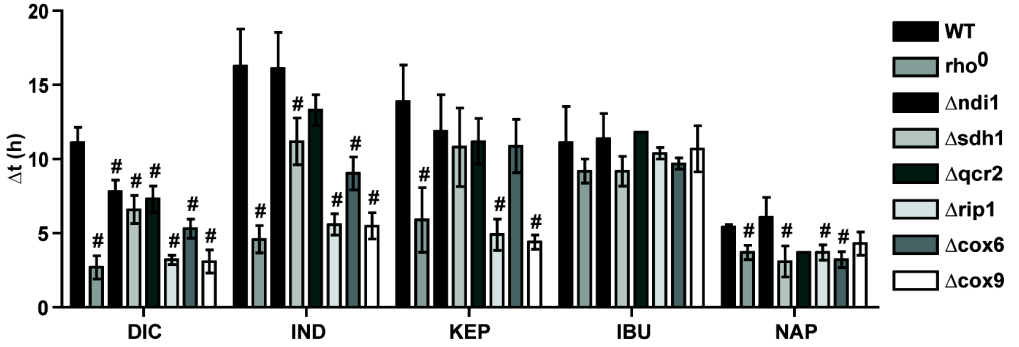

Fig. 3. Involvement of mitochondria in NSAID toxicity. Growth of BY4741 wild type (WT) cells, cells lacking mitochondrial DNA (rho^0) or cells in which a gene encoding a subunit of a respiratory chain complex has been deleted in YNB medium supplemented with glucose. The strains were grown in the presence of 50 μM diclofenac (DIC), 100 μM indomethacin (IND), 500 μM ketoprofen (KEP), 100 μM ibuprofen (IBU) or 500 μM naproxen (NAP). The delay in growth (Δt) is expressed in hours compared to unexposed cells ± SD. $^{\#}$ P<0.01 compared to WT cells treated with the same drug.

Previously, we have described a clear correlation between diclofenac toxicity and diclofenac-induced ROS formation (van Leeuwen et al., 2011a). Here, we investigated the ROS levels induced by the other NSAIDs in the various respiratory deficient strains at drug-concentrations where ROS levels were prominent. Indeed, rho^0 cells were not only more resistant to diclofenac, indomethacin and ketoprofen, the ROS levels were also significantly lower in this strain compared to wild type cells (Fig. 4). Interestingly, although rho^0 cells did not show

increased resistance to ibuprofen, the ROS levels induced by this drug were significantly lower in the rho^0 strain compared to wild type. Also Δrip1 and Δcox9 cells hardly showed any ROS formation at all for diclofenac, indomethacin and ibuprofen while the ROS levels in Δndi1, Δsdh1, Δqcr2 and Δcox6 were comparable to wild type levels (data not shown). Apparently, although Rip1p and Cox9p contribute to ibuprofen-induced ROS formation, this ROS formation is not the main cause of toxicity of ibuprofen. The ROS levels of sulindac-treated rho^0 cells were ~170% of those of untreated rho^0 cells, showing that sulindac-induced production of ROS is apparently not related to respiration. Ketorolac and zomepirac hardly induced ROS formation in wild type cells, so no decrease in ROS levels could be observed for these drugs.

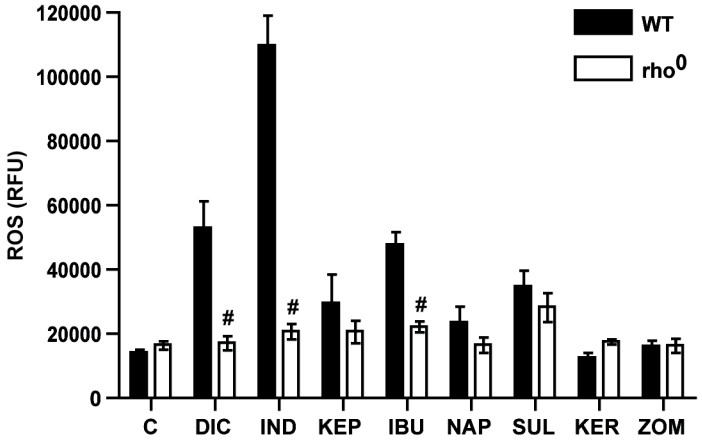

Fig. 4. Decreased ROS formation in rho^0 cells. Wild type cells (WT) and cells lacking mitochondrial DNA (rho^0) were incubated for 3 hours with 50 μM diclofenac (DIC), 50 μM indomethacin (IND), 500 μM ketoprofen (KEP), 100 μM ibuprofen (IBU), 500 μM naproxen (NAP), 250 μM sulindac (SUL), 500 μM ketorolac (KER), 250 μM zomepirac (ZOM) or without drug (C) in the presence of the ROS-sensitive, fluorescent 2',7'-dichlorodihydrofluorescein diacetate in YNB medium supplemented with glucose. The ROS levels are expressed as RFU, corrected for the cell density in the samples, ± SD. # P<0.01 compared to WT cells treated with the same drug.

Oxidative metabolism-dependent NSAID toxicity

Another suggested cause of NSAID toxicity is metabolism by cytochrome P450s (Agundez et al., 2009; Jurima-Romet et al., 1994). A previously established model system expressing cytochrome P450 BM3 M11 in yeast (van Leeuwen et al., 2011b) was used to examine the toxicity of NSAID oxidative metabolism. BM3 M11 is a model P450 that metabolizes various drugs, including the NSAIDs paracetamol and diclofenac, in a similar way as human P450s (Damsten et al., 2008; van Leeuwen et al., 2011b).

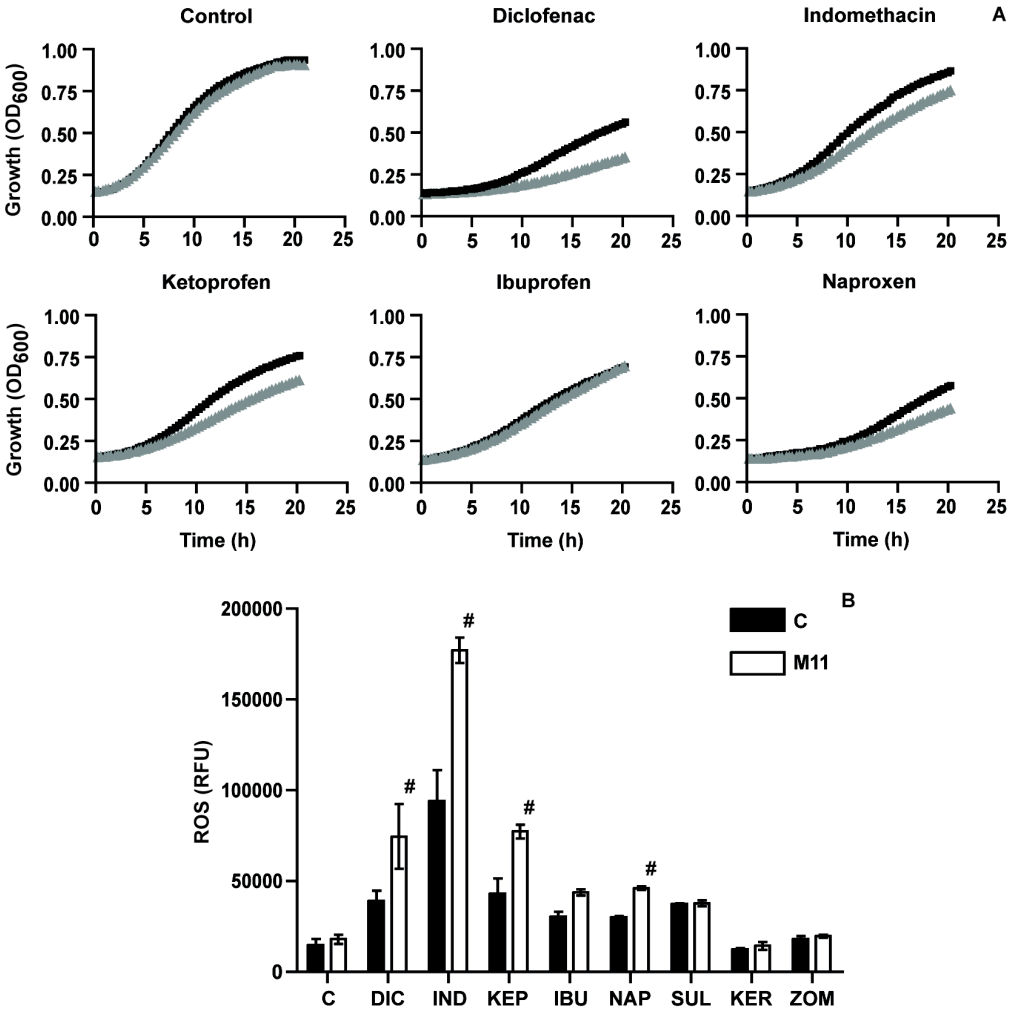

Fig. 5. Metabolism-dependent toxicity. (A) Growth curves of BY4741 strains expressing cytochrome P450 BM3 M11 (grey lines) or transformed with an empty vector (black lines). Growth is expressed as OD_{600}. Representative growth curves are shown. (B) ROS levels in BY4741 cells expressing BM3 M11 (M11) or transformed with an empty vector (C). The ROS levels are expressed as RFU, corrected for the cell density in the samples, ± SD. # $P<0.01$ compared to cells transformed with an empty vector treated with the same drug. The strains were incubated without drug or with 50 µM diclofenac (DIC), 50 µM indomethacin (IND), 500 µM ketoprofen (KEP), 50 µM ibuprofen (IBU), 500 µM naproxen (NAP), 250 µM sulindac (SUL), 500 µM ketorolac (KER) or 250 µM zomepirac (ZOM) in YNB medium supplemented with galactose.

Strains expressing BM3 M11 or control strains transformed with an empty vector were incubated with the NSAID and growth and ROS formation were examined (Fig. 5). In contrast to what we saw in the other experiments with glucose as carbon-source (Fig. 1A), on the galactose medium necessary to induce BM3 M11 expression, the NSAIDs did not just lead to a delay in growth but also decreased the growth rate (Fig. 5A). This might be due to the increased respiration on galactose-medium, which was shown to enhance diclofenac toxicity (van Leeuwen et al., 2011a). In the absence of NSAIDs, strains expressing BM3 M11 or controls had comparable growth and ROS levels. BM3 M11 activity increased the toxicity of diclofenac, indomethacin, ketoprofen and naproxen (Fig. 5A), which corresponded with increased ROS levels (Fig 5B). For diclofenac, indomethacin and ketoprofen, the increase in ROS due to BM3 M11 was most prominent (1.5-2 times). With ibuprofen, a slight increase in ROS formation was seen in the BM3 M11 expressing strain, however this difference was not significant ($p>0.01$), while the modest increase in ROS for naproxen was significant ($p<0.01$). BM3 M11 expression did not increase toxicity (data not shown) and ROS levels of cells incubated with sulindac, ketorolac or zomepirac.

Active transport and the multidrug resistance response

Previously, we showed that yeast ABC-transporter Pdr5p contributes to diclofenac-resistance (van Leeuwen et al., 2011c). In this research, we explored the role of the two main ABC-transporters Pdr5p and Snq2p in NSAID resistance in yeast. WT, Δpdr5 and Δsnq2 cells were incubated with the NSAIDs and growth was monitored (Fig. 6A). Indeed, lack of Pdr5p increased diclofenac toxicity. The Δpdr5 strain was also more sensitive to ketoprofen compared to wild type cells. Deletion of *SNQ2* increased the sensitivity to indomethacin, ketoprofen and sulindac. The toxicity of ibuprofen, naproxen, ketorolac and zomepirac was not enhanced by deletion of *PDR5* or *SNQ2*. These results show that the two promiscuous transporters show substrate-specificity towards these structurally related NSAIDs.

NSAIDs are known to induce the expression of various drug transporters in human cells (Mima et al., 2007; Takara et al., 2009; Tatebe et al., 2002). Previously, we showed that diclofenac also increased expression of multidrug resistance genes in yeast (van Leeuwen et al., 2011c). In yeast, transcription factors Pdr1p and Pdr3p regulate expression of multidrug resistance genes like *PDR5*, *SNQ2* and *RSB1*. Using a lacZ reporter construct we tested the effect of the NSAIDs on expression of *PDR5* (Fig. 6B). Expression of *TRP5*, encoding a protein involved in tryptophan biosynthesis, was assayed as a control (Fig. 6C). Diclofenac, indomethacin, ketoprofen, naproxen, sulindac, ketorolac and zomepirac all induced expression of *PDR5*, while *TRP5* expression levels were not increased. Diclofenac and indomethacin were also tested at 10 μM, however at this concentration *PDR5* expression was lower than at 25 μM (data not shown).

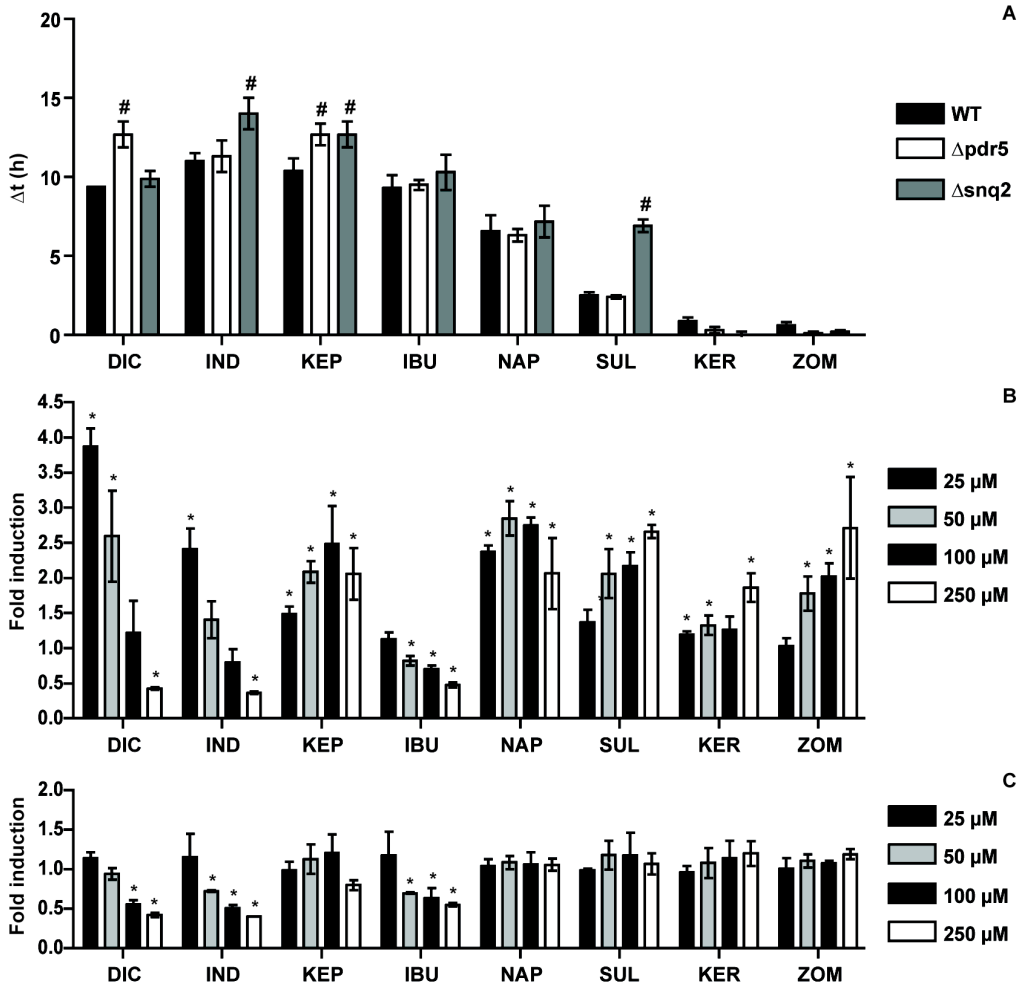

Fig. 6. Involvement of transporters and MDR in NSAID toxicity. (A) Growth of BY4741 wild type cells (WT) or cells lacking the genes encoding ABC-transporter Pdr5p (Δpdr5) or Snq2p (Δsnq2) incubated with 50 μM diclofenac (DIC), 100 μM indomethacin (IND), 500 μM ketoprofen (KEP), 100 μM ibuprofen (IBU), 500 μM naproxen (NAP), 250 μM sulindac (SUL), 500 μM ketorolac (KER) or 250 μM zomepirac (ZOM) in YNB medium supplemented with glucose. The delay in growth (Δt) is expressed in hours ± SD. # $P<0.01$ compared to WT cells treated with the same drug. (B,C) *PDR5*- (B) and *TRP5*-lacZ (C) expression in wild type strains incubated with 25, 50, 100 or 250 μM of the indicated NSAID. Data are expressed as fold induction of ß-galactosidase activity after correction for cell density in the samples ± SD. * $P<0.01$ compared to cells incubated without drug.

Higher concentrations of diclofenac, indomethacin and ibuprofen decreased *PDR5* and *TRP5* expression, probably due to decreased viability of the cells. *RSB1* and *SNQ2* showed a similar

induction-pattern as *PDR5*, although *SNQ2* induction was generally 2-fold lower (data not shown). Surprisingly, ibuprofen did not increase expression of multidrug resistance genes *PDR5*, *RSB1* or *SNQ2*. Also at 10 μM ibuprofen, no *PDR5* induction was observed (data not shown).

DISCUSSION

In this study we used yeast as eukaryotic model to investigate the toxicity of a set of structurally related NSAIDs containing a carboxylic acid group (Fig. S1). We have focused on the role of mitochondrial respiration, metabolism by cytochrome P450 BM3 M11 and active transport in NSAID toxicity, since these mechanisms have not only been implicated in NSAID toxicity in humans (Jurima-Romet et al., 1994; Lal et al., 2009; Mima et al., 2007) but we have also identified these as important in diclofenac toxicity in yeast (van Leeuwen et al., 2011a-c). We have used a set of yeast strains lacking mitochondrial respiration or transporter encoding genes or expressing bacterial cytochrome P450 BM3 mutant M11. By investigating these three toxicity mechanisms in a single study and model organism, we could compare the relative contribution of the processes to NSAID toxicity. The results obtained in yeast can provide a short cut into the investigation of the toxicity of these drugs on human cells. An example of such a spin-off is provided by Yu et al. (2008), who showed for structurally related imidalo-pyridines and -pyrimidines a differential involvement of mitochondrial dysfunction in their toxicity in yeast and confirmed these results in cultured human cells. Therefore, using a selected set of yeast mitochondrial deletion strains is not only a fast and cost-efficient way to determine the involvement of mitochondrial respiration in the toxicity of drugs, but potentially also relevant to humans.

Further support for the suitability of yeast is the observation that the general ‘order’ of NSAID-toxicity in yeast (Fig. 1) is comparable to what was found in rat hepatocytes and human epithelial cell cultures although the concentrations we used were generally ten-fold lower than what was used in the mammalian cells (Allen et al., 1991; Jurima-Romet et al., 1994; Masubuchi et al., 1998). In all model systems, the highest toxicity was observed with indomethacin and diclofenac. Interestingly, naproxen seems to be relatively more toxic to yeast than to the mammalian cells.

Historically, NSAIDs are classified based on their structure (i.e. acetic acid derivatives, salicylates) or on their ability to inhibit cyclooxygenase 2 (selective COX-2 inhibitors or ‘coxibs’). Based on the experimental results obtained in this study, we have grouped the NSAIDs in classes according to the mechanisms involved in their toxicity (Table 1). Class I is characterized by toxicity related to the generation of ROS at the mitochondrial respiratory

chain and ROS formation is enhanced by cytochrome P450 BM3 M11 metabolism. Class II is represented by compounds that mainly cause toxicity independent of ROS production and respiration. Class III is defined by a relative lack of toxicity at the tested concentrations in yeast, although a PDR response is elicited. Both class II and III NSAIDs show little or no P450 BM3 M11-related toxicity.

Mitochondrial toxicity of NSAIDs

The first class of NSAIDs consists of diclofenac, indomethacin and ketoprofen, whose toxicity is clearly linked to the mitochondrial respiratory chain with its subunits Rip1p and Cox9p as potential drug off-targets (Fig. 3). Deletion of the gene for either of these subunits or removal of the complete electron transfer chain by using rho^0 cells greatly diminished the toxicity of class I compounds. Although the mitochondrial effects of diclofenac and indomethacin have been widely described in literature using isolated rat liver mitochondria (Lal et al., 2009; Moreno-Sanchez et al., 1999), this is to our knowledge the first clear mechanistic understanding of the toxicity of ketoprofen in disturbing mitochondrial respiration. Translational research is needed to confirm the mitochondrial toxicity of ketoprofen in mammalian cells. Interference of the respiratory chain results in ROS production that has a negative impact on growth and viability of the yeast cells as shown for diclofenac in a survival assay (van Leeuwen et al, 2011a).

		Toxicity[a]			ROS[b]		P450[c]	Transporter[d]	MDR[e]
		WT	rho^0	Δrip1	WT	rho^0			
I	DIC	+++	+	+	++	-	++	PDR5	++
	IND	+++	+	+	++	-	++	SNQ2	++
	KEP	++	+	+	+	-	++	PDR5 + SNQ2	++
II	IBU	+++	+++	+++	++	-	-	-	-
	NAP	++	+	+	+	-	+	-	++
III	SUL	+	nd	nd	+	+	-	SNQ2	++
	KER	-	nd	nd	-	-	-	-	+
	ZOM	-	nd	nd	-	-	-	-	++

Table 1. Summary of toxicity parameters obtained in yeast. a: ability of the NSAID to delay growth of the WT, rho^0 or Δrip1 strain, b: ability of the NSAID to induce ROS formation in the WT or rho^0 strain, c: increase in toxicity in the cytochrome P450 BM3 M11 expressing strain compared to a control strain, d: ABC-transporter whose deletion increased toxicity of the NSAID, e: ability of the NSAID to induce expression of multidrug resistance genes. "+" indicates the relative increase compared to controls, "-" means no change compared to controls. "nd" = not determined.

Ibuprofen and naproxen are grouped as a separate class of NSAIDs that only slightly affect mitochondrial function. Although ibuprofen showed significantly lower ROS production in rho^0 cells, this did not lead to a major decrease in toxicity as for class I NSAIDs (Fig. 3 and 4). Additionally, ibuprofen (at 50 μM) and naproxen (at 250 μM) cause a growth delay in the absence of ROS induction, indicating that ROS formation is not a primary cause of toxicity of these drugs. Although for ibuprofen uncoupling has been described in isolated mitochondria (Lal et al., 2009; Moreno-Sanchez et al., 1999), mitochondrial dysfunction does not seem to be involved in toxicity in yeast. Previously, Campos et al. (2004) showed that mitochondrial dysfunction was also not involved in ibuprofen-induced cell death in mammalian tumor cells. Together these results show that another, as yet unknown target dominates the toxicity of ibuprofen and naproxen.

The class III NSAIDs, consisting of sulindac, ketorolac and zomepirac, are relatively non-toxic to yeast. However, sulindac did increase ROS formation in both WT and rho^0 cells. Apparently, an as yet unknown source and cellular localization (but not the ETC) of ROS formation does not result in a major growth defect. A possible other source of ROS could be the interference of NSAIDs with fatty acid oxidation (Yang et al., 1998). As the class III NSAIDs did induce expression of *PDR5*, the low toxicity is probably not a result of decreased cellular uptake of the drugs.

Metabolism-related toxicity of NSAIDs

All NSAIDs used in this study contain a carboxylic acid moiety that is readily glucuronidated. The resulting glucuronides are reactive and can form protein adducts associated with adverse immune reactions (Sawamura et al., 2010). In yeast, glucuronidation does not occur due to the lack of glucuronosyl-transferases. However, glucuronidation and protein-adduct formation do not correlate with hepatotoxicity (Obach et al., 2008) suggesting that another mechanism such as metabolism by cytochrome P450s may contribute to the toxicity.

Jurima-Romet et al. (1994) compared the effect of P450 activity on the toxicity of a range of NSAIDs in rat hepatocytes using a P450-inducer or inhibitor. They found that especially with diclofenac and ketoprofen differences in P450 expression had a large effect on toxicity. We expressed the versatile P450 BM3 M11 in yeast, which can metabolize a wide variety of drugs in a similar way as human P450s (Damsten et al., 2008). Using this model, we also found increased toxicity related to diclofenac and ketoprofen metabolism, but found additionally P450-related toxicity for indomethacin and naproxen (Fig. 5). This increase in toxicity could be indicative of the generation of a more toxic compound and can both be the result or the cause of the observed increase in ROS formation. No clear role for P450-metabolism in ibuprofen toxicity could be determined. Sulindac, zomepirac and ketorolac toxicity was clearly

not related to metabolism by cytochrome P450 BM3 M11 in yeast. In mammalian cells, sulindac is reduced by methionine sulfoxide reductases to the pharmacologically active sulindac sulfide that can cause mitochondrial uncoupling (Brunell et al., 2011; Leite et al, 2006). Although yeast contains two methionine sulfoxide reductases, Mxr1p and Mxr2p, it is unknown if these can metabolize sulindac. This may be useful to further investigate in future toxicity studies using yeast and sulindac.

The oxidative metabolism of diclofenac is extensively studied in various models, including the BM3 M11 enzyme used here, and multiple major and minor metabolites and their conjugates have been identified (Damsten et al., 2008; van Leeuwen et al., 2011b). However, less is known about the P450-related metabolism of the other NSAIDs, for which mainly the primary metabolic route has been described (Agundez et al., 2009). Both indomethacin and naproxen are subject to O-demethylation by human P450s (Nakajima et al., 1998; Rodriguez 1996). For diclofenac, indomethacin and ketoprofen a decarboxylated metabolite has been described, whose formation might be involved in toxicity (Grillo et al., 2008; Komuro et al., 1995, van Leeuwen et al., 2011b). The various NSAIDs can also be hydroxylated on the aromatic rings, which may proceed via potentially toxic epoxides as has been described for diclofenac and zomepirac (Chen et al., 2006; Yan et al., 2005). The resulting hydroxyl-metabolite may lead to quinone-like reactive species (Shen et al., 1999), however, diclofenac quinone-imines are not toxic in yeast (van Leeuwen et al., 2011b). Further research into the oxidative metabolite profiles of the NSAIDs will be needed to identify the metabolic route(s) responsible for the increase in toxicity.

MDR and transport

Decreased NSAID-efflux might also cause toxicity by increasing the cellular concentration of the drug. Indeed, polymorphisms in *ABCC2* (encoding MRP2) may contribute to toxicity in NSAID-users (Daly et al., 2007). Diclofenac, indomethacin, ketoprofen, ibuprofen, naproxen and sulindac were found to interact with the ABC-transporters MRP4, MRP2, MRP1 and/or BCRP (El-Sheikh et al., 2007; Nozaki et al., 2007; Reid et al., 2003). In yeast, overexpression of major facilitator family-member Tpo1p increases resistance to diclofenac, indomethacin and ibuprofen (Mima et al., 2007). Also overexpression of the mammalian Tpo1p homolog, TETRAN, conveys resistance to diclofenac and indomethacin in human adenocarcinoma gastric cells, while silencing increases sensitivity (Mima et al., 2007). We found that ABC-transporter Pdr5p contributes to the resistance to diclofenac and ketoprofen while Snq2p augments resistance to indomethacin, ketoprofen and sulindac.

Although sequence identity is relatively low, there is a strong functional analogy between MDR-regulators Pdr1p/Pdr3p in yeast and PXR in mammals (Thakur et al., 2008). Both

Pdr1p/Pdr3p and PXR can directly bind a large variety of xenobiotics and thereby induce expression of multidrug resistance transporters (Kliewer et al., 2002; Thakur et al., 2008). Both up- and downregulation of ABC-transporters by NSAIDs has been described in various mammalian cells, since expression levels of transporters are strongly affected by variations in cell type, COX-2 levels and incubation time (Patel et al., 2002; Roller et al., 1999; Zrieki et al., 2008). Diclofenac, indomethacin and sulindac can increase expression of MDR1, MRP1, MRP3 and/or TETRAN in human cancer cell lines (Mima et al., 2007; Takara et al., 2009; Tatebe et al., 2002). Additionally, we found that also ketoprofen, naproxen, ketorolac and zomepirac induce the MDR response (Fig. 6B). For ibuprofen, no increase in MDR1 expression was observed in human cells (Takara et al., 2009) and, interestingly, we neither observed an increase in PDR5 transporter-expression in yeast (Fig. 6B). Although both Pdr1p/Pdr3p and PXR are very promiscuous, ibuprofen is apparently unable to interact in such a way to promote MDR induction.

Conclusion

Using yeast as model system we investigated the involvement of mitochondrial dysfunction, cytochrome P450-related metabolism and active transport in NSAID toxicity and used these processes to classify the NSAIDs tested. We found that generation of ROS by the mitochondrial respiratory chain is the main toxicological event in diclofenac, indomethacin and ketoprofen toxicity, while this is not the primary cause of toxicity for ibuprofen or naproxen. Intracellular metabolism by cytochrome P450 BM3 M11 increased the toxicity of diclofenac, indomethacin, ketoprofen and naproxen but had no significant effect on the toxicity of the other NSAIDs tested. Finally, ABC-transporters Pdr5p and/or Snq2p were involved in the efflux of diclofenac, indomethacin, ketoprofen and sulindac. Ibuprofen is the only NSAID we tested that was unable to induce a pleiotropic drug response in yeast. Together, our results endorse that yeast provides a relatively easy, fast and cost-efficient way to determine various major mechanisms of drug toxicity.

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APPENDIX

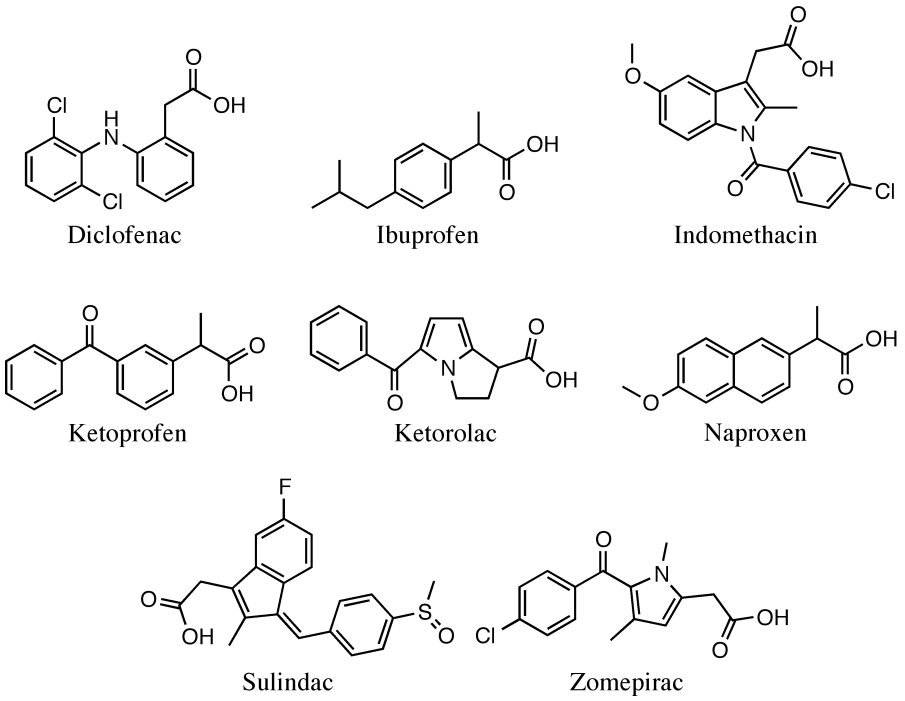

Fig. S1. Structures of the NSAIDs used in this study.

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VI

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.

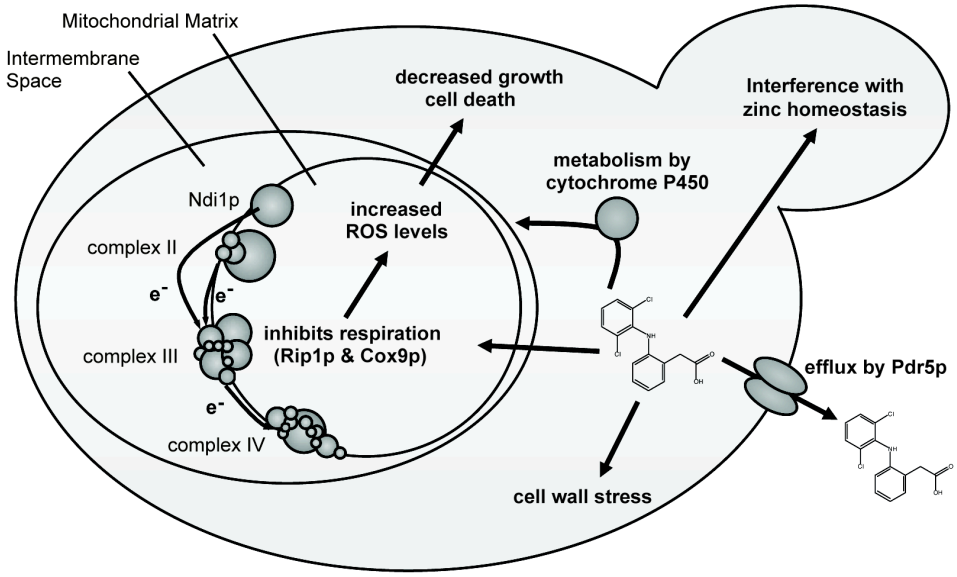

Fig. 1. Mechanisms of diclofenac toxicity described in this thesis. Inside the mitochondria, diclofenac inhibits respiration by interference with subunits Rip1p and Cox9p of the respiratory chain. This leads to increased levels of reactive oxygen species, causing growth inhibition and cell death. Metabolism 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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VII

NEDERLANDSE SAMENVATTING

GIST ALS MODEL EUKARYOOT IN ONDERZOEK NAAR DE VEILIGHEID VAN MEDICIJNEN: NIEUWE INZICHTEN IN DE TOXICITEIT VAN DICLOFENAC

De ontwikkeling van veel kandidaat-geneesmiddelen wordt vroegtijdig afgebroken doordat er schadelijke bijwerkingen optreden in proefdieren of tijdens klinische studies. Door de toenemende vraag naar veilige geneesmiddelen aan de ene kant en het terugdringen van proefdiergebruik aan de andere kant zijn er nieuwe testsystemen voor toxiciteit nodig. Bovendien eisen de EU REACH richtlijnen op korte termijn toxiciteitsprofielen van meer dan 30.000 chemicaliën, waarvoor testsystemen nodig zijn die in korte tijd de toxiciteit van een groot aantal stoffen nauwkeurig kunnen voorspellen. Het testen van kandidaat-geneesmiddelen en chemicaliën op genotoxiciteit wordt al veelvuldig toegepast. Echter, toxiciteit kan ook veroorzaakt worden door het binden van het geneesmiddel aan een "off-target" of kan aan metabolisme gerelateerd zijn. Metabolisme of biotransformatie verhoogt de wateroplosbaarheid van lichaamsvreemde stoffen en vergemakkelijkt daardoor de excretie via gal, feces en urine. Metabolisme kan echter ook leiden tot de vorming van reactieve metabolieten die toxiciteit kunnen veroorzaken. Cytochroom P450s (P450s) zijn de belangrijkste enzymen betrokken bij het metabolisme van geneesmiddelen, gevolgd door de fase II enzymen glucuronosyltransferases, sulfotransferases en glutathion S-transferases. Polymorfismen in deze enzymen veroorzaken grote individuele verschillen in geneesmiddelafbraak en toxiciteit is mede daardoor moeilijk te voorspellen. Proefdieren vertonen vaak andere absorptie, dispositie, metabolisme en excretie (ADME) profielen dan mensen. En zelfs als het ADME-profiel vergelijkbaar is, kunnen proefdieren in het beste geval een gemiddelde persoon nabootsen. Bovendien is het gebruik van proefdieren te duur en tijdrovend om vroeg in de ontwikkeling van een geneesmiddel te gebruiken. Daarom zijn cellulaire modelsystemen ontwikkelt om toxiciteit te testen.

Bakkersgist (*Saccharomyces cerevisiae*) is een veelgebruikt modelorganisme om eukaryote processen te bestuderen. Voordelen van het gebruik van deze gist zijn de relatief lage kosten, snelle groei en eenvoudige genetische modificatie. Bovendien is van een groot deel van de genen de functie bekend, waardoor gist een gewild model is voor studies die het hele genoom bestuderen. Met behulp van gist kunnen zo eiwitten worden geïdentificeerd die betrokken zijn bij de werking of de toxiciteit van geneesmiddelen. Gist wordt ook veel gebruikt om de genotoxiciteit of xeno-oestrogeniciteit van geneesmiddelen of andere stoffen te onderzoeken. Verschillende humane P450s zijn in gist tot expressie gebracht om aan metabolisme gerelateerde toxiciteit te kunnen onderzoeken.

In **hoofdstuk 1** worden de verschillende cellulaire modelsystemen die gebruikt kunnen worden om toxiciteit gerelateerd aan metabolisme te onderzoeken beschreven. Daarnaast wordt een

overzicht gegeven van de literatuur waarin gist als modelsysteem is gebruikt om biotransformatie en bioactivering te bestuderen. Gangbare cellulaire modelsystemen voor zulk soort onderzoek zijn gebaseerd op levercellen, zoals bijvoorbeeld primaire hepatocyten en de HepG2 cellijn. Een nadeel van deze modellen is de noodzaak om P450-remmers te gebruiken om verschil tussen wel en geen bioactivatie in toxiciteit te kunnen waarnemen, omdat deze remmers ook andere processen die relevant zijn voor toxiciteit, zoals transport en glucuronidering, kunnen beïnvloeden. Ook kunnen de P450-niveaus in deze cellen erg verschillen, snel afnemen of kunnen sommige P450s niet aanwezig zijn. Om de rol van P450s in metabolisme en bioactivatie beter te kunnen bestuderen zijn modelsystemen ontwikkelt, waarin humane P450s in *E. coli*, gist of humane cellen tot expressie zijn gebracht. Zoals hierboven beschreven is een groot voordeel van gist de combinatie van eukaryote eigenschappen met snelle groei, makkelijke hanteerbaarheid en relatief eenvoudige genetische manipulatie. De gisten die heterologe P450s tot expressie brengen zijn vaak gebruikt om mutageniciteit van stoffen te onderzoeken. Vooral aflatoxine B_1 is een veelgebruikte modelstof in deze studies. Daarnaast zijn P450-expresseerende gisten gebruikt in combinatie met deletiestammen of voor microarray experimenten om zo meer informatie over het mechanisme achter de toxiciteit te verkrijgen. Bovendien laten enkele groepen zien dat combinatie van P450s met andere metabole enzymen, zoals glutathion S-transferases en epoxide hydrolases, nuttig kan zijn in toxiciteit studies.

Zoals hierboven beschreven zijn gisten die heterologe P450s tot expressie brengen vooral gebruikt om genotoxiciteit te bestuderen. In dit proefschrift hebben we het gebruik van gist om niet-genotoxische toxiciteit te bestuderen onderzocht. We hebben het nut van gist als modelsysteem om cellulaire toxiciteitsmechanismen te ontrafelen en om de rol van P450s in biotransformatie gerelateerde toxiciteit te onderzoeken bestudeerd. Hiervoor hebben we diclofenac als modelgeneesmiddel gebruikt. Diclofenac wordt veel gebruik in de bestrijding van pijn en ontstekingen, bijvoorbeeld in reuma. Het gebruik van diclofenac kan echter ook tot zeldzame, maar ernstige bijwerkingen leiden. Vooral het bovenste deel van het maagdarmkanaal, de lever, het hart en de nieren kunnen schade ondervinden van diclofenac gebruik. Dit wordt mogelijk veroorzaakt door metabolisme door cytochroom P450s en schade aan de mitochondriën. De mechanismen, eiwitten en/of metabolieten betrokken bij de mitochondriële en P450-gerelateerde schade waren echter aan het begin van dit onderzoek nog niet bekend.

In **hoofdstuk 2** onderzoeken we allereerst de rol van mitochondriën in diclofenac toxiciteit in gist. In de mitochondriën doneren elektronrijke moleculen zoals NADH elektronen aan de ademhalingsketen. Deze elektronen worden uiteindelijk overgedragen aan zuurstof. Gedurende het elektronentransport worden protonen over het membraan gepompt wat tot een gradiënt

leidt dat gebruikt wordt voor energieproductie. Geneesmiddelen of andere lichaamsvreemde stoffen kunnen het elektrontransport remmen. De elektronen kunnen hierdoor weglekken waardoor onder andere ROS ("reactive oxygen species") ontstaat. We laten in gist duidelijk zien dat diclofenac toxiciteit gekoppeld is aan de ademhalingsketen. Het zuurstofverbruik van de gistcellen neemt af na incubatie met diclofenac en de diclofenac toxiciteit neemt toe in cellen die meer afhankelijk zijn van de ademhalingsketen voor hun energieproductie. Bovendien verhoogt diclofenac de productie van ROS. Vooral de eiwitten Rip1p en Cox9p die onderdeel uitmaken van respectievelijk complex III en IV van de ademhalingsketen zijn belangrijk in diclofenac toxiciteit. Als een van deze twee eiwitten niet aanwezig is, is de groeiremming door diclofenac aanmerkelijk lager en ook de hoeveelheid zuurstof radicalen is verlaagd. Mogelijk bindt diclofenac in de buurt van Rip1p in complex III, waardoor het elektrontransport wordt geremd en de elektronen gaan lekken. Aangezien Rip1p erg lijkt op het humane eiwit RISP (UQCRFS1) is het interessant om te onderzoeken of RISP betrokken is bij diclofenac toxiciteit in humane cellen. Meer onderzoek is nodig om te begrijpen hoe Rip1p en Cox9p betrokken zijn bij de interferentie van diclofenac met de ademhalingsketen.

In **hoofdstuk 3** hebben we de rol van metabolisme door cytochroom P450s in diclofenac toxiciteit onderzocht. Aangezien gist zelf geen P450s bevat die diclofenac kunnen metaboliseren, hebben we hiervoor P450 BM3 M11 in gist tot expressie gebracht. BM3 M11 is een model P450 dat een vergelijkbaar diclofenac-metabolietprofiel geeft als humane P450s. Gistcellen die BM3 M11 tot expressie brachten groeiden significant langzamer in aanwezigheid van diclofenac dan controle stammen die met een lege vector getransformeerd waren. Ook de hoeveelheid ROS was significant hoger in cellen met BM3 M11 dan in cellen zonder dit enzym. Dit bevestigt dat P450 activiteit de toxiciteit van diclofenac verhoogt. Opvallend was dat de diclofenac metabolieten 4'- en 5-hydroxydiclofenac geen effect hadden op celgroei en de hoeveelheid ROS in cellen die BM3 M11 tot expressie brachten. De aanwezigheid van reactieve metabolieten van 4'- en 5-hydroxydiclofenac, de quinone imines, in de cellen werd bevestigd door detectie van hun glutathion conjugaten. Kennelijk zijn 4'- en 5-hydroxydiclofenac en hun reactieve quinone imines niet betrokken bij diclofenac toxiciteit in gist. Andere reactieve metabolieten zoals arene oxides of radicalen kunnen gevormd worden gedurende het metabolisme en zijn mogelijk verantwoordelijk voor de toegenomen toxiciteit van diclofenac in aanwezigheid van een P450. Vorming van diclofenac arene oxides en radicalen is ook beschreven in humane lever microsomen. In toekomstig onderzoek zouden BM3 mutanten met een ander metaboliet profiel gebruikt kunnen worden om te onderzoeken welke metabolieten verantwoordelijk zijn voor de toename in toxiciteit. Bovendien zou gist genetica gebruikt kunnen worden om te achterhalen welke genen betrokken zijn bij de P450-gerelateerde toxiciteit.

In **hoofdstuk 4** beschrijven we dat gistcellen kunnen adapteren aan diclofenac toxiciteit en wat de onderliggende mechanismen daarvoor zijn. Als gistcellen na een dag incuberen met 100 µM diclofenac worden verdund in vers medium en er wordt nogmaals 100 µM diclofenac toegevoegd, is het de tweede keer niet meer toxisch. Alhoewel de meeste cellen dood gaan na een eerste confrontatie met 100 µM diclofenac, kunnen sommige cellen het overleven en weer gaan groeien. Met behulp van microarrays hebben we de genen bestudeerd die bij dit adaptatie proces betrokken zijn. We vonden dat vooral genen behorende tot de groep Pleiotropic Drug Resistance (PDR) genen en genen gereguleerd door Rlm1p opgereguleerd waren in cellen die aan diclofenac geadapteerd zijn. Rlm1p is een transcriptie factor die deel uitmaakt van de Protein Kinase C (PKC) signaleringsroute die reageert op celwand stress. Om na te gaan of deze processen ook direct betrokken zijn bij diclofenac toxiciteit maakten we gebruik van gist deletiestammen en overexpressie constructen. Van de PDR genen was vooral het ABC-transport eiwit Pdr5p belangrijk voor de diclofenac tolerantie van de cellen. Deletie van twee verschillende componenten van de PKC signaleringsroute verhoogde diclofenac toxiciteit, terwijl incubatie met calcofluor white, een stof die celwand stress veroorzaakt, diclofenac toxiciteit verlaagde. Bovendien vormden de cellen drijvende vlokken in het medium na toevoeging van diclofenac, een fenomeen dat mogelijk de oorzaak is van de celwand stress. De expressie van genen die gevoelig zijn voor de zink-concentratie in de cel en van genen die betrokken zijn bij ribosoom biogenese en rRNA processing was lager in diclofenac-geadapteerde cellen. Dit laatste proces is een veelvoorkomend verschijnsel in door chemicaliën of door andere factoren gestreste cellen en hebben we niet verder bestudeerd. Interessant genoeg veroorzaakte deletie van de zink-gevoelige transcriptie factor Zap1p of toevoeging van de zink-chelerende stof 1,10-phenantroline een significante toename van diclofenac toxiciteit. Dit suggereert dat diclofenac initieel een tekort aan zink veroorzaakt of dat zink nodig is om de cel tegen diclofenac te beschermen. Aangezien diclofenac gebruik ook de zink niveaus in patiënten kan verlagen is het interessant om dit verder te onderzoeken. In conclusie, we hebben drie nieuwe processen geïdentificeerd die betrokken zijn bij de resistentie van gist tegen diclofenac, namelijk transport door Pdr5p, celwand stress en zink homeostasis. Doordat we geadapteerde cellen hebben gebruikt voor de microarray analyse voorkomen we detectie van voornamelijk celdood- en stress-gerelateerde genen. Deze aanpak kan ook nuttig zijn voor studies naar resistentie tegen andere geneesmiddelen of chemicaliën.

In **hoofdstuk 5** hebben we onderzocht of de mechanismen en eiwitten betrokken bij diclofenac toxiciteit die we in eerdere hoofdstukken geïdentificeerd hebben ook betrokken zijn bij de toxiciteit van andere geneesmiddelen. Daarvoor hebben we zeven geneesmiddelen geselecteerd die allemaal net als diclofenac tot de groep niet-steroïde, ontstekingsremmende geneesmiddelen (NSAIDs) behoren en een carbonzuur groep bevatten. De volgorde van toxiciteit van de acht geneesmiddelen in gist was vergelijkbaar met de volgorde beschreven

voor rat en menselijke cellen. We ontdekten grote verschillen in de betrokkenheid van mitochondriële toxiciteit, metabolisme door P450s en actief transport in de toxiciteit van deze geneesmiddelen. Aan de hand van hun toxiciteit-mechanismen verdeelden we de geneesmiddelen in drie groepen. Groep I bestaat uit diclofenac, indomethacine en ketoprofen. Hun toxiciteit wordt gedomineerd door de mitochondriële ademhalingsketen en ROS. Metabolisme door P450s verhoogt hun toxiciteit verder, terwijl ABC-transport eiwitten voor resistentie zorgen. Groep II bestaat uit ibuprofen en naproxen. Alhoewel mitochondriën en P450s ook betrokken zijn bij de toxiciteit van groep II geneesmiddelen, wordt hun toxiciteit ergens anders door gedomineerd. De collectie gist deletiestammen zou gebruikt kunnen worden om verder te onderzoeken wat de belangrijkste oorzaak van de toxiciteit van deze groep is. Interessant genoeg was ibuprofen het enige geneesmiddel in de selectie dat niet in staat was opregulatie van de PDR-genen te veroorzaken. Het is mogelijk interessant om dit verder te onderzoeken. Groep III bestaat uit sulindac, ketorolac en zomepirac. Deze drie stoffen zijn vrijwel niet toxisch in gist. Dus we vonden een grote variatie in de toxiciteitsmechanismen van een groep in structuur vergelijkbare geneesmiddelen. Meer onderzoek is nodig om erachter te komen of deze mechanistische verschillen ook relevant zijn in humane cellen.

Tot slot trekken we in **hoofdstuk 6** algemene conclusies, bespreken we de relevantie van de resultaten en doen we suggesties voor toekomstig onderzoek. Door de toenemende vraag naar veilige medicijnen met minimaal gebruik van proefdieren zijn nieuwe testsystemen voor toxiciteit nodig. Gist is een veelgebruikt model om genotoxiciteit te detecteren. Onze resultaten laten zien dat gist ook goed gebruikt kan worden om andere toxiciteits-mechanismen te bepalen. Alhoewel we zelf onze resultaten niet direct in humane cellen geverifieerd hebben, zijn er meerdere sterke aanwijzingen in de literatuur dat enkele van onze vindingen ook relevant zijn voor patiënten. Vooral de rol van Rip1p in mitochondriële toxiciteit, de vinding dat quinone imines niet schadelijk zijn voor cellen en de ontdekking dat zink betrokken is bij diclofenac toxiciteit zijn mogelijk ook belangrijk in zoogdiercellen. Ontwikkelingen in het verwijderen van complete gist signaleringsroutes of het toevoegen van humane routes in wild type of deletie stammen maken het mogelijk steeds “menselijkere” gisten te ontwikkelen. Deze technieken en stammen zijn nieuwe en zeer nuttige hulpmiddelen in toxiciteitsonderzoek en in combinatie met zoogdiercellen kunnen zij onmisbare informatie opleveren met betrekking tot toxiciteitsmechanismen van geneesmiddelen en andere chemicaliën. Stammen die expressie van humane metabole enzymen en humane transporters combineren kunnen gebruikt worden om ADME te bestuderen in een humaan-relevant system. Vergelijkbare strategieën kunnen gebruikt worden om een “high-throughput” gist systeem op te zetten om voor farmacologische activiteit te screenen.

LIST OF PUBLICATIONS

van Leeuwen, J. S., Vredenburg, G., Dragovic, S., Tjong, T. F., Vos, J. C. and Vermeulen, N. P. (2011). Metabolism related toxicity of diclofenac in yeast as model system. *Toxicol Lett* **200**, 162-168.

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