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**Yeast as a model eukaryote in drug safety studies**

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# I

# 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).
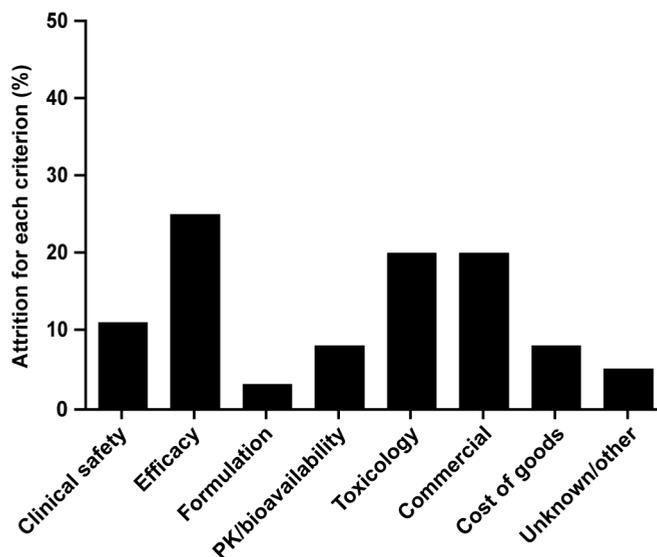


**Fig. 1.** Reasons for drug attrition in the year 2000. PK: pharmacokinetics. Adapted from Kola et al., 2004.

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.
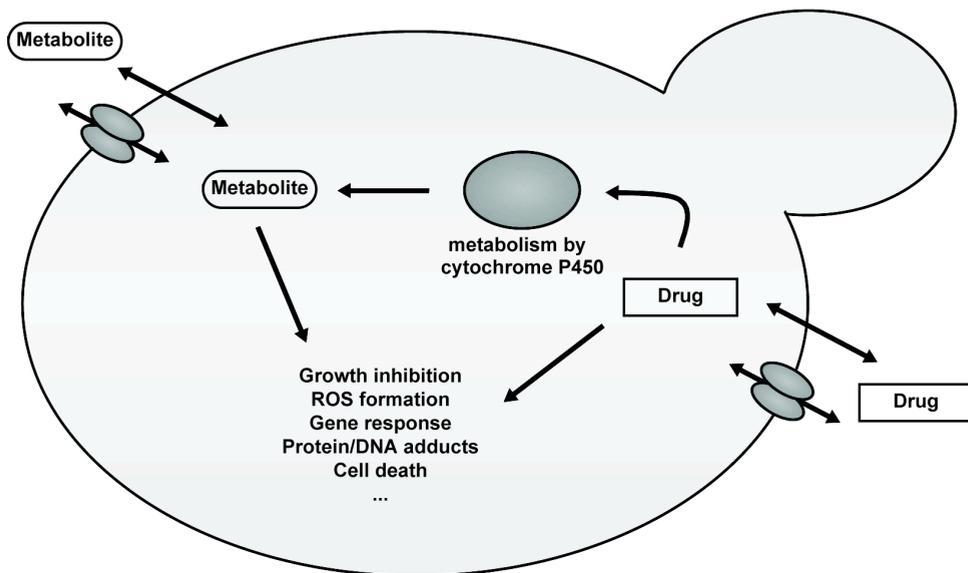


**Fig. 2.** Yeast expressing mammalian P450s as model in biotransformation-related toxicity studies. Inside the cell, the drug of interest is metabolized by the heterologously expressed P450, yielding a metabolite. Both the drug and the metabolite may affect cellular processes and thereby cause toxicity. 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 & Liver slices | + High resemblance to hepatocytes *in vivo* |
| | + 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 & Stem-cell derived hepatocytes | + Stable P450 expression |
| | + 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.

*Chapter 1*

# 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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*Chapter 1*