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Yeast as a Humanized Model Organism for Biotransformation-Related Toxicity

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Abstract: 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 (P450s) is one of the main causes of drug toxicity. As some of these enzymes are highly polymorphic leading to large differences is metabolic capacity, isotype-specific test systems are needed. In this review, we will discuss the use of yeast expressing (mammalian) P450s as a powerful, additional model system in drug safety. We will discuss the various cellular model systems for bioactivation-related toxicity and subsequently describe the properties of yeast as a model system, including the endogenous bioactivation enzymes present, the heterologous expression of (mammalian) P450s and the application of yeasts expressing heterologous P450s and/or other biotransformation enzymes in toxicity studies. All major human drug-metabolizing P450s have been successfully expressed in yeast and various mutagenicity tests have been performed with these humanized yeast strains. The few examples of non-mutagenic toxicity studies with these strains and of the combination of P450s with phase II or other human enzymes show the potential of yeast as a model system in metabolism-related toxicity studies. The wide variety of genome-wide screens available in yeast, combined with its well-annotated genome, also facilitate follow-up studies on the genes involved in toxicity. Unless indicated otherwise "yeast" will refer to baker's yeast *Saccharomyces cerevisiae*.

Keywords: Biotransformation, cytochrome P450 metabolism, model systems, yeast.

INTRODUCTION

Although new drug candidates are developed continuously, only few compounds make it through development and get approved by the regulatory authorities [1]. In the past 20 years, the 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 [1]. Despite years of research, accurately predicting human toxicity with either *in vitro* or animal models remains a challenge [2]. 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 1) the on-target (mechanism-based), 2) an off-target, 3) biological activation, 4) a hypersensitivity/immunological reaction or 5) can be idiosyncratic [3]. The main causes of attrition due to toxicity are biotransformation-related or based on an on- or off-target [4]. Around 75% of the top 200 drugs used in the US in 2002 are cleared via metabolism [5]. 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 carcinogenicity. Therefore, it is advised to also test the toxicity of major metabolites during drug development [6].

Around two-thirds of the drugs that are cleared via metabolism are metabolized by cytochrome P450s [5]. 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 the metabolism of xenobiotics [7]. CYP3A4 (partly overlapping in activity with CYP3A5), CYP2C9, CYP2C19, CYP2D6 and CYP1A2 are together responsible for >95% of drug metabolism by P450s [5]. 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 can cause liver toxicity [8]. Several P450s are also involved in the activation of pro-carcinogens. Especially CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2E1 and CYP3A4 contribute to carcinogen activation [7]. A famous example is the bioactivation of aflatoxin B_1 by CYP1A1, CYP1A2 and CYP3A4 into a carcinogenic metabolite [9].

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 [5]. 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 the bioactivation of dihaloalkanes by glutathione-S-transferases [10], the glucuronidation of non-steroidal anti-inflammatory drugs (NSAIDs) [11] and the sulfation of benzylic alcohols [12]. And finally, the interplay between ABC transporters and P450s has recently received considerable attention in toxicity studies [13, 14].

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 [15]. CYP2C19 and CYP2D6 are for example highly polymorphic, leading to large differences in pharmacokinetic parameters [15]. Additionally, idiosyncratic adverse drug reactions can occur, that are by definition difficult to

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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 [16]. Even if ADME profiles are similar, animal models can at best predict toxicity for an average population, as polymorphisms are not considered. 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 [17]. 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 [18]. 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 [19-21]. Yeast bioassays such as the yeast estrogen screen and the RadarScreen are widely applied to detect estrogenicity or genotoxicity [22, 23]. 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. **1**). Both the metabolite(s) and the parental drug may affect various cellular processes possibly leading to toxicity. Various bioactivation-dependent toxicity studies in yeast describe the genotoxicity of the natural toxin aflatoxin B_1 after bioactivation by CYP1A1, CYP1A2 or CYP3A4 [24-30]. 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 [31-35]. 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.

CELLULAR MODEL SYSTEMS FOR BIOTRANSFORMATION STUDIES

Model Systems Expressing Endogenous Bioactivation Enzymes

As described above, cellular model systems are useful in the determination of bioactivation-related toxicity. Friedberg *et al*. [18] 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**). The advantage of complex models is that they often closely resemble the human liver. However, limitations of complex cellular models in metabolism-related toxicity studies are the unstable and variable expression of cytochrome P450s and the limited possibilities for studying polymorphic variants of a P450 enzyme. 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 homogeneous cellular models is the apparent lack of toxicity of protein adducts that in higher organism may lead to immunological adverse effects.

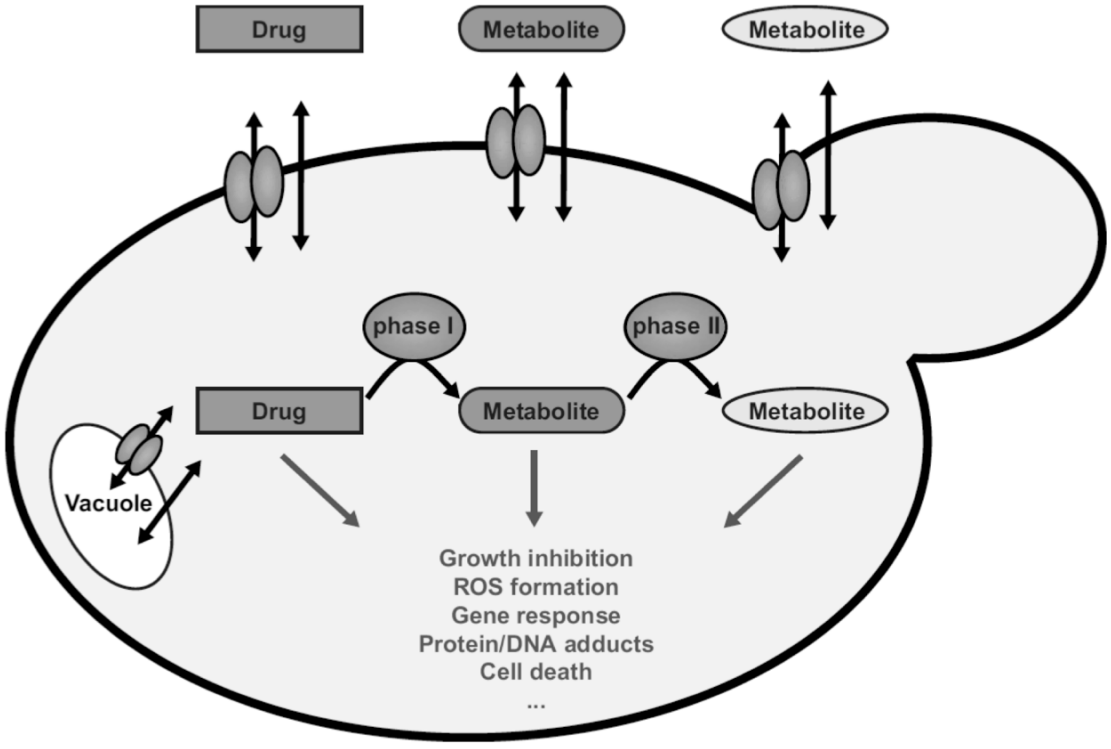

Fig. (1). Yeast expressing mammalian biotransformation enzymes as a model in biotransformation-related toxicity studies. Inside the cell, the drug of interest is metabolized by the heterologously expressed P450 (phase I), yielding a metabolite. This metabolite can be further metabolized by phase II enzymes such as UDP-glucuronosyltransferases, sulfotransferases or glutathione-S-transferases. Alternatively, the parental drug may be directly metabolized by one of the phase II enzymes or a phase II metabolite may be further metabolized by a cytochrome P450 (not shown). Both the drug and the metabolites may affect cellular processes and thereby cause toxicity. Additionally, both the drug and the metabolites may enter or leave the cell either by active transport or via diffusion or can be transported into the vacuole.

Table 1. Comparison of the Various Cellular Models to Study Bioactivation-Related Toxicity

Model	Remarks[a]
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

(a) Main advantages (+) and disadvantages (-) are indicated. Details are provided in the text.

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 [36]. However, either P450 inhibitors or simultaneous experiments with a metabolically incompetent cell line have to be used to study the effect of individual P450 enzymes on toxicity [37]. P450 inhibitors can also affect other cellular processes, such as drug transport and glucuronidation that may affect toxicity [38, 39]. Furthermore, P450 expression levels show high variation in primary hepatocytes obtained form different donors [40]. 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, thereby limiting exposure times [41]. An additional disadvantage of cryopreserved hepatocytes is that they contain drastically reduced glutathione (GSH) levels and can internalize transporters, which can both greatly affect toxicity [41, 42]. 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 [43, 44].

Alternative complex model systems are human hepatoma cell lines, like HepG2. These cells contain relatively stable levels of many phase I and II enzymes, which are lost in most cultured cell lines. However, several major P450s, such as CYP1A2, 2C19 and

Table 2. Endogenous Biotransformation Enzymes Present in *S. cerevisiae*

Class[a]	Gene	Biological Function	Reference
CYP51	*ERG11*	Biosynthesis of ergosterol	[86]
CYP56	*DIT2*	Biosynthesis of N,N′-bisformyl dityrosine	[87]
CYP61	*ERG5*	Biosynthesis of ergosterol	[88]
GST ω	*GTO1*	Unknown	[94]
GST ω	*ECM4*	Unknown	[94]
GST ω	*GTO3*	Unknown	[94]
GST	*GTT1*	Defense against various stresses	[90]
GST	*GTT2*	Defense against various stresses	[90]
GST	*GRX1*	Defense against various stresses	[91]
GST	*GRX2*	Defense against various stresses	[91]

(a) No homologs for sulfotransferases or glucuronosyltransferases have been found.

2D6, are hardly expressed in HepG2 cells [45]. New developments, using stem cell derived hepatocytes or HepaRG cells, show improvements in the number of P450s expressed, but expression levels are still low compared to primary hepatocytes [45, 46]. Recently, inducing 3D-cultures of hepatocytes has resulted in a significant upregulation of P450 metabolism [47, 48].

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 [49]. 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 [50]. 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 [50].

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 [51]. 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 [51]. Alternatively, a microfluidic device has been applied to simultaneously characterize metabolites generated by human liver microsomes using UV detection and test for their cytotoxicity on HepG2 cells [52]. However, metabolites can be too reactive or hydrophilic to penetrate into the cells, as has been described for the epoxide of benzo[a]pyrene [53]. 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 CYP2E1-expressing yeast cells [33].

Models using Heterologously Expressed Mammalian Bioactivation Enzymes

As alternative, many individual CYPs have been expressed in mammalian cells, as well as in *E. coli* and yeast. These microorganisms can be used as bioreactors to produce high amounts of metabolite and are useful for characterizing specific steps in the metabolism of a drug, studying polymorphisms, 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 is limited in many bacteria due to the relatively impermeable lipopolysaccharide layer found on the outer membrane [54]. However, several mutations can affect lipopolysaccharide structure and increase permeability [55]. Additionally, since *E. coli* lacks typical eukaryotic structures and pathways, 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 [56] 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 [4]. 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 [57]. 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 [58-60]. 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 a 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 Open Reading Frames 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 [61]. 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 [62].

The determination of drug targets is a very challenging issue [4]. 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 [19-21, 63-65]. Valuable tools are the collections of gene deletion strains [66] and overexpression constructs [67], 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*. [68] 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 [69, 70]. 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 [71-73].

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 [22, 74, 75]. 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 [23, 76, 77]. Following overnight exposure of the yeast culture to a test substance, the level of ß-galactosidase activity or GFP fluorescence induction gives a measure of the genotoxicity of the substance. Other DNA-damage sensitive promoters such as those of *RNR3* and *HUG1* have also been used for the detection of genotoxicity in yeast [78, 79]. 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 [80]. Additionally, assays for general cytotoxicity have been developed using the promoter of the housekeeping plasma membrane ATPase gene *PMA1* coupled to a reporter as biomarker [81]. Finally, yeast has been used as an indicator organism for the detection of the food toxin deoxynivalenol [82] and to examine the presence of toxicants in wastewater [83].

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 [74, 77, 81, 82]. 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 the multi-subunit mitochondrial respiratory complex I might interfere with the detection of mitochondrial toxicants. However, these problems can be partially solved, or turned into an advantage, by heterologous expression of the (e.g. polymorphic) 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 [84, 85]. 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 [86]. 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 [87]. The enzyme CYP61 (Erg5p) is a Δ22-desaturase in the membrane ergosterol biosynthesis pathway [88]. Besides having a housekeeping function, the *S. cerevisiae* CYP61 also metabolizes benzo[a]pyrene yielding 3-hydroxybenzo[a]pyrene [89]. To our knowledge, this is the only clear evidence of the involvement 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 [23, 76]. 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 [90, 91]. All four enzymes protect the cell against oxidative and heat stress and stress induced by xenobiotics [90-93]. Deletion of *GTT2* led to increased toxicity of menadione and decreased levels of the menadione-glutathione conjugate in the medium [92]. 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 [94]. Several additional proteins, including Ure2p, Mak16p and Yef3p,

show some homology to GSTs, although no catalytic GST activity has been observed for these proteins [95, 96].

Heterologous Expression of P450s in Yeast

Subsequent to the expression of rat CYP1A1 in baker's yeast in 1985 [97], 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* [98] and fission yeast *Schizosaccharomyces pombe* [99] 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 [100-103]. 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 [104] and the lack of an active import system to increase intracellular substrate concentrations of for example precursor steroids [105]. Expression of human CYP2C9 in both fission and baker's yeast yielded gram-scale amounts of the diclofenac metabolite 4'-hydroxydiclofenac [106, 107]. Sophisticated approaches resulted in the heterologous expression of complete biosynthetic pathways in yeast, consisting of up to eight enzymes including CYPs, for the production of complicated biological molecules such as sesquiterpenes, benzylisoquinoline alkoids or precursors for antimalarial or anticancer drugs [108-111].

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. 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 [112]. Importantly, the inhibitory values found in yeast were consistent with those found in human cells [112]. Also IC_{50} values for inhibition of human CYP17 and CYP21 expressed in fission yeast have been determined [113]. However, in this case the reported IC_{50} values in yeast cells were about one order in magnitude higher than those in human microsomes. Finally, expression of mammalian P450s in yeast has also been used to study CYP degradation (reviewed in [114]), localization [115] and polymorphisms [116].

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) [117]. 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 [109, 118, 119]. Also expression of human cytochrome b_5 may increase human CYP activity in yeast [120]. Additionally, yeast contains an ADR homolog, Ahr1p, which can supply electrons to mammalian mitochondrial CYPs, via mammalian ADX targeted to yeast mitochondria [121-123]. Also fission yeast *S. pombe* contains an ADX homolog, Etp1p, which can function with mammalian mitochondrial P450s and ADR [124, 125] although not as efficiently as mammalian ADX [126].

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* [124, 127]. 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 [124]. Also high expression levels of bacterial P450 BM3 mutant M11 decrease yeast cell growth (G. Vredenburg, 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*. [128] 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 (but not ER-targeted CYP2E1) already caused respiratory deficiency, possibly due to uncoupling leading to ROS formation.

P450 metabolism-related toxicity studies in yeast have mainly focused on the genotoxicity of metabolites 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 relative number of cells able to grow on canavanine is a direct measure for mutagenicity. Black *et al*. [31] 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 canavanine, increased in a dose-dependent manner over a control strain and was up to 16-fold higher at the highest doses used [31]. Also sterigmatocystin induced mutations in CYP2B1-expressing yeast cells [32].

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 [24, 25]. 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 [24-27, 30]. Also human CYP1A1 expression enhanced metabolism-dependent toxicity of aflatoxin B_1 in yeast [30]. Li and colleagues heterologously expressed carbohydrase genes in yeast cells expressing human CYP3A4 [28, 29]. 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 [129]. 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 [34]. 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 [33].

A nice example of the advantage of yeast as model system is provided by a study by Guo *et al.* [24] 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 [24]. 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 [9]. 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 [25, 130].

In the previously mentioned 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) [76]. 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 GreenScreen yeast strain [35]. 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.

Toxicity studies using mammalian CYP expressing yeasts have not solely focused on 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.* [131] 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 [132]. BM3 is a cytosolic, bacterial P450 with a coupled reductase domain [133]. Mutant M11 metabolizes several drugs, including diclofenac, in a similar way as human P450s [134]. 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 had a significant impact on two toxicity endpoints, namely decreased growth and increased ROS levels [132]. 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, in a study where yeast was used to classify the toxicity of structurally related NSAIDs, it was shown that the toxicity of indomethacin, ketoprofen and naproxen was also increased by BM3 M11 metabolism [135].

(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 [27], 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 drugs showing glucuronidation-related toxicity are the carboxylic NSAIDs, for which metabolism by UGTs is the main cause of protein-adduct formation leading to immune reactions [11]. Nine human UGTs belonging to the UGT1A, -2A and -2B families have been expressed in fission yeast *S. pombe* for production of glucuronides [136]. Expression of the UGTs alone did not produce a growth phenotype, however, co-expression with the co-factor-providing UDP-glucose dehydrogenase decreased biomass yield by 30-50% in the absence of a xenobiotic substrate [136]. A first spin-off is the production of ibuprofen-acyl glucosides using human UGT2B7 and demonstrating pronounced differences between two UGT2B7 isoforms [106]. Mouse and rat UGTs have been expressed in *S. cerevisiae* to study enzyme structure and function [137, 138]. Furthermore, human UGT1A6 and B3GAT3 have been expressed in the yeast *Pichia pastoris* for enzyme production and characterization [139-142]. Ikushiro *et al.* [143] 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 [12]. 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 [144-146]. Additionally, five animal-derived sulfotransferases have been expressed in the yeast *Kluyveromyces lactis* for the biosynthesis of heparin [147]. 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 (J.S. van Leeuwen, D.M. Vredenburg-Maasdijk, 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. However, also this group of enzymes can create potentially harmful reactive metabolites, as has been described for dihaloalkanes [10]. GSTs also play a regulatory role in cellular signaling by associating with several kinases [148, 149]. Notably, GST function in tumors may decrease the efficacy of anticancer drugs. This was confirmed by the expression of human GSTA1 and GSTP1 in *S. cerevisiae*, which in both cases resulted in a marked decrease in cytotoxicity of the anticancer drugs chlorambucil and adriamycin [150].

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-acetoxy-esters 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 [151].

CONCLUSIONS

We hope that this review will enhance the appreciation among drug safety scientists for yeast as a model organism, as it is apparent that it is a useful tool in toxicity 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. A few examples show the potential of bioactivation-competent yeast strains in non-genotoxic drug safety studies, such as our study on the metabolism-related toxicity of NSAIDs. A small number of studies on co-expression of P450s with phase II or other human enzymes have demonstrated the possibility to study a combination of enzymes in yeast. The occurrence of many polymorphisms in biotransformation enzymes requires test models that can reconstitute the genotype of human individuals. Yeast cells may provide a relatively clean and simple background for expression of variations of isotype for high-throughput screening. Furthermore, 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.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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