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## Metabolism related toxicity of diclofenac in yeast as model system

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## ABSTRACT

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

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## 1. Introduction

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

5-hydroxy-derivatives can form, in part spontaneously, reactive quinone imine intermediates (Shen et al., 1999; Tang et al., 1999a) that are suggested to cause the toxicity of diclofenac (Miyamoto et al., 1997; Poon et al., 2001; Shen et al., 1999; Tang et al., 1999b). Furthermore, arene oxides may be involved in the P450-dependent generation of 4'- and 5-hydroxydiclofenac (Masubuchi et al., 2002; Yan et al., 2005), leading to toxicity.

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

The yeast *Saccharomyces cerevisiae* is an excellent alternative model organism in pharmacological research (Menacho-Marquez and Murguia, 2007). Advantages of yeast over mammalian cell systems are its easy manipulation, cost-effectiveness, and rapid growth. Because wild type yeast strains lack drug-metabolizing cytochrome P450s and glucuronosyl-transferases, many biotrans-

Abbreviations: GSH, glutathione; HLM, human liver microsomes; NSAID, non-steroidal anti-inflammatory drug; P450, cytochrome P450; ROS, reactive oxygen species.

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**Table 1**

Metabolism of diclofenac and 4'- and 5-hydroxydiclofenac by BM3 M11 *in vitro* or *in vivo* in yeast. Metabolites were analyzed by HPLC with UV detection at 254 nm and by LC-MS. Presence (+) or absence (–) of the metabolite is indicated. N.d. = not detected.

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

<sup>a</sup> Single protonated molecular ion.

<sup>b</sup> Double protonated molecular ion.

<sup>c</sup> Triple protonated molecular ion.

formation enzymes have been heterologously expressed in yeast (Pompon et al., 1997; Renaud et al., 1993). Expression of mammalian P450s in yeast has been used to investigate the mutagenic effects of oxidative metabolites of xenobiotics, as has been described for N-alkylformamides and aflatoxine B<sub>1</sub> (Del Carratore et al., 2000; Guo et al., 2005).

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

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

## 2. Materials and methods

### 2.1. Chemicals and stock solutions

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

### 2.2. Strains and plasmids

The haploid *S. cerevisiae* strain W303 (MATa; ura3-52; trp1 Δ2; leu2-3,112; his3-11; ade2-1; can1-100) was used in this research. BM3 M11 was expressed in yeast using pTL26, a galactose inducible yeast expression vector carrying the *HIS3* selection marker and the CEN6/ARSH4 origin of replication (Lafontaine and Tollervey, 1996). The His-tagged BM3 M11 gene was obtained from the previously described pT1-M11 (Van Vugt-Lussenburg et al., 2007) and cloned into the pTL26 vector using standard procedures. The resulting pTL26-M11 and the empty pTL26 were transformed into yeast using the freeze-thaw method (Klebe et al., 1983).

### 2.3. Growth conditions and diclofenac treatment

Strains were grown on selective minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented amino acids and bases without histidine) and switched overnight to minimal media containing galactose to induce

BM3 expression. Overnight cultures were diluted and grown at 30 °C to OD<sub>600</sub> ~0.2. At this point, 0–50 μM diclofenac, 4'-hydroxydiclofenac or 5-hydroxydiclofenac was added. Controls were treated with equal amounts of DMSO or EtOH.

### 2.4. Western blotting of heterologously expressed BM3 M11

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

### 2.5. *In vitro* incubations using purified BM3 M11 and HPLC conditions

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

### 2.6. Detection of *in vivo* generated metabolites in yeast

Diclofenac treated cultures were centrifuged and cellular lysates were made in KPi buffer (100 mM potassium phosphate, pH 7.4) using glass beads. Proteins were precipitated by adding an equal volume of ice-cold MeOH and incubating 10 min on ice. Precipitate was removed by centrifugation (10 min, 14,000 rpm) and the supernatant was analyzed by HPLC.

### 2.7. ROS assay

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

### 2.8. Statistical analysis

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

## 3. Results and discussion

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

### 3.1. Cytochrome P450 BM3 M11 as model enzyme for oxidative diclofenac metabolism

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

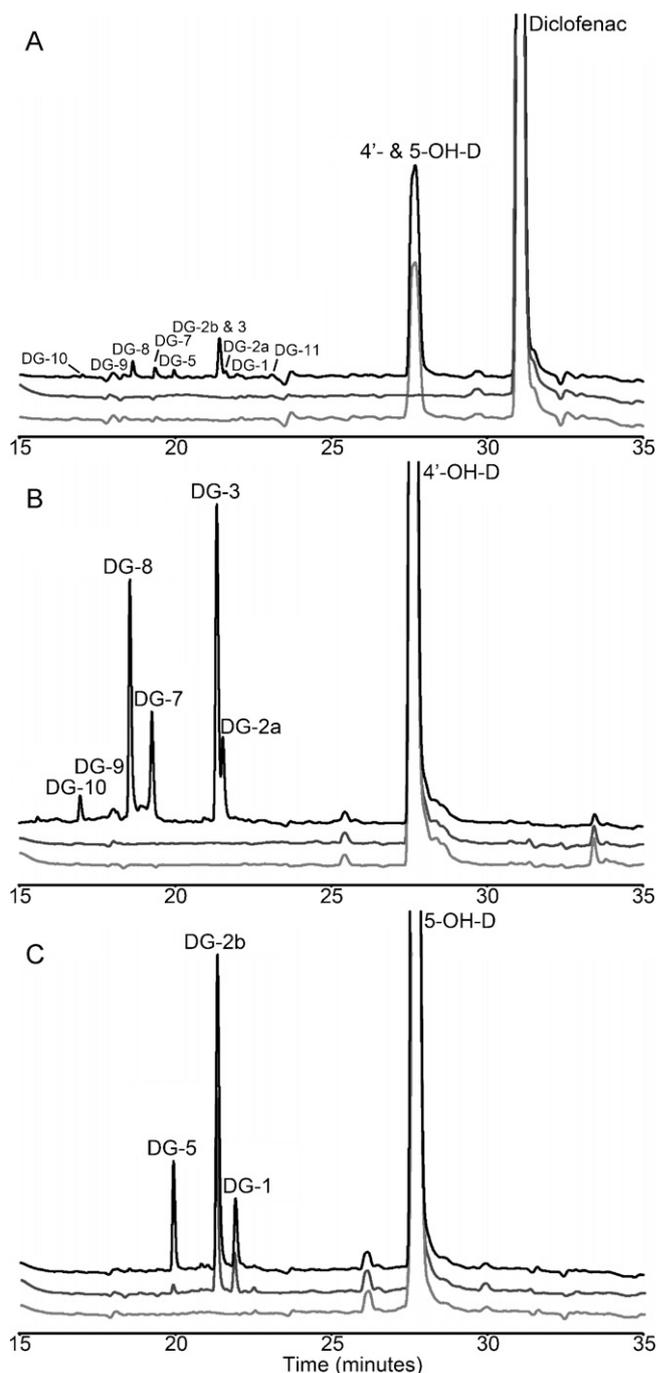
Damsten et al. (2008) showed that diclofenac was mainly metabolized by BM3 M11 into hydroxydiclofenac and several hydroxydiclofenac GSH-conjugates (DG-1, DG-2a, DG-2b, DG-3, DG-5, DG-7, DG-8, DG-9, DG-10; see Fig. 7 for the biotransformation scheme). Here, we confirmed these results and identified an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

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

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

### 3.2. Expression and activity of cytochrome P450 BM3 M11 in yeast

By expressing BM3 M11 in yeast, we aimed to establish a model system for investigating P450-related toxicity. Yeast was transformed with an episomal vector (pTL26-M11) containing the C-terminal His-tagged BM3 mutant M11 (Van Vugt-Lussenburg



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

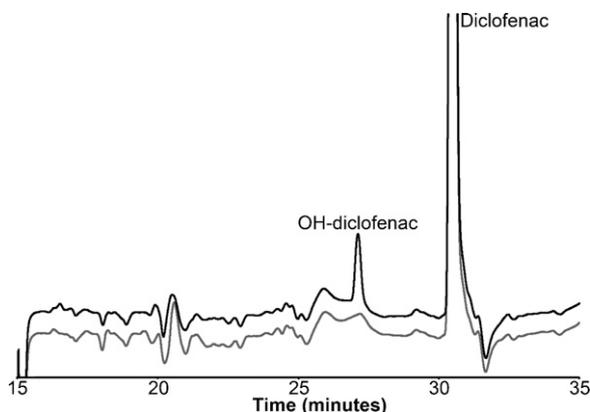
et al., 2007) behind a conditional GAL promoter. Switching the yeast cells from glucose- to galactose-containing medium induced BM3 M11 expression. The presence of BM3 M11 in the strain was determined by Western blotting (Fig. 2). The blot shows that when the yeast strain was grown on medium containing galactose, an antibody against His-tags bound to a protein with a similar size as BM3 M11 purified from *E. coli* (119 kDa). No protein was recognized in cells transformed with pTL26-M11 and grown on glucose



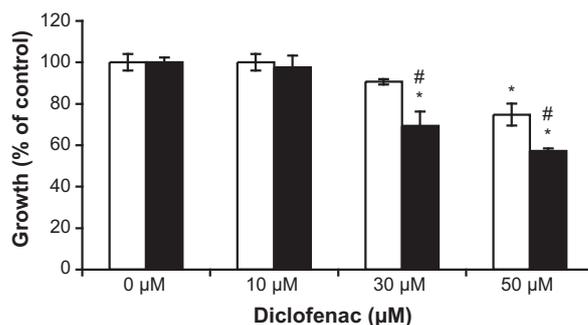
**Fig. 2.** Western blot of protein extracts of yeast strains transformed with pTL26-M11. An anti-His antibody was used against the His-tag of M11. Lane 1: purified BM3 M11 from *E. coli*; lanes 2 and 3: protein extract (25  $\mu$ g) of yeast cells transformed with pTL26-M11 grown in medium containing galactose; lanes 4 and 5: protein extract (25  $\mu$ g) of yeast cells transformed with pTL26-M11 grown in medium containing glucose.

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

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



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



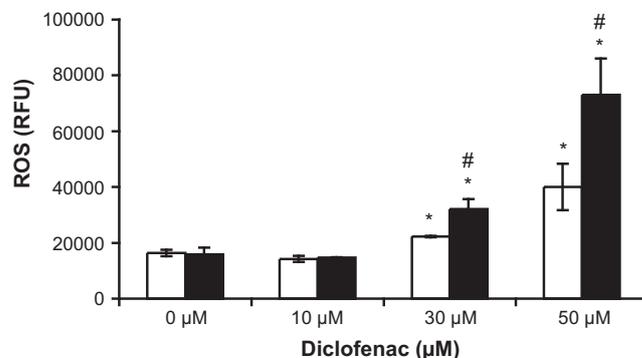
**Fig. 4.** Growth of yeast strains expressing BM3 M11 (black bars) or without BM3 M11 (white bars) after incubation for 6 h with different concentrations of diclofenac. The strains were grown in selective media containing galactose. The growth is expressed as % growth  $\pm$  S.D. compared to untreated cells (100%). \* $P$  < 0.01 compared to cells incubated without diclofenac, # $P$  < 0.01 compared to cells without BM3 M11.

and DG-10). Indeed, these metabolites could be detected in these *in vitro* incubations (data not shown). Since all GSH-conjugates were detected either inside the yeast cells or in incubations with yeast lysates, all reactive intermediates of diclofenac are expected to be generated inside the bioactivation competent yeast cells after incubation with diclofenac.

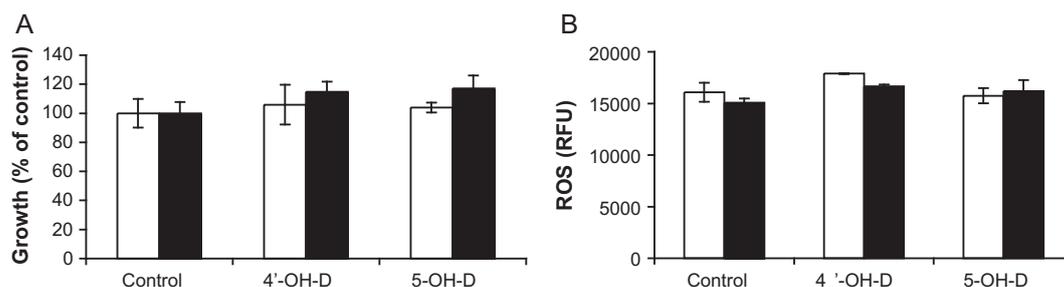
### 3.3. The effect of BM3 M11 expression on diclofenac toxicity in yeast

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

Diclofenac can induce the formation of ROS in both mammalian cells (Gomez-Lechon et al., 2003; Inoue et al., 2004; Lim et al.,



**Fig. 5.** ROS formation in yeast strains expressing BM3 M11 (black bars) or without BM3 M11 (white bars). The strains were incubated for 3 h with 10  $\mu$ M of the ROS-sensitive, fluorescent 2',7'-dichlorodihydrofluorescein diacetate and different concentrations of diclofenac in selective media containing galactose. The ROS levels are expressed as RFU  $\pm$  S.D. \* $P$  < 0.01 compared to cells incubated without diclofenac, # $P$  < 0.01 compared to cells without BM3 M11.



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

2006) and in yeast (van Leeuwen et al., 2010). In mammalian cells, it has been shown that sulfaphenazole (CYP2C9 inhibitor) and ketoconazole (CYP3A4 inhibitor) decrease toxic effects caused by diclofenac exposure, such as LDH release (Bort et al., 1999; Kretz-Rommel and Boelsterli, 1993) and ROS formation (Lim et al., 2006). To investigate if ROS levels were changed by the oxidative metabolism of diclofenac in the yeast system, we incubated the strain transformed with an empty vector and the BM3 M11 expressing strain with diclofenac and with the ROS-sensitive 2',7'-dichlorodihydrofluorescein diacetate. In the absence of diclofenac, ROS levels in both strains were comparable (Fig. 5). When incubated with 30 or 50  $\mu\text{M}$  diclofenac, the ROS levels were increased in both strains, but were significantly higher in the BM3 M11 strain than in the control strain. This shows that the increase in diclofenac toxicity observed in BM3 M11 expressing strains is accompanied by increased ROS formation. Similar results were obtained in another yeast strain, BY4741 (data not shown). Overall, these results demonstrate the direct involvement of cytochrome P450 activity in the cellular toxicity of diclofenac.

#### 3.4. 4'- and 5-hydroxydiclofenac metabolism is not toxic in yeast and does not increase ROS formation

The quinone imines formed from 4'- and 5-hydroxydiclofenac (Fig. 7) are considered to be responsible for the toxicity of diclofenac (Miyamoto et al., 1997; Poon et al., 2001; Shen et al., 1999; Tang et al., 1999b). To investigate if the quinone imines could be related to the P450-dependent toxicity of diclofenac in our yeast model, we tested the effect of 4'- and 5-hydroxydiclofenac on growth and ROS levels of yeast cells with or without BM3 M11. Interestingly, when either 4'- or 5-hydroxydiclofenac was added to the yeast strains at concentrations ranging between 0.3 and 50  $\mu\text{M}$ , no negative growth effect could be observed (Fig. 6(A), data shown for 30  $\mu\text{M}$ ), neither in control strains without BM3 M11, nor in strains expressing BM3 M11. Notably, 4'- and 5-hydroxydiclofenac (30  $\mu\text{M}$ ) did not induce ROS formation in control or M11 expressing strains (Fig. 6(B)) although GSH-conjugates were generated inside the cells (Section 3.2). Similar results were obtained in another yeast background (BY4741) or with 4'- and 5-hydroxydiclofenac obtained from another source (Toronto Research Chemicals, Canada; data not shown).

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

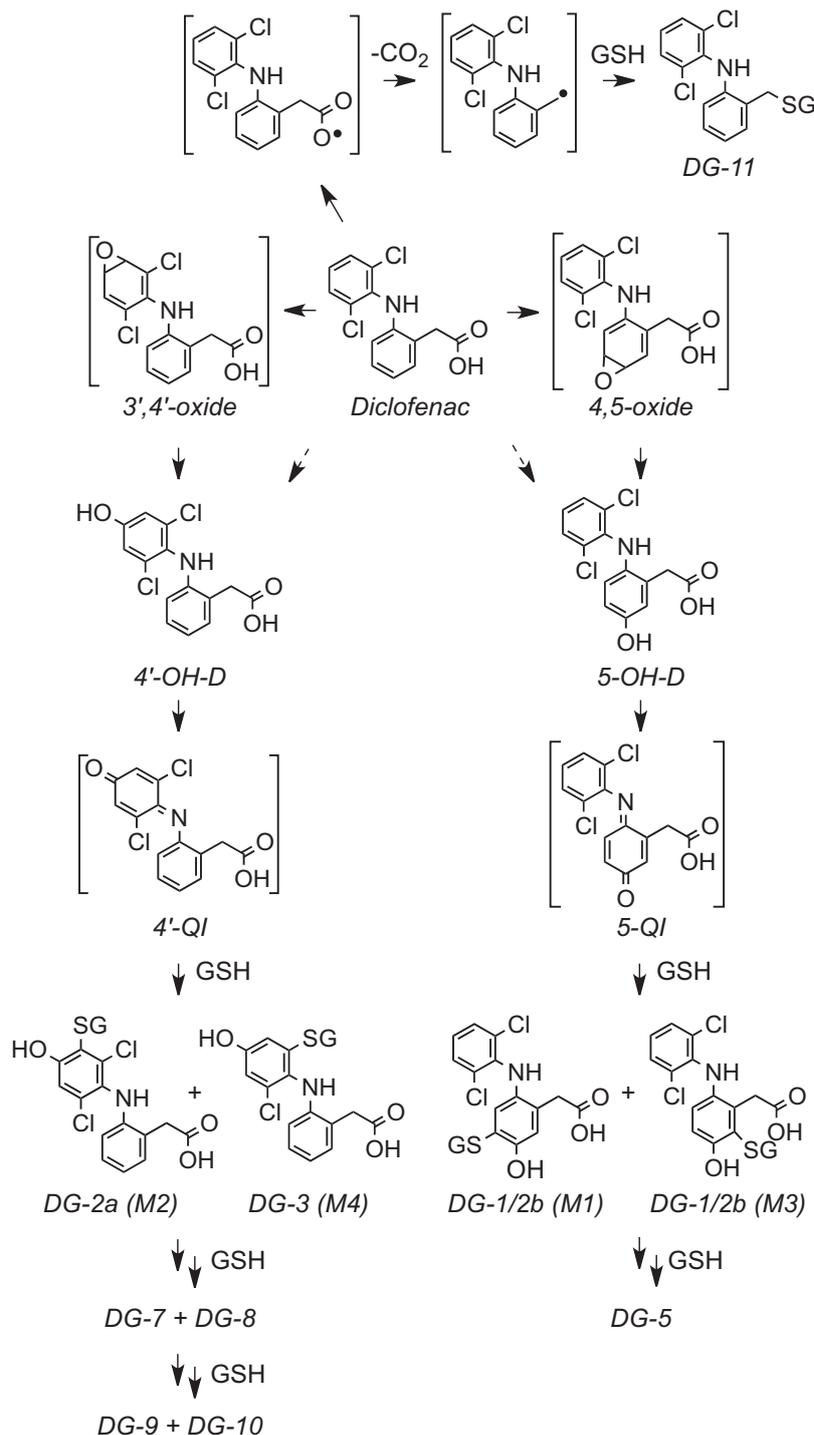
Collectively these results indicate that 4'- and 5-hydroxydiclofenac and their quinone imines, reflected by the marker GSH-conjugates DG1-10, are not involved in diclofenac toxicity in yeast. Interestingly, toxic effects such as uncoupling of oxidative phosphorylation, ATP depletion and inactivation of CYP3A4 are also mainly observed with diclofenac and not with isolated diclofenac metabolites (Bort et al., 1999; Masubuchi et al., 2000, 2002). On the other hand, 5-hydroxydiclofenac can bind to cellular protein in HLM incubations in the absence of GSH (Shen et al., 1999). Although the toxicological relevance of protein adducts in isolated cells such as yeast or hepatocytes may be limited (Kretz-Rommel and Boelsterli, 1993), *in vivo* protein adducts might result in an immunological response leading to toxicity (Naisbitt et al., 2007).

As hydroxydiclofenac is not toxic in bioactivation-competent yeast, the observed toxicity and ROS production is likely coupled to another P450-mediated diclofenac metabolite or reactive intermediate. Interestingly, the GSH-conjugate of the decarboxylated metabolite (DG-11) was the only metabolite we could detect in BM3 M11 incubations with diclofenac that was not formed in incubations with 4'- or 5-hydroxydiclofenac. Grillo et al. (2008) identified DG-11 previously in incubations with HLMs. Interestingly, also for the other NSAIDs ketoprofen and indomethacin oxidative decarboxylated metabolites have been identified (Komuro et al., 1995). Both ketoprofen and indomethacin caused a P450-dependent increase in ROS formation and toxicity in our yeast model system while other drugs like paracetamol and clozapine did not (data not shown). During oxidative decarboxylation of indomethacin, a carbon radical is formed (Komuro et al., 1995), which is also suggested to be formed in the oxidative decarboxylation of diclofenac (Grillo et al., 2008; Fig. 7). Since free radicals are toxic species, the formation of this decarboxylated metabolite might be responsible for the P450-dependent toxicity of diclofenac. Another possible explanation is that intermediate arene oxides that are involved in the formation of 4'- and 5-hydroxydiclofenac play a role in toxicity (Blum et al., 1996; Masubuchi et al., 2002; Fig. 7). Consistent with this hypothesis, the 2'-hydroxy-3'-GSH-monoclofenac metabolite,

**Table 2**

Concentration of diclofenac or hydroxydiclofenac in yeast cells expressing BM3 M11. The cells were incubated for 3 h with 30  $\mu\text{M}$  (hydroxy)diclofenac. Cellular lysates were analyzed by HPLC and LC-MS. Numbers indicate the amount of (hydroxy)diclofenac in pmol/ $\mu\text{g}$  protein.

	Concentration in lysate (pmol/ $\mu\text{g}$ )	
	Diclofenac	OH-diclofenac
30 $\mu\text{M}$ diclofenac	179 $\pm$ 5	0.3 $\pm$ 0.2
30 $\mu\text{M}$ 4'-OH-diclofenac	–	15 $\pm$ 2
30 $\mu\text{M}$ 5-OH-diclofenac	–	9 $\pm$ 3



**Fig. 7.** Biotransformation scheme of diclofenac. Diclofenac metabolism can yield potentially toxic reactive intermediates, including quinone imines, arene oxides and radical species.

identified in HLM incubations by Yan et al. (2005), is considered to result from the diclofenac 2',3'-arene oxide.

### 3.5. Conclusion

A crucial topic in examining the toxicity of drugs is distinguishing between the toxicity of the parent drug and its metabolites. Here, we created yeast strains with inducible expression of the cytosolic, stable and highly active P450 BM3 M11 and isogenic strains transformed with an empty vector as tool to study the P450-

dependent toxicity of diclofenac. Advantages of this yeast model over mammalian cellular systems are the controlled P450 expression levels, a defined eukaryotic background and the redundancy of P450 inhibitors. Both stable and GSH-conjugated metabolites of diclofenac were identified inside the BM3 M11 expressing yeast cells. We showed a significantly slower growth and higher ROS levels for strains expressing BM3 M11 when exposed to diclofenac compared to control strains without BM3. The metabolites 4'- and 5-hydroxydiclofenac had no effect on the cell growth and ROS formation in strains with or without BM3, showing that these

metabolites and their reactive quinone imines or other secondary metabolites are not responsible for P450-mediated diclofenac toxicity in yeast. Alternatively, either reactive arene oxides in the formation of hydroxydiclofenac or radical species in the formation of DG-11 might be responsible for the observed toxicity. Further research is needed to identify which of these two routes causes the toxicity, possibly by using BM3 mutants with an altered metabolite profile. Furthermore, yeast genetics can now be used to determine which genes are involved in the toxicity of diclofenac metabolism.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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