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

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Chapter 4

ABSTRACT

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

INTRODUCTION

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

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

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

**MATERIALS AND METHODS**

**Chemicals and stock solutions**

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

**Strains**

The haploid *Saccharomyces cerevisiae* strains W303-1A (MATa; ura3-52; trp1Δ2; leu2-3,112; his3-11,15; ade2-1; can1-100) and BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) were used. BY4741 deletion strains were obtained from EUROSCARF. W303-1A Δpkc1 and Δslt2 were a kind gift from Stefan Hohmann (Tamas et al., 1999).
Growth conditions and diclofenac treatment
Strains were grown overnight at 30°C in selective minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented amino acids and bases). Overnight cultures were diluted in minimal media and grown at 30°C to an OD$_{600}$ ~0.2. At this point, 0-100 µM diclofenac was added. Controls were treated with equal amounts of DMSO (max 0.1%). HPLC analysis of the medium or cellular lysates revealed no degradation products of diclofenac in wild type cells after incubating 24 hours (van Leeuwen et al., 2011a).

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

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

β-galactosidase reporter assay
The TRP5-, PDR3-, PDR5-, RSB1-, and SNQ2-lacZ reporter constructs in the low copy number vector pSEY102 were a kind gift from Scott Moye-Rowley (Decottignies et al., 1995; Hallstrom and Moye-Rowley, 2000; Katzmann et al., 1994; Panwar and Moye-Rowley, 2006). The plasmids were transformed into yeast strain BY4741 by using the freeze-thaw method (Klebe et al., 1983). Yeast cultures were grown at 30°C to an OD$_{600}$ ~0.2. Diclofenac was added and cultures were incubated for 2 hours. Protein extracts were made in LacZ buffer (40 mM Na$_2$HPO$_4$, 60 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 50 mM β-mercaptoethanol) by vortexing with glass beads. ~20 µg of protein was incubated with 24 µg chlorophenolred-β-
D-galactopyranoside at 30°C for ~30 minutes. Absorption at 575 nm was measured and corrected for the incubation time and the protein concentration in the samples.

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

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

RESULTS

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

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

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

Since loss of mitochondrial DNA (mtDNA) (rho⁰ cells) leads to decreased ROS formation and elevated resistance to diclofenac (van Leeuwen et al., 2011a), we investigated whether adapted strains lost their mitochondrial DNA. After several days of incubation with 100 µM diclofenac, cells were plated on plates containing either glucose or glycerol and ethanol as carbon sources. Only cells that are able to respire and therefore contain mtDNA can grow on glycerol/ethanol-plates. Approximately 95% of the adapted cells contained mtDNA, which was comparable to what was found for cells grown without diclofenac. Although rho⁰ cells have a higher resistance to diclofenac, no selection for rho⁰ cells occurred.

To further investigate the characteristics of adaptation, strains were adapted by exposure to 50 µM diclofenac for 72 hours. When these adapted cells were subsequently treated with 150 µM diclofenac, almost no toxicity was observed (Fig. 2). However, when the adapted strains were grown in the absence of diclofenac for 48 hours and were then treated with 150 µM diclofenac, the resistance was lost (Fig. 2). This reversibility of adaptation is consistent with the observation that adapted strains do not lose their mtDNA, and also excludes selection for a random DNA mutation as has been described for multidrug resistance genes (Carvajal et al., 1997).
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**Fig. 2.** Adaptation to diclofenac is reversible. W303 strains either were grown in the absence of diclofenac (squares), pretreated for 72h with 50 µM diclofenac and subsequently incubated with 150 µM diclofenac (circles), or first pretreated for 72h with 50 µM diclofenac, then incubated for 48h without diclofenac, and finally incubated with 150 µM diclofenac (triangles). Growth is expressed as OD at 600 nm ± SD.

<table>
<thead>
<tr>
<th>Motif</th>
<th>T-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>E-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genes&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>PDR</td>
<td>11.3</td>
<td>&lt;1.0E-15</td>
<td>AZR1, RSB1, PDR5, GRE2, RTA1, YGR035C, ADY2, PDR15, YPL088W, SNQ2, ICT1, YLR346c, YOR268C, YOR1, YHR140W, YKL071W, HXT1, PDR18, TPO1, YLL066W-B, PRM5</td>
</tr>
<tr>
<td>RLM1</td>
<td>4.5</td>
<td>8.6E-04</td>
<td>RTA1, YGR035C, YPL088W, YPS3, YCR101C, YGL258W-A, YAL067W-A, YNR066C, ADH2, CSM4, YPL067C, PRM5</td>
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<tr>
<td>ZAP1</td>
<td>-3.0</td>
<td>3.7E-02</td>
<td>YOR387C, ADH4, VEL1, ZPS1, YIL169C, ZAP1, CHA1, YLL053C, YGK3, HPF1</td>
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<tr>
<td>rRPE</td>
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<td>2.5E-09</td>
<td>YOR378C, ADH4, VEL1, CYB5, YAP7, BFR2, YDL063C, SUL1, GCV2, YOL029C, BIO2, REX4</td>
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<td>PAC</td>
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<td>Other (down)</td>
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<td></td>
<td>PCL1, PDC6, YGR079W, AQY2, SEO1, DAK2, IRC7, FTR1, GRE1, YNR063W, DAL80, FET3, HLR1</td>
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**Table 1.** Significantly enriched motif-groups in diclofenac-adapted strains compared with controls. a: T- and E-values were found using T-profiler analysis (Boorsma et al., 2005) on the complete dataset, b: genes, grouped by motif, that are >2 times up- or down-regulated in adapted strains compared with controls.

**Genome-wide analysis of cells adapted to diclofenac**

Because diclofenac-adaptation was reversible, it was probably related to altered gene expression. Genes whose expression is significantly altered in adapted cells might play an important role in tolerance to diclofenac. Therefore, we performed microarray analysis on
yeast cells adapted to diclofenac and compared the expression profile to that of controls grown without diclofenac. T-profiler analysis (Boorsma et al., 2005) was performed on the complete dataset to search for significantly enriched groups of genes containing upstream matches to a consensus transcription factor binding motif. The search for motifs revealed upregulation of pleiotropic drug resistance (PDR) genes and genes targeted by Rlm1p, a transcription factor in the protein kinase C (PKC) mediated MAP kinase pathway, which is responsive to cell wall stress (Table 1). Also genes containing an Msn2p/4p binding motif and responsive to oxidative or general environmental stress were slightly enriched. However, only Msn2p/4p responsive genes that also contain a PDR responsive element in their promoters were found to be upregulated. MSN2 and MSN4 themselves were both downregulated in the adapted cells (-1.1x and –1.4x, respectively) and probably are not involved in adaptation to diclofenac.

<table>
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<th>(drug) Transporter activity</th>
<th>T-value</th>
<th>E-value</th>
<th>Mean</th>
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<tr>
<td>F</td>
<td>6.1</td>
<td>1.2E-06</td>
<td>0.38</td>
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<tr>
<td>Endoplasmic reticulum</td>
<td>C</td>
<td>6.0</td>
<td>2.7E-06</td>
</tr>
<tr>
<td>Cell wall</td>
<td>C</td>
<td>4.4</td>
<td>1.4E-02</td>
</tr>
<tr>
<td>Transcription</td>
<td>P</td>
<td>-4.7</td>
<td>4.6E-03</td>
</tr>
<tr>
<td>Mitochondrion</td>
<td>C</td>
<td>-5.7</td>
<td>1.7E-05</td>
</tr>
<tr>
<td>rRNA processing</td>
<td>P</td>
<td>-11.4</td>
<td>&lt; 1.0E-15</td>
</tr>
<tr>
<td>Nucleus</td>
<td>C</td>
<td>-11.6</td>
<td>&lt; 1.0E-15</td>
</tr>
<tr>
<td>Ribosome biogenesis</td>
<td>P</td>
<td>-12.5</td>
<td>&lt; 1.0E-15</td>
</tr>
</tbody>
</table>

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

T-profiler was also used to analyze the data for significantly enriched classes or categories of genes using the Gene Ontology (GO) database (Table 2). Redundant or meaningless GO categories were left out. Interestingly, mRNA levels of proteins located in the ER or cell wall were increased, whereas mRNA levels of nuclear and mitochondrial proteins were lowered. Upregulation of the genes encoding cell wall components might be a consequence of RLM1.
and upregulation. In agreement with the results obtained by motif analysis, genes involved in drug transport (PDR motif) were upregulated and genes involved in rRNA processing and ribosome biogenesis were downregulated (rRPE and PAC motifs). Downregulation of Zap1p responsive genes did not lead to a significantly enriched GO category. In summary, the microarray data point to three pathways involved in diclofenac tolerance: the multidrug resistance response, cell wall stress and zinc homeostasis.

**Upregulation of multidrug resistance genes**

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

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

Furthermore, we tested diclofenac sensitivity of strains overexpressing RSB1, PDR5 or SNQ2. Only cells overexpressing PDR5 were more resistant to diclofenac (Fig. 4), whereas overexpression of the other genes had no effect (data not shown). Additionally, although ~10% of wild type cells survive incubating 3 hours with 100 µM diclofenac, less than 2% of the Δpdr5 cells and all PDR5 overexpressing cells survive these conditions. However, in a
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platereader assay, the minimum concentration of diclofenac that completely inhibited growth for 12 hours ranged from approximately 75 µM (Δpdr5) to 150 µM (WT) and 500 µM (PDR5 overexpressing strain) (data not shown). The relative small differences in these concentrations could indicate that Pdr5p does not actively transport diclofenac but rather indirectly affects diclofenac toxicity via an altered membrane composition (Shahi and Moye-Rowley, 2009). Regardless of the exact role of Pdr5p, the growth and survival data at 100 µM diclofenac clearly show the importance of Pdr5p dosage in diclofenac tolerance.

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

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

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

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

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

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

Altered zinc homeostasis during diclofenac exposure

The microarray analysis revealed that Zap1p responsive genes were strongly downregulated (Table 1). Zap1p is a transcription factor that regulates gene expression in response to changes in zinc levels (Herbig et al., 2005). Downregulation of Zap1p and Zap1p-responsive genes suggests that intracellular zinc levels are high in diclofenac-adapted cells. To further explore the role of zinc in diclofenac toxicity, we examined the toxicity of diclofenac in the presence of various zinc concentrations. Addition of 50-500 µM ZnSO4 to the medium one hour before diclofenac addition had no effect on diclofenac toxicity (Fig. 6A, data shown for 50 µm ZnSO4). However, addition of 50 µM of the zinc-chelator 1,10-phenantroline severely increased diclofenac toxicity (Fig. 6B). The increased toxicity was abolished by the addition of ZnSO4, indicating that the chelation of zinc indeed led to the increased toxicity (Fig. 6B). Since the multidrug resistance response regulators Pdr1p and Pdr3p are zinc transcription factors, addition of 1,10-phenantroline might lead to toxicity by preventing the multidrug resistance response. Therefore, we tested the effect of 1,10-phenantroline on PDR5-lacZ expression in the presence of 10 µM diclofenac. This diclofenac concentration is not toxic in combination with 50 µM 1,10-phenantroline. Both in the absence or presence of 1,10-phenantroline, PDR5-lacZ activity was induced ~6 times by 10 µM diclofenac, showing that 1,10-phenantroline does not interfere with the PDR response (data not shown).

Furthermore, we tested the diclofenac sensitivity of a Δzap1 deletion strain. Surprisingly, deletion of Zap1p increased diclofenac toxicity (Fig. 6C). In presence of 100 µM zinc, the diclofenac resistance of Δzap1 cells was increased to the level of wild type cells, showing that the enhanced diclofenac toxicity of Δzap1 strains was caused by a lack of zinc. Accordingly, the addition of 100 µM FeSO4 had no effect on the diclofenac sensitivity of wild type or Δzap1 strains (data not shown). These results point to a pivotal role for zinc in the ability of yeast cells to adapt to diclofenac exposure.
Yeast responses to diclofenac

**Fig. 6.** Diclofenac toxicity is increased under low zinc conditions. Wild type (A,B) or Δzap1 (C) BY4741 cells were incubated with 0 µM (squares) or 100 µM (triangles) diclofenac in minimal medium containing: (A) no additive (closed symbols) or 50 µM ZnSO₄ (open symbols); (B) 50 µM 1,10-phenanthroline (closed symbols) or 50 µM ZnSO₄ and 50 µM 1,10-phenanthroline (open symbols); (C) no additive (closed symbols) or 100 µM ZnSO₄ (open symbols). Data are expressed as OD at 600 nm ± SD.

**DISCUSSION**

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

The major group of upregulated genes in diclofenac-adapted cells consisted of pleiotropic drug resistance genes (Tables 1 and 2). Upregulation of multidrug resistance genes is a common response of yeast to xenobiotics. The resistance of yeast to around ~25% of all compounds tested (Hillenmeyer et al., 2008; Kemmer et al., 2009) is dependent on the upregulation of these genes. Although several multidrug resistance transporters are upregulated after diclofenac exposure, a single ABC transporter, Pdr5p, is crucial in diclofenac resistance (Fig. 3
This may be due either to direct transport of diclofenac by Pdr5p or to secondary effects of Pdr5p deletion or overexpression (Shahi and Moye-Rowley, 2009). Interestingly, a murine homolog of Pdr5p, BCRP1 (ABCG2), can efficiently transport diclofenac in vitro (Lagas et al., 2009). Previously, Mima et al. (2007) found that overexpression of the polyamine transporter TPO1 decreases sensitivity to diclofenac in yeast. However, deletion of TPO1 did not alter diclofenac toxicity in our assay. Since Pdr5p is the main transporter involved in diclofenac resistance, the continuous upregulation of PDR5 is likely to be the main mechanism protecting adapted cells from diclofenac-toxicity.

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

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

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

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

Remarkably, adaptation does not mimic typical weak acid adaptation with upregulation of H^+-ATPases and Msn2p/Msn4p-, Haa1p-, and War1p-regulons (Legras et al., 2010; Mira et al., 2010). Also, although in both yeast (van Leeuwen et al., 2011a) and mammalian cells (Gomez-Lechon et al., 2003) the toxicity of diclofenac is directed primarily toward mitochondria, resulting in increased ROS levels, no oxidative stress response with upregulated superoxide dismutases, peroxiredoxins, and catalases, is apparent. The lack of a mitochondrial retrograde response by upregulation of RTG1-3 indicates that the mitochondria are functional and indeed, we could not find selection for rho^0 strains during adaptation to diclofenac. T-profiler analysis, however, shows a reduced expression of mitochondrial genes, which might contribute to the reduced sensitivity. Apparently oxidative stress, and possibly also weak acid stress, is transient and is involved only in initial diclofenac toxicity. In agreement with this, ROS levels in adapted strains are lower than in freshly treated strains (Fig. 1B). Other cellular changes during adaptation, such as increased Pdr5p expression, may lower diclofenac levels sufficiently to prevent oxidative stress.
In conclusion, we used microarray analysis of diclofenac-adapted strains to identify several processes involved in diclofenac tolerance. By using yeast, we could directly verify the involvement of differentially expressed genes in diclofenac detoxification by applying yeast genetics. Limited transferability of transcriptionally altered genes to genes showing a growth phenotype has been described in many studies (Giaever et al., 2002; Zakrzewska et al., 2010). By using fully adapted strains for transcriptional analysis, we removed initial lethality responses from the results and linked several significantly enriched motif-groups and GO-categories to diclofenac toxicity. Especially, upregulation of multidrug transporter Pdr5p increases resistance to diclofenac. Changes in cell wall composition and zinc homeostasis further contribute to diclofenac tolerance. It remains to be seen whether the discovered importance of zinc for diclofenac tolerance in yeast, discovered here, is relevant for patients with a zinc deficiency (Shoji et al., 1993).

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APPENDIX

Upregulation of the PDR-response by diclofenac is dependent on Pdr1p
As described above, diclofenac severely upregulated the PDR-response in both diclofenac-adapted and freshly treated cells (Table 1, Fig. 3). Especially increased expression of multidrug transporter Pdr5p contributed to resistance to diclofenac (Fig. 4). In yeast, the multidrug resistance response is regulated by transcription factors Pdr1p and Pdr3p (reviewed by Moye-Rowley, 2003). We investigated which of these is involved in upregulation of the PDR-response by diclofenac. WT, Δpdr1, Δpdr3 and Δpdr5 cells were incubated with diclofenac (Fig. S1). Diclofenac-sensitivity of Δpdr3 cells was comparable to that of WT cells. However, Δpdr1 cells showed greatly decreased diclofenac-resistance and were nearly as sensitive to diclofenac as Δpdr5 cells. Additionally, PDR5 promoter activity was greatly lowered in the absence of Pdr1p, both in the absence and presence of diclofenac (Fig. S2). PDR5-lacZ expression was not altered in Δpdr3 cells compared to wild type cells. Other PDR-genes like RSB1 and SNQ2 showed a comparable expression pattern as PDR5, while expression of the control construct TRP5-lacZ was not altered in Δpdr1 or Δpdr3 cells compared to wild type both in presence or absence of diclofenac (not shown). These data strongly suggest that Pdr1p is the main regulator of diclofenac-induced upregulation of PDR5. Also for other xenobiotics, Pdr1p is the main transcription factor involved in upregulation of
The PDR-response (Fardeau et al., 2007). The role of Pdr3p seems to be more focused on upregulation of the PDR-genes in response to mitochondrial deficiencies (Devaux et al., 2002; Hallstrom and Moye-Rowley, 2000).

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

**Fig. S2.** Upregulation of PDR5-lacZ expression is dependent on Pdr1p. PDR5-lacZ expression in BY4741 wild type (black bars), Δpdr1 (grey bars) and Δpdr3 (white bars) cells incubated 3 hours with 0 µM or 30 µM diclofenac in minimal medium. LacZ expression is presented as β-galactosidase activity in units per minute per mg protein ± SD.
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Yeast responses to diclofenac reveal intestinal bacteria-dependent and -independent pathways associated with liver injury. J Pharmacol Exp Ther 327, 634-644.


Chapter 4