Acetaminophen toxicity and resistance in the yeast *Saccharomyces cerevisiae*

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Acetaminophen (paracetamol), one of the most widely used analgesics, is toxic under conditions of overdose or in certain disease conditions, but the mechanism of acetaminophen toxicity is still not entirely understood. To obtain fresh insights into acetaminophen toxicity, this phenomenon was investigated in yeast. Acetaminophen was found to be toxic to yeast cells, with erg mutants displaying hypersensitivity. Yeast cells grown in the presence of acetaminophen were found to accumulate intracellular acetaminophen, but no metabolic products of acetaminophen could be detected in these extracts. The toxicity response did not lead to an oxidative stress response, although it did involve Yap1p. The cytochrome P450 enzymes of yeast, Erg5p and Erg11p, did not appear to participate in this process, unlike the mammalian systems. Furthermore, we could not establish a central role for glutathione depletion or the cellular glutathione redox status in acetaminophen toxicity, suggesting differences from mammalian systems in the pathways causing toxicity. Investigations of the resistance mechanisms revealed that deletion of the glutathione-conjugate pumps Ycf1p (a target of Yap1p) and Bpt1p, surprisingly, led to acetaminophen resistance, while overexpression of the multidrug resistance pumps Snq2p and Flr1p (also targets of Yap1p) led to acetaminophen resistance. The Yap1p-dependent resistance to acetaminophen required a functional Pdr1p or Pdr3p protein, but not a functional Yrr1p. In contrast, resistance mediated by Pdr1p/Pdr3p did not require a functional Yap1p, and revealed a distinct hierarchy in the resistance to acetaminophen.

**INTRODUCTION**

Acetaminophen (N-acetyl-p-aminophenol, 4'-hydroxy-acetanilide, paracetamol) is one of the most widely used analgesics and antipyretics. Although it is generally considered safe at therapeutic doses, in overdose, or in conjunction with liver disease and other disease conditions, acetaminophen displays toxicity, leading to morbidity as well as mortality (Prescott, 1983; Davidson & Eastham, 1966). A great deal of work has gone into investigating the mechanisms by which acetaminophen is toxic (Howie *et al*., 1977; Prescott, 1983; Ray *et al*., 1993; Ruepp *et al*., 2002; Wu *et al*., 2004) and is detoxified in mammalian systems. The major pathway for the removal of acetaminophen appears to be through glucuronidation and sulphation, which make it more water soluble and allow its removal from the liver and the blood via the urine (Jollow *et al*., 1974). A third metabolic pathway involves the oxidation of acetaminophen by microsomal cytochrome P450 to NAPQI (N-acetyl-p-benzoquinone imine), a reactive intermediate. NAPQI appears to be detoxified via the formation of glutathione-conjugates followed by their subsequent excretion, since these conjugates and their degradatory products have been observed in the urine along with a few other oxidation products.

Despite extensive studies of the mechanisms of acetaminophen toxicity, the exact mechanism by which acetaminophen is toxic is surprisingly still controversial. Currently, two major theories have been proposed to explain the cytotoxicity. Although in both hypotheses the first step is the generation of the reactive intermediate NAPQI, the 'glutathione depletion theory' states that an excess of NAPQI (generated from acetaminophen by cytochrome P450) leads to depletion of glutathione, followed by oxidative stress, ultimately leading to cell death. The second theory, the 'covalent binding theory' or the 'macromolecular inhibition theory', considers that the major cause of cell death by acetaminophen is not the result of glutathione depletion per se, but the result of direct binding to macromolecules and inhibition of their function by NAPQI, eventually leading to cell death (Mitchell *et al*., 1973; Dahlin *et al*., 1984; Ruepp *et al*., 2002).

**Abbreviations:** MDR, multidrug resistance protein; MRP, multidrug resistance associated protein; NAPQI, N-acetyl-p-benzoquinone imine; ROS, reactive oxygen species.

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A second issue complicating studies of acetaminophen toxicity is the significant differences seen in the susceptibility of different species and even strains to acetaminophen toxicity (Potter et al., 1974; Hinson, 1980; Ioannides et al., 1983). It is not clear whether the increased drug resistance profiles are due to enhanced/reduced metabolism or other factors, hitherto unconsidered, such as increased efflux. Furthermore, the possible role of multidrug resistance associated proteins (MRPs) in these processes is unclear, although the involvement of MRPs has been indicated by one study (Xiong et al., 2000).

The yeast Saccharomyces cerevisiae is an excellent model system to investigate mechanisms of drug resistance and toxicity at the cellular level. Not only are most of the enzymic and cellular structures conserved, but the maintenance of the redox balance and oxidative stress response is also highly conserved, with glutathione being the major non-protein thiol compound present in yeasts as well as in higher eukaryotes. In addition, the family of drug resistance pumps found in mammalian cells (the multidrug resistance proteins, MDRs, and MRPs) are also present as a family of pumps in yeasts (Decottignies & Goffeau, 1997).

An earlier report investigating the effects of aniline and its metabolites in yeasts also investigated acetaminophen (an aniline derivative), and under the conditions in which it was investigated it was found to be non-toxic (Brennan & Schiestel, 1997). Considering the potential importance of yeast in resolving some of the controversial issues relating to acetaminophen toxicity, such as those described above, we considered it important to reinvestigate the toxicity of acetaminophen more rigorously and compare the mechanisms of toxicity and resistance with those of mammalian cells. We decided to investigate this by initially examining pleiotropically drug-sensitive mutants (certain erg mutants defective in ergosterol biosynthesis). Acetaminophen was found to be toxic in these yeast mutants. This allowed one to examine the possible mechanisms of drug detoxification as well as test the existing models concerning the mechanisms of acetaminophen-induced cell death. The results suggested that acetaminophen toxicity in yeast is not due to the generation of reactive oxygen species (ROS) and is also not dependent on the intracellular glutathione status. Resistance was conferred by the MRPs Snq2p and is also not dependent on the intracellular glutathione and is also not dependent on the intracellular glutathione

**METHODS**

**Chemicals.** The chemicals used were of analytical grade. Media components were purchased from Difco, chemicals from Sigma and Merck. Acetaminophen (4-acetamidophenol), 2-vinylpyridine and 2',7'-dichlorodihydrofluorescein diacetate were obtained from Sigma. Oligonucleotides were purchased from Biobasics Inc. (Canada).

**Strains, strain construction and growth conditions.** The yeast strains used in this study are listed in Table 1. The cells were routinely grown in YPD at 30 °C. For selection of transformants and β-galactosidase assays, minimal medium (SD medium) with supplements was used. Acetaminophen stock solutions were prepared in 30% methanol and the appropriate amounts were added to the media just prior to pouring the plates. Control plates contained equivalent amounts of 30% methanol. The strain ABC681 (snq2Δ) was constructed by PCR-mediated gene disruption of the SNQ2 gene of ABC154 using the KanMX2 module (Wach et al., 1994). The disruption cassette was amplified using the primers SNQ2-DEL1: 5’-AAGGTATTAAGCCATCAAAGGAGCAGCTGAAACCTCGTA-3’ and SNQ2-DEL2: 5’-TTTCTGATTGCCCATCGGTTTCTTTTTATGTAGCTGATG-3’. The disruption of the desired locus was confirmed by PCR using the primers SNQ2-FOR: 5’-GATGGAATGTCCTGACAAAGG-3’ and SNQ2-REV: 5’-CTTGGTCCCATGACACT-3’. The pdr15A strain (ABC668) was also constructed by PCR-mediated gene disruption using the KanMX2 module, using the primers PDR15-D1: 5’-GTCAGAAGTGCTTCTGTGTTAAGAGAGCAGTGATCCAGCTGACTGTAAGC3’ and PDR15-D2: 5’-TAAGGCAGTACAAAGTGGCCTGGTTTTAACCAACACTACGCTTCAT-AGGCGCTAATGTTGAT-3’. Disruptions were confirmed by PCR.

The ABC670 (pdr10A) strain was constructed by first cloning a 1-6 kb BglII–BamHI fragment of PDR10 that was amplified by PCR into pGEM7Z. A 4-5 kb LYS2 fragment containing the LYS2 gene was excised by PstI and cloned into the PstI site of PDR10 in pGEM7Z. A NsiI–SalI digestion of this pPDR10::LYS2 disruption plasmid was excised and used to transform ABC154. Transformants were selected on SD media without lysine, and disruptions were confirmed by PCR.

ABC936, YPH499-GV8-GSH1, a strain which contained an integrated copy of the GSH1 gene expressed downstream of the GPD promoter, was constructed by transforming a linearized DNA fragment obtained by digesting the pGV8-GSH1 plasmid with SacI. pGV8-GSH1 contained the yeast GSH1 gene, which was amplified by PCR and cloned into the HindIII–Bgl II sites downstream of the strong GPD promoter in a pBS306-derived integrating vector. Transformants were selected on plates containing SD media without uracil.

The SKN7 gene was disrupted in the ABC949 (wild-type) strain and ABC950 ( yap1Δ) to yield ABC1041 ( skn7A) and ABC1042 ( skn7A yap1Δ) by transforming a linearized fragment obtained by restriction digestion of an SKN7 disruption plasmid (skn7A:: TRP1) (Brown et al., 1993) with SacI. Disruptions were confirmed by PCR and by t-butylhydroperoxide sensitivity.

**Plasmids.** PDR5 on a multicopy plasmid (PDR5/Yeplac195) has been described earlier (Kaur & Bachhawat, 1999); YAPI on a multicopy plasmid and Yepl351–YAPI were obtained from Dr S. Moye-Rowley; FLR1 on multicopy plasmid p425GPD–FLR1 and the control plasmid (p425 GPD) from Dr M. Raymond; ATR1 on multicopy plasmid Yep74–Sc4018 and the corresponding control plasmid (Yrp74) from Dr K. Struhl; YOR1 on multicopy plasmid YepYRS1 was sent by Dr T. Miyakawa. The plasmid bearing pdr3-9 (hyperactive allele of Pdr3p) was obtained from Dr J. Subik. The plasmid bearing GADYR1* (a gene encoding the hyperactive allele of Yrr1p) was obtained from Dr C. Jacq.

The pSKN7 plasmid was isolated by library screen in the lab. (Sharma et al., 2003), pYC2–LacZ and pBPT–LacZ plasmids have been described previously (Sharma et al., 2002).
Table 1. List of yeast strains used in the study

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<th>Strain</th>
<th>Genotype</th>
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The ERG5 and ERG11 genes were ampliﬁed by PCR using vent DNA polymerase and cloned into the BamH–XhoI sites of the yeast expression vector pTEF-416, a single copy centromeric vector. The primers used for amplification were ERG5-gen-Bam-F (5’-ACA AAA gA A CC ATgAgT TCT gTC gCA gAA AAT ATA-3’) as well as ERG5 gen-Eco-R (5’-AAGACtgATCGTCCtCTCCGTggTCTCTC-3’) for ERG5, and ERG11-gen-Bam-F (5’-ACA Agg gA TCC ATgT TCT gCT gCA gAA AAT ATA-3’) as well as ERG11-gen-R (5’-TTA CAA gAA TCC ACC ACC TTA gAT CIT TTg TgC Tgg AT-3’) for ERG11. The genes were cloned downstream of the strong and constitutive TEF promoter and conﬁrmed by sequencing.

Growth experiments. Cells from overnight cultures of strains ABC154, ABC936 and ABC591 were reinoculated to an OD600 of 0–1 (2 × 10⁶ cells ml⁻¹) and allowed to grow to an OD600 of 0–5 to 0–6, and, at this stage, drug was introduced (16 mg ml⁻¹). In control experiments, an equivalent amount of 30% methanol solution was added. Growth of these cultures was followed by measuring OD600 values at diﬀerent time points (at 3 h intervals). At 12 and 24 h time points, a known number of cells was plated on YPD plates to check the number of viable cells.

Drug sensitivity experiments. Strains were transformed with the plasmids, and the transformants were grown in SD media with appropriate selection until they reached exponential phase, and then equal numbers of cells were harvested and resuspended in sterile water to a density of 1 × 10⁷ cells ml⁻¹. Portions (10 µl) of undiluted cell suspension, 1:10, 1:100 and 1:1000 dilutions were then spotted onto YPD plates containing diﬀerent concentrations of acetonophen. Growth was observed after 2 to 4 days at 30°C.

Glutathione estimation. The overnight cultures of strains ABC154, ABC591 (gsh1Δ) and ABC936 (strain overexpressing GSH1 gene) were reinoculated at OD600 = 0–1 and acetonophen was added when the OD600 reached 0–5–0–6. Glutathione estimation was carried out using the DTNB-glutathione reductase assay (Anderson & Meister, 1983) at different time points, as described earlier (Sharma et al., 2000). Oxidized glutathione levels were measured by using 2-vinyl pyridine to block the reduced glutathione (Anderson & Meister, 1983).

Induction conditions and β-galactosidase assays. ABC154 was transformed with plasmids pYCg-LacZ and pBPT-LacZ, and the
transformants were assayed for β-galactosidase in the presence or absence of acetaminophen (14 mg ml⁻¹). β-Galactosidase assays were carried out on permeabilized cells, as previously described (Guarente, 1983; Sharma et al., 2002).

Detection of intracellular acetaminophen. Cells of strain ABC154 were inoculated in YPD media and allowed to grow overnight, reinoculated at OD₆₀₀ = 0.2 and allowed to grow for 3–4 h. To this growing culture, acetaminophen was added at 8 mg ml⁻¹, and the incubation continued for 4–6 h. The cells from this culture were harvested, washed thoroughly (twice) with sterile distilled water, and lysed using 5% sulfosalicyclic acid and glass beads. The lysate was centrifuged at 5000 r.p.m. for 5 min to settle the unbroken cells and the cell debris. The supernatant was mixed with two volumes of ethyl acetate and vortexed vigorously for 1 min, the mixture was allowed to settle and the ethyl acetate layer was separated. The aqueous and ethyl acetate extract fractions were lyophilized, and the residues redissolved in small amounts of water and ethyl acetate, respectively. An aliquot of each of the ethyl acetate and aqueous extracts was subjected to mass spectroscopy (100–600 a.m.u.) through direct infusion under positive atmospheric pressure chemical ionization (APCI). A separate aliquot of the ethyl acetate fraction was used for LC-MS experiments (100–600 a.m.u., positive APCI) using a PDA detector.

Fluorescence assays using 2',7'-dichlorodihydrofluorescein diacetate. Overnight cultures of ABC154 (wild-type strain) and ABC681 (snq2Δ strain) were each subcultured into eight different flasks with 5 ml fresh medium at a concentration of 0.5 × 10⁴ cells ml⁻¹ and incubated at 30 °C at 200 r.p.m. for 2 h. The test chemicals H₂O₂ and acetaminophen (final concentrations of 4 mg ml⁻¹ and 18 mg ml⁻¹, respectively) were added to two flasks each of the wild-type and snq2Δ strain. The cultures were incubated for about 1 h and then 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) was added from a fresh 5 mM stock (prepared in ethanol) to a final concentration of 10 µM and the incubation was continued for a further 2 h. This permitted deacetylation of the dye and rendered it susceptible to oxidation in the presence of any ROS. About 1:5 ml of sample was removed from each of the above cultures and cells were harvested by centrifugation, washed twice with sterile water and resuspended in 100 µl 50 mM Tris/HCl buffer (pH 7.5). The cells were permeabilized by adding 50 µl chloroform and 20 µl 0-1% SDS and by vortexing at high speed for 20 s. The tubes were left to stand for 10 min to allow the dye to diffuse into the buffer. Cells were pelleted in a microcentrifuge and the fluorescence of the supernatant was measured using a Shimadzu fluorimeter (excitation, 502; emission, 521).

RESULTS

Yeast strains undergo acetaminophen-induced cell death in the presence of acetaminophen, with erg strains displaying hypersensitivity to acetaminophen

Although an earlier report had indicated a lack of acetaminophen toxicity in yeast (Brennan & Schiestel, 1997), we decided to re-examine the issue by initially using erg mutants, which display an increased sensitivity to a wide variety of drugs. Earlier studies have shown that mutants in the ergosterol biosynthetic pathway, particularly those disrupted in the latter half of the pathway, display an increased drug sensitivity which arises both from an increased influx of the drugs through the membranes, which are more permeable, and from decreased efflux by membrane pumps, which function less efficiently in an altered membrane environment (Kaur & Bachhawat, 1999).

As seen in Fig. 1, the erg2Δ and erg6Δ mutants in fact displayed a significant acetaminophen sensitivity. The erg3Δ and erg4Δ mutants showed slightly less sensitivity than the erg2Δ and erg6Δ mutants, but they also showed an increased sensitivity to the drug compared to the wild-type strains.

These observations prompted us to examine the issue of acetaminophen toxicity further and to examine the nature of the inhibition of growth by acetaminophen. Examination of different wild-type strains of S. cerevisiae in different genetic backgrounds revealed that wild-type strains were also sensitive to acetaminophen, although at increased concentrations, but the sensitivities differed in different backgrounds. We subsequently focused on the wild-type YPH499 (S288C background). We determined whether the growth inhibition observed in these strains was a consequence of growth stasis or of cell death. Cells were grown in YPD medium and acetaminophen was added to exponentially growing cells. At 24 h intervals, aliquots were plated to check for cell viability. After 72 h of growth in the presence of acetaminophen, there was a significant drop in cell viability, and the cells failed to recover even after removal of drug (data not shown). This was similar to the cell death observed in mammalian cells, and it further
suggested that we could use yeasts to examine the mechanism of acetaminophen-induced cell death.

Identification of intracellular acetaminophen (but no other metabolites of acetaminophen) in yeast cells grown in the presence of the drug

To determine if the acetaminophen-induced cell death was a result of acetaminophen or some other metabolite accumulating intracellularly, it was necessary to establish the accumulation of acetaminophen (or its metabolites) within the cell.

Whole-cell lysates of cells grown in the presence of acetaminophen were extracted with ethyl acetate (Methods) and the ethyl acetate and aqueous fractions were subjected to direct infusion mass spectroscopy. In each occasion, the peak at m/z 152 in the respective mass chromatogram indicated the presence of acetaminophen. However, the mass chromatogram did not reveal any new peaks of significant intensity. In order to investigate the accumulation of the drug inside the cell and to examine the possibility of any new peaks we further carried out LC-MS studies of the ethyl acetate fraction using a PDA detector. A major component with a retention time comparable to that of standard acetaminophen exhibited a peak at m/z 152 in the MS, revealing the presence of the drug in the ethyl acetate extract. The LC-MS did not reveal the presence of any new peak relative to the control, suggesting that the principal compound accumulating in these cells was acetaminophen and that no other transformed products of this drug were being generated. However, the possibility that other metabolic products (such as NAPQI) were being formed and rapidly removed from the cell, or conjugated to proteins preventing their extraction, still existed.

Overexpression of yeast-cytochrome-P450-encoding Erg5p (C22 sterol desaturase) and Erg11p (lanosterol demethylase) does not alter the acetaminophen resistance profiles in yeast

The inability to detect any other metabolites of acetaminophen suggested that acetaminophen was exerting its toxicity independently of a biotransformation step. This was in apparent contrast to mammalian cells, in which the activation of acetaminophen to the reactive intermediate has been shown to be dependent on the presence of specific cytochrome P450 enzymes. NAPQI is a very short-lived intermediate and, in the studies with mammalian cells, only 1% of the acetaminophen is converted into NAPQI through cytochrome P450. The possible involvement of the yeast cytochrome P450s in toxicity needed more thorough investigation. S. cerevisiae has three P450 enzymes which play important metabolic roles in the cell. Erg5p (Skaggs et al., 1996) and Erg11p (Aoyama et al., 1981) are involved in ergosterol biosynthesis, and homologues of these proteins are widely distributed in other yeasts as well. The third protein, Dtt2p, is involved in the spore wall formation of S. cerevisiae, and is unique to S. cerevisiae (Briza et al., 1990). Among these different P450 enzymes in yeast, only Erg5p has been implicated in also contributing to the detoxification pathway of some metabolites. To examine the possible role of Erg5p and Erg11p in the toxicity of (or resistance to) acetaminophen, we cloned and overexpressed these genes from a strong constitutive promoter. Both Erg5p and Erg11p overexpression could confer increased resistance to fluconazole, but we could not find any increased sensitivity or resistance to acetaminophen upon either Erg5p or Erg11p overexpression (data not shown).

Acetaminophen toxicity in yeast: absence of a role for glutathione

The inability to detect any intracellular metabolites other than acetaminophen in acetaminophen-treated cells and the lack of involvement of the yeast cytochrome P450s strongly suggested a toxicity mechanism that differed from the primary mechanism of toxicity observed in mammalian cells, in which reactive metabolites are generated through the action of specific cytochrome P450s. We decided to examine more rigorously whether the yeast cells were in fact subjected to an oxidative stress response in the presence of acetaminophen, and also if the glutathione status of the cell was important in the cellular response to acetaminophen.

We decided to initially examine this using 2’7’-dichlorodihydrofluorescein diacetate, a fluorogenic compound which has been used by several workers as a marker for oxidative stress and which is suggested to reflect the overall oxidative stress status of the cells, although its use as a marker of overall oxidative stress is still controversial. Experiments were carried out as described in Methods. Cells exposed to H$_2$O$_2$ displayed a significant increase in fluorescence intensity, but no increase in fluorescence intensity was observed when cells were treated with acetaminophen concentrations from 4 to 18 mg ml$^{-1}$ for a period of 1 to 2 h (Fig. 2). This confirmed that the cells were not being subjected to oxidative stress. However, the limitations of the assay in being responsive to, and therefore suitable for, only some oxidants (Myhre et al., 2003; Chignell & Sik, 2003) prompted us to investigate more carefully the role of glutathione, since glutathione depletion has been implicated in the acetaminophen toxicity of mammalian cells.

To examine if glutathione depletion played a crucial role in acetaminophen toxicity in yeast we constructed strains that had either depleted or elevated levels of glutathione. The strains with depleted levels were the result of the deletion of GSH1, the gene for the first enzyme in glutathione biosynthesis. These cells take up glutathione from the external medium through specific glutathione transporters (Bourbouloux et al., 2000), but the levels of intracellular glutathione rarely reach beyond 50% of the wild-type levels of glutathione (Sharma et al., 2000). The strains overproducing glutathione were constructed by integrating a copy of GSH1 that we had expressed.
downstream of the strong constitutive TEF promoter. The levels of GSH in these latter strains in YPD medium were threefold higher than wild-type levels (data not shown). We grew these cells in YPD medium, and at an OD$_{600}$ of about 0.5 to 0.6 we added acetaminophen to the cells and followed further growth. At 3 h intervals, we took aliquots to monitor the growth and in addition to monitor the glutathione levels of the different cells. In contrast to what we expected, irrespective of the intracellular glutathione content of the cells, there was no difference in the growth inhibition induced by acetaminophen. Furthermore, drug-treated cells did not show any significant decrease in

**Fig. 2.** Acetaminophen response detected by 2′,7′-dichlorodihydrofluorescein diacetate assay. Fluorescence of the cell extracts from strains ABC154 (wild-type) and ABC681 (snq2Δ) following 1 h exposure to H$_2$O$_2$ (bars 2 and 6) or different concentrations of acetaminophen (4 mg ml$^{-1}$, bars 3 and 7; 18 mg ml$^{-1}$, bars 4 and 8) in the presence of 2′,7′-dichlorodihydrofluorescein diacetate. The bars represent the fluorescence intensity observed at 521 nm in arbitrary units (A.U.). Each experiment was done at least in duplicate (details in Methods). WT, Wild-type.

**Fig. 3.** Effect of glutathione depletion on acetaminophen toxicity. (a) The strains ABC154 (wild-type) and ABC591 (gsh1Δ), were grown in YPD for 10 to 12 h and re-inoculated to OD$_{600}$=0.1. These cultures were allowed to grow to OD$_{600}$=0.5–0.6 and then acetaminophen was added at 16 mg ml$^{-1}$. ▲ and ■, ABC154 strain; × and •, gsh1Δ strain. (b) Effect of acetaminophen treatment on intracellular GSH levels. Intracellular GSH estimation was carried out as described in Methods. Bars represent intracellular GSH levels (nmol) of wild-type (white bars) and gsh1Δ cells (black bars). The various time points of the determination are indicated on the tops of the bars. +, Drug-treated samples; −, control samples without drug. The figure is representative of three independent experiments. WT, Wild-type.
glutathione levels compared to untreated cells (Fig. 3), providing further evidence against depletion of glutathione levels as the primary cause of the toxicity of the drug.

To examine if the glutathione redox status of the yeast cells might be important, we further examined the response of yeast cells disrupted for glutathione reductase (glr1Δ) to acetaminophen. Although the absence of glutathione reductase is not lethal for *S. cerevisiae*, such cells are much more sensitive to the presence of drugs that generate an oxidative stress response, owing to the elevated GSSG/GSH ratio (Grant et al., 1996). However, the glr1Δ cells failed to show an enhanced sensitivity to acetaminophen. Furthermore, acetaminophen-treated cells also failed to show an alteration in GSSG/GSH ratio compared to untreated cells (data not shown), clearly underlining firstly the fact that acetaminophen was not inducing an oxidative stress response in yeast, unlike the response observed in mammalian cells, and secondly that neither glutathione depletion nor the glutathione redox status was involved in the toxicity of acetaminophen.

**Yeast cell response to acetaminophen: role of Yap1p in resistance to acetaminophen**

Yap1p is a transcription factor known to play a central role in the oxidative stress response of yeast (Moye-Rowley et al., 1988) as well as in the response to several drugs that generate an oxidative stress response.

Acetaminophen toxicity was initially examined in strains deleted for *YAP1*. Our results clearly indicated that yap1Δ strains displayed an increased sensitivity to acetaminophen (Fig. 4a). Furthermore, overexpression of Yap1p in wild-type cells conferred increased resistance to acetaminophen (Fig. 4b), confirming the role of Yap1p in the response to cellular injury by acetaminophen. We also investigated if Skn7p, a second transcription factor also implicated in the cellular oxidative stress response (Morgan et al., 1997), might also be involved in the response to acetaminophen. However, neither the deletion of *SKN7* nor the overexpression of *SKN7* from a multicopy plasmid led to any discernable phenotype in the presence of acetaminophen (Fig. 4a, b). A deletion of *SKN7* in a yap1Δ background was also constructed to see if the phenotypes of an *skn7Δ* deletion might be seen in this background. However, no further increase in acetaminophen sensitivity was observed in the *skn7Δ* yap1Δ strains compared to yap1Δ strains. This indicated that, of the two oxidative-stress-responsive transcription factors, only Yap1p played a role in the response to acetaminophen. Although it has been widely used as an indicator of oxidative stress response in yeasts, recent studies have indicated that there are two independent mechanisms of Yap1p activation, one dependent on oxidative free radicals, and another which acts at an independent site of Yap1p which is activated by electrophiles (Azvedo et al., 2003). The lack of ROS suggested that the acetaminophen response of Yap1p was occurring through the latter mechanism. To further confirm this, we examined the effects of *gpx3Δ (orp1Δ)* on acetaminophen sensitivity. The oxidative response of Yap1p has been shown to be dependent on the presence of Gpx3p (Delaunay et al., 2002). We could not observe any effect of the presence of Gpx3p on the sensitivity to acetaminophen, further underlining that Yap1p was not functioning through this pathway and that it was being activated through an electrophilic compound which was not dependent on an oxidative stress response.

**Disruption of the yeast glutathione-conjugate pumps Ycf1p and Bpt1p leads to acetaminophen resistance**

The Yap1p transcriptional activator is known to activate a very large number of genes. Among these is a gene directly
implicated in the glutathione detoxification pathways, the yeast glutathione-conjugate pump Ycf1p.

Overexpression of Yap1p leads to a greater than 10-fold induction of YCF1 (Wemmie et al., 1994). We therefore decided to examine the role played by the yeast glutathione-conjugate pumps Ycf1p and Bpt1p. Bpt1p is a close homologue of Ycf1p, which has recently been also shown to function as a glutathione-conjugate pump but is not regulated by Yap1p (Sharma et al., 2002; Klein et al., 2002; Chaudhuri et al., 1997). These pumps have also been shown to transport unconjugated compounds (Pascolo et al., 2001; Petrovic et al., 2000). However, in contrast to what we expected, we observed that deletion of both YCF1 and BPT1 led to an increase in resistance to acetaminophen (Fig. 5). This resistance to acetaminophen was observed in a very narrow range of drug concentrations. The results were unexpected and were also in apparent conflict with the fact that Ycf1p is upregulated by Yap1p, as well as the observation, described above, that Yap1p leads to increased resistance to acetaminophen. To examine how acetaminophen affected the induction of YCF1 and BPT1, we checked the expression pattern of YCF1 and BPT1 using promoter–LacZ fusions in the presence of acetaminophen. Only YCF1 (and not BPT1 or the other members of the group) is known to be induced by Yap1p (Wemmie et al., 1994; Sharma et al., 2002, 2003). However, in the presence of acetaminophen we observed only a negligible (1.5-fold) increase in β-galactosidase activity in both YCF1 and BPT1 (Table 2). These results indicate that, although Yap1p does play a role in resistance to acetaminophen, the response of YCF1 (a target of Yap1p) might be influenced by other unknown regulatory factors in addition to Yap1p. Furthermore, it suggests that the H2O2-activated Yap1p and the thiol-compound-activated Yap1p show differential activation responses. The increased resistance of ycf1Δ bpt1Δ strains to acetaminophen, though small, possibly suggests some involvement of Ycf1p and Bpt1p in the process, although the exact manner in which this might be occurring is not clear. We also overexpressed the YCF1 gene from a multicopy plasmid, but no phenotypes on acetaminophen-containing plates could be discerned upon overexpression of YCF1 (data not shown).

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<th>Table 2. Induction of YCF1 and BPT1 in response to acetaminophen</th>
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**Role of the multidrug resistance pumps in acetaminophen resistance**

Although yap1Δ strains did possess an increased sensitivity to acetaminophen and Yap1p overexpression led to an increase in acetaminophen resistance, it did not appear that Yap1p-mediated resistance was dependent on the enzymes related to glutathione-mediated detoxification pathways. We therefore sought to examine if Yap1p might be acting through a completely different set of targets. Yap1p is also known to act on Snq2p, an ABC transporter involved in multidrug resistance (Sevos et al., 1993), as well as on other MRPs (DeRisi et al., 1997). Although work with mammalian cells has not implicated MRPs in acetaminophen resistance/sensitivity, we decided to examine the involvement of such proteins in yeast. We examined different strains deleted in the different ABC transporters to see if they might be involved in mediating drug resistance to acetaminophen. Pdr5p as well as Snq2p have been shown to mediate drug resistance to a number of different compounds (Balzi et al., 1994; Balzi & Goffeau, 1995; Decottignies et al., 1995; Decottignies & Goffeau, 1997). Close homologues of Pdr5p are Pdr10p and Pdr15p, but these proteins have not been demonstrated to confer resistance to drugs effluxed by Pdr5p. We nevertheless examined strains deleted in pdr5, pdr10, pdr15 as well as snq2 for their acetaminophen sensitivity. Only snq2Δ strains displayed a dramatic increase in acetaminophen sensitivity (Fig. 6a). This was further confirmed by Snq2p overexpression (Fig. 6b). We also examined the phenotypes of Pdr5p overexpression, as well as the overexpression of another ABC transporter, Yor1p, which belonged to the family of the YCF1 cluster of proteins. However, neither
Pdr5p overexpression nor Yor1p overexpression conferred any resistance to acetaminophen.

Although Snq2p clearly appeared to be the major pump involved in resistance to acetaminophen, it was of interest to examine if other pumps might also be involved. This was also prompted by our observation that when Yap1p was overexpressed in snq2Δ strains we still observed a small increase in drug resistance, even in this (snq2Δ) background (Fig. 7). This indicated that additional targets of Yap1p might be involved in efflux of the drug. In earlier studies, including a genome-wide analysis of genes induced by Yap1p, two other multidrug resistance pumps were observed to be targets of Yap1p (Alarco et al., 1997; DeRisi et al., 1997). These were Flr1p (Broco et al., 1999) and Atr1p, an aminotriazole resistance protein (Kanazawa et al., 1988).

We therefore sought to examine if these pumps might also contribute to resistance to acetaminophen. These genes were transformed into wild-type yeast strains on multicopy plasmids and the transformants checked for resistance to acetaminophen. Flr1p clearly contributed to acetaminophen resistance (Fig. 8), although Atr1p did not seem to play any role in acetaminophen resistance (data not shown). It thus appeared that Yap1p was possibly mediating its effects on acetaminophen resistance through the multidrug resistance pumps Snq2p and Flr1p.

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**Fig. 6.** The multidrug resistance pump Snq2p plays a role in acetaminophen resistance. (a) Wild-type strain ABC154 (WT) and deletion mutants for the MDR pumps snq2Δ (ABC681), pdr5Δ (ABC152), pdr15Δ (ABC668) and pdr10Δ (ABC670) were streaked on YPD control plates and YPD plates with acetaminophen (4 mg ml⁻¹, as indicated above). (b) ABC154 strain was transformed with control plasmid pRS426, and multicopy plasmids bearing either YOR1 (Yep-YRS1), SNO2 or PDR5 (PDR5/Yeplac195). The transformants were grown to exponential phase in SD media lacking uracil at 30°C and spotted on YPD or YPD plus acetaminophen (18 mg ml⁻¹) plates (Methods).

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**Fig. 7.** YAP1 overexpression confers resistance to acetaminophen in an snq2Δ background. The strain snq2Δ (ABC681) was transformed with plasmid Yep351-YAP1 (a multicopy plasmid harbouring the YAP1 gene). The transformants were grown in SD media lacking uracil and spotting was performed on YPD and YPD plus acetaminophen (10 mg ml⁻¹) plates as indicated in Methods.

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**Fig. 8.** FLR1 overproduction confers resistance to acetaminophen. ABC164 strain and snq2Δ (ABC681) were transformed with plasmids bearing the FLR1 gene (p425GPD-FLR1) and an empty plasmid (p425GPD). The transformants were spotted on acetaminophen (10 and 16 mg ml⁻¹) and control YPD plates as indicated in Methods.
The Yap1p response to acetaminophen is dependent on the presence of a functional Pdr1p or Pdr3p protein

Pdr1p and Pdr3p are among the primary regulators of pleiotropic drug resistance in yeast (Mamnun et al., 2002), and their targets include the multidrug efflux protein Snq2p, which is primarily responsible for acetaminophen resistance in yeast. A second transcription factor that has also been involved is Yrr1p. We therefore investigated if the Yap1p-mediated resistance to acetaminophen might be mediated through Pdr1p, Pdr3p or Yrr1p, or was functioning independently of these proteins. We observed that, while the pdr1Δ and yrr1Δ strains showed an increased sensitivity to acetaminophen, pdr3Δ strains displayed no increase in sensitivity. To determine if the Yap1p response was dependent on the presence of either of these proteins, we overexpressed Yap1p in wild-type strains as well as in the pdr1Δ, pdr3Δ and pdr1Δ pdr3Δ strains, and in yrr1Δ strains. Interestingly, while Yap1p overexpression continued to confer resistance to acetaminophen in a pdr1Δ as well as in a yrr1Δ background (Fig. 9), it failed to do so in a pdr1Δ pdr3Δ background. Yap1p-mediated resistance to acetaminophen was therefore dependent on the presence of either Pdr1p or Pdr3p, despite the fact that the absence of Pdr3p did not affect the acetaminophen resistance profile.

To determine if the activity of Pdr1p/Pdr3p or Yrr1p might be dependent on a functional Yap1p we expressed hyperactive alleles of Pdr3p (encoded by pdr3−9) (Kozovska

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**Fig. 9.** Yap1p-mediated resistance to acetaminophen requires Pdr1p or Pdr3p, while the resistance caused by Pdr3-9p is independent of Yap1p. (a) Yeast strains ABC949 (wt), ABC1374 (pdr1Δ), ABC1375 (pdr3Δ) and ABC1376 (pdr1Δ pdr3Δ) were transformed with plasmids overexpressing YAP1 and the corresponding empty vector. The transformants were picked and grown in the same selection media and then spotted on to YPD plates bearing different concentrations of acetaminophen, as described in Methods. (b) ABC949 (wt) and ABC950 (yap1Δ) were transformed with plasmids overexpressing YRR1, pdr3-9 and p416. The transformants were grown in minimal media and spotted on YPD plates containing different concentrations of acetaminophen. (c) ABC1376 (pdr1Δ pdr3Δ) and ABC1304 (yrr1Δ) strains were transformed with plasmids overexpressing YRM1, pdr3-9 and p416 (empty vector). The transformants were picked and grown in the same selection media and then spotted on to YPD plates bearing different concentrations of acetaminophen, as described in Methods.
et al., 2001) and Yrr1p (encoded by GAD–YRR1*) (Le Crom et al., 2002) in yap1Δ as well as yrr1Δ and pdr1Δ pdr3Δ backgrounds. We observed that pdr3–9 could confer resistance in a yap1Δ as well as a yrr1Δ background (Fig. 9). In contrast, GAD–YRR1 overexpression, while restoring resistance to the increased sensitivity of yrr1Δ strains, could not confer any resistance in either a yap1Δ or a pdr1Δ pdr3Δ background. In the case of acetaminophen resistance, therefore, the resistance is determined by a hierarchy of transcription factors, and appears to be distinct from the existing patterns of hierarchy for other reported drugs and targets.

**DISCUSSION**

In the present paper, we have examined acetaminophen toxicity in yeasts to see if it can shed light on the mechanisms of acetaminophen toxicity observed in mammalian cells. Considering the extensive use of this over-the-counter drug worldwide, any information on the mode of toxicity would be of crucial value in the judicious administration of this drug, especially in the presence of certain disease conditions (Prescott, 1983; Herzenberg et al., 1997).

The study clearly demonstrated the toxicity of acetaminophen in yeasts, although it became apparent only at higher concentrations of the drug or in ergosterol biosynthetic mutants.

Unlike mammalian cells, however, acetaminophen failed to induce an oxidative stress response in yeasts. The studies clearly show the involvement of Yap1p in acetaminophen resistance. However, Yap1p can be activated either by ROS or by electrophiles (Azevedo et al., 2003), and in the case of acetaminophen it appears that it is the generation of electrophiles, but not ROS, that is activating Yap1p. Acetaminophen itself is not considered an electrophile, while NAPQ1, a metabolic product of acetaminophen, is an electrophile. The inability to detect any metabolites of acetaminophen probably explains the relative lack of toxicity of this compound in yeast. Since the toxicity was also not enhanced by increasing the levels of the two cytochrome P450s (Erg5p and Erg11p), there are two possible explanations for the toxicity of acetaminophen in yeast:

(i) acetaminophen itself (independent of its activation to NAPQ1) can act as a weak electrophile;

(ii) the activation of acetaminophen occurs at exceedingly low levels in yeast, by a mechanism independent of the cytochrome P450s.

Both possibilities are intriguing, since they have not been considered in mammalian cells, and in the light of the results described here, these possibilities need to be seriously examined in mammalian cells too.

The experiments designed to evaluate the role of glutathione depletion or glutathione redox status on acetaminophen toxicity clearly argue against a role for glutathione depletion per se being the causative agent in acetaminophen toxicity.

Deletion of the genes for the glutathione conjugate pumps YCF1 and BPT1 surprisingly led to resistance to acetaminophen. This was an unexpected observation, since Ycf1p levels are actually enhanced by Yap1p. While a possible explanation is that the GSH conjugates in this case are more toxic, as has been suggested for some drugs (Monks & Lau, 1998), an alternative explanation is that accumulation of toxic intermediates and other cellular metabolic intermediates in a ycf1Δ bpt1Δ deletion strain may be causing a feedback inhibition of the enzymes responsible for the production of the toxic intermediate.

One of the surprising observations that was made in this study is that acetaminophen could be effluxed by the yeast multidrug resistance pump Snq2p (and to a lesser extent by Ffr1p). In addition to the relative lack of formation of reactive acetaminophen metabolites (such as NAPQI), the efflux of acetaminophen by multidrug resistance transporters might be a second reason for the relative lack of toxicity of these drugs to wild-type yeasts and the consequent toxicity of the drug only at elevated concentrations. Furthermore, the findings would suggest that one should examine the role of these pumps in mammalian cells more carefully. Although the relative difference in tissue and species specificity of the effects of acetaminophen have been attributed to differences in the metabolism of the drug, the possibility that differences in direct drug efflux are a cause also needs to be examined more carefully.

Interestingly, in addition to YAPI, deletions in YRR1 and PDR1 led to an increased sensitivity to acetaminophen, indicating the involvement of the drug-resistance regulatory network in acetaminophen resistance. While the Yap1p response required a functional Pdr1p or Pdr3p protein, the resistance conferred by Pdr1p/Pdr3p as seen through a hyperactive pdr3–9 allele could occur independently of either Yap1p or Yrr1p, suggesting a hierarchy of these transcription factors in the resistance to acetaminophen. A link between Yap1p and Pdr1p/Pdr3p has previously been shown for diazaborine resistance (Wendler et al., 1997; Jungwirth et al., 2000) and for benomyl resistance (Tenreiro et al., 2001). In the case of diazaborine resistance it was observed that the resistance due to Yap1p was dependent on a functional Pdr1p or Pdr3p protein, but in this case the pumps conferring resistance were Ycf1p and Ffr1p. More recently, it has been shown that the pdr3–33 mutation (a gain of function allele of PDR3) could specifically mediate resistance to diazaborine through Snq2p and Pdr3p, while a pdr1–12 mutant (a gain of function of PDR1) mediated resistance to the same drug through Ycf1p and Ffr1p (Wehrschatz-Sigl et al., 2004). In the case of benomyl resistance, Ffr1p appeared to be the primary pump involved in resistance, and was dependent on Yap1p and partially on a functional Pdr1p or Pdr3p (Tenreiro et al., 2001). Our investigations, while describing a quite different hierarchy in the resistance to acetaminophen, has
also opened up several other interesting issues and considerations on the toxicity of acetaminophen in relation to the pathways and networks that mediate resistance.

In conclusion, our studies investigating acetaminophen toxicity in yeast have demonstrated that acetaminophen can exert its toxicity in these unicellular eukaryotes by mechanisms quite distinct from those otherwise observed and described in mammalian systems. The possibility that toxic effects of acetaminophen through these pathways might also be operating (at a secondary level, perhaps) in mammalian cells thus needs to be examined, especially so in the light of the drug’s wide usage. The studies described here also throw light on important aspects of the resistance to the drug in yeast which might also help in resolving some of the conflicting issues regarding the toxicity in humans of this widely used drug.

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