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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′-hydroxyacetanilide, 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.

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 Flr1p and was mediated by Yap1p, Yrr1p and Pdr1p/Pdr3p. The hierarchy of these factors in the resistance to acetaminophen was also determined and found to be distinct from the existing knowledge about their hierarchies. Together, these findings demonstrate that acetaminophen can exert its toxicity at the cellular level by pathways completely different from those considered previously. The implications of these studies for mammalian cells are also presented.

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). Restriction enzymes were obtained from New England Biolabs.

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 media) 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 (*snq2*Δ) 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′-AAGGTATTAAGGCTAAGAGGCATCAAAAGATGAGAC-AGCTGAAGCTTCGTA-3′ and *SNQ2*-DEL2: 5′-TTTCGAATTC-CTCAGCGGTTCTTGGTACTTTATTTTCATAGGCCACTAGTGGA-TC-3′. The disruption of the desired locus was confirmed by PCR using the primers *SNQ2*-FOR: 5′-GATGCGAGTGCCCTAGAA-GG-3′ and *SNQ2*-REV: 5′-CTTGTTCCCAATATGACACT-3′. The *pdr15*Δ strain (ABC668) was also constructed by PCR-mediated gene disruption using the KanMX2 module, using the primers *PDR15*-D1: 5′-GTCAGAGGTGTTTCTGGTGGTGAAAGAAAGCG-TGTATCCAGCTGAAGCTTCGTACGC-3′ and *PDR15*-D2: 5′-TA-AGGCAGTCAAAGTGCCTGGTTTTACCCAACCATCTACGTTCAT-AGGCCACTAGTGGAT-3′. Disruptions were confirmed by PCR. The ABC670 (*pdr10*Δ) strain was constructed by first cloning a 1·6 kb *Bgl*II–*Bam*HI fragment of *PDR10* that was amplified by PCR into pGEM7Z. A 4·5 kb *LYS2* fragment containing the *LYS2* gene was excised by *Pst*I and cloned into the *Pst*I site of *PDR10* in pGEM7Z. A *Nsi*I–*Sca*I digestion of this p*PDR10*::*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 *Stu*I. pGV8-*GSH1* contained the yeast *GSH1* gene, which was amplified by PCR and cloned into the *Hin*dIII–*Bsp* DI sites downstream of the strong GPD promoter in a pRS306-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 (*yap1*Δ) to yield ABC1041 (*skn7*Δ) and ABC1042 (*skn7*Δ *yap1*Δ) by transforming a linearized fragment obtained by restriction digestion of an *SKN7* disruption plasmid (*skn7*Δ::*TRP1*) (Brown *et al.*, 1993) with *Sac*I. 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); *YAP1* on a multicopy plasmid and YEp351-*YAP1* 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 Yrp74-Sc4018 and the corresponding control plasmid (Yrp74) from Dr K. Struhl; *YOR1* on multicopy plasmid YEp*YRS1* was sent by Dr T. Miyakawa. The plasmid bearing *pdr3-9* (hyperactive allele of Pdr3p) was obtained from Dr J. Subik. The plasmid bearing *GADYRR1** (a gene encoding the hyperactive allele of Yrr1p) was obtained from Dr C. Jacq.

The p*SKN7* plasmid was isolated by library screen in the lab. (Sharma *et al.*, 2003). pYCF-LacZ and pBPT-LacZ plasmids have been described previously (Sharma *et al.*, 2002).

Table 1. List of yeast strains used in the study

Strain	Genotype	Source
ABC152 (YKKA)	Mat*a ura3-52α leu2-Δ1 his3-Δ200lys2-801 PDR5*::*TRP1*	K. Kuchler
ABC154 (YPH499)	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801*	K. Kuchler
ABC229	Mat*a ura3-52 leu2Δ1 his3-Δ200 erg2Δ*::*LEU2*	Lab. strain
ABC230	Mat*a ura3-52 leu2Δ1 his3-Δ200 erg4Δ*::*LEU2*	Lab. strain
ABC231	Mat*a ura3-52 leu2Δ1 his3-Δ200 erg6Δ*::*LEU2*	Lab. strain
ABC470	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801 ycf1Δ*::KanMX2	Lab. strain
ABC557	Mat*a ura3-52 leu2Δ1 lys2-801 his3-Δ200 erg3Δ*::*LEU2*	Lab. strain
ABC582	Mat*α ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 Lys2-801 gtt1*::*KanMX2 gtt2*::*KanMX2*	Lab. strain
ABC583	Mat*α ura3-52α leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 Lys2-801 gtt2*::*KanMX2*	Lab. strain
ABC584	Mat*a ura3-52 leu2Δ1 his3-Δ200 ade2-101lys2-801*	Lab. strain
ABC585	Mat*α ura3-52 leu2Δ1 his3-Δ200 ade2-101lys2-801 gtt1Δ*	Lab. strain
ABC591	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801 gsh1Δ*::*LEU2*	Lab. strain
ABC668	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801pdr5Δ*::*KanMX2*	Lab. strain
ABC670	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801pdr10Δ*::*LYS2*	Lab. strain
ABC681	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801snq2Δ*::*KanMX2*	Lab. strain
ABC709	*Mata ura3-52 lys2-801 leu2-Δ1his3-Δ200*	
ABC734 (BY4742)	Matα His3Δ*1 leu2Δ0 lys2Δ0 ura3Δ0*	Euroscarf
ABC791	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801 bpt1Δ*::*KanMX2*	Lab. strain
ABC794	Mat*α ura3-52 leu2Δ1 his3-Δ200 ade2-101 lys2-801bpt1Δ*::*KanMX2 ycf1Δ*::*KanMX2*	Lab. strain
ABC936	Mat*a ura3-52 leu2Δ1 his3-Δ200 trp-Δ63 ade2-101 lys2-801ura3Δ*::*GPD-GSH1-URA3*	K. Kuchler
ABC949 (SEY6210)	Mat*α ura3-52 leu2-13 his3-Δ200 trp1-Δ901lys2-801*	S. Moye-Rowley
ABC950	Mat*α ura3-52 leu2-13 his3-Δ200 trp1-Δ901 lys2-801 Yap1Δ*::*HisG*	S. Moye-Rowley
ABC1041	Mat*α ura3-52 leu2-13 his3-Δ200 trp1-Δ901 lys2-801 Skn7Δ*::*TRP1*	Lab. strain
ABC1042	Mat*α ura3-52 leu2-13 his3-Δ200 trp1-Δ901 lys2-801 Yap1Δ*::*HisG Skn7Δ* ::*TRP1*	Lab. strain
ABC1114	Mat*α* His3Δ*1 leu2Δ0 lys2Δ0 ura3Δ0 glr1Δ*::*KanMX4*	Euroscarf
ABC1139	*Mat a trp1 his3 leu2 Ura3 lys2 ade2 gpx 1*::*HIS3*	Y. Inoue
ABC1140	M*at a trp1 his3 leu2 Ura3 lys2 ade2 gpx 2*::*URA3*	Y. Inoue
ABC1141	M*at a trp1 his3 leu2 Ura3 lys2 ade2 gpx 3*::*LEU2*	Y. Inoue
ABC1142	M*at a trp1 his3 leu2 Ura3 lys2 ade2 gpx 1*::*HIS gpx 2*::*URA3 gpx 3*::*LEU23*	Y. Inoue
ABC1304	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yrr1Δ*::*KanMX4*	Euroscarf
ABC1374	*Matα leu2-3-112 ura3-52 his3-Δ200 trp1 pdr1Δ*::*HisG*	S. Moye-Rowley
ABC1374	*Matα leu2-3-112 ura3-52 his3-Δ200 trp1 pdr3Δ*::*HisG*	S. Moye-Rowley
ABC1376	*Matα leu2-3-112 ura3-52 his3-Δ200 trp1 pdr1Δ*::*HisG Pdr3Δ*::*HisG*	S. Moye-Rowley

The *ERG5* and *ERG11* genes were amplified by PCR using vent DNA polymerase and cloned into the *Bam*HI–*Xho*I 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 ggA TCC ATgAgT TCT gTC gCA gAA AAT ATA-3′) as well as *ERG5* gen-Eco-R (5′-AAgACTgAATTCTCTCCAgTAATTgggTCTCTC-3′) for *ERG5*, and *ERG11*-gen-Bam-F (5′-ACA Agg ggA TCC ATg TCT gCT ACC AAg TCA ATC-3′) as well as *ERG11*-gen-R1-R (5′-TTA CAA gAA TTC ACC TTA gAT CTT TTg TTC 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 OD_{600} of 0·1 (2×10^6 cells ml^{-1}) and allowed to grow to an OD_{600} 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 OD_{600} 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 1×10^7 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 acetaminophen. 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 $OD_{600} = 0{\cdot}1$ and acetaminophen was added when the OD_{600} 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 pYCF-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 $OD_{600}=0{\cdot}2$ 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′-dichorodihydrofluorescein diacetate. Overnight cultures of ABC154 (wild-type strain) and ABC681 (*snq2*Δ strain) were each subcultured into eight different flasks with 5 ml fresh medium at a concentration of $0{\cdot}5\times10^{7}$ cells ml^{-1} and incubated at 30 °C at 200 r.p.m. for 2 h. The test chemicals H_2O_2 and acetaminophen (at final concentrations of 4 mg ml^{-1} and 18 mg ml^{-1}, respectively) were added to two flasks each of the wild-type and *snq2*Δ 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 *erg2*Δ and *erg6*Δ mutants in fact displayed a significant acetaminophen sensitivity. The *erg3*Δ and *erg4*Δ mutants showed slightly less sensitivity than the *erg2*Δ and *erg6*Δ 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

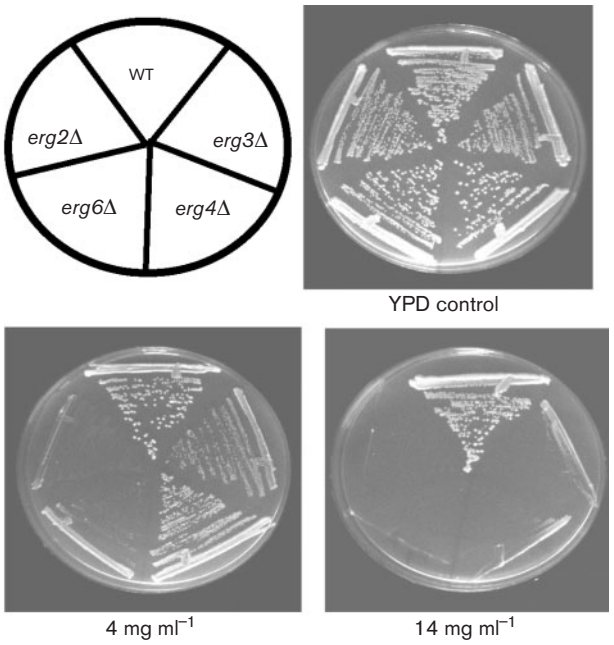

Fig. 1. *erg* strains are hypersensitive to acetaminophen. The strains *erg6*Δ (ABC231), *erg3*Δ (ABC557), *erg4*Δ (ABC230), *erg2*Δ (ABC229) and the corresponding wild-type strain (ABC709) were streaked on YPD plates containing 4 and 14 mg acetaminophen ml^{-1} and incubated at 30 °C. The YPD control plate contains an equivalent amount of 30 % methanol solution (Methods). WT, Wild-type.

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, Dit2p, 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 status is still controversial. Experiments were carried out as described in Methods. Cells exposed to H_2O_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

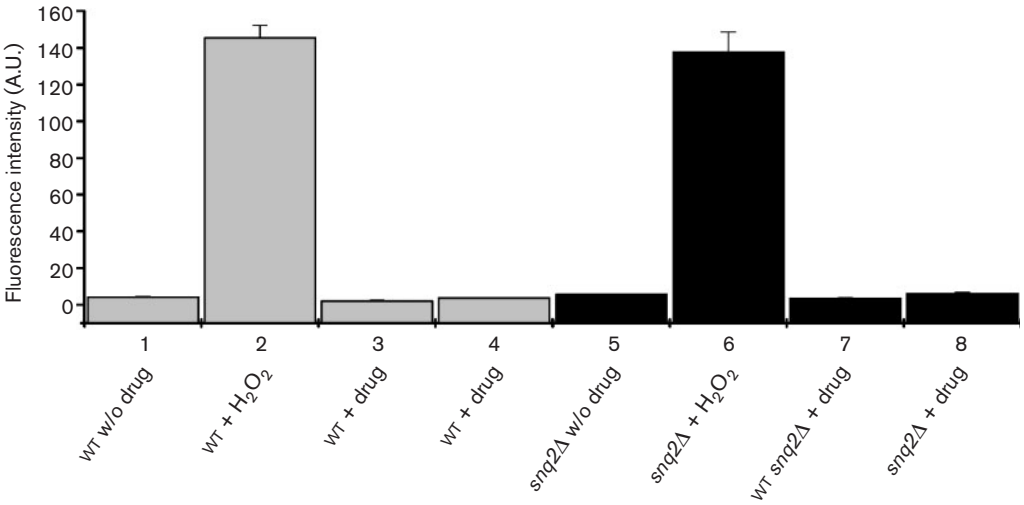

Fig. 2. Acetaminophen response detected by 2′,7′-dichlorodihydrofluorescein diacetate assay. Fluorescence of the cell extracts from strains ABC154 (wild-type) and ABC681 (*snq2*Δ) following 1 h exposure to H_2O_2 (bars 2 and 6) or different concentrations of acetaminophen (4 mg ml^{-1}, bars 3 and 7; 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.

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

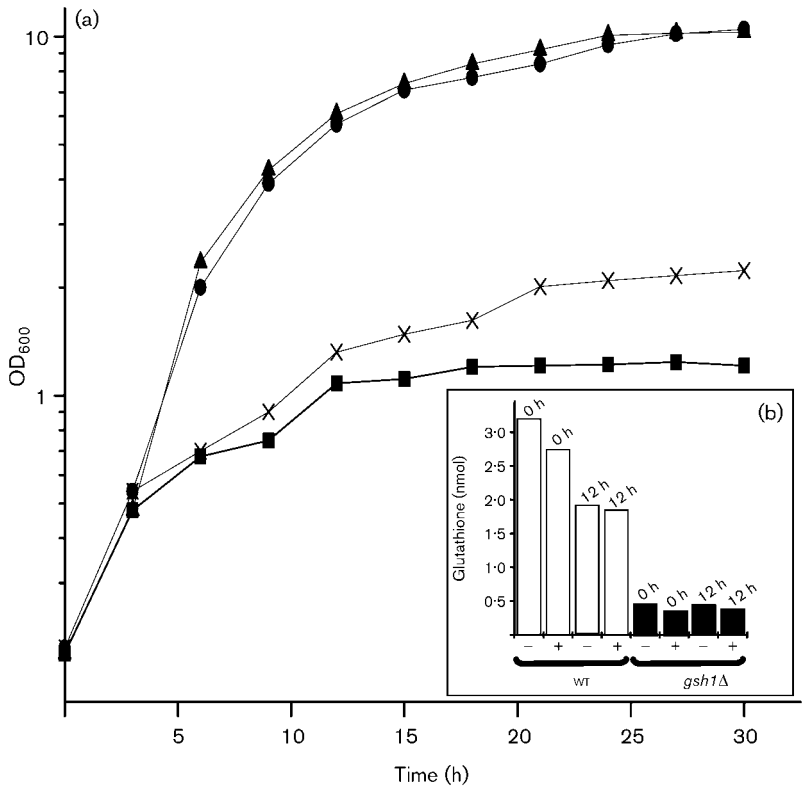

Fig. 3. Effect of glutathione depletion on acetaminophen toxicity. (a) The strains ABC154 (wild-type) and ABC591 (*gsh1*Δ), were grown in YPD for 10 to 12 h and re-inoculated to $OD_{600}=0·1$. These cultures were allowed to grow to $OD_{600}=0·5–0·6$ and then acetaminophen was added at 16 mg ml^{-1}. ▲ and ■, ABC154 strain; x and ●, *gsh1*Δ 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 *gsh1*Δ 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 (*glr1*Δ) 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 *glr1*Δ 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 *yap1*Δ 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 *yap1*Δ background was also constructed to see if the phenotypes of an *skn7*Δ deletion might be seen in this background. However, no further increase in acetaminophen sensitivity was observed in the *skn7*Δ *yap1*Δ strains compared to *yap1*Δ 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 (Azevedo *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

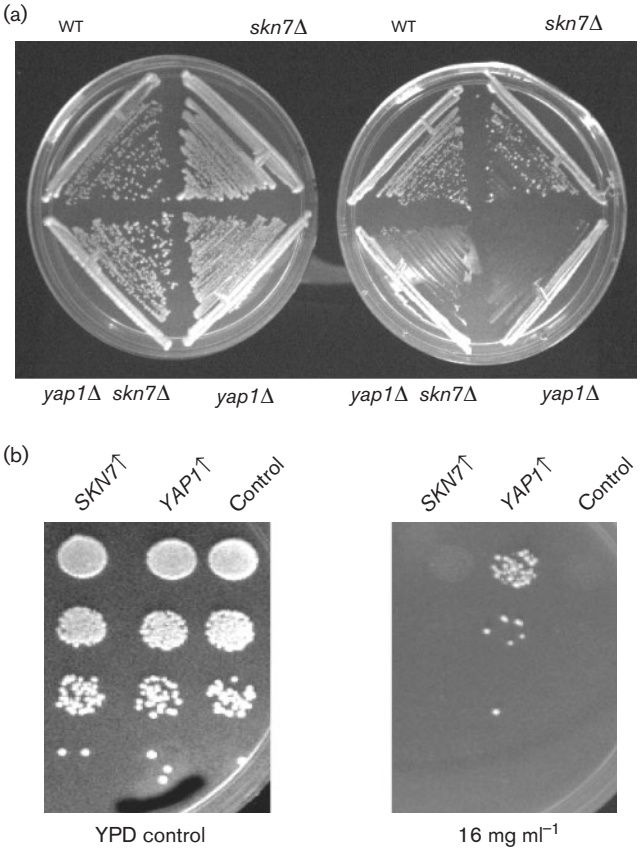

Fig. 4. The oxidative-stress-responsive transcription factor Yap1p, but not Skn7p, plays a role in acetaminophen resistance. (a) The deletion strains *skn7*Δ (ABC1041), *yap1*Δ (ABC950), *skn7*Δ *yap1*Δ (ABC1042) along with the corresponding wild-type (WT) strain (ABC949) were streaked on YPD plates with or without 14 mg acetaminophen ml^{-1}. Plates were incubated for 2 to 4 days at 30 °C and scored for growth. (b) Multicopy plasmids bearing *SKN7* or *YAP1*, and control vectors, were transformed into ABC154. Transformants were picked up and grown in SD media; uracil and spotting experiments were performed as indicated in Methods.

effects of *gpx3*Δ (*orp1*Δ) 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 H_2O_2-activated Yap1p and the thiol-compound-activated Yap1p show differential activation responses. The increased resistance of *ycf1*Δ *bpt1*Δ 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).

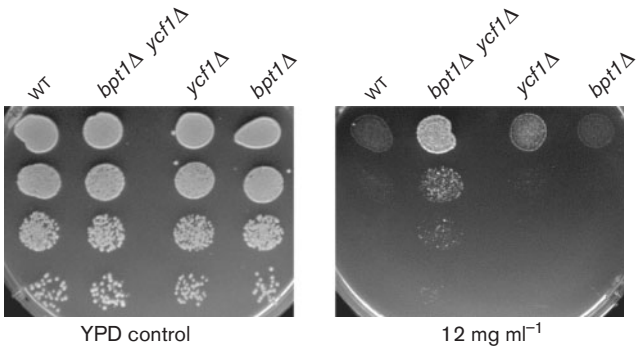

Fig. 5. Deletion of the GSH-conjugate pumps Ycf1p and Bpt1p leads to acetaminophen resistance. *ycf1*Δ (ABC470), *bpt1*Δ (ABC791), *ycf1*Δ *bpt1*Δ (ABC794) and the corresponding wild-type (WT) strains (ABC154) were spotted on YPD pates with or without 12 mg acetaminophen ml^{-1}.

Table 2. Induction of *YCF1* and *BPT1* in response to acetaminophen

β-Galactosidase reporter-gene assays were performed after induction (6 h) in medium containing 5 and 10 mg acetaminophen ml^{-1}, as described in Methods. Results shown are in β-galactosidase units, and are the mean of three independent transformants $\pm$ 1SD.

Plasmid	Acetaminophen concentration in medium (mg ml^{-1}):		
	0	5	10
pYCF1-lacZ	2·69 $\pm$ 0·12	4·05 $\pm$ 0·21	4·43 $\pm$ 0·17
pBPT1-lacZ	2·55 $\pm$ 0·12	3·72 $\pm$ 0·18	3·97 $\pm$ 0·04

Role of the multidrug resistance pumps in acetaminophen resistance

Although *yap1*Δ 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 *snq2*Δ 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

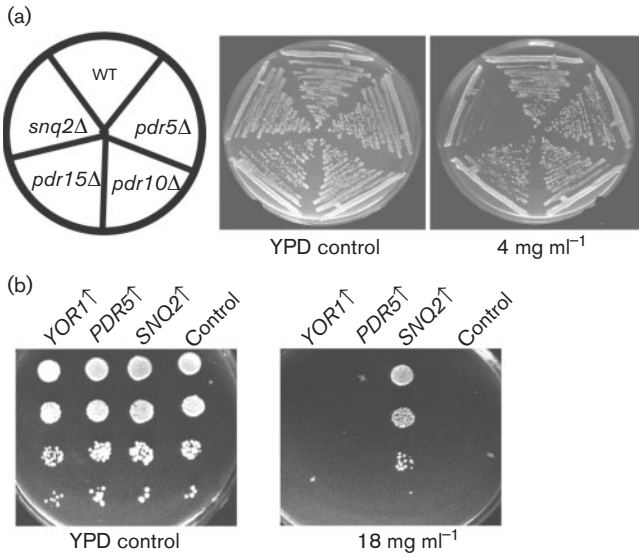

Fig. 6. The multidrug resistance pump Snq2p plays a role in acetaminophen resistance. (a) Wild-type strain ABC154 (WT) and deletion mutants for the MDR pumps *snq2*Δ (ABC681), *pdr5*Δ (ABC152), *pdr15*Δ (ABC668) and *pdr10*Δ (ABC670) were streaked on YPD control plates and YPD plates with acetaminophen (4 mg ml^{-1}, as indicated above). (b) ABC154 strain was transformed with control plasmid pRS426, and multicopy plasmids bearing either *YOR1* (Yep-*YRS1*), *SNQ2* 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).

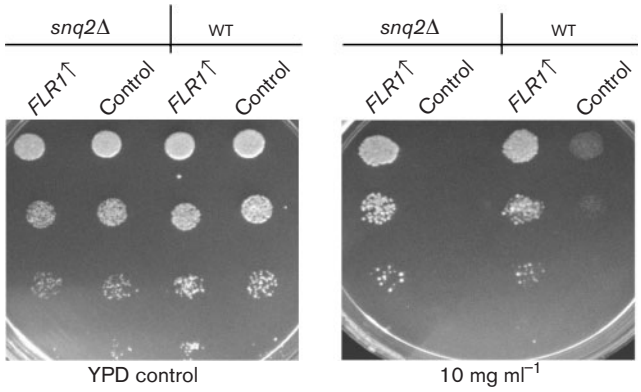

Fig. 8. *FLR1* overproduction confers resistance to acetaminophen. ABC154 strain and *snq2*Δ (ABC681) were transformed with plasmids bearing the *FLR1* gene (p425GPD-*FLR1*) and an empty plasmid (p425GPD). The transformants were spotted on acetaminophen (10 and 16 mg ml^{-1}) and control YPD plates as indicated in Methods.

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 *snq2*Δ strains we still observed a small increase in drug resistance, even in this (*snq2*Δ) 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 (Bróco *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.

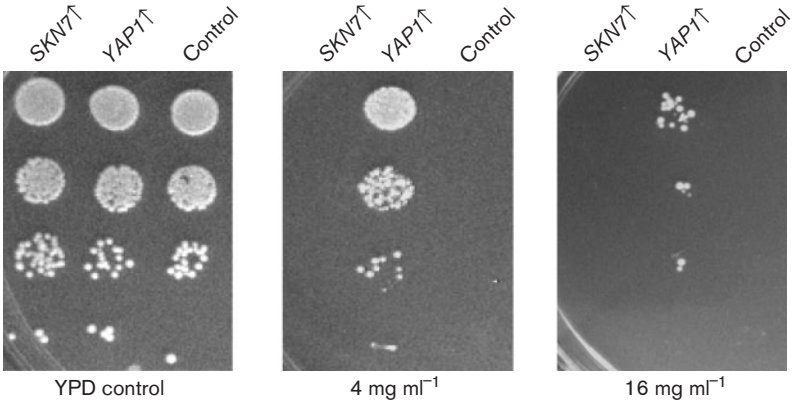

Fig. 7. *YAP1* overexpression confers resistance to acetaminophen in an *snq2*Δ background. The strain *snq2*Δ (ABC681) was transformed with plasmid YEp351-*YAP1* (a multicopy plasmid harbouring the *YAP1* gene). The transformants were grown in SD media lacking uracil and spotting was performed on YPD and YPD plus acetaminophen (10 mg ml^{-1}) plates as indicated in Methods.

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 *pdr1*Δ and *yrr1*Δ strains showed an increased sensitivity to acetaminophen, *pdr3*Δ 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 *pdr1*Δ, *pdr3*Δ and *pdr1*Δ *pdr3*Δ strains, and in *yrr1*Δ strains. Interestingly, while Yap1p overexpression continued to confer resistance to acetaminophen in a *pdr1*Δ as well as in a *yrr1*Δ background (Fig. 9), it failed to do so in a *pdr1*Δ *pdr3*Δ 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

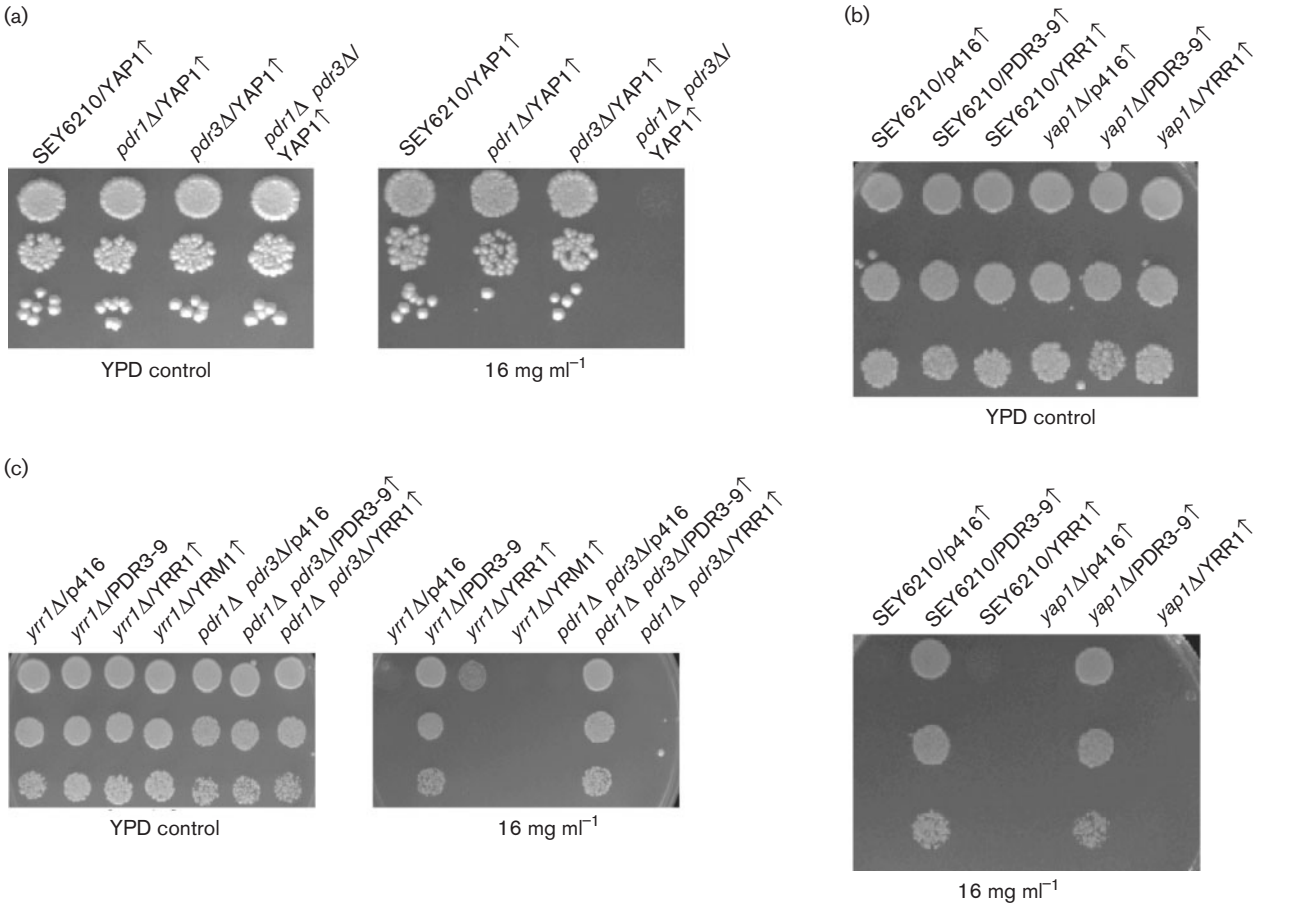

Fig. 9. Yap1p-mediated resistance to acetaminophen requires Pdr1p or Pdr3p, while the resistance caused by Pdr3-9p is independent of Yap1p. (a) Yeast strains ABC949 (WT), ABC1374 (*pdr1*Δ), ABC1375 (*pdr3*Δ) and ABC1376 (*pdr1*Δ *pdr3*Δ) were transformed with plasmids overexpressing *YAP1* and the corresponding 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 (*yap1*Δ) 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 (*pdr1*Δ *pdr3*Δ) and ABC1304 (*yrr1*Δ) 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 *yap1*Δ as well as *yrr1*Δ and *pdr1*Δ *pdr3*Δ backgrounds. We observed that *pdr3–9* could confer resistance in a *yap1*Δ as well as a *yrr1*Δ background (Fig. 9). In contrast, *GAD-YRR1* overexpression, while restoring resistance to the increased sensitivity of *yrr1*Δ strains, could not confer any resistance in either a *yap1*Δ or a *pdr1*Δ *pdr3*Δ 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 NAPQI, 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 NAPQI) 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 *ycf1*Δ *bpt1*Δ 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 Flr1p). 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 *YAP1*, 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 Flr1p. 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 Pdr5p, while a *pdr1–12* mutant (a gain of function of *PDR1*) mediated resistance to the same drug through Ycf1p and Flr1p (Wehrschutz-Sigl *et al.*, 2004). In the case of benomyl resistance, Flr1p 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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