Chapter 5

ACETAMINOPHEN TOXICITY AND RESISTANCE IN YEAST
INTRODUCTION

As described in the general introduction, GSH plays an important role in drug detoxification in eukaryotes, which possess this tripeptide. The first step of GSH mediated detoxification is usually the activation of the drug by cytochrome P450 followed by conjugation of the drug with GSH to form a GS-drug complex. The formation of this complex enables the faster elimination (e.g. in mammals through urine) since the water solubility of the complex is higher than the unconjugated drug. Various anticancer drugs (doxorubicin, daunorubicin, vinblastine, methotrexate and cisplatin) and toxic compounds are removed from the cell only after their conjugation with GSH (Muller et al., 1999). Later these conjugates are transported out of the cell by the action of glutathione conjugate (GS-X) pumps, which use ATP as their energy source (Hipfner et al., 1999). This pathway has been well characterized in mammalian cells. In yeasts, the GSH conjugate pumps have been identified and characterized but the role of the activation step (involving the CyP450) and the conjugating step (involving GSTs) has not been clearly demonstrated.

An important consequence of drug conjugation and removal by GSH is the depletion of intracellular GSH stores that make the cells highly susceptible to stress and free radical damage. The availability of mutants in the different GSH pathways in yeast allows one to carefully dissect the contributing factors in these processes, which are still poorly understood.

Acetaminophen is one of the most widely used analgesics and antipyretics. Although generally considered safe at therapeutic doses, overdoses or in conjunction with liver diseases and other disease conditions, acetaminophen displays toxicity leading to morbidity as well as mortality (Davidson et al., 1966; Jollow et al., 1974; Prescott, 1983). A great deal of work has gone into investigating the mechanisms by which acetaminophen is toxic (Howie et al., 1977; Prescott, 1983; Wu et al., 1985; Ray et al., 1993; Ruepp et al., 2002) and is detoxified in mammalian systems. The major pathway for the removal of acetaminophen appears to be through glucuronidation and sulphation which makes it more water soluble allowing it's removal from the liver and the blood via the urine (Jollow et al., 1974; Hinson et al., 1982). A third metabolic
pathway involves the oxidation of acetaminophen by microsomal cytochrome P450 to NAPQI (N-acetyl-p-benzoquinone imine), a reactive intermediate (Harvison et al., 1988, Myers et al., 1994). The NAPQI appears to be detoxified via the formation of glutathione-conjugates followed by their subsequent excretion since these conjugates and their degradation products have been observed in the urine along with a few other oxidation products.

Despite extensive studies on the mechanisms of acetaminophen toxicity, the exact mechanism by which acetaminophen is toxic, is surprisingly still controversial. Currently two major theories have been proposed explaining its 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 (that is generated from acetaminophen by cytochrome P450) leads to depletion of glutathione. This is followed by oxidative stress, ultimately leading to cell death (Fig. 5.1) (Thummel et al., 1993; Ruepp et al., 2002). The second theory 'the covalent binding theory' or the 'macromolecular inhibition theory' considers that the major cause of cell death by acetaminophen is not a result of glutathione depletion per se, but due to direct binding and inhibition of macromolecular function by NAPQI eventually leading to cell death (Pascolo et al., 1993; Mitchell et al., 1973; Dahlin et al., 1984; Ruepp et al., 2002).

Another issue, complicating studies on the toxicity of acetaminophen is the significant differences seen in the susceptibility of different species and even differences in various 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, none of the studies have indicated a possible role of multidrug resistance proteins (MDRs) in these processes, although a role for the multidrug resistance associated proteins (MRPs) has been indicated (Xiong et al., 2000).

An earlier report investigating the effects of aniline and its metabolites in yeasts, also investigated acetaminophen (an aniline derivative). Under the conditions in which this drug was investigated it was found to be non-toxic (Brennan and Schiestl, 1997). Considering the potential importance of yeast in resolving some of the controversial issues relating to acetaminophen
Figure 5.1. Schematic representation of the models of biotransformation of acetaminophen in mammalian systems
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 to mammalian cells. We decided to investigate this by initially examining pleiotropically drug sensitive mutants (certain \textit{erg} mutants defective in ergosterol biosynthesis). Acetaminophen was found to be toxic in these yeast mutants. This allowed us to investigate the possible mechanisms of drug detoxification as well as examine the existing models concerning the mechanisms of acetaminophen-induced cell death. These results are described in this chapter.

\textbf{RESULTS}

\textbf{5.1 Yeast strains undergo an acetaminophen-induced cell death with \textit{erg} strains displaying a hypersensitivity to acetaminophen}

Although an earlier report had indicated a lack of acetaminophen toxicity in yeast (Brennan and Schiestl, 1997), we decided to re-examine the issue by initially using \textit{erg} mutants, which are known to display an increased sensitivity to a wide variety of drugs (Kaur and Bachhawat, 1999). Earlier studies have shown that mutants in the ergosterol biosynthetic pathway, particularly those disrupted in the latter half of the pathway, display increased drug sensitivity. This sensitivity is due to an increased influx of drugs through the membranes which are more permeable, as well as through decreased efflux by membrane pumps, that function less efficiently in an altered membrane environment (Kaur and Bachhawat, 1999). The different \textit{erg} mutants were checked in a wide range of acetaminophen concentration (4mg/ml to 18mg/ml). As seen in \textbf{Fig. 5.2}, the \textit{erg}2Δ and \textit{erg}6Δ mutants in fact displayed significant acetaminophen sensitivity. The \textit{erg}3Δ and \textit{erg}4Δ mutants showed a slightly lesser sensitivity than the \textit{erg}2Δ and \textit{erg}6Δ 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 \textit{S. cerevisiae} in different genetic backgrounds revealed that WT strains were also sensitive to acetaminophen although at increased concentrations, but the sensitivities differed in
Figure 5.5: Prolactin represses sensitivity to actinomycin D by the interaction between the corresponding wild-type strain ABC and the non-ABC strains ARG7, ARG4, ARG1, and GAL10. YPD plates containing 1 M methanol or actinomycin D were streaked on YPD plates containing 0.5% methanol (see materials and methods).
different backgrounds. We have subsequently focused on the wild type YPH499 (S288C background). We examined whether the growth inhibition observed in these strains was a consequence of growth stasis or cell death. Cells were grown in YPD medium and acetaminophen added to exponentially growing cells. At 24-hour 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 (Fig. 5.3 and Table 5.1). This was similar to cell death observed in mammalian cells and it further suggested that we could possibly use yeast to examine the mechanism of acetaminophen-induced cell death.

**5.2 Identification of intracellular 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 examine 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 (materials and methods) and the ethyl acetate and aqueous fractions were subjected to direct infusion mass spectroscopy. On each occasion, the peak at m/z 152 in the respective mass chromatogram indicated the presence of acetaminophen (Fig. 5.4a, b). However, the mass chromatogram did not reveal any new peaks of significant intensity indicating the absence of any other chemical compound, which might have formed due to the biotransformation.

In order to investigate the accumulation of the drug inside the cell and to examine the possibility of any new peaks we further analyzed the ethyl acetate fractions of wild type strain by LC-MS using a PDA detector. The major component having the retention time comparable to that of the standard acetaminophen exhibited a peak at m/z 152 in the MS ascertained 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 sample. This suggests that the principal compound accumulating in these cells was acetaminophen, and no other transformed products of this drug were being generated (data not shown). However, the possibility that other metabolic products (such as NAPQI) were being
Figure 5.3. Effect of glutathione depletion on acetaminophen toxicity. The strains ABC 154 (wt.) and ABC591 (gsh1Δ), were grown in YPD for 10-12 hrs and reinnoculated at 0.1 OD₆₀₀. These cultures were allowed to grow till 0.5-0.6 OD and then acetaminophen was added (represented by an arrow) at 16 mg/ml. The growth of these strains were followed for 30 hrs. ▲ ■ are ABC154 strain and ×,● are gsh1Δ strain.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Estimated No. of cells plated</th>
<th>No. of colonies obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type. (ABC154) (16mg/ml drug)</td>
<td>650</td>
<td>&lt; 100</td>
</tr>
<tr>
<td>GSH1↑(ABC936) (16mg/ml drug)</td>
<td>5320</td>
<td>&lt;1000</td>
</tr>
<tr>
<td>gsh1Δ (16mg/ml drug)</td>
<td>3930</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Wild type) w/o drug</td>
<td>650</td>
<td>650</td>
</tr>
</tbody>
</table>

Table 5.1. Effect of Acetaminophen on yeast cell viability. The strains ABC 154 (wt.), ABC936 (GSH overproducing strain) and ABC591 (gsh1Δ), were grown in YPD for 10-12hrs and reinoculated to 0.1 OD_{600}. These cultures were allowed to grow till 0.5-0.6 OD and then acetaminophen was added at 16 mg/ml. After 16 hours of drug exposure the cells were plated on YPD media and colony number was scored after 2 days. Estimated cell number were based on the OD_{600} values (1 OD= 2 ×10^7 cells).
Figure 5.4. Detection of intracellular acetaminophen in yeast cells. Mass-spectrum of ethylacetate extracts of yeast cells (ABC154) treated with acetaminophen (a) and the untreated control cells (b). The spectrum is generated through the direct infusion of the extracts into the spectrometer that ionizes the samples by atmosphere pressure chemical ionization method (APCI). The peak corresponding to acetaminophen is shown by an arrow.
formed and were being rapidly removed from the cell or being conjugated to proteins preventing their extraction also exists.

5.3 Overexpression of yeast cytochrome P450 encoding Erg5p (C22 sterol desaturase) and Erg11p (Lanosterol demethylase) do not alter the acetaminophen resistance profiles in yeast

The inability to detect any other metabolites of acetaminophen suggested that the drug was exerting its toxicity independent of a biotransformation step. This was in apparent contrast to mammalian cells where the activation of acetaminophen to the reactive intermediate has been shown to be dependent on the presence of specific cytochrome P450 enzymes. As 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 being responsible for the toxicity needed thorough investigation. S. cerevisiae has 3 P450 enzymes that 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 is Dit2p involved in 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 contributing to the detoxification pathway of some metabolites. To examine the possible role of Erg5p and Erg11p in the toxicity (or resistance) to acetaminophen, we cloned and overexpressed these genes under a strong constitutive promoter. Both Erg5p and Erg11p overexpression could confer increased resistance to fluconazole (Fig. 5.5), but we could not find any increased sensitivity or resistance to acetaminophen upon overexpression of either Erg5p or Erg11p (data not shown).

5.4 Response of yeast to acetaminophen as detected by the dye 2’-7’-dichlorodihydrofluorescein diacetate

The inability to detect any other 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 where reactive metabolites were generated through the action of specific
Figure 5.5. Overexpression of \textit{ERG5} and \textit{ERG11} leads to resistance to fluconazole. Wild type yeast strain ABC154 was transformed with plasmids bearing \textit{ERG11} and \textit{ERG5} and the control plasmids. The transformants were grown in minimal media lacking uracil and were spotted on minimal media containing 5\,$\mu$g/ml fluconazole. Lane1: strains overexpressing \textit{ERG11}, Lane2: pTEF416 vector, Lane3: strains overexpressing \textit{ERG5}. 
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 that has been used by several workers as a marker for oxidative stress. It has been suggested to reflect the overall oxidative stress status in the cells, although its use as a marker for overall oxidative stress is still controversial. Experiments were carried out as described in materials and methods. Cells exposed to hydrogen peroxide displayed a significant increase in fluorescent intensity but no increase in fluorescence intensity was observed when cells were treated with acetaminophen concentrations from 4-18mg/ml for a period of 1-2 hours (Fig. 5.6). 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 and 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.

5.5 Acetaminophen toxicity in yeast: absence of a role for glutathione
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 a result of the deletion of *GSH1*, 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 3-fold higher than wild type levels (data not shown). We grew these cells in YPD medium, and at an OD$_{600}$ of about 0.5-0.6, we added acetaminophen to the cells and followed the growth further. At 3 hours intervals, we took aliquots to monitor the growth and glutathione levels of the different strains. In contrast to what we expected, irrespective of the intracellular glutathione content of the cells, there was no difference in the
Figure 5.6. Response of yeast cells to acetaminophen as detected by the dichlorofluorescein diacetate assay. Fluorescence of the cell extracts from strains ABC 154 (wild type) and ABC681 (snq2Δ) following 1 hour exposure to H₂O₂ (bars 2 and 6) or different concentrations of acetaminophen (4mg/ml bars 3, 7 and 18mg/ml bars 4, 8) in the presence of dichlorodihydrofluorescein diacetate. The bars represent the fluorescence intensity observed at 521nm in arbitrary units (A.U.). Wt. is wild type.
growth inhibition induced by acetaminophen. Furthermore, drug treated cells did not show any significant decrease in intracellular glutathione levels as compared to untreated cells (Fig. 5.7, 5.3), further suggesting against depletion of glutathione levels as the primary cause responsible for 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 strains disrupted for glutathione reductase (glr1Δ) to acetaminophen. Although the absence of glutathione reductase is not lethal for S. cerevisiae, these cells are much more sensitive to the presence of drugs that generate an oxidative stress response owing to the lowered GSH/GSSG ratios (Grant et al., 1996). However, the glr1Δ cells failed to show an enhanced sensitivity to acetaminophen (Data not shown). Furthermore, acetaminophen treated wild type cells also failed to show an alteration in GSH/GSSG ratios compared to untreated cells (Table 5.2) clearly underlining the fact that acetaminophen was not inducing an oxidative stress response in yeast unlike what is observed in mammalian cells and secondly, neither glutathione depletion nor the glutathione redox status was involved in the toxicity of acetaminophen.

5.6 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. The 2'-7'-dichlorodihydrofluorescein diacetate assay revealed that the acetaminophen toxicity was not due to oxidative stress. We tried to address this question using a genetic approach.

Acetaminophen toxicity was initially examined in strains deleted for YAP1. Our results clearly indicated that yap1Δ strains displayed an increased sensitivity to acetaminophen (Fig. 5.8a). Furthermore, overexpression of Yap1p in wild type cells conferred increased resistance to acetaminophen (Fig. 5.8b) 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 toxicity. However neither deletion of SKN7 nor overexpression of SKN7 from a multicopy plasmid led to any discernable phenotype in the presence of
Figure 5.7. Effect of acetaminophen treatment on intracellular GSH levels. Intracellular GSH estimation was carried out as described in materials and methods. The bars represent the intracellular GSH levels (in nmoles) of wild type cells and gsh1Δ. The various time points of the estimation are indicated on the top of the bars. The drug treated samples are represented by (+) and those without drug are represented by (-). The figure is a representative of 3 independent experiments.
Table 5.2. Effect of acetaminophen treatment on the GSH/GSSG ratio in yeast cells. The total intracellular GSH and the GSSG estimation was carried out following the protocol of Griffith (1980) (described in materials and methods). The values represent the intracellular GSH levels (in nmoles). Wt. is wild type.

<table>
<thead>
<tr>
<th>strain</th>
<th>GSH (nmoles)</th>
<th>GSSG (nmoles)</th>
<th>ratio</th>
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<tbody>
<tr>
<td>ABC734(Wt.)</td>
<td>3.2 ±0.2</td>
<td>0.8 ±0.2</td>
<td>4</td>
</tr>
<tr>
<td>ABC734 + drug</td>
<td>6.5 ±0.3</td>
<td>1.5 ±0.1</td>
<td>4.4</td>
</tr>
</tbody>
</table>
Figure 5.8. The oxidative stress responsive transcription factor, Yap1p, but not Skn7p, plays a role in acetaminophen resistance. (a) The deletion strains $\text{s}kn7\Delta$ (ABC1041), $\text{yap}1\Delta$ (ABC950), $\text{s}kn7\Delta \text{ y}ap1\Delta$ (ABC1042) along with the corresponding wild type (Wt.) strain (ABC949) were streaked on YPD plates and YPD plates containing 14 mg/ml of acetaminophen. (b) Multicopy plasmids bearing $\text{S}KN7$ (YEp13-SKN7) or $\text{Y}AP1$ (YEp351-YAP1) and control vectors were transformed into ABC154. The transformants were picked up and grown in SD media lacking uracil and spotting experiments were performed.
acetaminophen (Fig. 5.8a,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 as 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 alternate site of Yap1p which is activated by electrophiles (Azevedo et al., 2003). The lack of ROS suggested that the acetaminophen response on 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 difference in the presence of Gpx3p on the sensitivity to acetaminophen, thus indicating that Yap1p was not functioning through this pathway. It was in fact, being activated through an electrophilic compound that was not dependent on an oxidative stress response.

5.7 Disruption of the yeast glutathione conjugate pumps, Ycf1p and Bpt1p, lead to acetaminophen resistance

The cellular response to acetaminophen involved Yap1p. Since, Yap1p, a transcriptional activator, is known to activate a very large number of genes, it was important to identify the downstream targets. 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 that 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 demonstrated to transport unconjugated compounds (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. 5.9). This resistance to acetaminophen was observed in a very narrow range...
Figure 5.9. Deletions of the GSH-conjugate pumps, Ycf1p and Bpt1p, lead to acetaminophen resistance. ycf1Δ (ABC470), bpt1Δ (ABC791), ycf1Δ bpt1Δ (ABC794) and the corresponding wild type (Wt.) strains (ABC154) were spotted on (a) and streaked (b) on YPD plates or on YPD plates containing acetaminophen.
of drug concentrations. The results were unexpected and were also in apparent conflict with the fact that Ycf1p is up-regulated by Yap1p and the observation, described above, that Yap1p leads to increased resistance to acetaminophen. It may be mentioned here that the resistant colonies that were seen in case of ycf1Δ bpt1Δ strain were completely devoid of pigmentation (Fig. 5.9). These vacuolar glutathione-conjugate pumps have been reported to be deficient in vacuolar pigmentation (Chaudhuri et al., 1997). However, an enhanced pigmentation defect was observed in the resistant colonies (Fig. 5.9).

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; Sharma et al., 2003). However, in the presence of acetaminophen we observed only a negligible (1.5 fold) increase in β-gal activity in both YCF1 and BPT1 (Table 5.3). 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 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 (data not shown).

5.8 Role of the multidrug resistance pumps in acetaminophen resistance and involvement of snq2Δ

Although yap1Δ strains did lead to 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 other multidrug resistance proteins (DeRisi et al., 1997). Although work with mammalian cells has not implicated the multidrug resistance
Table 5.3. Expression of *YCF1* and *BPT1* in response to acetaminophen toxicity.
The wild type yeast strains (ABC154) were transformed with plasmids bearing *YCF1* promoter (pYCF1 LacZ) or *BPT1* promoter (pBPT1 LacZ) Lac Z fusions. These strains were exposed to acetaminophen (see methods) and were assayed for β-Galactosidase activity (see methods). The values given in the table are the average β-Galactosidase values of three independent samples.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No drug</th>
<th>5mg/ml drug (β-Gal units)</th>
<th>10mg/ml drug</th>
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<tbody>
<tr>
<td>pYCF1-lacZ</td>
<td>2.7 ± 0.1</td>
<td>4.0 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>pBPT1-lacZ</td>
<td>2.5 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>4 ± 0.0</td>
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</tbody>
</table>
proteins in acetaminophen resistance/sensitivity we decided to examine the involvement of such proteins in yeast. We examined yeast 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 and Goffeau, 1995; Decottignies et al., 1994; Decottignies et al., 1995; Decottignies et al., 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 pdr5Δ, pdr10Δ, pdr15Δ as well as snq2Δ for their acetaminophen sensitivity. Only snq2Δ strains displayed a very dramatic increase in acetaminophen sensitivity (Fig. 5.10). This was further confirmed by Snq2p overexpression (Fig. 5.10). 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 by Yap1p even in this (snq2Δ) background (Fig. 5.11). This indicated that additional targets of Yap1p might also 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; De Risi 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 drug 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. 5.12) although Atr1p did not seem to play any role in acetaminophen resistance. It thus appeared that Yap1p, was possibly mediating it's effects on acetaminophen resistance through the multidrug resistance pumps, Snq2p and Flr1p.
Figure 5.10. The multidrug resistance pump Snq2p is involved in detoxification of acetaminophen. (a) Wild type strain ABC154 (Wt.) and deletion mutants for the MDR pumps, snq2Δ (ABC681), pdr5Δ (ABC152), pdr15Δ (ABC668) and pdr10Δ (ABC670) strains were streaked on YPD control and YPD plates with acetaminophen (4mg/ml). (b) ABC154 strain was transformed with control plasmid Prs426, or multicopy plasmids bearing either YOR1 (YEp-YRS1), SNQ2 or PDR5 (PDR5/YEpplac195). The transformants were grown to exponential phase in SD media lacking uracil and were spotted on YPD or YPD + acetaminophen (18mg/ml) plates (see methods).
Fig 5.11. *YAPI* overexpression confers resistance to acetaminophen in a *snq2Δ* background. The strain *snq2Δ* (ABC 681) gene was transformed with plasmid YEp351-*YAPI* (a multicopy plasmid harboring *YAPI* gene). The transformants were grown in SD media lacking uracil and spotted on YPD and YPD + drug (4 and 16 mg/ml) plates (see methods). Lane 1: *SKN7*; Lane 2: *YAPI*; Lane 3: control plasmid.
Figure. 5.12. *FLR1* overproduction confers resistance to acetaminophen. ABC154 strain and *snq2Δ* (ABC681) were transformed with plasmids bearing *FLR1* gene (p425GPD-*FLR1*) and the control plasmids (p425GPD). The transformants were spotted on acetaminophen (10 mg/ml) and YPD plates as indicated in materials and methods.
5.9 Role of Pdr1p, Pdr3p, Yrm1p and Yrr1p in acetaminophen resistance

Pdr1p and Pdr3p are among the primary regulators of pleiotropic drug resistance in yeast (Manmun et al., 2002), and their targets include the multi drug efflux protein, Snq2p, primarily responsible for acetaminophen resistance in yeast. A generalized view of the action of some transcription factors along with their known interconnections is represented (Fig. 5.13). Another pair transcription factor, which are known to be involved in drug resistance, are Yrm1p and Yrr1p. We therefore investigated if the Yap1p mediated resistance to acetaminophen might be mediated through Pdr1p, Pdr3p, Yrm1p or Yrr1p, or was functioning independent 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 (Fig. 5.14 and Fig. 5.15).

To determine if the Yap1p response was dependent on the presence of either of these proteins, we first overexpressed Yap1p in wild type strains as well as the pdr1Δ, pdr3Δ and pdr1Δpdr3Δ 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 5.16), it failed to do so in a pdr1Δpdr3Δ 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.

5.10 The resistance conferred by Pdr3-9 is independent of Yap1p and Yrr1p

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 et al., 2001) and Yrr1p (encoded by GAD-YRR1*) (Le Crom et al., 2002) in yap1Δ as well as a yrr1Δ and pdr1Δ pdr3Δ backgrounds. We observed that pdr3-9 could confer resistance in yap1Δ as well as a yrr1Δ background (Fig 5.17 and 5.18). In contrast the GAD-YRRI 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.
Figure 5.13. Schematic representation of interconnection of known transcription factors, Yap1p, Yrr1p, Pdr3p/Pdr1p and their target genes. (taken from Le Corm et al., 2002)
Figure. 5.14. *pdr1Δ* strain is sensitive to acetaminophen. Yeast strains ABC949 (Wt.), ABC1374 (*pdr1Δ*) and ABC1375 (*pdr3Δ*) were transformed with plasmids overexpressing *YAPI* and the corresponding control 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 the methods.
Figure 5.15. The Pdr3p mediated resistance of Yap1p is independent of Yrr1p. Yeast strains ABC949 (Wt.) and ABC1304 (yrr1Δ) were transformed with plasmids overexpressing YRR1, PDR3, and YRM1. The transformants were picked and grown in the same selection media and then spotted on to YPD plates bearing different concentrations of acetaminophen (10, 14, 16 mg/ml) as described in the materials and methods. Lane1: Wt./ PDR3Δ, Lane2: Wt./ YRM1Δ, Lane 3: Wt./ YRR1Δ, Lane 4: yrr1Δ/ PDR3Δ, Lane 5: yrr1Δ/ YRM1Δ and Lane 6: yrr1Δ/ YRR1Δ.
Figure. 5.16. The Yap1p mediated resistance to acetaminophen requires at least one of Pdr1p or Pdr3p. Yeast strains ABC949 (Wt.), ABC1374 (pdr1Δ) and ABC1375 (pdr3Δ) and 1376 (pdr1Δ pdr3Δ) were transformed with plasmids overexpressing YAP1 and the corresponding control vector. The transformants were picked and grown in the same selection media and then spotted on to YPD plates bearing different concentrations of acetaminophen (only a representative plate is shown here) as described in the materials and methods. Lane 1: Wt./YAP1↑; Lane 2: pdr1Δ YAP1↑; Lane 3: pdr3Δ YAP1↑ and Lane 4: pdr1Δ pdr3Δ /YAP1↑. ↑ represents overexpression.
Fig 5.17. The resistance caused by hyperactive allele of Pdr3-9 is independent of Yap1p. Yeast strains ABC949 (Wt.) and ABC950 (yap1Δ) were transformed with plasmids overexpressing PDR3-9, YRR1 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 (only a representative plate is shown here) as described in the materials and methods. Lane 1: Wt./ cont. Vector; Lane 2: Wt. /PDR3-9↑; Lane 3: Wt. /YRR1↑ and Lane 4: yap1Δ/cont. Vector; Lane 5: yap1Δ/PDR3-9↑ and Lane 6: yap1Δ/YRR1. ↑ represents overexpression.
**Figure. 5.18.** *yrr1Δ* sensitivity to acetaminophen could be rescued by PDR3-9 overexpression allele. Yeast strains ABC 1304 (*yrr1Δ*) and ABC1376 (*pdr1Δ pdr3Δ*) were transformed with plasmids overexpressing *PDR3-9, YRR1 YRM1* and the corresponding empty vector (p416TEF). The transformants were picked and grown in the same selection media and then spotted on to YPD plates bearing different concentrations of acetaminophen (only a representative plate is shown here) as described in the materials and methods. Lane 1: *yrr1Δ*/control vector; Lane 2: *yrr1Δ/PDR3-9*; Lane 3: *yrr1Δ/YRR1* and Lane 4: *yrr1Δ/YRM1*; Lane 5: *pdr1Δ pdr3Δ* /control vector; Lane 6: *pdr1Δ pdr3Δ/PDR3-9* and Lane 7: *pdr1Δ pdr3Δ/YRR1*. ↑ represents overexpression.
DISCUSSION

In the present chapter we have examined the role of glutathione/glutathione-conjugate pumps in acetaminophen toxicity and resistance in yeasts and 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 association with 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 levels 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 by either ROS or electrophiles (Azevedo et al., 2003), and in the case of acetaminophen it appears that there is a 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 2 possible explanations for the toxicity of acetaminophen in yeast:

Either (i) acetaminophen itself (independent of it's activation to NAPQI) can weakly act as an electrophile (ii) the activation of acetaminophen occurs at exceeding 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 the 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 glutathione conjugate pumps, YCF1 and BPT1 surprisingly displayed resistance to acetaminophen. This was an unexpected observation since Ycf1p levels are actually enhanced by Yap1p. While a possible explanation is that either the GSH -conjugates in this case are more toxic as has been suggested for some drugs (Monks and Lau, 1998), an alternate 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 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 one to 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 may also be a possible cause also need to be examined more carefully.

Interestingly, in addition to YAPI, deletions in YRR1 and PDRI led to an increased sensitivity to acetaminophen indicating the involvement of the drug resistance regulatory network in acetaminophen resistance. While the Yap1p response required either 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 the Pdr1p/Pdr3p has earlier 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
allele of \textit{PDRI}) mediated resistance to the same drug through Ycf1p and Flr1p (Wehrschutz-Sigl \textit{et al.}, 2004). In the case of benomyl resistance, Flr1p appeared to be the primary pump involved in resistance that was dependent on Yap1p and partially on a functional Pdr1p or Pdr3p (Tenreiro \textit{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 it's resistance.

In conclusion, our studies investigating the role of glutathione in acetaminophen toxicity in yeast have demonstrated that acetaminophen can exert it's toxicity in these unicellular eukaryotes by mechanisms quite distinct from what has otherwise been observed and described in mammalian systems (Fig. 5.19). 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 it's wide usage. The studies described here also throw light on important aspects of the resistance of the drug in yeast that might also help in resolving some of the conflicting issues regarding the toxicity in humans of this widely used drug.
Figure 5.19. Schematic representation of the acetaminophen toxicity and the steps of detoxification in yeast. Ac. is acetaminophen. The cellular defense pathways are represented with green arrows while the acetaminophen entry and cellular damage is represented with red arrows.