Molecular Pharmacology Fast Forward. Published on November 8, 2006 as doi:10.1124/mol.106.030494
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MOL #30494

Title Page

Genomic screening *in vivo* reveals the role played by Vacuolar H^+ ATPase and cytosolic acidification in sensitivity to DNA damaging agents such as cisplatin.

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Molecular Pharmacology Fast Forward. Published on November 8, 2006 as DOI: 10.1124/mol.106.030494
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MOL #30494

Running title page.

Running title:

Pathways involved in sensitivity to cisplatin.

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Number of -

text pages: 24 (excluding title, running title & footnote pages)

tables: 2

figures: 6

references: 40

Words in -

Abstract: 245

Introduction: 744

Discussion: 1,481

Abbreviations: YPD, yeast extract peptone dextrose; NER, nucleotide excision repair; BER, base excision repair; RER: recombination dependant repair; RR: replication dependent repair; PR: post-replication repair. HU, hydroxyurea; MMS, methyl methanesulfonate.

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Abstract

Screening the *Saccharomyces cerevisiae* homozygous diploid deletion library against a sublethal concentration of cisplatin revealed seventy-six strains sensitive to the drug. As expected, the largest category of deletes, representing 40% of the sensitive strains, was composed of strains lacking genes involved in DNA replication and damage repair. Deletes lacking function of the highly conserved vacuolar H^+ translocating ATPase (V-ATPase) comprised the category representing the second largest number of sensitive strains. The effect on cell death exhibited by V-ATPase mutants was found to be a general response to various DNA damaging agents as opposed to being specific to cisplatin, as evidenced by sensitivity of the mutants to a DNA alkylating agent, hydroxyurea and UV irradiation. Loss of V-ATPase does not affect DNA repair, as double mutants defective for V-ATPase function and DNA repair pathways were more sensitive to cisplatin than the single mutants. V-ATPase mutants are more prone to DNA damage than wild type cells, indicated by enhanced activation of the DNA damage checkpoint. Vacuole function *per se* is not cisplatin sensitive, as vacuolar morphology and vacuolar acidification were unaffected by cisplatin in wild type cells. V-ATPase also controls cytoplasmic pH, so the enhanced sensitivity to DNA damage may be associated with the drop in pHi associated with V-ATPase mutants. The increased loss in cell viability induced by cisplatin at lower pH in V-ATPase mutants supports this hypothesis. The loss in viability seen in wild type cells under the same conditions was far less dramatic.

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Introduction

Cisplatin, cis-diamminedichloroplatinum(II), is one of the most widely used anticancer drugs. Platinum based chemotherapy cures most cases of advanced testicular cancer and has high efficacy in the treatment of other solid tumors such as ovarian and small-cell lung cancers. The principal cytotoxic mechanism associated with cisplatin is the generation of platinum-DNA adducts, the most significant DNA lesions being 1,2-intrastrand cross links that form across adjacent guanines (Wang and Lippard, 2005).

Unfortunately, acquired resistance to cisplatin can limit therapeutic potential (Perez, 1998). There are several resistance mechanisms including decreased intracellular drug accumulation, enhanced cellular detoxification by glutathione and metallothionein, altered DNA repair, and inhibition of apoptosis (Perez, 1998; Huang et al., 2005). However, these mechanisms do not completely account for the observed *in vivo* unresponsiveness of certain tumors to cisplatin (Niedner et al., 2001; Schenk et al., 2003). Accordingly, novel pathways mediating cisplatin resistance exist.

Use of model organisms such as the yeast *Saccharomyces cerevisiae*, have been instrumental in revealing the molecular basis of cisplatin toxicity. Complex systems driving signal transduction, DNA repair, and the cell cycle - are all highly conserved throughout the eukaryotic lineage. The range of mechanisms that can be probed using *S. cerevisiae* are those relating to maintenance of viability at the unicellular as opposed to the multicellular level. However, conclusions drawn from studies using these organisms are unambiguous because of the ability to disrupt expression of individual genes. Not surprisingly, these studies confirm the importance of DNA repair pathways including NER, RER, and PR (Grossmann et al., 2001; Beljanski et al., 2004; Wu et al., 2004). A significant advantage conferred by the use of *S.*

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cerevisiae genome-wide resources has been the identification of new genes, not associated with DNA repair, that mediate response to cisplatin. For example, elevated levels of phosphodiesterase 2 (Pde2), and the transcription factor Cin5, confer resistance to cisplatin (Burger et al., 2000; Furuchi et al., 2001). Cells lacking the serine/threonine kinase Sky1, the copper transporter Ctr1 and the nitrogen permease regulator Nrp2, are also resistant to cisplatin (Schenk et al., 2004; Ishida et al., 2002; Schenk et al., 2003). These strategies, exploiting gene overexpression from genomic libraries or transposon-mediated gene disruption, do not provide comprehensive coverage of the entire genome, since overexpression libraries rarely include all genes, and transposon insertion libraries do not disrupt genes in a random manner. Therefore, we screened the entire set of 4,728 homozygous deletion strains representing deletion of all non-essential open reading frames in *S. cerevisiae*, to identify genes that lead to sensitivity to cisplatin. A similar screen found 130 deletion strains that were sensitive to cisplatin (Wu et al., 2004). This screen, however, involved mixing all deletes followed by exposure to cisplatin and extraction of genomic DNA. Molecular barcodes identifying each delete were amplified, and hybridized to an oligonucleotide array, enabling abundance of each deletion strain to be determined (Wu et al., 2004). Cisplatin itself is a potent DNA damaging agent. Consequently, a DNA barcode associated with a strain that is sensitive to cisplatin might not be amplified, because it has sustained excessive damage. This would be the case in barcodes bearing consecutive guanines, given the nature of cisplatin-induced DNA damage. Lack of amplification is more likely in strains most sensitive to the drug. To avoid this problem, we individually assessed each delete, as previously described in screens for strains sensitive to the DNA alkylating agent methyl methanesulfonate and TPZ (Tirapazamine), a topoisomerase II inhibitor (Chang et al., 2002; Hellauer et al.,

2005). Our approach was vindicated because we present identification of forty-nine cisplatin sensitive deletes not identified by the screen involving amplification of molecular barcodes. Among the deletes novel to our screen are eight strains each lacking a component of the highly conserved vacuolar H^+ translocating ATPase (V-ATPase). Work in mammalian systems correlates V-ATPase activity with the response to cisplatin. Genes encoding sub-units of the V-ATPase are induced when human cell lines are treated with cisplatin, and are upregulated in cisplatin resistant cell lines (Murakami et al., 2001; Torigoe et al., 2002). Many genes are upregulated in tumors, though not all of the corresponding proteins play a role in tumor progression. Our data suggest that correlation between V-ATPase function and cisplatin sensitivity underpins a key relationship between the enzyme and sensitivity to the drug. Furthermore, we go on to show that reduction in V-ATPase activity increases susceptibility to DNA damage *per se*, explaining why V-ATPase inhibitors render tumors more sensitive to DNA damaging agents in general.

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Materials and Methods

Yeast Strains and media. The homozygous diploid deletion strains and individual haploid deletion strains were made by the *Saccharomyces* Gene Deletion Project (Winzeler et al., 1999). They were obtained from EUROSCARF (Frankfurt, Germany). The parental diploid strain BY4743 was used as control in the screening of the deletion library for sensitivity to cisplatin. BY4742 was used as the control for work using haploid strains. Genes encoding Vma6 or Vma8 sub-units of V-ATPase were deleted in the BY4742 background using a Hygromycin B resistance gene as selectable marker, as described by Goldstein et al. 1999. Combinations of mutants in the same strain were constructed by standard procedures (Rose et al., 1990). The genotypes of strains used in this study are listed in Table 1. Yeast was grown in YPD (1% yeast extract, 2% peptone and 2% dextrose) prepared as described in Rose et al., 1990. Cisplatin, HU, and MMS were obtained from Sigma (St. Louis, Missouri). Cisplatin stock solutions were prepared in YPD or phosphate buffered saline (PBS), and stored as aliquots at -20^{o}C.

Screen for cisplatin-sensitive yeast strains. We determined that the sublethal concentration of cisplatin required to result in visibly slower growth of the parental yeast diploid strain (BY4743) was 250µg/ml. Deletion strains were maintained by growth as arrays of 384 colonies on solid YPD. All replications were automated and were carried out using a 384-pin replicator operated by a Biomek® FX Laboratory Automation Workstation (Beckman Coulter, California). Colonies were picked and resuspended in 50µl YPD in 384 well plates and incubated for 48hrs at 30^{0}C (to stationary phase). Each culture (5µl) was transferred to fresh 50µl YPD in 384 well plates and grown to log-phase (14 hours at 30^{0}C with intermittent agitation). Replicas of these plates were made on solid YPD media with and without cisplatin (250µg/ml),

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followed by incubation at 30^0C. During incubation for 3 days, growth was scored by colony size compared to that of the wild type strain BY4743 (as described in Hellauer et al., 2005). Mutants showing a significant growth defect or absence of growth after 1 day in the presence of 250ug/ml cisplatin were scored as "x" or "xx". Mutants showing a significant growth defect or absence of growth after 3 days in the presence of 250ug/ml cisplatin were scored as "xxx" or "xxxx".

Assessing sensitivity or viability of individual strains to cisplatin. Cultures were grown in liquid YPD at 30°C to exponential phase and diluted to equal cell density. Six-fold serial dilutions were spotted across YPD, or YPD containing the concentration of cisplatin indicated in figure legends, followed by incubation at 30°C for 3 days. For viability assays, equal cell densities of exponential phase cultures were incubated in PBS (pH7.4) containing the concentration of cisplatin indicated in figure legends; aliquots were removed after 1 hour, diluted, and spread over YPD plates. Percentage viability was determined from the number of colonies that appeared after incubation for 3 days at 30°C - as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

Sensitivity to DNA damaging agents other than cisplatin. Cultures were grown at 30°C to exponential phase and diluted to equal cell density (1×10^7 cells/ml). Four successive six fold serial dilutions were spotted across YPD, or YPD plus 20mM hydroxyurea (HU) or 0.01% methyl methanesulfonate (MMS). For assessing sensitivity to UV irradiation, dilutions spotted across YPD were exposed to $40J/M^2$ UV using a UV crosslinker (Syngene, Maryland). All plates were subsequently incubated for 3 days at 30°C.

Vacuole staining. FM4-64 was obtained from Molecular Probes (Eugene, Oregon) and Quinacrine from Sigma (St. Louis, Missouri). Staining with FM4-64 was

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performed as described by Conibear and Stevens, 2002. FM4-64 was loaded into cells at 40μM for 15 min followed with one wash to remove free dye and a chase period for 60 min at 30^0C in YPD, or YPD with 100μg/ml cisplatin. Cells were examined by confocal laser microscopy using Texas Red filters. Staining with 200μM Quinacrine (in the presence or absence of 100μg/ml cisplatin) was performed as described by Roberts et al., 2002. Once stained, cells were visualized within 10 min by confocal laser microscopy using fluorescein filters.

Western blot analysis of Rad53. Exponential phase cultures grown at 30°C were incubated for 1 hour in the presence of cisplatin at concentrations indicated in the figure legends. Yeast protein extracts were prepared from TCA-treated cells and Rad53 was detected with a rabbit polyclonal antibody (Tercero et al., 2003).

Effect of pH on cisplatin sensitivity. Equal cell densities of exponential phase cells were incubated for 1 hour at 30^0C in PBS (pH7.4 or pH5.8) containing cisplatin, at the concentrations of drug indicated in figure legends, followed by dilution and inoculation over YPD plates. Percentage viability was determined from the number of colonies that appeared after incubation for 3 days at 30°C, as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

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Results

Screen for cisplatin-sensitive yeast strain. Hypersensitive strains revealed by screening with a sub-lethal concentration of cisplatin would reveal the identities of genes that normally function in pathways sensitive to this drug. We performed a high-throughput robot-aided screen for sensitivity to 250μg/ml cisplatin, using a collection of 4,728 homozygous diploid yeast deletion mutants, corresponding to non-essential yeast genes. Sensitive mutants were scored by comparison with colony size of the isogenic wild type strain, after incubation for one day. They same colonies were scored again after a further 2 days incubation. Overall, seventy-six strains were sensitive to cisplatin (Table 2). The sensitivity of these mutants was confirmed by individually pinning serial dilutions of deletion strains on 250μg/ml cisplatin and control plates.

Four of the seventy-six strains, however, correspond to deleted ORFs which overlap with other genes. These are the *YLR235c*, *YGL167c*, *YOR331c* and *YKL118w* deletes, which overlap with *TOP3*, *HUR1 VMA4* and *VPH2*, respectively. Two of the overlapped genes (*TOP3* and *HUR1*) are involved in DNA repair. The other two are involved in V-ATPase structure and function. In each case it is likely that the resistance is due to deletion of the overlapping gene (*TOP3*, *HUR1 VMA4* and *VPH2)*, given (a) the well-established importance of DNA repair mechanisms in the processing of cisplatin-DNA adducts, and (b) the importance of V-ATPase activity in resistance to cisplatin, demonstrated in the work presented here and inferred through correlative studies by others (Murakami et al., 2001; Torigoe et al, 2002). The genes are presented in categories based on known or inferred function (Table 2).

Exposure to cisplatin leads to DNA damage, so it was not surprising to find that the largest category, representing 42% of the sensitive strains, was composed of

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thirty-one strains deleted for genes involved in DNA replication and damage repair. These mainly fell into the following categories of DNA repair: Nucleotide excision repair - NER (6 genes, including *RAD1* and *RAD2*); Recombination repair of double strand breaks- RER (8 genes, including *RAD51* and *RAD52*); Replication-dependant repair – RR (8 genes, including *MUS81* and *POL32*); Post-replication repair - PR (3 genes, including *RAD18*). Deletes of two DNA helicases (*HPR5* and *SGS1*) and one topoisomerase (*TOP3*), were also sensitive. Twenty-three (of the thirty-one strains) were previously reported to be cisplatin sensitive (Wu et al 2004; Birrell et al., 2002); and seven were reported to be sensitive to TPZ, another anticancer drug that causes DNA damage (Hellauer et al., 2005). Deletion of genes in the following functional categories also gave rise to cisplatin sensitivity: Cell cycle (10 genes, Table 2), cell stress and signal transduction (*YDJ1*, *PPH3*, *SOD1*, *ZUO1*, *SEP1*, and *HAL5*), protein synthesis (*EAP1*, *MRF1*, *RPL13B*, *RPL7A*, and *SRO9*), transcription (*RPB9* and *HCM1*) and two transporters (*TPK1* and *GUP1*).

The category represented by the second largest number of cisplatin sensitive deletes was composed of strains lacking function of the vacuolar H^+ translocating ATPase (V-ATPase). The disruption of V-ATPase function is lethal in all eukaryotic organisms except *S. cerevisiae* (Graham et al, 2003). The lethality in higher systems is connected to aspects of V-ATPase function that are more significant in such organisms than they are in a unicellular organism like yeast, such as receptor mediated endocytosis. Yeast has one hormone receptor, the action of which is non-essential, this is in contrast to various essential receptors in membranes of complex organisms (Graham et al, 2003). This made budding yeast an ideal system to further investigate the role of V-ATPase in cisplatin resistance. We present data to explain

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why such mutants show more sensitivity to cisplatin as well as other DNA damaging agents.

V-ATPase mutants are sensitive to cisplatin. The V-ATPase is composed of thirteen subunits (Graham et al., 2003). Loss of any one of eight V-ATPase subunits led to cisplatin sensitivity (Table 2). Four strains lacked a component of the catalytic (V_1) domain responsible for ATP hydrolysis, and the other four lacked a component of the proton translocating (V_o) domain. Additionally, deletion of *VPS33*, a gene required for assembly of V-ATPase (though itself not a part of the enzyme) also led to cisplatin sensitivity. To confirm these results we generated two strains, one lacking a component of the V_o domain (*vma6Δ*, Table 1), the other lacking a component of the V_1 domain (*vma8Δ*, Table 1). As expected, both *vma6Δ* and *vma8Δ* strains exhibited severely impaired growth in the presence of cisplatin, compared with growth on media without the drug (Fig. 1A). In a cell survival assay, rapid loss of viability was exhibited by these mutants in the presence of cisplatin (Fig 1B).

Vacuolar morphology and acidity are not disrupted by cisplatin. The vacuole of budding yeast is required for protein turnover, nutrient recycling, osmoregulation, storage of amino acids and inorganic phosphate, and maintenance of cytoplasmic pH (Graham et al., 2003). The vacuole (or an aspect of vacuolar function) may be a target of cisplatin, as loss of V-ATPase function rendered cells hypersensitive to the drug. To investigate this possibility, we assessed the effect of cisplatin on vacuole morphology and vacuole acidification. Normally, one to five vacuoles can be visualised in wild type cells when stained with a fluorescent dye FM4-64 (Conibear and Stevens, 2002). Many mutants defective for vacuole function exhibit aberrant morphology of the vacuole itself (Raymond et al., 1992). Cisplatin at 100μg/ml had no effect on the viability of wild type cells but clearly led to loss of viability in the

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vma6Δ strain (Fig1B). However, under these conditions, vacuole morphology was unaltered in either strain, appearing normal in size, shape and number (Fig. 2A). Even though cisplatin does not affect vacuolar morphology, it could disrupt vacuole acidification. The lumen of the vacuole is more acidic than the surrounding cytoplasm, the resulting luminal pH drives numerous vacuole-associated processes (Graham et al., 2003). Cells were stained with Quinacrine, a fluorescent weak base which is only accumulated in the vacuolar lumen upon acidification (Roberts et al., 2002). Intensity of fluorescence is directly proportional to the degree of vacuolar acidification. Wild type cells treated with upto 200μg/ml cisplatin accumulate the same amount of Quinacrine as cells incubated without the drug (Fig 2B). As expected, *vma6Δ* cells could not be stained with Quinacrine due to the failure of V-ATPase-mediated vacuolar acidity (Fig 2B). These data indicate that vacuoles in wild type cells are acidified and morphologically normal in cells treated with cisplatin, suggesting that the vacuole *per se* is not the target of cisplatin.

V-ATPase mutants are hypersensitive to other DNA damaging agents. Cisplatin hypersensitivity in V-ATPase mutants could be due to increased levels of DNA damage, or may diminish the effectiveness of DNA repair mechanisms. We assessed the response of V-ATPase mutants to various DNA damaging agents. Methyl methanesulfonate (MMS) is a DNA alkylating agent, and hydroxyurea (HU) is a DNA replication inhibitor, giving rise to stalled replication forks that are sensed by the cell as abnormal DNA structures (Tercero et al., 2003). Both *vma6Δ* and *vma8Δ* strains are hypersensitive to both agents (Fig 3). The vacuole itself can act as a detoxification mechanism by accumulation of small molecules via the endocytic machinery. Both *vma6Δ* and *vma8Δ* strains, however, are also hypersensitive sensitive to UV irradiation in comparison with the wild type (Fig 3). Accordingly, it is likely

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that increased sensitivity to cisplatin, HU and MMS exhibited by V-ATPase mutants is not due to a defect in a mechanism that involves vacuolar sequestration of these cytotoxic agents. Instead, DNA could be more prone to damage in these cells, or, repair mechanisms may be less efficient.

Assessing sensitivity of V-ATPase/DNA repair double mutants to cisplatin. The cisplatin-induced DNA cross-linked adducts in *S. cerevisiae* are mainly repaired by three DNA repair mechanisms: the nucleotide excision repair (NER), recombination repair (RER), and postreplication (PR) pathways. To determine the possibility that V-ATPase would diminish the effectiveness of these repair pathways, we performed epitasis analysis using V-ATPase/DNA repair double mutants. *RAD1*, encoding a single-stranded DNA endonuclease, is a classical NER gene. *REV3* encodes a subunit of DNA polymerase zeta, which is involved in PR. *RAD52* encodes a protein that stimulates strand exchange during RER. Double mutants were constructed, that were defective for V-ATPase function and one DNA repair pathway. All three double mutants (*vma6Δrad1Δ*, *vma6Δrev3Δ*, and *vma6Δrad52Δ*) were more sensitive to cisplatin than the single mutants (Fig 4). This suggests that involvement of V-ATPase with DNA damage sensitivity is independent of DNA repair pathways. Instead, V-ATPase mutants could be more sensitive to cisplatin simply because more damage is caused by a given concentration of drug.

Activation of the DNA damage checkpoint in V-ATPase mutants. When yeast cells are treated with cisplatin, the DNA damage checkpoint is activated leading to cell cycle arrest at G2/M. Mutants in components of the checkpoint, display increased sensitivity to cisplatin (Grossmann et al., 1999). The sensitivity to cisplatin exhibited by strains lacking the S-phase checkpoint protein *MRC1*, is in agreement with this (Table 2). Rad53, the effector protein kinase in this pathway, is activated via

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hyperphosphorylation in response to DNA damage (Tercero et al., 2003). Phosphorylated Rad53 is detected as a smear of slowly migrating forms of the protein on western blots immunoprobed with anti-Rad53 antisera. We incubated cells in the presence of various concentrations of cisplatin for 1 hour. The incubation of both wild type and *vma6Δ* cells with 200μg/ml cisplatin led to the appearance of hyperphosphorylated forms of Rad53, suggesting that lack of V-ATPase activity did not impair the DNA damage checkpoint pathway (Fig. 5). A single band, which indicated the non-phosphorylated form of Rad53 was detected in the absence of the drug, in both wild type and *vma6Δ* cells (Fig. 5). In *vma6Δ* cells, however, hyperphosphorylated Rad53 was detected on incubation with 50μg/ml cisplatin. In contrast, hyperphosphorylated Rad53 was barely detectable in wild type cells treated in the same way, suggesting that V-ATPase mutants suffered more DNA damage than the wild type cells.

Effect of pH on cisplatin sensitivity. One of the major functions of V-ATPase is maintenance of intracellular pH, by translocating protons from the cytosol into the lumen of the vacuole (Graham et al., 2003). In both unicellular and multicellular organisms, cytoplasmic pH is reduced when function of the V-ATPase is compromised (Moreno et al., 1998; Murakami et al., 2001). The increased DNA-damage sensitivity in the V-ATPase mutants could be due to the acidified cytoplasm. To investigate the effect of pH on cisplatin sensitivity, we followed viability of cells after incubation with cisplatin in neutral (pH7.4) or acidic (pH5.8) conditions. Wild type yeast cells are efficient at maintaining a constant intracellular pH, even when they are incubated in media buffered to varying pH. This is principally due to the action of a plasma membrane H^+ translocating ATPase. This enzyme pumps H^+ out of the cell, consuming upto 40% of total cellular ATP. In order to remove the possibility

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that action of this enzyme would compensate for the effect of incubating cells in at varying pH, we incubated cells in the absence of a carbon source so that cells could not make ATP to drive H^+ extrusion by PM ATPase. Intracelllar pH of *S. cerevisiae* incubated under these conditions closely approaches the pH of the extracellular medium, with intracellular pH changes being due to H^+ leakage across the plasma membrane (Brett et al., 2005).

Both wild type and *vma6Δ* cells were more sensitive to cisplatin when incubated at lower pH. The effect of low pH, however, was more dramatic in *vma6Δ* cells (Fig 6). In the presence of 20μg/ml cisplatin, the viability of *vma6Δ* cells at pH5.8 was decreased by ca.76% (by ca.24.% at neutral pH), whereas the viability of the wild type cells at pH5.8 was decreased by ca.25% (by ca.10% at neutral pH). This implies that lower cytoplasmic pH increases sensitivity to DNA damage in V-ATPase mutants.

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Discussion

Pathways that modulate cisplatin sensitivity could be induced in tumor cells, and development of agents to inhibit these pathways can overcome the resistance that emerges frequently during treatment. Development of new platinum drugs by substitution of ligands or chloride leaving groups of cisplatin has not been successful in terms of overcoming drug resistance. New approaches can be developed based on discovery of mechanisms the mediate toxicity of this drug.

The budding yeast *S. cerevisiae* has been used as a powerful tool to identify and investigate pathways targeted by drugs. In the present study, we have applied a systematic approach to search for the nonessential genes in yeast which play a role in the response to cisplatin, leading to the identification of seventy-six deletion strains sensitive to this drug.

Over 40% of the cisplatin sensitive strains lacked various components involved in DNA damage repair. The deleted genes were mainly involved in three DNA repair pathways: replication/postreplication repair (RR and PR, respectively), recombination repair (RER) and nucleotide excision repair (NER). However, none of the components involved in base excision repair (BER) or mismatch repair (MR) were identified in this screen, suggesting that PR, RR and NER are the main mechanisms by which cells repair cisplatin-induced DNA damage, while BER and MR are not involved. This is in agreement with previous studies (Grossmann et al., 2001; Beljanski et al., 2004; Wu et al., 2004).

A novel insight from our screen is that sister chromatid cohesion plays a role in the response to cisplatin. Dcc1, Ctf8 and Ctf18 are components of a complex required for establishment of sister chromatid cohesion. Cells lacking the genes encoding any of these three proteins are sensitive to cisplatin. Cells lacking another

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gene involved in cohesion (Ctf4) are affected in the same way. Recruitment of the cohesin complex to sites of DNA damage is necessary for recombination-mediated repair of double strand breaks (Strom *et al.*, 2004). Therefore, it is likely that the Dcc1/Ctf8/Ctf18 complex and Ctf4, facilitate recombination repair (RER) of cisplatin-induced DNA damage. Also, two mutants (*asf1Δ* and *mrc1Δ*), known to impair the Rad53-dependent DNA damage checkpoint pathway, were sensitive to cisplatin. This is in agreement with work showing that cisplatin causes a checkpoint dependent G2/M arrest (Grossmann *et al.*, 1999). To support this notion, we showed that cisplatin resulted in hyperphosphorylation of Rad53, indicating the activation of this checkpoint pathway (Fig. 5).

Several mutants that compromised cell stress tolerance and signal transduction, were sensitive to cisplatin. In mammalian cells, the oxidative and osmolar stress responses protect cells from cisplatin-induced nephrotoxicity (Hanigan *et al.*, 2005). Also, genes involved with ribosomal function and protein synthesis were identified as cisplatin-resistant genes. In agreement with this are reports describing cisplatin-induced disruption of the translation initiation complex, and, overexpression of a ribosomal protein conferring resistance to cisplatin (Rosenberg and Sato, 1993; Shen et al., 2006).

Interestingly, a group of nine genes encoding V-ATPase subunits and an assembly factor for this enzyme, were identified as cisplatin-hypersensitive strains. It is not surprising that so many genes involved in V-ATPase function were identified, given that loss of *any* V-ATPase sub-unit or assembly factor is known to result in loss of V-ATPase activity (Graham et al., 2003). In yeast, this enzyme is localized to the membrane of the vacuole, with a smaller population of V-ATPase complexes localized to the endosomal network. In mammalian cells, the enzyme is similarly

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localized to the lysosome (equivalent to the yeast vacuole) and endocytic compartments. Additionally, the enzyme is localized to the membranes of specialized cells, notably the brush-border membranes of renal proximal tubules (Stevens and Forgac, 1997). In mammalian cells, one of the V-ATPase subunits is induced by cisplatin (Torigoe *et al.*, 2002). Furthermore, several genes encoding V-ATPase subunits are up-regulated in drug-resistant tumor cell lines (Martinez-Zaguilan *et al.*, 1999; Murakami *et al.*, 2001). This increase in levels of V-ATPase in cisplatin-resistant cells is correlative, and could be a consequence of drug treatment rather than a cause of resistance. The data we present, however, points towards V-ATPase activity directly contributing to drug tolerance.

V-ATPase translocates H^+ from the cytoplasm to the vacuole. Two consequences of this are regulation of cytoplasmic pH, and acidification of the vacuole – the latter being crucial for maintenance of processes associated with the vacuole lumen. We showed that cisplatin did not affect the morphology of vacuoles in wild type cells or a V-ATPase mutant. Furthermore, acidification of vacuoles in wild type cells was also unaffected. This implied sensitivity to cisplatin exhibited by V-ATPase mutants was associated with an effect on processes outside the vacuole, such as DNA repair, or the extent to which cisplatin damages DNA in the first place. V-ATPase mutants were also sensitive to the DNA alkylating agent methyl methanesulfonate (MMS), the DNA replication inhibitor hydroxyurea (HU), and UV-irradiation. This suggested that the activity of the V-ATPase was required for limiting the effects of DNA damaging agents in general. This concept is supported by a recent report describing the sensitivity of yeast V-ATPase mutants to Tirapazamine, an anticancer drug that targets topoisomerase II (Hellauer et al., 2005).

V-ATPase mutants may be defective in DNA damage repair mechanisms, or may lead to enhanced DNA damage. PR, RR and NER are the mechanisms that repair damage induced by cisplatin. Epistasis analysis ruled out the possibility that a defect in V-ATPase diminished the effect of repair pathways, since [V-ATPase/repair pathway] double mutants were far more sensitive to cisplatin than the single mutants (Fig 4). Activation of the DNA damage checkpoint by low concentrations of cisplatin was enhanced in V-ATPase mutants. This suggested that loss of the V-ATPase function facilitates the DNA damage caused by cisplatin.

V-ATPase is a regulator of cytoplasmic pH. Consequently, loss of V-ATPase activity leads to intracellular acidification, which may lead to greater levels of cisplatin mediated DNA damage. At lower pH, a greater proportion of hydrolysed cisplatin has an aqua ligand, rather than a hydroxo ligand. This enhances the reactivity of cisplatin as the aquated form is more labile. Chemical activity of cisplatin *in vitro* is greater at lower pH, promoting DNA platination (Murakami et al., 2001). The increased sensitivity to cisplatin in a V-ATPase mutant incubated at lower pH supports this hypothesis. At 50μg/ml cisplatin, it is notable that cell viability of the wild type in pH 5.8 buffer, is similar to cell viability of *vma6Δ* at pH 7.4. This may well reflect a similar intracellular pH in these cells.

The role played by lower pH, however, must be more complex than straightforward increases in chemical reactivity of drugs, because V-ATPase mutants were also hypersensitive to UV irradiation. Low pH can change DNA conformation (Robinson et al., 1992). Therefore, sensitivity to DNA damaging drugs or UV irradiation in V-ATPase mutants may be associated with altered DNA conformation at low cytosolic pH, rendering DNA more prone to damage. This may explain why

Molecular Pharmacology Fast Forward. Published on November 8, 2006 as DOI: 10.1124/mol.106.030494
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use of V-ATPase inhibitors in human cell lines renders them more sensitive to cisplatin (Laurencot et al., 1995; Murakami et al., 2001; Luciani et al., 2004).

In mammalian cells, cellular acidosis is an early event in apoptosis. Limiting the drop in cytoplasmic pH represses apoptosis, this being frequently associated with an upregulation of V-ATPase subunits in tumors (Torigoe et al., 2002; Izumi et al., 2003). Accordingly, enhanced cell death in yeast V-ATPase mutants incubated with cisplatin could be due, in part, to activation of apoptosis caused by lowering of intracellular pH. Some controversy does surround the concept of yeast apoptosis. However, yeast demonstrates several markers typical of apoptosis including DNA fragmentation, phosphatidylserine externalization, chromatin condensation and histone H2B phosphorylation (Madeo et al., 2004; Ahn et al., 2006). Furthermore, a growing list of genes that regulate apoptosis in mammalian cells have been identified in yeast, including the Yca1 caspase and the apoptosis inducing factor Aif1 (Madeo et al., 2004).

We also demonstrated that loss of four genes associated with ribosome function and protein synthesis rendered cells sensitive to cisplatin. Accordingly, we predict that enhancing protein synthesis would confer drug resistance. This is in agreement with recent work involving use of a human epidermoid carcinoma cell line that showed cisplatin resistance is induced by overexpression of the ribosomal protein gene *RPL36* (Shen et al., 2006). Another study revealed that inactivation of the Sky1 kinase in *S. cerevisiae* led to cisplatin resistance. Monitoring levels of the orthologous protein in testicular tumors revealed that expression of the kinase in cisplatin resistant cells was lower than in tumours from patients who were responding to platinum drug based therapy, so levels of the protein could predict the response to the drug (Schenk et al., 2004). Such work clearly demonstrates that conclusions drawn from use of the

Molecular Pharmacology Fast Forward. Published on November 8, 2006 as DOI: 10.1124/mol.106.030494
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S. cerevisiae model system are of direct relevance to mammalian systems. This indicates that other genes identified in our screen are worthy of further investigation, since they may be important for predicting the responsiveness of tumors to cisplatin.

Acknowledgements:

We thank Drs. Mehdi Mollapour and Nicholas L. Harris (University of Sheffield, UK) for assistance with re-arraying the genomic library from 96 to 384 colony format, and we thank Professor Peter W. Piper (University of Sheffield, UK) for allowing access to robotic facilities used for the re-arraying procedure. We are grateful to Dr. John F.X. Diffley (CRUK, Clare Hall, UK) for providing antiersa against Rad53. We also thank Dr. Katherine E. Bowers (University College London, UK) for advice pertaining to work with V-ATPase mutants.

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Footnotes:

This work was funded by the Royal Society (BP) and the BBSRC (BH).

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Molecular Pharmacology Fast Forward. Published on November 8, 2006 as DOI: 10.1124/mol.106.030494
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Figure 1. Sensitivity of the V-ATPase mutants to cisplatin. (A). Wild type and V-ATPase mutants were grown at 30°C to exponential phase and diluted to equal cell density. Six-fold serial dilutions were spotted across YPD, or YPD containing 150µg/ml cisplatin; plates were incubated at 30°C for 3 days. (B). Exponential phase wild type and V-ATPase mutants diluted to equal cell density were incubated in PBS (pH7.4) containing cisplatin at the concentration indicated; aliquots were removed after 1 hour, diluted, and spread over YPD plates. Viability was determined from the number of colonies that appeared after incubation at 30°C for 3 days – as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

Figure 2. Vacuolar morphology and acidity are not disrupted by cisplatin. Wild type and V-ATPase mutants were grown at 30°C to exponential phase and diluted to equal cell density, followed by incubation with cisplatin at the concentration indicated. Cells were stained with (A) FM4-64 for visualisation of vacuolar morphology or with (B) Quinacrine for assessing the acidity of the vacuole.

Figure 3. Sensitivity of V-ATPase mutants to various DNA damaging agents. Wild type and V-ATPase mutants were grown at 30°C to exponential phase and diluted to equal cell density. Six-fold serial dilutions were spotted across YPD, or YPD containing 20mM HU or 0.01% MMS or YPD followed by exposure to 40J/m^2 UV; plates were incubated at 30°C for 3 days.

Figure 4. Sensitivity of [V-ATPase/DNA repair] double mutants to cisplatin. Exponential phase wild type, single and double mutants were diluted to equal cell density and incubated in PBS (pH7.4) containing cisplatin at the concentration indicated; aliquots were removed after 1 hour, diluted, and spread over YPD plates. Viability was determined from the number of colonies that appeared after incubation at 30°C for 3 days - as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

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Figure 5. Phosphorylation of the Rad53 checkpoint effector kinase. Wild-type or V-ATPase mutant cells were grown to early log phase at 30°C, and then incubated for one hour with cisplatin at the concentration indicated. Rad53 was visualised by probing western blots with anti-Rad53 antibody. The positions of unphosphorylated Rad53 and phosphorylated Rad53 (Rad53-p) are shown on the right.

Figure 6. Effect of pH on cisplatin sensitivity. Exponential phase cultures diluted to equal cell density were incubated in PBS (pH7.4 or pH5.8) with cisplatin at the concentration indicated; aliquots were removed after 1 hour, diluted, and spread over YPD plates. Percentage viability was determined from the number of colonies that appeared after incubation at 25°C for 3 days - as a percentage of the number of colonies that appeared for each strain without cisplatin treatment.

Molecular Pharmacology Fast Forward. Published on November 8, 2006 as DOI: 10.1124/mol.106.030494
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Table 1. *S. cerevisiae* strains used in this study.

Strain	Genotype	Source
BY4742	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	EUROSCARF
BY4743	*Mat* a*/α; his3Δ2/ his3Δ2; leu2Δ0/ leu2Δ0; met15Δ0/MET15 LYS2/Lys2Δ0; ura3Δ0/ ura3Δ0*	EUROSCARF
rad52	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YML032c::kanMX4*	EUROSCARF
ku70	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YMR284w::kanMX4*	EUROSCARF
rad1	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YPL022w::kanMX4*	EUROSCARF
rev3	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YPL167c::kanMX4*	EUROSCARF
vma6	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YLR447c::* HygB	this study
vma8	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YEL051w::* HygB	this study
vma6/rad52	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YLR447c::* HygB *YML032c::kanMX4*	this study
*vma6/*ku70	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YLR447c::* HygB *YMR284w::kanMX4*	this study
vma6/rad1	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YLR447c::* HygB *YPL022w::kanMX4*	this study
vma6/rev3	*Mat a; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0;YLR447c::* HygB *YPL167c::kanMX4*	this study

EUROSCARF- European *Saccharomyces cerevisiae* Archive for Functional analysis

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Table 2. Cisplatin-sensitive deletion strains.
Mutants showing a significant growth defect or absence of growth after 1 day in the presence of 250ug/ml cisplatin scored as "×" or "××". Mutants showing a significant growth defect or absence of growth after 3 days in the presence of 250ug/ml cisplatin scored as "×××" or "××××".

ORF	Gene	sensitivity	Cellular function and comments
DNA replication and damage repair			
YJL092W	*HPR5*	××××	DNA helicase involved in DNA repair
YMR137C	*PSO2*	××××	DNA cross-link repair protein
YPL022W	*RAD1*	××××	Single-stranded DNA endonuclease involved in DNA repair
YGR258C	*RAD2*	××××	Single-stranded DNA endonuclease involved in DNA repair
YER162C	*RAD4*	××××	Nucleotide excision repair
YML095C	*RAD10*	××××	Single-stranded DNA endonuclease involved in DNA repair
YMR201C	*RAD14*	××××	Nucleotide excision repair
YCR066W	*RAD18*	××××	DNA postreplication repair
YNL250W	*RAD50*	××××	Subunit of MRX complex involved in DSBs repair
YER095W	*RAD51*	××××	DNA recombinational repair
YML032C	*RAD52*	××××	DNA DSBs repair
YDR076W	*RAD55*	××××	DNA recombinational repair
YDR004W	*RAD57*	××××	DNA recombinational repair
YLR234W	*TOP3*	××××	DNA Topoisomerase III
YDR369C	*XRS2*	××××	DNA recombinational repair
YLR235C		××××	ORF overlap with *TOP3*
YPR135W	*CTF4*	××××	Required for sister chromatid cohesion
YIL154C	*IMP2'*	××××	Transcriptional activator involved in protection against DNA damage
YBR098W	*MMS4*	×××	DNA endonuclease
YDR386W	*MUS81*	×××	DNA repair and replication fork stability
YJR043C	*POL32*	×××	Polymerase-associated gene
YLR032W	*RAD5*	×××	SS-DNA-dependent ATPase involved in postreplication repair
YDL059C	*RAD59*	×××	Involved in double strand break repair
YIL139C	*REV7*	×××	Subunit of DNA polymerase zeta involved in DNA repair
YJL115W	*ASF1*	××	Nucleosome assembly factor
YDL013W	*HEX3*	××	Involved in the DNA damage response with possible recombination role
YGL087C	*MMS2*	××	DNA postreplication repair
YGL175C	*SAE2*	××	Involved in meiotic recombination and chromosome metabolism
YMR190C	*SGS1*	××	Nucleolar DNA helicase of the RecQ family
YLR135W	*SLX4*	××	DNA repair and replication fork stability
YGL168W	*HUR1*	×	Required for hydroxyurea resistance and DNA replication
YCL060C	*MRC1*	×	Rrequired for DNA replication and Rad53 checkpoints activation
YGL167C	*PMR1*	×	ORF overlap with *HUR1*
Vacuolar V-ATPase and Vacuolar function			
YPR036W	*VMA13*	×××	Vacuolar ATPase V_1 domain subunit
YOR332W	*VMA4*	××	Vacuolar ATPase V_1 domain subunit
YLR447C	*VMA6*	××	Vacuolar ATPase V_0 domain subunit
YGR020C	*VMA7*	××	Vacuolar ATPase V_1 domain subunit
YEL051W	*VMA8*	××	Vacuolar ATPase V_1 domain subunit
YCL007C	*VMA9*	××	Vacuolar ATPase V_0 domain subunit
YHR039C-B	*VMA10*	××	Vacuolar ATPase V_0 domain subunit
YHR026W	*VMA16*	××	Vacuolar ATPase V_0 domain subunit
YKL119C	*VPH2*	××	Required for the biogenesis of a functional vacuolar ATPase
YLR396C	*VPS33*	××	Vacuolar sorting protein essential for vacuolar morphogenesis/function
YOR331C		××	ORF overlap with *VAM4*
YKL118W		××	ORF overlap with *VPH2*

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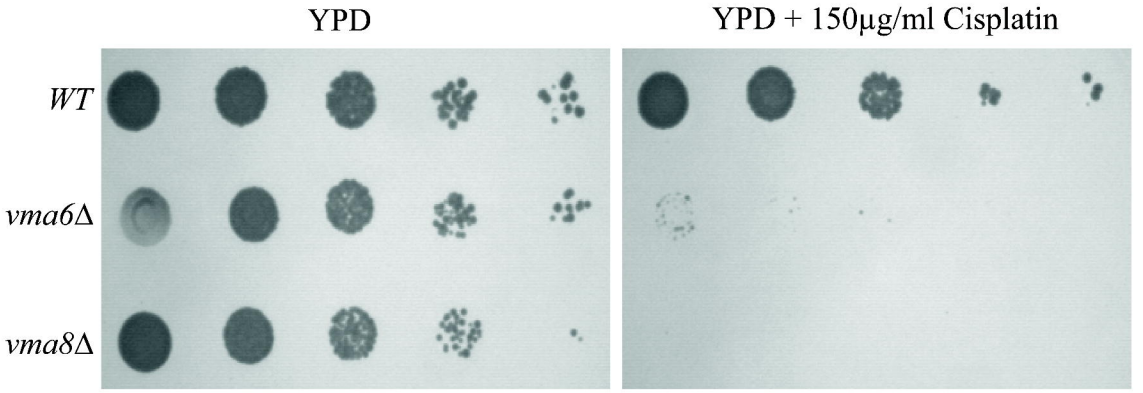
Table 2. continued

ORF	Gene	sensitivity	Cellular function and comments
Cell cycle			
YLR337C	*VRP1*	××××	Involved in cytoskeletal organization and cellular growth
YCR063W	*BUD31*	××	Involved in bud-site selection
YLR226W	*BUR2*	××	bypass UAS Requirement
YHR191C	*CTF8*	××	Required for sister chromatid cohesion
YMR078C	*CTF18*	××	Required for sister chromatid cohesion
YCL016C	*DCC1*	××	Required for sister chromatid cohesion
YGL240W	*DOC1*	××	Required for the activity of APC
YPR119W	*CLB2*	×	Involved in mitotic induction
YGR092W	*DBF2*	×	Kinase required for late nuclear division
YGR252W	*GCN5*	×	Histone acetyltransferase, acetylates lysine 14 on histone H3
Cell stress and signal transduction			
YNL064C	*YDJ1*	××××	Heat shock protein
YDR075W	*PPH3*	×××	Catalytic subunit of protein phosphatase PP2A
YJR104C	*SOD1*	×××	Cu, Zn superoxide dismutase
YGR285C	*ZUO1*	×××	Cytosolic ribosome-associated chaperone
YLR403W	*SFP1*	××	Transcription factor controls expression of ribosome biogenesis genes (in response to nutrients and stress)
YJL165C	*HAL5*	×	Putative protein kinase involved in sodium and lithium tolerance
Ribosomal protein and Protein synthesis			
YKL204W	*EAP1*	××	eIF4E-associated protein
YGL143C	*MRF1*	××	Mitochondrial polypeptide chain release factor
YMR142C	*RPL13B*	××	Component of the 60S ribosomal subunit
YGL076C	*RPL7A*	×	Component of the 60S ribosomal subunit
YCL037C	*SRO9*	×	RNA-binding protein which associates with translating ribosomes
Transcription			
YGL070C	*RPB9*	××	RNA polymerase II core subunit
YCR065W	*HCM1*	×	Transcription factor involved in cell cycle specific transcription
Transporter			
YJL129C	*TRK1*	××	180 kDa high affinity potassium transporter
YGL084C	*GUP1*	×	Membrane protein and putative glycerol transporter
Others			
YKR082W	*NUP133*	×××	Subunit of f the nuclear pore complex
YNL280C	*ERG24*	××	C-14 sterol reductase
YGL076C	*FYV5*	××	Unknown
YLR376C	*PSY3*	××	Unknown; deletion results in sensitivity to oxaliplatin & cisplatin
YJR121W	*ATP2*	×	F1 subunit of mitochondrial F_1F_0 ATP synthase
YDR245W	*MNN10*	×	Subunit of a Golgi mannosyltransferase complex

Figure 1

A.


YPD
YPD + 150μg/ml Cisplatin
WT
vma6Δ
vma8Δ

B.

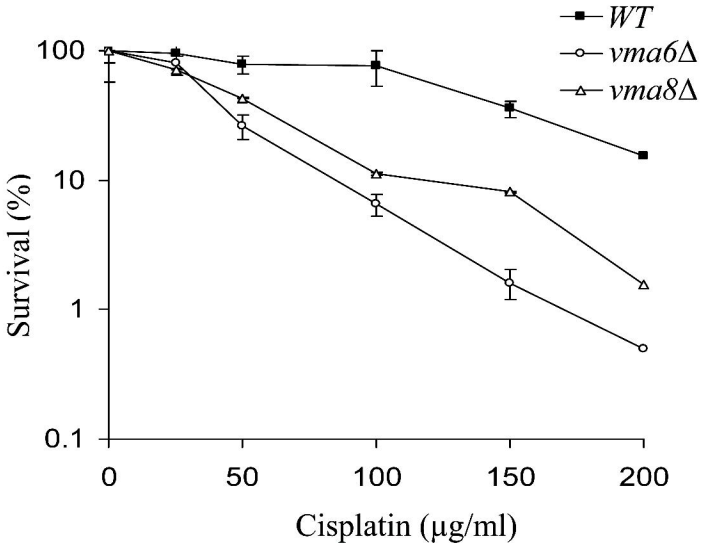
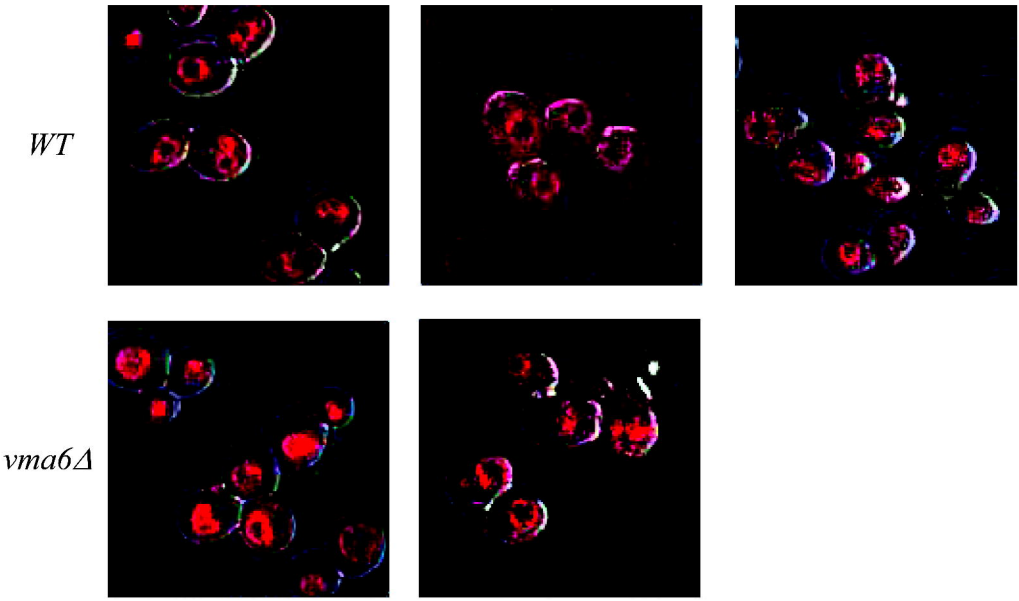

WT
vma6Δ
vma8Δ
100
10
1
0.1
Survival (%)
0
50
100
150
200
Cisplatin (μg/ml)

Figure 2

A.



B

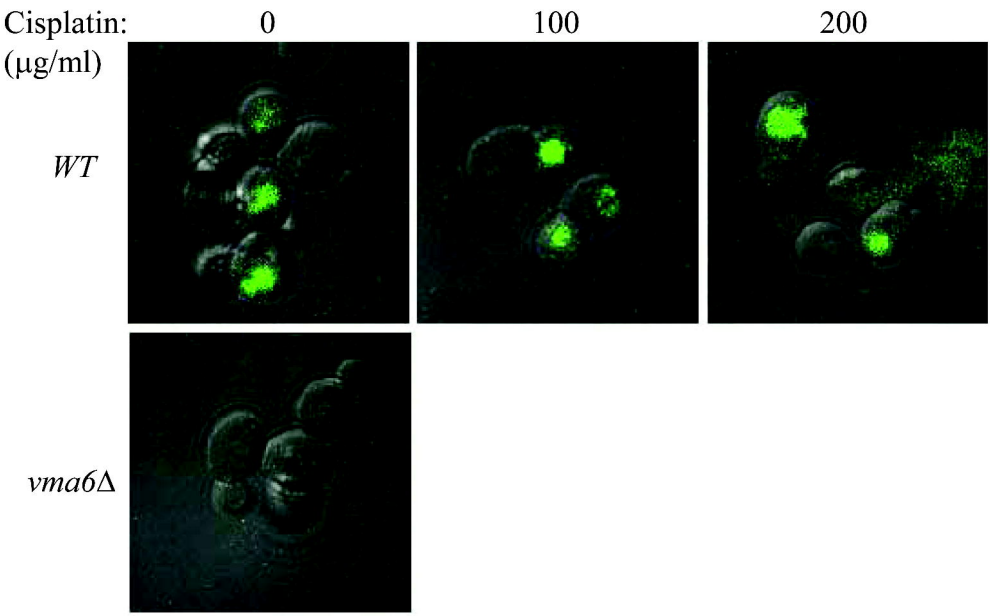


Figure 3

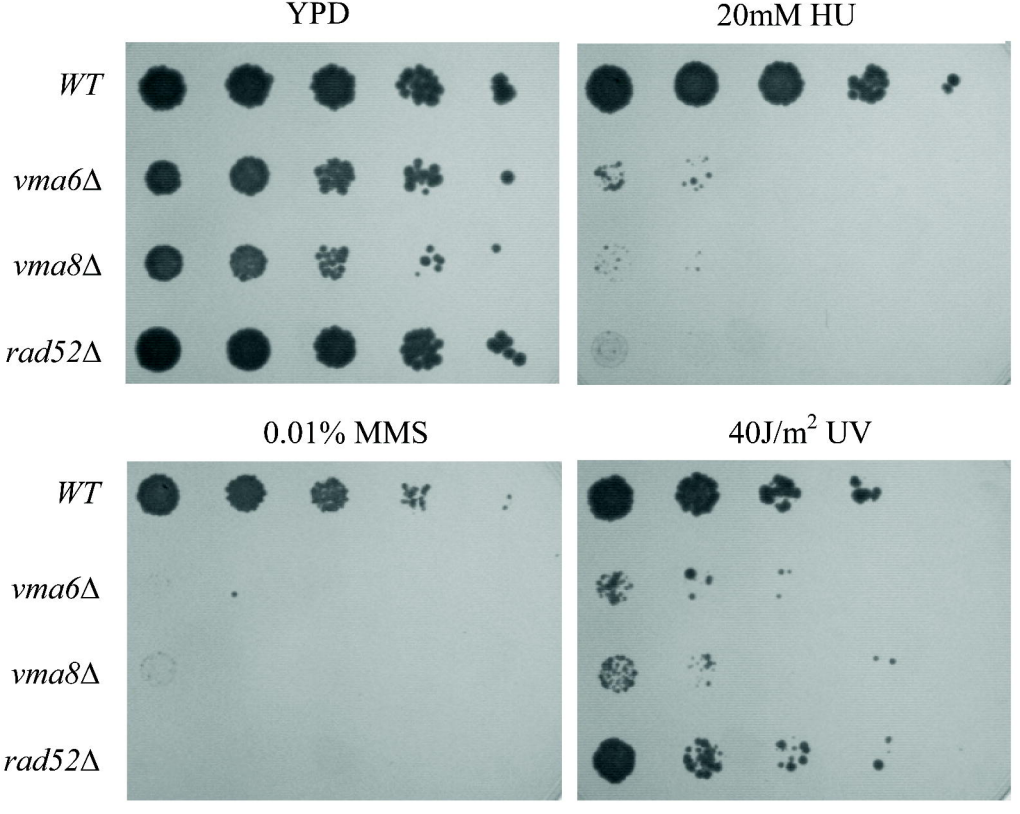

YPD
20mM HU
WT
vma6Δ
vma8Δ
rad52Δ
0.01% MMS
40J/m2 UV
WT
vma6Δ
vma8Δ
rad52Δ

Figure 4

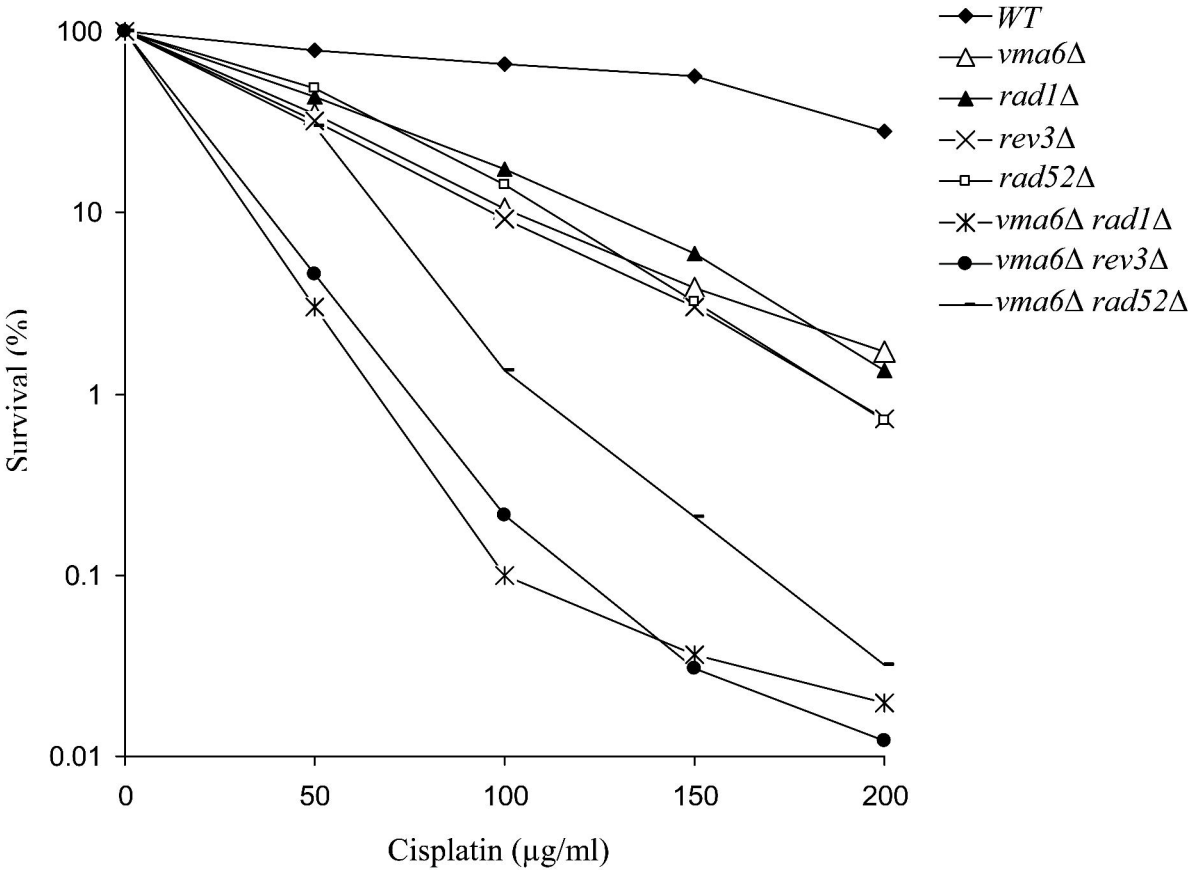

100
10
1
0.1
0.01
Survival (%)
0
50
100
150
200
Cisplatin (µg/ml)
WT
vma6Δ
rad1Δ
rev3Δ
rad52Δ
vma6Δ rad1Δ
vma6Δ rev3Δ
vma6Δ rad52Δ

Figure 5

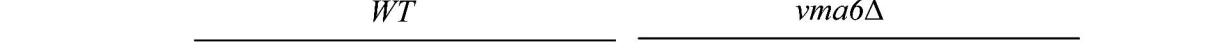
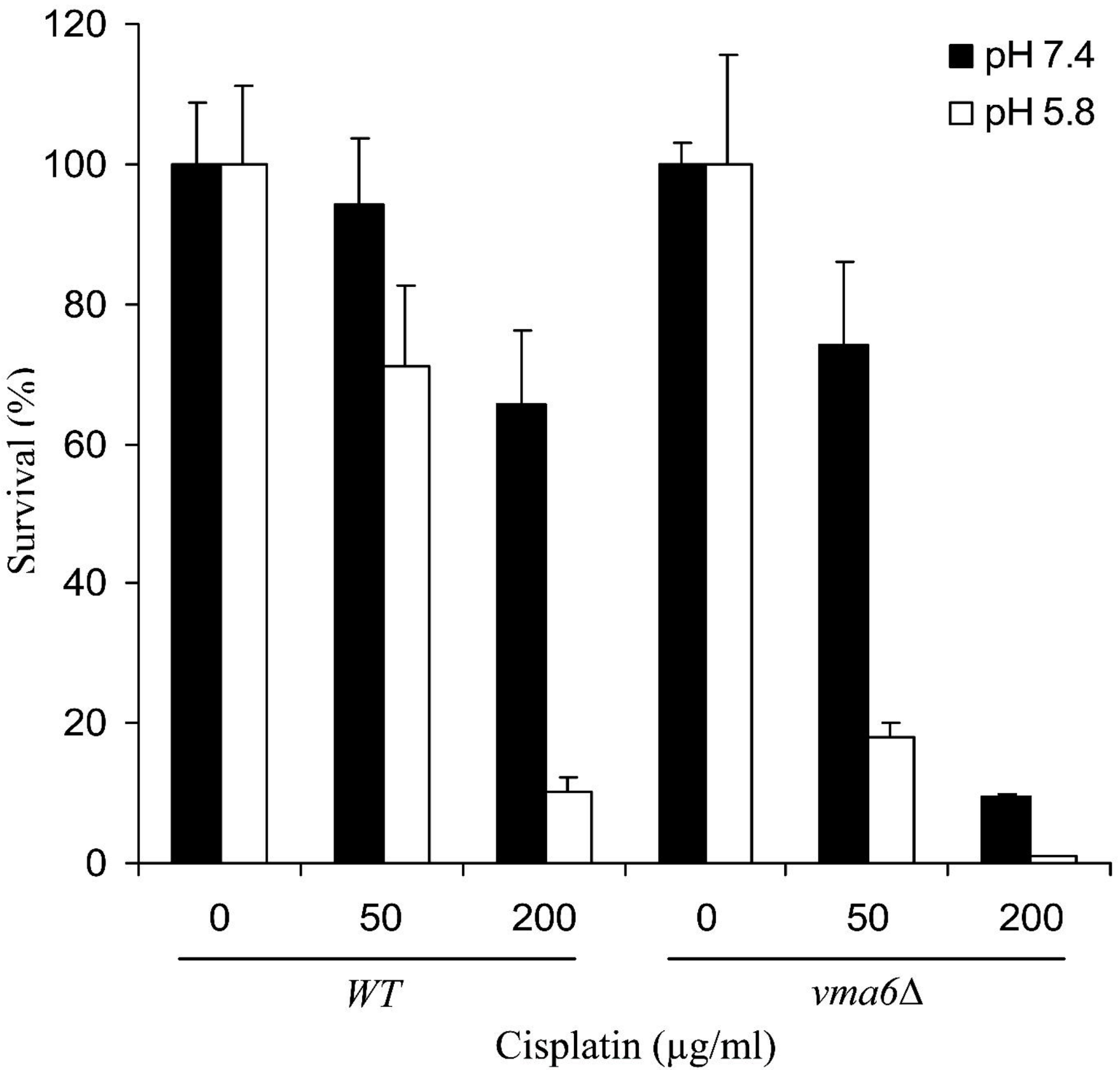


Figure 6


120
100
80
60
40
20
0
Survival (%)
pH 7.4
pH 5.8
0
50
200
0
50
200
WT
vma6Δ
Cisplatin (μg/ml)