MOL-048256

Title: Disregulation of Purine Nucleotide Biosynthesis Pathways Modulates Cisplatin Cytotoxicity in *Saccharomyces cerevisiae*

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MOL-048256

Running Title: purine nucleotide biosynthesis pathways and cisplatin cytotoxicity

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17 text pages

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236 words in the Abstract

630 words in the Introduction

1497 words in the Discussion

1 Supplemental Figure

Abbreviations:

cDDP;*cis*-diammine-dichloro-platinum II   (cisplatin); ADE, adenine; PCR, polymerase chain reaction; General: *YFG1,* your favorite gene 1; *yfg1⊿*, gene deletion lacking Yfg1; Yfg1, protein encoded by *YFG1*. dNTP, deoxbonucleotide triphosphate; FBS, fetal bovine serum.

Abstract

We previously found that inactivation of the *FCY2* gene, encoding a purine-cytosine permease, or the *HPT1 gene,* encoding the hypoxanthine guanine phosphoribosyl transferase, enhances cisplatin resistance in yeast cells. Here, we report that in addition to *fcy2Δ* and *hpt1Δ* mutants in the salvage pathway of purine nucleotide biosynthesis, mutants in the *de novo* pathway that disable the feedback inhibition of AMP and GMP biosynthesis also enhanced cisplatin resistance. An activity-enhancing mutant of the *ADE4* gene, which constitutively synthesizes AMP and excretes hypoxanthine, and a GMP kinase mutant (*guk1*), which accumulates GMP and feedback inhibits Hpt1 function, both enhanced resistance to cisplatin. Additionally, over-expression of the *ADE4* gene in wild-type cells, which increases *de novo* synthesis of purine nucleotides, also resulted in elevated cisplatin resistance. Cisplatin cytotoxicity in wild-type cells was abolished by low concentration of extracellular purines (adenine, hypoxanthine, and guanine), but not cytosine. Inhibition of cytotoxicity by exogenous adenine was accompanied by a reduction of DNA–bound cisplatin in wild-type cells. As a membrane permease, Fcy2 may mediate limited cisplatin transport since cisplatin accumulation in whole cells was slightly affected in the *fcy2Δ* mutant. However, the *fcy2Δ* mutant had a greater effect on the amount of DNA-bound cisplatin which decreased to 50-60% of that in the wild-type cells. Taken together, our results indicate that disregulation of the purine nucleotide biosynthesis pathways as well as addition of exogenous purines can modulate cisplatin cytotoxicity in *S. cerevisiae.*

Introduction:

Cisplatin (*cis*-diammine-dichloro-platinum II, cDDP) is one of the most frequently used chemotherapeutic agents for treating a wide spectrum of solid tumors. However, intrinsic and acquired resistance are major obstacles for the clinical use of these drugs. The development of resistance to cDDP in cancer treatment is believed to be caused by multiple mechanisms, including decreased intracellular drug accumulation, inactivation by glutathione or metallothioneins, increased DNA repair, enhanced tolerance, increased replicative bypass, and defects in pathways modulating cell death (Niedner et al., 2001; Perez, 1998). However, these mechanisms cannot fully account for resistance to cDDP-based treatment in clinical settings. In an effort to identify additional mechanisms of cDDP resistance and associated genes, we screened the yeast gene deletion collection and found several mutants that were more drug resistant than wild-type cells (Huang et al., 2005). A deletion mutant of the *FCY2* gene, a purine-cytosine permease which also transports protons through the plasma membrane was found most frequently and exhibited the strongest cDDP-resistant phenotype. A deletion mutant of a related gene, *HPT1,* encoding the hypoxanthine guanine phosphoribosyl transferase, was also identified (Huang et al., 2005). The *fcy2Δ* and *hpt1Δ* mutants also exhibit cross-resistance to 5-fluorouracil (5-FU) and doxorubicin. Since both Fcy2 and Hpt1 function in the nucleotide metabolism pathways, we sought to examine the possible involvement of these pathways in mediating cDDP-resistance.

AMP and GMP biosynthesis involves two interacting pathways, the *de novo* pathway and the salvage pathway. The *de novo* pathway synthesizes purine nucleotides from amino acids, carbon dioxide and ammonia, whereas the salvage pathway utilizes preformed nucleobases or nucleosides that are imported or present inside the cell. In *S. cerevisiae*, all the genes encoding enzymes required for *de novo* AMP synthesis (except *ADE16*) are repressed at the transcriptional level by the presence of extracellular adenine (Denis et al., 1998; Guetsova et al., 1997; Rebora et al., 2001). Both Fcy2 and Hpt1 function in the salvage pathway, and

inactivation of either gene causes derepression of the *de novo* pathway even in the presence of extracellular adenine (Guetsova et al., 1997). As a result, *de novo* synthesis of the purine nucleotides is constitutively active in *fcy2Δ* or *hpt1Δ* mutant. It has been demonstrated that excess intracellular purines are present in the *hpt1Δ* mutant and that purines, in particular hypoxanthine, are excreted from the cells (Lecoq et al., 2000). It is possible that enhanced cDDP resistance in the *fcy2Δ* and *hpt1Δ* mutants is due to constitutive activation of the *de novo* purine nucleotide synthesis or to a higher level of intracellular purines which may prevent cDDP from binding DNA, since cDDP binds strongly to guanine and adenine, and their nucleotides (Franska et al., 2005; Reedijk and Lohman, 1985). Surprisingly, while *fcy2Δ* cells excrete much smaller amounts of purines (Daignan-Fornier unpublished observations), they exhibit higher resistance to cDDP than *hpt1Δ* cells (Huang et al., 2005), suggesting that factors other than, or in addition to, purine excretion may be important. It has been shown that mutation in the *FCY2* gene results in resistance to purine and cytosine analogues and this was attributed to a defect in analogue influx (Guetsova et al., 1997). Thus, it is possible that Fcy2 may also transport cDDP. Another possibility is that mutation of *FCY2* somehow facilitates DNA repair.

In this study we found that the cDDP-resistant phenotype of the *fcy2Δ* mutant is not primarily due to reduced cDDP import or enhanced Rad52-mediated DNA repair activity. Instead, analysis of additional yeast mutants suggests that disregulation of the *de novo* pathway leading to purine nucleotide synthesis protects the *fcy2Δ* mutant from cDDP cytotoxicity, likely by limiting the amount of cDDP reaching the DNA in a reactive form. Our results thus suggest that disregulation of specific genes involved in purine nucleotide synthesis and elevated intra- or extra-cellular purine levels contribute to cDDP resistance.

Material and Methods

Yeast Strains and Media. Yeast strains are listed in Table 1. Haploid deletion strains were

obtained from Invitrogen (Carlsbad, California) or EUROSCARF (Frankfurt, Germany). Wild-

type PLY122 strain and the AMP synthesis mutants were as described previously (Guetsova et

al., 1997; Lecoq et al., 2000; Rebora et al., 2001). Standard yeast media and growth conditions

(Sherman, 1991) were used with minor modification. Briefly, yeast cells were streaked in plates

containing yeast extract peptone dextrose (YPD) media or synthetic defined yeast nitrogen base

media (SDM) supplemented with dextrose and appropriate amino acids for the auxotrophic

markers of the strains. Single colonies were inoculated overnight in SDM supplemented with

amino acid. All media containing cDDP, purines and the control solvents were SDM and the

tested agents were added immediately before pouring plates or treatment. We found no

difference in the cisplatin resistance phenotype between cells pre-grown in YPD and SDM

during the inoculation of single colony. For studies regarding pre-incubation of cisplatin with

adenine, plates were prepared as described in the figure legend.

Chemicals. Yeast nitrogen base, yeast extract, peptone and dextrose were purchased from

DIFCO Laboratories (Detroit, MI). cDDP, adenine, cytosine, guanine and hypoxanthine were

obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared as follows. cDDP

was prepared in DMSO (330 mM), stored as aliquots at -20°C, and used within 2 weeks. This

was further diluted in 0.9% NaCl (3.3 mM) before adding to the medium. Adenine and

hypoxanthine (200 mM in 0.5 N HCL) as well as cytosine and guanine (200 mM in 0.1 N NaOH)

were made freshly. All plates were made in SDM, stored in the dark, and used within 2-24 hours.

Plasmids, over-expression and gene replacements. Plasmids containing the wild-type (WT)

FCY2 gene and control constructs were generated as follows. The sequence containing the

open reading frame and 100-bp 3'-flanking region of the *FCY2* gene was PCR amplified and

cloned into the BamHI and XbaI sites of the pYES2 vector to create pFCY2-BX plasmid using the following primers: 5'-FCY2-BamHI (5'-ATCCGGATCCTGGAAGAGGGAAATAATGTTT-3') and 3'-FCY2-XbaI (5'-ATCCCTCTAGAAGCCGTGCAAATTGTCTT-3'). For monitoring the expression of the Fcy2 protein, the green fluorescence protein (GFP) gene containing a truncated *cup1* promoter at the C-terminal was PCR amplified from the pRS-cp-GFP-HA-YAP1 plasmid (Furuchi et al., 2001) using primers, 5'-SacI-GFP (5'-AAGCTGGAGCTCTCTTTTGCTGGCA) and 3'GFP-BamHI (TTAACCCTGGATCCAGGGAACAAA AG-3') and cloned into the pYES2 vector or fused to the start codon of the *FCY2* gene in the pFCY2-BX plasmid to create a control (pYES2G) or WT-FCY2 (pFCY2G) plasmid, respectively. In addition, the GFP-containing fragment was amplified using a mutant primer with one base deletion in the 3'GFP-BamHI primer and create a frame-shift-containing construct (p*fcy2m*). The GFP-fusion constructs were overexpressed in the wild-type (BY4741) and *fcy2Δ* strains under the induction of galactose and monitored using a fluorescence microscope. Plasmids used for over-expression of *ADE* genes were derivatives of YEp13 (Broach et al., 1979). YEp13:*(ADE1)*1 (Crowley and Kaback, 1984) and pPM13 (Mantsala and Zalkin, 1984) are *LEU2* 2 μm plasmids carrying *ADE1* and *ADE4* respectively. These plasmids were transformed into the BY4741 strain and selected on SDM plates without leucine. To create the *rad52Δ fcy2Δ* strain, a DNA fragment containing the *LEU2* marker flanked by upstream and downstream sequences of the *RAD52* ORF was PCR amplified using a plasmid containing the *LEU2* gene as a template. This fragment was transformed into the *fcy2Δ* strain and the correct gene replacement was verified by PCR of the genomic DNA isolated from the *LEU2*^{+} colonies.

Spot assay for cDDP cytotoxicity. Single yeast colonies were picked and grown overnight in liquid SDM at 30°C. Cultures were then diluted to a concentration of 5×10^6 cells/ml, and additional 5-fold serial dilutions were made. One microliter of each dilution was spotted onto SDM plates with or without cisplatin or tested compounds and grown for 2-3 days at 30°C. The

spot intensity at the second dilution for each strain was determined using densitometric analysis (Alpha Imager, Alpha Innotech) and was divided by the spot intensity of the corresponding untreated cells to determine the percent survival.

Platinum accumulation. For whole cell platinum accumulation, wild-type and $\Delta fcy2$ cells grown to log phase in SDM were treated with 100 µM cDDP for varying lengths of time and then washed 3 times with cold PBS. Whole cell extracts were prepared by the addition of 0.1% Triton X-100 and 0.01% SDS and vortexed with glass beads. Protein concentration was determined using Bradford assay and used for normalization. Platinum contents were measured by Atomic Absorption Spectrometry as described (Hector et al., 2001). For accumulation of DNA-bound platinum, cells were treated and washed as described above. DNA was isolated using yeast-breaking buffer [2%, (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0] and phenol extraction. After removal of RNA by RNaseA, DNA was hydrolyzed with 5% HCl and platinum content was measured as described (Hector et al., 2001). The units of measurements were pg-cDDP/ug-protein for accumulation into cells and pg-cDDP/µg-DNA for accumulation of DNA-bound platinum.

Statistical analysis. The data are presented as means ± SD (standard deviation). For a comparison of two means, Student's paired or unpaired t test (GraphPad Prism V4.03 software) was used. A probability value (p) of less than 0.05 was considered statistically significant.

Results:

cDDP resistance of *fcy2Δ* is not primarily due to an enhanced Rad52-mediated DNA repair activity

We have previously shown that deletion of the yeast *FCY2* or *HPT1* gene confers resistance to cDDP (Huang et al., 2005). To test whether the cDDP-resistant phenotype is due to the deletion of the *FCY2* gene, plasmid expressing the empty vector, wild-type Fcy2 or an inactive frameshift mutant, *fcy2mg*, was introduced into the *fcy2Δ* mutant. Figure 1A shows that the cDDP-resistant phenotype of the *fcy2Δ* mutant lacking the Fcy2 protein (Fcy2) can be greatly reduced by the expression of the wild-type protein, but not by expression of the inactive, mutated form, *fcy2mg* (see graph in Figure 1A). The data obtained in a spot assay quantified using densitometry were confirmed with a quantitative clonogenic survival assay (Supplemental Figure 1). Thus, the cDDP-resistant phenotype of the *fcy2Δ* strain is due to the absence of the Fcy2 protein. In addition, over-expression of the wild-type protein, but not the mutated form*,* is able to sensitize the wild-type cells to cDDP treatment. Thus, altering the level of Fcy2 protein can modulate cisplatin cytotoxicity.

Since one of the mechanisms by which cells may become resistant to cDDP is enhanced DNA repair activity (Perez, 1998), and RAD52p has been shown to be required for recombination repair of cisplatin-DNA lesions (Durant et al., 1999), we sought to determine whether the reduced cDDP sensitivity of the *fcy2Δ* mutant was due to enhanced recombination-mediated repair. We examined cDDP sensitivity in a *fcy2Δ* derivative incapable of recombinational repair because of deletion of *RAD52* (Shinohara and Ogawa, 1998). As expected, a yeast mutant lacking *RAD52* was hypersensitive to cDDP (Figure 1B), suggesting that Rad52p does mediate repair of cDDP-induced DNA lesions. Figure 1B also shows that deletion of the *RAD52* gene partially enhanced cDDP cytotoxicity in *fcy2Δ* cells; however, the *fcy2Δrad52Δ* strain was still more resistant than the wild-type strain. Densitometric analysis

indicates that *rad52Δ* increased the sensitivity of WT and *fcy2Δ* cells by 3 and 2 fold, respectively (graph in Figure 1B). These results thus suggest that cDDP resistance of *fcy2Δ* mutants is not primary due to an enhanced Rad52-mediated recombination repair activity and other factors play a major role in Fcy2-mediated cisplatin cytotoxicity.

Mutations of genes in the *de novo* purine nucleotide biosynthesis pathway modulate cDDP cytotoxicity

Fcy2 and Hpt1 function in the salvage pathway which overlaps and interacts with the *de novo* pathway for AMP and GMP biosynthesis (Figure 2). In the presence of extracellular purines, the *de novo* pathway is repressed by the purine nucleotide end products of the salvage pathway. One of the phenotypes of strains with a mutation in either *FCY2* or *HPT1* genes is derepressed *de novo* synthesis of purine nucleotides (Guetsova et al., 1997). In addition, previous studies have shown that intracellular purines are present in strains with a mutation in *FCY2* (Daignan-Fornier unpublished observations) or *HPT1* gene (Denis et al., 1998) and that cDDP reacts with all nucleobases and nucleotides to varying degrees (Reedijk and Lohman, 1985). It is possible that cDDP cytotoxicity may be compromised by disregulated *de novo* nucleotide synthesis. To test this possibility, we examined previously identified mutants in the purine nucleotide biosynthesis pathway (Figure 2) that also derepresses *ADE* gene expression (Denis et al., 1998) to see whether they exhibit resistance to cDDP. One of these is *ADE4[D]*, which is an activity-enhancing mutation of the *ADE4* gene encoding the glutamine PRPP amidotransferase (GPAT) and functions in the first step of the *de novo* pathway. The other mutant is *guk1* which carries mutation in the *GUK1* gene encoding GMP kinase and results in accumulation of GMP and feedback inhibition of the Hpt1 enzyme encoded by the *HPT1* gene (Escobar-Henriques and Daignan-Fornier, 2001; Lecoq et al., 2000). Figure 3A shows that the *ADE4[D]* mutant exhibited resistance to cDDP to a degree similar to that of the *hpt1Δ* mutant. The

guk1 mutant also exhibited a weak but significant resistant phenotype. Thus, activation of the *de novo* pathway through either the *ADE4^D* mutant or the *guk1* mutant leads to cDDP resistance. We also tested a mutant allele of the *ADE13* gene encoding adenylosuccinate lyase (Figure 2). The *ade13* mutant derepresses *ADE* gene expression but the *de novo* pathway is blocked at two steps (Figure 2) by the mutation, and purine nucleotides are not produced (Rebora et al., 2001). In contrast to the *ADE4^D* and *guk1* mutants that actively synthesize purine nucleotide via the *de novo* pathway, the *ade13* mutant was not resistant to cDDP. Taken together, these data suggest that derepression of *ADE* gene expression alone is not sufficient to cause cDDP resistance, and that elevated production of purine nucleotides by the *de novo* pathway is also important.

To further elucidate the role of the AMP biosynthesis pathway in modulating cDDP cytotoxicity, we tested additional mutants with defects in the *de novo* pathway for their sensitivity to cDDP. It has been shown that expression of the *ADE* genes is low in strains containing mutations that disable the first seven steps of the pathway (*ade4* to *ade1*), while it is constitutively derepressed in strains with mutations that disable later steps [Figure 2, (Rebora et al., 2001)]. We reasoned that if cDDP cytotoxicity is reduced in strains with elevated *ADE* gene expression, such as the *ADE4^D* mutant, mutants that disable later steps would exhibit resistance to cDDP while mutants that disable the first seven steps of the pathway would be expected to be sensitive. We tested several strains in this pathway. Deletion of the *ADE4* gene causes adenine auxotrophy which makes it impossible to assess its sensitivity to cDDP under our conditions (Figure 3B). However, viable strains containing a single deletion of either *ADE16* or *ADE17*, which encode isozymes that function in the last two steps of *de novo* IMP biosynthesis, were more resistant to cDDP than WT cells. Furthermore, it has been previously demonstrated that high expression of yeast AMP biosynthesis genes requires interaction between two transcription factors Bas1 and Bas2 (Rebora et al., 2001). Indeed, we found that mutants

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lacking either the Bas1 or Bas2 proteins were not significantly more resistant to cDDP than WT

cells (Figure 3B).

To address the contribution of the *ADE4* gene in cDDP cytotoxicity further, we examined

in WT cells the effect of overexpressing the *ADE4* gene, which also leads to derepression of the

ADE-genes, increased de novo purine nucleotide production, and purine excretion (Rebora et

al., 2001). As shown in Figure 3C, overexpressing the WT *ADE4* gene also resulted in

resistance to cDDP, at a level similar to that of the $ADE4^D$ dominant mutant. In contrast, over-

expression of the *ADE1* gene, which does not cause derepression of the *ADE* genes and

increased purine nucleotide production (Rebora et al., 2001), did not exhibit cDDP-resistance.

Thus these data indicate that mutations that result in increased *de novo* purine nucleotide

synthesis also confer cDDP resistance. Together, these results further support our hypothesis

that disregulation of the purine nucleotide biosynthesis pathway can modulate cDDP cytotoxicity

in yeast.

Effects of alterations in the purine salvage pathway on cDDP cytotoxicity

It has been reported that the presence of extracellular purines has a cytoprotective for

rat testes cells following cDDP-induced injury (Bhat et al., 2002) and platinum compounds bind

to purine nucleobases (Franska et al., 2005; Kerr et al., 2008; Sigel et al., 2001). We tested the

effect of exogenous purines on the cDDP-induced cytotoxicity in wild-type yeast cells. The wild-

type, *fcy2Δ*, and *hpt1Δ* strains were grown to log phase and spotted on plates containing 120

µM cDDP supplemented with or without adenine, hypoxanthine, or guanine. Figure 4A shows

that the cytotoxicity of cDDP to wild-type cells was remarkably diminished in the presence of

adenine (12.5 µM) or hypoxanthine (12.5 µM) relative to *fcy2Δ* cells. cDDP is known to interact

preferentially to guanine residues (Baik et al., 2003; Franska et al., 2005) and guanine is able to

cause moderate transcriptional repression of adenine biosynthetic genes (Guetsova et al.,

1997). cDDP cytotoxicity to WT cells was also found to be reduced by guanine (12.5 µM). In contrast, addition of cytosine, which is taken up by Fcy2 but is not involved in purine metabolism, had little or no effect on cDDP cytotoxicity in WT cells. These data confirm that addition of extracellular purines is able to protect yeast cells from cDDP-induced cell death. This effect is probably not due to feedback inhibition of *de novo* purine nucleotide synthesis since this would be expected to enhance sensitivity to cisplatin (Figure 3B). Instead, it is possible that exogenously supplied purines are sufficient to effectively neutralize cDDP either extra- or intracellularly before it binds to DNA. The ability of platinum to bind free nucleobases and the formation of resulting complexes has been previously analyzed (Kerr et al., 2008). Furthermore, when pre-complexed with nucleobases the cytotoxicity of platinum-adducts is reduced (Ali et al., 2005). To further confirm the deactivating effect of adenine on cDDP, increasing concentrations of cDDP were pre-incubated with adenine for 24 h before adding to the medium for making the plates. Similar to that shown in Figure 4A, the cytotoxicity of cDDP to wild-type cells was completely abrogated in the presence of both concentrations of adenine (12.5 and 50 µM) (Figure 4B). Together, these results indicate that cisplatin cytotoxicity is greatly compromised by exogenously supplied purines.

Within the cells, hypoxanthine is metabolized through Hpt1 to IMP then to AMP or GMP, and guanine is converted also through Hpt1 to GMP (see Figure 2). In contrast, adenine is metabolized directly into AMP by Apt1 (encoded by the *APT1* gene), or deaminated to hypoxanthine by Aah1 (encoded by the *AAH1* gene, Figure 2) and then transformed to IMP through Hpt1. We have demonstrated that mutation in Hpt1 reduced cDDP cytotoxicity. We next tested whether alterations at the steps in the conversion of salvaged adenine to nucleosides also protect yeast cells from cDDP toxicity. We compared cDDP sensitivity in three mutants: *apt1*, *aah1* and a double *aah1/apt1* mutant, which can take up adenine but cannot metabolize it. Figure 4C shows that neither single mutants nor the double mutant enhanced resistance to cDDP as compared with the *fcy2Δ* strain. These data were somewhat unexpected

since it has been demonstrated while repression of *de novo* AMP synthesis by exogenous adenine is intact in the *aah1* and *apt1* mutants, the regulation is abolished in *aah1/apt1* mutant strain (Guetsova et al., 1997). These data suggest that the major modulators for cDDP cytotoxicity in the yeast purine salvage pathway involve the Fcy2-Hpt1 route and provide possible explanation that *fcy2Δ* and *hpt1Δ* were found most frequently in our original cisplatin resistance screen (Huang et al., 2005).

Accumulation of DNA-bound cDDP but not whole-cell cDDP is significantly compromised in *fcy2Δ* mutants.

Resistance to cDDP in mammalian cells is often accompanied by impaired drug accumulation. Since Fcy2 is a membrane protein functioning as a nucleobase permease and proton transporter and the *fcy2Δ* mutant exhibited higher resistance than that of the *hpt1Δ* cells, we suspected that *fcy2Δ* mutants may have defects in cDDP uptake. To test this possibility the levels of cDDP accumulation in wild-type and *fcy2Δ* cells treated with 100 μM cDDP for 4 h were compared. Figure 5A shows that the level of whole cell accumulation of cDDP in the *fcy2Δ* mutants was mildly reduced as compared with that in wild-type cells. Because of the high standard deviation, the data suggest that Fcy2 does not function as a major cDDP transporter. In addition, although both wild-type and *fcy2Δ* strains were sensitized to cDDP cytotoxicity by the forced expression of Fcy2 (Figure 1), cDDP accumulation was not significantly altered in the Fcy2-overexpressing cells (data not shown).

Because the formation of cDDP-DNA adducts is the major mechanism by which cDDP causes cytotoxicity (Zamble and Lippard, 1995), we then tested the possibility that cDDP-DNA adduct formation was reduced in the cDDP-resistant *fcy2Δ* mutant. As shown in Figure 5B, the amount of cDDP bound to DNA during a 4-h (white bars) or 8-h (grey bars) incubation with 100 μM cDDP in the mutant cells was in fact reduced. The level was only 54 ± 8 (SD) % of that in

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wild-type cells at 4 h and 27 ± 11 (SD) % at 8 h. Thus, reduced cDDP-DNA adduct formation in the *fcy2Δ* mutant provides one of the likely explanations for the enhanced resistance to cDDP.

Since exogenous purines had striking effects on cDDP sensitivity in wild-type cells, we tested whether extracellular purines might somehow prevent cDDP and DNA binding. We measured the amount of cDDP-DNA adduct formation in both wild-type and *fcy2Δ* cells in the presence or absence of 150 µM adenine. As shown in Figure 5C, adenine reduced the accumulation of DNA-bound cDDP in the wild-type cells to 59% of that in the absence of adenine while that bound in *fcy2Δ* cells remained at the same low level seen in the absence of adenine.

Discussion:

Previously we identified the purine-cytosine permease gene, *FCY2*, and the hypoxanthine guanine phosphoribosyl transferase gene, *HPT1*, in a screen for yeast gene deletion strains that are more resistant than wild-type cells to cDDP (Huang et al., 2005). Inactivation of either of these salvage pathway genes derepresses expression of ADE genes, resulting in increased purine nucleotide synthesis via the *de novo* pathway (Denis et al., 1998; Guetsova et al., 1997; Rebora et al., 2001). Here we show that gene mutations in the *de novo* pathway that derepress *ADE* gene expression can also enhance cDDP-resistance, but only in mutants that increase production of purine nucleotides. Our results show that the cDDP-resistant phenotype caused by mutations in specific *ADE* genes (*ADE4, ADE16, ADE17*) is shared with mutations in particular salvage pathway genes (*FCY2, HPT1*). Also, overexpression of *ADE4* causes resistance to cDDP, while overexpression of *FCY2* has the opposite effect, causing sensitivity to the drug. Our data demonstrate that disregulation of specific genes in the purine nucleotide synthesis pathways by mutation or overexpression can modulate cDDP cytotoxicity in yeast.

How might activation of *de novo* purine nucleotide synthesis by gene disregulation enhance cDDP resistance of the cell? The *fcy2Δ* mutant is known to activate *de novo* purine nucleotide synthesis (Guetsova et al., 1997). We found that the level of cDDP-DNA adducts is substantially reduced in the *fcy2Δ* mutant (Figure 5). This suggests that reduced DNA-adduct formation contributes to cellular resistance because cDDP-DNA adducts are believed to be the main cause of cDDP cytotoxicity. cDDP can bind to purines and purine nucleotides (Chaney et al., 2005; Reedijk and Lohman, 1985). Thus, one possible mechanism is that intra-cellular purine nucleotides produced by the activated *de novo* pathway somehow interfere with cDDP binding to DNA. Whether this is a direct effect or whether it serves to activate additional cellular processes that interfere with cDDP binding to DNA is not clear (see below).

In addition to genetic alteration, we showed that cDDP cytotoxicity can be modulated by

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addition of exogenous purines. Wild-type cells became more resistant to cDDP in the presence

of extracellular purines (Figure 4A and 4B). The effect is specific for purines since cytosine,

which can also be transported into the cell by Fcy2 and can bind cDDP, had little or no effect.

cDDP adduct formation with DNA was reduced significantly in wild-type cells treated with

adenine, while the low level of adducts detected in the resistant *fcy2Δ* mutant in the absence of

adenine was not further affected by its presence. The salvage pathway is activated in the wild-

type cells by extracellular adenine but is inactive in the *fcy2Δ* mutant (Guetsova et al., 1997).

Our findings suggest that activation of the salvage pathway in wild-type cells by extracellular

adenine and the resulting formation of intracellular purine nucleotides can lead to cDDP

resistance. cDDP activity might be deactivated intracellularly or extracellularly because the

neutralizing effects of purines were seen in both experiments with (Figure 4B) and without

(Figure 4B) pre-incubation of cDDP with adenine for 24 h before pouring the plates. In addition,

the fact that the cDDP concentration (120 μM) used was in vast excess over that of the adenine

(12.5 μM) suggest that the detoxification exerted by exogenous purines can not be completely

attributed to interactions between cDDP and extracelllar purines alone. It is possible that purine-

cDDP adducts activate additional processes in the cells that somehow limits the reaction of

cDDP with DNA. It has been documented that binding of platinum to intra or extracellular

molecules can affect platinum-DNA adduct formation. For example, glutathione binds platinum

compounds (Jansen et al., 2002) and intracellular inactivation of cDDP by glutathione is one of

the well-known mechanisms of platinum resistance (Ishikawa and Ali-Osman, 1993). In contrast,

it has been recently demonstrated that extracellular carbonate interacts with carboplatin and

enhances its activity (Di Pasqua et al., 2007). Our findings that exogeneous purines and

intracellular production of puine nucleotides are capable of reducing cDDP cytotoxicity in yeast

provide a novel direction for future mechanistic study of cisplatin resistance in human cells.

Purines regulate the concentration of PRPP (5-phosphoribosyl 1-pyrophosphate) (Yoshida and Hoshi, 1984) which is a substrate common to both *de novo* and salvage pathways. PRPP is vital for cell function and cell proliferation through effects on DNA and RNA syntheses and ATP. Anticancer drugs reduce ATP concentration (Martin et al., 2001), resulting in cell stress. Exogenous purines or constitutive adenine nucleotide synthesis enables the cells to quickly replenish the level of intracellular ATP which, in turn, protects cells from drug-induced stress. It is remarkable that *fcy2Δ* cells exhibit reduced DNA platination in the absence of marked change of whole cell uptake. In mammalian cells the major copper influx transporter appears to mediate cisplatin transport via an endocytic process resulting in the accumulation of cDDP in vesicles (Holzer and Howell, 2006). It is possible that purine/nucleotide level might modulate the extent of DNA platination without affecting whole cell accumulation by influencing intracellular transporters that move cDDP out of intracellular vesicles. In addition, the level of purines may affect dNTP pools which are critical for cisplatin-induced DNA repair activity involving DNA polymerases (Chaney et al., 2005). It has been previously shown that depletion of purines in the medium greatly decrease the dATP pool and DNA synthesis in the V79 pur1, a purine auxotrophic mutant of the Chinese hamster lung cell line (Zannis-Hadjopoulos et al., 1980). Others also showed that genes in the nucleotide metabolism, including *purB*, *C/E*, *D*, and *H*, are greatly induced by cisplatin in *Dictyostelium* (Van Driessche et al., 2007). Further, treatment with hydroxyurea, an inhibitor of dNTP synthesis and DNA repair (Collins and Oates, 1987), enhances cisplatin cytotoxicity (Albain et al., 1992). Interestingly, deletion of the P2Y purine receptor gene in *Dictyostelium* also results in resistance to cisplatin (Li et al., 2000). Taken together, these studies support our hypothesis that the level of purines or purine nucleotides is one of the important modulators of cisplatin cytotoxicity.

Our data showing that deletion of *Fcy2* confers a weak protection against cDDP sensitivity of *rad52Δ* cells suggest that Fcy2 plays a minor role in Rad52-mediated DNA repair

of cDDP-induced DNA damage. How mutations in the purine nucleotide synthesis pathway or purine levels affect other DNA repair pathways requires further study. Whether purine levels affect checkpoint responses is also unclear. We have previously shown that *fcy2Δ* mutant is mildly cross-resistant to 5-Fu and doxorubicin (1.5-2.5 fold). However, *fcy2Δ* cells are not sensitive or resistant to camptothecin or MNNG (Huang et al., 2005). In addition, the rates of cell cycle progression in WT and *fcy2Δ* cells in response to cisplatin treatment are similar (data not shown). The implication of these data is that checkpoint response to DNA damage is unlikely to be involved in the cDDP-resistant phenotype of *fcy2Δ* cells.

It has recently been reported that the SAGA/SNF chromatin remodeling complexes are required for the activation of the *ADE* genes (Koehler et al., 2007). Given that, one would expect that defects in the SAGA/SNF chromatin remodeling complexes would result in increased cDDP sensitivity. However, our published data indicate that deletion of the *SNF6* gene also confers cDDP resistance (Huang et al., 2005). We also recently observed that defects in some other, but not all, genes coding for proteins in the SAGA/SNF complexes also confer resistance to cDDP (unpublished observations). Since the SAGA/SNF chromatin remodeling complexes are involved in many transcriptional processes, further studies will be required to delineate the exact roles played by this complex in cDDP cytotoxicity.

Purine nucleotide biosynthesis pathways are critically important for the normal functioning of cells and are conserved between yeast and humans. Whether there is an association between the level of purine nucleotide biosynthesis and resistance to cisplatin chemotherapy in human cancers is unknown. However, it is known that purine overproduction and defects of purine nucleotide biosynthesis enzymes lead to abnormal physical conditions. For example, purine overproduction and uric acid excretion occurs in about 20% of autistic patients (Page and Coleman, 2000) and could indeed be a consequence of purine nucleotide biosynthesis deregulation. Furthermore, human Lesch-Nyhan syndrome results from

inactivation of HPRT, the human functional homologue of the yeast *HPT1*. Patients with a partial

defect in HPRT develop hyperuricemia as a result of failure to salvage purine bases. The lack of

salvage of hypoxanthine and guanine by HPRT results in increased levels of PRPP which then

increases synthesis of purine nucleotides (Rosenbloom, 1968). Much has yet to be learned

about whether purine metabolism can modulate cDDP cytotoxicity in cancer patients. Whether

the activity of the human homologue of the yeast *ADE4* gene, GPAT (glutamine PRPP

amidotransferase), is higher in human cancer cells resistant to cDDP is also unknown. Ironically,

the frequency of mutations in the *HPRT* gene has been often employed to measure the effects

of chemotherapeutic agents used in the treatment of human malignancies including ovarian

cancer (Gercel-Taylor et al., 2005). The cause-and-effect relationship of mutations in the *HPRT*

gene and chemotherapeutic responses to cDDP merits further investigation.

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Legends for figures

Figure 1. (A) Over-expression of Fcy2 protein sensitizes wild-type and *fcy2Δ* cells to cDDP. Wild-type pFCY2G and its mutant form (p*fcy2mg*) fused to a green fluorescence protein carried on the pYES2 vector and the vector control (pYES2G) were expressed in the wild-type (WT, BY4741) and *fcy2Δ* strains. Cells were grown to late log phase in SDM and spotted on plates containing galactose and with or without 120 μM cDDP. Pictures were taken after incubation for 3 days at 30°C. Relative survival (%) was determined by densitometric measurement of the spots on the second column and is expressed relative to untreated (no drug) cells. Data shown are the mean of three independent experiments. Error bars indicates standard deviation and * indicates $P<0.05$ using paired t-test. (B) Deletion of *rad52* confers sensitivity to cDDP in both WT and *fcy2Δ* cells. Log phase yeast cells were diluted serially and spotted on plates with or without cDDP. Data shown are representative of three independent experiments. Bar graph depicts the relative survival (fold) determined by densitometric measurement of the spots on the second column and expressed after normalization to that of the WT cells. Error bars indicates standard deviation.

Figure 2. Schematic representation of purine metabolism in *S. cerevisiae*. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; SAICAR, phosphoribosyl-aminoimidazole-succinocarboxamide; IMP, inosine 5'-monophosphate; SAMP, adenylosuccinate; XMP, xanthosine 5'-monophosphate; AMP, adeninosine 5'-monophosphate; GMP, guanosine 5'-monophosphate. Gene names are italicized and genes tested are indicated with bold italic. For simplicity, some of the steps, products, and nucleosides are not depicted.

Figure 3. (A) Certain mutants that activate ADE gene expression are resistant to cDDP. These mutants were generated through mutagenesis and selected via bypass of repression of ADE gene expression by adenine (Guetsova et al., 1997; Lecoq et al., 2000). PLY121 is the parental strain of $Ade4^D$-dominant, *guk1* (purine excretion) *and ade13* strains. Five-fold serial dilutions of log phase cultures were spotted onto plates with or without 120 μM cDDP and incubated at 30°C for 3 days. Relative survival (%) was determined by densitometric measurement of the spots on the second column and is expressed relative to untreated (no drug) cells. Data shown are the mean of three independent experiments. Error bars indicates standard deviation and * indicates P<0.05 using paired t-test. (B) Cisplatin sensitivity of deletion mutants with defects in the AMP biosynthesis pathway. Relative survival was determined as above. (C) Over-expression of *ADE4* gene confers resistance to cDDP. The wild-type strain BY4741 transformed either with a vector plasmid, or with a plasmid expressing the *ADE4* gene or *ADE1* gene, was spotted on plates with or without cDDP.

Figure 4. (A) Effects of purines and cytosine on cDDP cytotoxicity. The wild-type (WT) strain BY4741, *fcy2Δ*, and *hpt1Δ* strains were spotted on plates with or without 120 μM cDDP and in the presence or absence of different purines (Ade, adenine; Hyp, hypoxanthine; Gua, guanine) or cytosine (Cyt) at a concentration of 12.5 μM. NaCl (saline) was used to dissolve cDDP and as a control, while HCl was the solvent for adenine and cytocine. Relative survival (%) was determined as described in Figure 1 and 3. Data shown are the mean of three independent experiments. (B) Effects of pre-incubation of adenine and cDDP on cDDP cytotoxicity. Adenine (333 or 1205 μM) or equal volume of control solvent (833 or 300 μM HCl) was incubated with cDDP (3.3 mM in 0.9% NaCl solution) for 24 h at room temperature in the dark and added to the SD medium before pouring the plates. The final concentration of cisplatin was 120 or 140 μm and adenine 12.5 or 50.5 μm. Cells were spotted on plates as described in Figure 1 and

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photographed at day 3. (C) Effects of mutations in *APT1* and *AAH1* on cDDP cytotoxicity.

Single mutant and double mutant strains along with their parental strain and *fcy2Δ* mutant were

spotted on plates with or without 120 μM cDDP. Data shown are representative of three

independent experiments

Figure 5. (A) Whole cell cDDP accumulation in wild-type and *fcy2Δ* strains. Wild-type (WT,

BY4741) and *fcy2Δ* cells were treated with 100μM cDDP for 4 h. The amount of platinum in

whole cell extracts was measured using atomic absorption spectrophotometry. Platinum

accumulation was normalized to the protein concentrations and relative level (% of WT) was

determined. Data shown are the mean of 6 independent experiments. Error bars indicates

standard deviation and * indicates $P<0.05$ using paired t-test. (B) DNA-bound cDDP in wild-type

and *fcy2Δ* strains. Cells were treated with 100μM cDDP for 4 h (white columns) or 8 h (grey

columns) and the amount of platinum in HCl-hydrolized genomic DNA was measured using

atomic absorption spectrophotometry. Platinum accumulation was normalized to the DNA

concentrations and the relative level (% of WT) was determined. Data shown are the mean of

three independent experiments. (C) Effects of adenine on the amount of DNA-bound cDDP in

wild-type and *fcy2Δ* strains. Wild-type (WT) and *fcy2Δ* cells were treated with 100 μM cDDP for

4 h in the presence or absence of 150 μM adenine. Platinum accumulation in DNA was

measured by atomic absorption spectrometry. Data shown are the mean of three independent

experiments.

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Table 1. Yeast strains used in this study.

Strain	Genotype	Source
PLY121	*MATα leu2-3,112 ura3-52 lys2-Δ201 his3-Δ200*	P. Ljungdahl
Y127[a]	*MATα leu2-3,112 ura3-52 lys2-Δ201 his3-Δ200 bra11-1*	Rebora et al.
Y1259	*MATα leu2-3 lys2Δ201 ura3-52 his3Δ200 ade13*	Rebora et al.
Y129[b]	*MATα leu2-3,112 ura3-52 lys2-Δ201 his3-Δ200*	Lecoq et al.
BY4741	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura-3Δ0::kanMX4*	EUROSCARF
Y00191	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura-3Δ fcy2::kanMX4*	EUROSCARF
Y04235	*MATa his3-Δ1 leu2-Δ0met15-Δ0 ura-3Δ0 hpt1::kanMX4*	EUROSCARF
Y00888	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 ade4::kanMX4*	EUROSCARF
Y11583	*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 ade16::kanMX4*	EUROSCARF
Y06561	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 ade17::kanMX4*	EUROSCARF
Y06015	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 bas1::kanMX4*	EUROSCARF
Y03803	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 bas2::kanMX4*	EUROSCARF
Y00540	*MATa his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 rad52::kanMX4*	EUROSCARF
PLY122	*MATa leu2-3,112 ura3-52 lys2-Δ201*	P. Ljungdahl
Y511	*MATa leu2-3,112 ura3-52 lys2-Δ201 apt1::URA3*	Guetsova et al.
Y520	*MATa leu2-3,112 ura3-52 lys2-Δ201 aah1::URA3*	Guetsova et al.
Y550	*MATa leu2-3,112 ura3-52 lys2-Δ201 apt1::URA3 aah1::URA3*	Guetsova et al.

[a] Y127 is the *ADE4^{D}* mutant.
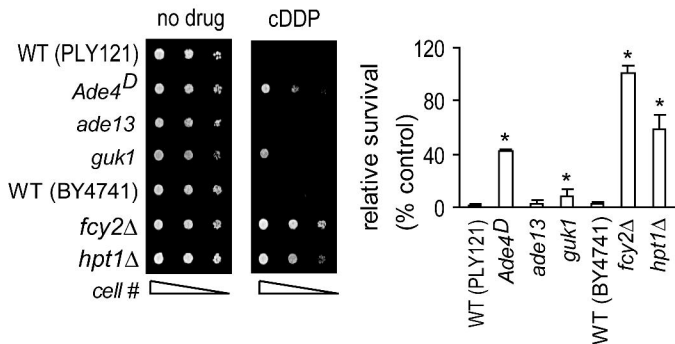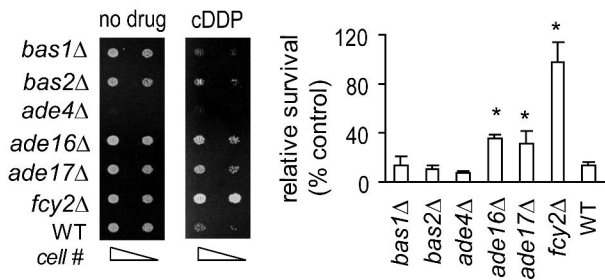
[b] Y129 is the *guk1* mutant.

Figure 1

A

B
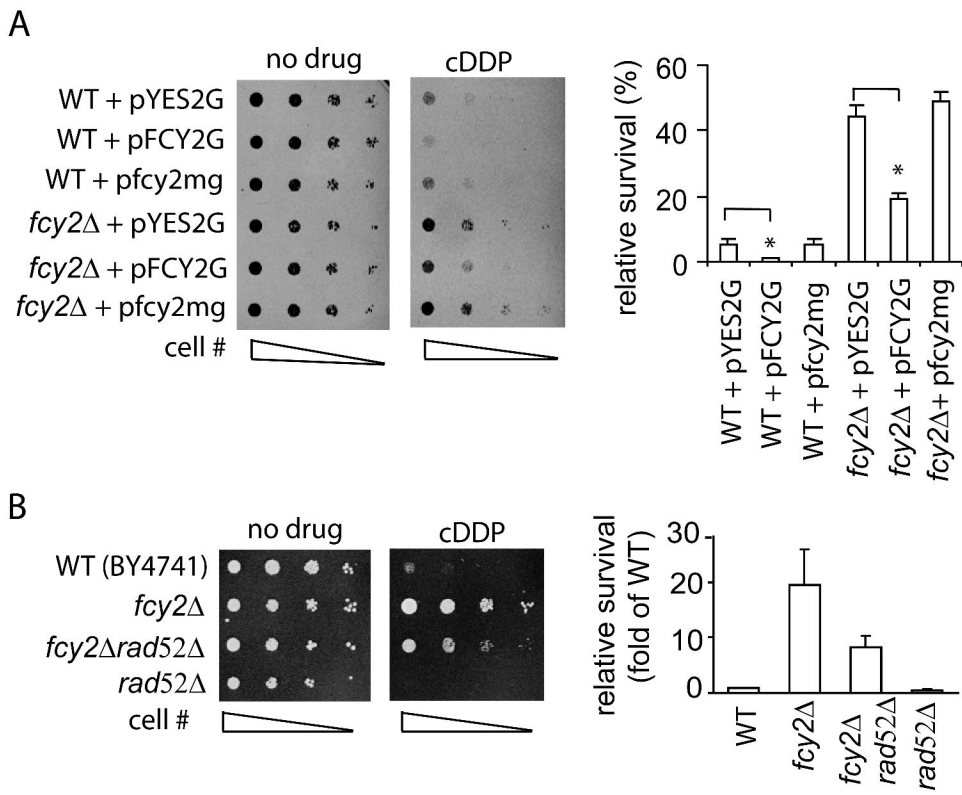
Figure 2

Figure 3

A

B

C

Figure 4

Figure 5