Small Ubiquitin-Related Modifier Pathway Is a Major Determinant of Doxorubicin Cytotoxicity in Saccharomyces cerevisiae

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Abstract

Development of drug resistance is a major challenge in cancer chemotherapy using doxorubicin. By screening the collection of Saccharomyces cerevisiae deletion strains to identify doxorubicin-resistant mutants, we have discovered that the small ubiquitin-related modifier (SUMO) pathway is a major determinant of doxorubicin cytotoxicity in yeast. Mutants lacking UBP2 (SUMO activating enzyme; E1), UBC9 (conjugating enzyme; E2), and ULPI and ULP2 (desumoylation peptidases) are all doxorubicin resistant, as are mutants lacking MLP1, UIP3, and NUP60, which all interact with ULP1. Most informatively, mutants lacking the SUMO E3 ligase Siz1 are strongly doxorubicin resistant, whereas mutants of other SUMO ligases are either weakly resistant (siz2) or hypersensitive (mms21) to doxorubicin. These results suggest that doxorubicin cytotoxicity is regulated by Siz1-dependent sumoylation of specific proteins. Eliminating SUMO attachment to proliferating cell nuclear antigen or topoisomerase II does not affect doxorubicin cytotoxicity, whereas reducing SUMO attachment to the bud neck–associated septin proteins has a modest effect. Consistent with these results, doxorubicin resistance in the siz1Δ strain does not seem to involve an effect on DNA repair. Instead, siz1Δ cells accumulate lower intracellular levels of doxorubicin than wild-type (WT) cells, suggesting that they are defective in doxorubicin retention. Although siz1Δ cells are cross-resistant to daunorubicin, they are hypersensitive to cisplatin and show near WT sensitivity to other drugs, suggesting that the siz1Δ mutation does not cause a general multidrug resistance phenotype. Cumulatively, these results reveal that SUMO modification of proteins mediates the doxorubicin cytotoxicity in yeast, at least partially, by modification of septins and of proteins that control the intracellular drug concentration. [Cancer Res 2007;67(2):765–72]

Introduction

The anthracycline doxorubicin is a topoisomerase II (Top2) inhibitor (1) that also inhibits DNA and RNA synthesis (2, 3) and produces ssDNA breaks (4, 5) and membrane damage (6). Doxorubicin is a major component of chemotherapy regimens for many cancers, including solid tumors, leukemias, and lymphomas (2, 3). However, the efficacy of doxorubicin-based regimens is compromised by the frequent occurrence of resistance, which can be either primary or acquired. Resistance to doxorubicin can occur by several mechanisms, including alteration of Top2 expression or activity (7), overexpression of classic and nonclassic pleiotropic drug/multidrug resistance (PDR/MDR) proteins (8), detoxification by glutathione transferase (9), and enhanced DNA repair (10). MDR is the major contributor to clinical resistance to anthracyclines identified to date, although evidence for the other mechanisms of resistance is less clear. In addition, doxorubicin causes cardiotoxicity in patients that has been linked to doxorubicin-induced generation of reactive oxygen species and the resulting damage to mitochondrial DNA (11).

To gain further insight into the mechanisms of doxorubicin resistance, we sought to identify additional genes and/or molecular pathways that participate in doxorubicin cytotoxicity. We have previously used a one-step selection method to screen the collection of deletion mutants generated by the Yeast Genome Deletion Project for nonessential genes that, when deleted, confer cisplatin resistance (12). In that study, we found that some deletion strains were cross-resistant to certain other cytotoxic agents, including doxorubicin, but were hypersensitive to others. These findings prompted us to screen for yeast mutants that are resistant to doxorubicin. Here, we report the analysis of the most prevalent pathway identified in the screen: the small ubiquitin-related modifier (SUMO) pathway.

Like ubiquitin, SUMO functions by becoming attached to other proteins as a post-translational modification. The SUMO family in vertebrates includes three distinct proteins, SUMO-1, SUMO-2, and SUMO-3, whereas Saccharomyces cerevisiae has a single SUMO gene (SMT3; ref. 13). The enzymatic cascade for attaching SUMO resembles the ubiquitin pathway but uses a distinct set of E1 (activation), E2 (conjugation), and E3 (ligation) enzymes (13). The E1 for activating SUMO is a heterodimer of Aos1 and Uba2 (also called Sae1-Sae2 in mammalian systems; ref. 13). Ubc9 is the only E2 enzyme identified for SUMO (13). E3 ligases collaborate with Ubc9 to confer substrate specificity on sumoylation. S. cerevisiae contains four known SUMO E3s: Siz1, Siz2/Nfi1 (13), Mms21 (14), and the meiotic E3 Zip3 (15). There are at least four types of SUMO E3 ligases in mammalian cells: the protein inhibitors of activated signal transducers and activators of transcription (PIAS) family, the nucleoporin RanBP2/Nup358, the polycomb group protein Pc2, and MMS21 (13, 16). Yeast Siz1 and Siz2 are members of the PIAS family. Sumoylation is a reversible modification, and a family of SUMO-specific proteases cleaves SUMO from protein conjugates and also generates mature SUMO from SUMO precursors (13). The SUMO-specific proteases in S. cerevisiae are ULP1 and ULP2/SMT4. In S. cerevisiae, most of the genes (SMT3, AOS1, UBA2, UBC9, MMS21, and ULPI) involved in sumoylation are essential for viability, although SIZ1, SIZ2, and ULP2 are not. SUMO conjugation is also required for viability of most other organisms (13, 17). The essential function of SUMO in yeast is unknown, but sumoylation has a variety of roles in maintaining genome integrity, including
roles in DNA repair pathways, chromosome cohesion, nucleolar and telomere structure, and recovery from checkpoint arrest induced by DNA damage or DNA replication defects (13). In our genome-wide doxorubicin resistance screen, the SIZ1 gene, which encodes a SUMO E3 ligase, was found most frequently. Further analysis indicated that mutations of other genes in the SUMO pathway, including the desumoylation enzymes, also confer doxorubicin resistance. Our data suggest that the SUMO pathway affects doxorubicin cytotoxicity at least partially through modification of septins as well as of proteins that control retention of the drug.

**Materials and Methods**

**Yeast strains and media.** The individual yeast strains used in this study are listed in Supplementary Table S1. Homozygous diploid deletion pool strains and individual haploid deletion strains (MATa met15-1 his3-11,15 leu2-3,112 lys1-1 ura3-1) generated by the Saccharomyces Gene Deletion Project (18) were obtained from Invitrogen (Carlsbad, CA) or EUROSCARF (Frankfurt, Germany). Standard yeast media and growth conditions were used (19). Yeast cultures were seeded from single colonies grown on yeast extract-peptone-dextrose (YPD) plates or synthetic defined yeast nitrogen base medium (SDM) supplemented with appropriate amino acids for the strain background at 25°C or 30°C. **Chemicals.** Yeast nitrogen base, yeast extract, peptone, and dextrose were purchased from Difco Laboratories (Detroit, MI). Doxorubicin, daunorubicin, dianorubicin, 5-fluorouracil (5-FU), camptothecin, etoposide and irinotecan, and cisplatin were obtained from Sigma-Aldrich (St. Louis, MO) or KLB Laboratory (St. Paul, MN). Stock solutions were prepared as follows. Doxorubicin, daunorubicin, camptothecin, etoposide, and irinotecan were made in DMSO and 5-FU in water and were stored at −20°C. Cisplatin was prepared in DMSO, stored as aliquots at −20°C (less than 2 weeks), and further diluted in 0.9% NaCl before adding to the medium. All plates were made in SDM or YPD and stored in the dark and used within 24 h. All other chemicals are from Sigma-Aldrich.

**High-throughput doxorubicin resistance screen.** A one-step selection method was used to screen the pool of deletion strains to isolate mutants that grew on SDM plates containing high concentrations of doxorubicin as described previously (21). Yeast cultures were purchased from Difco Laboratories and divided by the spot intensity of the corresponding untreated cells to identify yeast genes whose inactivation confers resistance to doxorubicin, we screened the mutant collection from the Yeast Genome Deletion Project for strains that are resistant to doxorubicin (see Materials and Methods). Of the genes identified, the SIZ1 gene, encoding a SUMO E3 ligase, was found most frequently (~16% of the colonies screened). Four other genes functionally related to the SUMO pathway were also found in the screen with a lower frequency (0.4–3%). These were the SIZ2 gene, which encodes another PIAS family E3 ligase, and three genes, MLP1, NUP60, and UTP3, which encode proteins that interact with the desumoylation enzyme Ulp1 (24, 25). The nucleoplasmin Nup60 and the nuclear pore-associated protein Mlp1 are required to localize and stabilize the desumoylation enzyme Ulp1 at the nuclear pore complex. Loss of either affects the level of Ulp1 and the pattern of SUMO conjugates (25). Several genes unrelated to the SUMO pathway were also obtained in the screen and will be described elsewhere.

To confirm that the doxorubicin resistance of these strains was due to the disruption of the intended genes, not to unrelated mutations acquired during the screening procedure, strains from the deletion collection were retested for doxorubicin resistance using a spot assay. haploid strains were examined because we found that lower levels of drugs were required to achieve similar degrees of toxicity compared with diploid strains. Figure 1A shows that siz1Δ, siz2Δ, mlp1Δ, and nup60Δ strains exhibited an approximately 1.6- to 6.5-fold resistance to doxorubicin (42.5 μmol/L) compared with the haploid WT strain BY4741 in a measure of spot growth. Thus, we have identified five genes in the SUMO pathway that, when deleted, confer resistance to doxorubicin.

We next asked whether defects in the other genes in the yeast SUMO pathway, such as UBA2, UBC9, MMS21, ULP1, and ULP2, also
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Figure 1. A, doxorubicin (DOX) resistance of individual deletion strains. Five-fold serial dilutions of exponentially growing WT (BY4741) and deletion mutant strains were spotted onto YPD plates with or without various concentrations of doxorubicin. Plates were photographed after 2 to 3 d of incubation at 30 °C. Representative pictures from plates containing 42.5 μmol/L doxorubicin. Percentage growth of strains is determined by densitometric measurement of the spots on the second column and is expressed relative to untreated cells. B, doxorubicin sensitivity of mutants in the SUMO pathway. JD52 is the parental strain of siz1Δ, siz2ΔT, siz1Δ, siz2Δ, mms21, smt3-allR, DF5 is the parental strain of ubc9ts and ulp1ts strains; DF5 is the parental strain of ubc9ts and ulp1ts strains. Five-fold serial dilutions of yeast cells were spotted on plates with or without doxorubicin (25 μmol/L) and incubated at 30 °C (semipermissive temperature for the temperature-sensitive strains) or 25 °C for 2 or 3 d, respectively. C, overexpression of Smt3 confers resistance to doxorubicin. WT (BY4741) and siz1Δ strains expressing the Smt3 protein under the control of the Gal10 promoter or the empty vector were diluted serially and spotted on plates with glucose (Glu) or galactose (Gal) and supplemented with or without 42.5 μmol/L doxorubicin.

Effect of siz1 on doxorubicin-induced sumoylation. Because the three SUMO E3 ligases have dramatically different effects on doxorubicin cytotoxicity, we wanted to examine cellular sumoylation patterns in the E3 ligase mutant strains. Whole-cell lysates from log-phase WT, siz1Δ, siz2Δ, and mms21 mutant cells were analyzed by SDS-PAGE and immunoblotting. The majority of the SUMO conjugates seen in WT cells were not present in the siz1Δ cells (Fig. 2), in agreement with published data (28). In contrast, SUMO conjugates in the siz2Δ and mms21 mutant strains were very similar to those in the WT strain. It has been shown that oxidative and ethanol stresses (29) and MMS (30) induce sumoylation of certain proteins in yeast. We next examined whether doxorubicin induces sumoylation and whether deletion of ULP2 affects doxorubicin cytotoxicity. With the exception of ULP2, these genes are essential for viability and, thus, are not present in the nonessential gene deletion collection used in our initial screen. Strains tested were temperature-sensitive mutants in uba2, ubc9, and ulp1, a point mutant in mms21, ulp2Δ, and a SMT3-allR strain, in which all lysines in yeast SUMO were replaced with arginine. The SMT3-allR strain has a defect in SUMO chain formation (26). These mutants and their parental strains were tested using the spot assay at both permissive (25 °C) and semipermissive (30 °C) temperatures. In addition to allowing growth of temperature-sensitive mutants, doing the assay at 25 °C increased the apparent doxorubicin sensitivity of the WT strain (Fig. 1B). This experiment showed that, first, SIZ2 does not play a significant role in doxorubicin sensitivity because deletion of this gene conferred only weak resistance (especially at 25 °C) and because no significant increase in resistance was observed when SIZ2 was deleted in combination with SIZ1. Second, mutation of MMS21, which encodes the third SUMO E3 ligase, did not confer resistance to doxorubicin. Instead, it resulted in hypersensitivity to doxorubicin treatment. Finally, mutations in all other genes in the SUMO pathway, including the activating enzyme (uba2ts), conjugating enzyme (ubc9ts), and desumoylation enzymes (ulp2Δ and ulp1ts), caused doxorubicin resistance at 25 °C. It has been shown that uba2ts and ubc9ts mutants have reduced SUMO conjugates even at 25 °C (20, 27). We were somewhat surprised that mutants with both decreased and increased global sumoylation all exhibited the doxorubicin resistance phenotype. However, ulp2Δ and ulp1ts mutants have complex effects on sumoylation because of their roles both in generating free SUMO and in cleaving SUMO conjugates. Consequently, in each of these mutants, sumoylation of some proteins is increased whereas that of others is decreased. To test whether oversumoylation can result in doxorubicin resistance, yeast SUMO was overexpressed under the control of a GAL10 promoter. This technique increases levels of many SUMO conjugates.4 We found that increased levels of SUMO in WT cells also resulted in doxorubicin resistance (Fig. 1C). Finally, a defect in the SUMO chain formation (Smt3-allR) conferred only weak resistance (Fig. 1B), consistent with a previous finding that chain formation does not play an essential role in growth or stress responses in yeast (26). Thus, our results showed that many different perturbations of the SUMO pathway result in doxorubicin resistance. Because deletion of the SIZ1 gene exhibited the most significant resistance to doxorubicin, we focused on identifying the possible mechanisms by which Siz1 mediates doxorubicin cytotoxicity.

4 Orr and Johnson, unpublished data.
The HA-tagged Top2 strain and the mutant top2-SNM-HA (21) that carries mutations at the sumoylation sites were first analyzed for sensitivity to doxorubicin. As a reference, we used the top2-4ts allele, which has defects in chromosomal segregation at the nonpermissive temperature (35°C; ref. 36) and is resistant to doxorubicin (37). Figure 3A shows that the top2-4ts strain was indeed resistant to doxorubicin at the semipermissive temperature (30°C), whereas mutation of the SUMO attachment sites in Top2 did not affect doxorubicin cytotoxicity in either the WT or siz1A background. Thus, reduced sumoylation of Top2 is not responsible for the doxorubicin resistance phenotype in siz1A mutant.

Septin cytoskeletal proteins form filaments at the yeast bud neck, and the septins Cdc3, Cdc11, and Shs1/Sep7 are the most abundant SUMO conjugates in G2-M-arrested yeast cells (31). SIZ1 is absolutely required for SUMO attachment to septins in vivo (28). To test whether sumoylation of septins has any effect on doxorubicin resistance, doxorubicin sensitivity was tested in a mutant (cdc3-1294-HA) carrying a deletion of the 94 NH2-terminal residues of Cdc3, which harbors its four major SUMO attachment sites, and in a mutant carrying mutations at the major SUMO attachment sites of all three sumoylated septins (cdc3-4,11,30,63-HA, cdc11-R412-HA, shs1-R426, 437-HA; ref. 31). We found that these septin mutants were slightly resistant to doxorubicin compared with WT but were much less resistant than siz1A cells (Fig. 3B). We inferred from these results that SUMO modification of septins mediates some aspects of doxorubicin cytotoxicity but that SUMO attachment to other proteins is also involved.

PCNA (Pol30) is an essential processivity factor for DNA replication and repair (30). PCNA is both ubiquitylated and sumoylated on treatment of cells with the DNA-damaging agent MMS. PCNA has two SUMO conjugation sites: K127 and K164 (30), where K164 is also the site of RAD6-mediated ubiquitylation. Ubiquitylation at this site is required for bypass of replication-blocking lesions (30, 38, 39), whereas sumoylation acts separately from ubiquitylation and inhibits recruitment of the Rad52 pathway recombination machinery. We found that mutations at K127 (pol30/K127R), K164 (pol30/K164R), or both sites (pol30/K127R/K164R) of PCNA confer hypersensitivity to doxorubicin (Fig. 3B). Deletion of the SIZ1 gene in these PCNA mutant strains resulted in a resistance phenotype similar to that of siz1A cells (Fig. 3B). Together, these results show that neither sumoylation of PCNA at either site nor ubiquitylation at K164, which is also eliminated by the lysine to arginine mutation, is involved in the resistance of siz1A cells to doxorubicin.

During the course of these experiments, we also tested the effect of siz1A on doxorubicin sensitivity of rad6:jA and rad52:jA mutants because sumoylation and ubiquitylation of PCNA have effects on both the RAD6- and RAD52-dependent DNA repair pathways. Both rad6:jA and rad52:jA mutants are hypersensitive to doxorubicin, but remarkably, deleting SIZ1 completely suppressed the hypersensitivity of both of these mutants (Fig. 3C). In fact, the siz1A rad6:jA and siz1A rad52:jA mutants were nearly as resistant to doxorubicin as the siz1A strain. These results indicate that doxorubicin-dependent DNA damage is dramatically reduced in these mutants and suggest that the siz1A mutation is affecting a step upstream from DNA damage, possibly by reducing the ability of doxorubicin to affect its intracellular target(s).

Doxorubicin-induced cell cycle arrest is slightly compromised in the siz1 deletion strain. In mammalian cells, doxorubicin induces cell cycle arrest in the G2-M phase (40). We wanted to test whether doxorubicin causes a G2-M arrest in WT
yeast and, if so, whether siz1A cells respond differently. WT and siz1A cells were grown to log phase and treated with doxorubicin for 12 h. FACS analysis showed that WT and siz1A cells had similar cell cycle profiles in the absence of doxorubicin (Fig. 4A; Supplementary Table S2). In the presence of doxorubicin, a portion of the WT cells accumulated in the S and G2-M phases. Although some siz1A cells also accumulated at S and G2-M phases in response to doxorubicin treatment, the fraction of siz1A cells arrested in S phase were significantly lower than that of WT cells (Fig. 4A; Supplementary Table S2). Thus, the doxorubicin resistance of siz1A cells is associated with reduced cell cycle arrest.

Deletion of the SIZ1 gene impairs doxorubicin retention. The results that siz1A strongly suppresses the doxorubicin hypersensitivity of DNA repair mutants and reduces doxorubicin-induced cell cycle arrest, combined with the observation that sumoylation of the plasma membrane-associated septin proteins plays a role in doxorubicin cytotoxicity, led us to test whether siz1A cells exhibit a defect in doxorubicin uptake or retention. The cellular content of doxorubicin, which is fluorescent, was measured in WT and siz1A cells by flow cytometry. As shown in Fig. 4B, there was little difference in the MFI between the WT and siz1A mutant cells treated with doxorubicin for 1 h, suggesting that siz1A cells had no defects in drug uptake. In addition, the rate of doxorubicin efflux was similar in WT and siz1A cells because the MFIs measured at 1 h (Fig. 4B; data not shown) were similar in both strains. However, when the cells were treated for 4 or 6 h, the MFI in the siz1A mutant was significantly lower than that in WT cells. The MFI measurement was confirmed by the D values as calculated by the Kolmogorov-Smirnov statistic (see Materials and Methods). The D values were 0.2 at 4 h and 0.3 at 6 h (Fig. 4B), which are considered as significant. The difference in MFI between the siz1A and WT cells at these time points was not due to cell death in WT cells because the amount of cells stained with propidium iodide, which is indicative of leaky membrane, was similarly low (~13%) in both strains. These data, in conjunction with the fact that treatment with doxorubicin for 1 h did not increase significant amount of SUMO conjugates (Supplementary Fig. S3), suggest that doxorubicin resistance of siz1A cells is, at least in part, due to the impairment of drug accumulation.

To investigate whether doxorubicin resistance in the siz1A mutant is related to increased activity of PDR/MDR ATP-binding cassette (ABC) drug transporters, we asked whether mutations in yeast PDR/MDR genes affect this phenotype. The yeast ABC transporters with the best-characterized roles in drug resistance are Pdr5 and Snq2, although yeast contains 14 other full-length ABC cassette (ABC) drug transporters with roles in drug resistance and other processes. Recent mass spectrometry data identified Pdr5 as a SUMO substrate (34). Furthermore, a yeast mutant lacking PBD5 is sensitive to doxorubicin (Fig. 4C; ref. 41), showing that Pdr5 does mediate doxorubicin efflux. In contrast, the snq2Δ mutant was not hypersensitive to doxorubicin (Fig. 4C, top), indicating a different substrate specificity. To test whether Pdr5 is responsible for doxorubicin resistance in siz1A cells, we deleted the PDR5 gene in the siz1A strain. Figure 4C (bottom) shows deletion of the PDR5 gene partially enhanced doxorubicin cytotoxicity in siz1A cells, although the siz1AΔpdr5Δ strain was still more resistant than the WT strain. This experiment does not conclusively address whether higher Pdr5 activity partially contributes to the doxorubicin resistance of siz1A cells because pdr5Δ increased the sensitivity of both WT and siz1A cells. To further address this issue, we next tested sensitivity of siz1A mutant to the known
Pdr5 substrate cycloheximide. Increased Pdr5 activity results in cycloheximide resistance (42), so if the doxorubicin resistance of the siz1Δ strain is partially mediated by elevated Pdr5 activity, this strain should also show cycloheximide resistance. However, the siz1Δ strain was not resistant to cycloheximide (Fig. 4D). Together, the results that siz1Δ is sensitive to cycloheximide and that the siz1Δpdr5A strain still shows significant doxorubicin resistance clearly show that factors other than Pdr5 are involved in the doxorubicin resistance of the siz1Δ mutant.

**Sensitivity of the siz1Δ mutant to other cytotoxic agents.** One characteristic of PDR/MDR phenotypes is cross-resistance to different classes of cytotoxic agents. We next examined whether siz1Δ cells have this phenotype by treating WT and siz1Δ cells with several clinically used anticancer agents: the Top2 inhibitors daunorubicin and etoposide (VP16), the Top1 inhibitors camptothecin and irinotecan (CPT-11), cisplatin, and 5-FU. The results are summarized in Table 1. Although siz1Δ cells were cross-resistant to daunorubicin, they were hypersensitive to cisplatin. In addition, the siz1Δ mutant and the WT cells were equally sensitive to 5-FU. Because the yeast strains used were relatively insensitive to VP16, camptothecin, and CPT-11 even at an extremely high concentration (e.g., 1 mmol/L camptothecin), we introduced into the WT and siz1Δ mutant cells a targeted disruption of the ERG6 (ISE1) gene to ablate ergosterol biosynthesis, which has been shown to enhance permeability to some anticancer drugs (43). erg6Δ cells exhibited slow growth but showed significantly enhanced sensitivity to these drugs (Table 1; Supplementary Fig. S2). Curiously, the siz1Δ mutation seemed to partially suppress the slow growth phenotype of the erg6Δ mutant. These experiments showed that, although siz1Aerg6Δ cells were 20-fold more resistant than erg6Δ cells to doxorubicin, they were approximately equally sensitive to VP16 and CPT-11 and were, if anything, slightly hypersensitive to camptothecin (3-fold). Thus, these data suggest that reduced global sumoylation may play different roles in mediating the cytotoxicity of different anticancer agents.

**Discussion**

The present study provides the first demonstration that defects in many steps of the SUMO pathway result in resistance to doxorubicin treatment. Alterations in the SUMO conjugation machinery have been shown to cause hypersensitivity to a variety of DNA-damaging agents, such as Top1 poisons, UV light, HU, or MMS (21, 44–46), but this is the first example of drug resistance in SUMO pathway mutants. Interestingly, defects in either sumoylation (siz1Δ, siz2Δ, ssa2ts, and uba9ts) or desumoylation (ulp1ts, ulp2Δ, nap60Δ, mlp1Δ, and ulp3Δ) cause resistance to doxorubicin as does overexpression of SUMO. Similar apparently contradictory results have been reported previously. For example, overexpression of both SUMO (SMT3) and ULP2 (SMT4) suppresses the growth defects of a mif2 mutant (which encodes a centromere protein; ref. 47). This is probably partly related to the fact that ulp2Δ and ulp1ts mutants each strongly increase sumoylation of some proteins while decreasing sumoylation of others (26, 46, 48). Thus, it seems most likely that there is a protein or proteins that must undergo Siz1-dependent sumoylation for cells to exhibit WT doxorubicin sensitivity and that sumoylation of this protein is also reduced in the ulp2Δ and ulp1ts mutants. An alternative possibility is that both undersumoylation and oversumoylation of the same or different protein(s) result in the same phenotype, possibly by

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**Figure 4.** A, cell cycle progression of WT and siz1Δ cells treated with doxorubicin. DNA content (N) was measured in log-phase haploid yeast cells treated with doxorubicin for 12 h by FACS. B, doxorubicin retention and efflux in WT and siz1Δ cells. Cells were treated for 1, 4, or 6 h and washed twice with phosphate buffer. The MFI was measured with a FACScan flow cytometer. For efflux assay (1 hr-ef), an aliquot of the washed, 1-h treated cells was returned to drug-free medium for another hour and FACS analysis was done. Columns, mean of three independent experiments; bars, SD. Asterisk, significant difference in D values as calculated by Kolmogorov-Smirnov statistic as described in Materials and Methods. C, deletion of Pdr5 confers sensitivity to doxorubicin in both SIZ1 and siz1 cells. Log-phase yeast cells were diluted serially and spotted on plates with or without doxorubicin. Deletion of Sqq2 in SIZ1 cells was included for comparison. D, deletion of SIZ1 does not confer resistance to cycloheximide. Log-phase yeast cells (siz1Δ, pdr5Δ, and siz1Δpdr5Δ) were diluted serially and spotted on plates with or without cycloheximide (CHX) for 2 to 3 d. Data are representative of three independent experiments.
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Table 1. Relative resistance and sensitivity of siz1A mutant to various cytotoxic agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Doxorubicin</th>
<th>Daunorubicin</th>
<th>Cisplatin</th>
<th>5-FU</th>
<th>VP16</th>
<th>Camptothecin</th>
<th>CPT-11</th>
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<tr>
<td>Fold</td>
<td>+6.5</td>
<td>+4.5</td>
<td>−7.5</td>
<td>1</td>
<td>−1.4</td>
<td>−3</td>
<td>+1.3</td>
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NOTE: Sensitivity to doxorubicin, daunorubicin, cisplatin, and 5-FU was assessed in isogenic BY4741 (WT) and siz1A cells using a semiquantitative spot assay. Sensitivity to VP16, camptothecin, and CPT-11 was assessed in erg6Δ cells with or without deletion of the Siz1. Fold represents fold resistance (+) or sensitivity (−) relative to the corresponding WT cells and was calculated using the percentage growth from duplicate spots at the concentration at which 10% to 20% (doxorubicin, daunorubicin, cisplatin, 5-FU, and camptothecin) or 70% to 80% (VP16 and CPT-11) of the WT (SIZ1+) cells survived. A relative sensitivity of +1 represents similar sensitivity in the WT and siz1A strains. The values were derived from averages of at least three independent experiments. Concentrations of the drugs were as follows: doxorubicin, 425 μmol/L; daunorubicin, 44 μmol/L; cisplatin, 100 μmol/L; 5-FU, 190 μmol/L; camptothecin, 0.5 mmol/L; VP16 (etoposide), 0.25 mmol/L; and CPT-11 (irinotecan), 75 μmol/L.

References


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disrupting a cycle of sumoylation and desumoylation that is necessary for a doxorubicin-related process to occur (13, 49). These are questions that will be addressed once the relevant SUMO substrate(s) has been identified.

SUMO modifies hundreds of yeast proteins that participate in diverse cellular processes (13), so it is not immediately apparent which substrate is involved in doxorubicin resistance. Many SUMO conjugates are absent in the siz1A mutants, and it is not known how many of these mediate cytotoxic activities of anticancer drugs. Our studies clearly show that septins are among the Siz1 substrates that mediate the cytotoxic effect of doxorubicin, but the mechanism is not clear. In yeast, septins form a ring at the bud neck that is required for normal bud growth and cytokinesis serves as a scaffold for binding of many other proteins and forms a barrier in the plasma membrane between the mother cell and the bud (50). In addition, septins are involved in the control of secretion, particularly vesicle fusions and exocytosis (51). It is tantalizing to speculate that sumoylation of septins may be involved in doxorubicin retention or intracellular trafficking. The septin mutants examined here, however, did not have defects in the uptake of doxorubicin (data not shown). Furthermore, the mutant lacking septin sumoylation is only weakly doxorubicin resistant, showing that other SUMO substrates are also involved.

Our data also suggest that reduced sumoylation of Top2 and PCNA does not contribute to resistance to doxorubicin. It has been shown that the formation of human SUMO-1-Top2 conjugates occurs in leukemia CEM cells but not in CEM/VM-1 cells, which are resistant to the Top2 poison VM-26 (52). However, it is unclear whether this is due to a defect in the sumoylation pathway in VM-26-resistant cells, a decrease in the amount of Top2 to be sumoylated, or whether drugs simply are not retained in these resistant cells.

Finally, our data suggest that reduced doxorubicin retention, but not uptake nor immediate drug efflux, is at least partially responsible for its doxorubicin resistance in the siz1A mutant, but the mechanism for this defect remains to be determined. There are several possibilities. One is that siz1A increases the level or activity of ABC transporters. We tested the two major yeast transporters involved in drug efflux, Pdr5 and Sng2, and found that they are not major contributors to doxorubicin resistance in siz1A cells. A snq2A mutant was not hypersensitive to doxorubicin, suggesting that it does not mediate doxorubicin efflux. The pdr5A mutant was hypersensitive to doxorubicin, and the pdr5Asiz1A double mutant was more drug sensitive than the siz1 single mutant, but two lines of evidence argue against Pdr5 being a major factor in doxorubicin resistance in siz1A cells. First, siz1A cells remained more resistant than the WT strain to doxorubicin after deleting PDR5 (Fig. 4C), showing that other factors are at least partially responsible for the resistance. Second, siz1A cells were distinctively cross-resistant only to daunorubicin but not to other classes of anticancer agents (Table 1; Supplementary Fig. S2) or to the known Pdr5 substrate cycloheximide (Fig. 4D). Increased activity of Pdr5 should result in cycloheximide resistance. These data correlate with the presence of immediate drug efflux activity (Fig. 4B), possibly mediated by the major drug efflux pump Pdr5, in siz1A cells. Yeast also contains 14 other full-length ABC transporters, some of which could mediate the SUMO pathway effect on doxorubicin cytotoxicity. Some of these genes are coregulated transcriptionally (53), making the transcriptional regulation another potential target for the SUMO pathway. Further studies are needed to determine which of these mechanisms are in effect and to identify other proteins whose sumoylation status affects doxorubicin cytotoxicity.
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