The Na\textsuperscript{+}/H\textsuperscript{+} Exchanger Nhx1 of \textit{Saccharomyces Cerevisiae} is Essential to Limit Drug Toxicity

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Abstract

Nhx1 is an intracellular Na\textsuperscript{+}/H\textsuperscript{+} exchanger localized to the late endosomes and believed to play a major role in pH-mediated vesicle trafficking, as \textit{nhx1}\textsuperscript{Δ} mutant is defective in maintaining the intracellular pH in the vacuoles and cytoplasm when grown in low osmolarity media. In this work, we report novel drug sensitivities of the \textit{nhx1}\textsuperscript{Δ} mutant to a range of cationic and anionic agents when cells are grown in rich media. Unlike the low osmolarity media, the \textit{nhx1}\textsuperscript{Δ} mutant showed no sensitivity to salt. We further show that the drug phenotypes of the \textit{nhx1}\textsuperscript{Δ} mutant, as well as the secretion of the vacuolar protein carboxypeptidase Y, were not rescued by either altering the pH or salt concentration. Genetic analysis reveal that the \textit{nhx1}\textsuperscript{Δ} mutant displayed distinct drug phenotypes in comparison to mutants that are defective in retrograde trafficking from the prevacuole to the late Golgi, excluding the possibility that the drug sensitivity of the \textit{nhx1}\textsuperscript{Δ} mutant is related to retrograde trafficking.

Keywords: Bleomycin, Endocytic pathway, Prevacuolar compartment, Golgi, Transporters
INTRODUCTION

Bleomycin (BLM) is a glycopeptide antibiotic which acts by damaging the DNA [3]. It is effective in treating various cancers including testicular and non-Hodgkin’s lymphomas [2]. However, it is unclear how certain cancers such as colon carcinoma remain refractory to BLM therapy. To better understand the cellular response to BLM, we previously conducted a genome-wide screen with the *Saccharomyces cerevisiae* haploid mutant collection to identify mutants that exhibit resistance or hypersensitivity to the drug when grown in rich media [2]. Our screen identified several genes encoding proteins that are involved in many biological processes including DNA repair and chromatin remodeling, and the proper functional maintenance of the mitochondria and vacuoles [2]. The group consisting of the largest number of BLM-sensitive genes belongs to the endosomal pathway [2]. Deletion of any one of these genes resulted in varying sensitivity to BLM implying that this pathway plays a crucial role in preventing the toxicity of the drug [2]. Indeed, we show that fluorescently labeled bleomycin is transported and accumulate into the vacuoles [3].

We decided to conduct additional characterization of one of the endosomal pathway genes, namely *NHX1*, as deletion of this gene resulted in mutant with severe sensitivity to BLM (unpublished data). Nhx1 encodes an intracellular Na⁺/H⁺ exchanger, which is located in the late endosomes/prevacuolar compartment [15]. It is involved in regulating vesicle trafficking out of the prevacuolar compartment to the vacuoles [6]. Nhxl is also required to mediate vacuolar sequestration of Na⁺ and K⁺ by coupling their transport to the H⁺ gradient created by the vacuolar H⁺-ATPase [16]. Thus, mutant lacking Nhxl1 is reported to be sensitive to NaCl in media with low ionic strength [25]. Nhxl1 contains 12 transmembrane segments, as well as a C-terminal hydrophilic domain that is involved in protein–protein interactions [13]. The C-terminal tail of Nhxl1 was shown to interact with C-terminal region of Gyp6, a GTPase-activating protein involved in Ypt6-mediated retrograde traffic to the Golgi [1]. Evidence suggests that Gyp6 negatively regulates Nhxl1 function and that Nhxl1 may exert a role to allow trafficking from the prevacuole to the late Golgi via Ypt6 [1].

To date, the phenotypes reported for *nhx1Δ* mutant include sensitivity to NaCl, hygromycin B (HYG), BLM and gentamicin, an aminoglycoside antibiotic used for killing Gram-negative bacteria [1, 5, 23]. In addition, *nhx1Δ* mutant displayed missorting of carboxypeptidase Y (CPY) causing its secretion into the growth media [1]. The NaCl and HYG sensitivities were reported under conditions of a special growth media (APG) containing low salt where the investigators suggested that these phenotypes could be correlated with a defect in the mutant ability to maintain salt and pH homeostasis [5]. In contrast, the sensitivity to BLM and gentamicin were
observed with rich growth media [2]. These combined studies raised the possibility that \textit{nhx1}Δ mutant phenotypes may dependent upon the growth media. In this study, we examined the \textit{nhx1}Δ mutant for sensitivity to cationic and anionic drugs that enter the cell via both receptor-mediated and fluid endocytosis, as well as to various DNA damaging agents with different mode of action, when the cells were cultured only in rich growth media. We show that \textit{nhx1}Δ mutant is sensitive to several of these agents, but the sensitivity was not influence either by pH, NaCl or KCl. We further show that \textit{nhx1}Δ mutant secretes CPY, but which was also not prevented by changes in pH or NaCl concentrations. We conclude that Nhx1 plays a general role to detoxify those drugs that are channeled to the vacuole via the endosomal pathway, and that in its absence there is likely a leak from the prevacuole into the cytosol resulting in the observed toxicity.

**MATERIALS AND METHODS**

\textbf{Yeast strains, media, and plasmid.} The wild-type strain BY4741 (\textit{MATa his3Δ leu2Δ met15Δ ura3Δ}) and the isogenic \textit{nhx1Δ::KAN} null mutant were used in this study. Cells were grown at 30°C in either yeast peptone dextrose (YPD) (1\% (w/v) yeast extract, 2\% (w/v) peptone, 2\% (w/v) dextrose) or minimal synthetic (SD: 0.65 % yeast nitrogen base without amino acids, 2 % dextrose, 0.17% dropout mix) used for transformation [9]. pRin73, a 2\ μ plasmid harbouring NHX1 tagged with a C-terminal triple hemagglutinin epitope (NHX1-HA), under control of its endogenous promoter, was kindly provided by Dr. Rajini Rao (Johns Hopkins, USA). Yeast cells were transformed by the lithium acetate method [8].

\textbf{Spot tests.} Spot test analysis was previously described [12].

\textbf{Spermidine uptake analysis.} Prior to the polyamine uptake assay, cells were grown to the mid logarithmic phase, washed three times with uptake buffer A (50 mM sodium citrate, pH 5.5, 2\% D-glucose), and resuspended in 100 \μl of the same buffer at 2 X 10\textsuperscript{7} cells/ml. The uptake assay was initiated by the addition of 2.5 \μM [\textsuperscript{14}C] spermidine followed by incubation in 30°C with shaking. The reaction was stopped at predetermined intervals by adding 1 ml of ice-cold uptake buffer. Cells were washed three times with uptake buffer and resuspended in 100 \μl of the this buffer. Five ml of scintillation mixture (Amersham Bioscience) were added to each sample, and the retained radioactivity was determined by liquid scintillation spectrometry.

\textbf{Immunoblot analysis.} Immunoblot analysis was performed as previously described [22]. Membranes were blocked and then incubated with anti-HA monoclonal antibody (Santa Cruz Biotechnology) at a dilution of 1:5000, and the secondary
antibodies were anti-mouse (Amersham Bioscience). Protein bands were revealed by using PerkinElmer Chemiluminescence Reagent Plus followed by exposure to Kodak double emulsion film.

**Coomassie staining.** Samples were resolved by SDS-PAGE gels. SDS–polyacrylamide gels were stained with coomassie for 30 min in 4.6% methanol (v/v), 9.2% acetic acid, and 0.25% (w/v) coomassie Brilliant Blue R-25 and then destained in 7.5% acetic acid and 12% methanol.

**CPY plate assay.** Exponentially growing cells OD_{600} \approx 0.6 were serially diluted. Five μl aliquots were spotted on a nitrocellulose membrane (Amersham Biosciences) which was placed on the surface of YPD or SC plates and incubated at 30°C for 12-18 h. The membrane was lifted and washed with deionized and distilled water to remove all cells. Proteins absorbed on the membrane were detected by immunoblotting using monoclonal CPY antibody (Molecular Probes; 1:2500 dilution). The data represent results from three independent experiments.

**Quinacrine vacuole staining.** Vacuolar accumulation of quinacrine was examined as described previously [19]. Briefly, 3 X 10^7 log-phase yeast cells were harvested and resuspended in 500 μl of YPD buffered with 50mM Na₂HPO₄, pH 7.6, and containing 200 μM quinacrine. After incubation at the room temperature for 5 min. Cells were sedimented at 10,000 ×g for 5 s, washed once with 500 μl of 2% glucose buffered with 50 mM Na₂HPO₄ pH 7.6, and resuspended in 100 μl of the same solution. Samples were applied to a microscope slide and photographed by a fluorescent microscope.

**UVC treatment.** Overnight cultures were sub-cultured into 4 ml YPD and cells at O.D_{600} \approx 1.0 were harvested and resuspended in 4 ml of 20 mM PBS buffer. The cells were diluted 10^4 and 100 μl plated on YPD agar. Plates were irradiated with 254nm UVC (0-90 J/cm²) and scored for survival after 2 days of growth at 30°C.

**RNA extraction and RT-PCR.** Total RNA was prepared using the RiboPure-Yeast extraction kit (Ambion) from 3 ml of overnight culture, treated with the TURBO DNA-free kit (Ambion) to eliminate the genomic DNA contamination. Total RNA was quantified by absorption at 260 nm. cDNA was synthesized using M-MLVRT (Reverse transcriptase) from Invitrogen. To detect NHX1 expression two PCR primers were chosen: RT-PCR-NHX1-F1 (5'-TTATTTGAATGCTGTTTGAAATAGGATGTGCCCGGGCA-3') and RT-PCR-NHX1-R1 (5'-CAATTTGAGGATGCGCCCTTATTGAGTGTGTTTCAACAG-3').
RESULTS

**Deletion of the NHX1 gene confers hypersensitive to BLM-A5.** We verified if the \textit{nhx1Δ} mutant identified by the genome-wide screen was indeed sensitive to BLM-A5 by using an independent approach [2]. Spot test analysis revealed that the mutant was extremely sensitive to BLM-A5, when compared to the parent or the BLM-A5 resistant \textit{agp2Δ} mutant (Fig. 1A) [2]. In fact, deletion of the \textit{NHX1} gene in another wild-type strain W303 also resulted in \textit{nhx1Δ} mutant displaying hypersensitivity to BLM-A5 (data not shown). The \textit{nhx1}/\textit{NHX1} diploid retained parental resistance to BLM-A5, suggesting that the \textit{nhx1} allele is recessive.

We examined if a multicopy plasmid pNHX1 designed to overexpress the \textit{NHX1} fusion gene \textit{NHXI-HA}, as determined by RT-PCR (Fig. 1B) and the corresponding protein Nhx1-HA assessed by Western blot analysis (Fig. 1C), would restore BLM-A5 resistance to the \textit{nhx1Δ} mutant. The overexpressed Nhx1-HA conferred nearly full resistance to the \textit{nhx1Δ} mutant towards BLM-A5 at the lower drug concentration (Fig. 1A), while at a 2-fold higher concentration (0.5 \mu g/ml) only partial resistance was observed (data not shown). These findings demonstrated that the \textit{NHX1} gene product is required to protect cells against the toxicity caused by BLM-A5. We note that the overexpression of Nhx1-HA showed no additional BLM-A5-resistance to the parent strain (Fig. 1A), suggesting that Nhx1 function is not limiting in the cells.

\textit{nhx1Δ} mutant is hypersensitive to polyamines. Because BLM-A5 contains a polyamine moiety [3], we reasoned that \textit{nhx1Δ} mutant sensitivity to this drug might be a result of its chemical composition. We tested if the \textit{nhx1Δ} mutant would display cross-sensitivity to polyamines such as spermine (SPM) and spermidine (SPD). Spot test analysis revealed that the \textit{nhx1Δ} mutant was hypersensitive to both SPM and SPD, and that these phenotypes were rescued by the introduction of the plasmid pNHX1 (Fig. 1A). Thus, it appears that Nhx1 function is also required to prevent toxicity caused by polyamines. Since polyamine sensitivity can be explained by an increase accumulation in the cell, as observed for \textit{end3Δ} mutant lacking a protein involved in the initial phase of endocytosis [4], we monitored the uptake of \text{^{14}C}-labeled spermidine in the \textit{nhx1Δ} mutant. In comparison to the parent, the \textit{nhx1Δ} mutant showed only a modest increase in the accumulation of the labeled SPD (Fig. 2), excluding the possibility that the sensitivity of the mutant is due to enhanced uptake.

\textit{nhx1Δ} mutant is also hypersensitive to other agents. Previous studies documented that \textit{nhx1Δ} mutant show sensitivity towards HYG [7], a cationic amino glycoside that accumulates in the cytosol of the cell [18]. Since BLM-A5 and polyamines are
ations as well, we therefore tested if the mutant sensitivity was related to the charge by examining a panel of drugs that are both cationic and anionic. We indeed reproduced \( \text{nhx1}\Delta \) mutant sensitivity to HYG in YPD media (Fig. 3). Interestingly, the mutant was hypersensitive to the anionic drug deoxycholine, as well as to calcofluor white, a related anionic agent which interferes with the construction and stress response of the cell wall (Fig. 3) [20]. The \( \text{nhx1}\Delta \) mutant was also hypersensitive to two other species of BLM, BLM-A2 and BLM-B2, which lack the polyamine moiety (data not shown). These data suggest that Nhx1 function is required to prevent toxicity caused by a range of charged compounds and not limited to either cationic or anionic agents. Thus, it is unlikely that the initial sensitivity observed for \( \text{nhx1}\Delta \) mutant towards BLM is related to the polyamine moiety.

Since BLM is known to damage the DNA, we checked if the \( \text{nhx1}\Delta \) mutant would be sensitive to other DNA damaging agents. In comparison to the parent, the \( \text{nhx1}\Delta \) mutant showed no additional sensitivity to 254-nm ultraviolet light, which creates pyrimidine dimers (data not shown). Thus, it would appear that drugs that are known to be detoxified by the endosomal pathway would illicit sensitivity to the \( \text{nhx1}\Delta \) mutant, consistent with the identification of Nhx1 as a prevacuolar protein [15].

It is noteworthy that the \( \text{nhx1}\Delta \) mutant was reported to be sensitive to NaCl under the special growth media APG, which has a low osmolarity and acidic pH [25]. However, we found no sensitivity of the \( \text{nhx1}\Delta \) mutant to NaCl when grown on YPD (Fig. 5). In fact, the \( \text{nhx1}\Delta \) mutant exhibited sensitivity to CaCl\(_2\), but not to MgCl\(_2\), when grown on solid YPD media (Fig. 5 and data not shown). We did not perform any analysis for drug sensitivity with the APG media, as the mutant exhibited sensitivity to HYG whether grown in APG or YPD media.

**\( \text{nhx1}\Delta \) mutant is not sensitive to pH when grown in YPD media.** Mutants defective in vacuolar membrane H\(^+\)-ATPase activity such as \( \text{vma12}\Delta \) are unable to grow on solid YPD media with increasing pH [10]. Since it has been reported that \( \text{nhx1}\Delta \) mutant has a defect in maintaining the intracellular pH in the vacuoles and cytoplasm when grown in APG media [5], we therefore tested if the mutant can grow on YPD media with varying pH. Like the parent, \( \text{nhx1}\Delta \) mutant was able to grow and tolerate a wide range of pH (pH 4 to 8) (data not shown). In contrast, the vacuolar defective mutant \( \text{vma12}\Delta \) was unable to grow efficiently at the higher pH (data not shown)[10]. Thus, under this growth condition Nhx1 does not appear to play a key role in maintaining the vacuolar pH, although the previous report showing \( \text{nhx1}\Delta \) mutant sensitivity to pH may be related to the specific growth media, APG [5].

To further examine if the \( \text{nhx1}\Delta \) mutant has a defect in vacuolar acidification, we tested if the vital dye quinacrine can accumulate in the vacuole. Quinacrine is a basic dye that accumulates in acidic compartments in yeast and is used to rapidly
monitor vacuolar acidification [24]. No defect was observed in the accumulation of quinacrine into the vacuoles of the nhx1Δ mutant, as compared to the parent (Fig. 7), confirming that the vacuole was acidified and functional. Consistent with this observation it has been shown that blocking endocytosis in end4Δ mutant does not affect vacuolar acidification [17]. Thus, it would appear that the sensitivity of the nhx1Δ mutant to the drugs is likely unrelated to the maintenance of vacuolar pH.

**CPY secretion by the nhx1Δ mutant is not blocked by varying the pH or NaCl concentrations.** nhx1Δ mutant has been documented to secrete carboxypeptidase Y (CPY) [1]. If the nhx1Δ mutant phenotypes such as CPY secretion are associated with a defect in maintaining the pH of the prevacuolar compartment, then culturing cells in low pH is likely to block CPY secretion. In this experiment, the cells were grown in a range of pH under minimal media and tested for CPY secretion using nitrocellulose lift that was probed with anti-CPY antibodies. Interestingly, the low pH did not prevent CPY secretion by the nhx1Δ mutant, while introduction of the pNHX1 plasmid into the mutant inhibited the secretion (Fig. 5A). Since Nhx1 is a Na⁺ antiporter, we also checked if the CPY secretion could be blocked by increasing NaCl concentration. However, we found that increasing NaCl concentrations also did not suppress CPY secretion (Fig. 5B), raising the possibility that Nhx1 may execute an additional function that is crucial to prevent CPY secretion.

**The Nhx1 variant Glu355Ala, but not Glu371Ala, is unable to rescue the drug phenotypes of nhx1Δ mutant.** To examine if Nhx1 plays a functional or structural role in protecting cells against drug toxicity, we introduced into the nhx1Δ mutant a plasmid expressing either the Glu355Ala or Glu371Ala variant of Nhx1. Both variants are located on an extracellular loop that is flanked by two transmembrane helices predicted to be inserted into the membrane [13]. Glu355, but not Glu371, was shown to be essential for Nhx1 function in tolerating growth in APG media at low pH [13]. As shown in Fig. 6, the E355A variant did not rescue the nhx1Δ sensitivity to either BLM or HYG. In contrast, the E371A variant complemented these defects to the same extent as the native Nhx1. If indeed Glu355 interferes with the activity of Nhx1, it is possible that the function rather than the structure of the exchanger is critical for drug tolerance.

**Nhx1 acts independently of Ypt6 and Ypt7 in drug resistance.** Previous study suggests that Nhx1 and Ypt6 may function along the same trafficking pathway as the nhx1Δ and ypt6Δ single mutants showed similar sensitivity to HYG in the APG media [1]. As such, we tested if the ypt6Δ mutant would display similar sensitivities as nhx1Δ mutant towards various drugs. Surprisingly, although ypt6Δ mutant showed hypersensitivity to SPM and calcofluor white, it did not reveal any significant
sensitivity to BLM or HYG on YPD media (Fig. 7A). On the basis of these findings, it is unlikely that Nhx1 and Ypt6 are functioning along the same pathway in drug detoxification, at least for BLM. Interestingly, deletion of the \textit{YPT6} gene in the \textit{nhx1}Δ mutant resulting in the \textit{nhx1}Δ\textit{ypt6}Δ double mutant that was more resistant to BLM, HYG and SPM (Fig. 7B), suggesting that accumulation of these drugs in the prevacuole may leak into the Golgi via functional Ypt6.

It is noteworthy that the \textit{ypt7}Δ mutant lacking a protein involved in fusion of the vesicles derived from endosomes with the vacuoles [1] also did not show any significant sensitivity towards either BLM or HYG (Fig. 7A). This is also in contrast to the HYG sensitivity observed for \textit{ypt7}Δ mutant when tested on APG media, suggesting that the type of media might influence the phenotypes displayed by these mutants.

**DISCUSSION**

We report novel phenotypes of the \textit{nhx1}Δ mutant that raise additional interesting questions regarding the function of Nhx1. The initial experiments done to characterize the physiological role of Nhx1 were performed mainly in APG medium with low osmolarity, which is essential in order to reveal the osmotolerance defect and to accurately measure the intracellular pH of \textit{nhx1}Δ mutant [5]. This media was also used to demonstrate that the \textit{nhx1}Δ mutant is sensitive to NaCl [25]. In accordance with the previous studies, we show that \textit{nhx1}Δ mutant revealed hypersensitivity to HYG, as well as displayed the CPY missorting phenotype even when grown in the rich media YPD. However, under the YPD growth media, the \textit{nhx1}Δ mutant no longer retained sensitivity to NaCl even at high concentration (1 M in YPD). Moreover, addition of NaCl to the YPD media did not suppress the \textit{nhx1}Δ sensitivities to any of the drugs (data not shown). These observations clearly uncoupled the drug sensitivities, as well as CPY missorting, from a need to maintain osmolarity, suggesting that any deficiency of Na⁺ in the late endosome can not account for the drug phenotypes displayed by the \textit{nhx1}Δ mutant.

Another unique observation reported in this study is that neither \textit{ypt6}Δ mutant nor \textit{ypt7}Δ mutant was sensitive to HYG when grown in YPD media as compare to the sensitivity seen with the APG media [1]. If indeed Nhx1 and Ypt6 function along the same pathway as proposed [1], it is expected that \textit{ypt6}Δ mutant should show at least some level of sensitivity to HYG particularly since \textit{nhx1}Δ mutant is dramatically sensitive to the drug. One explanation for this observation is that under YPD media, an alternative member of the Ypt proteins, of which there are at least eleven [11], could be expressed to substitute for Ypt6. Likewise, a similar explanation may hold true for the lack of sensitivity of \textit{ypt7}Δ mutant to HYG in YPD media.
As pointed out above, one of our main interests is to understand how BLM is detoxified in yeast cells. Our data to date clearly indicate that the key detoxification pathway involves the endosomal process, as fluorescently labeled BLM accumulates in the vacuoles and mutants defective in this pathway are sensitive to BLM [2]. However, we do not know if the main reason for cytotoxicity or genotoxicity is a result of leak from the endosomal process. The data presented here indicate that defect in the late endosome is a major factor leading to toxicity. It is possible that the toxicity could arise as a result of drug leak from the late endosome to the cytosol or the leak is via retrograde trafficking from the late endosome to the nucleus through the Golgi and the endoplasmic reticulum. In the latter situation, we would expect that blocking Ypt6 function (involved in recycling of cargoes from the late endosome to the Golgi) would cause elevated resistance to BLM. Instead, we observed that ypt6Δ mutant was weakly sensitive to BLM. Therefore, it is unlikely that leak from the late endosome to Golgi is a major contributing factor for severe toxicity caused by BLM. The modestly increased resistance to BLM observed when YPT6 is deleted in the nhx1Δ mutant may be due to lack of interaction between the prevacuole and the Golgi.

What could be the role of Nhx1, if it is not involved in osmotolerance or pH homeostasis in YPD media? Nhx1 could play a role in mediating the tethering of the late endosome to the vacuoles for efficient drug delivery. For example, the HOPS (hormotypic vacuole fusion and protein sorting) complex presented on the vacuoles consists of several proteins and some of which could participate in the fusion process with the late endosome, as reported for Ypt7 which is required for fusion of vesicles derived from endosome with the vacuole, through an interaction with the HOPS complex and Vam7 [21]. In this manner, the fusion process might occur by tethering of the HOPS protein directly or indirectly with Nhx1. Consistent with this notion, Nhx1 has been shown to participate in protein-protein interaction whereby its C-terminal tail interacts with Gyp6, an activator of the Ypt6 GTPase activity [1]. However, further studies would be needed to clarify if this is the actual mechanism by which Nhx1 functions in drug tolerance when cells are grown in YDP media. It is noteworthy that Nhx1-related proteins, NHE6, 7, 8 and 9 are present in human cells [14], although it is not known if any of these, when knockdown separately, would cause sensitivity to the same class of drugs as the nhx1Δ mutant. Perhaps targeting this unique isoform of the human NHEs would provide a way of improving the chemotherapeutic benefits of BLM.

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References

Novel drug phenotypes of NhX1-deficient mutants


LEGENDS

Figure 1. Effect of the NHX1 gene deletion and NhX1-HA expression on sensitivity of the indicated strains towards BLM and polyamines. A, Spot test analysis. Exponentially growing cells were serially diluted and spotted onto YPD solid media without and with the indicated drugs. Plates were photographed after 48 h incubation at 30°C. The data is representative of two independent analyses. B, RT-PCR analysis to detect NHX1 and ACT1 expression in the indicated strains. C, Expression of NhX1-HA fusion by Western blot analysis. Total extracts (150 μg) were subjected to Western blot analysis and probed with anti-HA monoclonal antibodies. D, Coomassie staining to monitor protein loading.

Figure 2. nhx1Δ mutant shows no significant enhancement of spermidine uptake. Exponentially growing cells were incubated with [14C] spermidine and processed for the uptake as described in “Materials and methods”. The data represent results from three independent experiments.

Figure 3. nhx1Δ mutant displays wide sensitivity to various drugs and CaCl2, but not to NaCl. Cells were spotted on YPD plates without and with the indicated concentration of HYG, calcofluor white, deoxycholic acid, NaCl and CaCl2 and photographed as in Fig. 1A after 48 h of incubation at 30°C. The data is representative of three independent analyses.

Figure 4. Positive staining of the vital dye quinacrine in the nhx1Δ mutant. Vacuolar acidification in parent strain (A and B) and nhx1Δ mutant (C and D) were assessed by visualizing quinacrine accumulation in this organelle as described in “Materials and methods”. Figure showed both the bright field (A and C) and fluorescent (B and D) images. The data is representative of three independent analyses.

Figure 5. CPY secretion by the nhx1Δ mutant is not inhibited by varying either the pH or NaCl concentrations. Exponentially growing cells OD600 ~0.6 were serially diluted, spotted onto nitrocellulose membrane followed by incubation of the membrane on top of: A, SD media with varying pH, B, YPD media with increasing NaCl concentrations. After incubating the plates at 30°C for 12-18 hours, the
membranes were subjected to immunoblotting with mouse anti-CPY. The data is representative of three independent analyses.

**Figure 6.** The variant E355A, but not E371A, is defective in rescuing \( \text{nhx1}^{-}\) mutant from drug toxicities. Exponentially cultures were serially diluted and spotted as in Fig. 1A.

**Figure 7.** Comparison of the sensitivities of the parent and the indicated mutants towards various drugs. Exponentially cultures were serially diluted and spotted as in Fig. 1A.

![Image of dilution plates with captions](image-url)

**Fig. 1A** Khodami-Pour et al., 2009
Novel drug phenotypes of Nhx1-deficient mutants

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Fig. 1B-D: Khodami-Pour et al., 2009
Fig. 2: Khodami-Pour et al., 2009
Novel drug phenotypes of Nhx1-deficient mutants

Fig. 3: Khodami-Pour et al., 2009
Fig. 4: Khodami-Pour et al., 2009
Novel drug phenotypes of Nhx1-deficient mutants

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Fig. 5: Khodami-Pour et al., 2009
Fig. 6: Khodami-Pour et al., 2009
Novel drug phenotypes of Nhx1-deficient mutants

Fig. 7A: Khodami-Pour et al., 2009

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YPD       | HLM 0.25 μg/ml | SPM 1.75 mM |

HYG 20 μg/ml | HYG 60 μg/ml | Calcefluor 50 μg/ml
Fig. 7B: Khodami-Pour et al., 2009

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