The antidepressant sertraline targets intracellular vesiculogenic membranes in yeast

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Running head: Sertraline mechanism of action in yeast

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ABSTRACT

Numerous studies have shown that the clinical antidepressant sertraline (Zoloft®) is biologically active in model systems, including fungi, which do not express its putative protein target, the serotonin/5-HT transporter, thus demonstrating the existence of one or more secondary targets. Here we show that in the absence of its putative protein target, sertraline targets phospholipid membranes that comprise the acidic organelles of the intracellular vesicle transport system by a mechanism consistent with the bilayer couple hypothesis. Based on a combination of drug-resistance selection and chemical-genomic screening, we hypothesize that loss of vacuolar ATPase activity reduces uptake of sertraline into cells, whereas dysregulation of clathrin function reduces the affinity of membranes for sertraline. Remarkably, sub-lethal doses of sertraline stimulate growth of mutants with impaired clathrin function. Ultrastructural studies of sertraline-treated cells revealed a phenotype that resembles phospholipidosis induced by cationic amphiphilic drugs in mammalian cells. Using reconstituted enzyme assays, we also demonstrated that sertraline inhibits phospholipase A₁ and phospholipase D, exhibits mixed effects on phospholipase C and activates phospholipase A₂. Overall, our study identifies two evolutionarily conserved membrane-active processes – vacuolar acidification and clathrin-coat formation – as modulators of sertraline’s action at membranes.
INTRODUCTION

Sertraline, whose trade name is Zoloft®, is a Food and Drug Administration (FDA) approved drug that belongs to the pharmacological class of antidepressants called selective serotonin reuptake inhibitors (SSRIs) (KOE et al. 1983). The putative therapeutic target of SSRIs is the human serotonin/5-HT transporter (hSERT/5-HTT/SLC6A4), an integral membrane protein that mediates sodium-dependent reuptake of the monoamine neurotransmitter serotonin at presynaptic nerve terminals in the brain (MEYER et al. 2004; MORTENSEN et al. 2001; TATSUMI et al. 1997). Indeed, the recent crystal structure of sertraline bound to a bacterial leucine transporter, LeuT – a homolog of mammalian monoamine transporters – has led to the proposal of a corresponding high-affinity binding site for sertraline in hSERT (ZHOU et al. 2009). Inhibition of hSERT-dependent reuptake by sertraline that is thought to underlie its antidepressant effect in people, as documented extensively over the last two decades in the clinical psychiatric literature (reviewed in (CIPRIANI et al. 2009)).

However, there are numerous examples of sertraline’s effects on cellular physiology, including, most curiously, cytotoxicity in yeast (LASS-FLÖRL et al. 2001a; LASS-FLÖRL et al. 2001b), by an unknown mechanism that does not involve hSERT, so must involve one or more heretofore unrecognized secondary drug targets that may be evolutionarily conserved (LEVKOVITZ et al. 2007; REDDY et al. 2008). Those observations, coupled with reports that antidepressants can accumulate in the brains of people to concentrations at which “off-target” effects are seen (LEVKOVITZ et al. 2007), suggest that sertraline’s
interactions with targets besides hSERT may contribute to its antidepressant and/or “side-effect” properties in people. Clues as to the identity of such targets emerge when one connects sertraline to the vast literature on a class of compounds called cationic amphiphilic drugs, or CADs, to which sertraline belongs. CADs are defined by two physicochemical attributes: (i) a hydrophobic ring system (in the case of sertraline, a naphthalene scaffold); (ii) a basic, nitrogen-containing group (in the case of sertraline, a secondary amine). Many FDA-approved small-molecule drugs, including a large number of so called psychoactives, are classified as cationic amphiphiles. In addition to their known and/or putative interactions with proteinaceous targets, it has long been recognized that CADs interact with phospholipid membranes; this interaction is traditionally understood through the lens of the decades-old bilayer couple hypothesis.

The bilayer couple hypothesis (Sheetz and Singer 1974) arose from studies of the effects of CADs on the shape of human erythrocytes visualized by scanning electron microscopy. It specified the following molecular mechanism of action: cationic amphiphiles preferentially localize to the inner leaflet of the plasma membrane, which is more negatively charged relative to the outer leaflet in red blood cells, resulting in its asymmetric expansion. This in turn leads to membrane invagination across the plasma membrane, or cup-shaped cellular morphology. Conversely, anionic amphiphiles preferentially localize to the outer leaflet, resulting in membrane evaginations, or crenated cellular morphology. Critically, simultaneous addition of both a cationic amphiphile and an anionic amphiphile results in no net structural change to the bilayer, as leaflet expansion is balanced.
Subsequent studies using artificial membranes have shown that CADs induce changes in the biophysical properties of both planar and vesicular phospholipid bilayers that are consistent with the bilayer couple hypothesis and depend on factors like lipid composition, membrane curvature and pH (ERIKSSON 1987; VERKLEIJ et al. 1982). However, because drug-lipid interactions are much more poorly understood than drug-protein interactions at the atomic level, the former are often simply described as intercalations. That said, CADs appear to have pleiotropic effects on membrane-active processes whether one examines these effects on proteins, cells or even whole organisms. For example, CADs modulate the activity of structurally and catalytically unrelated proteins whose proper function depends on being either in, on or near membranes, including mechanosensitive ion channels (MARTINAC et al. 1990), phospholipases (LELI and HAUSER 1987a; STURTON and BRINDLEY 1977), and even the GTPase dynamin (OTOMO et al. 2008). Indeed, it has long been thought that inhibition by CADs of phospholipases is responsible for drug-induced phospholipidosis in mammalian cells (STURTON and BRINDLEY 1977). Phospholipidosis is defined as an accumulation of multilamellar vesicles or arrays that leads to disruption of organellar integrity, altered phospholipid metabolism, and in some instances cell death (ANDERSON and BORLAK 2006).

Given the broad swathe of CAD-induced perturbations of membrane biophysical properties in vitro and membrane-active processes in vivo, these drugs have been the focus of investigators in diverse fields, including yeast genetics. Two examples that are
especially pertinent to our present study involve the identification of fungal genetic determinants of resistance/sensitivity to the oft-studied phenothiazine antipsychotic drugs trifluoperazine (SHIH et al. 1988) and chlorpromazine (Thorazine®) (FILIPPI et al. 2007), the latter being perhaps the most well-studied CAD in a variety of experiment types and model systems. The older study showed that resistance to trifluoperazine in yeast is caused by mutations in subunits comprising the evolutionarily conserved vacuolar ATPase (V-ATPase), which is composed of two functional units, a soluble V₁ and membrane-associated V₀. These subunits assemble into an ATP-dependent proton-translocating complex that acidifies the lumens of dynamic organelles like the vacuole (or lysosome, in mammalian cells), thereby energizing intracellular vesicular transport. The resistance-conferring effects of loss of V-ATPase complex activity was explained using a lysosomal trapping/basicicity model: because acidified vacuoles/lysosomes act as sinks for weak bases, loss of acidification reduces intracellular CAD accumulation. The more recent study showed that deletion of genes involved in vesicle trafficking – a number of which have mammalian homologs – causes hypersensitivity to chlorpromazine. A similar gene set enriched in processes involved in the intracellular vesicle transport system was found to modulate sensitivity to a diverse collection of psychoactive drugs by a group using an unbiased chemical-genomic approach (ERICSON et al. 2008).

In an effort to understand why sertraline exhibits cytotoxic effects in a model system lacking the putative protein target, we began by combining the unparalleled specificity of a drug-resistance selection with rapid and cost-effective microarray-enabled polymorphism detection. In other words, we used whole-genome tiling microarrays, as
opposed to cloning by complementation, to locate rapidly resistance-conferring mutations. The advent of microarray-enable polymorphism detection (GRESHAM et al. 2006) greatly reduces the expenditure of cost and time traditionally associated with identifying drug resistance-conferring loci, thereby modernizing ad hoc cloning-by-complementation approaches, and complementing high-throughput chemical genomic screening of engineered deletion libraries (LEHÁR et al. 2008). And throughout the present study, we also examined the effects of the classic CAD chlorpromazine in order to strengthen the prima facie linkage between sertraline and the pleiotropic effects of cationic amphiphiles on biological membranes.

**RESULTS**

**Sertraline-resistant mutants target clathrin and V-ATPase function**

We selected BY4712 and BY4716 as parental strains. 16 spontaneous sertraline-resistant (sert\textsuperscript{R}) mutants were isolated: four of the mutants are dominant; the remaining 12 are recessive. A complementation test revealed that eight of the 12 recessive mutants belong to a single complementation group; two of the remaining four mutants comprise a second complementation group; the other two mutants are orphans, i.e., were not assigned to any complementation group. We hybridized fragmented and labeled genomic DNA (gDNA) derived from a subset of sert\textsuperscript{R} mutants to Affymetrix\textsuperscript{®} whole-genome tiling arrays. The resulting genome-wide hybridization intensity measurements were then analyzed by the SNPscanner prediction algorithm (GRESHAM et al. 2006) in order to identify candidate resistance-conferring mutations, which we expected to reside in protein-coding regions. SNPscanner predicted mutations in all cases save one orphan recessive BY4716 sert\textsuperscript{R}
mutant, which we did not analyze further. A complete catalogue of 16 (15 single mutants and one double mutant) sert\textsuperscript{R} strains is presented in Table 1. We recovered mutants affecting two evolutionarily conserved membrane-active processes: clathrin-coat formation and the vacuolar ATPase (V-ATPase) complex.

The largest complementation group corresponds to the gene \textit{SWA2 (YDR320C)}, which encodes the yeast homolog of auxilin, a clathrin-binding protein with co-chaperone function that mediates uncoating of clathrin assemblies through activation of the Hsp70 chaperone (\textit{Gall \textit{et al.} 2000}). We independently observed twice a nonsense mutation (C603STOP) that results in truncation of the critical C-terminal J domain that is strongly conserved from yeast to human, and without which Swa2p cannot activate the clathrin-remodeling function of Hsp70p (\textit{Xiao \textit{et al.} 2006}). Deletion of \textit{SWA2} in yeast (\textit{Xiao \textit{et al.} 2006}), or RNAi-knockdown of the \textit{SWA2} homolog auxilin in mammalian cells (\textit{Hirst \textit{et al.} 2008}), prevents the uncoating reaction from disassembling clathrin-coated vesicles, which shifts the equilibrium of clathrin from the disassembled state to the assembled state.

Unexpectedly, one of the mutants that we originally assigned to the \textit{swa2} complementation group actually encodes wildtype \textit{SWA2}, as borne out by targeted resequencing. In other words, this mutant failed to complement sertraline resistance when mated to a \textit{swa2} mutant. Upon closer inspection, this mutant harbors a partially dominant missense mutation (E292K) in \textit{CHC1/SWA5 (YGL206C)}, which encodes clathrin heavy chain (\textit{Lemon \textit{et al.} 1991}). The residue affected by the missense mutation resides in the
N-terminal domain of Chc1p, which is thought to interact with clathrin adaptor complexes, but is not directly involved in trimerization of clathrin heavy chain molecules into triskelia, the building block of clathrin coats. The fact that we only isolated one chc1 mutant indicates that our selection was not performed to saturation. To our knowledge, neither SWA2 nor CHC1 has been isolated as a mutant in the context of drug-resistance screening or selection, though mutant alleles of both loci were previously shown to be synthetically lethal with deletion of ARF1, which encodes a small soluble GTPase that initiates clathrin-coat formation at the Golgi (CHEN and GRAHAM 1998).

The second complementation group corresponds to the gene CUP5/VMA3 (YEL027W), which encodes the c subunit of the V₀ sector of the V-ATPase. Interestingly, the four dominant sert⁸ mutations occur in two other V-ATPase complex subunits: TFP1 and VMA9. TFP1 (YDL185W) encodes the A subunit of the eight-subunit V₁ peripheral membrane domain. VMA9 (YCL005W-A) encodes the e subunit of V₀ sector. As expected, phenotypic analysis of sert⁸ mutants affecting V-ATPase complex activity reveals that they exhibit phenotypes characteristic of Vma- mutants: sensitivity to high extracellular calcium and inability to grow in alkaline (pH 8) media (Figure S1). TFP1 is noteworthy because dominant alleles of it were previously shown to confer resistance to the phenothiazine antipsychotic trifluoperazine (SHIH et al. 1988).

Next we tested whether the potent and specific chemical inhibitor of the V-ATPase, bafilomycin A1, which binds to the c subunit encoded by CUP5/VMA3, suppresses sertraline-induced cytotoxicity in wildtype cells. This is indeed the case (Figure S2).
What’s more, the proton ionophore gramicidin also suppresses sertraline-induced cytotoxicity in wildtype cells. Mutations in \textit{CUP5/VMA3} that convert the V\textsubscript{0} subunit into a passive proton channel would have the same effect as gramicidin, which inserts into membranes and forms proton-conducting channels. These results reinforce the lysosomal trapping/basicicity model. We further assessed the role of acidification by testing the effect of environmental pH on sertraline-induced cytotoxicity in wildtype cells. Just as was observed in a recent study of chlorpromazine resistance (Filippi et al. 2007), in acidic YPD media (pH 4.0) sertraline does not exhibit any antifungal activity, while in alkaline YPD media (pH 8.0) sertraline’s antifungal activity is exacerbated (Figure S2). Thus the protonated form of sertraline is inactive because it may be impermeable to cells. These results reinforce the notion that vacuolar acidification and external pH are common determinants of cellular response to CADs in yeast.

**V-ATPase and clathrin mutants confer partial resistance non-additively**

In addition to the known role of the V-ATPase in shaping the cellular response to CADs, our drug-resistance selection uncovered a novel mechanism of resistance: dysregulation of clathrin function. In order to characterize further the two mechanisms, we quantified the half-maximal inhibitory concentration (IC\textsubscript{50}) of sertraline in the wildtype strain, BY4716, and two sert\textsuperscript{R} mutants, one from each of the two resistant classes: \textit{CUP5\textsuperscript{R46I}} and \textit{SWA2\textsuperscript{C603STOP}}. These experiments yielded three important observations. First, the steepness of the dose-response curves of all three strains suggests that sertraline exhibits cooperative binding at its sites of action. Second, the IC\textsubscript{50} of sertraline in BY4716 is 15\textmu M, and this value increases only two-fold in all sert\textsuperscript{R} strains (Figure 1A). Partial
resistance demonstrates that the binding sites for sertraline are not completely extinguished by mutation. Third, sertR strains exhibit cross-resistance to chlorpromazine (Figure 1B), but are not cross-resistant – if anything the clathrin pathway mutants are more sensitive than wildtype – to the structurally and mechanistically unrelated translation inhibitor cycloheximide (Figure 1C).

Next, we tested whether CUP5 and SWA2 function in the same pathway or in parallel pathways. We generated a cup5 swa2 double mutant by crossing the aforementioned CUP5R46I and SWA2C603STOP strains. Tetrad analysis revealed that a cup5 swa2 double mutant exhibits modest synthetic sickness in the absence of sertraline; some allelic combinations resulted in greater synthetic sickness. Surprisingly, the cup5 swa2 double mutant initially appeared to be more sensitive to sertraline than either single mutant with an IC50 of ~6.5µM (Figure 2A). However, if the double mutant was incubated for an additional 12-24 hours its dose response assumed an appearance closer to that of either of the single mutants, indicating a kinetic defect in the emergence of resistance, as opposed to a diminution of absolute resistance (Figure 2B). Interestingly, the dose response of a cup5 swa2 double mutant is better fit by a less steep curve, suggesting that the kinetic defect may affect the cooperativity of sertraline binding at its sites of action. Similar results were obtained using a cup5 swa2 double mutant comprised of different single mutant alleles. As a control, we generated a cup5 tfp1 double mutant; these two genes encode interacting subunits in the V-ATPase complex. Two cup5 tfp1 double mutants comprised of different single mutant alleles yielded similar results, namely the double
mutant is as resistant as the average of both single mutants (Figure 2A); moreover there is no change in the steepness of the dose-response curves or the kinetics of resistance.

In order to dissect further the resistance-conferring role of the V-ATPase, we conducted a second round of drug-resistance selection, but this time we started with the $CUP5^{R46I}$ mutant, as opposed to the parental strain. We increased the stringency of selection to ~60µM sertraline given the two-fold increased basal resistance of the $CUP5^{R46I}$ mutant. Using the aforementioned microarray-enabled polymorphism detection approach we identified, and subsequently validated by resequencing and linkage analysis (data not shown), two point mutations in the gene $CSG2$ ($YBR036C$) (Table 1 and Figure S3). A deletion of a single adenine base in a short run of adenosines is located three nucleotides upstream of a T→C transition point mutation. The single-base deletion is predicted to cause a frame shift and subsequent premature STOP codon at residue 280. $CSG2$ encodes an integral membrane protein that localizes to the endoplasmic reticulum and is involved in calcium-ion homeostasis and sphingolipid metabolism (Beeler et al. 1994).

Interestingly, a $cup5$ $csg2$ double mutant was previously shown to be more sensitive than a $cup5$ single mutant to the calcineurin inhibitor FK506 (Tanida et al. 1996). We show that a $cup5$ $csg2$ mutant is more resistant to sertraline than a $cup5$ mutant, while a $csg2$ mutant exhibits wildtype sensitivity (Figure S3). Consistent with a role for calcium in determining sertraline sensitivity, we also observed that calcium suppresses, while the calcium-ion chelator EGTA enhances, sertraline-induced cytotoxicity of wildtype cells (data not shown). Thus, $CSG2$ is genetic modifier of $CUP5$, which suggests that
modulation of calcium-ion homeostasis may in part be driving the selection of V-ATPase hypomorphs.

**Growth-stimulatory effects of sertraline on mutants with impaired clathrin function**

CHC1\(^{E292K}\) and SWA2\(^{C603STOP}\) mutants exhibit non-allelic noncomplementation. Therefore, we posit that these mutations exert similar effects on clathrin function. As swa2 mutants accumulate assembled clathrin (GALL et al. 2000), a similar shift in the equilibrium of clathrin would be predicted to occur in the dominant CHC1\(^{E292K}\) mutant. In order to examine more closely how dysregulation of clathrin function engenders resistance to sertraline, we included a loss-of-function allele of clathrin heavy chain (chc1-521) that is conditionally assembly-incompetent (BENSEN et al. 2000). Again, dose-response experiments yielded three important observations (Figure 3A). First, the CHC1\(^{E292K}\) mutant, like the other sert\(^{R}\) strains, exhibits a two-fold increase in resistance compared to wildtype. Second, sertraline exhibits concentration-dependent, or biphasic, effects over its bioactive concentration range. We define a sub-lethal regime and a lethal regime. The lethal regime is any concentration greater than or equal to 30µM and results in maximal growth inhibition of wildtype and all sert\(^{R}\) strains. The sub-lethal regime spans 0.1µM to 10µM and results in suppression of the constitutive growth defect of the CHC1\(^{E292K}\) mutant that may arise from the failure of clathrin assemblies to recycle properly, or downstream effects thereof. Third, the temperature-dependent growth defect of the chc1-521 strain is surprisingly also suppressed in the sublethal regime. These results demonstrate that sertraline and clathrin exhibit complex competition at sertraline’s sites of action.
Interestingly, chlorpromazine also exhibits biphasic effects, though not as potently as sertraline (Figure 3B). Thus, concentration-dependent rescue of growth defects of mutants with altered clathrin function may be a general feature of psychoactive CADs, which is consistent with previous observations that some CADs affect clathrin self-assembly in vitro (Di Cerbo et al. 1984; Nandi et al. 1981) as well as clathrin-dependent endocytosis in vivo (Wang et al. 1993). Importantly, the concentration-dependent effects of sertraline appear to be specific to clathrin function, because at no concentration does cycloheximide rescue the growth defect of either mutant with altered clathrin function (Figure 3C). The specificity for clathrin function is further supported by the observation that the moderately slow growing VMA9^{S35R} mutant strain does not experience growth-rate enhancement in the low-dose sertraline regime (data not shown).

**Clathrin and V-ATPase mutant exhibit distinct kinetics of resistance**

Because dose-response experiments capture the endpoint of the cellular response to sertraline, we quantified the kinetics of resistance to sertraline using an automated plate reader that incubates a 96-well plate at constant temperature with agitation. We first assessed the baseline growth rate of the wildtype and several representative sert^{R} strains, as well as the chc1-521 mutant. SWA2^{C603STOP}, CHC1^{E292K} and the dominant V-ATPase mutant VMA9^{S35R} grow slowly in the absence of sertraline, indicating that a fitness penalty sometimes accompanies sertraline resistance. On the other hand, the recessive CUP5^{R46I} mutant grows at rate comparable to wildtype. Next we tested two concentration
regimes of sertraline: one two-fold above (30µM) and a second two-fold below (7.5µM) the IC$_{50}$ of wildtype cells.

At 30µM sertraline, the $CHC1^{E292K}$ mutant grows at the same rate in the presence or absence of sertraline, while the $CUP5^{R46I}$ mutant exhibits a reduced growth rate relative to its steady state that is still faster than wildtype, whose growth rate is completely inhibited (Figure 4A-C). At 7.5µM sertraline, we observe rescue of the growth defects of the $CHC1^{E292K}$ and chc1-521 mutants (Figure 4C, D), consistent with the results of dose-response experiments. These results support the notion that the $CUP5^{R46I}$ mutant appears to exhibit reduced uptake of sertraline compared to wildtype. However, the behavior of the $CHC1^{E292K}$ mutant is more complex, and appears not to involve uptake given the growth-stimulatory effects of sub-lethal doses of sertraline. Rather, the downstream, cytotoxic effects of sertraline accumulation are suppressed, suggesting that the affinity of membranes for sertraline is reduced.

**Sertraline induces all-or-none cytotoxicity**

The aforementioned experiments all involve populations of cells. In order to verify that the observed changes in absolute resistance or the kinetics of resistance occur at the level of single cells, we assessed cellular response to sertraline using colony-forming unit assays. These experiments unequivocally show that sertraline-induced cytotoxicity is all-or-none and irreversible. Viability of wildtype BY4716 cells after 100 minutes of sertraline treatment followed by wash out is 56% of untreated isogenic controls; after 250 minutes is 28% of untreated isogenic controls (Figure 5). The rate of cell death seems to
approximate 50% of the population per cell cycle at the cell densities and sertraline concentration tested, i.e., in the linear range of this assay, with the caveats that these are asynchronous batch cultures, and that increasing drug concentration while holding cell density constant accelerates the rate of cell death ultimately to ~100%. Also, the surviving clones do not exhibit a growth lag after wash out, indicating negligible after-effects of sertraline treatment.

As a control, we used micromanipulation to physically isolate individual cells post-treatment and wash out, and independently calculated loss of viability as a function of time. We observed nearly identical results using both methods. Following micromanipulation of single cells, 55% (53/96) yielded colony-forming units after 100 minutes of sertraline treatment followed by wash out; 23% (22/96) yielded colony-forming units after 250 minutes; untreated cells yielded 99% (94/96) colony-forming units. Furthermore, visual inspection revealed that single cells that failed to form colonies were terminally arrested at the one-cell stage but showed no evidence of lysis. As a negative control, wildtype cells treated with cycloheximide, which is cytostatic, and then washed, recover with nearly no loss of viability. These results suggest that there is a non-uniform distribution of drug uptake or downstream compensatory mechanisms or both in individual cells.

Next, we assayed the viability of \( \text{CUP5}^{R46I} \) and \( \text{CHC1}^{E292K} \) mutants under the same assay conditions (Figure 5). The \( \text{CUP5}^{R46I} \) mutant demonstrably loses viability in response to sertraline treatment but at 60% of the wildtype rate. Viability of \( \text{CUP5}^{R46I} \) cells after 100
minutes of sertraline treatment followed by wash out is 80% of untreated isogenic controls; after 250 minutes is 56% of untreated isogenic controls. On the other hand, nearly no loss of viability was measured for the \(\text{CHC1}^{E292K}\) mutant. In fact, colony-forming units increase to 130% of untreated control levels after exposure of \(\text{CHC1}^{E292K}\) cells to sertraline, though this increase in viability only becomes apparent after 350 minutes of sertraline treatment. Taking all growth-based assays together, we hypothesize that the V-ATPase and clathrin modulate resistance to sertraline in two fundamentally different ways. On the one hand, loss of V-ATPase complex activity appears to lower but not extinguish the probability that individual clones accumulate the minimum-required lethal dose of sertraline. On the other hand, dysregulation of clathrin function appears to raise uniformly the minimum-required lethal dose of sertraline across the population, thereby protecting clones that would otherwise succumb.

**Chemical-genomic screening also implicates clathrin-coat formation**

In order to complement the genetic landscape determined by an examination of \textit{de novo} variation, we performed two chemical-genomic screens. The first involved identifying strains in the complete homozygous knockout collection (~4600 strains) that are hypersensitive to sertraline. This type of synthetic-lethal screen has been used to identify non-essential cellular pathways that function in parallel to the pathway(s) affected by a small-molecule drug of interest. The second experiment involved a screen of the complete heterozygous deletion collection (~6000 strains) for strains that are haploinsufficient with respect to sertraline-induced cytotoxicity. Haploinsufficiency screens have been used to identify essential protein targets of small-molecule drugs, and
are much more stringent than synthetic-lethal screens. Response to sertraline has already been assessed using these knockout libraries, though in those experiments pools of deletions mutants were assayed in bulk as opposed to individually in separate wells (ERICSON et al. 2008).

The top 20 most hypersensitive deletion strains (Table S1) are notably enriched for genes involved in the process Gene Ontology (GO) term “vesicle-mediated transport” (p-value, 1.40e⁻⁵), the function GO term “clathrin binding” (p-value, 4.54e⁻⁵), and the component GO term “AP-1 adaptor complex” (p-value, 4.55e⁻⁸). Similar enrichments were reported by the aforementioned chemical-genomic study. The AP-1 complex localizes to the Golgi and endosomes, where it nucleates clathrin assembly. It is composed of two large subunits (APL2 and APL4), one of two medium subunits (APL1 and APM2) and one small subunit (APS1). There are three known AP complexes in yeast, but we only observe genetic interactions with AP-1 subunit deletion strains. Other sertraline-hypersensitive strains include vps1Δ, which normally encodes a fungal dynamin-like protein, a GTPase involved in vesicle scission; laa1Δ, which normally encodes an AP-1 complex accessory protein; mpk1Δ, which normally encodes serine/threonine kinase that is regulated by the PKC1 signaling pathway.

The heterozygous deletion collection yielded two heterozygous deletion strains, PIK1/pik1Δ and NEO1/neo1Δ, which are haploinsufficient in sertraline. PIK1, an essential gene, encodes phosphatidylinositol 4-kinase, which catalyzes the formation of phosphatidylinositol 4-phosphate, PI(4)P, a precursor of PIP2 and an effector molecule in
its own right that concentrates in the trans-Golgi network (TGN), where it recruits the
AP-1 complex in one of the early steps of clathrin-coated vesicle biogenesis. NEO1, also
an essential gene, encodes a P-type ATPase and putative flippase of unknown substrate
specificity, and also localizes to the Golgi and endosomes. Both strains exhibit a two-fold
increase in sensitivity to sertraline compared to wildtype (Figure S4). NEO1 was
originally isolated as a multicopy suppressor of neomycin cytotoxicity (PREZANT et al.
1996); neomycin is an aminoglycoside antibiotic that has been shown to bind to the
phosphoinositide PIP2. These complementary genetic and chemical-genomic interaction
data suggest that sertraline-induced cytotoxicity is modulated by phosphoinositide
metabolism or phospholipid asymmetry (or both) at sites of vesicle formation in the
TGN. Interestingly, some CADs, including chlorpromazine, have been shown to affect
phosphoinositide metabolism at concentrations relevant to the present study (LELI and
HAUSER 1987b).

**Sertraline induces ultrastructural hallmarks of phospholipidosis**

Transmission electron microscopy (TEM) of yeast cells treated with chlorpromazine
previously showed evidence of perturbed Golgi- and vacuolar-membrane organization,
including the appearance of so-called Berkeley bodies, or aberrant vesicular structures
that take the place of normal Golgi (FILIPPI et al. 2007). We conducted a similar analysis
of organellar membrane ultrastructure in response to sertraline treatment. First we
examined wildtype cells treated with high-dose sertraline for one hour. We used a
membrane-preserving protocol previously employed to study membrane ultrastructure in
*VPT* mutants (BANTA et al. 1988). Comparison of fields of untreated and sertraline-
treated cells immediately reveals a vacuolar defect reminiscent of Class C VPT/VPS mutants (Figure 6). Specifically, four changes in membrane ultrastructure following drug treatment are noteworthy. First, electron-dense vacuoles, which typically number one to three per cell, were not observed. After scoring fields of cells for the presence or absence of vacuoles, we determined that 60% of untreated cells contain one or more vacuoles, while none of the sertraline-treated cells contain a normal, electron-dense vacuole. Vacuoles that are visible in sertraline-treated cells are much more electron-transparent compared to wildtype vacuoles, and often they contain large inclusions that appear to be incompletely digested autophagosomes (Figure 7B, D). The increase in vacuolar electron transparency and accompanying presence of autophagosomal inclusions suggests a defect in delivery of vacuolar hydrolases, loss of vacuolar acidification or some combination of both. Second, a surprising number of sertraline-treated cells also lack nuclei. Nuclei are visible in 60% of untreated cells, but only in 30% of sertraline-treated cells. And when the nuclear membrane is present in sertraline-treated cells, it appears swollen and has many gaps. The loss of nuclei could be a sign of apoptotic death, and would explain the irreversibility of sertraline-induced cytotoxicity.

Third, every sertraline-treated cell examined contained at least one of several classes of aberrant membrane-enclosed structures, among which 35% contain autophagosomal intermediates (e.g., dumbbell-shaped vesicles), indicating elevated rates of autophagy. Upon higher magnification, compacted multilamellar structures were observed encapsulated by double-membrane structures that are clearly autophagosomes (Figure 7E), indicating active phospholipidosis in sertraline-treated cells. Although direct
comparison between yeast and mammalian cells requires caution, the compacted, often crescent-shaped multilamellar arrays we observed in sertraline-treated yeast cells are remarkably similar to those previously observed in CAD-induced phospholipidosis in mammalian cells. Fourth, in 33% of sertraline-treated cells but never in untreated cells we observed 250-500 nm multilamellar bodies with complex morphology (Figure 7A). Upon higher magnification, some these multilamellar bodies appeared highly disordered, and contain numerous osmiophilic deposits (Figure 7C). Others display striking organization and resemble kiwi slices: a modestly electron-dense core encircled by a ring of osmiophilic puncta that is surrounded by a starburst of multilamellar ruffles (Figure S5).

As a comparison, we analyzed cup5 mutant cells using the same membrane-preserving protocol. At steady state, cup5 mutant cells appear similar to wildtype cells with at least one notable exception: the vacuoles of some cup5 mutant cells have a more electron-transparent, mottled appearance consistent with alkalinization due to inactivation of V-ATPase complex activity (Figure S6). (cup5 mutant cells also appear to have exaggerated endoplasmic reticulum, but this phenotype was not investigated further). Surprisingly, although all cup5 cells harbor the mutant allele, only 56% (23/41) of cells exhibit the vacuolar defect at steady state. In order to maximize differences between wildtype and cup5 mutant cells, we used a threefold lower dose of sertraline than what was used in the previous TEM experiment. After one hour of sertraline treatment of cup5 mutant cells, we observed fewer (8.5% vs. 16%) of what appear to be dead or dying cells – cells lacking both nuclei and vacuoles – compared to sertraline-treated wildtype cells.
This approximately twofold difference in the number of dead cells is consistent with the data obtained using colony-forming unit assays (Figure 5). Furthermore, these results are validate the hypothesis that sertraline uptake is not uniformly distributed among a clonal population of \textit{cup5} mutant cells, with approximately half of the population appearing to require higher concentrations of sertraline to achieve the same degree of cytotoxicity.

\textbf{Sertraline modulates phospholipase activity in vitro}

Inhibition of phospholipases by CADs may be responsible for the accumulation of multilamellar bodies observed in drug-induced phospholipidosis in mammalian cells, and may be responsible for sertraline-induced phospholipidosis we observed in yeast cells. In order to test whether sertraline directly affects phospholipases, we screened a panel of four different phospholipases using zwitterionic phosphatidylcholine vesicles as substrates. Phospholipase A$_1$ hydrolyzes the ester bond at the \textit{sn}-1 position, yielding a fatty acid and a lysophospholipid; phospholipase A$_2$ hydrolyzes the ester bond at the \textit{sn}-2 position, also yielding a fatty acid and a lysophospholipid. Phospholipase C hydrolyzes the ester bond before the phosphate group, releasing diacylglycerol and a phosphate-containing head group. Phospholipase D hydrolyzes the ester bond after the phosphate group, releasing phosphatidic acid and the head group choline.

If, as expected, sertraline intercalates into phospholipid bilayers, thereby altering membrane organization, then the substrate availability of individual membrane phospholipids might be changed. Indeed, chlorpromazine is known to affect the activity of reconstituted phospholipases, exhibiting biphasic or concentration-dependent effects,
such that at low doses it stimulates phospholipase activity but at high doses it inhibits phospholipase activity (LELI and HAUSER 1987a). For this reason, we also included chlorpromazine in these assays.

We screened both compounds across six orders of magnitude, from $10^{-9}$ to $10^{-3}$ M, encompassing the concentration range in which sertraline exhibits activity in yeast. Our results, summarized in Figure 8, clearly demonstrate a distinct pattern of activity across the panel of phospholipases, including stimulation, inhibition and mixed effects. Phospholipase A1 activity is inhibited equipotently by both sertraline and chlorpromazine above 10µM. Strikingly, phospholipase A2 activity is stimulated by both drugs to varying degrees. Sertraline’s stimulatory effects begin at 0.1µM and level off at 10µM, while chlorpromazine’s stimulatory effects are negligible below 10µM but increase exponentially thereafter. Both sertraline and chlorpromazine appreciably inhibit phospholipase C activity at or above ~50µM, chlorpromazine is more potent than sertraline, but only sertraline exhibits a modest stimulatory bump in the 1µM - 10µM range that is not fit by a classic sigmoidal function. Notably, the Hill coefficient for phospholipase C inhibition by both drugs is closer to -2, indicating negative cooperativity. This stands in contrast to the Hill coefficient for phospholipase A1 inhibition by both drugs of -1. Finally, sertraline and chlorpromazine are weak inhibitors of phospholipase D activity, achieving 50% inhibition at 100µM. As a negative control, we used a coupled-enzyme assay for the detection of inorganic phosphate that does not contain lipid in the reaction mixture. The effects of sertraline are indistinguishable from
those of vehicle, while chlorpromazine exhibits non-specific inhibitory effects but only in the high micromolar range (Figure S7).

The following trend emerges from these data with respect to the modulatory effects of both drugs on enzymatic activity: \( \text{PLA}_1 = \text{PLA}_2 > \text{PLC} > \text{PLD} \). Put another way, there is a positive correlation between the distance of the cleaved bond from the head group and the strength of drug-induced effects. This trend demonstrates that both sertraline and chlorpromazine intercalate into phospholipid membranes and, not unexpectedly, reside at the interface between the polar head groups and the apolar/hydrophobic acyl chains. The most striking result, however, is that the \( sn-1 \) and \( sn-2 \) ester linkages are only angstroms apart yet both drugs have opposite effects on their cleavage, suggesting a specific three-dimensional orientation of sertraline in the bilayer.

**DISCUSSION**

Upon integrating genetic, ultrastructural and biochemical observations in a model organism that does not express its putative protein target, we propose that the selective serotonin reuptake inhibitor sertraline (Zoloft\textsuperscript{®}) intercalates into the polar/apolar interface of the cytoplasmic leaflets of V-ATPase-acidified organelles involved in intracellular vesicle transport, e.g., the trans-Golgi network (TGN), endosomes and vacuole. The main physiological consequence of a lethal dose of sertraline is the induction of phospholipidosis, possibly because sertraline-intercalated membranes are indigestible to phospholipases. Phospholipidosis is correlated with membrane ultrastructural changes consistent with irreversible programmed cell death, e.g., increased autophagy and loss of
nuclei. Intriguingly, at sub-lethal concentrations, sertraline rescues the growth defect caused by dysregulation of clathrin function, raising the possibility that both sertraline and clathrin consume a limiting reagent in or associated with membranes (per anonymous reviewer’s suggestion).

By exploiting the cytotoxicity of sertraline in a drug-resistance selection, we identified two evolutionarily conserved membrane-active pathways that confer two-fold partial resistance to sertraline by completely different mechanisms. The first membrane-active pathway is vacuolar acidification, which was already known to be a determinant of resistance/sensitivity in yeast to compounds with physicochemical and pharmacological properties shared with sertraline. Mutations affecting V-ATPase activity appear to reduce the probability that an individual clone will uptake, i.e., accumulate intracellularly, the minimum lethal dose of sertraline without changing the underlying affinity of membranes for sertraline. However, the second membrane-active pathway is clathrin-coat formation, which was not previously known to affect drug resistance in yeast. Mutations in this pathway appear to reduce the underlying affinity of membranes for sertraline compared to wildtype without changing the rate of uptake.

In other words, the rate of sertraline uptake into vesiculogenic membranes and the total binding capacity of these membranes for sertraline can be regulated by at least two compensatory/protective mechanisms. The V-ATPase and clathrin may normally function in wildtype cells to alleviate sertraline-induced membrane stress but in the absence of mutation these buffering systems become overwhelmed. The point at which
individual cells succumb varies in a clonal population, as evidenced by the single-cell viability study. Epigenetic factors that could account for clonal variation in the penetrance of sertraline-induced cytotoxicity include intracellular pH, natural cycles of vacuole fission and fusion, or other periodicities associated with vesicular transport (Bishop et al. 2007).

The V-ATPase has already been shown to play a protective role in the cellular response to CADs, but clathrin constitutes a novel axis of protection. By incorporating this new axis, our model improves upon the drug-accumulation model proffered by previous studies of cellular response to CADs (Filippi et al. 2007; Shih et al. 1990; Shih et al. 1988). With respect to the specific role of the V-ATPase, our model also rests on a lysosome-trapping/basicicity argument. However, as hinted above, the results of the epistasis analysis with the cup5 swa2 double mutant indicate that a basicicity argument alone is insufficient. The synthetic sickness of that double mutant in the absence of sertraline, as well as the kinetic defect in the emergence of resistance, suggest that the V-ATPase and clathrin chaperones function in a context-dependent manner to regulate the equilibrium of clathrin assembly/disassembly, a claim for which there are precedents in the literature (Forgac et al. 1983; Liu et al. 1994). It is also possible that clathrin-mediated transport affects residual proton-translocating activity or another aspect of V-ATPase activity.

How might dysregulation of clathrin function alter the underlying affinity of vesiculogenic membranes for sertraline? To this answer question, we must return to the
aforementioned limiting reagent common to both sertraline and clathrin function. An attractive candidate is the phosphoinositide PI(4)P, which is generated by the gene product of PIK1, and is essential for viability. The haploinsufficiency of the PIK1/pik1Δ heterozygous deletion strain indicates that PI(4)P levels are limiting during sertraline-induced membrane stress. Reduced PI(4)P levels may sensitize cells to sertraline by several mechanisms: (i) PI(4)P may be a “receptor” for sertraline, in which case sertraline binding to it further depletes the active pool; (ii) the biosynthesis of PI(4)P is particularly sensitive to sertraline intercalation; (iii) the presence of PI(4)P is correlated with other membrane biophysical properties, such as membrane curvature, that favor sertraline intercalation (Mulet et al. 2008).

But what does sertraline intercalation, or CAD intercalation more broadly, entail at the atomic level? A recent study demonstrating that the σ-2 receptor agonist siramesine binds specifically to phosphatidic acid shows that lipids can be specific targets of small-molecules drugs (Parry et al. 2008). Also, studies of some CADs and membrane-disrupting antimicrobial cationic peptides reveal two phases of membrane binding. CADs are initially recruited to membranes through electrostatic interactions with head groups before penetrating into the hydrophobic region of the bilayer. It is tempting to hypothesize that the V-ATPase may affect the rate of the first phase, while clathrin may affect the affinity of the second phase.

In conclusion, this study demonstrates that sertraline, an antidepressant developed to have high potency and selectivity at a single protein target, also targets phospholipid
membranes by a molecular mechanism that may be common to many psychoactive cationic amphiphiles, even those that exhibit high degrees of polypharmacy at protein targets. Detailed atomic-level studies will be required to illuminate the exact nature of drug-lipid interactions. Future work will also be required to determine the extent to which the V-ATPase and clathrin shape the cellular response to sertraline in higher eukaryotes, and especially in neurons.

MATERIALS AND METHODS

Yeast strains and genetic analysis:
Standard genetic manipulations of S. cerevisiae were performed as previously described (GUTHRIE and FINK 1991). Standard growth conditions were YPD (1% yeast extract, 2% peptone and 2% dextrose) media at 30°C. BY4712 (leu2Δ0) and BY4716 (lys2Δ0) were chosen as the parental strains and are congenic to S288C (BRACHMANN et al. 1998). The chc1-521 temperature-sensitive strain and isogenic control strain (BY4742) were kindly provided by G. Payne (UCLA) (BENSEN et al. 2000).

Chemical compounds:
Sertraline hydrochloride (cat. # S6319) was purchased from Sigma Aldrich, resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 25 mg/mL (~73mM), and 100µL aliquots were stored in glass vials at -20°C until use and subjected to a maximum of one freeze/thaw cycle. Chlorpromazine hydrochloride (cat. # C8138), cycloheximide
(cat. # C7698), gramicidin (cat. # G5002), and bafilomycin A1 (cat. # B1793) were also purchased from Sigma Aldrich and treated similarly.

**Drug-resistance selection and hypersensitivity screen:**

We plated 100µL of unmutagenized overnight cultures inoculated from single colonies of BY4712 and BY4716 on YPD plates (2% agar) supplemented with ~45µM sertraline, which is approximately three times the half-maximal inhibitory concentration (IC50) of sertraline as determined by dose-response experiments in liquid culture. Mutagenesis was not performed so as to minimize the background polymorphism rate. Plates were grown for 3-5 days at 30°C until distinct sertraline-resistant colonies appeared. On average, independent cultures plated on selection plates yielded approximately a dozen large to medium colonies, and up to a dozen micro-colonies, depending on the exact incubation time. We note that the optimal selective concentration range is exceedingly narrow: lowering the selection concentration from ~45µM to ~42µM resulted, on average, in a 10-fold increase in the number of resistant colonies. 16 sertraline-resistant mutants were colony purified: eight each of BY4712 and BY4716. All BY4712 mutants were backcrossed to the parental BY4716, and vice versa, in a dominance test. Copies of the complete homozygous and heterozygous deletion collections originally purchased from Invitrogen were screened in 384-well plates in duplicate as previously described (PERLSTEIN *et al.* 2006). P-values associated with Gene Ontology (GO) enrichments were calculated using the GO Term Finder, which is accessible on the Saccharomyces Genome Database (www.yeastgenome.org).
**Microarray-based polymorphism detection:**

Genomic DNA (gDNA) was prepared for hybridization to Affymetrix® whole-genome S. cerevisiae tiling arrays (1.0R) as previously described (GRESHAM et al. 2006). However, the gDNA labeling protocol was modified: a BioPrime DNA labeling system (Invitrogen) was used to generate labeled gDNA fragments for hybridization accordingly to manufacturer’s protocol. We validated SNPscanner predictions by targeted resequencing of PCR amplicons corresponding to the entire open reading frame containing a candidate mutation. We then verified causality by showing 2:2 cosegregation of validated mutations with sertraline resistance in linkage analysis of tetrads. PCR was performed using the Accuprime Pfx polymerase kit (Invitrogen). For the analysis of recessive mutants, inclusion of two independent members of the same complementation group effectively excluded false positives. For dominant mutants, microarray-based polymorphism detection using a single hybridization worked reliably enough that independent biological replicates were not necessary. Open reading frames (ORFs) in which a polymorphism was predicted were amplified using the “A” and “D” primers designed by the Stanford Deletion Consortium for each ORF in the yeast genome. Resequencing of the immediate regions surrounding the candidate polymorphisms was performed with sequencing primers whose sequences are available upon request.

**Growth-related assays of sertraline-resistant strains:**

Dose-response experiments were performed in 96- or 384-well plates as previously described (PERLSTEIN et al. 2006). Growth-rate time courses were generated using a TECAN plate reader (Tecan Group Ltd); these experiments were performed at two
concentrations of sertraline in YPD over a ~16 hour observation period. We selected 30µM (high-dose regime) and 7.5µM (low-dose regime) because they are approximately two-fold higher and lower than sertraline’s IC50, respectively. Data was analyzed using Prism 4 (GraphPad Software, Inc). Cytotoxicity assays (n=3 per strain) were performed as follows: 3 x10^7 wildtype, but 1.5 x10^7 cup5 and chc1 mutant cells, were treated with 120µM sertraline, quickly pelleted by centrifugation (500 x g), and then washed 3 times. Half as many cup5 or chc1 mutant cells were used because these mutants exhibit two-fold greater resistance to sertraline compared to wildtype cells. Regardless of strain, high cell densities required more sertraline to achieve growth inhibition comparable to that observed at low cell densities. Cell concentration was determined by Coulter counter and 200µL of cells at a concentration of 10^3 cells/mL were plated on YPD plates and colony forming units were counted after 2-3 days incubation at 30ºC. Data were normalized to basal, pre-treatment viability measurements. Micromanipulation of single cells was performed using a benchtop dissecting microscope.

**Transmission electron microscopy and phospholipase assays:**

Samples were prepared as previously described (BANTA et al. 1988). For the high-dose treatment, mid-log phase cells at OD600 0.5 were treated with 120µM sertraline for one hour; for the cup5 comparison, mid-log phase cells at OD600 0.5 were treated with 45µM sertraline for one hour. For the 120µM dataset, quantification of vacuole number was performed using ImageJ on multi-cell, low magnification fields composed of 214 untreated or 77 treated cells; quantification of all other ultrastructural phenotypes was performed on a collection of single-cell, high magnification images containing 71
untreated or 52 sertraline-treated individual cells. For the 45µM dataset, quantification of dead cells was performed on 57 wildtype cells and 35 cup5 mutant cells. (Cell totals not listed here are directly listed in the Results). The following kits for assaying phospholipase activity was purchased from Invitrogen: EnzChek® Phospholipase A1 (cat. # E10219); EnzChek® Phospholipase A2 (cat. # E10217); EnzChek® Phospholipase Phospholipase C (cat. # E10215); Amplex® Red Phospholipase D (cat. # A12219). Assays were performed according to manufacturer’s specifications in clear-bottom, black 384-well plates (Corning). The PiPer Phosphate Assay Kit (cat. # P22061) was also purchased from Invitrogen. Assay plates were read in a SynergyMx multimode spectrophotometer (Biotek). Curve-fitting and analysis was performed using the Prism 4 (GraphPad Software, Inc.).

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FIGURE LEGENDS
FIGURE 1. Summary of dose-response experiments and cross-resistance test. For all panels the strains are color-coded as follows: BY4716 - black; $CUP5^{R46I}$ - red; $SWA2^{C603STOP}$ - blue. Strains were treated either with sertraline (A), chlorpromazine (B), or cycloheximide (C). Small-molecule drug concentration is given as log [M]. All strains were grown at 30ºC. Experimental OD$_{600}$ values were normalized to OD$_{600}$ values of untreated isogenic controls. Data points are the average of quadruplicate OD$_{600}$ measurements. Points were fit with a variable slope sigmoidal dose-response equation. Error bars indicate standard deviation.

FIGURE 2. Epistasis analysis of $cup5$ $swa2$ and $cup5$ $tfp1$ double mutants. (A). IC$_{50}$ values normalized by the wildtype tetratype of three spores derived from a representative tetrad. The three spores are color-coded as follows: $cup5$ – red; $swa2$ and $tfp1$ – blue; $cup5$ $swa2$ and $cup5$ $tfp1$ – purple. (B). Time course of $cup5$ $swa2$ double mutant (purple-filled circles) and the wildtype strain (black-filled circles). Both strains were treated with sertraline and grown at 30ºC. Data points are the average of six replicates. Error bars indicate standard deviation.

FIGURE 3. Biphasic effects of sertraline and chlorpromazine on mutants with dysregulated clathrin function. For all panels the strains are color-coded as follows: BY4716 - black; $CHC1^{E292K}$ - blue; $chc1-521$ - red. Strains were treated either with sertraline (A), chlorpromazine (B), or cycloheximide (C). Small-molecule drug concentration is given as log [M]. All strains were grown at 30ºC, except as indicated. Experimental OD$_{600}$ values were normalized to OD$_{600}$ values of untreated isogenic controls. Data points are the average of quadruplicate OD$_{600}$ measurements. Points were fit with a variable slope sigmoidal dose-response equation. Error bars indicate standard deviation.

FIGURE 4. Kinetic time-course experiments with select sert$^R$ strains grown in YPD supplemented with 0µM (blue-filled circles), 7.5µM (green-filled circles) or 30µM (red-filled circles) sertraline reveal two modes of resistance. (A). Baseline and sertraline-treated growth curves of the wildtype BY4716. (B). Baseline and sertraline-treated growth curves of the $CUP5^{R46I}$ mutant. (C). Baseline
and sertraline-treated growth curves of the \( CHC1^{E292K} \) mutant. (D). Baseline and sertraline-treated growth curves of the \( chc1-521 \) temperature-sensitive mutant. Cells were grown in 96-well plates and incubated with shaking at 30ºC in a TECAN plate reader except for (D) which was grown at 37ºC. 10^4 seconds equals approximately 2.8 hours. All OD_{595} measurements represent the average of six experimental replicates. Error bars indicate standard deviation.

FIGURE 5. Single-cell viability assay shows that sertraline is irreversibly cytotoxic. The percentage of colony-forming units is plotted as a function of the duration of sertraline treatment in minutes. Data corresponds to BY4716 (blue-filled circles); \( CHC1^{E292K} \) (red-filled circles); \( CUP5^{R46I} \) (green-filled circles).

FIGURE 6. Transmission electron micrographs reveal loss of vacuoles. (A). Representative thin section of a field of untreated wildtype BY4716 cells. (B). Representative thin section of a field of sertraline-treated wildtype BY4716 cells. Scale bars = 2.0 microns.

FIGURE 7. Transmission electron micrographs reveal sertraline-induced phospholipidosis. Wildtype BY4716 cells in mid-log phase were treated with 120µM for 1 hour (A). Thin section of a sertraline-treated cell containing multilamellar bodies. (B). Thin section of a sertraline-treated cell illustrating change in vacuolar contents and accumulation of inclusions. (C). High-magnification image of multilamellar body indicated by asterisk in panel (A). (D) High-magnification image of distended vacuole-like structure containing undigested autophagosomes indicated by asterisk in panel (B). (E). High-magnification image of a representative crescent-shaped multilamellar array encapsulated by an autophagosome. Scale bars are included in each panel.

FIGURE 8. Effects of sertraline and chlorpromazine on enzymatic activity of a panel of phospholipases. Data corresponding to sertraline treatment is shown in blue circles; chlorpromazine treatment is shown in red triangles. (A) Dose-response curve of phospholipase A1 activity. (B) Dose-response curve of phospholipase A2 activity. (C) Dose-response curve of
phospholipase C activity. (D) Dose-response curve of phospholipase D activity. All data points are normalized to baseline enzymatic activity in untreated wells and represent the mean of five experimental replicates. Error bars indicate standard deviation.

**FIGURE S1.** Summary of phenotypic analyses of sert^R tetrads. Each box corresponds to four spores (“a”, “b”, “c”, “d”) dissected from a representative tetrad generated from a heterozygous diploid strain (labeled below each box). Red indicates that the spore is sensitive, i.e., growth-inhibited, in a condition; green indicates that a spore is resistant, i.e., not growth-inhibited, in a condition; gray indicates that a spore was not assayed in a condition.

**FIGURE S2.** Intracellular and extracellular pH affects sertraline response. (A) Dose-response curve of wildtype BY4716 cells treated with 30µM sertraline and two-fold dilutions of bafilomycin A1. (B) Dose-response curve of wildtype BY4716 cells treated with 30µM sertraline and two-fold dilutions of gramicidin. (C) Dose-response curve of wildtype BY4716 cells in three different media: unbuffered YPD (yellow); YPD pH 4.0 (red); YPD pH 8.0 (blue). All strains were grown at 30°C. Experimental OD600 values were normalized to OD600 values of untreated isogenic controls. Data points are the average of quadruplicate OD600 measurements. Data points were fit with a variable slope sigmoidal dose-response equation. Error bars indicate standard deviation.

**FIGURE S3.** Isolation and characterization of mutation in *CSG2* that increases sertraline resistance in a *cup5* background. (A) Sequence chromatogram of an PCR product amplified from genomic DNA corresponding to a portion of the *CSG2* coding region that contains two polymorphisms predicted by the SNPscanner algorithm. Sequences derived from the *csg2* strain (“mutant”) and the parental strain BY4712 (“wildtype”) are depicted. (B). Final yields of genotyped spores from tetrads derived from a *Csg2/csg2 CUP5/cup5* diploid were measured at optical density 600 nm (OD600). Cells were grown in 96-well plates in YPD supplemented with 60µM sertraline and observed 24-48 hours post-inoculation. Solid black bars indicate average final yield
values of eight wildtype CSG2 CUP5 spores, four single mutant CSG2 cup5 spores, four single mutant csg2 CUP5 spores, and eight double mutant cup5 csg2 spores.

**FIGURE S4.** Phosphoinositide metabolism implicated in sertraline hypersensitivity. Dose-response curve of wildtype diploid BY4743 (black), PIK1/pik1 (blue), and NEO1/neo1 (green) treated with sertraline. All strains were grown at 30ºC. Experimental OD$_{600}$ values were normalized to OD$_{600}$ values of untreated isogenic controls. Data points are the average of quadruplicate OD$_{600}$ measurements. Data points were fit with a variable slope sigmoidal dose-response equation. Error bars indicate standard deviation.

**FIGURE S5.** Transmission electron micrograph of sertraline-treated BY4716 cell at high magnification. Asterisk indicates structure referred to as a kiwi. Scale bar is included.

**FIGURE S6.** Transmission electron micrograph of cup5 mutant cell at high magnification. Scale bar is included.

**FIGURE S7.** Results of enzymatic assay for the detection of inorganic phosphate. Sertraline (blue-filled circles), chlorpromazine (red-filled circles) and DMSO (black-filled circles) were tested at the concentrations indicated.

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<sup>a</sup> 3 clones of this allele were isolated; <sup>b</sup> 2 clones of this allele were isolated; <sup>c</sup> frameshift mutation at residue 277 results in premature stop at residue 280. n.d. is not determined.