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Running title: Synergy of colistin and echinocandin antifungals

Synopsis

Objectives: *Candida albicans* is the most prevalent fungal pathogen of humans, causing a wide range of infections from harmless superficial to severe systemic infections. Improvement of the antifungal arsenal is needed since existing antifungals can be associated with limited efficacy, toxicity and antifungal resistance. In this respect, we aimed to identify compounds to identify compounds acting synergistically with echinocandin antifungals and that could contribute to a faster reduction of the fungal burden.

Methods: 38,758 compounds were tested for their ability to act synergistically with aminocandin, a β-□□□-glucan synthase inhibitor of the echinocandin family of antifungals. The synergy between echinocandins and an identified hit was studied with chemogenomic screens and test of individual *Saccharomyces cerevisiae* and *C. albicans* mutant strains.

Results: We found that colistin, an antibiotic that targets membranes in Gram negative bacteria, is synergistic with drugs of the echinocandin family on all *Candida* species tested. The combination of colistin and aminocandin led to faster and increased permeabilization of *C. albicans* cells than either colistin or aminocandin alone. Echinocandin sensitivity was a pre-requisite to be able to observe the synergy. A large-scale screen for genes involved in natural resistance of yeast cells to low doses of the drugs, alone or in combination, identified efficient sphingolipid and chitin biosynthesis as necessary to protect *S. cerevisiae* and *C. albicans* cells against the antifungal combination.

Conclusions: These results suggest that echinocandin-mediated weakening of the cell wall facilitates colistin targeting of fungal membranes that in turn reinforces the antifungal activity of echinocandins.

Key-words:

Candida albicans, polymyxin, combination therapy, caspofungin, sphingolipid

Introduction

Invasive fungal infections (IFI) have emerged as a major cause of infectious complications in hospitalized patients with serious underlying diseases, especially those undergoing treatment of haematological malignancies or hospitalized in ICUs.[1,2] Recent and comprehensive epidemiological surveys have reported that invasive candidiasis accounts for approximately 75% of all IFI[3,4], and *Candida* species are now recurrently found among the five leading causes of nosocomial bloodstream infections.[5,6] Invasive candidiasis remains associated with a significant mortality, in the range of 40%[5,7], even in patients receiving antifungals of the echinocandin class. However, recent studies suggest that early antifungal treatment and the use of echinocandins might be optimal for patient survival and therapeutic success [1,8-10]. In this respect, it can be postulated that compounds that enhance the efficacy of echinocandins could contribute to a faster reduction of the fungal burden and have the potential to provide a breakthrough in the management of invasive candidiasis.

The main objective of the present study was to identify compounds acting synergistically with echinocandins, *ie* compounds that when combined with echinocandins would have a greater antifungal effect than the sum of the effects of the two individual compounds.[11] We have screened a collection of 38,758 compounds in order to identify those that would increase the antifungal activity of aminocandin (IP960; HMR3270; NXL201)[12], a non-marketed echinocandin to which we had easy access. We found that the antibiotic colistin showed synergism with aminocandin and another echinocandin, caspofungin[13] in all *Candida* species tested. Colistin (polymyxin E) is a member of the polymyxin family of antibiotics that show efficacy towards Gram-negative bacteria.[14] Polymyxins are cationic cyclic heptapeptides with a hydrophobic tail that interacts with the bacterial cytoplasmic membrane, therefore changing its permeability and triggering cell death.[14] Notably, weak antifungal activity of colistin and polymyxin B has been previously reported.[15,16] Moreover, polymyxin B was shown to

synergize with polyenes and azole antifungals and it has been proposed that azole-induced changes in the ergosterol content of fungal membranes might enhance the pore-forming activity of polymyxins.[17-19] In contrast to azoles and polyenes that impact fungal membranes, echinocandins are lipopeptides that target cell wall biosynthesis through non-competitive inhibition of β-1,3-glucan synthase, the product of the *FKS1* gene in *C. albicans*.[20] Moreover, echinocandins are proposed to act at the outer face of the plasma membrane[21] making it unlikely that their synergy with colistin has the same basis as that observed with azoles. Hence, we have further investigated the synergy between echinocandins and colistin with chemogenomic screens and test of individual mutant strains. Our data suggest that echinocandin-mediated alteration of the cell wall might facilitate colistin access to and perturbation of fungal membranes that in turn facilitate echinocandin activity.

Materials and methods

Strains and media

Clinical or reference isolates of *C. albicans* (SC5314[22]), clinical isolates of *Candida lusitaniae* (NEM_7104301), *Candida parapsilosis* (NEM_7103546), *Candida glabrata* (NEM_7118432), *Candida tropicalis* (NEM_7114236), *Candida krusei* (ATCC6258; LGC Standards, Molsheim, France), *Yarrowia lipolytica* (NEM_7115511) and laboratory isolates of *S. cerevisiae* (BY4741, BY4743[23]) were used in this study. Fluconazole- and caspofungin-resistant strains used in this study are listed in Table 1 and were obtained from D. Perlin (PHRI, New Jersey, USA) and D. Sanglard (CHUV, Lausanne, Switzerland). Strains were routinely grown at 30 °C in Yeast Peptone Dextrose medium (YPD; 1% yeast extract, 2% peptone, 2% glucose), or Synthetic Dextrose minimal medium (SD; 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose) and 2% agar for solid media. RPMI 1640 glutamax medium (Invitrogen) buffered with 50 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7 was used for screening compounds that synergize with aminocandin and for chequerboard assays. Alternatively RPMI 1640 glutamax medium buffered with 0.165 M MOPS (3-(N-morpholino)propanesulfonic acid) was used. Aminocandin (a kind gift from Dr. Dominique Lebeller), caspofungin (MSD), colistin sulfate (Sigma-Aldrich, Saint-Quentin Fallavier, France), colistin sodium methanesulfonate (Sigma-Aldrich), hygromycin B (Sigma-Aldrich), nourseothricin (Werner BioAgents, Jena, Germany) and 5-fluorocytosine (Sigma-Aldrich) were dissolved in H_2O. Fluconazole (Sigma-Aldrich) and amphotericin B (Sigma-Aldrich) were dissolved in DMSO.

Screening of a chemical library for synergy with aminocandin

A large-scale screen for antifungal synergy between a sub-inhibitory concentration of aminocandin and a collection of 38,758 chemicals obtained from Prestwick, ChemDiv and the

French “Chimiothèque Nationale”[24] was performed in 96-well plates using a Tecan Freedom EVO 200 platform. *C. albicans* wild-type cells of strain SC5314 were grown in the presence of 0.0156 mg/L aminocandin and 50 µM (on average) of each tested compound (dissolved in dimethyl sulfoxide, DMSO) in 100µl RPMI medium, starting from an OD_{600} of 0.01. Columns 1 and 12 were used for controls: DMSO was used as a negative control to define growth of the cells without compound; 10 mg/L of aminocandin was used as a positive control to kill all cells. Cells were incubated for 24 h at 30°C and cell viability was measured using a resazurin assay[25] through addition of resazurin-sodium salt to a final concentration of 100 mg/L. Plates were incubated for 2 h at room temperature (RT). While resazurin is naturally blue, its oxidation is associated with a change to red colour. To measure the colour change a dual wavelength measurement was performed (reference wavelength 604 nm, measurement wavelength 570 nm) using a Tecan Safire^2 microplate reader. For each plate the Z´factor[26] was calculated and was above 0.55 (Supplemental Figure 1A). The data were normalized using the following formula: % viability = 100 x (sample value - average value of positive controls) / (average of negative controls - average of positive controls). Active compounds were defined as compounds causing <1% viability. Chemical families were defined using clustering methods based on similarity concept using the Tanimoto coefficient and the K-modes clustering algorithm[27-29]. The best compounds were ordered from the different libraries and validated using minimum inhibitory concentration (MIC) assays.

MIC and chequerboard microdilution assays

In order to define the antifungal activity of a compound, MIC assays were performed in RPMI 1640 glutamax medium buffered with 50 mM Hepes. Compounds were diluted two fold in the concentration range 0.1 mg/L - 50 mg/L and $1.3x10^5$ cells.mL^{-1} of the *C. albicans* wild-type

strain SC5314 were added. The MIC assays were incubated for 24 h at 30°C and viability of the cells was measured using the resazurin assay as described above.

In order to assess the synergy between two compounds, checkerboad microdilution assays were performed in RPMI 1640 glutamax medium using 96-well plates.[30] The tested concentrations were 0.01-1 mg/L for aminocandin and caspofungin, 0.02-1.25 mg/L for fluconazole, 0.4-2.5 mg/L for amphotericin B, 0.008-0.5 mg/L for 5-fluorocytosine, 8-500 mg/L for hygromycin B, 0.08-5 mg/L for nourseothricin and 0.4-25 mg/L for colistin. The starting inoculum was $2.5x10^3$ cells/mL. Chequerboards were incubated for 24 h at 30°C and viability of the cells was measured using the resazurin assay as described above. The Fractional Inhibitory Concentration index (FICI) was calculated by using the formula: MIC of drug A tested in combination/MIC of drug A alone + MIC of drug B tested in combination/MIC of drug B alone and compounds were qualified as synergistic when the FICI was below or equal to 0.5.[11]

Fitness assay with barcoded *S. cerevisiae* knock-out collections

Fitness assays were performed with a homozygous and a heterozygous *S. cerevisiae* knock-out collection: 4885 *S. cerevisiae* mutants from the systematic deletion collection of Giaever *et al*. (strain BY4741[31]) were grown in individual wells in 96 deep-well plates at 30°C for 2 days in YPD medium, pooled and aliquots were stored at -80°C. A diploid heterozygous mutant collection (Invitrogen 95401.H4Pool; Life Technologies, Saint Aubin, France) consisting of 5936 mutants was grown for 15 generations in YPD, pooled and aliquots were stored at -80°C.

For each chemogenomics experiment, aliquots were thawed and cells were allowed to recover in YPD medium for 8 h at 30°C, under shaking. Pools of $3x10^6$ cells of the homozygous mutants were transferred to 100 ml flasks in YPD and cells were grown for about 11

generations at 30°C under the following conditions: With (1) no compound, (2) 0.00125 mg/L aminocandin, (3) 20 mg/L colistin and (4) the combination of 0.00125 mg/L aminocandin and 5 mg/L colistin. The pool of heterozygous mutants was grown with (1) no compound, (2) 0.002 mg/L aminocandin, (3) 30 mg/L colistin and (4) the combination of 0.002 mg/L aminocandin and 10 mg/L colistin.

Subsequently, $7x10^7$ cells were recovered by centrifugation, washed once with cold sterile water and stored at -80°C until processing. The growth of individual strains in the different cultures was determined by amplifying, labeling and hybridizing the barcodes on custom barcode microarrays (Agilent G2509F - AMADID N°026035; Agilent, Massy, France). Briefly, genomic DNA from the collected cells was extracted with phenol-chloroform by extensive vortexing in the presence of glass beads (425-600 nm size). Primers U1 and KU (see Supplemental Table 1 for primer sequences) were used to amplify the upstream barcodes and primers KD and D1 to amplify the downstream barcodes. 25 cycles of PCR with an annealing temperature of 50°C were used. The resulting PCR products were verified by electrophoresis on an agarose gel and used in a labeling PCR reaction with the Cy3 or Cy5 5'-labeled oligonucleotides U2comp for the upstream tags and D2comp for the downstream tags and unlabeled U1 and D1 as a control. Only 15 cycles of amplification were used in the labeling step. The labeled PCR products were mixed and precipitated in the presence of linear acrylamide and of a mixture of complementary oligonucleotides (U1, D1, U2block, D2block) in four fold molar excess to avoid binding of the fluorescently labeled oligonucleotides to the microarray probes. Hybridization was performed using the DIG Easy Hyb buffer (Roche Applied Science, Meylan, France), at 24°C, overnight, in a rotating Agilent hybridization chamber. The slides were washed in decreasing concentrations of SSPE buffer (10 mM potassium phosphate (pH 7.4), 150 mM NaCl, 0.5 mM EDTA, 0.05% (w/v) Triton X100) down to 0.2 x SSPE, dried and treated immediately with the Agilent Stabilization and Drying

Solution to avoid ozone-induced degradation of the Cy5 fluorophore. Scanning was performed on a Genepix 4200AL scanner (Molecular Devices, Saint Grégoire, France) and the images were analysed using Axon Genepix Pro 7 (Molecular Devices). We filtered the data according to our previous estimates of the reliability of the microarray signal.[32] Filtered data were normalized using the Loess algorithm (R package marray included in Bioconductor[33]) separately for signals coming from upstream or downstream barcodes. The average of the values for the upstream barcode and the downstream barcode was calculated. The log2 of the ratio between the signal obtained for a given mutant growing with and without drug was used as an estimate of the drug's effect on the growth rate of the mutant.

Confirmation of selected *S. cerevisiae* and *C. albicans* mutants

In order to confirm the data of the fitness screen we selected several strains of the homozygous and the heterozygous deletion mutant collections (Table 1) and retested them singularly. The respective strains were streaked on YPD plates and single colonies were used to isolate genomic DNA of the strains using the Epicentre MasterPure Yeast DNA purification kit (Tebu-bio, Le Perray en Yvelines, France). PCRs were performed using oligonucleotides binding to the up-stream region of the respective gene and on the KANMX4 cassette, which was used to delete the genes. Confirmed strains, *i e* those harbouring a correctly integrated marker cassette, were inoculated from an overnight culture to an OD_{600}=0.002 in YPD medium and grown with (1) no compound, (2) 0.00125 mg/L aminocandin, (3) 20 mg/L colistin and (4) the combination of 0.00125 mg/L aminocandin and 5 mg/L colistin over night at 30°C (16 h). Subsequently, the OD_{600} of the strains was measured to determine the sensitivity of the respective strains. *C. albicans* mutant strains (Table 1) were tested using the same procedure except that cells were grown with (1) no compound, (2) 0.00125 mg/L aminocandin, (3) 30 mg/L colistin and (4) the combination of 0.00125 mg/L aminocandin and

20 mg/L colistin.

Analysis of cell permeability using Propidium-Iodide staining

$2x10^5$ CFU/mL of the wild-type strain SC5314 were inoculated from an over-night culture in RPMI and grown for 1 h at 30°C. Subsequently, cells were grown in RPMI with (1) no compound, (2) 0.00125 mg/L aminocandin, (3) 20 mg/L colistin and (4) the combination of 0.00125 mg/L aminocandin and 20 mg/L colistin. After 10, 30, 50 and 70 minutes growth at 30°C, $4x10^6$ cells were harvested, washed with 1x PBS, resuspended in 1x PBS and incubated for 3 min with 5µg Propidium Iodide (PI; Sigma-Aldrich) in the dark. Subsequently, the cells were washed with 1x PBS, resuspended in 1x PBS and analysed using a Miltenyi Biotec MACS QUANT FACS machine (Miltenyi, Paris, France). Cells treated with 70% ethanol for 1 h were used as positive control while untreated cells were used as negative control. In order to analyse the data and to calculate the percentage of PI-stained cells, the software FlowJo version 7.6.5 (TreeStar Inc, Oregon, USA) was used. The raw data were analysed using the statistical test Chi2-Mantel-Haenszel.

Mouse model of systemic candidiasis

All animal experiments adhered to the EU Directive 86/609 on the approximation of laws, regulations and administrative provisions of Member States regarding the protection of animals used for experimental and other scientific purposes, and to related national regulations. All experiments were performed according to the guidelines of the European Convention For The Protection Of Vertebrate Animals Used For Experimental And Other Scientific Purposes (ETS No. 123). The protocol was approved by Institut Pasteur Health Center Animal Care Committee (Protocol number:10.455). Recovery of organs was performed following euthanasia of animals, and all efforts were made to minimize suffering.

In vivo interaction between colistin and caspofungin was evaluated using a mouse model of systemic infection. Ten weeks old BALB/c mice (Charles River, L'Arbresle, France) were immuno-supressed with 200mg/kg endoxan (cyclophosphamide; Baxter, Maurepas, France) 4 days before infection and were infected intravenously with 10^4 colony forming units (CFUs) per mouse of *C. albicans* strain SC5314. Treatment with antifungals was performed intraperitoneally in a volume of 200 µl and started 24 hours after infection. For *in vivo* studies the less toxic prodrug colistin sodium methanesulfonate was used. Colistin sodium methanesulfonate (Sigma-Aldrich) was injected two times per day at a concentration of 40 mg/kg[34] while Cancidas™ (caspofungin; MSD) was used at a concentration of 0.3 mg/kg and injected once per day[35]. Mice were kept in groups of 10 for each treatment group. One group of mice was treated with colistin alone, one with caspofungin alone and one with caspofungin and colistin, at the given concentrations. The group of mice not treated received 200 µl of sterile saline i.p. At 3 days postinfection (p.i.), mice were euthanized by CO_2 asphyxiation. Kidneys of each animal were removed, weighed and homogenized in 1ml of sterile 0.15 M NaCl. Serial 10-fold dilutions of the homogenates were plated onto SD agar containing 50 mg/L ticarcillin and 10 mg/L gentamycin and incubated for two days at 30°C. The fungal burden was determined by counting the CFUs and analysed using the software GraphPad Prism5. Data shown in Figure 6 represent pooled results of two independent experiments.

Results

Colistin is synergistic with echinocandins

In order to find compounds acting synergistically with the echinocandin aminocandin[12], a high-throughput screen was performed on a total of 38,758 molecules with a large diversity of chemical backbones. The compound collection comprised 16,224 commercial compounds [1,120 obtained from Prestwick Chemical (http://www.prestwickchemical.com/index.php?

pa=26) and 15,104 obtained from ChemDiv (http://chemistryondemand.com/compound-library)] and 22,534 compounds from the French academic chemical library (Chimiothèque Nationale[24], http://chimiotheque-nationale.enscm.fr/). Each compound at a final concentration around 50µM was combined with a sub-inhibitory dose of aminocandin (0.0156 mg/L) and growth of *C. albicans* SC5314 was recorded after 24h at 30°C using a resazurin assay for measuring cell viability. Data were normalized as percentage of viability relative to positive and negative controls. The Z' factor[26], which is a statistical test to define the quality of a large-scale screen was above 0.55 for all plates, indicating the robustness and reliability of the assay (Supplemental Figure 1A). Five hundred and fifty five compounds were identified whose combination with aminocandin resulted in 100% growth inhibition (Supplemental Figure 1B) and were clustered into chemical families. A hundred and fifty six compounds selected within the different families were reevaluated using MIC tests and chequerboard assays. Among these, 98 showed antifungal activity independently of the addition of aminocandin while 58 had no significant antifungal activity on their own but acted synergistically with aminocandin (data not shown).

Colistin sulfate (referred to below as colistin) was chosen for further study since it exhibited the highest synergy with aminocandin in a chequerboard microdilution assay (FICI=0.14 in RPMI-Hepes, FICI=0.25 in RPMI-MOPS; Table 2). Colistin showed antifungal activity towards *C. albicans* strain SC5314 at concentrations equal or above 60 mg/L (data not shown). Colistin was also tested in combination with different classes of antifungals using chequerboard-assays and the FIC indices were calculated for each combination (Table 2). We found synergy with the other β-1,3-glucan synthase inhibitor caspofungin (FICI=0.26). With both caspofungin and aminocandin, synergy was observed from a concentration of 0.4 mg/L colistin and *C. albicans* growth was prevented at all echinocandin concentrations tested in combination with a concentration of 3.1 mg/L colistin and above (data not shown). Our data

also indicated synergy of colistin with amphotericin B (FICI=0.27) and fluconazole (FICI=0.47), consistent with previously published data that showed synergy between these antifungals and polymyxin B.[17,18] In contrast, colistin did not show synergism with nourseothricin and hygromycin B that act on protein synthesis (FICI=0.55 and data not shown) and 5-fluorocytosine that acts on DNA and RNA synthesis (FICI=1).

We also tested whether colistin exhibited synergistic activity with aminocandin in other yeast species. We found synergism in all species tested with FICI ranging from 0.07 to 0.28 (Table 2). Colistin also showed weak antifungal activity in *C. lusitaniae*, *C. krusei, C. tropicalis* and *Y. lipolytica* whereas MICs were equal or above 50 mg/L for the other species (Table 2).

Taken together, these results showed that colistin, an antibiotic in clinical use, slightly increases the antifungal activity of amphotericin B and fluconazole, two drugs that target fungal membranes, and strongly potentiates the antifungal activity of cell wall β-1,3-glucan synthase inhibitors of the echinocandin family in yeasts.

Synergism of colistin and caspofungin in caspofungin- and fluconazole-resistant strains

The rising number of caspofungin- and fluconazole- resistant strains occurring in hospitals is of concern.[5] Therefore, we tested whether colistin acted synergistically with caspofungin in *C. albicans* caspofungin- and fluconazole-resistant strains (Table 1). While caspofungin-resistant strains harboured mutations in the *FKS1* gene that encodes β-1,3-glucan synthase targeted by echinocandins[36], fluconazole-resistant strains had mutations in *ERG11* that encodes cytochrome P450 lanosterol 14-alpha-demethylase targeted by azoles[37] and/or *TAC1* that encodes a transcription factor regulating the expression of the drug efflux pump-encoding genes *CDR1* and *CDR2*.[38] Consistent with results obtained using *C. albicans* strain SC5314, we found that colistin acted synergistically with caspofungin or fluconazole in all *C. albicans* wild-type isolates tested (Table 2 and data not shown). Colistin acted synergistically with

caspofungin in fluconazole-resistant strains but not in caspofungin-resistant strains (Table 2 and data not shown). This suggested that efficacy of caspofungin towards its target was a prerequisite to observe synergy with colistin. Moreover, it indicated that colistin toxicity was not affected by increased expression of drug efflux proteins including Cdr1 and Cdr2.

Genome-wide fitness profiling of *S. cerevisiae* knock-out mutants reveals a role of sphingolipid and chitin biosynthesis in tolerance to the aminocandin/colistin combination

Collections of molecularly-barcoded heterozygous and homozygous knock-out *S. cerevisiae* mutants are available [31]. Exposure of pools of both types of mutants to a given compound allows revealing those strains that show reduced or increased sensitivity to this compound. This strategy has been used successfully to obtain insights in the mode-of-action of antifungals [39]. Hence, we screened a collection of 4885 barcoded *S. cerevisiae* haploid KO mutants (excluding genes essential for *S. cerevisiae* growth) and a collection of 5936 barcoded *S. cerevisiae* heterozygous diploid KO mutants (including essential genes) for sensitivity to colistin, aminocandin, and the combination of aminocandin and colistin. Pools of deletion mutants were grown in YPD without compound, in the presence of 20 mg/L colistin (that led to a decrease in growth rate of about 10% allowing us to test the sensitivity of *S. cerevisiae* KO mutants to colistin alone), in the presence of 0.00125 mg/L aminocandin and in the presence of the combination of 0.00125 mg/L aminocandin and 5 mg/L colistin. Subsequently, cells treated with the different compounds or combination of compounds were compared to non-treated cells in order to identify those mutants that showed increased or decreased fitness in one of the three conditions tested (Supplemental Tables 2 and 3). Selected mutants identified in this manner were then tested individually in order to confirm the data of the high throughput experiments (Supplemental Figure 2).

Table 3 lists *S. cerevisiae* genes whose corresponding haploid gene deletion strains were most affected by treatment with colistin [$\log_2$(treated/untreated) < -0.7], aminocandin [$\log_2$(treated/untreated) < -0.7] or the combination of the drugs [$\log_2$(treated/untreated) < -1.0]. Enriched functional annotations for haploid mutants showing hypersensitivity to drugs were evaluated using the generic GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder). "Glycosphingolipid biosynthetic process" was the only term significantly enriched (corrected p value 0.00119) among the 14 strains most affected by colistin treatment. The genes annotated to this term code for Sur1/Csg1 and Csg2, two proteins that act in a complex to catalyse the formation of mannose-inositol-P-ceramide, one of the sphingolipid components that concentrate with ergosterol in lipid rafts of the plasma membrane.[40-42] Similarly, the term "Glycosphingolipid biosynthetic process" was enriched when functional annotations for the 20 heterozygous diploid mutants most sensitive to colistin were analysed (p=0.00598). Deletion of one copy of *SUR1/CSG1* led to hypersensitivity of the heterozygous diploid strain to colistin as did the deletion of one copy of *IPT1*, coding for the enzyme that catalyses the next step in this pathway *ie* synthesis of mannose-(inositol-P)2-ceramide from mannose-inositol-P-ceramide[43], and the deletion of *SUR4/ELO3* that encodes an elongase involved in sphingolipid biosynthesis[44] (Supplemental Table 3). As shown in Fig. 1 and Table 3, growth of haploid strains with deletions in sphingolipid biosynthesis genes was not altered in the presence of aminocandin. Therefore, the combination of colistin and aminocandin did not aggravate the fitness defect of these strains observed upon treatment with colistin alone even though "Glycosphingolipid biosynthetic process" was among the enriched GO terms (p=0.0085) found upon analysis of the 25 genes whose deletion conferred highest sensitivity to the aminocandin/colistin combination treatment.

We could not find any significant enrichment for a common annotation for the most affected haploid or heterozygous diploid strains from the screen using aminocandin alone. In contrast,

two GO terms were highly enriched among the 25 haploid strains most sensitive to the combination of aminocandin and colistin: "fungal-type cell wall chitin biosynthetic process" ($p=1.35x10^{-5}$, 4 genes - *CHS3, CHS7, CHS6* and *SKT5*) and the broader category "cellular cell wall organization or biogenesis" ($p=1.51x10^{-5}$, 9 genes, including chitin biosynthesis genes and *SLT2, YPK1, FKS1, ROM2* and *TUS1*). As shown in Fig. 1, inactivation of these genes also resulted in increased sensitivity to aminocandin alone but to a lesser extent than the sensitivity observed upon treatment with the combination. The observation that chitin-synthesis mutants with a decreased amount of chitin in their cell wall[45-49] have increased sensitivity to aminocandin is consistent with previously published data[39] and the role of chitin synthesis in protecting yeast cells against the cell wall alterations resulting from β-1,3-glucan synthase inhibition.[50] However, the enhanced reduction in fitness observed with the combination of aminocandin and colistin suggested that combined defects in β-1,3-glucan and chitin synthesis might facilitate colistin antifungal activity. Intriguingly, strains deleted for the *FKS1* and *ROM2* genes, the former encoding one of the two catalytic subunits of the *S. cerevisiae* β-1,3-glucan synthase and the latter encoding a GDP/GTP exchange protein for the Rho1 regulatory subunit of β-1,3-glucan synthase[51], were less affected than the average population of mutants by colistin treatment while they were hypersensitive to aminocandin and aminocandin/colistin combination treatments (Fig. 1).

Mutations in sphingolipid and chitin biosynthesis result in increased sensitivity to colistin in *C. albicans*

Results presented above indicated that defects in sphingolipid biosynthesis increased sensitivity of *S. cerevisiae* to colistin and the colistin/aminocandin combination while defects in chitin biosynthesis increased sensitivity to this combination and aminocandin alone. In order to test whether this held true in *C. albicans*, we took advantage of existing *C. albicans*

knock-out mutants in the *MIT1, CSG2, IPT1, CHS3, CHS5* and *CHS7* genes that encode orthologues of *S. cerevisiae SUR1/CSG1, CSG2, IPT1, CHS3, CHS5,* and *CHS7*.[52-56] Inactivation of *MIT1* and *CSG2* did not increase the sensitivity of *C. albicans* to colistin, aminocandin or their combination (data not shown). In contrast, inactivation of *IPT1* resulted in enhanced sensitivity of *C. albicans* to colistin alone or in combination with aminocandin (Fig. 2A). Furthermore, this increased susceptibility was abolished upon reintroduction of a wild-type allele of *IPT1* in the *ipt1*Δ mutant (Fig. 2A). Data presented in Fig. 2B showed that inactivation of *CHS3* increased the sensitivity of *C. albicans* to the aminocandin/colistin combination while having no significant effect on the susceptibility to the individual drugs. Inactivation of *CHS5* enhanced sensitivity of *C. albicans* to colistin alone or in combination with aminocandin while inactivation of *CHS7* had no effect. Taken together, these results indicated that, as observed in *S. cerevisiae,* efficient sphingolipid and chitin biosynthesis protected *C. albicans* against the antifungal activity of colistin and the aminocandin/colistin combination, although the impact of inactivating orthologous genes in *S. cerevisiae* and *C. albicans* had somewhat different outcomes.

Colistin treatment increases membrane permeability of aminocandin-treated *C. albicans* cells

Colistin might achieve its antifungal activity through targeting of biological membranes, as described for its antibacterial activity.[14] In order to test this hypothesis, we assessed the permeability of colistin- and/or aminocandin-treated *C. albicans* cells using the fluorescent dye propidium-iodide (PI) that enters cells only when the membrane has been permeabilized.[57] *C. albicans* strain SC5314 cells were grown for 1 h at 30°C, treated for different durations with (1) no compound, (2) 0.00125 mg/L aminocandin, (3) 20 mg/L colistin and (4) the combination of 0.00125 mg/L aminocandin and 20 mg/L colistin, and the

proportion of PI-stained cells was determined by flow cytometry. We found that a significantly larger amount of cells became stained with PI when treated with aminocandin and colistin relative to aminocandin alone ($P < 0.0001$; Fig. 3). Untreated cells and cells treated with colistin alone were not stained by the dye. Therefore, these results indicated that colistin treatment increased the permeability of *C. albicans* cells provided that these had been exposed to aminocandin.

The colistin/caspofungin combination reduces fungal burden in kidneys of mice infected with *C. albicans*

We tested whether the synergistic effect of colistin with echinocandins *in vitro* could be recapitulated during systemic *C. albicans* infection in mice. We could not identify any condition where the combination between Cancidas™, the clinical formulation of caspofungin, and the less toxic prodrug colistin sodium methanesulfonate allowed improved survival of *C. albicans*-infected mice (data not shown). However, when we used a combination of 0.3 mg/kg Cancidas™ and 40 mg/kg colistin sodium methanesulfonate, we observed a slightly but significantly lower fungal burden in kidneys after 3 days of infection relative to the fungal burden in mice that had been treated with 0.3 mg/kg caspofungin alone (Fig. 4; Mann Whitney test, p-value <0.05). Taken together, our results indicated that the combination between caspofungin and colistin might have the potential to decrease fungal burden during early stages of infection.

Discussion

Colistin targets the fungal membrane

In this study, we have found that the antibiotic colistin has weak, if any, antifungal activity towards several hemiascomycetous yeasts but that this activity is highly enhanced through

combination with echinocandins. Colistin is a member of the family of polymyxin antibiotics that target bacterial membranes and is currently used for the treatment of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections as well as infections caused by multi-resistant bacteria such as the recently emerged NDM-1 *Escherichia coli* and *Klebsiella pneumoniae.*[58] Weak antifungal activity of colistin and polymyxin B towards several distantly related fungal species has already been reported[15,16,18,34] and it has been proposed that they act at the fungal membrane. Indeed, colistin could trigger rapid efflux of ATP from germlings of *Mucorales* and alterations of membrane structure.[34] Moreover, polymyxin B showed synergy with membrane-targeting antifungals such as azoles and polyenes[17,18], an observation that we have extended to colistin in this study. Our results are consistent with this antifungal mode of action. Indeed, we could show that under conditions where the antifungal activity towards *C. albicans* could be manifested, *e g* in combination with the β-1,3-glucan synthase inhibitor aminocandin, *C. albicans* cells became significantly more permeable to the membrane impermeable fluorescent dye propidium-iodide[57], than when treated with aminocandin alone. Furthermore, fitness profiling experiments performed using *S. cerevisiae* haploid and heterozygous diploid knock-out mutants showed that alteration of sphingolipid biosynthesis results in increased sensitivity to colistin. Similarly, a *C. albicans ipt1*ΔΔ mutant defective for one of the enzymes of sphingolipid biosynthesis showed increased sensitivity to colistin and the aminocandin/colistin combination. Sphingolipids are essential components of the eukaryotic plasma membrane, concentrating together with sterols at so-called lipid rafts where they play a variety of functions.[42] Notably, *S. cerevisiae csg1, csg2* and *ipt1* and *C. albicans ipt1* mutants that are impaired in the latest steps of mannose-inositol-phosphoceramide (MIPC) and mannose-(inositol phosphate)2-ceramide (M(IP)2C) biosynthesis show increased sensitivity to azoles and amphotericin B.[39,53] Synergism of colistin with these antifungals and

mutations that alter sphingolipid biosynthesis are therefore consistent with its targeting of fungal membranes and possibly lipid rafts within these membranes.

Synergy of colistin with echinocandins: a rationale?

We have shown that colistin is synergistic with echinocandins. Analysis of the topology of *S. cerevisiae* β-1,3-glucan synthase and the location of aminoacids whose changes render the enzyme resistant to echinocandins suggest that these drugs interact with regions of the β-1,3-glucan synthase that are exposed to the external milieu or embedded in the outer leaflet of the plasma membrane.[21] Therefore, synergy between colistin and echinocandins is unlikely to result from colistin-mediated increased influx of echinocandins into cells. Here, we would like to propose that echinocandin-mediated weakening of the cell wall facilitates colistin targeting of fungal membranes that might in turn reinforce the antifungal activity of echinocandins. As mentioned above, combination of aminocandin with colistin rendered *C. albicans* cells highly permeable to propidium iodide while colistin-treated cells appeared impermeable to this compound. This suggests that echinocandin treatment facilitated access of colistin to membranes and its membrane-targeted antifungal activity, similar to what has been shown for echinocandin-facilitated entry of antimicrobial peptides (AMP).[59] Consistently, synergy between colistin and echinocandins was lost in strains harbouring mutations in the *FKS1* gene and rendering *C. albicans* resistant to echinocandins, suggesting that weakening of the cell wall through inhibition of β□□□□-glucan synthase was a prerequisite for colistin antifungal activity. Moreover, we have shown that sensitivity to aminocandin of *S. cerevisiae* and *C. albicans* mutants with defects in chitin synthesis was markedly aggravated when colistin was added. Chitin synthesis is triggered upon treatment of yeast cells by echinocandins and contributes to the tolerance to these drugs.[50,60,61] Our results are consistent with this observation and suggest that colistin might enhance the activity of

aminocandin and the chitin biosynthesis requirement for survival of aminocandin-treated cells. While this might also be explained by the impact of impaired chitin biosynthesis on colistin access to the plasma membrane and its antifungal activity, it is probably not the case, as chitin-defective mutants did not show increased sensitivity to colistin alone. In contrast, *S. cerevisiae* mutants with defects in β-1,3-glucan synthesis showed intrinsically high tolerance to colistin that might reflect adaptative changes in the cell wall in response to constitutive lower β-1,3-glucan levels[62], consequently reducing colistin ability to reach membranes.

Mechanisms by which colistin could enhance the activity of echinocandins have not been investigated here. However, it can be proposed that colistin-induced perturbation of the plasma membrane might impair β□□□□-glucan synthase activity, rendering it more susceptible to echinocandins, or might facilitate membrane insertion of echinocandins. Yet, it should be noted that not all membrane-targeting drugs are synergistic with echinocandins. For instance, azoles and polyenes are not generally regarded as synergistic with echinocandins in *Candida* spp. although some examples of synergy have been reported.[63-65] As mentioned above, synergy between cationic AMP and echinocandins has been observed.[59,66] Polymyxins are cationic cyclic heptapeptides with a hydrophobic tail suggesting that they share properties with other AMP that could specifically impact fungal membranes and β□□□□-glucan synthase activity. Interestingly, we did not observe any synergy between colistin and several cell wall-targeting drugs (Congo red, calcofluor white, nykkomycin Z; data not shown) suggesting that there is specificity in the colistin/echinocandin synergy.

Synergism of colistin and echinocandins in diverse fungal species and in a mouse model of systemic candidiasis

In this study, we have observed *in vitro* synergy between colistin and echinocandins in several pathogenic yeasts, namely *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C.*

krusei, as well as in fluconazole-resistant *C. albicans* strains. In contrast, no synergy could be observed between aminocandin and colistin in *A. fumigatus* (data not shown). Using a mouse model, we have shown that the colistin/caspofungin combination could possibly reduce fungal burden at early stages of systemic candidiasis relative caspofungin alone. However, this observation did not translate into an improvement of survival of animals suggesting that further demonstration of the efficacy of this drug combination in the treatment of systemic candidiasis will probably require optimizing delivery routes and evaluating pharmacodynamic interactions of caspofungin and colistin sodium methanesulfonate. Moreover, we believe that our results suggest that more emphasis should be put on the search and development of compounds that specifically target fungal membranes and could be synergistic with echinocandins.

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References

1. Pfaller MA, Diekema DJ. Epidemiology of invasive mycoses in North America. *Crit Rev Microbiol* 2010; **36**: 1-53.

2. Perlroth J, Choi B, Spellberg B. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med Mycol* 2007; **45**: 321-46.

3. Azie N, Neofytos D, Pfaller M *et al*. The PATH (Prospective Antifungal Therapy) Alliance(R) registry and invasive fungal infections: update 2012. *Diag Microbiol Infec Dis* 2012; **73**: 293-300.

4. Horn DL, Fishman JA, Steinbach WJ *et al*. Presentation of the PATH Alliance registry for prospective data collection and analysis of the epidemiology, therapy, and outcomes of invasive fungal infections. *Diag Microbiol Infec Dis* 2007; **59**: 407-14.

5. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 2007; **20**: 133-63.

6. Gudlaugsson O, Gillespie S, Lee K *et al*. Attributable mortality of nosocomial candidemia, revisited. *Clin Infect Dis* 2003; **37**: 1172-7.

7. Lass-Florl C. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* 2009; **52**: 197-205.

8. Garey KW, Rege M, Pai MP *et al.* Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin Infect Dis* 2006; **43**: 25-31.

9. Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of Candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob Agents Chemother* 2005; **49**: 3640-5.

10. Andes DR, Safdar N, Baddley JW *et al.* Impact of treatment strategy on outcomes in patients with candidemia and other forms of invasive candidiasis: a patient-level quantitative review of randomized trials. *Clin Infect Dis* 2012; **54**: 1110-22.

11. Hall MJ, Middleton RF, Westmacott D. The fractional inhibitory concentration (FIC) index as a measure of synergy. *J Antimicrob Chemother* 1983; **11**: 427-33.

12. Pasqualotto AC, Denning DW. New and emerging treatments for fungal infections. *J Antimicrob Chemother* 2008; **61 Suppl 1**: i19-30.

13. Groll AH, Walsh TJ. Caspofungin: pharmacology, safety and therapeutic potential in superficial and invasive fungal infections. *Expert Opin Investig Drugs* 2001; **10**: 1545-58.

14. Newton BA. The properties and mode of action of the polymyxins. *Bacteriol Rev* 1956; **20**: 14-27.

15. Nicholls MW. Polymyxin sensitivity of *Candida tropicalis*. *J Med Microbiol* 1970; **3**: 529-38.

16. Schwartz SN, Medoff G, Kobayashi GS *et al*. Antifungal properties of polymyxin B and its potentiation of tetracycline as an antifungal agent. *Antimicrob Agents Chemother* 1972; **2**: 36-40.

17. Moneib NA. In-vitro activity of commonly used antifungal agents in the presence of rifampin, polymyxin B and norfloxacin against *Candida albicans*. *J Chemother* 1995; **7**: 525-9.

18. Zhai B, Zhou H, Yang L *et al*. Polymyxin B, in combination with fluconazole, exerts a potent fungicidal effect. *J Antimicrob Chemother* 2010; **65**: 931-8.

19. Ogita A, Konishi Y, Borjihan B *et al*. Synergistic fungicidal activities of polymyxin B and ionophores, and their dependence on direct disruptive action of polymyxin B on fungal vacuole. *J Antibiot (Tokyo)* 2009; **62**: 81-7.

20. Denning DW. Echinocandin antifungal drugs. *Lancet* 2003; **362**: 1142-51.

21. Johnson ME, Edlind TD. Topological and Mutational Analysis of *Saccharomyces cerevisiae* Fks1. *Eukaryot Cell* 2012; **11**: 952-60.

22. Gillum AM, Tsay EY, Kirsch DR. Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. *Mol Gen Genet* 1984; **198**: 179-82.

23. Brachmann CB, Davies A, Cost GJ *et al*. Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 1998; **14**: 115-32.

24. Hibert MF. French/European academic compound library initiative. *Drug Discov Today* 2009; **14**: 723-5.

25. Tiballi RN, He X, Zarins LT *et al*. Use of a colorimetric system for yeast susceptibility testing. *J Clin Microbiol* 1995; **33**: 915-7.

26. Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* 1999; **4**: 67-73.

27. Chatuverdi A, Green PE, Carroll JD. K-modes clustering. *J Classification* 2001; **18**: 35-56.

28. Huang Z. Extensions to the k-means algorithm for clustering large data sets with categorical values. *Data Min Knowl Discov* 1998; **2**: 283-304.

29. Willett P. *Similarity and clustering in chemical information systems*. Letchworth, Hertfordshire, England: Research Studies Press Ltd., 1987.

30. Scott EM, Tariq VN, McCrory RM. Demonstration of synergy with fluconazole and either ibuprofen, sodium salicylate, or propylparaben against *Candida albicans* in vitro. *Antimicrob Agents Chemother* 1995; **39**: 2610-4.

31. Giaever G, Chu AM, Ni L *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* 2002; **418**: 387-91.

32. Peyroche G, Saveanu C, Dauplais M *et al.* Sodium selenide toxicity is mediated by O2-dependent DNA breaks. *PLoS ONE* 2012; **7**: e36343.

33. Gentleman RC, Carey VJ, Bates DM *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004; **5**: R80.

34. Ben-Ami R, Lewis RE, Tarrand J *et al.* Antifungal activity of colistin against mucorales species in vitro and in a murine model of *Rhizopus oryzae* pulmonary infection. *Antimicrob Agents Chemother* 2010; **54**: 484-90.

35. Louie A, Deziel M, Liu W *et al.* Pharmacodynamics of caspofungin in a murine model of systemic candidiasis: importance of persistence of caspofungin in tissues to understanding drug activity. *Antimic Agents Chemother* 2005; **49**: 5058-68.

36. Perlin DS. Resistance to echinocandin-class antifungal drugs. *Drug Resist Updat* 2007; **10**: 121-30.

37. Vanden Bossche H, Koymans L, Moereels H. P450 inhibitors of use in medical treatment: focus on mechanisms of action. *Pharmacol Ther* 1995; **67**: 79-100.

38. Coste AT, Karababa M, Ischer F *et al*. *TAC1*, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. *Eukaryot Cell* 2004; **3**: 1639-52.

39. Hillenmeyer ME, Fung E, Wildenhain J *et al*. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* 2008; **320**: 362-5.

40. Uemura S, Kihara A, Iwaki S *et al*. Regulation of the transport and protein levels of the inositol phosphorylceramide mannosyltransferases Csg1 and Csh1 by the Ca2+-binding protein Csg2. *J Biol Chem* 2007; **282**: 8613-21.

41. Uemura S, Kihara A, Inokuchi J *et al*. Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. *J Biol Chem* 2003; **278**: 45049-55.

42. Dickson RC. Thematic review series: sphingolipids. New insights into sphingolipid metabolism and function in budding yeast. *J Lipid Res* 2008; **49**: 909-21.

43. Dickson RC, Nagiec EE, Wells GB *et al*. Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in *Saccharomyces cerevisiae*, requires the *IPT1* (*YDR072c*) gene. *J Biol Chem* 1997; **272**: 29620-5.

44. Oh CS, Toke DA, Mandala S *et al*. *ELO2* and *ELO3*, homologues of the *Saccharomyces cerevisiae ELO1* gene, function in fatty acid elongation and are required for sphingolipid formation. *J Biol Chem* 1997; **272**: 17376-84.

45. Cid VJ, Duran A, del Rey F *et al*. Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiol Rev* 1995; **59**: 345-86.

46. Ziman M, Chuang JS, Tsung M *et al*. Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*. *Mol Biol Cell* 1998; **9**: 1565-76.

47. Trilla JA, Duran A, Roncero C. Chs7p, a new protein involved in the control of protein export from the endoplasmic reticulum that is specifically engaged in the regulation of chitin synthesis in *Saccharomyces cerevisiae*. *J Cell Biol* 1999; **145**: 1153-63.

48. DeMarini DJ, Adams AE, Fares H *et al*. A septin-based hierarchy of proteins required for localized deposition of chitin in the S*accharomyces cerevisiae* cell wall. *J Cell Biol* 1997; **139**: 75-93.

49. Lam KK, Davey M, Sun B *et al*. Palmitoylation by the DHHC protein Pfa4 regulates the ER exit of Chs3. *J Cell Biol* 2006; **174**: 19-25.

50. Walker LA, Munro CA, de Bruijn I *et al*. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog* 2008; **4**: e1000040.

51. Ozaki K, Tanaka K, Imamura H *et al*. Rom1p and Rom2p are GDP/GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. *The EMBO journal* 1996; **15**: 2196-207.

52. Mille C, Janbon G, Delplace F *et al*. Inactivation of *CaMIT1* inhibits *Candida albicans* phospholipomannan beta-mannosylation, reduces virulence, and alters cell wall protein beta-mannosylation. *J Biol Chem* 2004; **279**: 47952-60.

53. Prasad T, Saini P, Gaur NA *et al*. Functional analysis of *CaIPT1*, a sphingolipid biosynthetic gene involved in multidrug resistance and morphogenesis of *Candida albicans*. *Antimicrob Agents Chemother* 2005; **49**: 3442-52.

54. Noble SM, French S, Kohn LA *et al*. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nat Genet* 2010; **42**: 590-8.

55. Sanz M, Carrano L, Jimenez C *et al*. *Candida albicans* strains deficient in *CHS7*, a key regulator of chitin synthase III, exhibit morphogenetic alterations and attenuated virulence. *Microbiology* 2005; **151**: 2623-36.

56. Bulawa CE, Miller DW, Henry LK *et al*. Attenuated virulence of chitin-deficient mutants of *Candida albicans*. *Proc Natl Acad Sci* 1995; **92**: 10570-4.

57. Deere D, Shen J, Vesey G *et al*. Flow cytometry and cell sorting for yeast viability assessment and cell selection. *Yeast* 1998; **14**: 147-60.

58. Li J, Nation RL, Turnidge JD *et al*. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis* 2006; **6**: 589-601.

59. Harris MR, Coote PJ. Combination of caspofungin or anidulafungin with antimicrobial peptides results in potent synergistic killing of *Candida albicans* and *Candida glabrata* in vitro. *Int J Antimic Ag* 2010; **35**: 347-56.

60. Markovich S, Yekutiel A, Shalit I *et al*. Genomic approach to identification of mutations affecting caspofungin susceptibility in *Saccharomyces cerevisiae*. *Antimicrob Agents Chemother* 2004; **48**: 3871-6.

61. Lee KK, Maccallum DM, Jacobsen MD *et al*. Elevated cell wall chitin in *Candida albicans* confers echinocandin resistance in vivo. *Antimicrob Agents Chemother* 2010.

62. Lesage G, Sdicu AM, Menard P *et al*. Analysis of beta-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. *Genetics* 2004; **167**: 35-49.

63. Kiraz N, Dag I, Yamac M *et al*. Synergistic activities of three triazoles with caspofungin against *Candida glabrata* isolates determined by time-kill, Etest, and disk diffusion methods. *Antimicrob Agents Chemother* 2010; **54**: 2244-7.

64. Oliveira ER, Fothergill AW, Kirkpatrick WR *et al*. In vitro interaction of posaconazole and caspofungin against clinical isolates of *Candida glabrata*. *Antimicrob Agents Chemother* 2005; **49**: 3544-5.

65. Roling EE, Klepser ME, Wasson A *et al*. Antifungal activities of fluconazole, caspofungin (MK0991), and anidulafungin (LY 303366) alone and in combination against

Candida spp. and Crytococcus neoformans via time-kill methods. *Diagn Microbiol Infect Dis* 2002; **43**: 13-7.

66. Rossignol T, Kelly B, Dobson C *et al*. Endocytosis-mediated vacuolar accumulation of the human ApoE apolipoprotein-derived ApoEdpL-W antimicrobial peptide contributes to is antifungal activity in *Candida albicans*. *Antimicrob Agents Chemother* 2011; **55**: 4670-81.

67. Balashov SV, Park S, Perlin DS. Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in *FKS1*. *Antimicrob Agents Chemother* 2006; **50**: 2058-63.

68. Karababa M, Coste AT, Rognon B *et al*. Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. *Antimicrob Agents Chemother* 2004; **48**: 3064-79.

69. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 1993; **134**: 717-28.

70. Noble SM, Johnson AD. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot Cell* 2005; **4**: 298-309.

71. Cherry JM, Hong EL, Amundsen C *et al*. Saccharomyces Genome Database: the genomics resource of budding yeast. *Nucl Acids Res* 2012; **40**: D700-5.

Legends to Figures

Figure 1: Colistin affects sphingholipid biosynthesis deficient strains and increases the sensitivity of chitin synthesis mutants to aminocandin. A pool of *S. cerevisiae* haploid deletion strains was treated with colistin (20 mg/L), aminocandin (0.00125 mg/L) or the combination of 0.00125 mg/L aminocandin and 5 mg/L colistin and grown for 11 generations in liquid medium. Relative growth rates, estimated using barcode-specific microarrays, were

normalized and represented as log2 ratios of the signal obtained with treated versus untreated culture. Mutant strain behaviour in the different conditions is illustrated for genes that belong to functional categories that were found to be significantly enriched (sphingolipid biosynthesis, green; cell wall organization and biogenesis, blue and chitin synthesis, red, see text for details).

Figure 2: Efficient sphingolipid or chitin biosynthesis protects *C. albicans* cells of the combined antifungal activity of aminocandin and colistin. *C. albicans* strains with mutations in the *IPT1* gene involved in sphingolipid biosynthesis (deltaIPT1, TPIPT1-4; deltaIPT1+IPT1, TPIPT1-4; Table 1) and in the *CHS3, CHS5* and *CHS7* genes involved in chitin biosynthesis (CACB3B-5, ΔCHS5_ARGplu, ΔCHS3_ARGplus, respectively; Table 1) and their parent strains (CAI4 and SN250_ARGplus; Table 1) were grown in the absence of drugs (untreated) or in the presence of aminocandin alone (AMC; 0.00125 mg/L), colistin alone (COL; 30 mg/L), and the combination of aminocandin (0.00125 mg/L) and colistin (20 mg/L) (AMC + COL). Optical density was recorded after 16 h growth and is represented relative to the optical density obtained for the untreated wild-type cells. Data are mean of 2 independent experiments, with bars indicating the range of values.

Figure 3: Colistin increases permeability of echinocandin-treated cells

Cells were left untreated or treated with colistin alone (COL, 20 mg/L), aminocandin alone (AMC, 0.00125 mg/L) or with the combination of both drugs (AMC + COL) and the proportion of propidium-iodide stained cells was measured by flow cytometry. The statistical test Chi2-Mantel-Haenszel was used to analyse the difference between cells treated with aminocandin alone and with the combination. At all time points the difference was significant ($p < 0.0001$).

Figure 4: The colistin/caspofungin combination reduces fungal burden in kidneys of *C. albicans* infected mice. Immuno-supressed female BALB/c mice were infected intravenously with 10^4 CFU/mouse of *C. albicans* strain SC5314. One group of mice was not treated, one was treated with colistin alone (40 mg/kg), one with caspofungin alone (0.3 mg/kg) and one with caspofungin and colistin at the given concentrations. Mice were killed three days after infection and fungal burden in kidneys of mice was evaluated. * $P<0.05$. Note that only 7 mice untreated or treated with colistin alone were still alive on day 3 after infection, therefore the CFUs of only 7 kidneys are represented in the graph.

Supplemental Figure 1:

High-throughput screening for compounds acting synergistically with aminocandin. (A) Evaluation of the test using the calculated Z'-factor value plotted for each plate. (B) Distribution of the number of compounds as a function of the percentage of viability as defined in the material and methods section.

Supplemental Figure 2:

Confirmation of *S. cerevisiae* mutants exhibiting sensitivity towards the treatment in a fitness assay. Heterozygous- and homozygous mutants were grown for 16 h at 30°C without compound (-Amc -CS), in presence of aminocandin only (+Amc -CS), colistin alone (-Amc +CS) and the combination of both compounds (+Amc +CS).

Table 1: Yeast strains used in this study

Species	Strain	Type of strain	Genotype	Reference
C. albicans	DPL1000	WT, parent of 20S, 22S and 36S		D. Perlin
C. albicans	20S	Caspofungin-resistant, Fks1^{S645F}	*FKS1*^{C1934T}/*FKS1*^{C1934T}	67
C. albicans	22S	Caspofungin-resistant, Fks1^{S645Y}	*FKS1*^{C1934A}/*FKS1*^{C1934A}	67
C. albicans	36S	Caspofungin-resistant,Fks1^{S645P}	*FKS1*^{T1933C}/*FKS1*^{T1933C}	67
C. albicans	M70	WT, parent of C42		
C. albicans	C42	Caspfungin-resistant, Fks1^{F641S}	*FKS1/FKS1*	
C. albicans	DSY294	Fluconazole-susceptible clinical strain	*TAC1-3/TAC1-4 ERG11-3/ERG11-4*	68
C. albicans	DSY296	Fluconazole-resistant	*TAC1-5/TAC1-5 ERG11-5/ERG11-5*	68
C. albicans	DSY3987	Fluconazole-resistant	*ura3*Δ*::FRT/ura3*Δ*::FRT TAC1-5/TAC1-5 ERG11-5/ERG11-5* RP10::CIp10	D. Sanglard
C. albicans	DSY3988	Fluconazole-resistant	ura3Δ::*FRT/ ura3*Δ*::FRT tac1-5*Δ*::hisG/tac1-5*Δ*::hisG ERG11-5/ERG11-5* RP10::CIp10	D. Sanglard
C. albicans	CAI4		Δ*ura3::λimm434/*Δ*ura3::λimm434*	69
C. albicans	TPIPT1-4		Δ*ura3::λimm434/*Δ*ura3::λimm434* Δ*ipt1::hisG/*Δ*ipt1::hisG*	53
C. albicans	TPIPT1-5		Δ*ura3::λimm434/*Δ*ura3::λimm434* Δ*ipt1::hisG/*Δ*ipt1::hisG::IPT1-URA3*	53
C. albicans	CACB3B-5		Δ*ura3:: λimm434/1*Δ*ura3:: λimm434* Δ*chs3-2::hisG/*Δ*chs3-3::hisG_URA3_hisG_URA3_hisG*	56
C. albicans	SN152		*arg4*Δ*/arg4*Δ *leu2*Δ*/leu2*Δ *his1*Δ*/his1*Δ *URA3/ura3*Δ*::λimm434 IRO1/iro1*Δ*::λimm434*	70
C. albicans	SN250_ARGplus		*his1Δ/his1Δ leu2Δ::CdHIS1/leu2Δ::CmLEU2 arg4Δ::CaARG4/arg4Δ URA3/ura3Δ::imm434 IRO1/iro1Δ::imm434*	54
C. albicans	ΔCHS5_ARGplus		SN152 *chs5*Δ*::CmLEU2 /chs5*Δ*::CdHIS1 arg4Δ::CaARG4*	54
C. albicans	ΔCHS7_ARGplus		SN152 *chs7*Δ*::CmLEU2 /chs7*Δ*::CdHIS1 arg4Δ::CaARG4*	54
C. albicans	ΔCSG2_ARGplus		SN152 *csg2*Δ*::CmLEU2 /csg2*Δ*::CdHIS1 arg4Δ::CaARG4*	54
C. albicans	S11.A14		*mit1-*Δ*::hisG/mit1-*Δ*::hisG-URA3-hisG*	52

S. cerevisiae	BY4741	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	[23]
S. cerevisiae	BY4743	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*/ MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	[23]
S. cerevisiae	*sur*?Δ	BY4741 *sur1*Δ??KanMX4	[31]
S. cerevisiae	*sur1*Δ het	BY4743 *sur1*Δ??KanMX4/*SUR1*	Invitrogen
S. cerevisiae	*csg2*Δ	BY4741 *csg2*Δ??KanMX4	[31]
S. cerevisiae	*gef1*Δ	BY4741 *gef1*Δ??KanMX4	[31]
S. cerevisiae	*erg6*Δ	BY4741 *erg6*Δ??KanMX4	[31]
S. cerevisiae	*vps74*Δ	BY4741 *vps74*Δ??KanMX4	[31]
S. cerevisiae	*trp1*Δ	BY4741 *trp1*Δ??KanMX4	[31]
S. cerevisiae	*chs3*Δ	BY4741 *chs3*Δ??KanMX4	[31]
S. cerevisiae	*chs5*Δ	BY4741 *chs5*Δ??KanMX4	[31]
S. cerevisiae	*chs6*Δ	BY4741 *chs6*Δ??KanMX4	[31]
S. cerevisiae	*chs7*Δ	BY4741 *chs7*Δ??KanMX4	[31]
S. cerevisiae	*skt5*Δ	BY4741 *skt5*Δ??KanMX4	[31]
S. cerevisiae	*ipt1*Δ?het	BY4743 *ipt1*Δ??KanMX4/*IPT1*	Invitrogen
S. cerevisiae	*sur4*Δ?het	BY4743 *sur4*Δ??KanMX4/*SUR4*	Invitrogen

Table 2: Synergistic activity of colistin with echinocandins in different yeast species

Strain (Phenotype)		MIC of individual compound		Optimal compound concentrations for observing synergy		
	Test compound	Test compound (mg/L)	Colistin (mg/L)	Test compound (mg/L)	Colistin (mg/L)	FICI[1]
Candida albicans SC5314	Aminocandin	0.5	60	0.06	0.39	0.14
Candida albicans SC5314	Caspofungin	0.5	60	0.125	0.39	0.26
Candida albicans SC5314	Fluconazole	0.31	60	0.08	12.5	0.47
Candida albicans SC5314	AmphotericinB	0.63	60	0.16	0.8	0.27
Candida albicans SC5314	Nourseothricin	2.5	60	1.25	3.1	0.55
Candida albicans SC5314	5-Fluorocytosine	0.25	60	0.25	0.39	1
Candida albicans DPL1000 (Cas^S Flu^S)	Caspofungin	2.0	250	0.25	0.78	0.12
Candida albicans 36S (Cas^R Flu^S)	Caspofungin	20	250	0.5	25	1.1
Candida albicans DSY294 (Cas^S Flu^S)	Caspofungin	1.0	50	0.25	0.78	0.26
Candida albicans DSY3987 (Cas^S Flu^R)	Caspofungin	0.63	125	0.16	0.39	0.26
Candida albicans DSY3988 (Cas^S Flu^R)	Caspofungin	0.63	125	0.16	0.39	0.26
Candida glabrata NEM_7118432	Aminocandin	0.5	50	0.06	0.39	0.14
Candida lusitaniae NEM_7104301	Aminocandin	1.0	25	0.03	0.39	0.07
Candida krusei ATCC6258	Aminocandin	5.0	25	0.06	0.78	0.09
Candida tropicalis NEM_7114236	Aminocandin	2.5	12.5	0.63	0.39	0.14
Candida parapsilosis NEM_7103546	Aminocandin	5.0	100	0.08	1.56	0.15
Yarrowia lipolytica NEM_7115511	Aminocandin	12.5	12.5	1.25	1.56	0.25
Saccharomyces cerevisiae BY4741	Aminocandin	0.13	25	0.13	0.39	0.28

[1] Fractional Inhibitory Concentration index

Table 3: *Saccharomyces cerevisiae* haploid gene deletion strains most affected by treatment with colistin, aminocandin or combination of the drugs. Selection was done using a threshold value of -0.7 for single drug treatment and -1.0 for the combination.

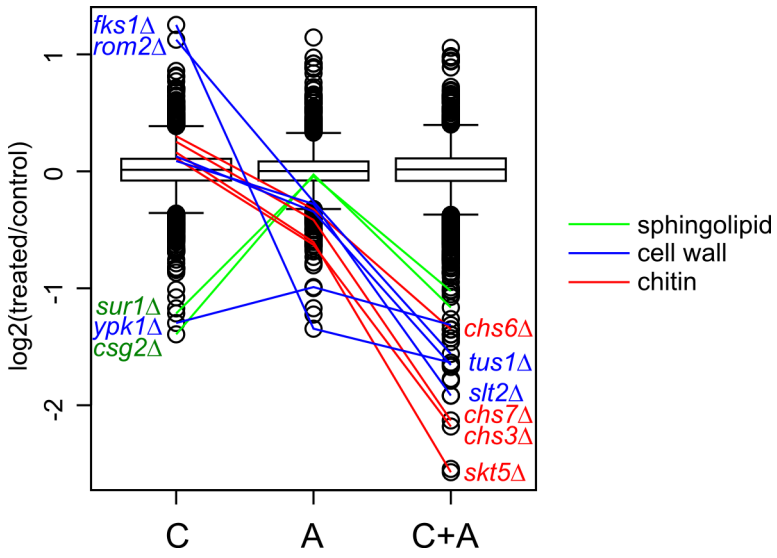
ORF	Name	Colistin[1]	Aminocandin[1]	Combined[1]	Description[2]
Glucan synthesis					
YKL126W	*YPK1*	-1.3	-1.0	-1.3	Protein kinase that affects FKS1 - sphingolipid mediated cell integrity signalling
YLR425W	*TUS1*	0.1	-0.3	-1.7	GEF that modulates Rho1 GTPase
YLR371W	*ROM2*	1.1	-0.3	-1.6	GDP/GTP exchange protein (GEP) for Rho1p and Rho2p
YLR342W	*FKS1*	1.3	-1.3	-1.6	Catalytic subunit of 1,3-beta-D-glucan synthase; binds regulatory subunit Rho1
Chitin synthesis					
YBL062W	overlaps *SKT5*	-0.1	-0.6	-1.4	Overlaps MRM1 - chitin synthesis
YBR023C	*CHS3*	0.1	-0.6	-2.2	Chitin synthase
YBL061C	*SKT5*	0.2	-0.6	-2.6	Activator ot Chs3 - chitin biosynthesis
YHR142W	*CHS7*	0.3	-0.4	-2.1	Unknown function, chitin biosynthesis
YJL099W	*CHS6*	0.3	-0.3	-1.4	Mediates export or cargo proteins, including Chs3, from Golgi to plasma membrane
PKC pathway					
YJL095W	*BCK1*	-0.1	-0.4	-2.5	MAP kinase kinase kinase in the PKC pathway
YHR030C	*SLT2*	0.1	-0.3	-1.9	MAP kinase in the PKC pathway
Sphingolipid synthesis					
YBR036C	*CSG2*	-1.4	0.0	-1.2	ER membrane protein required for mannosylation of inositolphosphorylceramide
YPL057C	*SUR1*	-1.2	0.0	-1.0	Mannosylinositol phosphorylceramide (MIPC) synthase in complex with Csg2

YLR372W	*SUR4*	-0.6	-0.4	-1.1	Fatty acid and sphingolipid biosynthesis
Golgi function					
YER151C	*UBP3*	-1.1	-0.4	-0.9	Ubiquitin-specific protease that acts in complex with Bre5 to co-regulate transport between the ER and Golgi
YKR001C	*VPS1*	-0.9	0.0	-0.5	Dynamin-like GTPase required for vacuolar sorting
YDR372C	*VPS74*	-0.8	0.0	-0.7	Protein required for Golgi localization of glycosyltransferases
YFL025C	*BST1*	-0.8	-0.5	-1.4	GPI inositol deacylase in the ER
YAL026C	*DRS2*	-0.6	0.0	-1.3	Aminophospholipid translocase (flippase)
YJL183W	*MNN11*	-0.2	-0.6	-1.9	Golgi mannosyltransferase complex component
YOR327C	*SNC2*	0.6	-0.8	0.0	Vesicle membrane receptor protein (v-SNARE)
Aromatic aminoacid metabolism					
YBR069C	*TAT1*	-1.2	-0.2	-1.0	Aminoacid transporter
YER090W	*TRP2*	-0.8	-0.1	-0.5	Anthranilate synthase - tryptophan biosynthesis
YGL148W	*ARO2*	-0.8	-0.2	-0.7	Chorismate synthase - aromatic acid biosynthesis
Other roles					
YOR200W	*MRM1**	-1.0	-1.2	-0.6	Overlaps MRM1 - mitochondrial enzyme
YIR023W	*DAL81*	-0.8	0.8	0.0	Positive regulator of genes in multiple nitrogen degradation pathways
YKL048C	*ELM1*	-0.8	-0.3	-0.6	Protein kinase involved in cellular morphogenesis
YCR044C	*PER1*	-0.5	-0.1	-1.4	ER protein required for GPI-phospholipase A2 activity
YOR080W	*DIA2*	-0.3	-1.0	0.0	Origin-binding F-box protein that forms an SCF ubiquitin ligase complex with Skp1p and Cdc53
YDR146C	*SWI5*	-0.2	-0.6	-1.5	Transcription factor
YER111C	*SWI4*	-0.1	0.0	-1.8	Transcription factor

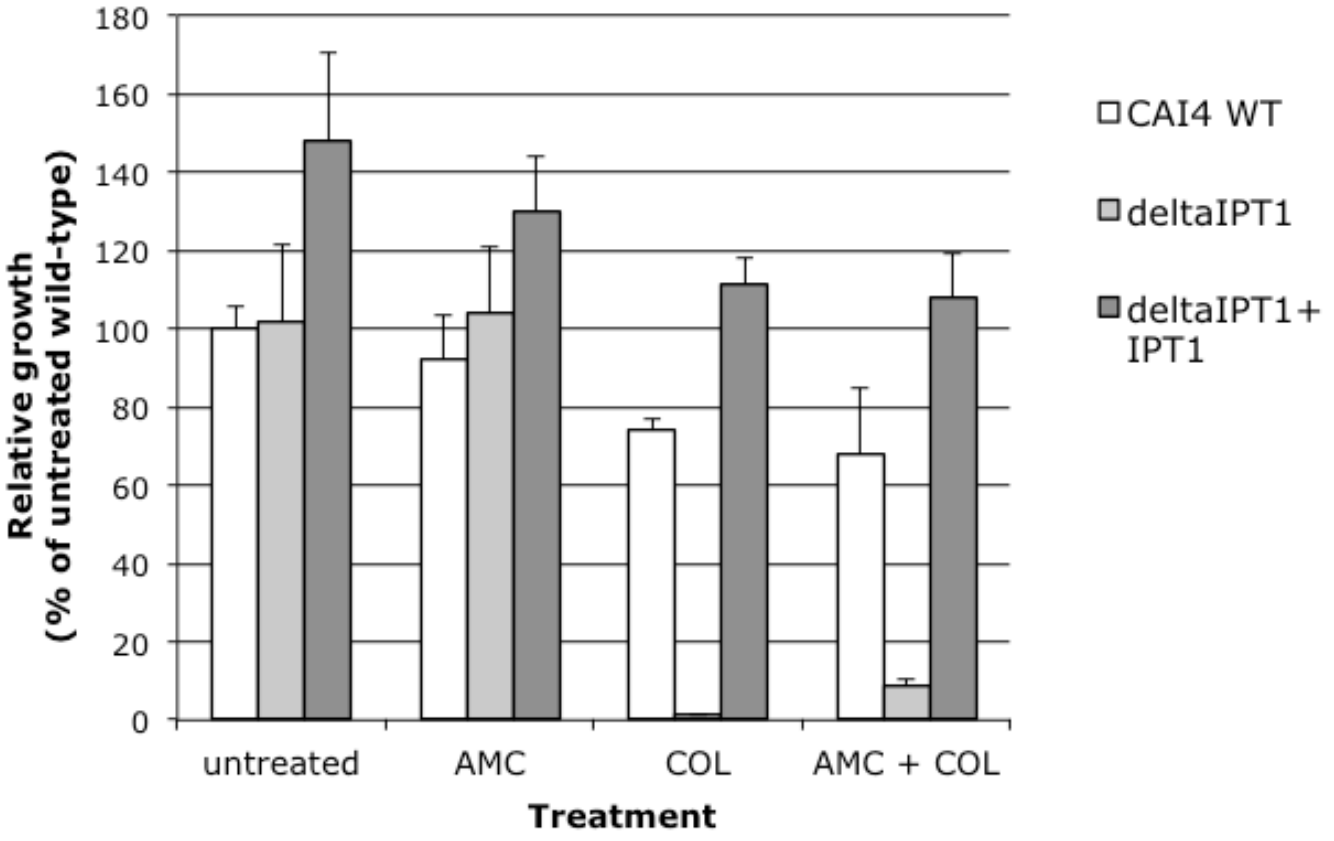
YPL066W		0.0	-0.7	-1.8	Unknown function
YPL086C	*ELP3*	0.1	-0.7	-0.4	Subunit of Elongator complex - modifies tRNAs
YPL158C	*AIM44*	0.1	-0.6	-1.4	Unknown function
YGR228W		0.2	-0.2	-1.1	Overlaps SMI1 - involved in the regulation of cell wall synthesis
YFL001W	*DEG1*	0.3	-0.8	0.1	tRNA:pseudouridine synthase
YLR140W	*RRN5**	0.3	-1.2	0.0	Overlaps RRN5 - involved in pol I transcription of rDNA
YBR200W	*BEM1*	0.7	-0.2	-1.7	Involved in establishing cell polarity and morphogenesis

[1] Numbers represent $\log_2$ of the ratio treated over control for each strain

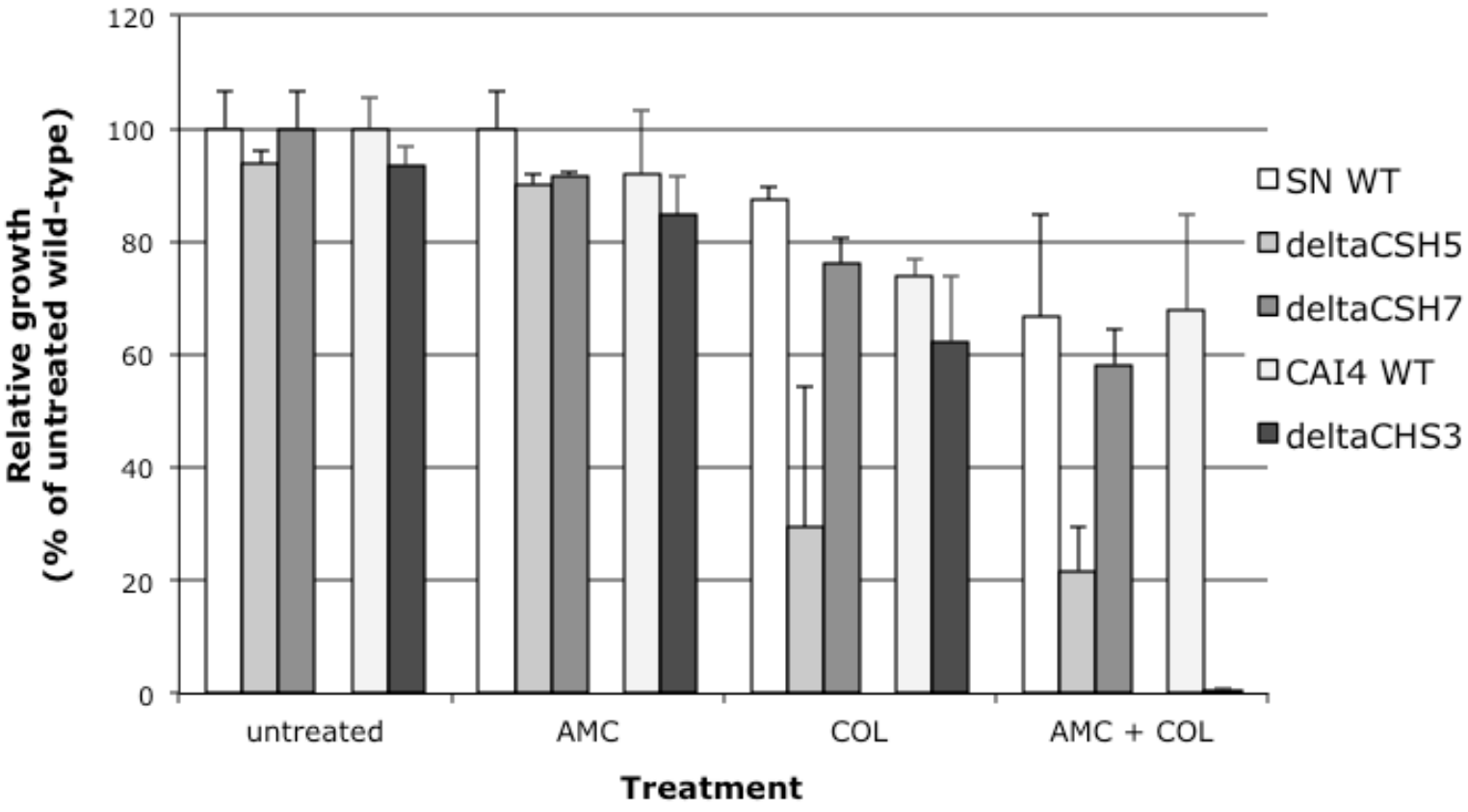
[2] Descriptions were retrieved from the *Saccharomyces* Genome Database [71] and simplified

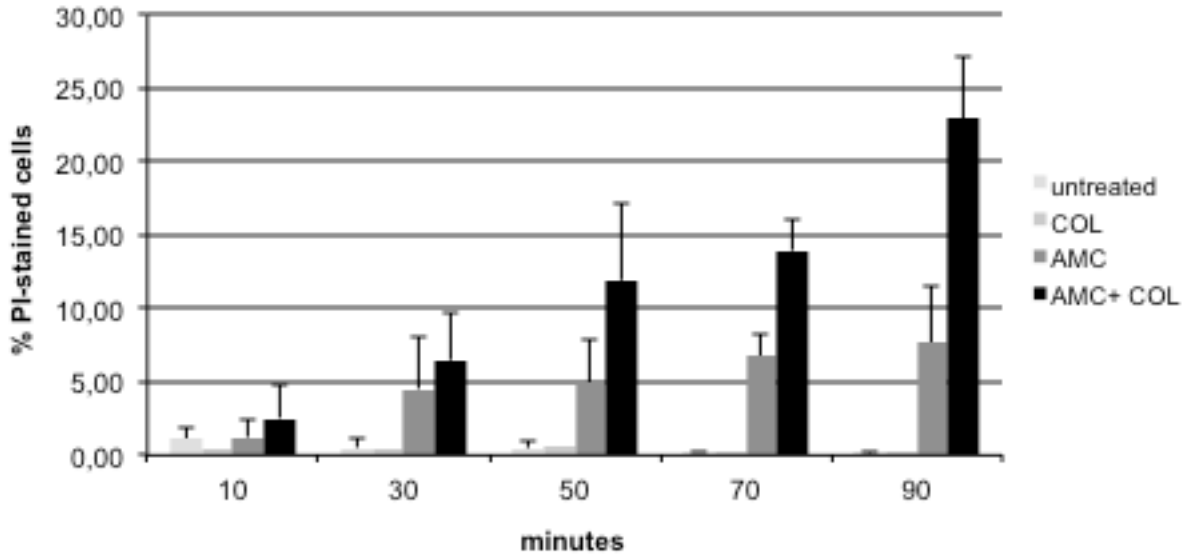

log2(treated/control)
1
0
-1
-2
C
A
C+A
fks1Δ
rom2Δ
sur1Δ
ypk1Δ
csg2Δ
chs6Δ
tus1Δ
slt2Δ
chs7Δ
chs3Δ
skt5Δ
sphingolipid
cell wall
chitin

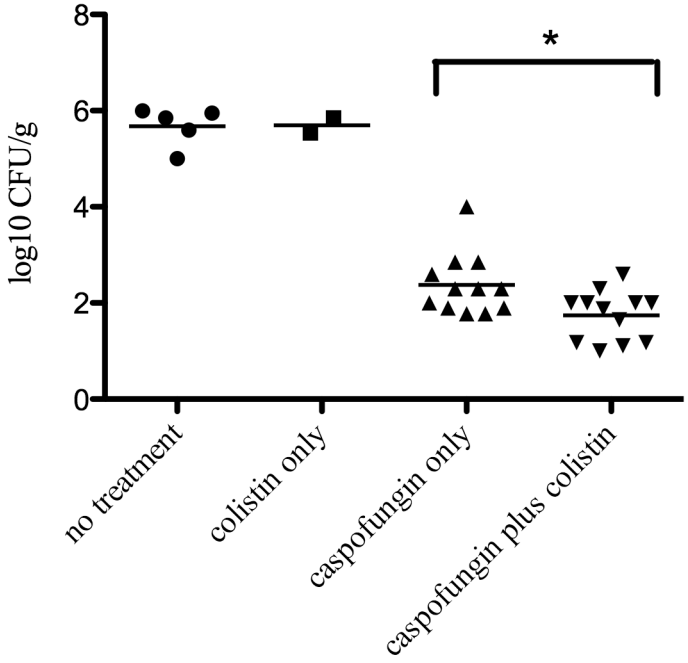
A


180
160
140
120
100
80
60
40
20
0
Relative growth
(% of untreated wild-type)
untreated
AMC
COL
AMC + COL
Treatment
CAI4 WT
deltaIPT1
deltaIPT1+
IPT1

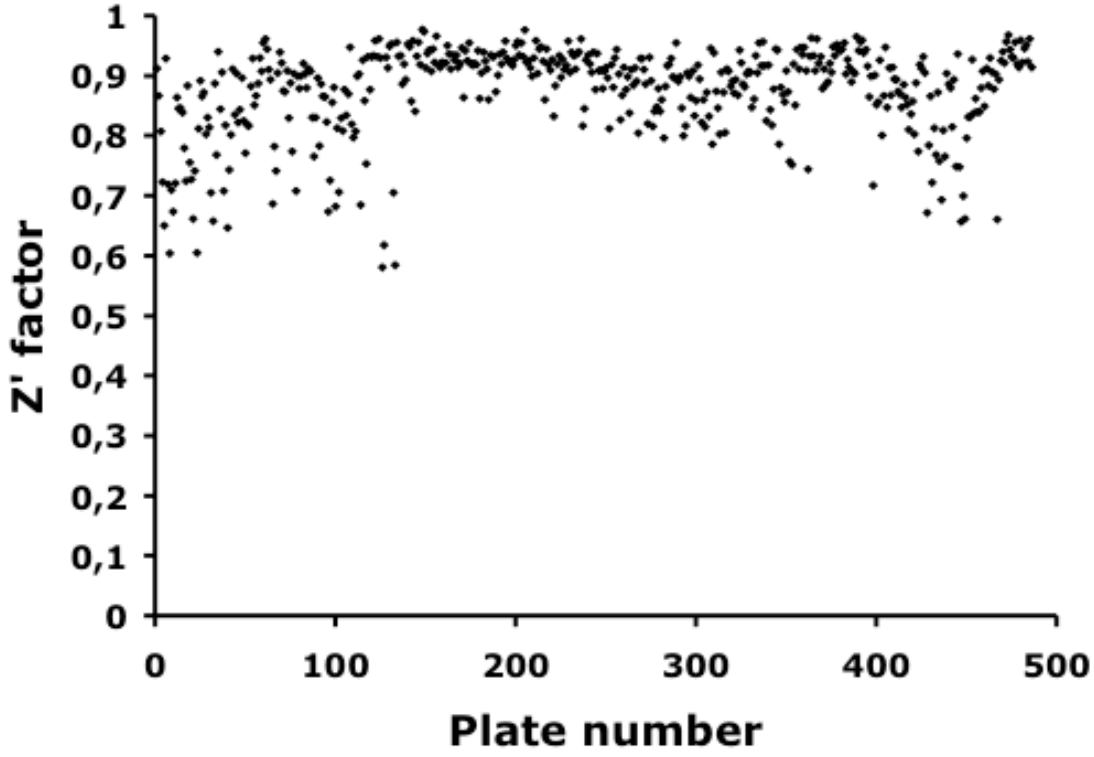
B


120
100
80
60
40
20
0
Relative growth
(% of untreated wild-type)
untreated
AMC
COL
AMC + COL
Treatment
SN WT
deltaCSH5
deltaCSH7
CAI4 WT
deltaCHS3


30,00
25,00
20,00
15,00
10,00
5,00
0,00
% PI-stained cells
10
30
50
70
90
minutes
untreated
COL
AMC
AMC+ COL


8
6
4
2
0
log10 CFU/g
*
no treatment
colistin only
caspofungin only
caspofungin plus colistin

A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0,7
0,6
0,5
0,4
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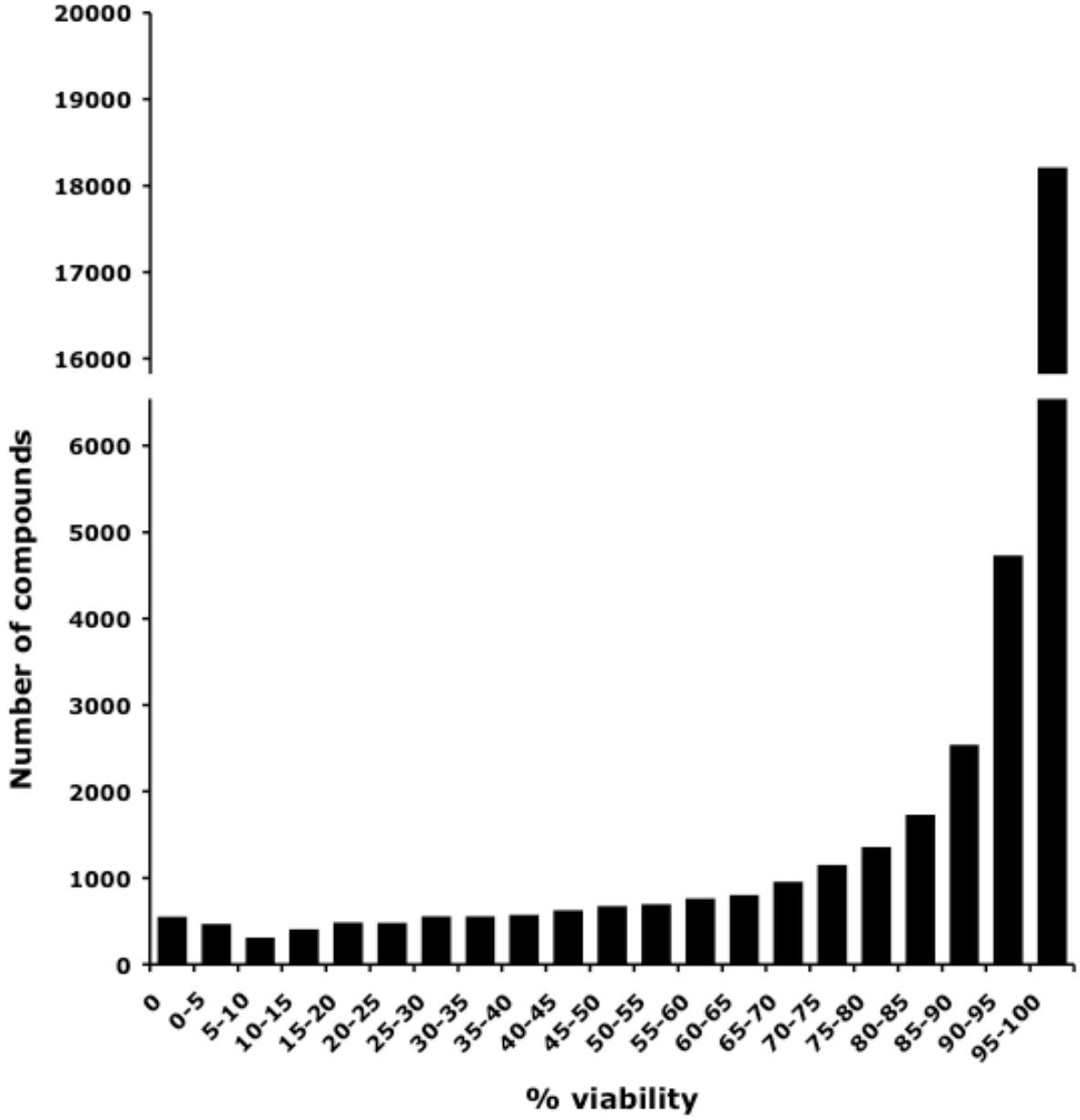
0,2
0,1
0
Z' factor
0
100
200
300
400
500
Plate number

B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18000
17000
16000
6000
5000
4000
3000
2000
1000
0
Number of compounds
0
0-5
5-10
10-15
15-20
20-25
25-30
30-35
35-40
40-45
45-50
50-55
55-60
60-65
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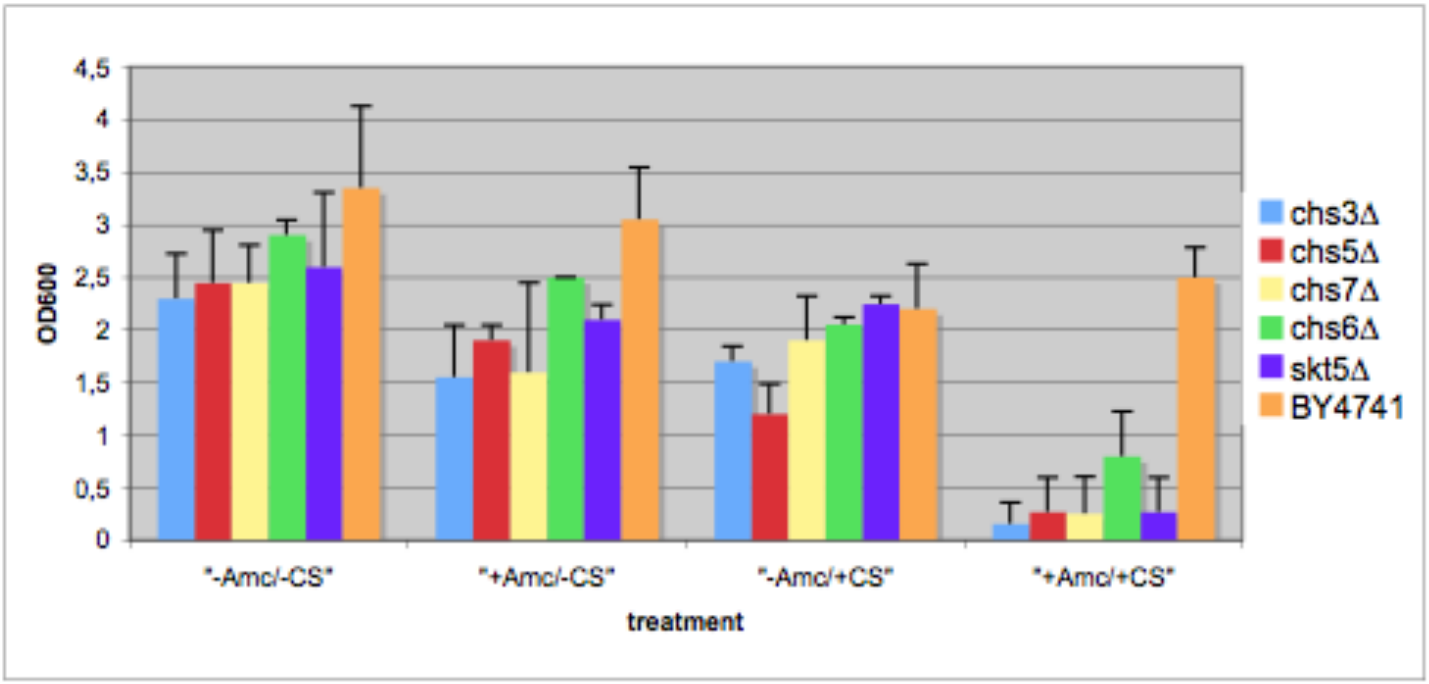
75-80
80-85
85-90
90-95
95-100
% viability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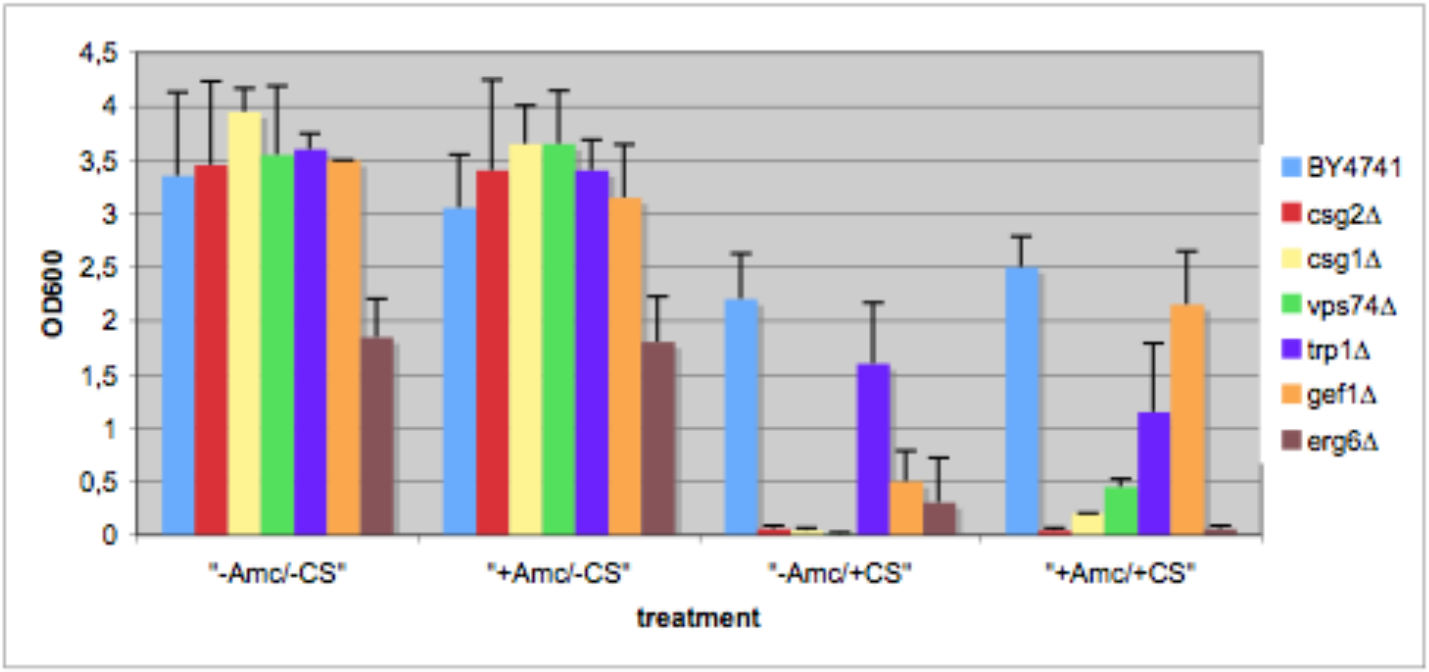
4
3,5
3
2,5
2
1,5
1
0,5
0
OD600
"-Amc/-CS"
"+Amc/-CS"
"-Amc/+CS"
"+Amc/+CS"
treatment
chs3Δ
chs5Δ
chs7Δ
chs6Δ
skt5Δ
BY4741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4
3,5
3
2,5
2
1,5
1
0,5
0
OD600
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"+Amc/-CS"
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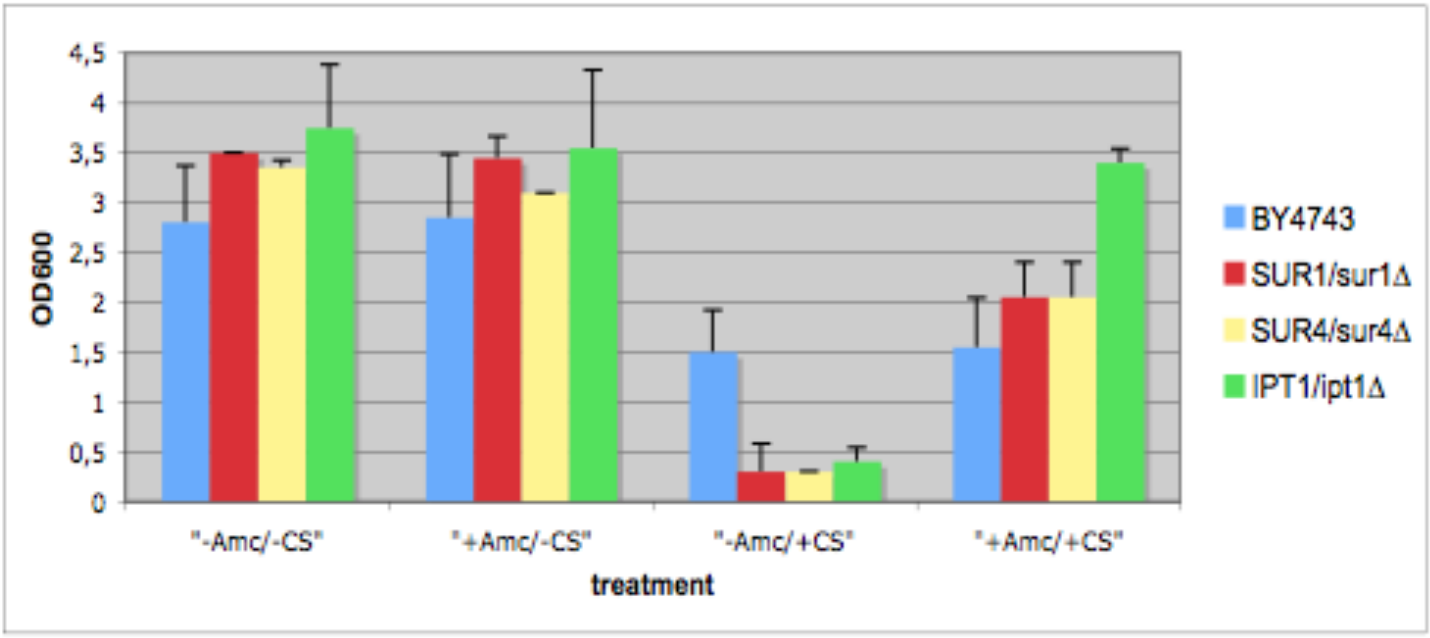
"+Amc/+CS"
treatment
BY4741
csg2Δ
csg1Δ
vps74Δ
trp1Δ
gef1Δ
erg6Δ


4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
OD600
"-Amc/-CS"
"+Amc/-CS"
"-Amc/+CS"
"+Amc/+CS"
treatment
BY4743
SUR1/sur1Δ
SUR4/sur4Δ
IPT1/ipt1Δ

Supplemental Table 1: Oligonucleotides used in this study

Name	Application	Sequence
U1	Amplification of up-stream barcode	GAT GTC CAC GAG GTC TCT
KU	Amplification of up-stream barcode; confirmation of mutants	AAG AAG AAC CTC AGT GGC
KD	Amplification of down-stream barcode	GGA TCT TGC CAT CCT ATG
D1	Amplification of down-stream barcode	CGG TGT CGG TCT CGT AG
U2comp	Labelling of up-stream barcode	GTC GAC CTG CAG CGT ACG
D2comp	Labelling of down-stream barcode	CGA GCT CGA ATT CAT CGA T
U2block	Block fluorescently labelled primers	CGT ACG CTG CAG GTC GAC
D2block	Block fluorescently labelled primers	ATC GAT GAA TTC GAG CTC G
ScSUR1-up	confirm homozygous and heterozygous *S. cerevisiae sur1*Δ strain	CGG AAG ACA CTT TTC ATT TTC
ScCSG2-up	confirm *S. cerevisiae csg2*Δ strain	GGA GGC TAT GTG AAC GTG
ScGEF1-up	confirm *S. cerevisiae gef1*Δ strain	CAC CGC CTG TTC TCC AGT
ScERG6-up	confirm *S. cerevisiae erg6*Δ strain	CCC TTA TCT GTT TTA CTT TCG
ScVPS74-up	confirm *S. cerevisiae vps74*Δ strain	GCA GTT TCA TTG TTG GCT ATA
ScTRP1-up	confirm *S. cerevisiae trp1*Δ strain	GGG AGG GCA TTG GTG ACT
ScCHS3-up	confirm *S. cerevisiae chs3*Δ strain	CCG ATT TGC AAA GTT CTC GA
ScCHS5-up	confirm *S. cerevisiae chs5*Δ strain	GCG TAG ATG CTA AAT GTT ATC
ScCHS6-up	confirm *S. cerevisiae chs6*Δ strain	GGA ATA GAG GAT CTT AAC AAA
ScCHS7-up	confirm *S. cerevisiae chs7*Δ strain	GCT GTC TCC ACG TGG AAA
ScSKT5-up	confirm *S. cerevisiae skt5*Δ strain	GTG TTG GCA CTT GAC TTT AG
ScIPT1-up	confirm heterozygous *S. cerevisiae ipt1*Δ strain	CCA CCC TAA CTG TTC CTT T
ScSUR4-up	confirm heterozygous *S. cerevisiae sur4*Δ strain	GAC AGC TCT TCA CTC GCT T