

RESEARCH ARTICLE

Antibiotic saving effect of combination therapy through synergistic interactions between well-characterized chito-oligosaccharides and commercial antifungals against medically relevant yeasts

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Abstract

Combination therapies can be a help to overcome resistance to current antifungals in humans. The combined activity of commercial antifungals and soluble and well-defined low molecular weight chitosan with average degrees of polymerization (DP_n) of 17–62 (abbreviated C17–C62) and fraction of acetylation (F_A) of 0.15 against medically relevant yeast strains was studied. The minimal inhibitory concentration (MIC) of C32 varied greatly among strains, ranging from $> 5000 \mu\text{g mL}^{-1}$ (*Candida albicans* and *C. glabrata*) to < 4.9 (*C. tropicalis*). A synergistic effect was observed between C32 and the different antifungals tested for most of the strains. Testing of several CHOS preparations indicated that the highest synergistic effects are obtained for fractions with a DP_n in the 30–50 range. Pre-exposure to C32 enhanced the antifungal effect of fluconazole and amphotericin B. A concentration-dependent post-antifungal effect conserved even 24 h after C32 removal was observed. The combination of C32 and commercial antifungals together or as part of a sequential therapy opens new therapeutic perspectives for treating yeast infections in humans.

Introduction

With the increased competence of medical science to extend the lives of immunocompromised hosts, the incidence of systemic fungal infections has raised dramatically. For years, amphotericin B has been considered the “gold” standard for the treatment of invasive fungal infections, but toxicity limits its usefulness. Nowadays, less toxic triazole antifungals, such as fluconazole and itraconazole, are considered reasonable substitutes. However, in spite of amphotericin B or triazole monotherapy treatments, mortality associated with fungal infections remains to be substantial. There is a great interest in using combined therapies in an attempt of improving survival rates and of reducing fungal resistance. For this reason, a great number of studies have investigated the synergistic activity of commercial antifungals (CA) [1–4].

Competing interests: The authors have declared that no competing interests exist.

Antifungal resistance makes infections harder to treat and is an increasing problem. *Candida* spp are increasingly resistant to antifungal treatment with azoles and echinocandins. The most resistant *Candida* spp are *C. glabrata*, *C. krusei* and the emerging new species *C. auris* [5–7]. In less common fungal infections as with the mold *Aspergillus fumigatus*, emerging resistance to azoles threatens the effectiveness of life-saving medications. Resistant *Aspergillus* infections can develop in people who use antifungals and agricultural use of azole fungicides to treat crop diseases, lead to the growth of resistant strains of *Aspergillus* and people with weakened immune systems is at risk to be infected [8, 9]. Other molds as *Fusarium* spp. [10] and *Scedosporium* spp have been increasingly recognized as cause of resistant life-threatening infections. Treatment of filamentous fungi species, is particularly challenging because of their resistance to many antifungal agents [11].

Chitin is a polysaccharide that consists of $\beta(1\rightarrow4)$ linked N-acetyl-D-glucosamine residues. It is the second most abundant biopolymer in nature after cellulose, as it is an important component of the cell walls of fungi, and yeasts, and of the shells of insects and crustaceans. Chitosan is a cationic polymer obtained by the alkaline partial of full deacetylation of chitin. The use of oligomers of chitosan, known as chito-oligosaccharides or CHOS, is of major interest since CHOS have a variety of interesting biological activities and are more soluble than chitosan [12]. The number of monomeric units defines the polymerization degree of the CHOS (DP), and the fraction of acetylation (F_A) refers to the average fraction of acetylated monomers (GlcNAc units). These two features determine important physical-chemical properties of the CHOS, like solubility and conformation [13].

In recent years, chitosan and CHOS have received remarkable attention due to their potential use in medicine, and since they are considered to be biodegradable, non-toxic, non-immunogenic and non-carcinogenic. Chitosan has been proposed as delivery system for different antifungals, including amphotericin B [14, 15] and fluconazole [16, 17]. Moreover, the polymer has a well-documented antifungal activity itself [18–20]. Recently, we demonstrated antifungal activity of well-defined chito-oligosaccharide preparations against medically relevant yeasts [21]. The aim of the work presented in this report is to study the combined antifungal pharmacodynamics of a these well-characterized CHOS and commercial antifungals, in the inhibition of medically relevant yeasts. Thus, we addressed the potential of CHOS for use in combination therapy.

Materials and methods

Enzymatic production of CHOS

Chitosan (KitoNor, F_A 0.15, DP_n 206) was obtained from Norwegian Chitosan, Gardermoen, Norway. CHOS were produced by enzymatic hydrolysis of the chitosan by chitosanase ScCsn46A and the resulting CHOS (abbreviated C32) were determined to be of CHOS with DP_n of 32 and F_A of 0.15 as described previously [22].

For further fractionation, C32 was dissolved in water to a concentration of 20 mg/mL and dialyzed against distilled water using Spectra/Por 6 dialysis membranes with cutoffs of 3.5 kDa, 8.0 kDa, 10 kDa, or 15 kDa, (Spectrumlabs, Rancho Dominguez, CA, USA). Each dialysis step was performed at 4°C with stirring for 48h. At the end of each dialysis step, the retentate and/or permeate was collected and lyophilized. Prior to use in biological experiments, the CHOS-powder was dissolved in two-fold concentrated culture medium and sterilized by filtration [21].

Determination of the average degree of polymerization (DP_n) with ¹H-NMR spectroscopy

¹H NMR experiments were performed on an Avance™ 400 instrument from Bruker. The DP_n was calculated by the equation $(D\alpha+D\beta+D+A\alpha+A\beta+A)/(D\alpha+D\beta+A\alpha+A\beta)$, where $D\alpha$, $D\beta$,

$A\alpha$ and $A\beta$ are the integrals of the reducing end signals of the α and β anomers of the deacetylated (GlcN, D) and acetylated (GlcNAc, A) units, D is the integral of the signals from the internal and non-reducing end deacetylated units and A is the integral of the signals from the internal and non-reducing end acetylated units [23].

Determination of relative molecular weights of CHOS fractions

Size exclusion chromatography was performed on a Dionex Ultimate 3000RSLC system (ThermoScientific, Sunnyvale USA) with RI detection. The columns were TOSOH TSKgel G3000PWXL-CP (7.8 x 300 mm, 7 μ m) and TOSOH TSKgel G-oligoPW (7.8 x 300 mm, 7 μ m) coupled in series and operated isocratically at 1 mL/min with 0.1 M NaNO_3 as the mobile phase. Samples were dissolved in the mobile phase. The system was calibrated with DIN-pullulan standards with molecular masses of 6 kDa, 12 kDa, 22 kDa, 50 kDa and 110 kDa (PSS Polymer Standards Service, Mainz, Germany). Chromatography data were exported and treated by WinGPC Scientific v 6.20 software for estimation of average molecular weights, degree of polymerization, average molecular mass, and dispersity [21].

Antifungals

Commercial antifungals (CA), fluconazole (Flu), amphotericin B (Amp), voriconazole (Vor), flucytosine (Fcs), and miconazole (Mcz), were purchased from Sigma (St. Louis, MO).

Yeast strains

Growth inhibition tests were performed using *Candida parapsilosis* 220919, *Candida tropicalis* 13803, and *Candida norvegensis* 22977 strains from the American Type Culture Collection (ATCC). Additionally, the clinical isolates *Candida albicans* (1581), *Candida guilliermondii* (12146), *Candida lusitanae* (20949), *Candida glabrata* (3808), *Rhodotorula glutinis* (909700100), and *Rhodotorula mucilaginosa* (37016), belonging to the Oslo University Hospital collection were used. The strains were kept frozen in YPD broth at -70°C until testing.

Preparation of inocula

Yeast strains were cultured in Sabouraud agar and incubated for 48 h at 37°C . Yeast suspensions were prepared in sterile water by touching ten colonies from a culture plate and adjusting the resulting suspension to 0.5 McFarland turbidity standard (approximately 5.5×10^6 CFU mL^{-1}) using spectrophotometric methods. One milliliter of the fungal suspension was added to 9 mL of RPMI (pH 6), providing the starting inoculum of approximately 5.5×10^5 CFU mL^{-1} .

Analysis of synergistic effects

Checkerboard synergy testing was performed in triplicate using combinations of CHOS and CA as follows. Briefly, 100 μL of yeast inoculum obtained as previously described were added to a 96-well microplate containing different combinations of C32 and CA in potato dextrose agar (PDA) to a total volume of 200 μL , yielding final concentrations of 4.9 to 5000 $\mu\text{g mL}^{-1}$ (CHOS) and 0.01 to 64 $\mu\text{g mL}^{-1}$ (CA). Positive growth controls were performed in wells not containing antifungals. The minimal inhibitory concentration (MIC) was defined as the lowest drug concentration at which there was no visible growth after 48 h incubation at 37°C . The minimal inhibitory concentration in combination (MICC) was the lowest concentration of the drug and CHOS, respectively, when used in combination at which there was no visible growth after 24 h or 48 h incubation at 37°C . To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antifungal (i.e. CHOS and CA) in each

combination. The following formulas were used to calculate the FIC index: FIC of antifungal A equals MICC of A divided by MIC of A; FIC of antifungal B equals MICC of B divided by MIC of B; and FIC index equals FIC of antifungal A + FIC of antifungal B. Synergy was defined as an FIC index of ≤ 0.5 . Indifference was defined as an FIC index of > 0.5 and ≤ 4 . Antagonism was defined as an FIC index of > 4 [24]. Time-kill curves were determined incubating Flu and Amp at concentrations at $0.25 \times \text{MIC}$ or MICC, separately, in the presence of C32 or absence of C32 (control), for a period of 48 h at 37°C . Samples were taken at different times and cultured on Sabouraud agar and incubated at 37°C for 48 h.

Sequential therapy

Inocula obtained as previously described were incubated in the absence (control) and presence of C32 ($0.25 \times \text{MIC}$) for 12 h at 37°C . The cells were then washed three times, and cell suspensions were adjusted to 0.5 McFarland turbidity standard in PDA and inoculated into flasks containing Flu or Amp ($0.25 \times \text{MIC}$). Samples were taken at regular intervals to record survival. Numbers of living cells were determined by incubation on Sabouraud agar at 37°C for 48 h.

Post-antifungal effects

Continuous antifungal effect (CAFE) and post-antifungal effect (PAFE) were determined *in vitro* as follows. Yeast inocula were exposed to $0.5 \times \text{MIC}$, $1.0 \times \text{MIC}$, and $2.0 \times \text{MIC}$ of C32 for 2 h at 37°C . For the control samples, no C32 was used. Afterwards, cells were collected by centrifugation, washed twice with PDA, and resuspended in fresh medium containing no antifungals. At different times, samples were taken, plated on Sabouraud agar and incubated for 48 h at 37°C . The PAFE was calculated using the formula $\text{PAFE} = T - C$, where T is the time required for the titer to increase 1 log₁₀ over the post-washing titer for cells grown in the presence of C32, and C is the time required for the titer to increase 1 log₁₀ over the post-washing titer for cells grown the absence of C32. For determining of the CAFE, yeasts were grown in the presence of $0.5 \times \text{MIC}$, $1.0 \times \text{MIC}$, and $2.0 \times \text{MIC}$ of C32 for 24 h at 37°C . Samples were taken at different times during this 24h period and plated on Sabouraud agar. Calculations were made using the formula $\text{CAFE} = T - C$, where T is the time required for the titer to increase 1 log₁₀ in the presence of C32, and C was the time required for the titer to increase 1 log₁₀ in the absence of C32.

Statistical analysis

Experiments were done in triplicate. Experimental data was analyzed using Minitab version 16 (Minitab 16, State College, PA). Student's *t*-tests were performed to identify differences between samples. Differences were considered to be significant when $p \leq 0.05$. Tukey's range test was used to assess differences between pairs of means.

Results

In this study, the *in vitro* antifungal activity of C32, a chito-oligosaccharide mixture with DP_n 32 and F_A 0.15, was analyzed against clinical-relevant yeast strains, and the effect of the CHOS preparation was also determined when combined with five commercial antifungals: amphotericin B (Amp), fluconazole (Flu), voriconazole (Vor), flucytosine (Fcs), and miconazole (Mcz) (Table 1). The MIC of C32 varied greatly among strains, ranging from $> 5000 \mu\text{g mL}^{-1}$ (*C. albicans* and *C. glabrata*) to $< 4.9 \mu\text{g mL}^{-1}$ (*C. tropicalis*). Inhibitory effect of the CAs also varied among strains. In the case of Amp, the MIC ranged from $0.25 \mu\text{g mL}^{-1}$ (*C. guilliermondii* and *C.*

norvegensis) to $32 \mu\text{g mL}^{-1}$ (*C. lusitanae*), whilst for Flu the values ranged from $4 \mu\text{g mL}^{-1}$ (*C. tropicalis*) to $>64 \mu\text{g mL}^{-1}$ (*C. norvegensis*). The MIC was between $0.12 \mu\text{g mL}^{-1}$ (*C. albicans*) and $4 \mu\text{g mL}^{-1}$ (*C. lusitanae*) for Vor and Fcs, and for Mcz, the MIC was $>16 \mu\text{g mL}^{-1}$ for both tested yeasts, *C. albicans* and *C. glabrata*.

Table 1 further shows that in most cases the combination of C32 and CA had clear synergistic effects and that some observed synergies were very strong. For example, the MIC for C32 and Mcz acting on *C. albicans* were reduced from $>5000 \mu\text{g mL}^{-1}$ to $4.9 \mu\text{g mL}^{-1}$ and $>16 \mu\text{g mL}^{-1}$ to $0.5 \mu\text{g mL}^{-1}$, respectively, when combined.

A time-kill study was performed on *C. albicans* and *C. guilliermondii*, in order to analyze the combined effect of C32 and Flu or Amp in more detail (Fig 1). When using CA concentrations that only weakly inhibited growth of *C. albicans*, the combination with C32 at 0.25 MIC yielded a dramatic decrease in cell viability (Fig 1A), the enhancing effect being higher for Flu than for Amp. A similar, but less pronounced effect was observed after combining the CA with C32 at its MIC (Fig 1B). Similar results were obtained for the C32-sensitive strain *C. guilliermondii*. In both cases we observed that the combination of C32 and Amp or Flu reduced yeast growth to a higher extent than what one would expect based on the sum of the reductions caused by each individual antifungal.

The effect of Flu and Amp on yeasts previously exposed to C32 (0.25 x MIC) for 12 h was studied (Fig 2). Pre exposure to C32 enhanced the antifungal effect of Flu and Amp even after 24 h incubation. Growth curves of *C. guilliermondii* pre-exposed to various concentrations of C32 (0.5, 1.0, and 2.0 x MIC) further showed a concentration-dependent post-exposure antifungal effect, even after 24 h. Harmonizing with these results, PAFE values (Table 2) showed that a certain degree of antifungal activity was maintained after removal of C32. However, the activity was significantly ($p \leq 0.05$) lower than before C32 removal (CAFE).

To assess the size-dependency of CHOS with respect to antifungal and synergistic effects, the C32 preparation was fractionated using dialysis [21]. Four new mixtures were prepared: i) below 3.5 kDa, ii) between 3.5 kDa and 8.0 kDa, iii) above 3.5 kDa, and iv) above 10 kDa. Table 3 shows the properties of the various preparations, including the average degree of polymerization (DP_n) was determined by $^1\text{H NMR}$ [23] and sample naming. Table 3 also shows the relative molecular weight average (MW) estimated from analytical size-exclusion chromatography using pullulan standards with molecular weights of 6 kDa, 12 kDa, 22 kDa, 50 kDa, and 110 kDa (Table 3). The DP_n values of the obtained four fractions were 17, 31, 54 and 62.

The inhibitory effects of the four CHOS mixtures were studied *in vitro* on 4 clinically relevant yeast strains (Table 4), using two CAs. The data show a clear size dependency both for the inhibitory effect of CHOS alone and the overall impact and synergistic effects of combining CHOS and CA. The C31 fraction and, to a slightly lesser extent, the C54 fraction stand out as superior, relative to the C17 and C62 fractions.

Discussion

Combined antifungal therapies have received great interest due to their potential of overcoming fungal resistance to conventional treatments. In this study, the feasibility of using a combined therapy of a well-characterized CHOS (C32) and five well-established CA against medically relevant yeasts strains was analyzed *in vitro*. We show that combining C32 with CA yield synergistic effects. The magnitude of these effects differs between yeasts and CA: in some cases, the synergistic effects seemed very strong.

Some reports have suggested that the polycationic character of chitosan is responsible for its antifungal activity, since these cationic groups may interact with anionic components of the cell wall of the fungi and destabilize their membrane [21, 25, 26]. It thus seems reasonable to

Table 1. Combined effect of C32 and CA on the growth of clinically relevant yeasts after 24 h of incubation. MIC: minimum inhibitory concentration; MICC: minimum inhibitory concentration in combination (= MIC of the compound in the presence of the other compound); S: Synergy; I: Indifference. All values are in $\mu\text{g mL}^{-1}$.

	CA	MIC CA	MICC CA	MIC C32	MICC C32	Interaction
<i>C. albicans</i>				>5000		
	Amp	0.50	0.60		156.3	S
	Flu	64.0	0.50		19.5	S
	Vor	0.12	0.06		39..1	S
	Fcs	0.12	0.06		312.5	S
	Mcz	>16.0	0.50		4.9	S
<i>C. guilliermondii</i>				39.1		
	Amp	0.25	0.01		<4.9	S
	Flu	32.0	0.01		<4.9	S
	Vor	1.0	0.06		<4.9	S
	Fcs	1.0	0.06		<4.9	S
<i>C. glabrata</i>				>5000		
	Flu	>32.0	32.0		5000	I
	Mcz	>16.0	1.0		156.3	S
<i>C. parapsilosis</i>				4.9		
	Amp	1.0	0.25		<4.9	I
	Flu	32.0	4.0		<4.9	S
	Vor	0.50	0.25		<4.9	I
	Fcs	0.50	0.06		<4.9	S
<i>C. tropicalis</i>				4.9		
	Amp	1.0	0.13		4.9	I
	Flu	4.0	1.0		4.9	I
<i>C. norvegensis</i>				9.8		
	Amp	0.25	0.03		<4.9	S
	Flu	>64	8.0		<4.9	S
<i>C. lusitaneae</i>				78.1		
	Amp	32.0			9.8	S
	Flu	32.0			19.5	S
	Vor	4.0			<4.9	S
	Fcs	4.0			<4.9	S
<i>R. glutinis</i>				78.1		
	Amp	1.0	0.06		9.8	S
	Flu	>64.0	16.0		9.8	S
	Vor	2.0	0.06		<4.9	S
	Fcs	2.0	0.06		<4.9	S
<i>R. mucilaginosa</i>				4.9		
	Amp	0.50	0.13		<4.9	S
	Flu	>64.0	>64.0		<4.9	I
	Vor	0.50	0.50		4.9	I
	Fcs	0.50	0.06		<4.9	S

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hypothesize that increased membrane permeability promoted by C32 might allow the CA to penetrate the target cells more easily. In the case of the azoles tested (Flu and Vor), this increased CA flux into the cell cytosol might increase the inhibition of the production of 14 α -demethylase resulting in reduction of the membrane fluidity and an increase in the production of toxic sterols [27, 28]. Likewise, for Fcs, increased membrane permeability could improve its

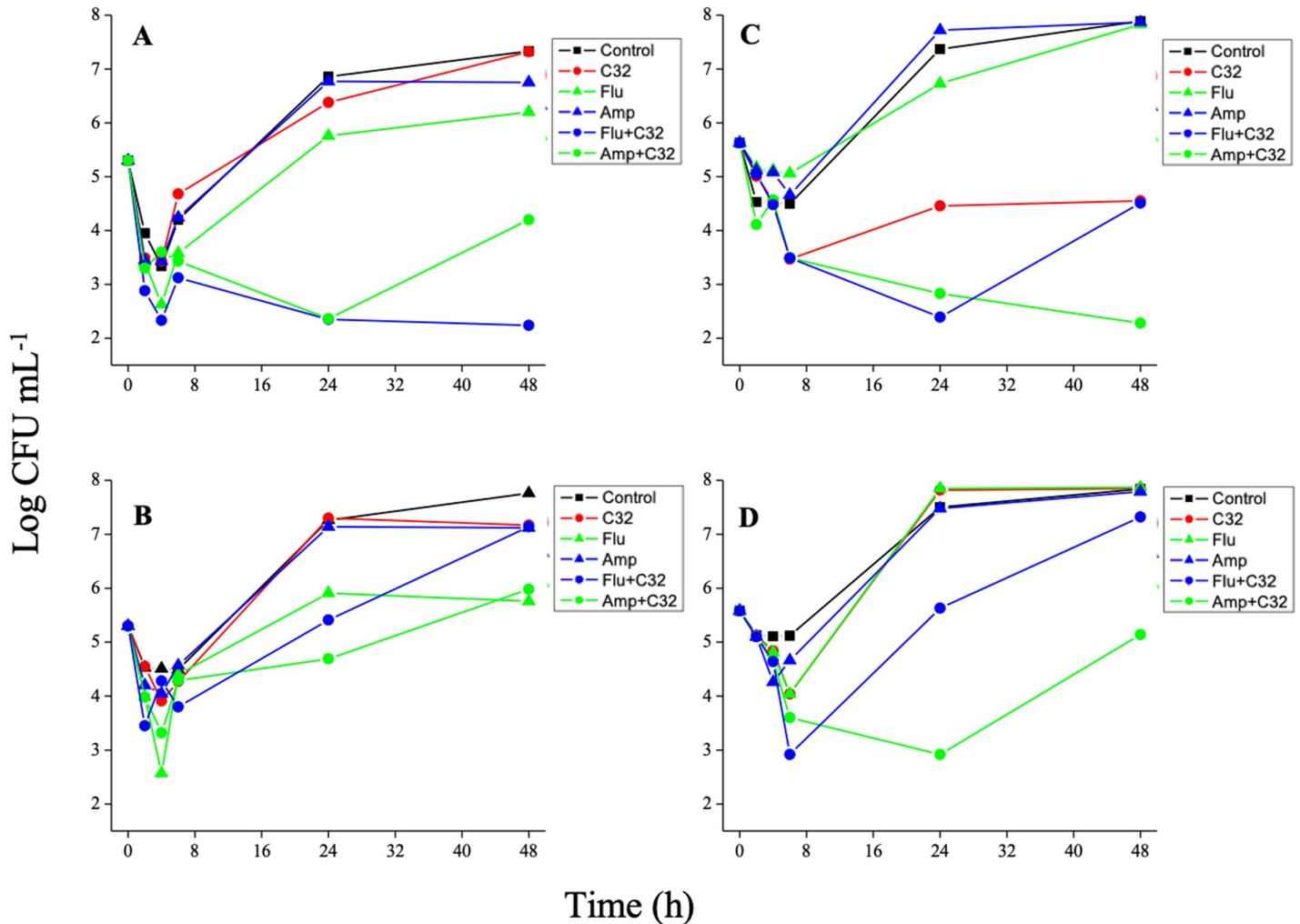


Fig 1. Inhibition of *C. albicans* (a, b) and *C. guilliermondii* (c, d) when exposed to sub-inhibitory concentrations of CA and C32 at 0.25 x MIC (a, c) or MIC (b, d). The CA were applied at MIC, i.e. at concentrations that hardly inhibit the yeasts. The graphs show the amount of viable cells over time. Color coding is provided in the Figure. Standard deviations are omitted for clarity. Normally, these were between 0.1 to 0.5 Log CFU ml⁻¹ units.

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flux into the cytosol where Fcs is suggested to interact with RNA biosynthesis [29]. For Amp, one may speculate that alterations in the cell membrane caused by C32 contribute in a synergistic way to the membrane destabilization caused by the formation of Amp-driven transmembrane channels, leading to the collapse and death of the cell [30].

Interestingly, synergy was observed even in the case of *C. albicans*, for which C32 was not effective when used alone. This result suggests that the CHOS might, to some extent, be disturbing the cell membrane, even if it does not affect the cell viability. Similar results were obtained in a previous study conducted by Palmeira-de-Oliveira *et al.* [31]. Through a cytometric analysis the authors showed that a chitosan hydrogel induced primary lesions on the cell membrane of *Candida* spp. even under conditions that did not reduce cell viability.

The present results agree with those reported by Jaime *et al.* [27] on *Saccharomyces cerevisiae*, who found that the combination of CHOS (84 μg mL⁻¹, 5.44 kDa and 97% degree of deacetylation) and Flu (20 μg mL⁻¹) had a synergistic effects on growth inhibition. Contrarily, in a different study Calamari *et al.* [32] studied the activity of fluconazole (50-100-150 μg mL⁻¹),

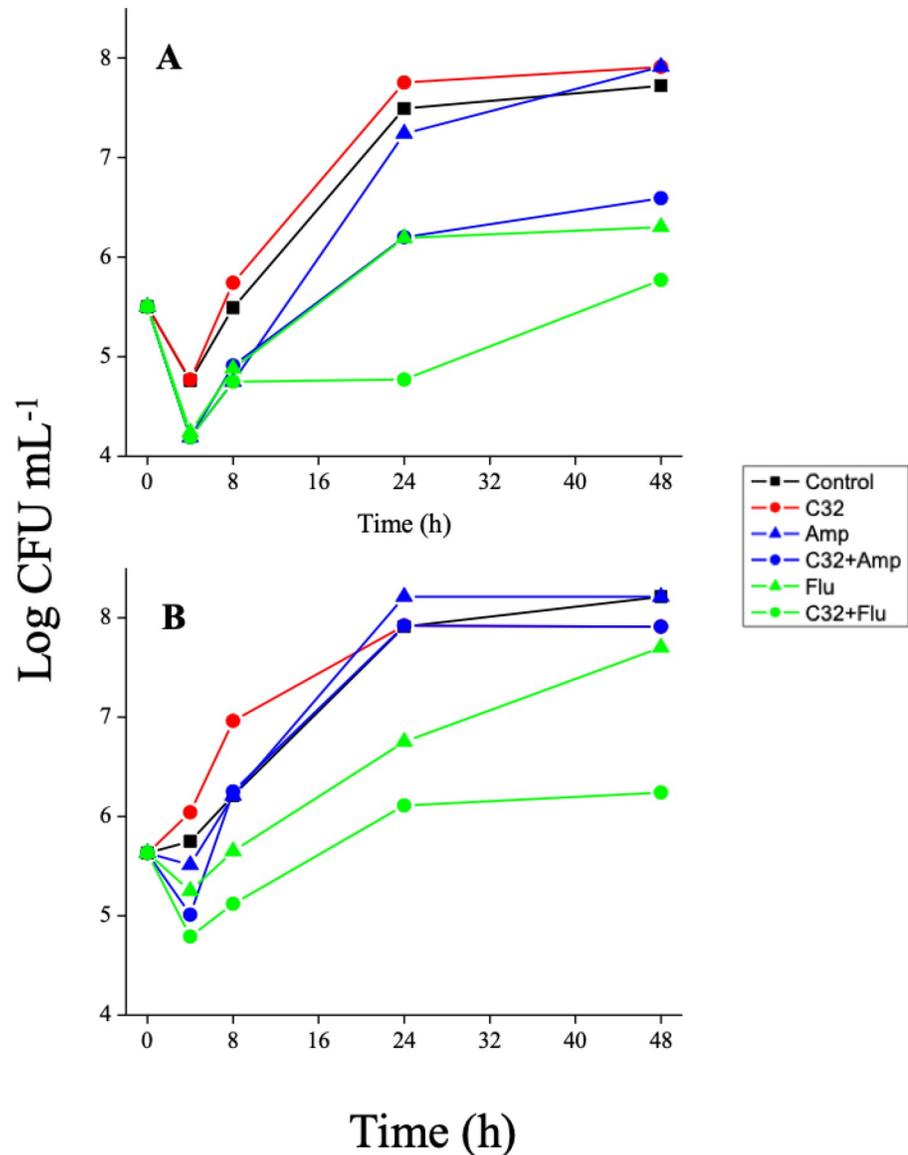


Fig 2. Sequential therapy time-kill curve for *C. albicans* (a) and *C. guilliermondii* (b) in the presence of amphotericin B or fluconazole (concentration = 0.25 x MIC) after 12 h pre exposure to C32 (concentration = 0.25 x MIC). Color coding is provided in the Figure. Standard deviations are omitted for clarity. Normally, these were between 0.1 to 0.5 Log CFU ml⁻¹ units.

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Table 2. PAFE and CAFE for *C. guilliermondii* in the presence of 0.5, 1.0 and 2.0 x MIC C32. Results expressed as average ± standard deviation. Results are expressed in time (h). Values that do not share the same letter are significantly different.

Concentration (x MIC)	CAFE	PAFE
0.5	2.52 ± 0.06	0.23 ± 0.11
1.0	4.20 ± 0.25	0.42 ± 0.44
2.0	6.06 ± 0.36	0.74 ± 0.03

<https://doi.org/10.1371/journal.pone.0227098.t002>

Table 3. Molecular size determination of chitosan fractions by 1H-NMR and SEC.

Fraction	C32	C17	C31	C54	C62
cutoff (kDa) ^a	n.a.	<3.5	>3.5<8	>3.5	>10
1H-NMR (DP _n) ^b	32	17	31	54	62
MW (kDa) ^c	15	7.6	15	21	24

^a Dialysis cut-offs used to separate hydrolyzed chitosan into different CHOS preparations.

^b Standard NMR method used to characterize CHOS fractions. These DP_n values are used to name the CHOS fractions.

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chitosan (0.25%, 300 kDa, 90% F_A), chlorhexidine (12.5-25-50 µg mL⁻¹) and their combinations against *C. albicans* and observed no synergistic effects. Additionally, in a different study, no apparent synergistic activity between commercial available chitosan (Mw = 70 kDa and Fa = 0.25) and fluconazole was reported on clinical *Candida* strains [18]. These results combined with those obtained in the present study suggest that both DP_n and Fa are important to obtain synergy with CAs.

Increase in antifungal activity of a compound due to the presence of chitosan has been observed previously. For instance, a chitosan gel was shown to increase the antifungal activity of a membrane-destabilizer chlorhexidine-gluconate [33]. Additionally, the combination of chitosan acetate (ChA) and another membrane-disrupting compound (EDTA) showed a synergistic effect on *C. albicans*. El-Sharif and Hussain reported a dramatic reduction in MIC values when using chitosan acetate and EDTA in combination (MICC ChA = 0.5 µg mL⁻¹, MICC EDTA = 0.5 µg mL⁻¹) compared the individual compounds (EDTA = 850 µg/ml, ChA = 500 µg mL⁻¹) [34].

The present study shows that synergistic effects between CHOS and CA are strongest for the C31 and C54 fraction. In a previous study, Rahman *et al.* [22] studied the effect of CHOS with different DP_n on germination of *Botrytis cinerea* and *Mucor piriformis*. The authors found that CHOS with DP_n 23 and 40 had the strongest inhibitory effect against the tested pathogens. The original CHOS (DP_n 206) and shorter CHOS were considerably less effective. Thus, accumulating data indicate that CHOS with a DP_n near 30 are of particular interest for application as anti-fungals. As discussed above, it is believed that CHOS adsorbs to the cell surface, disturbs membrane integrity, and may accumulate inside the cells. Our results indicate that this membrane disturbance depends on the length of the CHOS. Although C17 and C62 show inhibitory effects (MIC, Table 4) on most of the strains and clearly display synergies with commercial antifungals on all strains (MICC, Table 4), the effects are less pronounced than what is observed for i.e. C31 and C54.

Additionally to combined treatments, sequential therapy has been intensively studied as an alternative for fighting resistant yeast infections [35]. The present study shows that pre exposure to C32 enhances the inhibitory effects of subsequent administration of Flu and Amp, even 24 h after C32 removal. Combined and sequential antifungal therapy outcomes are related to the presence of a PAFE, which is a term used to describe the persistent suppression of fungal growth after limited exposure to an antifungal. This feature is useful for the evaluation of the pharmacokinetic and pharmacodynamic indices, which are closely associated with the efficacy of the antifungal agents *in vivo*. In this sense, it can be expected that antifungals with long PAFE may be administered less frequently than those with short ones, which may require more frequent administration [36, 37]. A common assumption is that the PAFE is the result of the inhibition of microbial growth with a consequent prolongation of the lag time. However, antifungals with long PAFE are capable of exerting many different effects on surviving fungi, detectable after the drug has been removed, including prolonged changes in cell morphology,

Table 4. Combined effect of CA and CHOS with different DPn on the growth of yeast strains. CA: commercial antifungals, MIC: minimum inhibitory concentration, MICC: minimum inhibitory concentration in combination when administered simultaneously, Flu: fluconazole, Amp: amphotericin B. Data expressed in $\mu\text{g mL}^{-1}$.

CA	CHOS	MIC CA	MICC CA	MIC CHOS	MICC CHOS
<i>C. albicans</i>					
Amp		0.5			
	C17		0.06	>5000	156.3
	C31		0.06	>5000	156.3
	C54		0.06	>5000	156.3
	C62		0.06	>5000	2500
Flu		>16.0			
	C17		2.0	>5000	156.3
	C31		0.50	>5000	19.5
	C54		0.50	>5000	19.5
	C62		4.0	>5000	2500
<i>C. guillermondii</i>					
Amp		0.25			
	C17		0.06	78.1	19.5
	C31		0.01	39.1	<4.9
	C54		0.03	39.1	<4.9
	C62		0.12	>312.5	156.3
Flu		>16.0			
	C17		0.06	78.1	9.8
	C31		0.01	39.1	<4.9
	C54		0.03	39.1	<4.9
	C62		0.12	>312.5	39.1
<i>C. lusitanae</i>					
Amp		0.50			
	C17		4.0	156.3	78.1
	C31		1.0	78.1	9.8
	C54		2.0	78.1	19.5
	C62		4.0	>312.5	19.5
Flu		>16.0			
	C17		4.0	156.3	78.1
	C31		1.0	78.1	9.8
	C54		2.0	78.1	19.5
	C62		4.0	>312.5	19.5
<i>C. parasilopsis</i>					
Amp		1.0			
	C17		0.25	19.5	9.8
	C31		0.50	4.9	<4.9
	C54		0.25	19.5	9.8
	C62		0.25	>312.5	156.3
Flu		>16.0			
	C17		16.0	19.5	9.8
	C31		8.0	4.9	<4.9
	C54		16.0	39.1	9.8
	C62		16.0	>312.5	156.3

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metabolism, growth and generation time as well as delayed protein synthesis and altered susceptibility to other antifungals [38].

Recently, Wang *et al.* [39] studied the post-antifungal effect of a chitosan/nano-ZnO nanofibrous membrane on *C. albicans* and reported concentration-dependent PAFE of 4.1 ± 0.2 h, 8.2 ± 0.2 h, 10.2 ± 0.2 h, for 0.5 x MIC; 1.0 x MIC and 1.5 x MIC, respectively. Additionally, several studies have evaluated the PAFE of commercial drugs. For instance, Ernst *et al.* [36] reported Flu concentration-dependent PAFE for *C. albicans* that ranged between 4 h and >12 h (0.25 x MIC). Also, Manavathu *et al.* [40] reported a PAFE for Amp ($\mu\text{g mL}^{-1}$) on *C. albicans* of 5.3 ± 1.15 h, while Egusa *et al.* [41] reported that the mean duration of PAFE of amphotericin for *C. albicans* was 8.73 ± 0.93 h (2 x MIC). Interestingly, PAFE values obtained in our study were considerably lower, in spite of C32 maintaining its positive effect of the efficacy of some CAs for up to 48 h after removal. This observation reinforces our theory of C32 causing a perturbation of the cell membrane without causing altering of the growth rate.

The combination of CHOS and conventional antifungals, together or as part of a sequential therapy, opens new therapeutic perspectives for treating human candidiasis. The synergistic effects described above may be useful to reduce antifungal dosages without substantially compromising the efficacy, to broaden the spectrum of anti-fungal activity, and/or to improve the efficacy of current antifungals. Overcoming the rising resistance of yeasts to current treatments is another perspective of the results described above.

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