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# Combination of caspofungin or anidulafungin with antimicrobial peptides results in potent synergistic killing of and in vitro

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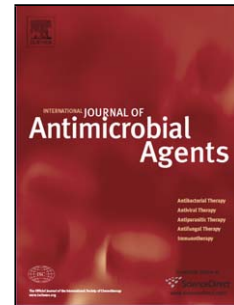
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## Accepted Manuscript

Title: Combination of caspofungin or anidulafungin with antimicrobial peptides results in potent synergistic killing of *Candida albicans* and *Candida glabrata* in vitro

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1           **Combination of caspofungin or anidulafungin with antimicrobial**  
2           **peptides results in potent, synergistic killing of *Candida albicans* and**  
3                           ***Candida glabrata* in vitro**

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5  
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19  
20  
21    **Keywords:** echinocandins; antifungals; synergy; combination therapy; candidiasis

22  
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25  
26  
27  
28    **Abstract**

29 Administering synergistic combinations of antifungals could be a route to overcome problems with  
30 toxicity and the development of resistance. Combination of the echinocandins, caspofungin or  
31 anidulafungin, with a range of structurally-diverse antimicrobial peptides resulted in potent,  
32 synergistic killing of *Candida* species in vitro. Fungicidal synergy was measured by calculating FIC  
33 values from checkerboard assays and loss of viability. Inhibitory combinations of the antifungals  
34 did not induce cytotoxicity in vitro. However, in a murine-model of systemic candidiasis, co-  
35 administration of caspofungin with one example of the cationic peptides tested, ranalexin, did not  
36 show enhanced efficacy compared to the single treatments alone. Further study using alternative  
37 peptides will identify whether this combination approach could represent a novel treatment for  
38 fungal pathogens.

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56 **1. Introduction**

57 Over the past thirty years fungi have emerged as significant causes of human disease with  
58 attendant morbidity and mortality [8, 9]. A large proportion of these cases occur in  
59 immunocompromised patients because fungal pathogens are readily able to overwhelm the  
60 already weakened host defence mechanisms. Hospitalised patients with serious underlying  
61 disease are increasingly exposed to invasive surgical procedures, or chemotherapeutic treatments,  
62 such as broad-spectrum antibiotics, or immunosuppressants after organ transplantation and cancer  
63 chemotherapy that readily promote mycotic disease. Infections caused by *Candida albicans* have a  
64 population incidence of between 1.1 and 24 cases per 100 000 individuals with an associated  
65 mortality rate of greater than 30% [10, 29]. In the USA this equates to between 3 000 and 11 000  
66 deaths annually from nosocomial candidaemia [23]. Notably, treatment with many antifungal drugs  
67 is complicated by: high toxicity and low tolerability; ineffectiveness against new or re-emerging  
68 fungi; or the development of drug-resistant strains in patients undergoing treatment [23]. Thus,  
69 there is a justifiable need for more research to develop new antifungal treatments.

70  
71 One possible approach to improve the range and scope of antifungal therapy is the use of  
72 combinations of antifungals. Benefits could include: improved efficacy over the use of single drugs,  
73 consequently allowing lower, less costly, doses of each individual drug; reduced toxicity and  
74 improved tolerance; broader spectrum of efficacy; and finally, the potential to prevent resistant  
75 organisms arising as can occur with monotherapy.

76  
77 A potential source of new antifungals is cationic antimicrobial peptides that are ubiquitous  
78 throughout nature and protect against invasive microbial pathogens. To date, more than 800 have  
79 been described [11]. Potential advantages of cationic peptides for future therapy include; a broad  
80 spectrum of activity, rapid killing of microbes, unaffected by classical antibiotic resistance  
81 mechanisms, neutralise endotoxin and are active in animal models [11]. Disadvantages include;  
82 sensitivity to proteolytic degradation by serum proteases in vivo; potential toxicity at high doses  
83 necessary to show efficacy in vivo, including haemolytic activity; and the high cost of synthesising  
84 and producing large quantities of these molecules [13]. Employing cationic peptides in

85 combinations with other clinically-approved drugs could overcome some of the problems  
86 associated with the clinical application of peptides.

87 Echinocandins inhibit the synthesis of  $\beta$ -D-glucan in fungal cell walls and are fungicidal against  
88 *Candida* and fungistatic against *Aspergillus* species due to loss of cell wall integrity [7]. Importantly,  
89 an intact cell wall also represents a potential barrier that cationic peptides must interact with and  
90 pass through before they can interact with the plasma membrane beneath. Supporting this, recent  
91 data from our laboratory has demonstrated that disruption of the outer cell-wall phosphomannan  
92 content has a significant influence on the degree of susceptibility of *Candida albicans* to a cationic  
93 peptide [12]. Thus, due to the complementary inhibitory action of the echinocandins and cationic  
94 peptides, we reasoned that the application of combinations of these molecules could result in  
95 additive or synergistic inhibition of fungal pathogens. In this study, we combined the three clinically-  
96 approved echinocandins with a range of structurally diverse antimicrobial peptides and observed  
97 potent, synergistic killing of *Candida* species *in vitro*.

98

99

## 100 2. Material and Methods

101

### 102 2.1. Yeast strains and growth media.

103 Yeast strains used in this work were; *Candida albicans* SC5314; and clinical isolates of *C. albicans*  
104 and *C. glabrata* provided by Dr. Cyril Lafong, Fife Area Laboratory, Victoria Hospital, Kirkcaldy. All  
105 strains were cultured in Malt Extract Broth (MEB), pH 7 (1% glucose, 0.6% malt extract, 0.12%  
106 yeast extract) at 30°C with shaking. For enumerating cell survival after exposure to antifungals,  
107 yeasts were plated on YEPD agar (2% glucose, 2% agar, 1% bactopectone, 1% yeast extract).  
108 Numbers of cells used in the various assays described below were calculated using an optical  
109 density (OD<sub>600</sub>) versus cell numbers calibration curve for each yeast strain used (data not shown).

110

### 111 2.2. Antifungals.

112 Magainin 2 [31], ranalexin [3], cyclic peptide 6752 [6], cyclic peptide GS14K4 [14], gomesin [24]  
113 and dermaseptin S3(1-16) [18] were synthesized according to their published sequences by  
114 Peptide Research Ltd, Wickham, UK, to greater than 95% purity and verified by HPLC and mass  
115 spectrometry. **Biochemical properties of the peptides employed in this study are listed in Table 1.**  
116 Peptides were solubilised in d.H<sub>2</sub>O at stock concentrations of 50 mg/mL. Caspofungin (Merck) and  
117 micafungin (Astellas) were diluted into 1 mg/mL and 10mg/mL aliquots with sterile d.H<sub>2</sub>O,  
118 respectively. Anidulafungin (Pfizer) was diluted into 10mg/mL aliquots with 20% v/v ethanol.

119

### 120 2.3. Checkerboard assay of yeast growth inhibition.

121 96-well microplate (Greiner Bio-one Ltd, Stonehouse, UK) wells were filled with 150µl RPMI-1640  
122 (Sigma) with 2% MOPS (Sigma), pH 6.4. Appropriate concentrations of individual antifungals or  
123 combinations of peptides with the echinocandins were added. Approximately 500 cells from mid-  
124 exponential phase cultures of each yeast strain were then added to each well. Plates were  
125 incubated at 30°C for 48 h and scanned using an ImageScanner (GE Healthcare UK Ltd, Chalfont  
126 St Giles, UK) with ImageMaster Labscan v 3.00 software (GE Healthcare UK Ltd). Images were  
127 annotated using Microsoft PhotoDraw. The MIC for each antifungal tested was defined as the  
128 concentration present in the first optically clear well after 48 h.

129

130 *2.4. Disc diffusion assay of yeast growth inhibition.*

131 Sterile paper discs (6mm, Aa, Whatman) were impregnated with the relevant peptide (5 to 25 µg)  
132 with or without echinocandin. These were left in sterile Petri dishes overnight at room temperature  
133 to dry. 100µl of mid-exponential phase cultures (OD<sub>600</sub> of 0.3) were spread onto RPMI-1640  
134 agarose plates (2% agarose, 1% RPMI-1640 medium Auto-mod™ (Sigma), 0.4% sodium  
135 bicarbonate (Sigma), 2% MOPS (Sigma)) and left to dry for 1 h at room temperature. The discs  
136 containing the various antifungal combinations were then applied to the surface of the plates and  
137 incubated at 30°C for 48 h. Images showing the zones of inhibition were obtained by scanning the  
138 plates as described in section 2.3.

139

140 *2.5. Assay of yeast cell viability.*

141 Starter cultures were incubated overnight in MEB, pH 7 at 30 °C with shaking. 1 mL of this culture  
142 was diluted with fresh MEB, pH 7 to give a starting OD<sub>600</sub> of 0.05 prior to incubation at 30°C with  
143 shaking at 200 rpm. OD<sub>600</sub> readings were taken every 60 min until an OD<sub>600</sub> of 0.3 was reached.  
144 Cultures were then exposed to peptide with or without caspofungin. Culture viability was measured  
145 every 30 min by serial dilution and plating onto YEPD agar plates. Plates were incubated at 30°C  
146 for 48 h prior to enumeration of survivors.

147

148 *2.6. In vitro mammalian cell cytotoxicity assay.*

149 The method is as described in Yang *et al.* [30] and employed non-cancerous Vero cells (African  
150 green monkey kidney fibroblasts). Vero cells were exposed to echinocandins, antimicrobial  
151 peptides or combinations of both for 48 h and viability determined by the neutral red procedure.  
152 Viable cells were expressed as the proportion retaining neutral red after exposure to antifungals  
153 relative to untreated controls. CCCP (Sigma) was used as a positive control (0.15 µg/mL).

154

155 *2.7. Intracellular localisation of fluorescein tagged dermaseptin S3(1-16) using fluorescence*  
156 *microscopy.*

157 The phase-contrast and fluorescent peptide images were generated using the FITC filter (Ex λ =  
158 490/20 nm, Em λ = 528/38 nm). Propidium iodide (PI; Invitrogen) was used to identify dead cells



159 (RD-TR-PE, Ex  $\lambda$  = 490/20 nm, Em  $\lambda$  = 528/38 nm). Cells were cultured in MEB and harvested at  
160 mid exponential phase ( $OD_{600}$  0.6). A volume equivalent to  $2.0 \times 10^6$  cells/ml was removed for each  
161 assay, centrifuged for 2 min at 10 000 rpm, washed in ddH<sub>2</sub>O and resuspended in 1 ml RPMI-  
162 1640. Echinocandin and/or DsS3(1-16)-fluorescein were added with PI (1.8  $\mu$ M) and incubated for  
163 2 h at 30°C in the dark. Unbound dye and peptide were removed by harvesting for 8 min at 3 000 x  
164 g, washing the pellet in ddH<sub>2</sub>O, harvesting and resuspension in 20  $\mu$ l RPMI-1640. 2  $\mu$ l of the  
165 stained cells were fixed with 2  $\mu$ l of 1 % low-melting-point agarose (Biogene). Samples were  
166 placed on ice and protected from light until analysis. Images were captured on an Olympus IX70  
167 DeltaVision microscope (Applied Precision LLC). SoftWoRx Explorer 1.3 (Applied Precision LLC)  
168 was used for image processing and analysis.

169

170 *2.8. Efficacy in vivo of the combination of caspofungin with ranalexin in a murine model of*  
171 *disseminated Candidiasis.*

172 Basic details of the intravenous challenge model for disseminated *C. albicans* infection have been  
173 published previously [17]. Briefly, female BALB/c mice were infected intravenously with *C. albicans*  
174 at a dose of  $3 \pm 1 \times 10^4$  CFU/g body weight. Animals were humanely terminated 72 h after challenge,  
175 and body weight changes, which correlate with survival times [16], and fungal burdens in kidney  
176 homogenates were determined.

177

178 Caspofungin IV preparation was diluted in saline as required. Combination therapy with  
179 caspofungin and ranalexin was investigated in two experiments. In the first, caspofungin was  
180 dosed at 0.01 mg/kg IP, qd, and ranalexin at 10 mg/kg IV, qd. Saline IV and IP was used as  
181 placebo, and animals given caspofungin or ranalexin monotherapy were concomitantly dosed with  
182 saline by the IV and IP routes, respectively. The first treatments were given 1 h after challenge and  
183 repeated at 24 and 48 h. In the second experiment, caspofungin was dosed at 0.05 mg/kg IV, qd,  
184 and ranalexin was dosed IV at 10 mg/kg bid. The first treatments were given 1 h after challenge.  
185 Saline IV was used as placebo.

186 All animal experimentation was approved by the local ethical review committee and was performed  
187 under UK Home Office regulations. Statistical analysis of the data was by Mann-Whitney *U*-test.

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190 **3. Results**

191 *3.1. Combination of caspofungin or anidulafungin with antimicrobial peptides results in potent,*  
192 *synergistic inhibition of C. albicans and C. glabrata in vitro.*

193 Exposing *C. albicans* SC5314 to a combination of peptides with caspofungin (Figure 1), or  
194 anidulafungin (data not shown), resulted in significant enhancement of the inhibitory activity of the  
195 peptides when compared with peptide treatment alone. FICs calculated from the checkerboard  
196 assays (Table 2) showed that combination of all five peptides with caspofungin resulted in clear  
197 synergy (<0.5), particularly versus the *C. albicans* strains. Synergy against *C. glabrata* was evident  
198 to a lesser extent with all the peptides tested with the exception of ranalexin. The FIC values  
199 generated from combining anidulafungin with the same peptides revealed a similar pattern of  
200 synergy as occurred with caspofungin with the exception of cyclic peptide 6752 that displayed no  
201 synergy. As with caspofungin, the synergistic effect induced by combining the peptides with  
202 anidulafungin was most evident versus the *C. albicans* strains.

203

204 Similar data were also obtained from identical checkerboard assays using the hospital isolates of  
205 *C. albicans* and *C. glabrata* (data not shown). One peptide, gomesin, showed no enhanced  
206 inhibition of any of the strains tested in combination with any of the echinocandins (data not  
207 shown). Also, no enhancement of the inhibitory effect of all the peptides tested was observed when  
208 they were combined with micafungin (data not shown).

209

210 To determine if the synergistic inhibition of the combinations enhanced the fungicidal effect of the  
211 peptides or the echinocandins we tested the effect of the combination of caspofungin with  
212 ranalexin, magainin 2 and DsS3(1-16) on the viability of growing cells of *C. albicans* SC5314  
213 (Figure 2). Addition of ranalexin (9 mg/L: Figure 2a), magainin 2 (10 mg/L: Figure 2b) or DsS3(1-  
214 16) (4 mg/L: Figure 2c) alone to exponentially growing cells had **little** effect on viability compared to  
215 untreated controls. In contrast, exposure to caspofungin alone (0.01 mg/L: Figure 2a, b and c)  
216 resulted in minor inhibition of growth rate. Notably, with cultures exposed to the individual peptides  
217 (at identical concentrations as described above), but in the presence of 0.01 mg/L caspofungin, in  
218 each case there was complete cessation of growth and **notable** loss of viability. Thus, the

219 combination of antimicrobial peptides with either caspofungin or anidulafungin resulted in potent,  
220 synergistic fungicidal activity.

221

222 3.2. *In vitro* toxicity testing of antimicrobials peptides alone and in combination with caspofungin.

223 In preparation for *in vivo* testing of the combination of antimicrobial peptides with caspofungin in a  
224 mouse model of systemic candidiasis, the *in vitro* toxicity of the antifungals on noncancerous Vero  
225 cells was evaluated (Figure 3). Exposure to caspofungin or anidulafungin resulted in a minor loss  
226 of cell viability at a concentration an order of magnitude higher than necessary to completely inhibit  
227 growth of *C. albicans* *in vitro* (Figure 3a). The antimicrobial peptides had no measurable toxic  
228 effect on Vero cells up to a concentration of 50 mg/L. However, exposure to 100 mg/L of the cyclic  
229 peptide GS24K4 and ranalexin resulted in approximately 70% and 20% loss of viability respectively  
230 (Figure 3b). Peptides 6752, DsS3(1-16) and magainin 2 induced no significant loss of cell viability  
231 even at the highest concentration tested: 100 mg/L. Following this, we tested the effect of  
232 combinations of either caspofungin (Figure 3c) or anidulafungin (Figure 3d) with each of the  
233 peptides. Combination of 10 mg/L caspofungin with 30 mg/L of any of the antimicrobial peptides  
234 had no detectable effect on Vero cell viability. However, caspofungin at 15 mg/L in combination  
235 with 50 mg/L of ranalexin showed some cytotoxicity and 100 mg/L caspofungin with 100 mg/L  
236 GS14K4, ranalexin and DsS3(1-16) induced significant loss of viability. Identical experiments  
237 testing the cytotoxicity of anidulafungin also showed that combination of 10 mg/L anidulafungin  
238 with 30 mg/L of any of the antimicrobial peptides had only a minor effect on Vero cell viability. At  
239 higher concentrations of anidulafungin and peptides toxicity increased significantly, demonstrating  
240 that peptide combinations with anidulafungin were potentially more cytotoxic than those with  
241 caspofungin. Nonetheless, the study revealed that concentrations of the antifungals in combination  
242 that are much higher than concentrations necessary to completely inhibit growth of *C. albicans in*  
243 *vitro* (Figure 1) did not induce significant cytotoxicity *in vitro*. Thus, these results indicated that  
244 there may be a therapeutic 'window' where the combination treatment may show efficacy versus *C.*  
245 *albicans* without inducing toxicity.

246

247 *3.3 Exposure to an antimicrobial peptide in combination with caspofungin results in increased*  
248 *intracellular accumulation of the peptide and enhanced killing of C. albicans.*

249 Previous work with DsS3(1-16) tagged with fluorescein revealed that the peptide binds transiently  
250 to the outer cell wall prior to being taken up by the cell and sequestered into the vacuole before  
251 finally disseminating throughout the cytosol [12]. Thus, we used fluorescence microscopy to study  
252 how exposure to caspofungin affected the uptake of DsS3(1-16)-fluorescein. In addition to  
253 exposing the cells to DsS3(1-16)-fluorescein, we simultaneously stained with PI to correlate stages  
254 of peptide uptake with loss of viability (Figure 4). Exposure to 20 mg/L DsS3(1-16)-fluorescein for 2  
255 hours resulted in approximately 5% of the population displaying fluorescence throughout the entire  
256 cell, 20% with fluorescence localised to the vacuole, and 75% of the population showing no peptide  
257 uptake (Figure 4a). As before [12], when the peptide was disseminated throughout the entire cell  
258 this correlated with PI uptake into the cells indicating that these cells were unviable (data not  
259 shown). In contrast, cells with the peptide concentrated inside the vacuole were not PI-positive and  
260 were viable (Figure 4b).

261 Exposure to 0.001 mg/L caspofungin for 2 h resulted in approximately 15% of the population  
262 becoming PI-positive. However, if the cells were exposed to the caspofungin in the presence of  
263 DsS3(1-16)-fluorescein, the proportion of cells that were PI-positive increased to approximately  
264 35% (Figure 4a). As before, all these PI-positive cells also displayed green fluorescence  
265 throughout the entire cell indicating complete peptide entry into the cells.

266 Exposure to 0.01 or 0.05 mg/L caspofungin alone resulted in approximately 24 and 26%,  
267 respectively, of the population becoming PI-positive after 2 h (Figure 4a). Paradoxically, at higher  
268 concentrations of caspofungin, 0.1 and 1 mg/L respectively, the proportion of cells that were PI-  
269 positive decreased to approximately 19% in each case. This phenomenon was seen in many  
270 repeat experiments with both caspofungin and anidulafungin (data not shown).

271 Upon addition of DsS3(1-16)-fluorescein to increasing concentrations of caspofungin (0.01 – 1  
272 mg/L) we observed a similar trend to that observed previously with an increase in the proportion of  
273 cells in the population becoming PI-positive and simultaneously displaying total green fluorescence  
274 throughout the cell. Coincident with the increase in the proportion of cells displaying total  
275 dissemination of DsS3(1-16)-fluorescein throughout the entire cell, at every concentration of

276 caspofungin tested, addition of DsS3(1-16)-fluorescein resulted in a smaller proportion of the  
277 population displaying only vacuolar localisation of the peptide. Thus, the presence of caspofungin  
278 results in greater, or accelerated, uptake of the peptide into the cytosol, which in turn, leads to  
279 enhanced loss of cell viability.

280

281 In identical experiments using anidulafungin instead of caspofungin, similar trends with regard to  
282 peptide uptake were observed (data not shown).

283

284 *3.4. Combination of caspofungin with ranalexin did not show synergistic inhibition of C. albicans in*  
285 *a murine model of disseminated Candidiasis.*

286 The results of the mouse experiments are summarized in Table 3. In the first experiment, with  
287 caspofungin given IP at 0.01 mg/kg, the 72 h kidney burden was reduced significantly below  
288 placebo ( $p < 0.05$ ) in mice given the caspofungin/ranalexin combination: however, the extent of the  
289 effect was less than a 1-log burden reduction. No significant reduction in weight loss relative to  
290 placebo-treated mice was seen for any of the test agents. In the second experiment, with a higher  
291 caspofungin dose of 0.05 mg/kg, IV dosing of both test agents, and BID dosing of ranalexin, highly  
292 significant ( $p < 0.01$ ) reductions in kidney burdens and 3-day weight loss were seen for groups of  
293 animals receiving treatments that included caspofungin. However, progressive necrosis of the tails  
294 of animals given ranalexin alone or in combination meant that treatments with this agent were  
295 stopped after 36 h in the interests of animal welfare.

296

297 *3.5. Combination of antimicrobial peptides with caspofungin results in potent, synergistic killing of*  
298 *C. albicans on a porous, solid surface.*

299 To explore whether the combination treatment displayed synergy in a topical application, lawns of  
300 *C. albicans* SC5314 (Figure 5a) and the *C. albicans* hospital isolate (Figure 5b) covering the  
301 surface of RPMI1640 agarose plates were exposed to sterile, filter paper discs impregnated with  
302 caspofungin, ranalexin, magainin 2 or DsS3(1-16) alone and combinations of caspofungin with  
303 each peptide. With both strains, caspofungin impregnated discs showed only minor zones of  
304 inhibition at the higher concentrations tested of 0.075, 0.1 and 0.124  $\mu\text{g}$ . Exposure of both strains

305 to discs impregnated with each peptide alone up to the highest concentration of 25  $\mu\text{g}$  resulted in  
306 no detectable zones of inhibition. However, exposure of both strains to discs impregnated with  
307 combinations of each peptide with caspofungin clearly revealed synergistic inhibition, with larger,  
308 more significant zones of inhibition after 48 h exposure. Similar results were observed for both  
309 yeast strains exposed to combinations of the same peptides with anidulafungin (data not shown).

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312 **4. Discussion**

313 Combination therapy using clinically-approved antifungals is commonly employed to treat mycotic  
314 disease (reviewed by Mukherjee *et al.* [19]) eg., combinations of intravenous amphotericin B or oral  
315 fluconazole with 5-fluorocytosine have been proposed for treatment of candidiasis [22]. Synergy  
316 between caspofungin and amphotericin B against *Aspergillus* spp. has been described in vitro [1],  
317 and in a murine model [15], and resulted in improved therapeutic outcome in a murine model of  
318 systemic *C. glabrata* infection [21]. However, controversy surrounds whether or not combination  
319 therapy is superior to monotherapy in clinical situations due to a lack of evidence from clinical trials  
320 [4].

321  
322 There are examples in the literature of apparent synergy, or enhanced efficacy, between various  
323 antimicrobial peptides and clinically-approved antifungals eg., the combination of Peptide 2, a  
324 derivative of lactoferrin, with amphotericin B resulted in enhanced survival of mice in a model of  
325 systemic candidiasis [25]. Notably, there are no published reports of synergistic interactions  
326 between antimicrobial peptides and the clinically-approved echinocandins.

327  
328 Despite the introduction of new antifungals, such as voriconazole and caspofungin, in some cases  
329 antifungal efficacies remain sub-optimal [5] and there is increasing evidence of antifungal therapy  
330 failing due to the selection in vivo of resistant fungal pathogens eg., reduced susceptibility to  
331 caspofungin in patients receiving therapy for invasive candidiasis [26]. Thus, the use of  
332 combinations of antifungals in therapy could be an effective alternative approach to treat invasive  
333 fungal infections that are not responsive to standard monotherapy.

334 Here, we describe how combination of the echinocandins, caspofungin and anidulafungin, with  
335 antimicrobial peptides results in significant synergy against *Candida* spp. in vitro. Notably, we  
336 detected no apparent synergy using identical combinations of peptides with the other clinically-  
337 approved echinocandin, micafungin. This is surprising considering the similarity in structure, and  
338 apparent inhibitory action, between all of these drugs and merits further investigation. The FIC  
339 values calculated from the checkerboard experiments revealed that all the antimicrobial peptides  
340 tested (with the exception of the cyclic peptide 6752 in combination with anidulafungin) displayed



341 some degree of synergy against the three yeasts tested in combination with either caspofungin or  
342 anidulafungin (Table 2). With each combination tested, the level of synergy against the hospital-  
343 isolate of *C. glabrata* was reduced compared to that shown against the two *C. albicans* strains.  
344 This is not surprising considering *C. glabrata* has been shown to show greater resistance than *C.*  
345 *albicans* to many antifungal drugs, particularly the triazoles [23].

346

347 Study of the nature of the inhibitory mechanism of the synergistic combination using fluorescence  
348 microscopy revealed that exposure to the cell-wall degrading effect of caspofungin lead to  
349 enhanced uptake of the cationic peptide into the cell, resulting in enhanced killing. Intriguingly,  
350 there was evidence of paradoxical resistance to increasing concentrations of caspofungin as the  
351 degree of peptide uptake into the cells initially increased with higher concentrations but then  
352 declined again as the concentration was raised further. Evidence of a paradoxical resistance  
353 phenotype to echinocandins has been presented before with *Candida* spp., particularly with those  
354 isolates exposed to caspofungin [2]. Possible explanations for this paradoxical effect include; the  
355 induction of stress-response pathways [28], or, the elevation in the chitin content of the cell walls of  
356 *C. albicans* cells exposed to caspofungin reducing the penetration of the peptide into the cells [27].  
357 Ideally, whether or not a combination treatment could prove efficacious in vivo would be assessed  
358 using animal models followed by clinical trials. Thus, we employed a murine model of disseminated  
359 candidiasis to determine if the combination treatment demonstrated enhanced efficacy in vivo. We  
360 chose to use caspofungin and ranalexin due to previous experience of in vivo dosing with these  
361 two compounds. Disappointingly, the combination treatment did not result in enhanced efficacy  
362 compared to the single treatments. However, resources only permitted a preliminary experiment  
363 with only one dose of each of the compounds tested and we were only able to test the in vivo  
364 efficacy of one echinocandin, caspofungin, with one peptide, ranalexin. As the experiment  
365 progressed there was evidence of ranalexin toxicity which was manifest as tail vein necrosis and  
366 ulceration at the site(s) of administration. Clearly, the pharmacokinetics and interactions of  
367 antifungal peptides, where little is known, is difficult to optimise in an animal model. Furthermore, it  
368 is even more difficult to replicate conditions present in humans. Nonetheless, there is additional  
369 scope to use the mouse model to study the effects of alternative, non-toxic peptides in combination

370 with other echinocandins, with varied dosing regimens and alternative routes of administration,  
371 versus additional fungal pathogens such as *Aspergillus* spp.. Thus, despite this preliminary failure,  
372 we believe that a combination of an echinocandin with an antimicrobial peptide can be identified  
373 that could still result in enhanced efficacy in humans. Importantly, we have demonstrated  
374 synergistic inhibition of yeast growing on the surface of an agarose plate and if intravenous  
375 administration of the combination treatment is not efficacious then topical administration to treat  
376 conditions such as oral or mucosal candidiasis could prove to be more effective.

377

378

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384

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472 **Legends for Figures**

473

474 **Figure 1.** – Checkerboard growth assay(s) of; antimicrobial peptide MIC's alone, and each of the  
475 peptides in combination with caspofungin. The assays were performed on *C. albicans* SC5314 in  
476 RPMI-1640 (with 2% MOPS, pH 6.4) media and incubated at 30°C for 48 h. The black lines  
477 indicate the first well where no growth was observed. Representative results from at least triplicate  
478 experiments are shown.

479

480 **Figure 2.** – The effect of combination of antimicrobial peptides with caspofungin on the growth and  
481 viability of *C. albicans* SC5314. Cultures were grown in MEB, pH 7 at 30°C with shaking and  
482 viability ( $\log_{10}$ CFU/mL) is shown as open symbols and growth (culture optical density at 600 nm) as  
483 filled symbols - control culture (squares), treated with peptide alone (diamonds), treated with  
484 caspofungin alone (triangles) and treated with a combination of peptide and caspofungin (circles).  
485 The effect of caspofungin (0.01 mg/L) in combination with: a) ranalexin (9 mg/L); b) magainin 2 (10  
486 mg/L); and c) DsS3(1-16) (4 mg/L) is shown. Representative results from at least triplicate  
487 experiments are shown.

488

489 **Figure 3.** – *In vitro* toxicity of the echinocandins, antimicrobial peptides and combinations of  
490 peptides and echinocandins on mammalian cells. The effect on the viability of Vero cells in RPMI-  
491 1640 media of a 48 h exposure to increasing concentrations of: a) caspofungin (squares) or  
492 anidulafungin (circles); b) peptide 6752 (open diamond), GS14K4 (open triangle), DsS3(1-16)  
493 (open circle), ranalexin (filled square) and magainin 2 (filled diamond); combinations of, c)  
494 caspofungin, and d) anidulafungin, with: DsS3(1-16) (open square), ranalexin (open diamond),  
495 magainin 2 (open triangle), 6752 (filled square) and GS14K4 (filled diamond). Cell viability was  
496 expressed as the proportion of cells within the population that retained neutral red after exposure  
497 to the antifungals relative to untreated controls. Released dye was measured  
498 spectrophotometrically at 540nm. Representative results from at least triplicate experiments are  
499 shown.

500 Figure 4. – Microscopic study on how the presence of caspofungin effects the uptake of DsS3(1-  
501 16)-fluorescein into *C. albicans* SC5314. a) Yeast cells in RPMI-1640, supplemented with 1.8  $\mu$ M  
502 PI, were exposed to the following conditions for 2 h at 30°C: DsS3(1-16)-fluorescein (20 mg/L)  
503 alone; increasing concentrations of caspofungin (0.001 – 1 mg/L); or, combinations of DsS3(1-16)-  
504 fluorescein (20 mg/L) with increasing concentrations of caspofungin (0.001 – 1 mg/L).  
505 After harvesting and washing, the proportions of cells within the population that showed no  
506 fluorescence, were PI +ve and showed either vacuolar green fluorescence, or whole-cell green  
507 fluorescence were calculated. For each tested condition the fluorescent state of over 300 cells was  
508 ascertained from multiple captured images. A representative result of a duplicate experiment is  
509 shown. b) A representative image of stained cells after exposure to a combination of 0.05 mg/L  
510 caspofungin and 20 mg/L DsS3(1-16)-fluorescein. Cells staining PI +ve (inviable) co-stain with  
511 cells that show green fluorescence throughout the entire cytosol (full uptake of DsS3(1-16)-  
512 fluorescein). Cells showing no fluorescence at all, and cells that show only green fluorescence  
513 concentrated in the vacuole, do not co-stain with PI and are thus viable.

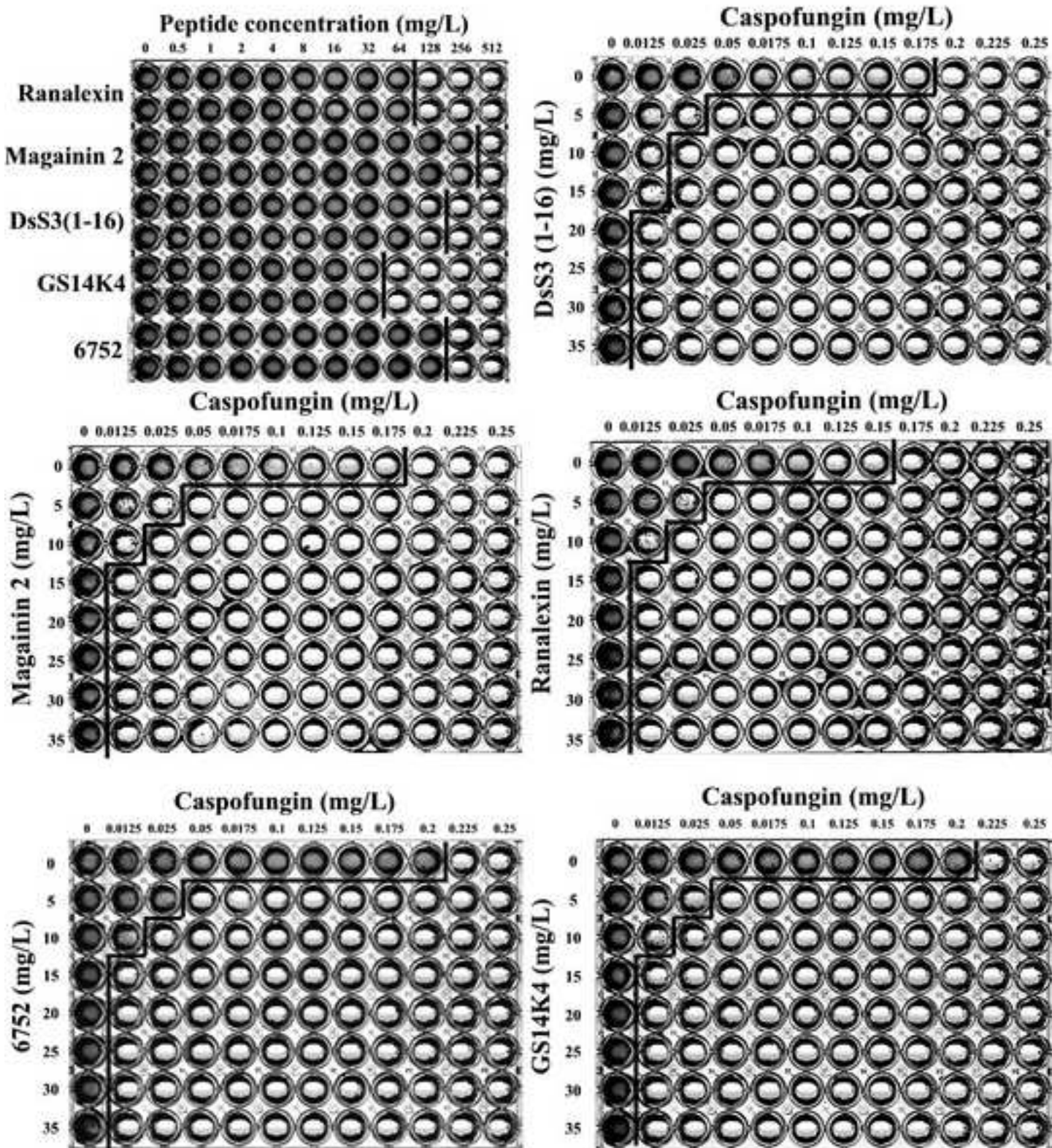
514

515 Figure 5. – The effect of antimicrobial peptides and/or caspofungin impregnated into filter paper  
516 discs on the surface growth of *C. albicans* on MEB/agarose. Shown is the effect of increasing  
517 caspofungin alone; increasing DsS3(1-16), magainin 2 and ranalexin alone; and combination of  
518 caspofungin with each peptide. The effect of these treatments on surface growth of; a) *C. albicans*  
519 SC5314, and b) a hospital isolate of *C. albicans*, after 48 h at 30°C is shown. Zones of clearing in  
520 the yeast lawn are evident as black zones around the filter paper discs. Representative results  
521 from at least triplicate experiments are shown.

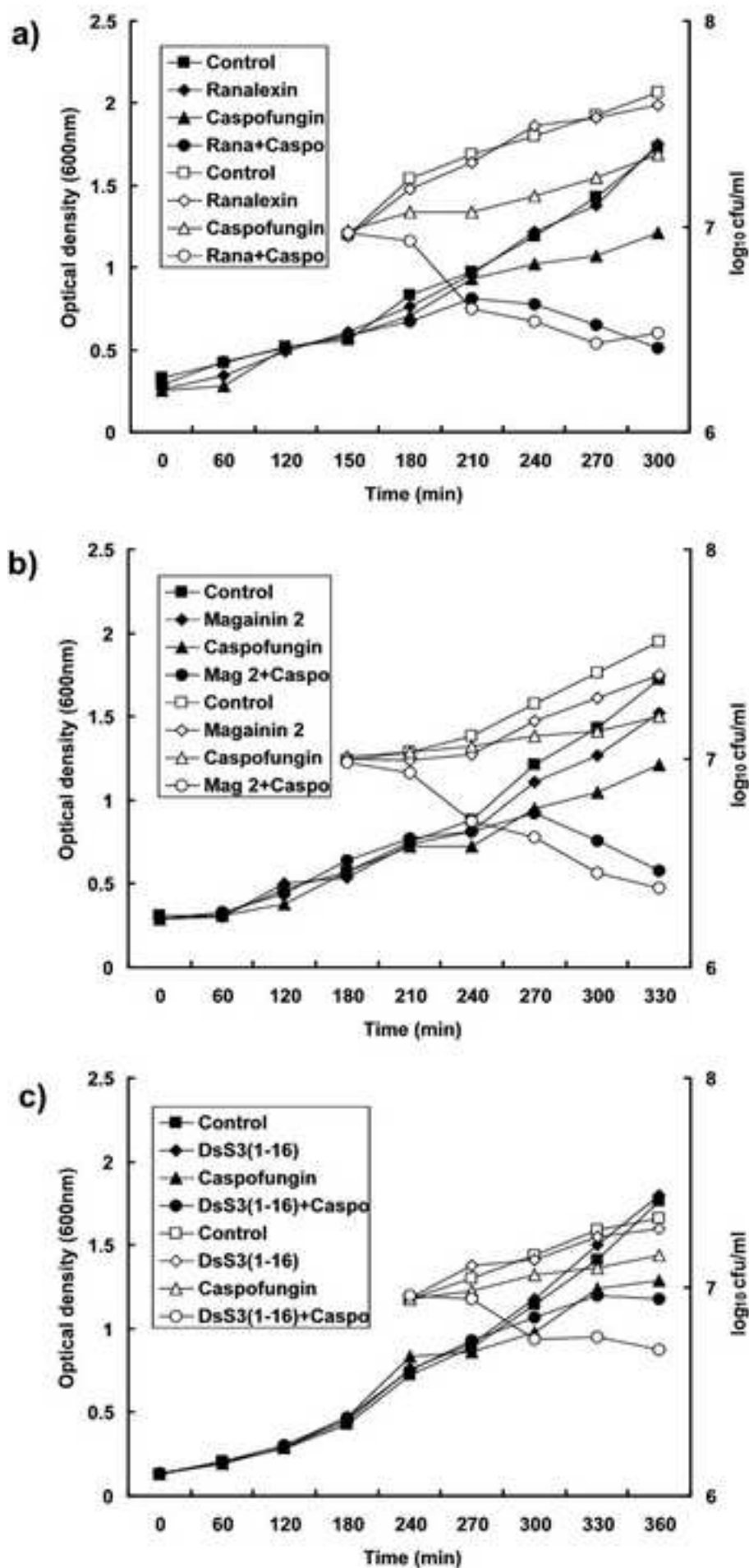
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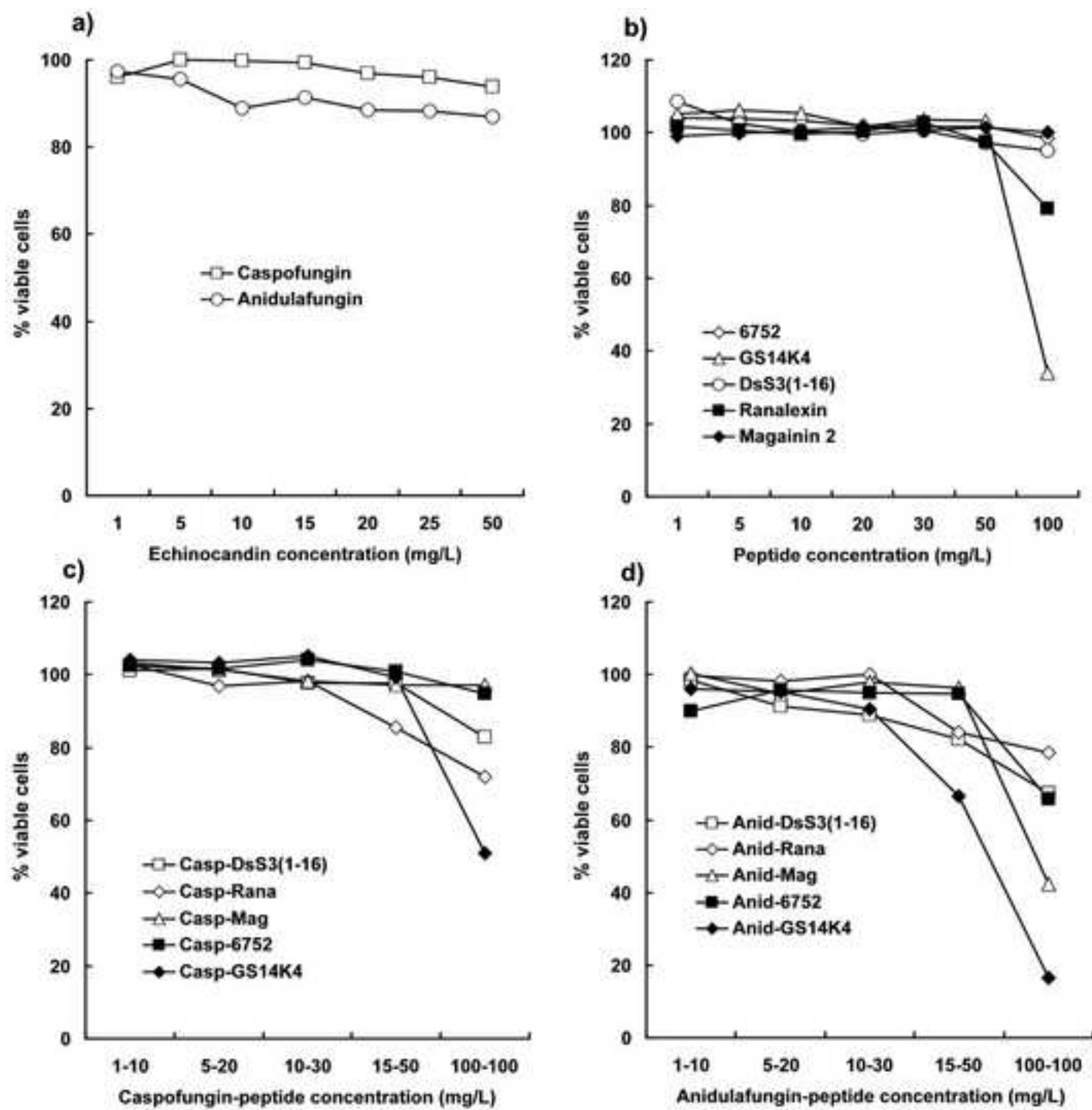
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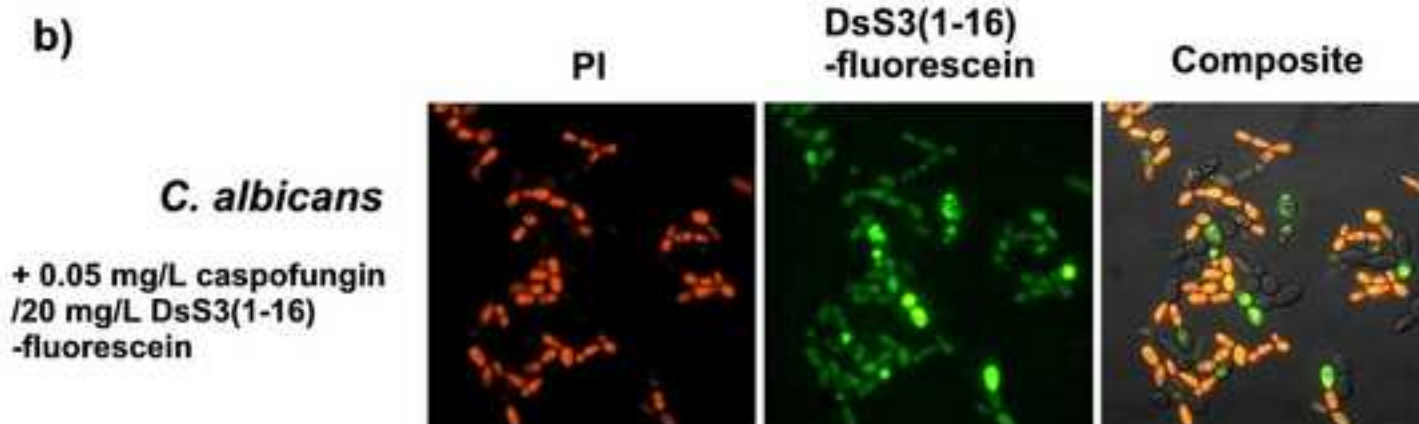
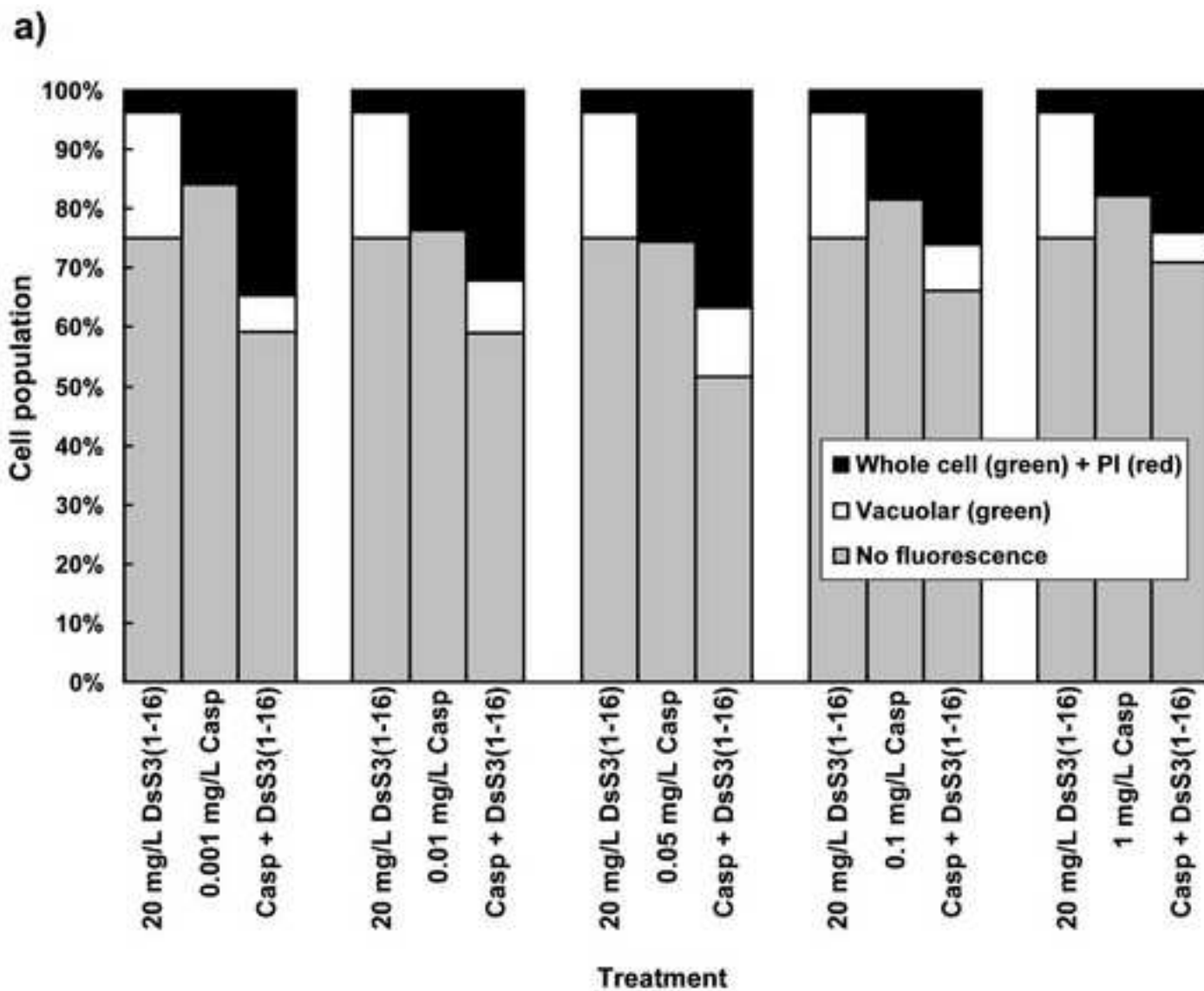
*C. albicans* SC5314 RPMI 1640

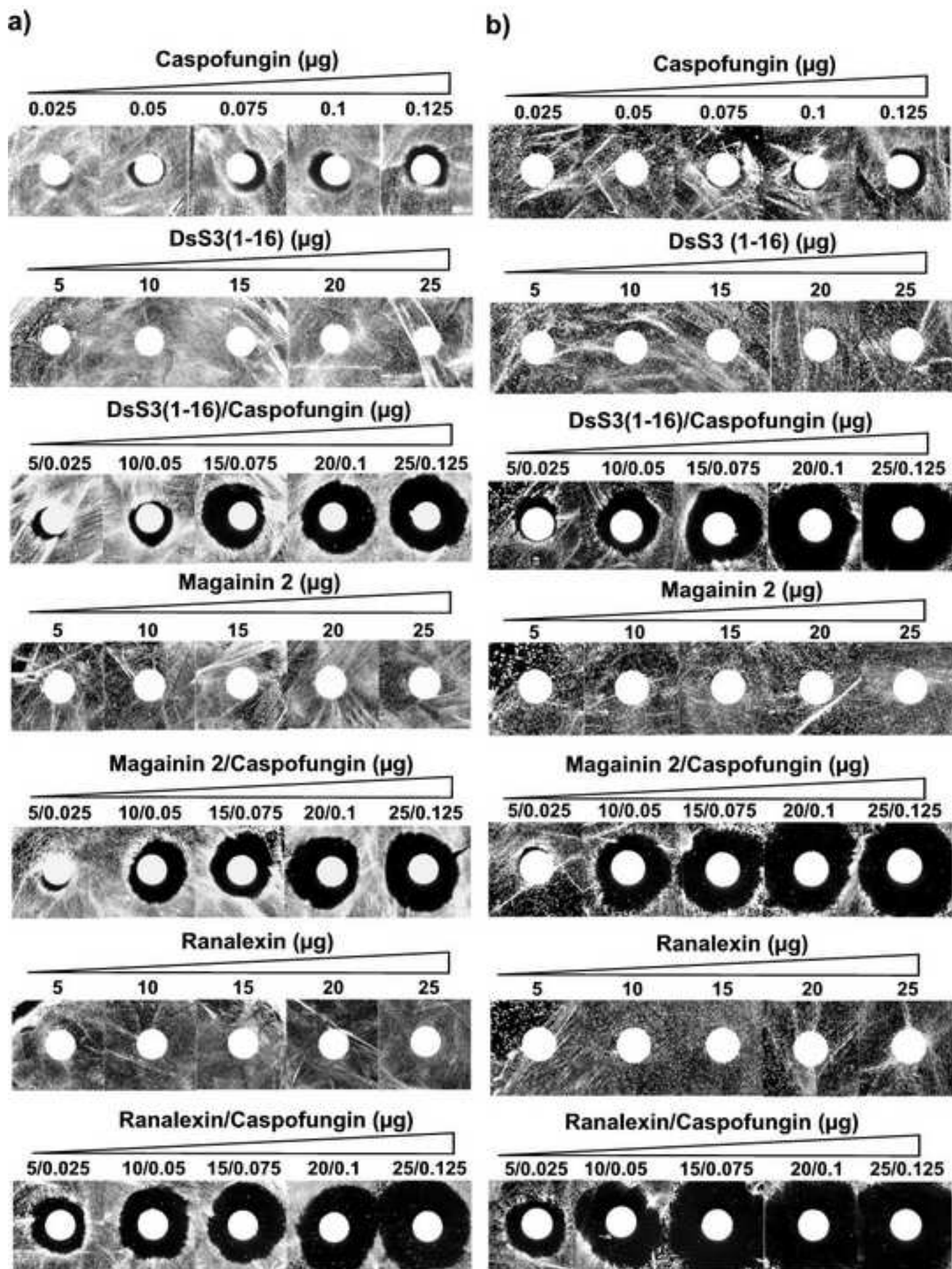












**Table 1.** – Biochemical properties of the antimicrobial peptides used in this study. In addition to the listed references, data was also obtained from the Antimicrobial Peptide Database (<http://aps.unmc.edu/AP/main.php>).

Peptide	Sequence <sup>a</sup>	Length	Structure	Net Charge	% Hydrophobic residues	100% haemolysis of RBCs	Reference
Dermaseptin S3(1-16)	ALWKNMLKGIGKLAGK	16	Helix	+4	50	>150 $\mu$ M	[18]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	23	Helix	+4	43	>150 $\mu$ g/ml	[31]
Ranalexin	FLGGLIKIVPAMICAVTKKC	20	Helix with heptapeptide loop from 1 disulphide bridge	+3	65	>500 $\mu$ g/ml	[3]
6752 (synthetic)	<u>S</u> <u>W</u> <u>F</u> <u>K</u> <u>T</u> <u>K</u> <u>S</u> <u>K</u>	8	Cyclic	+3	25	>400 $\mu$ g/ml	[6]
GS14K4	V <u>K</u> L <u>K</u> V <u>Y</u> PLKVKLY <u>P</u>	14	Cyclic	+4	42	200 $\mu$ g/ml	[14]
Gomesin	ECRRLCYKQRCVTYCRGR	18	$\beta$ -hairpin with 2 disulphide bridges	+6	33	Approx. 20% haemolysis at 100 $\mu$ M	[24]

<sup>a</sup>Amino acid residues underlined represent D amino acids.

**Table 2.** – Fractional inhibitory concentration (FIC) indices for combinations of caspofungin or anidulafungin with various antifungal peptides calculated from checkerboard assays of visible growth inhibition of three *Candida* strains. FICs were calculated according to Odds [20] where values <0.5 indicate synergy and 0.5 to 4 no interaction. FIC values highlighted in grey indicate combinations where no synergy was observed.

Peptide	Caspofungin			Anidulafungin		
	<i>C. albicans</i> SC5314	<i>C. albicans</i> (Hospital isolate)	<i>C. glabrata</i> (Hospital isolate)	<i>C. albicans</i> SC5314	<i>C. albicans</i> (Hospital isolate)	<i>C. glabrata</i> (Hospital isolate)
<b>DsS3(1-16)</b>	0.164	0.164	0.282	0.107	0.111	0.287
<b>Ranalexin</b>	0.221	0.164	0.186	0.106	0.111	0.443
<b>Magainin 2</b>	0.144	0.135	0.278	0.095	0.087	0.376
<b>6752</b>	0.150	0.289	0.347	0.773	0.563	0.737
<b>GS14K4</b>	0.267	0.139	0.269	0.142	0.287	0.341

**Table 3.** – The effect of combination treatment with caspofungin and ranalexin compared to the individual treatments alone on kidney burden of *C. albicans* SC5314 and animal weight change in a mouse model of disseminated candidiasis.

<b>Treatment</b>	<b>Kidney log<sub>10</sub> CFU/g Mean±SD</b>	<b>Weight change (%) day 0–day 3 Mean±SD</b>
saline IP/saline IV	4.1±0.6	0.3±3.5
caspofungin (0.01 mg/kg) IP/saline IV	3.4±0.4	1.4±2.5
saline IP/ranalexin IV (10 mg/kg)	3.8±0.6	-1.0±2.4
caspofungin IP/ranalexin IV	3.4±0.3*	-1.1±1.9
saline IV	4.0±0.3	-5.8±0.9
caspofungin (0.05 mg/kg) IV	2.5±0.3**	-2.2±1.4**
ranalexin (10 mg/kg) IV	4.5±0.7	-5.0±3.3
caspofungin+ranalexin IV	3.2±0.2**	-1.5±1.6**

\* $p < 0.05$  and \*\* $p < 0.01$  relative to placebo-treated group (Mann-Whitney  $U$  test).

**Combination of caspofungin or anidulafungin with antimicrobial peptides results in potent synergistic killing of *Candida albicans* and *Candida glabrata* in vitro**

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## ABSTRACT

Administering synergistic combinations of antifungals could be a route to overcome problems with toxicity and the development of resistance. Combination of the echinocandins caspofungin or anidulafungin with a range of structurally diverse antimicrobial peptides resulted in potent synergistic killing of *Candida* spp. in vitro. Fungicidal synergy was measured by calculating fractional inhibitory concentration indices from checkerboard assays as well as loss of viability. Inhibitory combinations of the antifungals did not induce cytotoxicity in vitro. However, in a murine model of systemic candidiasis, co-administration of caspofungin with one example of the cationic peptides tested, ranalexin, did not show enhanced efficacy compared with the single treatments alone. Further study using alternative peptides will identify whether this combination approach could represent a novel treatment for fungal pathogens.

## 1. Introduction

Over the past 30 years, fungi have emerged as significant causes of human disease, with attendant morbidity and mortality [1,2]. A large proportion of these infections occur in immunocompromised patients, as fungal pathogens are readily able to overwhelm the already weakened host defence mechanisms. Hospitalised patients with serious underlying diseases are increasingly exposed to invasive surgical procedures or chemotherapeutic treatments, such as broad-spectrum antibiotics or immunosuppressants following organ transplantation and cancer chemotherapy, that readily promote mycotic disease. Infections caused by *Candida albicans* have a population incidence of between 1.1 and 24 cases per 100 000 individuals with an associated mortality rate of >30% [3,4]. In the USA this equates to between 3000 and 11 000 deaths annually from nosocomial candidaemia [5]. Notably, treatment with many antifungal drugs is complicated by high toxicity and low tolerability, ineffectiveness against new or re-emerging fungi, or the development of drug-resistant strains in patients undergoing treatment [5]. Thus, there is a justifiable need for more research to develop new antifungal treatments.

One possible approach to improve the range and scope of antifungal therapy is the use of combinations of antifungals. Benefits could include: improved efficacy over the use of single drugs, consequently allowing lower, less costly doses of each individual drug; reduced toxicity and improved tolerance; broader spectrum of efficacy; and finally, the potential to prevent resistant organisms arising as can occur with monotherapy.

A potential source of new antifungals is the cationic antimicrobial peptides (AMPs), which are ubiquitous throughout nature and protect against invasive microbial pathogens. To date, more than 800 AMPs have been described [6]. Potential advantages of cationic

peptides for future therapy include: a broad spectrum of activity; rapid killing of microbes; they are unaffected by classical antibiotic resistance mechanisms; they can neutralise endotoxin; and they are active in animal models [6]. Disadvantages include: sensitivity to proteolytic degradation by serum proteases in vivo; potential toxicity at high doses necessary to show efficacy in vivo, including haemolytic activity; and the high cost of synthesising and producing large quantities of these molecules [7]. Employing cationic peptides in combination with other clinically approved drugs could overcome some of the problems associated with the clinical application of peptides.

Echinocandins inhibit the synthesis of  $\beta$ -D-glucan in fungal cell walls and are fungicidal against *Candida* spp. and fungistatic against *Aspergillus* spp. owing to loss of cell wall integrity [8]. Importantly, an intact cell wall also represents a potential barrier that cationic peptides must interact with and pass through before they can interact with the plasma membrane beneath. Supporting this, recent data from our laboratory have demonstrated that disruption of the outer cell wall phosphomannan content has a significant influence on the degree of susceptibility of *C. albicans* to a cationic peptide [9]. Thus, owing to the complementary inhibitory action of the echinocandins and cationic peptides, we reasoned that application of combinations of these molecules could result in additive or synergistic inhibition of fungal pathogens. In this study, we combined the three clinically approved echinocandins with a range of structurally diverse AMPs and observed potent synergistic killing of *Candida* spp. in vitro.

## 2. Material and methods

### 2.1. Yeast strains and growth media

Yeast strains used in this work were *C. albicans* SC5314 and clinical isolates of *C. albicans* and *Candida glabrata* provided by Dr Cyril Lafong (Fife Area Laboratory, Victoria Hospital, Kirkcaldy, UK). All strains were cultured in malt extract broth (MEB) (pH 7) (1% glucose, 0.6% malt extract and 0.12% yeast extract) at 30 °C with shaking. To enumerate cell survival after exposure to antifungals, yeasts were plated on YEPD agar (2% glucose, 2% agar, 1% Bacto Peptone and 1% yeast extract). Numbers of cells used in the various assays described below were calculated using an optical density at 600 nm (OD<sub>600</sub>) versus a cell number calibration curve for each yeast strain used (data not shown).

### 2.2. Antifungals

Magainin 2 [10], ranalexin [11], cyclic peptide 6752 [12], cyclic peptide GS14K4 [13], gomesin [14] and dermaseptin S3(1-16) [DsS3(1-16)] [15] were synthesised according to their published sequences by Peptide Research Ltd. (Wickham, UK) to >95% purity and were verified by high-performance liquid chromatography (HPLC) and mass spectrometry. Biochemical properties of the peptides employed in this study are listed in Table 1. Peptides were solubilised in d.H<sub>2</sub>O at stock concentrations of 50 mg/mL. Caspofungin (Merck, Whitehouse Station, NJ) and micafungin (Astellas, Staines, UK) were diluted into 1 mg/mL and 10 mg/mL aliquots, respectively, with sterile d.H<sub>2</sub>O. Anidulafungin (Pfizer, New York, NY) was diluted into 10 mg/mL aliquots with 20% v/v ethanol.

### 2.3. Checkerboard assay of yeast growth inhibition

For the checkerboard assay, 96-well microplate wells (Greiner Bio-One Ltd., Stonehouse, UK) were filled with 150  $\mu$ L of RPMI 1640 (Sigma, Irvine, UK) with 2% 3-(*N*-morpholino) propane sulfonic acid (MOPS) (Sigma) (pH 6.4). Appropriate concentrations of individual antifungals or combinations of peptides with the echinocandins were added. Approximately 500 cells from mid exponential phase cultures of each yeast strain were then added to each well. Plates were incubated at 30 °C for 48 h and were scanned using an ImageScanner (GE Healthcare UK Ltd., Chalfont St Giles, UK) with ImageMaster Labscan v3.00 software (GE Healthcare UK Ltd.). Images were annotated using Microsoft PhotoDraw. The minimum inhibitory concentration for each antifungal tested was defined as the concentration present in the first optically clear well after 48 h.

### 2.4. Disk diffusion assay of yeast growth inhibition

Sterile paper disks (6 mm; Whatman International Ltd. Maidstone, UK) were impregnated with the relevant peptide (5–25  $\mu$ g) with or without echinocandin. Disks were left in sterile Petri dishes overnight at room temperature to dry. Then, 100  $\mu$ L of mid exponential phase cultures ( $OD_{600} = 0.3$ ) were spread onto RPMI 1640 agarose plates [2% agarose, 1% RPMI 1640 medium Auto-Mod™ (Sigma), 0.4% sodium bicarbonate (Sigma), 2% MOPS (Sigma)] and left to dry for 1 h at room temperature. Disks containing the various antifungal combinations were then applied to the surface of the plates and incubated at 30 °C for 48 h. Images showing the zones of inhibition were obtained by scanning the plates as described in Section 2.3.

### 2.5. Assay of yeast cell viability

Starter cultures were incubated overnight in MEB (pH 7) at 30 °C with shaking. Then, 1 mL of this culture was diluted with fresh MEB (pH 7) to give a starting OD<sub>600</sub> of 0.05 prior to incubation at 30 °C with shaking at 200 rpm. OD<sub>600</sub> readings were taken every 60 min until an OD<sub>600</sub> of 0.3 was reached. Cultures were then exposed to peptide with or without caspofungin. Culture viability was measured every 30 min by serial dilution and plating onto YEPD agar plates. Plates were incubated at 30 °C for 48 h prior to enumeration of survivors.

### 2.6. In vitro mammalian cell cytotoxicity assay

The method for the in vitro mammalian cell cytotoxicity assay is as described by Yang et al. [16] and employed non-cancerous Vero cells (African green monkey kidney fibroblasts). Vero cells were exposed to echinocandins, AMPs or combinations of both for 48 h and viability was determined by the neutral red procedure. Viable cells were expressed as the proportion retaining neutral red after exposure to antifungals relative to untreated controls. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) 0.15 µg/mL (Sigma) was used as a positive control.

### 2.7. Intracellular localisation of fluorescein-tagged dermaseptin S3(1-16) using fluorescence microscopy

Phase contrast and fluorescent peptide images were generated using the fluorescein isothiocyanate filter (Ex  $\lambda$  = 490/20 nm, Em  $\lambda$  = 528/38 nm). Propidium iodide (PI) (Invitrogen, Paisley, UK) was used to identify dead cells (RD-TR-PE, Ex  $\lambda$  = 490/20 nm, Em  $\lambda$  = 528/38 nm). Cells were cultured in MEB and were harvested at mid exponential

phase ( $OD_{600} = 0.6$ ). A volume equivalent to  $2.0 \times 10^6$  cells/mL was removed for each assay, centrifuged for 2 min at 10 000 rpm, washed in ddH<sub>2</sub>O and re-suspended in 1 mL of RPMI 1640. Echinocandin and/or DsS3(1-16)-fluorescein were added with PI (1.8  $\mu$ M) and incubated for 2 h at 30 °C in the dark. Unbound dye and peptide were removed by harvesting for 8 min at  $3000 \times g$ , washing the pellet in ddH<sub>2</sub>O, harvesting and re-suspension in 20  $\mu$ L of RPMI 1640. Then, 2  $\mu$ L of the stained cells were fixed with 2  $\mu$ L of 1% low-melting-point agarose (BioGene, Huntingdon, UK). Samples were placed on ice and protected from light until analysis. Images were captured on an Olympus IX70 DeltaVision microscope (Applied Precision LLC, Washington, DC). SoftWoRx<sup>®</sup> Explorer 1.3 (Applied Precision LLC) was used for image processing and analysis.

### *2.8. In vivo efficacy of the combination of caspofungin with ranalexin in a murine model of disseminated candidiasis*

Basic details of the intravenous (i.v.) challenge model for disseminated *C. albicans* infection have been published previously [17]. Briefly, female BALB/c mice were infected intravenously with *C. albicans* at a dose of  $3 \pm 1 \times 10^4$  colony-forming units/g body weight. Animals were humanely terminated 72 h after challenge and body weight changes, which correlate with survival times [18], as well as fungal burdens in kidney homogenates were determined.

Caspofungin i.v. preparation was diluted in saline as required. Combination therapy with caspofungin and ranalexin was investigated in two experiments. In the first experiment, caspofungin was dosed at 0.01 mg/kg via the intraperitoneal (i.p.) route once daily and ranalexin was dosed at 10 mg/kg i.v. once daily. Saline i.v. and i.p. was used as placebo, and animals given caspofungin or ranalexin monotherapy were concomitantly dosed with

saline by the i.v. and i.p. routes, respectively. The first treatments were given 1 h after challenge and treatments were repeated at 24 h and 48 h. In the second experiment, caspofungin was dosed at 0.05 mg/kg i.v. once daily and ranalexin was dosed at 10 mg/kg i.v. twice daily. The first treatments were given 1 h after challenge. Saline i.v. was used as placebo.

Statistical analysis of the data was performed by the Mann–Whitney *U*-test.

### 3. Results

#### 3.1. Combination of caspofungin or anidulafungin with antimicrobial peptides results in potent synergistic inhibition of *Candida albicans* and *Candida glabrata* in vitro

Exposing *C. albicans* SC5314 to a combination of peptides with caspofungin (Fig. 1) or anidulafungin (data not shown) resulted in significant enhancement of the inhibitory activity of the peptides compared with peptide treatment alone. Similar data were also obtained from identical checkerboard assays using the hospital isolates of *C. albicans* and *C. glabrata* (data not shown). Fractional inhibitory concentration indices (FICIs) calculated from the checkerboard assays (Table 2) showed that combination of all five peptides with caspofungin resulted in clear synergy (<0.5), particularly versus the *C. albicans* strains. Synergy against *C. glabrata* was evident to a lesser extent with all the peptides tested with the exception of ranalexin. The FICI values generated from combining anidulafungin with the same peptides revealed a similar pattern of synergy as occurred with caspofungin, with the exception of cyclic peptide 6752 that displayed no synergy. As with caspofungin, the synergistic effect induced by combining the peptides with anidulafungin was most evident against the *C. albicans* strains.



One peptide, gomesin, showed no enhancement of inhibition of any of the strains tested in combination with any of the echinocandins (data not shown). Also, no enhancement of the inhibitory effect of all the peptides tested was observed when they were combined with micafungin (data not shown).

To determine whether the synergistic inhibition of the combinations enhanced the fungicidal effect of the peptides or the echinocandins, the effect of the combination of caspofungin with ranalexin, magainin 2 and DsS3(1-16) on the viability of growing cells of *C. albicans* SC5314 was tested (Fig. 2). Addition of 9 mg/L ranalexin (Fig. 2a), 10 mg/L magainin 2 (Fig. 2b) or 4 mg/L DsS3(1-16) (Fig. 2c) alone to exponentially growing cells had little effect on viability compared with untreated controls. In contrast, exposure to caspofungin alone at 0.01 mg/L (Fig. 2a–c) resulted in minor inhibition of the growth rate. Notably, with cultures exposed to the individual peptides (at identical concentrations as described above) but in the presence of 0.01 mg/L caspofungin, in each case there was complete cessation of growth and notable loss of viability. Thus, the combination of AMPs with either caspofungin or anidulafungin resulted in potent synergistic fungicidal activity.

### *3.2. In vitro toxicity testing of antimicrobials peptides alone and in combination with caspofungin*

In preparation for in vivo testing of the combination of AMPs with caspofungin in a mouse model of systemic candidiasis, the in vitro toxicity of the antifungals on non-cancerous Vero cells was evaluated (Fig. 3). Exposure to caspofungin or anidulafungin resulted in a minor loss of cell viability at a concentration an order of magnitude higher than necessary to completely inhibit growth of *C. albicans* in vitro (Fig. 3a). The AMPs had no measurable toxic effect on Vero cells up to a concentration of 50 mg/L. However, exposure to 100 mg/L of the cyclic peptide GS14K4 and ranalexin resulted in ca. 70% and 20% loss of

viability, respectively (Fig. 3b). Peptides 6752, DsS3(1-16) and magainin 2 induced no significant loss of cell viability even at the highest concentration tested (100 mg/L). Following this, the effect of combinations of either caspofungin (Fig. 3c) or anidulafungin (Fig. 3d) with each of the peptides was tested. Combination of 10 mg/L caspofungin with 30 mg/L of any of the AMPs had no detectable effect on Vero cell viability. However, 15 mg/L caspofungin in combination with 50 mg/L ranalexin showed some cytotoxicity, and 100 mg/L caspofungin with 100 mg/L GS14K4, ranalexin and DsS3(1-16) induced significant loss of viability. Identical experiments testing the cytotoxicity of anidulafungin also showed that combination of 10 mg/L anidulafungin with 30 mg/L of any of the AMPs had only a minor effect on Vero cell viability. At higher concentrations of anidulafungin and peptides, toxicity increased significantly, demonstrating that peptide combinations with anidulafungin were potentially more cytotoxic than those with caspofungin. None the less, the study revealed that concentrations of antifungals in combination that are much higher than concentrations necessary to completely inhibit growth of *C. albicans* in vitro (Fig. 1) did not induce significant cytotoxicity in vitro. Thus, these results indicated that there may be a therapeutic 'window' where the combination treatment may show efficacy versus *C. albicans* without inducing toxicity.

### 3.3. Exposure to an antimicrobial peptide in combination with caspofungin results in increased intracellular accumulation of the peptide and enhanced killing of *Candida albicans*

Previous work with DsS3(1-16) tagged with fluorescein revealed that the peptide binds transiently to the outer cell wall prior to being taken up by the cell and sequestered into the vacuole before finally disseminating throughout the cytosol [9]. Thus, fluorescence microscopy was used to study how exposure to caspofungin affected the uptake of DsS3(1-16)–fluorescein. In addition to exposing cells to DsS3(1-16)–fluorescein, cells

were simultaneously stained with PI to correlate stages of peptide uptake with loss of viability (Fig. 4). Exposure to 20 mg/L DsS3(1-16)–fluorescein for 2 h resulted in ca. 5% of the population displaying fluorescence throughout the entire cell, 20% with fluorescence localised to the vacuole and 75% of the population showing no peptide uptake (Fig. 4a). As reported previously [9], when the peptide was disseminated throughout the entire cell this correlated with PI uptake into the cells, indicating that these cells were non-viable (data not shown). In contrast, cells with the peptide concentrated inside the vacuole were not PI-positive and were viable (Fig. 4b).

Exposure to 0.001 mg/L caspofungin for 2 h resulted in ca. 15% of the population becoming PI-positive. However, if the cells were exposed to caspofungin in the presence of DsS3(1-16)–fluorescein, the proportion of cells that were PI-positive increased to ca. 35% (Fig. 4a). As before, all these PI-positive cells also displayed green fluorescence throughout the entire cell indicating complete peptide entry into the cells.

Exposure to 0.01 mg/L or 0.05 mg/L caspofungin alone resulted in ca. 24% and 26%, respectively, of the population becoming PI-positive after 2 h (Fig. 4a). Paradoxically, at higher concentrations of caspofungin (0.1 mg/L and 1 mg/L), the proportion of cells that were PI-positive decreased to ca. 19% in each case. This phenomenon was seen in many repeat experiments both with caspofungin and anidulafungin (data not shown).

Upon addition of DsS3(1-16)–fluorescein to increasing concentrations of caspofungin (0.01–1 mg/L), a similar trend to that observed previously was observed, with an increase in the proportion of cells in the population becoming PI-positive and simultaneously displaying total green fluorescence throughout the cell. Coincident with the increase in the proportion of cells displaying total dissemination of DsS3(1-16)–fluorescein throughout the

entire cell, at every concentration of caspofungin tested addition of DsS3(1-16)–fluorescein resulted in a smaller proportion of the population displaying only vacuolar localisation of the peptide. Thus, the presence of caspofungin results in greater, or accelerated, uptake of the peptide into the cytosol, which in turn leads to enhanced loss of cell viability.

In identical experiments using anidulafungin instead of caspofungin, similar trends with regard to peptide uptake were observed (data not shown).

#### *3.4. Combination of caspofungin with ranalexin did not show synergistic inhibition of *Candida albicans* in a murine model of disseminated candidiasis*

Results of the mouse experiments are summarised in Table 3. In the first experiment with caspofungin given i.p. at 0.01 mg/kg, the 72 h kidney burden was reduced significantly below placebo ( $P < 0.05$ ) in mice given the caspofungin/ranalexin combination; however, the extent of the effect was  $<1$  log burden reduction. No significant reduction in weight loss relative to placebo-treated mice was seen for any of the test agents. In the second experiment with a higher caspofungin dose of 0.05 mg/kg, i.v. dosing of both test agents and twice daily dosing of ranalexin, highly significant ( $P < 0.01$ ) reductions in kidney burdens and 3-day weight loss were seen for groups of animals receiving treatments that included caspofungin. However, progressive necrosis of the tails of animals given ranalexin alone or in combination meant that treatments with this agent were stopped after 36 h in the interests of animal welfare.

### 3.5. Combination of antimicrobial peptides with caspofungin results in potent synergistic killing of *Candida albicans* on a porous solid surface

To explore whether the combination treatment displayed synergy in a topical application, lawns of *C. albicans* SC5314 (Fig. 5a) and the *C. albicans* hospital isolate (Fig. 5b) covering the surface of RPMI 1640 agarose plates were exposed to sterile filter paper disks impregnated with caspofungin, ranalexin, magainin 2 or DsS3(1-16) alone as well as combinations of caspofungin with each peptide. For both strains, caspofungin-impregnated disks showed only minor zones of inhibition at the higher concentrations tested of 0.075, 0.1 and 0.124  $\mu\text{g}$ . Exposure of both strains to disks impregnated with each peptide alone up to the highest concentration of 25  $\mu\text{g}$  resulted in no detectable zones of inhibition. However, exposure of both strains to disks impregnated with combinations of each peptide with caspofungin clearly revealed synergistic inhibition, with larger, more significant zones of inhibition after 48 h exposure. Similar results were observed for both yeast strains exposed to combinations of the same peptides with anidulafungin (data not shown).

## 4. Discussion

Combination therapy using clinically approved antifungals is commonly employed to treat mycotic disease (reviewed by Mukherjee et al. [20]), e.g. combinations of i.v. amphotericin B or oral fluconazole with 5-fluorocytosine have been proposed for the treatment of candidiasis [21]. Synergy between caspofungin and amphotericin B against *Aspergillus* spp. has been described in vitro [22] and in a murine model [23] and resulted in improved therapeutic outcome in a murine model of systemic *C. glabrata* infection [24]. However, controversy surrounds whether or not combination therapy is superior to monotherapy in clinical situations owing to a lack of evidence from clinical trials [25].

There are examples in the literature of apparent synergy or enhanced efficacy between various AMPs and clinically approved antifungals, e.g. the combination of peptide 2, a derivative of lactoferrin, with amphotericin B resulted in enhanced survival of mice in a model of systemic candidiasis [26]. Notably, there are no published reports of synergistic interactions between AMPs and the clinically approved echinocandins.

Despite the introduction of new antifungals such as voriconazole and caspofungin, in some cases antifungal efficacies remain suboptimal [27] and there is increasing evidence of antifungal therapy failing due to the selection in vivo of resistant fungal pathogens, e.g. reduced susceptibility to caspofungin in patients receiving therapy for invasive candidiasis [28]. Thus, the use of combinations of antifungals in therapy could be an effective alternative approach to treat invasive fungal infections that are not responsive to standard monotherapy.

Here we describe how combination of the echinocandins caspofungin and anidulafungin with AMPs results in significant synergy against *Candida* spp. in vitro. Notably, no apparent synergy was detected using identical combinations of peptides with the other clinically approved echinocandin, micafungin. This is surprising considering the similarity in structure and apparent inhibitory action between all of these drugs and merits further investigation. The FICI values calculated from the checkerboard experiments revealed that all the AMPs tested (with the exception of the cyclic peptide 6752 in combination with anidulafungin) displayed some degree of synergy against the three yeasts tested in combination with either caspofungin or anidulafungin (Table 2). With each combination tested, the level of synergy against the hospital isolate of *C. glabrata* was reduced compared with that shown against the two *C. albicans* strains. This is not surprising

considering that *C. glabrata* has been shown to show greater resistance than *C. albicans* to many antifungal drugs, particularly the triazoles [5].

Study of the nature of the inhibitory mechanism of the synergistic combination using fluorescence microscopy revealed that exposure to the cell wall-degrading effect of caspofungin led to enhanced uptake of the cationic peptide into the cell, resulting in enhanced killing. Intriguingly, there was evidence of paradoxical resistance to increasing concentrations of caspofungin, as the degree of peptide uptake into the cells initially increased with higher concentrations but then declined again as the concentration was raised further. Evidence of a paradoxical resistance phenotype to echinocandins has been presented previously for *Candida* spp., particularly with those isolates exposed to caspofungin [29]. Possible explanations for this paradoxical effect include the induction of stress response pathways [30] or elevation in the chitin content of the cell walls of *C. albicans* cells exposed to caspofungin, reducing penetration of the peptide into the cells [31].

Ideally, whether or not a combination treatment could prove efficacious in vivo would be assessed using animal models followed by clinical trials. Thus, a murine model of disseminated candidiasis was employed to determine whether the combination treatment demonstrated enhanced efficacy in vivo. Caspofungin and ranalexin were chosen owing to previous experience of in vivo dosing with these two compounds. Disappointingly, the combination treatment did not result in enhanced efficacy compared with the single treatments. However, resources only permitted a preliminary experiment with only one dose of each of the compounds tested and it was only possible to test the in vivo efficacy of one echinocandin, caspofungin, with one peptide, ranalexin. As the experiment progressed there was evidence of ranalexin toxicity, which was manifest as tail vein

necrosis and ulceration at the site(s) of administration. Clearly, the pharmacokinetics and interactions of antifungal peptides, about which little is known, are difficult to optimise in an animal model. Furthermore, it is even more difficult to replicate conditions present in humans. None the less, there is additional scope to use the mouse model to study the effects of alternative non-toxic peptides in combination with other echinocandins, with varied dosing regimens and alternative routes of administration, against additional fungal pathogens such as *Aspergillus* spp. Thus, despite this preliminary failure, we believe that a combination of an echinocandin with an AMP can be identified that could still result in enhanced efficacy in humans. Importantly, we have demonstrated synergistic inhibition of yeast growing on the surface of an agarose plate, and if i.v. administration of the combination treatment is not efficacious then topical administration to treat conditions such as oral or mucosal candidiasis could prove to be more effective.

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### **Competing interests**

None declared.



**Ethical approval**

All animal experimentation was approved by the local ethical review committee and was performed under UK Home Office regulations.

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**Fig. 1.** Checkerboard growth assays of minimum inhibitory concentrations of each antimicrobial peptide (AMP) alone and for each of the AMPs in combination with caspofungin. Assays were performed on *Candida albicans* SC5314 in RPMI 1640 with 2% MOPS (pH 6.4) and incubated at 30 °C for 48 h. Black lines indicate the first well where no growth was observed. Representative results from at least triplicate experiments are shown. DsS3(1-16), dermaseptin S3(1-16).

**Fig. 2.** Effect of the combination of antimicrobial peptides (AMPs) with caspofungin (0.01 mg/L) on the growth and viability of *Candida albicans* SC5314: (a) caspofungin plus 9 mg/L ranalexin; (b) caspofungin plus 10 mg/L magainin 2; and (c) caspofungin plus 4 mg/L dermaseptin S3(1-16). Cultures were grown in malt extract broth (MEB) (pH 7) at 30 °C with shaking. Viability [ $\log_{10}$  colony-forming units CFU/mL] is shown as open symbols and growth [culture optical density at 600 nm] as filled symbols. Representative results from at least triplicate experiments are shown.

**Fig. 3.** In vitro toxicity of the echinocandins, the antimicrobial peptides (AMPs) and combinations of AMPs and echinocandins on mammalian cells. Effect on the viability of Vero cells in RPMI 1640 media of 48 h exposure to increasing concentrations of (a) caspofungin ( $\square$ ) or anidulafungin ( $\circ$ ), (b) the AMPs alone and (c,d) combinations of caspofungin (c) and anidulafungin (d) with the AMPs. Cell viability was expressed as the proportion of cells within the population that retained neutral red after exposure to the antifungals relative to untreated controls. Released dye was measured spectrophotometrically at 540 nm. DsS3(1-16), dermaseptin S3(1-16); Rana, ranalexin; Mag, magainin 2.

**Fig. 4.** Microscopic study of the effect of the presence of caspofungin on uptake of DsS3(1-16)–fluorescein into *Candida albicans* SC5314. (a) Yeast cells in RPMI 1640 supplemented with 1.8  $\mu$ M propidium iodide (PI) were exposed to the following conditions for 2 h at 30 °C: DsS3(1-16)–fluorescein (20 mg/L) alone; increasing concentrations of caspofungin (0.001–1 mg/L); or combinations of DsS3(1-16)–fluorescein (20 mg/L) with increasing concentrations of caspofungin (0.001–1 mg/L). After harvesting and washing, the proportions of cells within the population that showed no fluorescence, were PI-positive and showed either vacuolar green fluorescence or whole-cell green fluorescence were calculated. For each tested condition, the fluorescent state of >300 cells was ascertained from multiple captured images. A representative result of a duplicate experiment is shown. (b) A representative image of stained cells after exposure to a combination of 0.05 mg/L caspofungin and 20 mg/L DsS3(1-16)–fluorescein. Cells staining PI-positive (non-viable) co-stain with cells that show green fluorescence throughout the entire cytosol [full uptake of DsS3(1-16)–fluorescein]. Cells showing no fluorescence at all and cells that show only green fluorescence concentrated in the vacuole do not co-stain with PI and are thus viable. DsS3(1-16), dermaseptin S3(1-16).

**Fig. 5.** Effect of antimicrobial peptides (AMPs) and/or caspofungin impregnated into filter paper disks on the surface growth of *Candida albicans* on malt extract broth/agarose. Shown is the effect of increasing caspofungin alone; increasing dermaseptin S3(1-16) [DsS3(1-16)], magainin 2 and ranalexin alone; and combination of caspofungin with each AMP. The effect of these treatments on surface growth of (a) *C. albicans* SC5314 and (b) a hospital isolate of *C. albicans* after 48 h at 30 °C is shown. Zones of clearing in the yeast lawn are evident as black zones around the filter paper disks. Representative results from at least triplicate experiments are shown.

**Table 1**

Biochemical properties of the antimicrobial peptides used in this study

Peptide	Sequence <sup>a</sup>	Length (amino acids)	Structure	Net charge	% Hydrophobic residues	100% haemolysis of RBC	Reference <sup>b</sup>
Dermaseptin S3(1-16)	ALWKNMLKGIGKLAGK	16	Helix	+4	50	>150 $\mu$ M	[15]
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	23	Helix	+4	43	>150 $\mu$ g/mL	[10]
Ranalexin	FLGGLIKIVPAMICAVTKKC	20	Helix with heptapeptide loop from one disulphide bridge	+3	65	>500 $\mu$ g/mL	[11]
6752 (synthetic)	<u>S</u> <u>W</u> <u>F</u> <u>K</u> <u>T</u> <u>K</u> <u>S</u> <u>K</u>	8	Cyclic	+3	25	>400 $\mu$ g/mL	[12]
GS14K4	V <u>K</u> L <u>K</u> V <u>Y</u> PLKVKL <u>Y</u> P	14	Cyclic	+4	42	200 $\mu$ g/mL	[13]
Gomesin	ECRRLCYKQRCVTYCRGR	18	$\beta$ -Hairpin with two disulphide bridges	+6	33	ca. 20% haemolysis at 100 $\mu$ M	[14]

RBC, red blood cells.

<sup>a</sup> Amino acid residues underlined represent D amino acids.



<sup>b</sup> In addition to the listed references, data were also obtained from the Antimicrobial Peptide Database (<http://aps.unmc.edu/AP/main.php>).

**Table 2**

Fractional inhibitory concentration indices (FICIs) for combinations of caspofungin or anidulafungin with various antifungal peptides calculated from chequerboard assays of visible growth inhibition of three *Candida* strains <sup>a</sup>

Peptide	Caspofungin			Anidulafungin		
	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. albicans</i>	<i>C. albicans</i>	<i>C. glabrata</i>
	SC5314	hospital isolate	hospital isolate	SC5314	hospital isolate	hospital isolate
Dermaseptin	0.164	0.164	0.282	0.107	0.111	0.287
S3(1-16)						
Ranalexin	0.221	0.164	0.186	0.106	0.111	0.443
Magainin 2	0.144	0.135	0.278	0.095	0.087	0.376
6752	0.150	0.289	0.347	<b>0.773</b> <sup>b</sup>	<b>0.563</b> <sup>b</sup>	<b>0.737</b> <sup>b</sup>
GS14K4	0.267	0.139	0.269	0.142	0.287	0.341

<sup>a</sup> FICIs were calculated according to Odds [19], where values <0.5 indicate synergy and values 0.5–4 indicate no interaction.

<sup>b</sup> FICI values in bold indicate combinations where no synergy was observed.

**Table 3**

Effect of combination treatment with caspofungin and ranalexin compared with individual treatments alone on kidney burden of *Candida albicans* SC5314 and animal weight change in a mouse model of disseminated candidiasis

Treatment	Kidney log <sub>10</sub> CFU/g (mean ± S.D.)	Weight change (%) Day 0– Day 3 (mean ± S.D.)
Experiment 1		
Saline i.p./saline i.v.	4.1 ± 0.6	0.3 ± 3.5
Caspofungin (0.01 mg/kg) i.p./saline i.v.	3.4 ± 0.4	1.4 ± 2.5
Saline i.p./ranalexin (10 mg/kg) i.v.	3.8 ± 0.6	−1.0 ± 2.4
Caspofungin i.p./ranalexin i.v.	3.4 ± 0.3 *	−1.1 ± 1.9
Experiment 2		
Saline i.v.	4.0 ± 0.3	−5.8 ± 0.9
Caspofungin (0.05 mg/kg) i.v.	2.5 ± 0.3 **	−2.2 ± 1.4 **
Ranalexin (10 mg/kg) i.v.	4.5 ± 0.7	−5.0 ± 3.3
Caspofungin + ranalexin i.v.	3.2 ± 0.2 **	−1.5 ± 1.6 **

CFU, colony-forming units; S.D., standard deviation; i.p., intraperitoneal; i.v.

intravenous.

\*  $P < 0.05$  and \*\*  $P < 0.01$  relative to placebo-treated group (Mann–Whitney *U*-test).

*C. albicans* SC5314 RPMI 1640

