Rapamycin and caspofungin show synergistic antifungal effects in caspofungin-susceptible and caspofungin-resistant *Candida* strains *in vitro*

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Running title : *In vitro* synergy of rapamycin and caspofungin in *Candida*

Abstract

Objectives: Caspofungin is an echinocandin antifungal agent that inhibits synthesis of glucan required for the fungal cell wall. Resistance is mediated by mutation of Fks1 glucan synthase, among which S645P is the most common resistance-associated polymorphism. Rapamycin is a macrolide that inhibits the mechanistic target of rapamycin (mTOR) protein kinase activity. This study investigated the interaction between rapamycin and caspofungin in inhibiting the growth of wild type *Candida albicans* and Fks1 S645P mutant clinical isolate and wild type *Candida lusitaniae* and genetically engineered isogenic strain with Fks1 S645P mutation at equivalent position.

Methods: Interactions between caspofungin and rapamycin were evaluated using the microdilution checkerboard method in liquid medium. The results were analysed using the Loewe additivity model (fractional inhibitory concentration index, FICI) and the Bliss independence model (response surface, RS, analysis).

Results: Synergy between rapamycin and caspofungin was shown for *C. albicans* and *C. lusitaniae* strains by RS analysis of the checkerboard tests. Synergy was observed in strains susceptible and resistant to caspofungin. Weak subinhibitory concentrations of rapamycin were sufficient to restore caspofungin susceptibility.

Conclusions: We report here for the first time synergy between caspofungin and rapamycin in *Candida* species. Synergy was shown for strains susceptible and resistant to caspofungin. This study highlights the possible implication of the TOR pathway in sensing antifungal-mediated cell wall stress and in modulating the cellular response to echinocandins in *Candida* yeasts.

Introduction

Invasive fungal infections are a major cause of global morbidity and mortality, accounting for nearly 1.4 million deaths every year.[1] Echinocandins have become the first-line therapy for invasive candidiasis in most patients because of their excellent safety profile and good *in vitro* fungicidal activity against *Candida* species, including azole-resistant clinical isolates.[2] They act as noncompetitive inhibitors of the fungal β(1,3)-glucan synthase, and thus disturb fungal cell wall synthesis.[3] In *Candida albicans*, the main mechanism of echinocandin resistance involves nonsynonymous mutations in two hotspot regions of the *FKS1* gene, HS1 and HS2, which decrease the binding affinity of glucan synthase to echinocandins.[4] Substitutions involving Ser-645 (S645P/F/Y) are the most common polymorphisms and are responsible for the most pronounced MIC elevations.[5,6]

Tolerance and resistance to echinocandins have also been shown to involve regulatory pathways dependent on the Hsp90 chaperone and its client protein, calcineurin, in several fungal species.[7–10] A previous study on *Candida lusitaniae* reported that the calcineurin inhibitor tacrolimus increased susceptibility to caspofungin of strains that were susceptible and resistant to echinocandins, notably in a strain harbouring an Fks1 mutation at the equivalent S645 position.[11] The cellular receptor of tacrolimus is Fpr1p, a peptidyl-prolyl isomerase. Fpr1p also binds rapamycin,[12] a natural product of *Streptomyces hygroscopicus*, which was first described as an antifungal agent capable of inhibiting *C. albicans* growth [13] but was later shown to have strong immunosuppressive activity.[14] The cellular target of rapamycin is target of rapamycin complex 1 (TORC1), which is involved in the regulation of many cellular processes, including protein translation, autophagy, and stress responses.[12,15,16]

To the best of our knowledge, few studies have been conducted on the impact of rapamycin activity on the susceptibility and resistance of *Candida* species to other antifungal agents.[17] To gain insights into the possible interaction between the Fpr1–

rapamycin complex and echinocandins, we evaluated the *in vitro* interaction between rapamycin and caspofungin against *C. albicans* wild type strain and Fks1 S645P mutant clinical isolate, and *C. lusitaniae* wild type strain and a genetically engineered isogenic strain with Fks1 S645P mutation at the equivalent position.

Materials and methods

Candida strains

The different strains used in this study are listed in Table 1. *C. albicans* ATCC 90029 and *C. lusitaniae* CBS 6936 (ATCC 38553) were used as susceptible reference strains. The echinocandin-resistant *C. albicans* strain was a clinical isolate obtained from the Laboratory of Mycology of Bordeaux University Hospital and identified by MALDI-TOF MS (Microflex LT system; Bruker Daltonics, Billerica, MA, USA). The nucleotide sequence of the entire *FKS1* gene was determined, and comparison with the sequence of the same gene of *C. albicans* ATCC 90029 revealed a heterozygous mutation T1933C resulting in substitution of a serine by a proline (S645P) in the HS1 region (amino acids 641–649) of glucan synthase (GenBank accession no. D88815). Then the same mutation was introduced at the equivalent position in the *FKS1* allele (T1912C substitution) in a genetically engineered strain of *C. lusitaniae* derived from the wild-type strain CBS 6936, as described previously.[18]

In vitro susceptibility testing

Stock solutions of 10 mg/mL caspofungin (Euromedex, Souffelweyersheim, France) and 10 mg/mL rapamycin (Euromedex) were prepared in DMSO and kept at –20°C until use. The checkerboard method used for drug combination studies was based on microdilution CLSI standards[19] in Roswell Park Memorial Institute medium (RPMI) 1640, pH 7.0, buffered with 0.165 M MOPS.

MIC determination and interpretation

Caspofungin at concentrations ranging from 0.0625 to 4 mg/L was combined with

rapamycin at concentrations ranging from 0.001 to 1 or 0.01 to 10 mg/L depending on the susceptibility of the strains. Yeast suspensions were diluted with RPMI 1640 at a final cell density of 1×10^3 cells/mL in 96-well plates and incubated for 48 h at 35°C; growth was measured with a microplate reader at 450 nm. All experiments were performed in triplicate in independent assays, and growth variation did not exceed 10%. The MIC was defined at 90% growth inhibition for both drugs tested alone and in combination. High off-scale MICs were converted into the next twofold highest concentration. Two different methods were used to analyse the drug interactions: one based on the Loewe additivity model (calculation of the fractional inhibitory concentration index (FICI)) and another based on the Bliss independence model (response surface modelling (RS)).

The FICI was calculated as follows: FICI = (MIC combination/MIC alone) caspofungin + (MIC combination/MIC alone) rapamycin. The FICI data were interpreted as: synergy, FICI ≤ 0.5; no interaction, FICI > 0.5–4; and antagonism, FICI > 4.[20]

For RS analyses, the experimental data generated were expressed for each well as a percentage of growth in the presence of drugs compared to the growth control in drug-free medium and then transformed into a dose–response curve for each drug alone. We used the Bliss independence model, which is based on the hypothesis that drugs act independently from each other. Using this model, a theoretical response surface of the combination corresponding to no interaction was calculated using the dose-response curves of both drugs. To calculate the synergy distribution, the modelled response surface was compared to experimental data. The effect of the drugs in combination was defined as synergistic or antagonistic if the observed effect lay below or above the predicted no interaction dose–response surface, respectively. All calculations were performed with Combenefit software.[21] *In vitro* caspofungin susceptibility was also determined using Etest (bioMérieux, Marcy-l'Étoile, France) in accordance with the manufacturer's instructions (Table 1).

Results

Table 2 shows the results regarding the *in vitro* interaction between caspofungin and rapamycin against strains of *C. albicans* and *C. lusitaniae* susceptible and resistant to caspofungin. Caspofungin MICs and rapamycin MICs for each strain were within ± $2\log_2$ dilutions in all experiments.

The *C. albicans* and *C. lusitaniae* reference strains exhibited caspofungin MICs of 0.25 and 1 mg/L and rapamycin MICs of 0.5 and 10 mg/L, respectively. As expected, strains bearing the S645P mutation in Fks1 had higher caspofungin MIC values: 2 mg/L for *C. albicans* and 8 mg/L for *C. lusitaniae* (8 mg/L being the high-off scale MIC). The MICs of rapamycin for the *FKS1* mutant strains were identical to those observed in the corresponding reference strains (Table 2), i.e., 0.5 mg/L for *C. albicans* Fks1 S645P and 10 mg/L for *C. lusitaniae* Fks1 S638P.

Analysis of the results of the checkerboard tests with the response surface approach based on the Bliss model revealed synergy between caspofungin and rapamycin for all strains. To visualise the results, the synergy levels were mapped onto the experimental combination dose–response surface. To summarise the synergy distribution, the SUM-SYN-ANT metric was used, which represents the sum of synergy and antagonism observed in the concentration range. As described elsewhere,[22] for interpreting the SUM-SYN-ANT metric, a control plate with combinations of different concentrations of caspofungin alone was used for each reference strain of *Candida*. For *C. albicans* ATCC90029, the SUM-SYN-ANT of the control plate was 13.41%. Synergy and antagonism between caspofungin and rapamycin were assumed when the SUM-SYN-ANT was > 13.41% and < –13.41%, respectively. No drug interaction was considered for values between –13.41% and 13.41%. For *C. lusitaniae* CBS 6936, this metric was 25.11%.

Using caspofungin and rapamycin in combination, the SUM-SYN-ANT metric for *C. albicans* reference strain and *C. albicans* Fks1 S645P were 49.77% and 36.38%, respectively, indicating synergy between the two agents. For *C. albicans* Fks1 S645P, the

presence of rapamycin at a concentration of 0.25 mg/L (i.e., half the MIC) reduced the MIC of caspofungin by a factor of 16 from 2 to 0.125 mg/L, thus restoring the susceptibility of the *C. albicans FKS1* mutant to caspofungin (Figure 1). The restoration of susceptibility to caspofungin in the presence of a low dose of rapamycin was confirmed in *C. lusitaniae* Fks1 S638P, in which a dose of 0.31 mg/L rapamycin (32 times less than the MIC) resulted in a decrease in caspofungin MIC from 8 to 1 mg/L (8 mg/L being the high-off scale MIC) (Figure 2).

This observation was confirmed by RS analysis showing high SUM-SYN-ANT values of 75.36% and 69.53% for *C. lusitaniae* CBS 6936 and *C. lusitaniae* Fks1 S638P, respectively, reflecting a strong synergistic effect between caspofungin and rapamycin for both strains. Analysis of the checkerboard results via FICI revealed synergy between caspofungin and rapamycin only for *C. lusitaniae* CBS 6936 and *C. lusitaniae* Fks1 S638P, with an FICI of 0.249 and 0.27, respectively. For *C. albicans* strains, even if the FICI values were low, ranging from 0.504 to 0.765, the result was interpreted as no interaction (Table 2). These results suggest that the FICI method is not sufficiently discriminating to detect synergistic effects when the MICs for both drugs are low.

Discussion

The interactions between rapamycin and antifungals have been little studied, probably because rapamycin, first described for its antifungal properties,[13] has also been shown to be an immunosuppressant[14] thus making its use for the treatment of opportunistic fungal infections inconceivable. However, this perspective cannot be completely dismissed, as there are rapamycin analogues that have reduced immunosuppressive properties and that have retained their antifungal activity through TOR inhibition.[23,24]

Other immunosuppressants show synergistic activity with echinocandins or azole antifungals, such as cyclosporin or tacrolimus, which inhibit calcineurin[25–27]. Tacrolimus binds to the Fpr1 protein, a peptidyl-prolyl isomerase equivalent to the human

189 immunophilin FKBP12, which also binds rapamycin.[12] Therefore, we were interested in
190 the possible interactions between rapamycin and echinocandins, particularly
191 caspofungin.
192 Accordingly, this study represents the first demonstration of a synergistic relationship
193 between rapamycin and caspofungin in the inhibition of the growth of *C. albicans* and *C.*
194 *lusitaniae* strains susceptible and resistant to caspofungin. Synergy between rapamycin
195 and caspofungin was already reported in some strains of the filamentous fungi belonging
196 to Mucorales.[28] A positive interaction between these two molecules was also reported in
197 *Aspergillus* species based on the agar disk diffusion method[29] but it was not subsequently
198 confirmed via the checkerboard dilution method.[30,31] This was likely because analysis of
199 the results of a checkerboard test using the FICI is not suitable depending on the
200 molecules tested, particularly when the MICs for one or both molecules involved in the
201 association are low, which is the case here for rapamycin and caspofungin in *C. albicans*
202 with MICs of 0.5 mg/L and 0.25 mg/L, respectively. In the present study, RS analysis made
203 it possible to detect the synergy for the two strains of *C. albicans* and to confirm the effect
204 for the two strains of *C. lusitaniae*. This method seems to have greater discriminatory
205 power because it is independent of the end point unlike the FICI method.
206 Synergy was observable not only for strains susceptible to caspofungin but also for
207 resistant strains. For this demonstration, we used a clinical strain of *C. albicans* carrying
208 one of the most common polymorphisms responsible for resistance to echinocandins in
209 one allele of the *FKS1* gene, a nonsynonymous mutation in the HS1 region resulting in the
210 S645P glucan synthase variant.[32] This mutation is responsible for a decrease in binding
211 affinity of the glucan synthase target to the inhibitor.[33] We found that a rapamycin
212 concentration as low as 0.25 mg/L was sufficient to restore caspofungin susceptibility.
213 To confirm this observation, we introduced the equivalent S638P mutation in the Fks1
214 protein of a laboratory yeast model, *C. lusitaniae*,[18] and demonstrated that rapamycin also
215 restored susceptibility to caspofungin in an isogenic background. This indicates that the

decrease in caspofungin binding affinity to glucan synthase was probably not the only mechanism involved in the resistance of strains with the S645P or equivalent substitution, and that there may be other mechanisms depending on the action of rapamycin.

From a molecular viewpoint, the synergy between rapamycin and caspofungin suggests that the two underlying signalling pathways, the TOR pathway for rapamycin [34,35] and the PKC pathway for caspofungin,[36] are interconnected and share common effectors. In *Saccharomyces cerevisiae*, rapamycin activates the cell wall integrity (CWI) salvage pathway by phosphorylation of Slt2, the orthologous protein of Mkc1 in *C. albicans*.[15] This observation strongly suggests crosstalk between the TOR and CWI pathways. Other work in *S. cerevisiae* has established a link between parietal stress induced by caffeine and Stl2 phosphorylation in a TOR-dependent manner.[37] Other observations indicated that small G proteins from the RAS and Rho families may be good candidates at the intersection of these signalling pathways. In *C. albicans*, the Rhb1 protein belonging to the RAS superfamily could be involved in the activation of TOR because a homozygous mutant, *rhb1Δ/Δ*, showed increased susceptibility to rapamycin.[38] In a more direct but also more complex way, it has been shown in *S. cerevisiae* that the Rho1 protein is involved in both negative regulation of the TORC1 complex and also that it could itself be activated by the TORC1 complex, depending on the environmental stresses to which yeasts are exposed.[39] The information obtained in *S. cerevisiae* may not be directly transferrable to *C. albicans* because this species has only one copy of Tor, unlike *S. cerevisiae*, which has two Tor kinases.[40] Nevertheless, Rho1 is located upstream of the PKC pathway. Reduced expression of *RHO1* in *C. albicans* (haploinsufficiency) results in increased susceptibility to caspofungin and calcofluor white.[41,42] Rho1 has the dual function of regulating the glucan synthase protein complex and also of activating the CWI pathway under conditions of parietal stress, such as exposure to echinocandins.[43,44]

The finding of synergistic interactions between caspofungin and rapamycin represents

additional evidence for the involvement of the TOR pathway in the cellular response to parietal stress due to inhibition of glucan synthase by echinocandins.

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Transparency declarations

None to declare.

Author contributions

M. L., I. A. and T. N. designed the experiments. M. L., V.F.-O. and N. B. performed the experiments. M. L., I. A. and T. N. analysed the results. M. L., I. A. and T. N. wrote the manuscript.

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Table 1. Name, genotype, phenotype, and caspofungin susceptibility of the strains used in this study

Strain	Origin[a]	*FKS1* allele/mutation	Phenotype	Agar diffusion assay MIC (mg/L)	Reference
Candida albicans ATCC 90029	ATCC	WT/WT	WT, Cas^S	0.125	
Candida albicans Fks1 S645P	Clinical isolate	WT/*FKS1*^{T1933C}	$Fks1p^{S645P}$, Cas^R	2	
Candida lusitaniae CBS 6936	CBS	WT	WT, Cas^S	0.125	[45]
Candida lusitaniae Fks1 S638P	CBS 6936	*FKS1*^{T1912C}	$Fks1p^{S638P}$, Cas^R	> 32	[18]

[a]CBS, Centraal Bureau voor Schimmelcultures, renamed Westerdijk Fungal Biodiversity Institute.

Table 2. *In vitro* interaction between caspofungin and rapamycin against strains of *C. albicans* and *C. lusitaniae* susceptible and resistant to caspofungin

Strains	**Checkerboard MICs (mg/L)**			**FICI[a]**		**Response surface analysis**	
	Caspofungin alone	**Rapamycin alone**	**Caspofungin and rapamycin in combination**	**Value**	**INT**	**SUM-SYN-ANT**	**INT**
***C. albicans* ATCC 90029**	0.25	0.5	0.125/0.002	0.504	IND	49.77	SYN
***C. albicans* Fks1 S645P**	2	0.5	0.125/0.25	0.765	IND	36.38	SYN
***C. lusitaniae* CBS 6936**	1	10	0.064/0.31	0.249	SYN	75.36	SYN
***C. lusitaniae* Fks1 S638P**	8	10	1/0.31	0.27	SYN	69.53	SYN

[a]FICI, fractional inhibitory concentration index; INT, interpretation; SYN, synergistic; IND, indifference

A

Caspofungin (µg/mL) \ Rapamycin (µg/mL)	1	0.5	0.25	0.125	0.06	0.03	0.016	0.008	0.004	0.002	0.001	0
4	3.1	3.1	3.2	3.3	2.7	3.1	3.6	4.2	4.5	3.7	3.9	4.9
2	2.2	2.0	1.3	1.1	0.7	0.9	1.1	2.6	2.1	4.7	5.5	7.1
1	2.7	2.5	1.1	4.0	13.6	29.2	25.7	28.9	38.7	52.6	54.9	44.3
0.5	2.6	2.3	3.2	13.3	48.5	53.0	46.5	49.5	59.3	96.7	99.4	92.1
0.25	2.0	1.8	4.2	20.0	73.1	74.6	77.0	83.5	85.4	99.7	98.3	98.6
0.125	2.4	2.5	6.9	66.0	82.5	86.0	88.0	91.1	87.7	99.9	98.6	99.4
0.0625	1.3	2.1	15.1	83.1	90.5	89.4	90.5	96.0	91.9	100.2	97.8	100.0
0	2.1	4.2	43.4	77.4	78.2	72.7	68.0	62.3	61.3	92.9	92.7	100.0

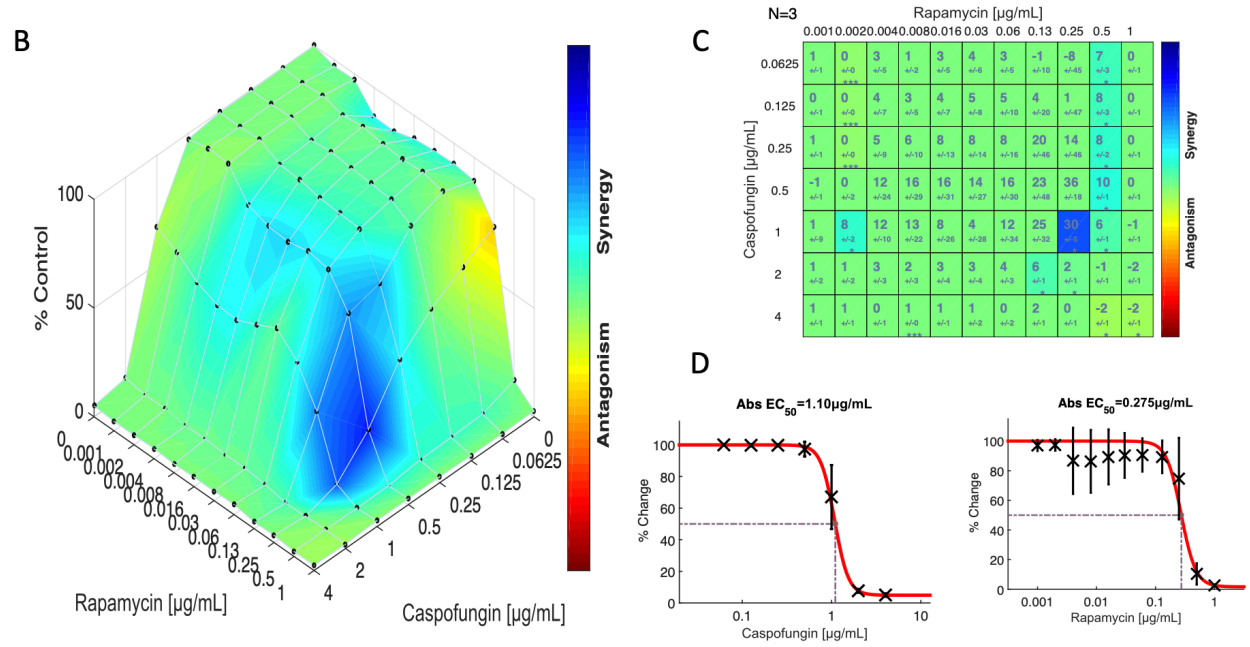

Figure 1. Checkerboard test using caspofungin and rapamycin with the *C. albicans* Fks1 S645P strain. (A) Percentage of yeast growth compared to the growth in drug-free medium on 96-well microplates. (B) Response surface analysis showing the mapping of the synergy levels based on the Bliss model. (C) Matrix of the synergy distribution derived from the combination dose–response and from the reference dose–response. (D) Dose–response curve of each drug alone. Panels B, C, and D were generated with Combenefit, version 2.021.

A

Caspofungin (µg/mL) \ Rapamycin (µg/mL)	10	5	3	1,250	0.625	0.31	0.16	0.08	0.04	0.02	0.01	0
4	1.9	1.4	1.8	2.7	3.0	4.8	37.5	51.7	82.4	87.5	87.0	90.7
2	1.5	2.0	1.8	1.8	2.8	4.8	35.3	52.5	84.4	88.6	87.2	92.8
1	1.2	1.7	0.7	0.4	0.4	5.2	39.2	67.5	89.5	91.7	91.4	94.0
0.5	1.6	1.6	1.4	2.2	4.6	21.6	60.7	75.1	89.7	94.3	94.3	96.9
0.25	2.3	3.8	3.1	7.6	15.7	35.6	66.1	81.7	87.5	92.7	93.3	98.6
0.125	2.2	7.7	9.6	12.7	20.0	40.9	67.2	80.9	90.2	94.8	95.5	100.7
0.0625	2.6	9.0	10.8	13.6	18.0	38.3	68.9	82.2	90.5	92.4	93.9	100.3
0	3.3	10.3	12.7	14.7	18.2	34.5	70.7	90.0	94.6	95.2	96.5	100.0

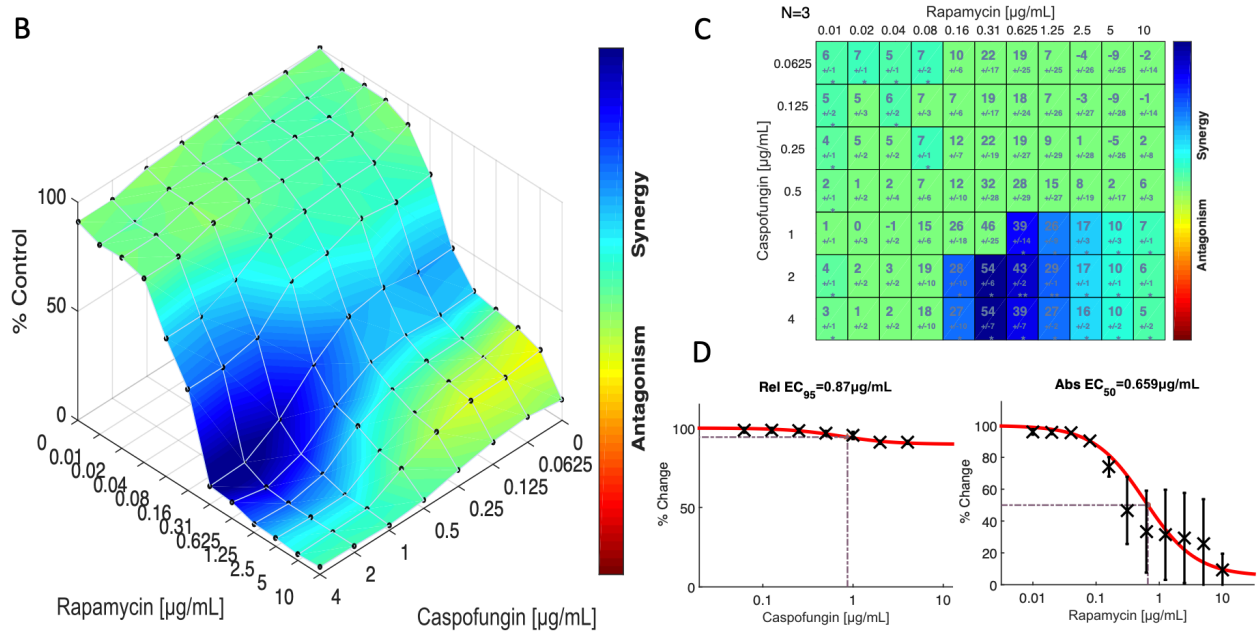

Figure 2. Checkerboard test using caspofungin and rapamycin with the *C. lusitaniae* Fks1 S638P strain. (A) Percentage of yeast growth compared to the growth in drug-free medium on 96-well microplates. (B) Response surface analysis showing the mapping of the synergy levels based on the Bliss model. (C) Matrix of the synergy distribution derived from the combination dose–response and from the reference dose–response. (D) Dose–response curve of each drug alone. Panels B, C, and D were generated with Combenefit, version 2.021.