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Letter

Hyper-Synergistic Antifungal Activity of Rapamycin and Peptide-Like Compounds against *Candida albicans* Orthogonally via Tor1 Kinase

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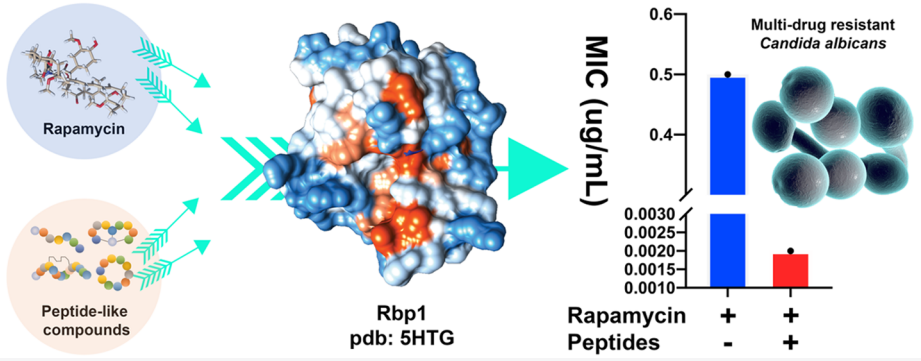

ABSTRACT: *Candida albicans* is a life-threatening, opportunistic fungal pathogen with a high mortality rate, especially within the immunocompromised populations. Multidrug resistance combined with limited antifungal drugs even worsens the situation. Given the facts that the current drug discovery strategies fail to deliver sufficient antifungals for the emerging multidrug resistance, we urgently need to develop novel approaches. By systematically investigating what caused the different antifungal activity of rapamycin in RPMI 1640 and YPD, we discovered that peptide-like compounds can generate a hyper-synergistic antifungal effect with rapamycin on both azole-resistant and sensitive clinical *C. albicans* isolates. The minimum inhibitory concentration (MIC) of rapamycin reaches as low as 2.14 nM (2^{-9} μg/mL), distinguishing this drug combination as a hyper-synergism by having a fractional inhibitory concentration (FIC) index ≤ 0.05 from the traditional defined synergism with an FIC index < 0.5. Further studies reveal that this hyper-synergism orthogonally targets the protein Tor1 and affects the TOR signaling pathway in *C. albicans*, very likely without crosstalk to the stress response, Ras/cAMP/PKA, or calcineurin signaling pathways. These results lead to a novel strategy of controlling drug resistant *C. albicans* infection in the immunocompromised populations. Instead of prophylactically administering other antifungals with undesirable side-effects for extended durations, we now only need to coadminister some nontoxic peptide additives. The novel antifungal strategy approached in this study not only provides a new therapeutic method to control fungal infections in rapamycin-taking immunocompromised patients but also mitigates the immunosuppressive side-effects of rapamycin, repurposing rapamycin as an antifungal agent with wide applications.

KEYWORDS: *Candida albicans, multidrug resistance, rapamycin, drug combination, synergism, TOR kinase*

C. albicans is one of the most common fungal pathogens and a major cause of systemic infections in immunocompromised patients. It is responsible for about 30% of severe infections with a mortality rate of higher than 40%.[1,2] Given the fact that more and more people are becoming immunocompromised, from causes such as transplantations of bone marrow, organs, and hemopoietic stem cells; to intensive chemotherapy or other immunosuppressive treatments; and to infections such as HIV,[1−3] one major consequence of being immunocompromised is hyper-susceptibility to infectious diseases, including fungal infections. However, only a very limited number of antifungal drugs are available in the clinic.[4] (i) Azoles such as fluconazole can inhibit Erg11, the key enzyme of ergosterol biosynthesis. (ii) Polyenes such as amphotericin B can bind to

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Table 1. Synergism Evaluations of Rapamycin and Selected Compounds

	MIC (μg/mL), SC5314 in RPMI 1640				
	alone	with rapamycin	rapamycin	FICI[a]	definition[b]
rapamycin	0.5				
cyclosporin A	>100	3.125	0.0078	<0.047	HS
cyclosporin B	>100	1.56	0.0078	<0.031	HS
cyclosporin C	>100	3.125	0.0156	<0.063	S
cyclosporin D	>100	6.25	0.0078	<0.078	S
cyclosporin H	>100	6.25	0.0156	<0.094	S
FK520	>100	0.39	0.0313	<0.66	S
FK506	>100	0.39	0.0313	<0.066	S
surfactin	>100	1.56	0.0020	<0.020	HS
arthrofactin	>100	0.39	0.0078	<0.020	HS
A23187	8	2	0.2500	0.750	NS
beauvericin	8	2	0.5000	1.250	NS
ionomycin	16	4	0.0313	0.3125	S
berberine	64	16	0.0625	0.375	S
nigericin	3.2	0.8	0.5000	1.25	NS
daptomycin	50	12.5	0.5000	1.25	NS
polymyxinb	25	6.25	0.2500	0.75	NS
mizoribine	3.2	0.8	0.1250	0.5	S
nikkomycin	0.8	0.2	0.1250	0.5	S
mycophenolic acid	6.4	1.6	0.5000	1.25	NS
valinomycin	1.6	0.4	0.5000	1.25	NS
amphotericin B	0.5	0.125	0.5000	1.25	NS

[a]When MIC alone >100 μg/mL, use 100 to calculate FIC index; if MIC alone >100 μg/mL, the start concentration is 25 μg/mL in the checkerboard assay; the possible minimal FIC index was shown. [b]HS, Hyper-Synergism; S, Synergism; NS, No Synergism.

ergosterol, form pores in cell membranes, and make cells leaky. (iii) Echinocandins, such as caspofungin, can inhibit β-(1,3)-D-glucan synthase and then disrupt cell-wall integrity. (iv) Fluoropyrimidines, such as 5-flucytosine, can inhibit DNA and RNA synthesis. And, (v) griseofulvin can deteriorate the spindles and cytoplasmic microtubules to influence mitosis.

Since fungal pathogens are eukaryotes, all antifungal drugs possess some degree of undesirable side effects/toxicity. However, prophylactic and excessive use of the limited variety of available antifungals has rapidly developed multidrug resistance, and a lack of anticipation of this development has dramatically increased the frequency of clinical treatment failures.[5] This serious situation demands more effective antifungal drugs and novel therapies.[5−7] Toward this end, only one antifungal drug with a new mechanism of action has been discovered and used clinically in the past few decades.[8] As the process of de novo antifungal discovery fails to meet clinical needs, the approach of repurposing approved drugs has drawn much attention. In fact, the value of this approach has been widely demonstrated. For example, sildenafil (Viagra) was initially developed for angina and was repurposed as a medication for erectile dysfunction. Another example is metformin (Glucophage), which was discovered as an antidiabetes drug and is now the active chemical in over 100 ongoing phase II and III clinical trials as a cancer therapeutic agent.[9,10]

Rapamycin (Figure S1), a natural product of *Streptomyces hygroscopicus*, was first reported as an anti-*C. albicans* compound in 1975, but later it was repurposed to an immunosuppressive drug, for organ transplant patients due to its strong immunosuppressive activity.[11−14]

After entering cells, rapamycin binds to its conserved cellular receptor, immunophilin FKBP12 (FK506-binding protein of 12 kDa), then forms the FKBP12-rapamycin complex. This complex binds to the FRB (FKBP12-rapamycin binding) domain, a small region in Tor protein kinases, and inhibits the activity of Tor protein kinases. Tor protein kinases are key components of TORC1 (target of rapamycin complex 1) and are central regulators in the conserved nutrient-sensing TOR signaling pathway. The TOR signaling pathway regulates many important cellular processes such as protein translation, autophagy, and apoptosis.[15,16] Two Tor proteins, Tor1 and Tor2, were identified within *Saccharomyces cerevisiae*, while only Tor1 was found in both *C. albicans* and humans.[15,17−19] Resistance to rapamycin is known to be caused either by the absence of the FKBP12 protein or mutations to the FRB domain.[20,21] In *C. albicans*, Rbp1 was identified as a homologue of FKBP12, and the null mutant of *rbp1* expectedly showed rapamycin resistance.[22,23]

Rapamycin has been widely used in clinics to prevent rejections in transplanted marrow and organ tissue. However, taking immunosuppressive drugs makes patients highly susceptible to opportunistic infections. Although rapamycin was initially discovered as an antifungal agent, it showed very weak antifungal activity on its own (Table 1), and dosage to suppress immune response in patients is generally not enough to also control fungal infections.[14,24] If acting prophylactically or infected by fungi, a patient would need to take additional antifungal drugs and may suffer the corresponding side effects.

Historically, drug combinations have been frequently adopted in the clinic, especially in traditional Chinese medicine, where it traces back thousands of years. Not only can taking drugs in combination enhance activity through synergism, it decreases unwanted side effects by permitting lower dosages. Drug combinations can also slow down or avoid the emergence of drug resistance[25−27] by simultaneously affecting multiple drug targets. This approach extends to

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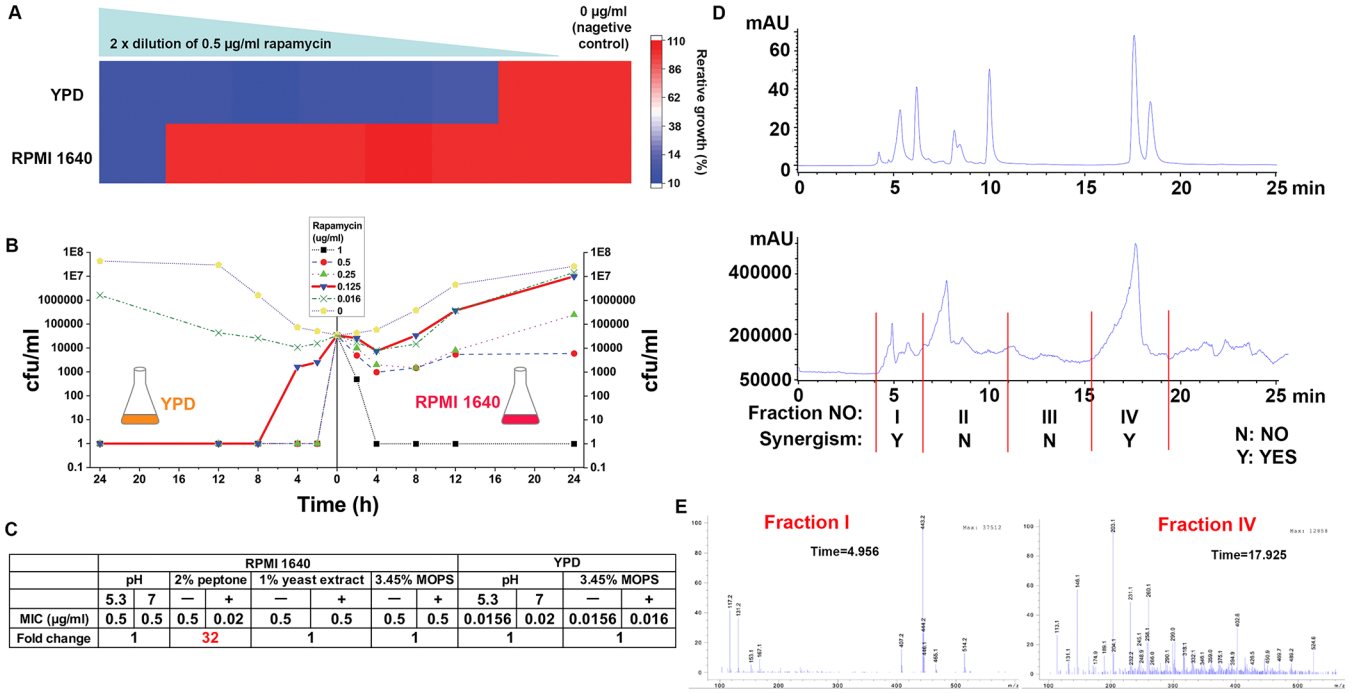

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	RPMI 1640								YPD			
	pH		2% peptone		1% yeast extract		3.45% MOPS		pH		3.45% MOPS	
	5.3	7	−	+	−	+	−	+	5.3	7	−	+
MIC (μg/ml)	0.5	0.5	0.5	0.02	0.5	0.5	0.5	0.5	0.0156	0.02	0.0156	0.016
Fold change	1		32		1		1		1		1	

Figure 1. Rapamycin is much more active against *C. albicans* in YPD than in 1640. (A) Different MICs of rapamycin in RPMI 1640 and YPD from a broth microdilution assay. Colors were based on the cell viabilities. Blue indicated the inhibition of the cell growth, while red indicated no inhibition of the cell growth. (B) Time−kill curves of rapamycin in RPMI 1640 and in YPD during a 24-h incubation. Results of the 0.125 μg/mL rapamycin treatment are highlighted as a solid bold red line. (C) Peptone is the key component of YPD that caused the hyper-synergistic antifungal activity of rapamycin. (D) Fractions I and IV of BD Bacto Peptone from HPLC-MS showed synergistic antifungal activity with rapamycin in RPMI 1640. (E) ^{1}H NMR analysis showed that the composition of fractions I and IV was mainly peptide.

antifungals, generating synergism through the extended application of drugs both with or without antifungal activities.[4]

Our approach in this study is to bring together these strategies of "repurposing approved drugs" and "leveraging drug combinations" to improve the efficacy of rapamycin. We target clinical isolates of both drug-sensitive and multi-drug-resistant *C. albicans* in this study. It shows that applying noncytotoxic peptide-like compounds without anti-*C. albicans* activity with another compound can lead to hyper-synergistic antifungal interactions. We demonstrate that the hyper-synergism orthogonally targets Tor1 and inhibits cell growth by interfering with the TOR signaling pathway. Our presented findings provide clinicians a novel effective strategy to control fungal infections in rapamycin-taking immunocompromised patients. Further, our strategy broadens the spectrum of antifungal drugs and allows repurposing rapamycin as antifungals without concern for its major side effects, which can shed light on other classes of drug discovery.

The Clinical and Laboratory Standards Institute (CLSI) provided a reference standard method, M-27A3,[28] for antifungal susceptibility testing of yeasts. The recommended medium is RPMI 1640 with L-glutamine, additionally supplemented with 2% glucose and buffered with 3.45% MOPS. The pH is adjusted to 7.0. This CLSI standard method has been widely and successfully used in minimal inhibitory concentration (MIC) evaluation of many antifungal agents. However, the MICs could be different with different testing media.[29,30] Yeast extract peptone dextrose (YEPD or YPD) is a complete medium for yeast growth. YPD is comprised of 1% yeast extract, 2% Bacto Peptone (BD Biosciences, US), and 2% glucose with natural pH (about 5.5). It is one of the most commonly used media for yeast growth. In 1975, rapamycin was first reported as an antifungal compound through anti-*C. albicans* screening, using a medium called sabouraud dextrose broth.[14] In this study, we found that the MIC of the database strain SC5314 in RPMI 1640 was 0.5 μg/mL, which is 32-fold higher than that in YPD (Figure 1A). The different susceptibility was also demonstrated by time−kill curves. In general, the time−kill curves had different patterns. After a 24-h treatment with 0.125 μg/mL of rapamycin, the population of the testing strain in YPD was more than 7 orders of magnitude less than that in RPMI 1640 (bold red line in Figure 1B). In fact, where the rapamycin concentration was above 0.25 μg/mL, all cells in YPD were killed within 2 h (Figure 1B).

The above results indicated that there must be a certain component(s) in YPD that could synergize with rapamycin to generate a much higher antifungal activity. Therefore, we listed the main differences between YPD and RPMI 1640, assumed a single variance principle, and tested the MIC of rapamycin in the mixed media (Figure 1C). Interestingly, the MIC of rapamycin in "RPMI 1640 plus 2% Bacto Peptone" reached 0.016 μg/mL, the same MIC as in YPD (Figure 1C). However, Bacto Peptone, a source of nutrition, could not have any antifungal activity by itself. Our hypothesis then was that a synergism must occur between rapamycin and some unknown components of Bacto Peptone. To discern which components were creating this interaction, we used High Performance Liquid Chromatography−Mass Spectrometry (HPLC-MS) to analyze the Bacto Peptone. Four fractions were collected (Figure 1D). We then evaluated the synergistic antifungal activity of rapamycin with each fraction in RPMI 1640. Fractions I and IV were observed to have synergism with rapamycin (Figure 1D). To identify which kind of molecule(s) in these fractions was responsible for the synergism, we further

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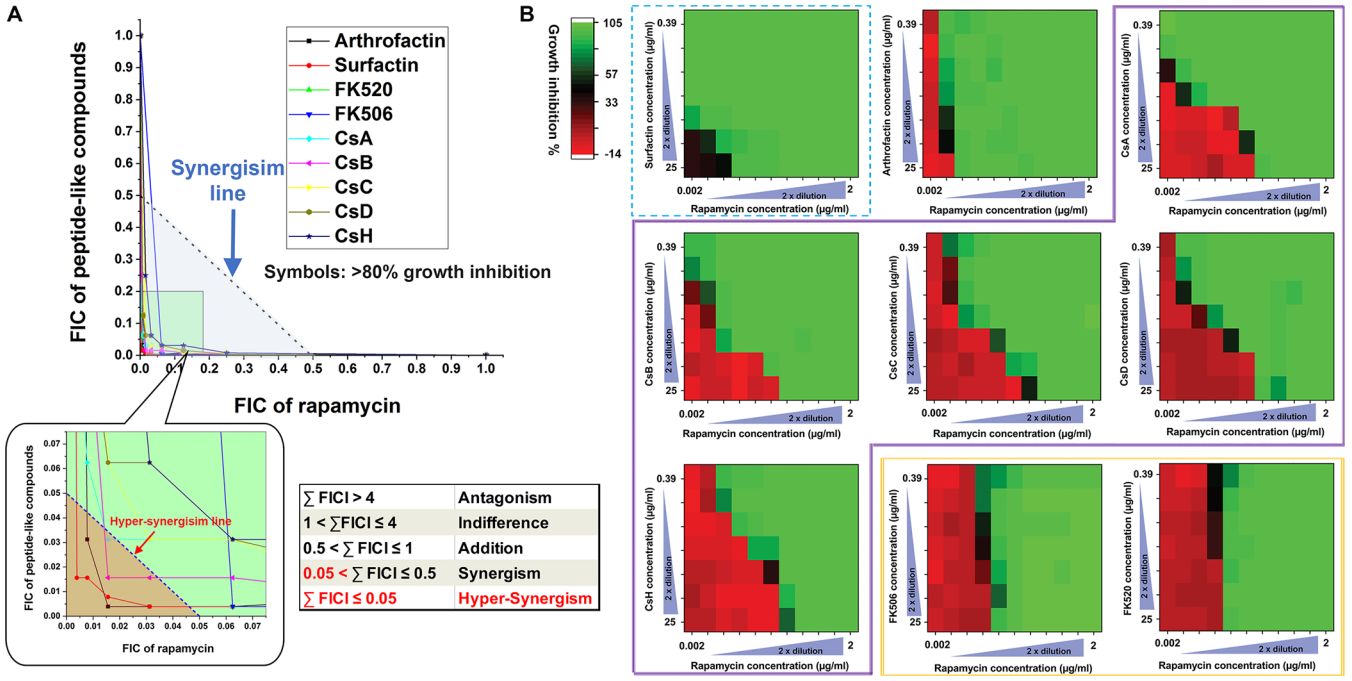

Figure 2. Hyper-synergism of rapamycin and nine selected peptide-like compounds against *C. albicans* SC5314. (A) The black dotted line in the upper panel and blue dotted line in the lower panel indicate an ideal isobole. Data points over the upper black dotted line indicate additive effects. Data points below this line indicate normal synergism (FIC index ≤ 0.5, the light blue area), and data points below the lower blue dotted line indicate hyper-synergism (FIC index ≤ 0.05, the light red area). To distinguish the data below the blue dotted line, we redefined synergism as 0.05 < FIC index ≤ 0.5. (B) The checkerboard assay (dose response matrix) was performed in the RPMI 1640 medium with gradients of rapamycin and the nine selected peptide-like compounds as indicated. Data were analyzed after 24 h of incubation at 30 °C. Similar patterns of the heat plots are boxed.

analyzed them with nuclear magnetic resonance (^{1}H NMR). The results indicated that these fractions were composed of peptide-like compounds (Figure 1E).

After successfully narrowing down the synergistic factors into peptide-like compounds, we had wanted to find defined compounds that also could cause hyper-synergistic antifungal activity with rapamycin. To achieve this goal, some well-known, commercial, and medicinal peptides were selected for testing. Our results showed multiple classes of compounds created effective synergism with rapamycin: five cyclosporine compounds, cyclosporine A, B, C, D, and H (cyclic peptides); two immunosuppressive agents, FK506 and FK520 (macrolides); and two biosurfactants, surfactin and arthrofactin (lipopeptides; Table 1). Chemical structures are shown in Figure S1. Notably, these nine compounds showed no antifungal activities at all by themselves (MICs > 100 μg/mL). Checkerboard assays were used to obtain the minimum synergistic concentrations with rapamycin based on the fractional inhibitory concentration (FIC) index.[31,32] To our surprise, for achieving 90% growth inhibition, only 0.002 μg/mL of rapamycin was required together with a very low amount (1.56 μg/mL) of surfactin (Table 1).

From the checkerboard assays, we observed some very low FIC indexes (Table 1), in order to distinguish them from normal definition of synergism (FIC index ≤ 0.5).[31,32] On the basis of the isobolograms of the selected drug combinations (Figure 2A), we defined hyper-synergism as an FIC index ≤ 0.05, and redefined normal synergism as 0.05 < FIC index ≤ 0.5. Remarkably, the MIC of rapamycin in the hyper-synergism could be as low as 0.00195 μg/mL (2.14 nM). Notably, this synergistic MIC is 1/256 times the MIC of rapamycin alone in RPMI 1640, which is a level that mitigates the concern of side effects. The interactions between rapamycin and the nine synergistic and hyper-synergistic peptide-like compounds against *C. albicans* are depicted in Figure 2B. From the heat plots of the growth inhibition data set, we observed that the cyclic peptides, macrolides, and lipopeptides each created different patterns (Figure 2B). However, the basic patterns formed by all nine compounds are very similar, indicating that they might share the same mode of action in the hyper-synergism.

We initially observed the hyper-synergism of rapamycin in a rich medium, and the conserved TOR signaling pathway is nutrient-sensitive, which motivated us to discover the internal link. We know that the TOR signaling pathway can be blocked by inhibiting either the binding of rapamycin to FKBP12, or the binding of the FKBP12−rapamycin complex to Tor1. In *C. albicans*, the homologue of the FKBP12 protein is Rbp1. As expected, the deletion of *RBP1* or the disruption of the FRB domain made the resulting *C. albicans* resistant to rapamycin (Table 2), which was consistent with previous reports.[20,21] Moreover, here we also observed that the hyper-synergism of rapamycin disappeared in these mutants (Table 2). These results indicated that the binding of rapamycin and its receptor Rbp1 (FKBP12) was the precondition of hyper-synergism, while the following binding of the rapamycin−Rbp1 complex and Tor1 actually caused the antifungal effects.

From a systems biology perspective, many of the intercellular signaling pathways are cross talking.[33,34] Since orthogonality is one of the most important concerns for being a successful drug target, we would like to evaluate whether other signaling pathways could also contribute or be influenced by the hyper-synergism of rapamycin. To this end, three major

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Table 2. Evaluation of Hyper-Synergism Target

strains	Genotype	MIC of rapamycin (μg/mL) RPMI 1640	YPD	RPMI 1640 +1/4MIC of CsA
SC5314	parental strain	0.5	0.016	<0.008
RBP1	*rbp1*Δ::*MX3*/*RBP1*	0.5	0.016	<0.008
Δrbp1	*rbp1*Δ::*MX3*/ *rbp1*Δ::*CaURA3MX3R*	>128	>128	>128
Tor1−1	SC5314 *TOR1*-1 (S1972R)/*TOR1*	>128	>128	>128
Tor1−2	BWP17 *TOR1*-2 (S1972R)/*TOR1*	>128	>128	>128

signaling pathways (Ras/cAMP/PKA, calcineurin, and stress response) were picked for observation.

Studies have demonstrated that the Ras/cAMP/PKA signaling pathway was critical in *C. albicans* life cycle. This signaling pathway could influence virulence, pathogenicity, morphological changes and gene expression profiles and has been considered as an antifungal target.[35−39] However, in our testing, we did not observe any changes in rapamycin's MIC on null mutants of this signaling pathway compared with their parental strain (Figure 3).

The calcineurin pathway is highly conserved from yeast to humans and mediates many important cellular processes.[40] Again, we did not see changes in the MICs of rapamycin after mutating the calcineurin signaling pathway.

As TOR is considered having a connection to stress response pathways, some important stress response pathway related mutants were subjected to the assay for testing, but we saw almost no changes in susceptibility of these mutants compared to the wild-type strain (Figure 3). The above results indicated that the hyper-synergism of rapamycin was orthogonal to Ras/cAMP/PKA, calcineurin, and some stress response pathways in *C. albicans*.

To address the value of the hyper-synergism of rapamycin in clinical applications, we would like to evaluate the efficacy of rapamycin hyper-synergism on clinical *C. albicans* isolates other than laboratory strains. *C. albicans* can colonize on the mucosal surfaces of different tissues of the human body and can also disseminate to internal organs and cause severe systemic infections.[41−43] Clinical isolates and laboratory strains will have different behaviors due to the different habitats. As a result, drug susceptibility data from clinical isolates will be a better indicator of future clinical applications.

To reduce bias, strains were isolated from different patients and different habitats of the patients' bodies. From Beijing

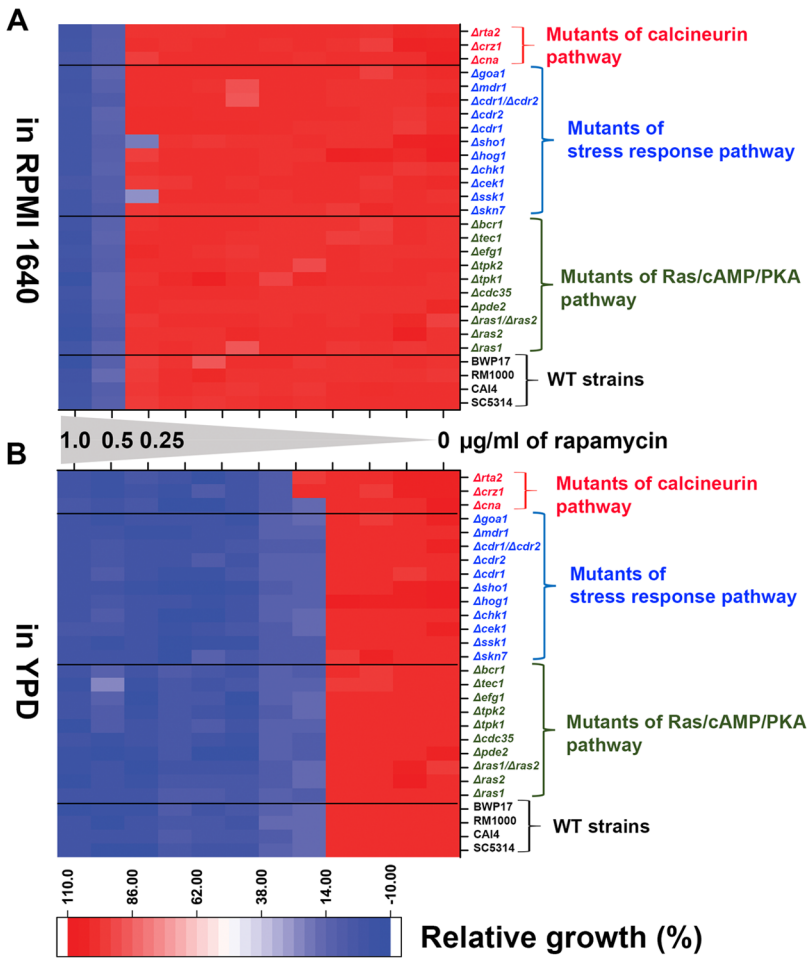

Figure 3. Hyper-synergism of rapamycin has no cross-talk to other important signaling pathways in *C. albicans*. The relative growth of the indicated *C. albicans* strains was calculated by setting the OD600 of 24 h drug-free SC5314 culture as 100%. The rapamycin dose response assay was performed in RPMI 1640 medium (A) and in YPD (B) with 2-fold dilution of rapamycin. Data were analyzed after 24 h of incubation at 30 °C. The strains were clustered by different pathways.

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Chaoyang Hospital, we obtained 10 isolates from blood, 10 from bile, 10 from ascetic fluid, and 10 from oral cavities. In total, 40 isolates were identified as fluconazole-sensitive strains. We then tested the sensitivity of rapamycin on these 40 isolates both in RPMI 1640 and in YPD. We found that the hyper-synergism of rapamycin in YPD mirrored our findings to the laboratory strain SC5314 (Table S3). Furthermore, we wanted to know whether our analyzed hyper-synergism worked on drug-resistant clinical isolates. Two different series of drug resistant clinical isolates were used for testing. One series of 12 isolates was obtained from the vaginas of different recurrent vulvovaginal candidiasis (RVVC) patients in the Peking University Third Hospital. These isolates were identified as fluconazole-resistant (Table S4). The other series of eight drug resistant clinical isolates was isolated from a single HIV-infected patient over two years. During that time period, the MIC of fluconazole increased over 200-fold.[44] However, the MIC of rapamycin did not change on these isolates compared with SC5314 (Table S4). All of these results indicated that normal drug resistant isolates do not generate cross resistance to rapamycin. The hyper-synergism of rapamycin can work very well against both drug sensitive and drug resistant clinical *C. albicans* isolates.

For clinical applications, the cytotoxicity of the helper compounds facilitating the hyper-synergism with rapamycin must be evaluated. Two tumor cell lines (BGC-823 and HeLa) and a control cell line (normal human liver cells HL-7702) were used to test the cytotoxicity. We found that besides cyclosporin B and rapamycin itself, the other compounds had relatively low cytotoxicity (Figure 4), which indicates that findings in this study make not only academic but also clinic sense.

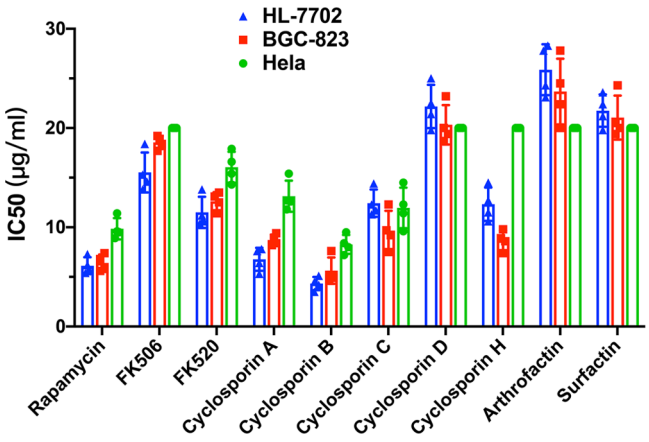

Figure 4. Cytotoxicity evaluation of rapamycin and the other nine peptide-like compounds. IC50 values of each tested compounds are depicteded as a bar chart. Means ± S.D. of four biological replicates are shown.

We are entering the postantibiotic era. As eukaryotes, fungal cells and human cells share many similarities, which results in direct application of antifungals coming with some degree of unwanted side effects. This difficulty has heavily restricted the discovery of novel antifungals, but the need for novel therapies continues to grow. While technological advances in modern medicine have enabled marrow and organ transplants to become more commonplace, this practice has expanded the immunocompromised population and led to increased preventive use of the small variety of known antifungal drugs. For these people, prevalence of side effects and emerging drug resistance remain issues. The traditional approach to overcome drug resistance is to first increase dosage, then to find a substitute. This leads to an endless cycle of problems, with the opportunity for pathogens to gain resistance more rapidly than we can discover substitutes—indeed, only one class of antifungal agents with a novel mechanism of action has been discovered and successfully adopted for clinical use in the past several decades.[8] We badly need new antifungal drugs and novel therapeutic strategies to break this vicious cycle.[45,46]

We have successfully combined two drugs together to generate a synergistic effect and control fungal infection.[27] Synergy could be generated by administering two antifungal agents at decreased dosage, by one antifungal agent at decreased dosage with another nonantifungal compound, as was found to be effective in this study, or potentially by two nonantifungal compounds. Additionally, synergy can overcome the disadvantage of single target drugs which can easily generate drug resistance.

Despite being initially discovered as an anti-*C. albicans* compound, patients taking rapamycin today must also take another antifungal and suffer both side effects. This is due to rapamycin's direct antifungal activity being low compared to its serious immunosuppression side-effect. It was famously later developed to focus on this effect for immunosuppressive treatments such as transplantations, but the drug serves to increase vulnerability to fungal infections and cannot manage them on its own. In this study, we introduced a new antifungal strategy—instead of taking other antifungal drugs, only a nontoxic peptide drug additive is needed to hyper-synergistically enable rapamycin's activity against fungal pathogens. The case of an opportunistic fungal infection caused by *C. albicans* could be controlled from the very beginning. We call this strategy "one stone, two birds" (Figure 5).

Furthermore, as the effective dosage is as low as 2 nM, the previous concern of immunosuppressive side effects from applying rapamycin as an anti-*C. albicans* antifungal is greatly lessened, as direct pharmacological inhibition of Tor1 is too high a cost on the host's immune system. This concern was stopping further development of rapamycin as an antifungal, but now, with nontoxic additives, we could confidently repurpose rapamycin to fulfill its original goal as an antifungal.

METHODS

Strains Used in This Study. The list of laboratory *C. albicans* strains used in this study is provided in Table S1, while the clinic isolates (obtained from local hospitals: Peking University Third Hospital and Beijing Chaoyang Hospital) are listed in Table S2. Three cell lines, HL-7702, BGC-823, and Hela, were ordered from a cell bank, Shanghai Institutes for Biological Sciences. All strains were stored in 25% glycerol at −80 °C. Cell lines were stored in liquid nitrogen.

Chemicals and Culture Media. The chemicals were reconstituted according to the manufacturers' directions. Cyclosporine A, cyclosporine B, cyclosporine C, cyclosporine D, cyclosporine H, surfactin, FK520, FK506, arthrofactin, daptomycin, mizoribine, and amphotericin B were purchased from a local chemical pharmacy (purity >98%), while rapamycin, beauvericin, berberine hydrochloride, nikkomycin, polymyxin B, and valinomycin were purchased from Sigma. A23187, nigericin, and ionomycin were purchased from Alexis.

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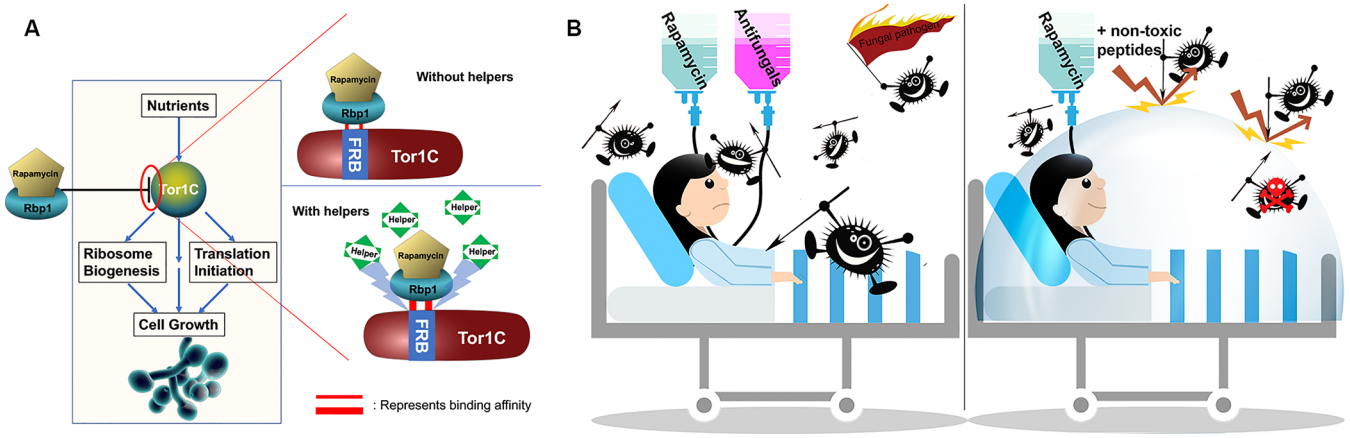

Figure 5. A proposed working model of the rapamycin hyper-synergism. (A) Nontoxic peptide-like compounds serve as helpers that can make the rapamycin−Rbp1 complex tightly bind to the Tor1 kinase, orthogonally targeting Rbp1 to inhibit the TOC pathway. (B) For controlling the fungal infections in the rapamycin-taking patients, instead of co-taking other antifungal drugs, our proposed novel "one stone, two birds" strategy only requires coadministering nontoxic peptides.

RPMI 1640 was purchased from Invitrogen and was used according to the manufacturer's protocol; in short, it was prepared by supplementing 2% glucose and 3.45% MOS, then adjusting the pH to 7.0. YPD liquid medium consisted of 1% yeast extract (w/v), 2% peptone (w/v), and 2% dextrose (w/v), with 2% (w/v) agar to make a solid medium when needed.

Antifungal Susceptibility and Synergistic Antifungal Testing Assay. Antifungal susceptibility testing was carried out as described previously[27] in flat-bottomed, 96-well microtiter plates (Greiner, Germany), using a broth microdilution protocol modified from the Clinical and Laboratory Standards Institute M-27A3 methods.[28] Approximately 5×10^3 fungal cells were inoculated into the testing media of RPMI 1640 and YPD. Pipette tips were changed after every dilution step; otherwise, we noticed that the MIC would be greatly different due to the poor water-solubility of rapamycin. MIC was determined as the drug concentration that inhibits fungal growth by >80% relative to the corresponding drug-free growth control. For the synergistic antifungal testing, 1/4 MIC of one compound was preadded into the medium, with the procedures being otherwise carried out in the same fashion.

Checkerboard Assay. Assays were carried out as described previously.[47] Basic procedures were the same as tose for antifungal susceptibility testing. Final concentrations ranged from 0.002 to 2 μg/mL for rapamycin and 0.39 to 25 μg/mL for peptide-like compounds, respectively. Rapamycin was 2-fold diluted from 1 to 11 (column), while peptide-like compounds were 2-fold diluted from A to G (row) of the 96-well microtiter plate. The fractional inhibitory concentration (FIC) index is defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. Synergism and antagonism were defined by FIC indices of $\leq$0.5 and >4, respectively. An FIC index result of >0.5 but $\leq$4 was additive.[31,32] In this study, we defined hyper-synergism as having an FIC index < 0.05.

Time−Kill Assay. Time−kill experiments were carried out as described previously,[48] using RPMI 1640 and YPD as the testing media. Then, 0, 0.016, 0.125, 0.25, 0.5, and 1 μg/mL of rapamycin were tested. About 3.4×10^3 cells of SC5314 were inoculated into flat-bottomed, 96-well microtiter plates (Greiner, Germany). The experiments were carried out in two media simultaneously. A 1 μL suspension of each well was removed and diluted appropriately at 0, 2, 4, 8, 12, and 24 h, then plated on YPD agar plates and incubated at 30 °C until the colony could be seen and counted.

Chemical Analysis of the BD Bacto Peptone. The aqueous solution of BD Bacto Peptone was analyzed using an Agilent 1200 RP-HPLC system-MS and using an Atlantis HILIC Silica column (4.6 mm × 250 mm, pore size, 5 μm) with a linear gradient from 90% to 70% of acetonitrile. The column was eluted with the mobile phase at 1 mL/min for 30 min at room temperature, and the elution was monitored at 280 nm. The retention time (RT) alignment method was used in LC-MS profiles analysis. Background subtraction was accomplished by first detecting features in the LC-MS map of a blank sample, aligning these features with those in LC-MS maps of peptone samples, and then excluding any shared features between them.

^{1}H NMR spectral data were acquired with a Bruker Avance-500 spectrometer in D_2O.

Cytotoxicity Evaluation Using MTT Assay. The protocol was modified from ref 49. Briefly speaking, the testing cells were seeded in a 96-well flat-bottomed microtiter plate with 1×10^4 cells/well and allowed to adhere for 24 h at 37 °C in a CO_2 incubator. Cells were gently washed with fresh medium. Cells were then treated with various concentrations of the target compounds for 24 h under the same cultivation conditions. Cells were gently washed with fresh medium again. Subsequently, 10 μL of MTT working solution (5 mg/mL in phosphate buffer solution) was added to each well, and the plate was incubated for 4 h at 37 °C in a CO_2 incubator. The medium was then aspirated, and the formed formazan crystals were solubilized by adding 50 μL of DMSO per well for 30 min at 37 °C in a CO_2 incubator. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using the ELISA plate reader at 540 nm.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00448.

Figure S1, chemical structures of rapamycin and other nine peptide-like compounds used in Figure 2; Table S1, all laboratory *C. albicans* strains used in this study; Table S2, all clinical *C. albicans* isolates used in this study;

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Table S3, MIC data of rapamycin on drug sensitive clinical isolates with both YPD and RPMI 1640 media; Table S4, MIC data of rapamycin on drug resistant clinical isolates with both YPD and RPMI 1640 media; SI references (PDF)

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Author Contributions

Y.T., H.D., and L.Z. conceived the project. Y.T., J.Z., N.S., G.L., B.Z., F.S., J.Z., G.A., H.D., and L.Z. designed the experiments and analyzed the data. Y.T., J.Z., N.S., G.L., F.S., L.W., Q.W., H.H., X.C., Q.Z., and H.L. performed the experiments. Y.T. and L.Z. wrote the manuscript with input from the other authors.

Author Contributions

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MIC, minimal inhibitory concentrations; CLSI, Clinical and Laboratory Standards Institute; FIC, fractional inhibitory concentration; FKBP12, FK506-binding protein of 12 kDa; TOR, target of rapamycin; HPLC-MS, High Performance Liquid Chromatography−Mass Spectrometry; RVVC, recurrent vulvovaginal candidiasis

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