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天然素材 Tryptanthrin 對隱球菌的抗菌效果及細胞週期之影響

Tryptanthrin, a natural product, exhibits novel antifungal activity and cell cycle arrest against *Cryptococcus* species

張雅琳

Ya-Lin Chang

指導教授：陳穎練 博士

Advisor: Ying-Lien Chen, Ph.D.

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本論文係張雅琳君（R03633014）在國立臺灣大學植物病理與微生物學系完成之碩（博）士學位論文，於民國 106 年 02 月 20 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

似乎經歷了一個比其他人更長的碩士生涯，長到回到校園後每個人都疑惑你怎麼還在這。回想起來還是完成不少事，研討會、學術發表、論文口試、海外實習，因為有許多人的幫助才得以圓滿達成這些。當初念碩士班最自己最大的期許就是培養獨立思考與解決問題的能力，目標太抽象，至今不確定自己在經過碩士班訓練後是否有符合預期，畢竟研究從來不是獨立可以完成的；而問題也鮮少真的被完全解決，學術的道路太漫長，往往只留下更多需要被探究的未知。學到更多的是如何與人溝通，在每次討論後，能夠更務實的找出有價值的資訊，從中找到最適切的研究方向。

謝謝指導教授陳穎練老師，在這三年多的日子提供這麼多的協助，給我許多機會磨練，成就了一些，也搞砸了不少。謝謝口試委員，曾祥洸醫師、楊玉良老師、薛雁冰老師，對於我的研究成果給予的指導與肯定。謝謝 novel 305，你們給予的心靈上的支持與生活中的調劑永遠是讓人繼續前行的動力。謝謝家人，雖然總是難以與你們分享研究上的苦難與喜悅，一無所知仍無條件支持是讓我覺得自己一點也沒資格放棄的理由。

在臺大的日子終要告一段落，希望走過的日子不會白費，下一站，還要更努力。

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中文摘要

本研究致力於尋找對人類病原真菌念珠菌及隱球菌具抗真菌效果之活性物質，希望能找出具抑制能力或和現有藥物有協同作用的化合物。透過快速大量篩選小分子化合物，包括天然素材與美國食品藥品管理局核准之藥品，找出有抗真菌能力的化合物。從篩選結果中發現，來自蜜蜂腸胃道共生之放線菌所分泌的次級代謝物色胺酮 tryptanthrin 對隱球菌屬有抑制能力，對新型隱球菌的最小抑制濃度為 2 μg/mL，對格特隱球菌為 4 μg/mL。進一步實驗結果發現，tryptanthrin 與鈣調蛋白免疫抑制劑(FK506)有協同作用。Tryptanthrin 的抗隱球菌能力為劑量依賴性的，並且為抑菌型藥物而非殺菌型。我們也發現 tryptanthrin 在 37 ºC 時抑菌能力比 30 ºC 或 25 ºC 更佳。同時，tryptanthrin 對臨床抗藥性隱球菌菌株 T1 和 89-610 也具抑菌作用。為了找到 tryptanthrin 可能參與的藥物標靶或途徑，我們用藥物敏感性的測試篩選突變株庫。相較於野生株，49 個突變株對藥物更敏感或有抗性，其中有一部分突變株參與在細胞週期的調控。螢光激發流式細胞分選和基因表現定量結果顯示隱球菌的細胞週期調控可能和 tryptanthrin 呈現的抑制作用相關。綜合以上研究，天然素材 tryptanthrin 具有抗真菌能力並和 FK506 有協同作用，有潛力作為隱球菌症的治療策略，尤其是抗藥性菌株。

關鍵字: 色胺酮、隱球菌、天然素材、病原真菌、抗真菌藥劑

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ABSTRACT

This study aims to identify bioactive compounds that exert novel antifungal activity alone or exhibit synergistic effect with an existing antifungal agent against human fungal pathogens *Cryptococcus* and *Candida*. We screened several compound libraries including natural products, agricultural fungicides and FDA-approved drugs in order to identify compounds that exert antifungal activity. Among selected compounds, tryptanthrin secreted from *Nocardiopsis alba*, an actinobacterium existed in the intestine of honeybee, was chosen for further characterization because of its potent inhibition activity against *Cryptococcus* species with the minimal inhibition concentration (MIC) of 2 μg/mL for *C. neoformans* and 4 μg/mL for *C. gattii*. We further found that tryptanthrin showed synergistic effect with FK506, an immunosuppressant, based on broth dilution and checkerboard assays. Tryptanthrin inhibits the growth of *Cryptococcus* cells in a dose-dependent manner and shows fungistatic activity instead of fungicidal. We also found that tryptanthrin is more effective at 37 ºC compared with to 30 ºC or 25 ºC. Meanwhile, tryptanthrin demonstrated antifungal activity against two clinical azole-resistant *C. neoformans* isolates, T1 and 89-610. In order to identify potential targets or pathways that tryptanthrin involves in, we screened the *Cryptococcus* deletion mutant library by drug susceptibility test. Forty-nine deletion mutants were found to be more susceptible or resistant than that of the wild-type, and some of these mutants were involved in in cell cycle regulation. Fluorescence-activated cell sorting and gene

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expression quantification results support that cell cycle regulation in *C. neoformans* may be linked to tryptanthrin. In summary, the natural product tryptanthrin shows novel antifungal activity alone or in combination with FK506, leading possible therapeutic strategies for cryptococcosis caused by *Cryptococcus* species, especially the azole-resistant isolates.

Keywords: Tryptanthrin, *Cryptococcus*, Natural product, Fungal pathogen, Antifungal agent

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1. Introduction

1.1 Antifungal drug research and development

Approximately 1.2 billion individuals worldwide suffer from fungal infections, and the occurrence of these infections has significantly increased in recent years due to a rise in the number of immunocompromised patients, such as patients with AIDS or those with cancer, organ transplant, or autoimmune disease who require immunosuppressive therapy [1]. Unlike superficial infections that cause local, benign, or self-limiting diseases, invasive fungal infections (IFIs) are deep-seated and include bloodstream and systemic infections as well as infection of specific organs. IFIs are frequently caused by yeast pathogens such as *Candida* and *Cryptococcus*, filamentous fungi such as *Aspergillus*, *Fusarium*, or *Mucor*, or, less frequently, dimorphic fungi, including *Coccidioides*, *Blastomyces*, or *Histoplasma*. Currently, only three main classes of antifungals are approved for treatment of patients with IFIs: polyenes, triazoles, and echinocandins. These agents target ergosterol, lanosterol 14-α-demethylase, and β-1,3 glucan synthase, respectively [2]. Because our current antifungal therapies have problems like great efficacy but with significant toxicities, emergence of drug resistance and low potency against specific fungal pathogen, new drugs are needed to extend the limited antifungal arsenal [3].

1.2 *Cryptococcus neoformans*

The pathogenic yeast *Cryptococcus* species belong to Basidiomycota and are divergent from other human fungal pathogens *Candida* and *Aspergillus*, which belong to ascomycetous fungi. Two major *Cryptococcus* species including *C. neoformans* and *C. gattii* attribute to cryptococcosis in human. The virulence factors of the pathogen include the capability of survival in host temperature (37 ℃), the production of capsule and

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melanin which are specific structures for escaping from immune system and restricted environment [4]. Current studies also reported quorum sensing between fungal populations and unique changing in cell morphology as important virulence factors in host [5]. *Cryptococcus* spp. are ubiquitous in the environment and usually abundant in avian excreta and various trees with waxier cuticles [6]. Therefore, the epidemiology of cryptococcosis demonstrated the worldwide distribution of clinical cases. It is estimated that one million cases of cryptococcosis were diagnosed each year and over half of the patients were died from cryptococcal meningoencephalitis [7, 8]. Meanwhile, high mortality in different countries is noticed. Two main reasons contributed to the high mortality possibly include drug shortage in low-resources countries and strong association with human immunodeficiency virus infection [9]. Others people at risk are the immunocompromised patients who take immunosuppressive medications [9]. Hence, the adequate treatment and new antifungal for prophylaxis and management of cryptococcosis is important.

1.3 Currently used antifungal agents for *Cryptococcus*

C. neoformans is an opportunistic pathogen that colonizes in different parts of human body and is life-threatening if no appropriate therapeutic treatments performed. The pathogen infects individuals by inhalation into lungs and thus cause pulmonary cryptococcosis. Afterward, *C. neoformans* can spread quickly to other organs and disseminate to central nerve system and lead to overwhelming cryptococcal meningoencephalitis. Cryptococcosis is a challenging disease due to limited utilizable antifungal drugs. Currently, the practical guideline for management of cryptococcosis only articulate two regimens: fluconazole monotherapy and amphotericin B with flucytosine as combination therapy [10]. Besides,

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amphotericin B can cause severe nephrotoxicity, thus newer formulation liposomal amphotericin B is used when it comes to renal impairment patients. Fluconazole is a fungistatic agents widely used as an initial therapy. It is a safer and cheaper antifungal drugs compared with others. However, the cases of fluconazole resistance in patients with cryptococcal meningitis have more frequently been reported in Africa [11]. Due to the risk of terrible side effects and emergence of drug resistant isolates [12], more novel antifungal agents are urgent to be discovered and applied to the clinic.

Multiple approaches have been launched to discover lead compounds for antifungal investigation. We could follow the target-based or phenotype-based methods to find out the potential drug targets or the candidate compounds. Nowadays, several antifungal agents are in pre-clinical stages. T-2307, an arylamidines derivatives, has been reported its broad-spectrum antifungal activity [13, 14]. Structure of arylamidine is similar to pentamidine which is utilized in anti-parasitic and anti-pneumocystis. Though the modes of action are still unclear, the *in vitro* and *in vivo* results both demonstrated that T-2307 was more effective than the conventional echinocandins or polyenes against human fungal pathogens [14]. Besides, a series of 1,2-benzisothiazol-3(*2H*)-one derivatives (BZT) were also reported to exhibit antifungal activity. The mode of action of BZT was proposed by interfering fungal mitochondrial respiration system [13, 15]. In addition, a group of phosphoinositide dependent kinase inhibitors KP-372-1, OSU-03012 and UCN-01 have been discovered as potent antifungal compounds against *Candida* and *Cryptococcus* species [16]. BHBM and D0 have been demonstrated as new class of antifungal agents with the ability to target the fungal sphingolipids, GlcCer [17]. It has been demonstrated that these compounds exhibit antifungal activity against wide ranges of human fungal pathogens and are highly effective in *in vivo*

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test.

Repurposing of off-patent drugs is another strategy for antifungal discovery. Zhai et al. screened the Johns Hopkins Clinical Compound Library and found an early stage antibiotics, polymyxin B, for Gram-negative bacterial infection and later for, human fungal infections [18, 19]. Furthermore, polymyxin B is synergistic with fluconazole and exhibits fungicidal effect at lower concentration. Other repurposing cases such as tamoxifen and toremifene, acted as estrogen receptor antagonists in breast cancer therapy, have been found to exhibit anti-cryptococcal activity [20]. Miltefosine which is a medication for treating leishmaniasis was reported its *in vitro* activity against *Cryptococcus* species [21]. The miltefosine analogs also exhibits broad-spectrum activity to human fungal pathogen [22]. While the *in vivo* test of miltefosine and its analogs revealed unsuccessful efficacy. In spite of the fact that more and more antifungal agents have been discovered and identified, none of them represents as a new class of antifungal agent approved in pharmacy market yet.

1.4 Natural product tryptanthrin

Natural alkaloid tryptanthrin is a yellow needle-like crystal which could be purified from several natural sources or be synthesized through organic procedure. Tryptanthrin sublimated from a plant *Indigo* is first identified in 1879 by Sommargua [23, 24]. Afterward, it is denominated tryptanthrin due to the high production of this compound by culturing *Candida lipolytica* in tryptophan-contained media [25, 26]. The empirical formula of tryptanthrin is $C_{15}H_8N_2O_2$ (M.W. = 248.24) and its chemical structure is planar. Tryptanthrin could be isolated from many organisms, from microorganism *Schizophyllum commune, Leucopaxillus cerealis* to higher plants *Isatis indigotica, Polygonum tinctorium*

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and *Wrightia tinctoria*. In this study, we identify tryptanthrin from the secondary metabolites of *Nocardiopsis alba*, an actinobacterium existed symbiotically in the intestine of honeybee. Tryptanthrin has been mentioned to its various medical activities, including anti-microbial, anti-trypanosomal, anti-inflammatory and anti-cancer properties.[25, 27, 28] Tryptanthrin possesses anti-bacterial activity against both Gram positive and negative bacteria including *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Porphyromonas gingivalis* and methicillin-resistant *Staphylococcus aureus*. It could inhibit the growth of different pathogenic protozoa such as *Trypanosoma brucei*, *Leishmania donovani* and *Plasmodium falciparum*. Otherwise, tryptanthrin inhibits cellular expression of inflammation-related enzyme COX-2 and 5-LOX. Tryptanthrin exhibits various anti-cancer activity via decrease the secretion of growth factor, inhibition of multidrug resistance gene, cytotoxicity to specific cancer cells. All of the above demonstrated that tryptanthrin is a multifunction bioactive compound potential for pharmacological use. But little of studies reported that tryptanthrin also exhibits anti-fungal activity [29, 30]. Few studies described that tryptanthrin exhibits antifungal activity to topical infection fungus such as *Trichophyton mentagrophytes* and *Malassezia furfur* [31, 32]. As to systemic infection fungus, none of the report has mentioned tryptanthrin exhibits antifungal activity.

Studies about the mechanism of tryptanthrin bioactivity are still finite and incomplete. Bandekar et al. indicated that tryptanthrin inhibits the growth of *Escherichia coli* by DNA intercalation [33]. On the other hand, few studies reported that tryptanthrin exhibits anti-cancer activity by arresting cell cycle at G_0/G_1 phase and inhibiting the proliferation on myeloid leukemia and neuroblastoma cells [34-36]. Conversely, tryptanthrin exhibits anti-angiogenesis by arresting cell cycle at G_2/M phase [37]. However, the definite drug targets or

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associated pathways that tryptanthrin interacts with remain elusive [38].

2. Materials and methods

2.1 Strains, media and chemicals

Fungal strains used in this study were listed in Table 1. YPD (1% yeast extract, 2% peptone and 2% glucose) and RPMI-1640 medium (Sigma) buffered to PH 7.0 with 0.2% glucose and 0.165 M MOPS were used in this study. Compound libraries (Selleckchem Chemicals) of natural products, FDA-approved drugs were used for antifungal activity screening. Tryptanthrin used in this study was obtained from the laboratory of Dr. Yu-Liang Yang in Academia Sinica. FK506 (Astellas Pharma Inc.), fluconazole (FLC, Bedford Laboratories), amphotericin B (AmB, Sigma), rapamycin (Selleckchem Chemicals) were used for drug susceptibility test and cell cycle analysis.

2.2 Drug library screening

C. albicans and *C. neoformans* cells were grown overnight at 30 ºC then washed twice with sterile water. The concentration of cells suspension was adjusted to 0.0005 OD/mL in RPMI-1640 medium. 98 μL of the strain culture was added to each well in a 96 well plate. 2 μL of drug library compounds were added into the indicated wells to 10 μM in final volume. The plates were incubated at 35 ºC for 24 or 48 hr. The results were read visually. The potential candidates with obvious antifungal activity were chosen for further drug susceptible test.

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2.3 Disc diffusion assay

Yeast cells were grown overnight at 30 ºC, and 0.1 OD_{600} (in 100 μL) was spread on the surface of YPD medium. Paper discs (6 mm) were placed on the surface of the medium, and 12.5 μL of tryptanthrin (25 μg) and 5 μL of DMSO (as a control) were added to each disc. The plates were incubated at 37 ºC for 48 hr and photographed.

2.4 Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

We determined MIC by the Clinical and Laboratory Standards Institute (CLSI) protocol M27-A3. Testing was done in RPMI-1640, buffered to pH 7.0 with 0.165 M MOPS. Yeast strains were grown in YPD medium overnight, incubated at 30 ºC with shaking, and washed twice with ddH_2O. The OD_{600} was measured and each strain was diluted to 1 OD_{600} /mL. This inoculum was diluted to 0.0005 OD_{600} /mL in RPMI-1640. 98 μL of the strain culture was added to each well in a 96 well plate format. 2 μL of serially diluted drugs were added to the wells, yielding a final volume of 100 μL per well. The concentration of tryptanthrin which ranged from 0.25–64 μg/mL was added across the plate with the highest to the lowest from the left to the right well. The plates were incubated for 48 hr at 35 ºC, and OD_{600} value of each plate was read by spectrophotometer. The *in vitro* drug studies were performed at least twice. The minimum inhibitory concentration (MIC_{100}) of drug was defined as the clear well with the lowest concentration of each drug. The minimum fungicidal concentration were determined by transferring 3 μL of solution from indicated well to drug-free YPD plates. The viability of fungus cells was checked after the plates were incubated for 48 hr at 30 ºC.

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2.5 Fractional inhibitory concentration assay for antifungal activity

Fractional inhibitory concentration (FIC) was assessed via checkerboard titration assays. The inoculum were prepared as MIC method. Tryptanthrin was added across the plate with the highest concentration in the left well and the lowest concentration in the right well. The complement antifungal agents FK506, fluconazole or amphotericin B with concentration ranged from 0.25-16 μg/mL was added from top to bottom, with the highest concentration in the top row and the lowest concentration in the bottom row. This manipulation demonstrated 70 different combined drug concentration to be tested on one plate. The plates were incubated for 48 hr at 35 ºC and the MIC value was based on spectrophotometric determination at OD_{600} comparing to the control well. The FIC was calculated by: (MIC combined drug A/MIC alone drug A) + (MIC combined drug B/MIC alone drug B). A FIC index of < 0.5 indicates synergy, > 4.0 indicates antagonism, and an index between 0.5 and 4 indicates no interaction.

2.6 Growth kinetics assay

Cells were grown overnight at 30 ºC and washed twice with sterile water and then diluted to 0.0005 OD/mL with fresh YPD broth. 10 mL of mixtures were incubated at 37 ºC (200 rpm) with 1, 2, 4 and 8 μg/mL tryptanthrin. The cell density were measured after 0, 4, 8, 12, 24 and every 12 hr later using SpectraMax190 microplate reader (Molecular Devices). The experiments were performed in triplicate and the results were plotted using Prism 5.03.

2.7 Serial dilution growth assay

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Cells were grown overnight at 30 ºC and washed twice with sterile water. The optical density of cells was measured and then the fungus cells were diluted to 1 OD_{600}/mL in sterile water. 5-fold serial dilution of each strain was accomplished in 96-well microplate. 3 μL of serial diluted suspension was spotted onto the YPD soli agar. The plates were incubated at 25, 30 and 37 ºC for 72 hr and photographed.

2.8 Mutant library screening

The *C. neoformans* mutant library was used in the fitness test. This deletion mutant library contains 1201 genes knockout strains and cover ~30% genes in *C. neoformans* [39]. Mutants were recovered from frozen stock to liquid YPD medium and incubated at 30 ºC for 48 hr. Cells were washed twice with sterile water. Mutants in each well were diluted to 0.05 OD/mL and transferred by pin replicator to one well YPD-agar plate without or with tryptanthrin (1, 2 or 4 μg/mL). Plates were incubated at 35 ºC for 48 hr. Mutants that displayed growth defect in plate with 1 μg/mL tryptanthrin were defined as hypersensitive (HS) and mutants which could be able to grow on YPD plate containing 4 μg/mL tryptanthrin were considered as resistant (R).

2.9 Fluorescence-activated cell sorting assay

Yeast cells treated with 0, 1, 2, 4, 8 μg/mL tryptanthrin for 12 hr were harvested and washed twice with PBS. Cells were fixed in 70% ethanol overnight at -20 ºC. Cells were centrifuged at 850 g for 5 min, washed twice with PBS and later re-suspended in 1 mL Tris/NaCl/MgCl2 buffer (200 mM Tris PH7.5, 211 mM NaCl, 78 mM $MgCl_2$).RNase was added to final concentration of 100 μg/mL and incubated at 37 ºC for 2 hr. Propidium

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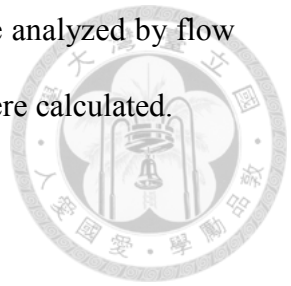
iodide was added to final concentration of 5 μg/mL. The samples were analyzed by flow cytometry (Beckman Coulter FC500) and percentages of each phase were calculated.



2.10 Quantitative real-time RT-PCR

Strains were grown overnight at 30 ºC and washed twice with sterile water. The yeast cells were diluted to 0.02 OD/mL in 50 mL fresh YPD containing indicated compounds. Cells were harvested after 12 h incubation at 37 ºC (200 rpm), washed twice and lyophilized overnight. The total RNAs were extracted using TRIZOL (Thermo Fisher Scientific) and chloroform (Fisher Scientific). The RNA was precipitated in pre-cooled isopropanol, washed by 75% ethanol and finally eluted in RNase-free dH_2O. Total RNA were evaluated the quality and quantity by RNA electrophoresis and Nanodrop spectrophotometer. The qualified RNA was treated with TURBO DNA-free Kit (Invitrogen) to remove the remaining genomic DNA. The DNase-treated RNA was reverse-transcribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR reactions of 20 μl included 6 μl cDNA (6 ng), 10 μl of 2x qPCR master mix (Fast SYBR Green Master Mix; Applied Biosystems), 2 μl of 2.5 μM forward primer and 2 μl of 2.5 μM reverse primer. Primers were designed using Primer 3 (Applied Biosystems) and listed in Table 5. Quantitative PCR conditions were shown below: 95°C /10 min for denaturation; 95°C /3 sec, 60°C /30 sec (40 cycles); 95°C /15 sec, 60°C /60 sec, 95°C /15 sec (melting curve). The StepOnePlus System and StepOne v2.2 (Applied Biosystems) were used to determine the cycle threshold (Ct) and the relative expression levels were calculated based on 2 -ΔΔCt. The bar graphs of *ACT1* normalized relative quantity compared with drug-untreated samples were created with Prism 5.03. The P-values between samples were

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determined by using unpaired t test.

2.11 Murine model and antifungal treatment

Four-week male outbred ICR mice (BioLasco Co. Ltd, Taiwan) were utilized in this study. Mice were housed five mice per cage and maintained with sufficient food and water until weighing approximately 27 g. To establish disseminated cryptococcosis, mice were inoculated intravenously via the lateral tail vein with *C. neoformans* (H99). *C. neoformans* were grown in 10 mL YPD medium overnight at 30 ºC. The cells were washed twice with sterile water, counted with hemocytometer and adjusted the concentration to obtain an infection inocula concentration of 5 x 10^6 cells/mL. 200 μL were inoculated in mice by lateral tail vein injection. The inocula were appropriate diluted and spread on YPD medium for confirmation of viability. Antifungal treatment with tryptanthrin was initiated at 4, 24, 48 and 96 hr post inoculation at doses 2, 8, 32 mg/kg of body weight by oral gavage or intraperitoneal injection. Tryptanthrin were dissolved in DMSO and prepared to indicated concentration with phosphate buffered saline (DMSO: PBS=1:9, DMSO should not above 10% of the final volume).

3. Results and discussion

3.1 Screening results of compound libraries

The high-throughput screening of nearly 1060 natural products and FDA-approved drugs provided several compounds with novel antifungal activity against *C. neoformans* (H99) and *C. albicans* (SC5314) (Fig. 1). Screening ~140 natural products, 12 compounds with antifungal activity against *C. neoformans* (H99) and *C. albicans* (SC5314) were identified

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and four of them including sclareol, azomycin, parthenolide and tryptanthrin have not been reported. These four compounds could inhibit the growth of *Cryptococcus neoformans* but not *Candida albicans*. Meanwhile, 61 FDA-approved drugs demonstrated anti-*Candida* or anti-cryptococcal activity and five of them such as carmofur, ponatinib, eltrombopag, penfluridol and otilonium bromide were unpublished.

3.2 Tryptanthrin exhibits novel antifungal activity against *Cryptococcus* species

Several compounds including tryptanthrin were selected for further investigation based on the results of the high-throughput screenings. We validated the antifungal activity of tryptanthrin against *C. neoformans*. The disc diffusion assay indicated that tryptanthrin has antifungal activity against *C. neoformans* and *Saccharomyces cerevisiae*, but not *Candida albicans* (Fig. 2). The obvious inhibition zone produced by tryptanthrin could be seen in *C. neoformans*. However, tryptanthrin showed a slight inhibition against S. *cerevisiae*. Therefore, we focused on the anti-cryptococcal activity of tryptanthrin. In *C. neoformans*, we first tested serotype A strain, which is the most widespread strain causing human fungal disease [40]. Serotype D, a lesser virulent strain and causing fewer cases of cryptococcosis than serotype A, was also tested [41, 42]. The antifungal susceptibility test via broth ilution assays showed that tryptanthrin exhibited equivalent growth inhibition for both A and D serotypes (Table 1), suggesting no serotype susceptibility differences in response to tryptanthrin and it is consistent to other antifungal drugs [43]. In *Cryptococcus gattii*, we have tested three strains including two isolates (R265 and R272) from Vancouver Island outbreak and one isolate (WM276) from the environment (Table 1). The MIC values for *C. neoformans* and *C. gattii* is 2 μg/mL and 4 μg/mL, respectively. The MFC values of *C.*

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neoformans and *C. gattii* strains were all above 64 μg/mL, indicating tryptanthrin may be a fungistatic agent. The growth kinetic assay of *C. neoformans* in different concentration demonstrated that tryptanthrin exhibits antifungal activity at 2 μg/mL and in a dose-dependent manner (Fig. 3). Besides, *C. neoformans* continued to grow at 2 μg/mL after incubating for 60 hr but still inhibited at 4 μg/mL. These result may resulted from different nutrient conditions. Nevertheless, the growth curve indicated that tryptanthrin exhibits potent growth inhibition activity against *C. neoformans* but cannot kill the fungal cells.

Emergence of drug resistant isolates is a serious problem in clinical therapy and leads to treatment failure. Thus finding a potential antifungal agent that could conquer clinically drug-resistant isolates is important. Surprisingly, the MIC value for tryptanthrin against *C. neoformans* T1 and 89-610, azole-resistant isolates, is 2 μg/mL (Table 3), which is the same as the azole sensitive *C. neoformans* (H99), leaving a hint that tryptanthrin may target *C. neoformans* via a pathway distinct from ergosterol biosynthesis pathway. Nevertheless, it needs more clinical isolates from different sources for antifungal susceptibility assay to validate the potency of tryptanthrin. Our results suggested that tryptanthrin revealed a narrow-spectrum antifungal activity due to ineffective inhibition activity against other human fungal pathogen such as *Aspergillus fumigatus*, *Fusarium solani*, *Candida krusei*, and *Candida parapsilosis*. Interestingly, tryptanthrin exhibits antifungal activity against *Trichophyton rubrum*, a filamentous fungus causing onychomycosis, as well as the previous study in 1979 against *Trichophyton mentagrophytes* (Table 3) [32].

3.3 Growth of *C. neoformans* is sensitive to tryptanthrin at 37 ºC but not at 25 ºC

C. neoformans is capable to grow at the human body temperature of 37 ºC which is an

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important virulence factors. To determine the impact of tryptanthrin on thermotolerance of *C. neoformans*, we tested the susceptibility of drug at different temperatures by disc diffusion and spotting assay. The results demonstrated that *C. neoformans* is more sensitive to tryptanthrin at higher temperature (Fig. 4). The agar plate incubated at 37 ºC exhibits a clear inhibition zone and the MIC value is 1 μg/mL which reveals great antifungal activity. Interestingly, the disc diffusion assay on solid agar demonstrated slightly inhibition of growth at 30 ºC and 25 ºC but tryptanthrin could still exhibit antifungal activity in liquid medium (Fig. 4). These results may be caused by the differences between liquid and solid-based test [44]. The spotting assay demonstrated the similar results as shown above (Fig. 5). In solid YPD agar plate containing tryptanthrin (2 μg/mL), *C. neoformans* could hardly grow at 37 ºC but appeared a normal growth at 25 ºC. Otherwise, *C. gattii* demonstrated increased sensitivity to tryptanthrin at higher temperature as well. Multiple genes involve with stress responses were up-regulated at high temperature for altering the tough conditions [45]. FK506 is an example of antifungal agent that targets calcineurin which requires for fungal growth at 37 ºC. It demonstrated toxicity to *C. neoformans* at 37 ºC but not at 24 ºC [46]. In addition to calcineurin, pathways such as Ras, HOG and MAPK are also play the role in temperature-sensing signaling [47]. Therefore, we hypothesized that tryptanthrin may involve in specific pathways essential at high temperature.

3.4 Tryptanthrin exhibits synergistic activity with the calcineurin inhibitor FK506 against *Cryptococcus* species

Combination therapy is a strategy that aim to enhance the clinical outcome by blocking the drug resistance, declining dosage of drugs and improving drug efficacy [48]. Therefore, we

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tested the interactive effects between tryptanthrin and current antifungal agents for cryptococcosis treatment. Our results suggested that there is no interaction between tryptanthrin and fluconazole or amphotericin B against the tested *Cryptococcus* species (Table 3). Surprisingly, the calcineurin inhibitor FK506, which can inhibit the growth of *C. neoformans* at 37 ºC, exhibits synergistic activity with tryptanthrin against *C. neoformans* H99 and *C. gattii* R265 and R272 (FIC<0.5; Table 3) [49]. FK506 has been reported of the synergistic effect with bafilomycin A_1 or fluconazole against *C. neoformans* and with posaconazole against *C. albicans* [50, 51]. We also tested another calcineurin inhibitor, cyclosporine A, by disc diffusion assay, but cyclosporine A has no synergy with tryptanthrin (Fig. 6A) [52]. As the evidence of synergistic effect between tryptanthrin and FK506, we further investigated that whether this combination may transform fungistatic activity of tryptanthrin into fungicidal effect. However, the medium transferring from the checkerboard onto drug-free YPD solid agar has grown into colonies and this result indicated that the combination treatment cannot kill cryptococcal cells (Fig. 6B).

3.5 Fitness test of *Cryptococcus* deletion mutant library

The modes of action for each lead compound should be heavily focused on during the drug development and targets at some elsewhere in fungus cell would be a pioneering innovation for antifungal agents [53]. We utilized the deletion collection mutants of *C. neoformans* to identify their susceptibility to tryptanthrin and aim to find the direct or indirect clues for drug target identification. In these fitness assay conditions, we defined mutants unable to grow on the YPD plate with 1 μg/mL tryptanthrin as hypersensitive and able to grow on YPD plate with 4 μg/mL as resistant strains. Our results demonstrated 49 mutants revealed

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differential drug susceptibility; 27 of them were resistant and 22 were hypersensitive. Microplate antifungal susceptibility assays were used to verify the fitness results from agar plates. The final results were classified by the function of the deleted mutants. A category of genes were identified and the may related directly or indirectly to the pathway interfering by tryptanthrin (Table 4). We noticed that a group of cell cycle-related genes such as *RAD53*, *SSN801*, *MLN2*, *BUB1* (CNAG_03184), *ACP2* (CNAG_03900), and *KIP2* (CNAG_06335).

3.6 Tryptanthrin exhibits G_1/S cell cycle arrest in *C. neoformans*

Based on the fitness test above, we suggest that the variation of susceptibility in mutants compared with wild-type may reflect cell cycle dysfunction in tryptanthrin-treated cells, thus the following experiments related cell cycle analysis were conducted. The DNA contents of each single cell were measured by fluorescence-activated cell sorting (FACS) assay and the distribution of cell cycle were plotted. Our results showed that tryptanthrin exhibits a dose dependent manner in arresting the G_1/S phase of *C. neoformans* (H99). The percentage of G_2 was declined when the concentration of tryptanthrin increased (i.e. 8 μg/mL) in culture medium (Fig. 7). Meanwhile, rapamycin which induces G_1 arrest in yeast were used as a positive control, and it demonstrated a pattern similar to tryptanthrin (Fig.7 and Fig. S1A) [54]. Surprisingly, fluconazole also exhibits G_1 arrest as well as tryptanthrin (Fig. S1A). These results suggested that arresting in cell cycle of *C. neoformans* may occur when treating with fungistatic agents. By treating with fungistatic agents, cells were impeded to enter the G_2 phase and thus the budding process were inhibited [55].

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3.7 Cell cycle associated genes were up-regulated after treating with tryptanthrin

Due to the fact that tryptanthrin blocks cell cycle of *C. neoformans* in G_1/S phase, we further examine whether the regulation of cell cycle was interfered by tryptanthrin. We analyzed the mRNA expression of genes associate with the regulation of cell cycle, especially focused on the G_1 and G_1/S transition genes. Tryptanthrin exerted a dose dependent effect on the mRNA levels of *CLN1*, *PCL103*, *WHI5*, *MBS1*, CNAG_00995 and *ERG11* (Fig. 8B). Cln1 and Mbs1 have been reported to play important roles on cell cycle progression in *C. neoformans* [56]. Mbs1 is a transcription factor homologous to the yeast Mbp1 and Swi4 [57]. Proteins Mbp1 and Swi4 in *S. cerevisiae* form the MBF and SBF complex with Swi6 and involve in the G_1/S transition by activating the G1 cyclins, Cln1 and Cln2 [58]. Our data suggested that tryptanthrin blocks the cell cycle in G_1 phase and therefore causes the up-regulated the expression of G_1/S transition genes. CNAG_00995 encoded a hypothetical protein that predicted to function in meiotic recombination was also up-regulated [54, 59]. Since fluconazole and rapamycin revealed the cell cycle arrest effect in G_1/S phase, as well as tryptanthrin, we wondered the mRNA expression of *ERG11* and *TAP42* which associated with ergosterol biosynthesis and TOR pathway, target of fluconazole and rapamycin, respectively. Interestingly, *ERG11* was up-regulated after tryptanthrin treatment and is similar to the results of fluconazole [59]. Conversely, expression of *TAP42*, encodes an essential protein implicated with TOR pathway, was not affected by tryptanthrin [60]. Our data suggested that tryptanthrin and fluconazole may regulate cell cycle in a similar way than rapamycin even though these three fungistatic agents share the same pattern in cell cycle analysis.

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3.8 Limited anti-cryptococcal activity of tryptanthrin in murine infection model

To evaluate the application potential of tryptanthrin, we tested the efficacy of tryptanthrin against disseminated cryptococcosis. The intravenous injection of *C. neoformans* in murine model could simulate the situation of cryptococcosis. Tryptanthrin was treated 4 hr post-inoculation and once-daily dosing for three days. Due to the uncertainty of the best regimen for the delivery of tryptanthrin, we delivered tryptanthrin via oral gavage or intraperitoneal injection (IP). As the results of two delivery strategies, tryptanthrin did not improve the survival rate at dose of 2, 8 or 32 mg/kg as compared to PBS control group (Fig. 9). In the oral gavage administration group, every tryptanthrin-treated group died earlier than PBS group, indicating the potential toxicity of tryptanthrin to mice during cryptococcal infection. Furthermore, we found that mortality rate of tryptanthrin at 32 mg/kg dose at the fastest pace and the *P* value is 0.0313 (Log-rank test) as compared with the PBS group. In the IP administration group, all of the drug treatment group demonstrated no anti-cryptococcal activity and had equivalent survival rate. Our results indicated that high dose of tryptanthrin administration via oral gavage may cause negative effect in murine infection model.

The failure of tryptanthrin therapeutic test in murine model may be due to three possible disadvantages of tryptanthrin as an antifungal agent in animal bodies. First, tryptanthrin was slightly precipitated and crystallized in PBS solvent, which may cause a difficulty in diffusion of tryptanthrin to the bloodstream. Second, *C. neoformans* could transmigrate into brain of murine model in 24 hr post-inoculation and it is the major cause of fatality[61]. Jähne et al. indicated that tryptanthrin has high blood-brain barrier penetration activity based on the *in vitro* human and animal models [62]. Nevertheless, an *in vivo* pharmacokinetic study of tryptanthrin in rats reported that tryptanthrin remained in liver the most and the

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longest time, but did not detect any in brain [63]. Thus, the inefficacious therapeutic results may be attributed to the difficulty of tryptanthrin crossing blood-brain barrier. Third, on the other hand, the toxicity and side effect of tryptanthrin could also be concerned.

4. Conclusion

This study demonstrated that tryptanthrin exhibits anti-cryptococcal activity against *C. neoformans* and *C. gattii* which has not been reported in the past. It also exhibits antifungal activity against clinical azole-resistant isolates. We found the synergistic effect between tryptanthrin and FK506. Though the direct tryptanthrin-involved pathways has not been identified, we proved that the cell cycle regulation is interfered by tryptanthrin and cause cell cycle arrest in G_1/S phase. The phenomenon of cell cycle arrest and the change in mRNA expression of cell-cycle related genes may attribute to other upstream pathways or transcription factors. Besides, we also tested the potential therapeutic efficacy in murine model. In further experiments, we could modify its chemical structure or invent a better formulation to make tryptanthrin an utilizable natural product.

5. Future work

Our data suggested that tryptanthrin exhibits anti-cryptococcal activity *in vitro*, but only few isolates were tested. If more clinical isolates could be obtained and tested, the antifungal activity against *Cryptococcus* would be more solid. We found that tryptanthrin is synergistic with FK506 *in vitro*. Meanwhile, FK506 is an immunosuppressant which was reported its drawback outweighed antifungal activity *in vivo*. The combination therapy of tryptanthrin and FK506 may improve the efficacy. As to the toxicity of tryptanthrin in mice,

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we may conduct toxicology assay in mammalian cell line. In addition, the derivatives of tryptanthrin could also be tested for antifungal activity. Derivatives with great solubility and better potency is our expectation. We have verified the effect of tryptanthrin on cell cycle related genes in *C. neoformans*, the further confirmation related to the changes in protein level is needed. Hopefully a clarified pathway of the growth inhibition by tryptanthrin can be established.

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Table.1 Fungal strains used in this study.

Strain	Description	Background	Reference
Cryptococcus neoformans			
H99	Wild-type MATa, serotype A	Clinical isolate	64
JEC21	Wild-type MATa, serotype D	Clinical isolate	65
T1	fluconazole resistant	Clinical isolate	66
89-610	fluconazole resistant	Clinical isolate	66
Cryptococcus gattii			
R265	Wild-type VG IIa MATa, serotype B	Clinical isolate	67
R272	Wild-type VG IIb MATa, serotype B	Clinical isolate	67
WM276	Wild-type VG I	Environmental isolate	68
Candida albicans			
SC5314	Wild-type	Clinical isolate	69
Candida krusei			
ATCC 6258	Wild-type	Clinical isolate	70
Candida parapsilosis			
ATCC 22019	Wild-type	Clinical isolate	70
Saccharomyces cerevisiae			
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Laboratory strain	71
Aspergillus fumigatus			
AF293	Wild-type	Clinical isolate	72
Trichophyton rubrum			
MYA-4438	Wild-type	Clinical isolate	73
Fusarium solani			
Fungus III-6	Wild-type	Clinical isolate	

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Table 2. Minimum inhibitory concentration of tryptanthrin against human fungal pathogen.

Strain	MIC_{100} (μg/mL)	MFC (μg/mL)
C. neoformans (4)	2	4 - >64
C. gattii (3)	4	>64
C. albicans (1)	>64	-
C. krusei (1)	>64	-
C. parapsilosis (1)	>64	-
A. fumigatus (1)	>64	-
F. solani (1)	>64	-
T. rubrum (1)	8	32

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Table 3. Fractional inhibitory concentration of tryptanthrin with FK506, fluconazole (FLC) or amphotericin B (AmB).

	MIC				MIC combined			FIC index		
Strain	**TRP**	**FK506**	**FLC**	**AmB**	**(TRP;FK506)**	**(TRP;FLC)**	**(TRP;AmB)**	**(TRP; FK506)**	**(TRP; FLC)**	**(TRP; AmB)**
Cryptococcus neoformans										
H99	2	>16	16	2	0.25; 0.03	1; 8	1; 1	**0.125**	1	1
T1	2	>16	>32	0.5	1; 0.12	2; 0.06	1; 0.5	0.503	1	1.5
89-610	2	>16	32	1	1; 0.12	2;0.06	1; 0.5	0.503	1	1
Cryptococcus gattii										
R265	4	>16	16	2	1; 0.03	4; 0.06	2; 1	**0.251**	1	1
R272	4	>16	>32	0.5	0.25; 0.06	4; 0.06	2; 0.5	**0.062**	1	1.5
WM276	4	>16	32	0.5	2; 0.03	4; 0.06	2; 0.5	0.501	1	1.5

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Table 4. Deletion mutants of *C. neoformans* with altered susceptibility to tryptanthrin.

deletion mutant CNAG_*****	*C.n.* name	homolog	fitness	description of function
Enzymes				
03981		*PFA4(S.c.)*	HS	palmitoyltransferase PFA4
01232	*PMC1*		HS	calcium-translocating P-type ATPase, PMCA-type
03525	*NTH2*		R	alpha,alpha-trehalase
04735	*MEP1*		R	metalloproteinase
Metabolism				
01981		*HMT2(S.p.)*	HS	sulfide:quinone oxidoreductase
02833			R	NADH dehydrogenase (ubiquinone) subcomplex 7
05540	*URE1*		R	urease
07180			R	NAD-dependent histone deacetylase
Transporter				
04704		*JEN1(S.c.)*	R	MFS transporter, SHS family, lactate transporter
03664	*NIC1*		R	high-affinity nickel-transporter
05330		*MPH2(S.c.)*	R	MFS transporter, SP family, glucoside: H+ symporter
05812		*KHA1(S.c.)*	R	potassium: hydrogen antiporter
Cell cycle				
03184		*BUB1(S.p.)*	R	BUB protein kinase
04162	*PKA2*		R	AGC/PKA protein kinase
05216	*RAD53*		R	non-specific serine/threonine protein kinase
03900		*ACP2(S.p.)*	R	F-actin-capping protein beta subunit
06335		*KIP2(S.c.)*	R	centromeric protein E
MAPK signaling pathway				
04090	*ATF1*		R	activating transcription factor
03938	*CPR2*		R	pheromone a factor receptor
02531	*CPK1*		R	mitogen-activated protein kinase
Transcription				
04545		*LSG1(S.p.)*	R	CTD kinase subunit gamma
00440	*SSN801*		R	cycin C /Cyclin subunit of Mediator subcomplex
01217		*MPE1(S.c.)*	R	protein MPE1
05221		*HTZ1(S.c.)*	R	histone H2A.Z
00073		*FAR8(S.p.)*	R	nuclear mRNA splicing protein
02215	*HAP3*		R	transcriptional activator
01551	*GAT201*		R	transcription factor
03409	*SKN7*		R	osomolarity two-component system, response regulator
Nucleus				
06224	*MLN2*		HS	nuclear movement protein nudC
01747	*PMS1*		R	DNA mismatch repair protein PMS1

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05301	*CRN1*	R	deoxyuridine 5'-triphosphate nucleotidohydrolase
02090	*GPA3*	R	guanine nucleotide-binding protein subunit alpha
00445	*NHP6B01*	R	non-histone chromosomal protein
Ubiquitin system			
03151	*CSN4*	HS	COP9 signalosome complex subunit 4
05765	*UBC6*	R	ubiquitin--protein ligase
04208		R	Ataxin-3

doi:10.6342/NTU201704288

Table 5. Oligonucleotide primers used in this study.

Primer	Use	Sequence (5’ →3’)
JC1052	*C.n. ACT1*	CCACACTGTCCCCATTTACGA
JC1053	*C.n. ACT1*	CAGCAAGATCGATACGGAGGAT
JC1054	*C.n. CLN1*	CATTCCCATAGCATTGATTTCCT
JC1055	*C.n. CLN1*	TTCGGACATCCCATACCATGAT
JC1363	*C.n. CNAG_00995*	CGCCACTTCTGCTCCTTACCTT
JC1364	*C.n. CNAG_00995*	TAGTCTCTCGGATGGCAGCAAG
JC1367	*C.n. MBS1*	CGCCCGAATGTATGTGGTATGT
JC1368	*C.n. MBS1*	TTGTCGAAACCGGCTACTTTCA
JC1411	*C.n. ERG11*	AACTTGCCCCTTCCCAGTTACA
JC1412	*C.n. ERG11*	CTTGCAGCTCTGGAGGTTTTCA
JC1415	*C.n. PCL103*	TGTTTCCGGTTAGCGAGATCAA
JC1416	*C.n. PCL103*	CAAGAACGGTTGGAAGTTGTCG
JC1417	*C.n. WHI5*	TGCATCAACTGCAACAACGACT
JC1418	*C.n. WHI5*	TTGAAAGGGAGTCGAAGGAAGC
JC1413	*C.n. TAP42*	TGTCTGCTTCAACTCCCGTCTC
JC1414	*C.n. TAP42*	TCTTCCGCCATGTCATCTTCAT

doi:10.6342/NTU201704288

Figure 1

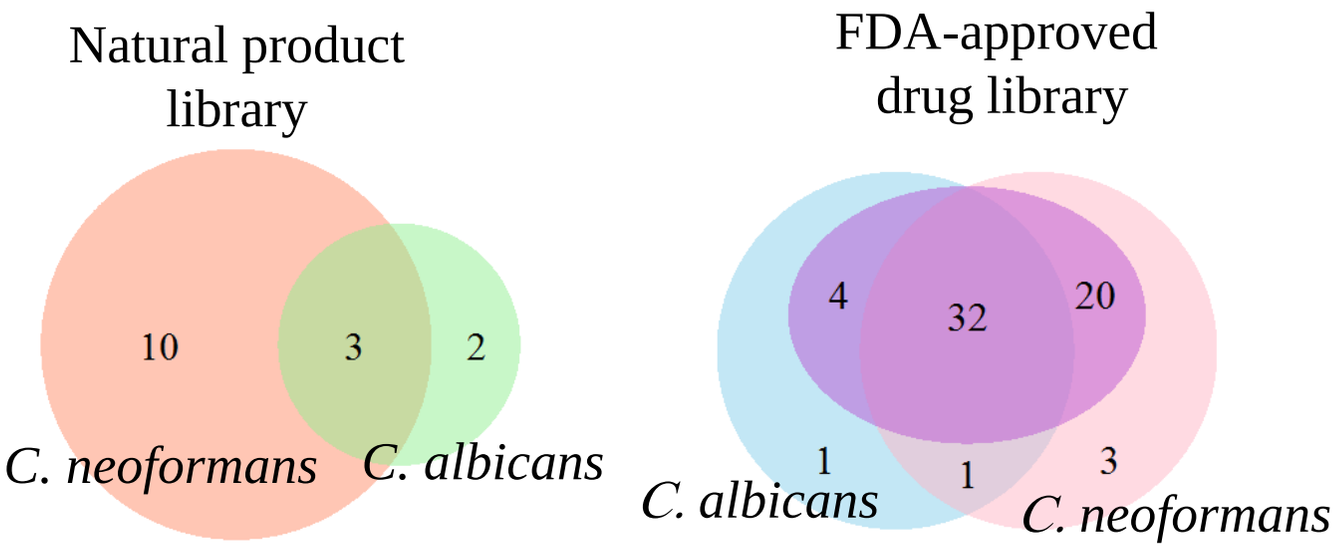

Figure 1. Screening results of the compounds libraries against *Cryptococcus neoformans* and *Candida albicans*. 12 of the natural products and 61 of FDA-approved drugs exhibit antifungal activity. The orange and pink charts indicated compounds with anti-cryptococcal activity. The green and blue charts revealed compounds with anti-*Candida* activity. The purple chart represents the compounds which have been reported their anti-fungal activities.

Figure 2

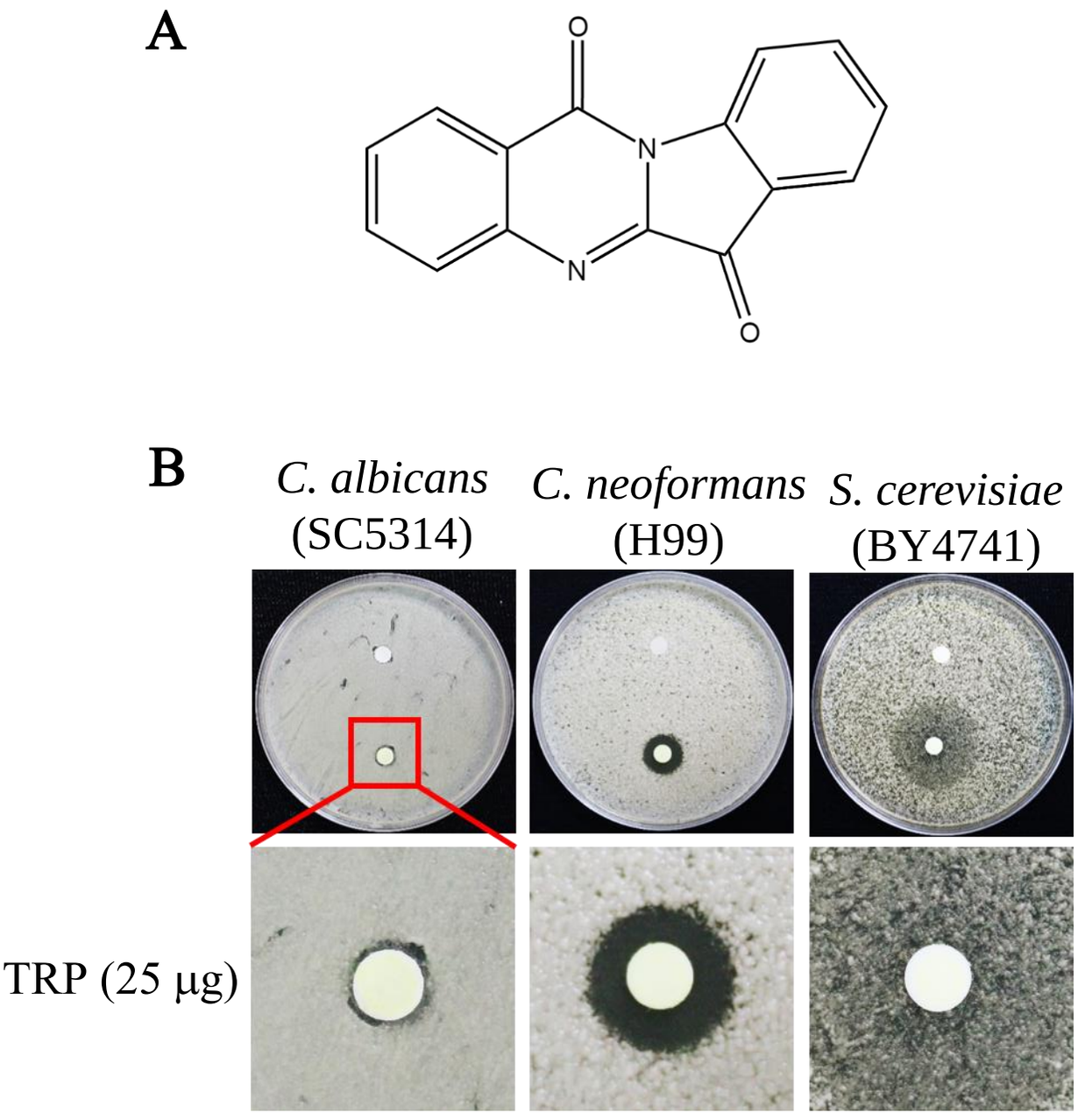

Figure 2. The structure and antifungal activity of tryptanthrin. (A) Structure of tryptanthrin (B) Tryptanthrin exhibits anti-cryptococcal activity. Tryptanthrin (TRP) exhibits antifungal activity, including *C. neoformans* and *Saccharomyces cerevisiae*, but not *C. albicans*. The obvious clearing zone of inhibition by tryptanthrin could be seen in *C. neoformans* (H99). Tryptanthrin showed a slight inhibition activity against *S. cerevisiae*. The plates were incubated at 37 ℃ for 48 hr and photographed.

Figure 3

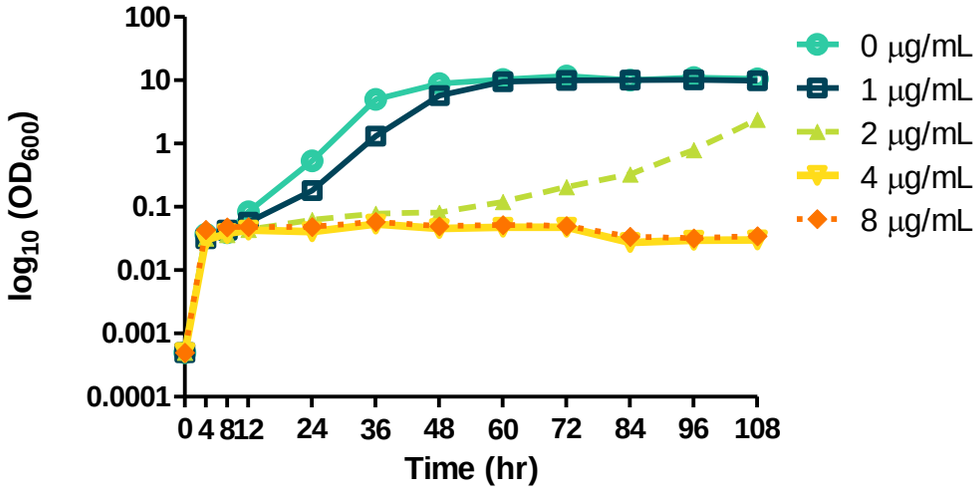

Figure 3. Tryptanthrin exhibits dose-dependent inhibition against *C. neoformans* (H99). Cells were grown overnight at 30 ℃ and diluted to 0.0005 OD/mL with 10 mL fresh YPD broth with 1, 2, 4 and 8 μg/mL tryptanthrin, incubated at 37 ℃. The cell density were measured at 0, 4, 8, 12, 24 and every 12 hr later using SpectraMax190 microplate reader. The experiments were performed in triplicate.

Figure 4

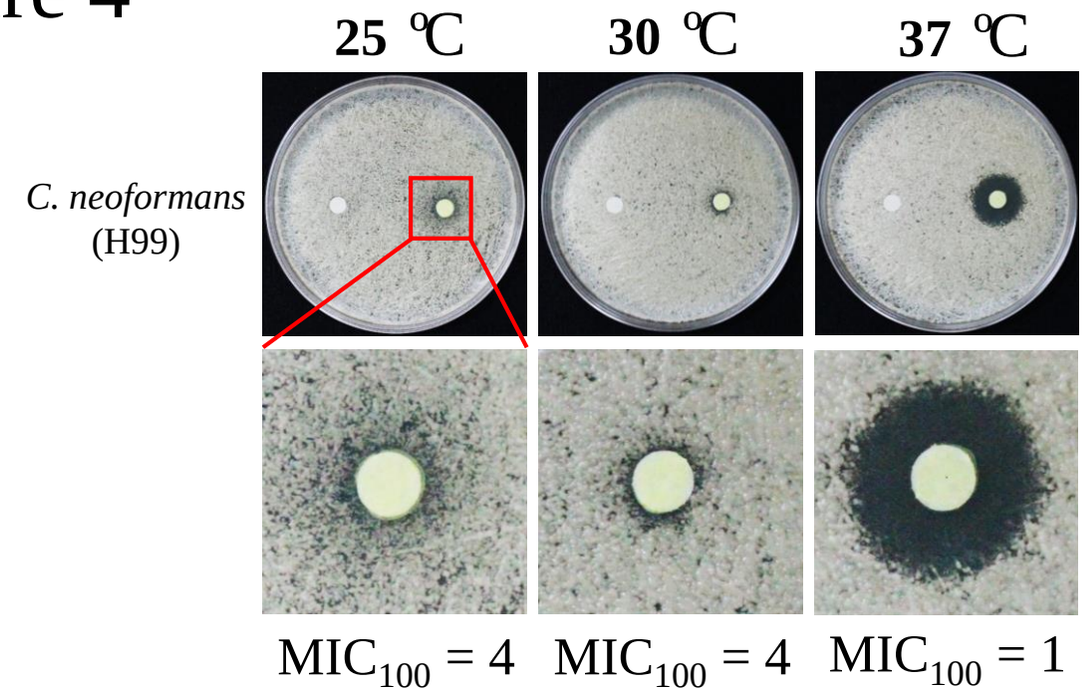

Figure 4. Growth of *C. neoformans* is sensitive to tryptanthrin at 37 ℃ but not at 30 or 25 ℃. Cells were grown overnight at 30 ℃, and 0.1 OD_{600} cells were spread on the YPD media. A 6 mm disc was placed on the surface of the medium and 25 mg tryptanthrin was added. The plates were incubated at 37, 30, 25 ℃ for 48 hr and photographed.

Figure 5

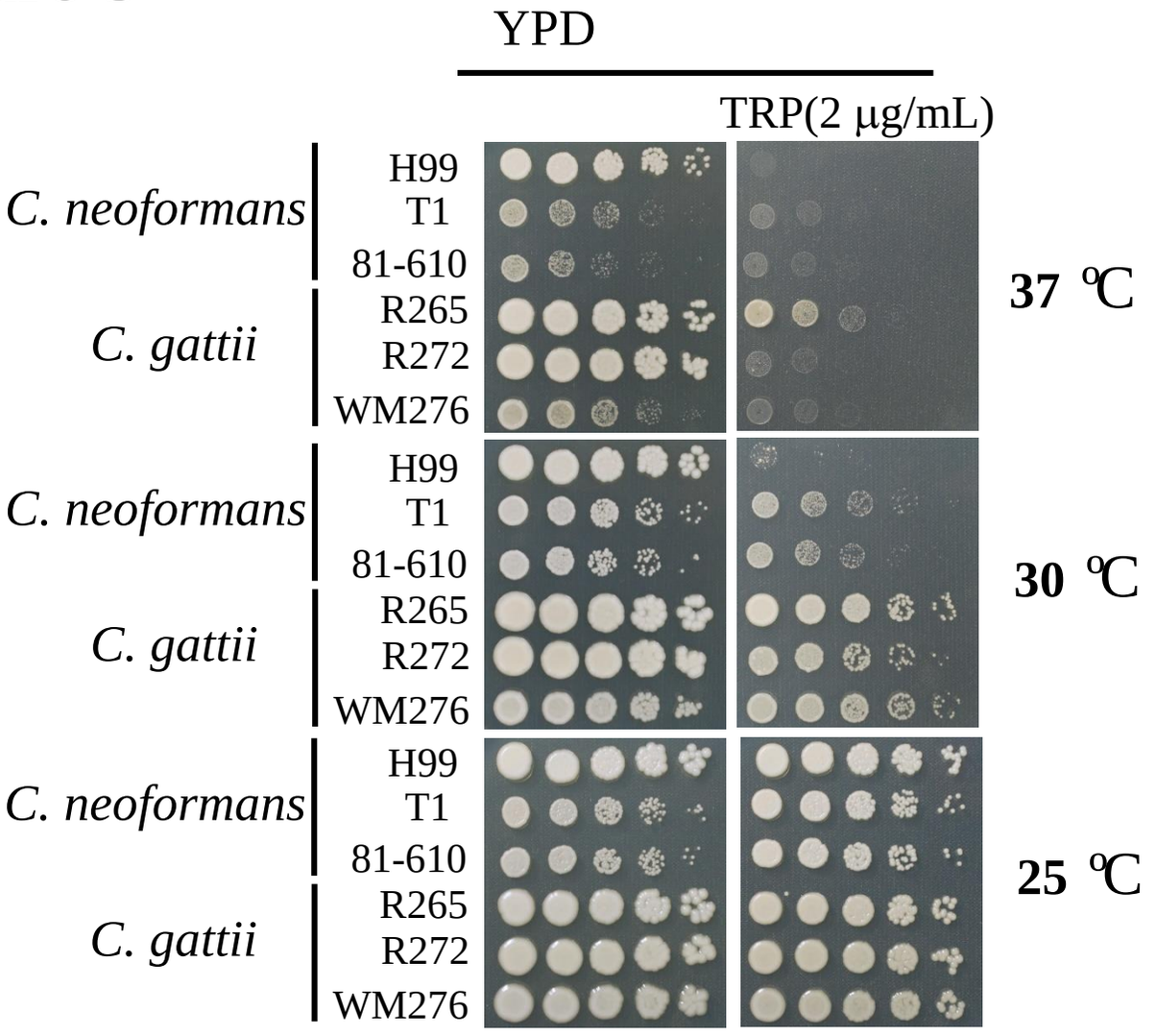

Figure 5. Tryptanthrin can inhibit drug-resistant *C. neoformans*, and clinical or environmental *C. gattii* isolates at 37 ℃. Cells were grown overnight at 30 ℃, 5-fold serially diluted and spotted onto YPD containing tryptanthrin and incubated at indicated temperature for 48 hr.

Figure 6

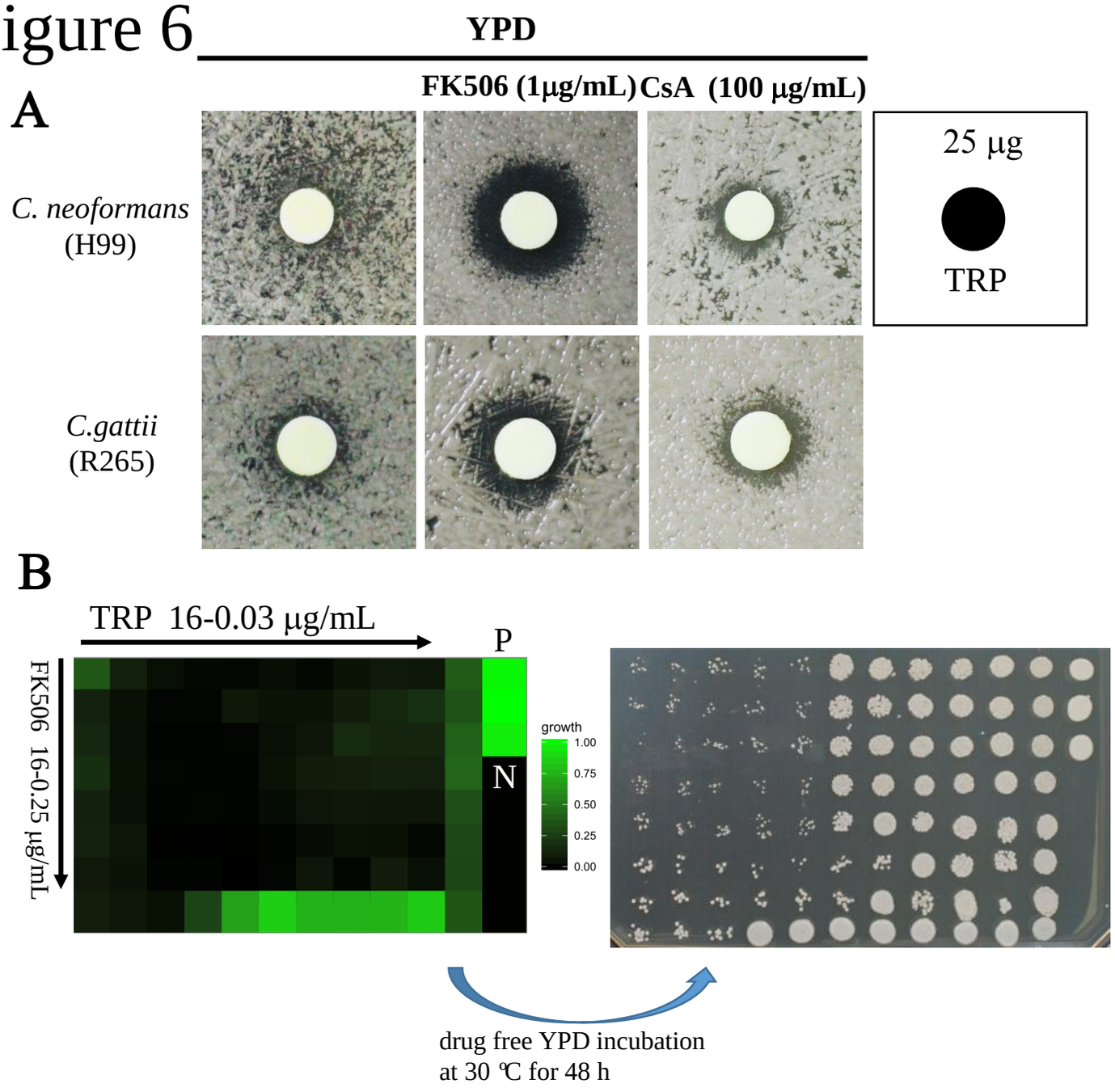

Figure 6. Tryptanthrin exhibits synergistic effect with FK506 against *Cryptococcus* species. (A) Disc diffusion assays were used to determine the combination effect of two drugs against *Cryptococcus* species. Cells were grown overnight at 30 ℃, 0.1 OD_{600} cells were spread on the surface of YPD media with or without FK506 (1 μg/mL), cyclosporin A (CsA; 100 μg/mL).Disc was placed on the surface of the medium and 25 mg tryptanthrin or DMSO was added to each. Plates were incubated at 30 ℃ for 48 hr and then photographed. (B)Tryptanthrin and FK506 are synergistic but fungistatic against *C. neoformans* (H99). The cells after checkerboard assay were transferred to drug-free YPD medium for viability confirmation.

Figure 7

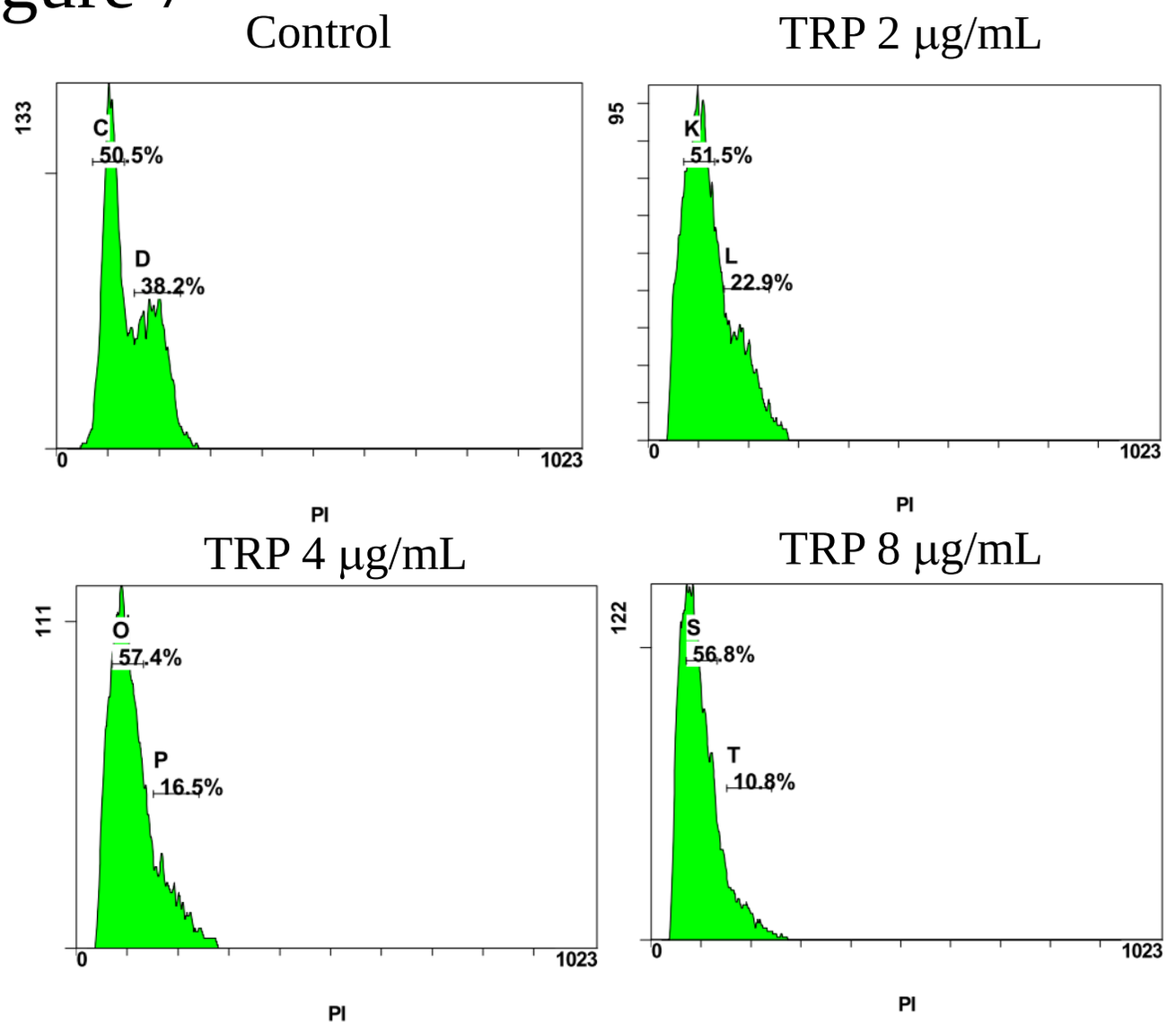

Figure 7. Tryptanthrin exhibits dose-dependent manner on resting of G_1 /S phase in *C. neoformans* (H99). Yeast cells treated with 0, 2, 4, 8 μg/mL tryptanthrin for 12 hr were harvested and washed twice with PBS. Cells were fixed and stained with propidium iodide. The samples were analyzed by flow cytometry and percentages of each phase were calculated.

Figure 8

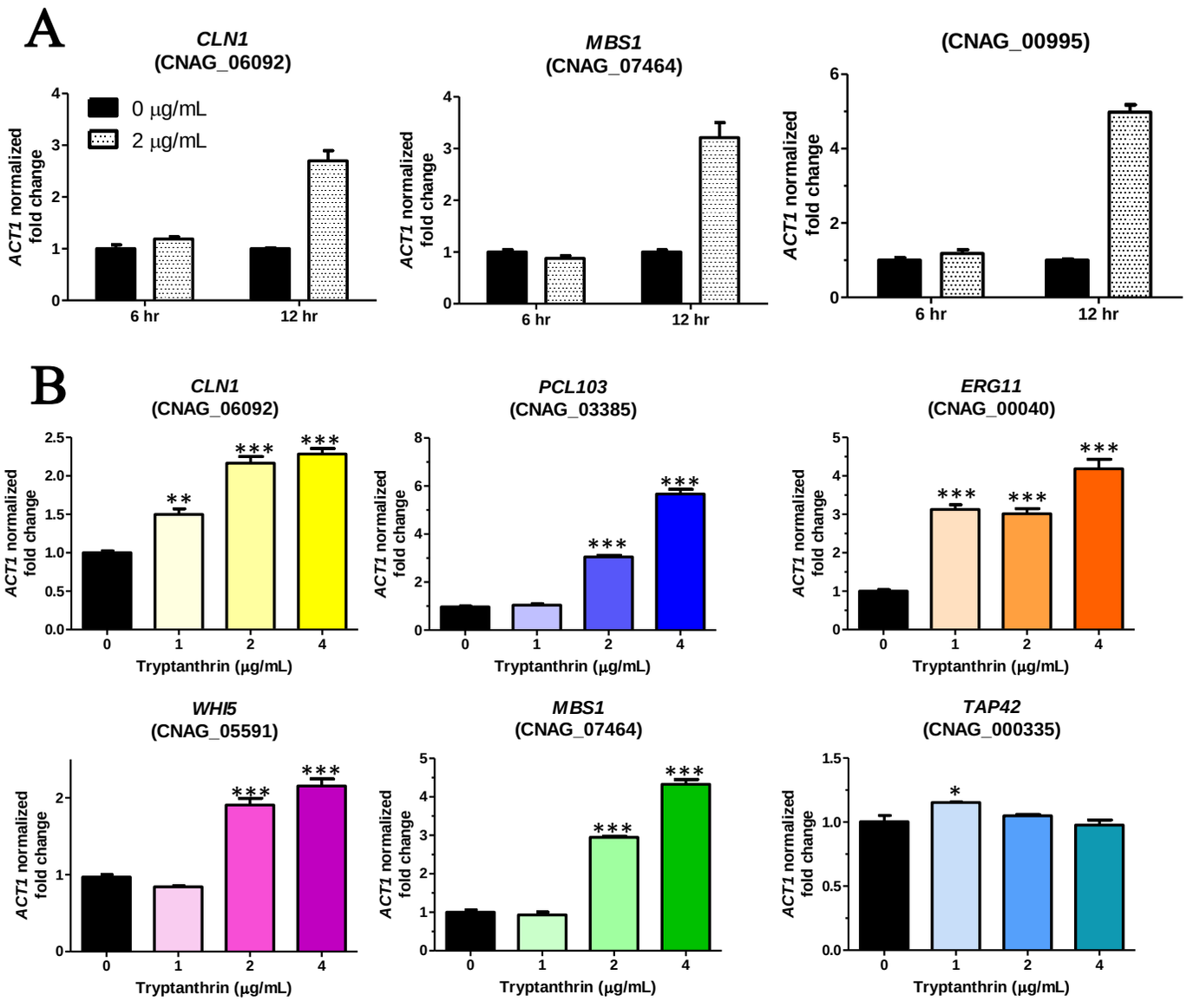

Figure 8. Cell cycle associated genes were up-regulated after treating with tryptanthrin. (A) *C. neoformans* were grown in fresh YPD broth with or without tryptanthrin (2 μg/mL) and harvested at 6 and 12 hr. (B) *C. neoformans* were grown in fresh YPD broth in indicated concentration of tryptanthrin (1,2, 4 μg/mL) and harvested at 12 hr. The mRNA expression level was measured by real-time RT-PCR. The bar graphs of *ACT1* normalized relative quantity compared with drug-untreated samples were created with Prism 5.03. Statistically significant differences (P<0.05, 0.01, 0.001 based on unpaired t tests) compared with the untreated group are indicated by the asterisks.

Figure 9

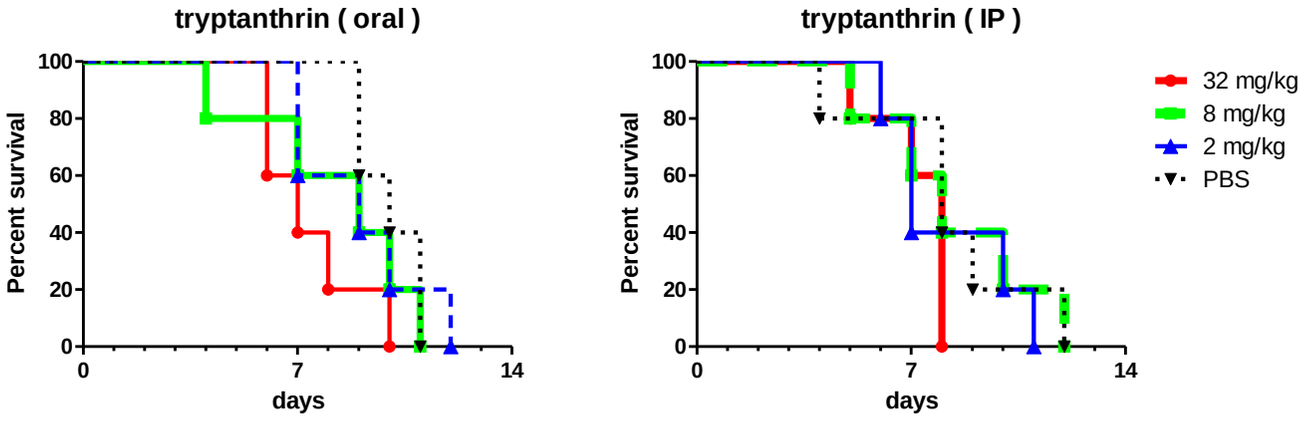

Figure 9. Limited anti-cryptococcal activity of tryptanthrin in murine infection model. Five 4-week male ICR mice per group were intravenous injected with 10^6 cells *C. neoformans* (H99) in 200 mL of sterile water. Tryptanthrin treatment was initiated at 4, 24, 48 and 96 hr post inoculation at doses 2, 8, 32 mg/kg of body weight by oral gavage or intraperitoneal injection. Survival rate was monitored for 12 days.

Figure S1

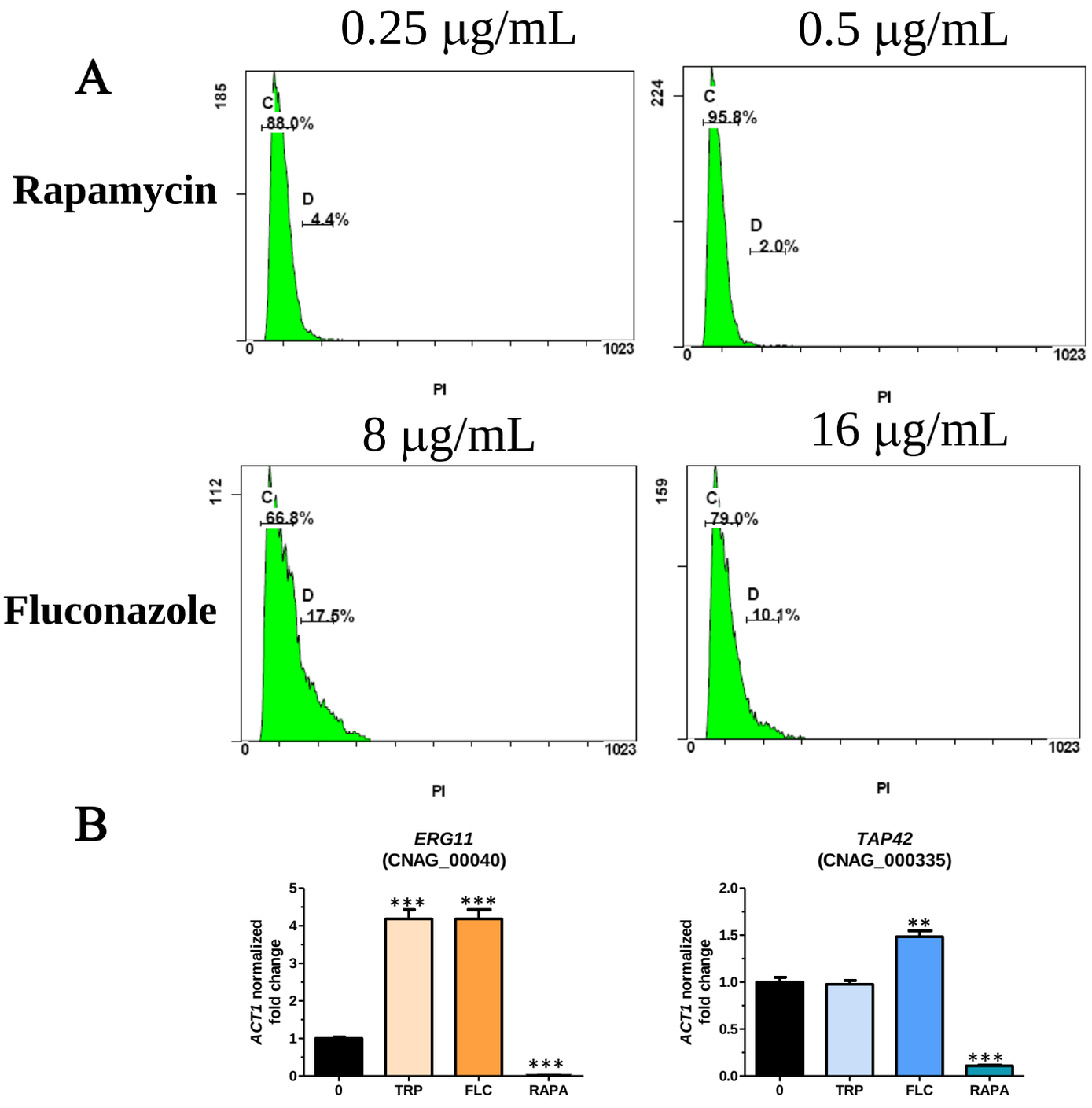

Figure S1. Rapamycin and fluconazole cause cell cycle arrest in G_1 /S phase but exhibit different gene expression pattern. (A) Yeast cells treated with 0, 2, 4, 8 μg/mL tryptanthrin for 12 hr were harvested and washed twice with PBS. Cells were fixed and stained with propidium iodide. The samples were analyzed by flow cytometry and percentages of each phase were calculated. (B) C. neoformans were treated with indicated tryptanthrin (4 μg/mL), fluconazole (16 μg/mL), or rapamycin (0.5 μg/mL) and harvested at 12 hr. The mRNA expression level was measured by real-time RT-PCR.

8. Appendix

Virulence

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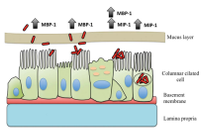

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REVIEW

New facets of antifungal therapy

Ya-Lin Chang[a], Shang-Jie Yu[a], Joseph Heitman[b], Melanie Wellington[c], and Ying-Lien Chen[a]

[a]Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan; [b]Department of Molecular Genetics and Microbiology, Duke University, Durham, NC, USA; [c]Department of Pediatrics, University of Rochester Medical Center, Rochester, NY, USA

ABSTRACT

Invasive fungal infections remain a major cause of morbidity and mortality in immunocompromised patients, and such infections are a substantial burden to healthcare systems around the world. However, the clinically available armamentarium for invasive fungal diseases is limited to 3 main classes (*i.e.*, polyenes, triazoles, and echinocandins), and each has defined limitations related to spectrum of activity, development of resistance, and toxicity. Further, current antifungal therapies are hampered by limited clinical efficacy, high rates of toxicity, and significant variability in pharmacokinetic properties. New antifungal agents, new formulations, and novel combination regimens may improve the care of patients in the future by providing improved strategies to combat challenges associated with currently available antifungal agents. Likewise, therapeutic drug monitoring may be helpful, but its present use remains controversial due to the lack of available data. This article discusses new facets of antifungal therapy with a focus on new antifungal formulations and the synergistic effects between drugs used in combination therapy.

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KEYWORDS
combination therapy; fungal pathogens; new antifungal formulations

Introduction

Approximately 1.2 billion individuals worldwide suffer from fungal infections, and the occurrence of these infections has significantly increased in recent years due to a rise in the number of immunocompromised patients, such as patients with AIDS or those with cancer, organ transplant, or autoimmune disease who require immunosuppressive therapy.[1,2] Unlike superficial infections that cause local, benign, or self-limiting diseases, invasive fungal infections (IFIs) are deep-seated and include bloodstream and systemic infections as well as infection of specific organs. IFIs are frequently caused by yeast pathogens such as *Candida* and *Cryptococcus*; filamentous fungi such as *Aspergillus, Fusarium*, or *Mucor*; or less frequently dimorphic fungi, including *Coccidioides, Blastomyces*, or *Histoplasma*.[3-6] Currently, only 3 main classes of antifungals are approved for treatment of patients with IFIs: polyenes, triazoles, and echinocandins. These agents target ergosterol, lanosterol 14-α-demethylase, and β-1,3 glucan synthase, respectively.[7]

Because our current antifungal therapies have only modest efficacy with significant toxicities, newer antifungal formulations have been developed that ideally will reduce the occurrence of adverse effects associated with the original formulations.[8,9] The newest antifungal drug, isavuconazonium sulfate, is now commercially available in the United States and Europe. Isavuconazonium sulfate is a new member of the triazole class and provides an additional option for the treatment of aspergillosis and mucormycosis in adult patients.[10-12]

Due to the slow pace of novel antifungal drug development, combination therapy has been suggested as an alternative approach to increase fungicidal potency, combat emerging drug resistance, and improve spectrum of activity. Unfortunately, combination antifungal therapy has been shown to improve outcomes in few clinical scenarios.[13] Furthermore, adverse drug effects and drug interactions are more likely with combination therapy.

In this article, we review the most recent antifungal formulations and discuss antifungal combination therapy from a clinical perspective.

Antifungals currently in clinical use

Azoles

Azoles inhibit the fungal cytochrome P450 enzyme, lanosterol 14α-demethylase (CYP51), a key enzyme in ergosterol synthesis.[14] Unlike mammalian membranes, which are rich in cholesterol, ergosterol is the predominant sterol in the cell membrane of fungi. Thus, targeting ergosterol synthesis results in selectivity against fungi. The azoles also have higher affinity for fungal P450

CONTACT Melanie Wellington melanie_wellington@urmc.rochester.edu University of Rochester Medical Center, 601 Elmwood Ave., Box 690, Rochester, NY 14642, USA; Ying-Lien Chen ychen28@ntu.edu.tw National Taiwan University, No. 1, Sec. 4, Roosevelt Road, Taipei 10617, Taiwan.
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enzymes than the mammalian counterparts, adding to their selectivity.[15] Nevertheless, azoles do affect human cytochrome P450 (CYP) enzymes, resulting in significant drug interactions.[16] Interestingly, the human CYP isoforms that are affected vary depending on the azole, underlining the importance of evaluating each patient for potential drug interactions prior to azole use.

The first imidazole-based antifungal drugs, miconazole and ketoconazole, became available for systemic use in 1978 and 1981, respectively.[17,18] Ketoconazole became the standard drug used to treat candidiasis and infections caused by dimorphic fungi (Table 1). However, ketoconazole is associated with significant liver toxicity. Greenblatt *et al.* demonstrated that approximately 1 in 500 patients were at risk of liver injury after ketoconazole administration.[14] In the early 1990s, when the triazoles became available for systemic use, they rapidly supplanted ketoconazole.

Resistance to azole antifungals can be intrinsic (primary) or evolved. *Candida krusei* has strong intrinsic resistance to fluconazole, whereas *Candida glabrata* has intrinsic reduced susceptibility to fluconazole and, with increasing frequency, is evolving high-level fluconazole resistance.[19,20] The widespread use of fluconazole may be contributing to the increased incidence of *Candida* infections with evolved resistance and/or infections with intrinsically resistant non-*albicans Candida*. The antifungal drug resistance mechanisms of azoles include: (1) decreased effective drug concentration due to the activation of efflux transporters such as *CDR1* and *CDR2* in *C. albicans*, or overexpressing the drug target Erg11; (2) alteration of drug targets, such as *erg11* mutation, which has been shown to decrease the affinity of the target to azoles.[21] Prior to effective anti-retroviral therapy, patients with AIDS often required treatment with very long courses of triazoles to treat or suppress oropharyngeal candidiasis, resulting in a clinically significant increase in evolved resistance to triazoles among *Candida* species.[20] However, the current rate of evolved azole resistance remains low in intrinsically susceptible *Candida* species with the exception of *C. glabrata*. Rates of high-level resistance to azoles in *C. glabrata* have been steadily increasing, which is of particular concern because many of these isolates are also resistant to echinocandins.[19] Recently, resistance of *Aspergillus* to azoles has been described. The evolution of voriconazole resistance in *Aspergillus* appears to be due in part to the use of agricultural fungicides.[22] Unfortunately, cross-resistance among azoles is relatively common and develops rapidly in *Candida* species.[19] Therefore, if a *Candida* isolate is resistant to fluconazole, development of resistance to newer-generation triazoles during treatment should be expected, even if the organism appears to be "susceptible" *in vitro*. If a *Candida* isolate is fluconazole-resistant, it is unlikely that use of later-generation triazoles would be of significant clinical benefit.

Because the target of triazoles is a cytochrome P450 enzyme and the triazoles are substrates for human CYP3A, CYP2C19, and CYP2C9 enzymes.[23,24], concurrent treatment with triazoles and other drugs that are substrates for the CYP450 enzymes may lead to significant drug interactions. One particularly challenging drug interaction occurs because patients who are on calcineurin and/or mTOR inhibitors are typically at high risk for IFI and often require treatment with triazoles. By inhibiting the clearance of immunosuppressive agents, triazoles can cause accumulation of the immunosuppressive agent and prolonged immunosuppression[25] Individual azoles have varied drug interactions based on their individual binding affinity for the CYP450 isozymes. For example, azole-induced QT interval prolongation is of significant clinical concern. Meanwhile, fluconazole and voriconazole can affect the QT interval of patients and cause Torsade de Pointes (TdP).[26,27], an uncommon but dangerous cardiac arrhythmia. However, this adverse effect is rare in patients treated with posaconazole[28,29] Therefore, antifungal drugs should be carefully used, and patients with TdP should be therapeutically monitored.

First generation triazoles: Fluconazole and itraconazole

Fluconazole and itraconazole, the first-generation triazoles, became available in the early 1990s (Table 1). Both have a substantially improved safety profile compare with the imidazoles. Fluconazole, which is highly water soluble and available in both oral and intravenous formulations[18], is the only member of the first- and second-generation triazoles with high, reliable bioavailability and minimal variation in absorption. It is also the only triazole drug that is excreted unchanged in the urine, making it the treatment of choice for *Candida* urinary tract infections.[30] Importantly, fluconazole enters the cerebral spinal fluid (CSF) well.

Fluconazole has activity against many *Candida* species, but *C. krusei* and some strains of *C. glabrata* are inherently resistant. It is also highly active against *Cryptococcus neoformans*, but has no activity against filamentous fungi.[24] Fluconazole is currently used as first-line therapy for mucocutaneous candidiasis, empiric therapy for candidemia in non-neutropenic patients with mild-moderate illness, and for "step-down" treatment of candidemia in clinically stable patients with an isolate that is likely fluconazole-susceptible.[31] Recently, a meta-analysis demonstrated that non-neutropenic patients with invasive candidiasis who were treated with an echinocandin had lower mortality rates than patients treated with

doi:10.6342/NTU201704288

Table 1. New antifungal formulations or agents approved by US Food and Drug Administration.

Agent	Original Formulation(s)	Year	New Formulation(s)	Year	Indications	Ref.
Azoles						
Ketoconazole	Oral tablet*	1981	*N/A*		Blastomycosis, coccidioidomycosis, histoplasmosis, chromomycosis and paracoccidioidomycosis	9,34
Fluconazole	Intravenous injection Oral tablet	1990	Oral suspension	1993	Invasive and mucosal candidiasis, cryptococcal meningitis, and prophylaxis of *Candida* infections	17,34,123
Itraconazole	Oral capsule	1992	Oral solution	1997	Coccidioidomycosis, blastomycosis, histoplasmosis, onychomycosis, mucosal candidiasis, sporotrichosis, paracoccidioidomycosis, chromomycosis, and dermatomycosis Alternative agent: Aspergillosis	17,34
Voriconazole	Intravenous injection Oral tablet Oral suspension	2002	*N/A*		Aspergillosis, invasive and mucosal candidiasis Alternative agent: Fusariosis and Scedosporiosis	34,105,123
Posaconazole	Oral suspension	2006	Delayed-release oral tablet Intravenous injection	2013 2014	Mucosal candidiasis and prophylaxis of invasive fungal infections	34,105,123
Isavuconazole	Intravenous injection Oral capsule	2015	*N/A*		Invasive aspergillosis and mucormycosis	11,49
Echinocandins						
Caspofungin	Intravenous injection	2001	*N/A*		Invasive and mucosal candidiasis; empiric antifungal therapy in patients with fever and neutropenia, Alternative agent: Aspergillosis	81,123
Micafungin	Intravenous injection	2005	*N/A*		Invasive and mucosal candidiasis, Prophylaxis of *Candida* infections	81,123,124
Anidulafungin	Intravenous injection	2006	*N/A*		Invasive and mucosal candidiasis	81,123
Polyenes						
Amphotericin B deoxycholate	Intravenous injection	1958	AmB lipid complex	1995	Aspergillosis, cryptococcosis, blastomycosis, invasive candidiasis, Coccidioidomycosis, histoplasmosis, mucormycosis, sporotrichosis, phaeohyphomycosis	71,105,123
			AmB colloidal dispersion	1996		
			Liposomal AmB	1997		

Note. *The oral tablet was initially approved but later withdrawn in 2013; *N/A*: not available

triazoles or amphotericin B.[32] Fluconazole is also used as a primary therapy for the treatment of pulmonary cryptococcosis and consolidation therapy for patients with cryptococcal meningitis after induction therapy with amphotericin B.[24] In resource-limited regions that lack the ability to safely treat patients with amphotericin B, fluconazole is the mainstay of anti-cryptococcal therapy. Fluconazole is also frequently used in high-risk patients to provide prophylaxis against *Candida* infections. As one would expect given its spectrum of activity, it is not as effective as second-generation triazoles at preventing aspergillosis.[24]

Unlike fluconazole, itraconazole has generally lower bioavailability that is complicated by substantial variation in absorption.[24] Furthermore, itraconazole has poor central nervous system (CNS) penetration and urinary metabolites are inactive.[24] Thus, clinical use of itraconazole is primarily limited to treatment of fungi that do not cause CNS disease. Itraconazole is currently available in 2 formulations: oral capsule and oral solution (Table 1). The oral solution is superior to capsules because of improved bioavailability, but it is not tolerated as well as the capsules.[33] Importantly, the solution should be taken on an empty stomach, while capsules should be taken with food to maximize absorption. An intravenous formulation was once FDA-approved, but its approval was withdrawn in 2007 due to cardiac toxicity.[34]

Itraconazole has a much wider spectrum of activity than fluconazole. It is active against fluconazole-susceptible *Candida* species, *Cryptococcus*, and many dimorphic fungi including *Coccidioides*, and has some activity against filamentous fungi such as *Aspergillus*.[35] Importantly, itraconazole is not active against *Fusarium* species or Zygomycetes. Due to its less favorable pharmacokinetics and more prominent drug-drug interactions, itraconazole has widely been replaced by second-generation triazoles for most clinical uses. Currently, itraconazole is still used to treat patients with dimorphic mycoses, including coccidiomycosis, blastomycosis, and histoplasmosis.[4-6] Interestingly, itraconazole, with known safety and tolerability, has the potential to be developed into an anti-cancer agent.[36,37] However, the drug interactions of

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itraconazole and existing anti-cancer agents remain unclear and require further investigation.

Second generation triazoles: Voriconazole and posaconazole

The second-generation triazoles were developed with the goal of improving pharmacokinetics and spectrum of activity and decreasing drug-drug interactions. The chemical structure of voriconazole is similar to fluconazole, whereas posaconazole is more closely related to itraconazole.[30] Variation in blood levels is an issue with both voriconazole and posaconazole. The predominant source of variability for voriconazole is due to individual variations in metabolism, whereas absorption of the posaconazole oral suspension from the GI tract is highly variable.[30] Both voriconazole and posaconazole have poor solubility in water. Thus, intravenous solutions require the addition of cyclodextrins to improve solubility. Although neither voriconazole nor posaconazole are renally eliminated, the cyclodextrin component is and it accumulates in patients with renal failure.[30]

Voriconazole is available in both intravenous and oral forms (Table 1). The oral forms have excellent bioavailability.[24] Similar to fluconazole, it has excellent CNS penetration. Voriconazole is primarily metabolized by the cytochrome P450 enzyme CYP2C19.[24] The safety profile of voriconazole is excellent, but treatment-related visual disturbances occur in approximately 20% of patients. Voriconazole has a substantially broader spectrum of activity than the first-generation azoles. Its activity against *Candida* species and *Cryptococcus* mirrors that of fluconazole, but voriconazole also has activity against dimorphic fungi, *Fusarium*, and *Scedosporium*, and most importantly, it has very high activity against most *Aspergillus* species.[24] However, it is not active against Zygomycetes. Voriconazole has become the first-line treatment for invasive aspergillosis as it has better efficacy and substantially fewer drug-related toxicities than amphotericin B.[38] Voriconazole is FDA-approved for treatment of mucocutaneous and systemic candidiasis, but it is not frequently used for these indications because it is not substantially better than fluconazole for these diseases. Voriconazole is commonly used for prophylaxis against yeast and mold infections in high-risk patients such as those undergoing bone marrow transplantation.[30] One possible difficulty with the use of voriconazole for prophylaxis against IFIs is that it does not protect against Zygomycetes.

Posaconazole was originally available only in oral suspension form. However, the bioavailability of the oral suspension depends on food intake. Ingestion of high-fat meals or nutritional supplements is required for good absorption.[24] In 2013, a new oral delayed release tablet formulation was introduced (Table 1) that is given once daily; its bioavailability is independent of food intake.[39,40] The delayed release oral tablet is considered a more reliable option for the prophylaxis or treatment of IFIs. Studies have suggested that the posaconazole oral tablet has higher azole plasma levels[41], better absorption[42], and improved bioavailability[43] than that of oral suspension. Furthermore, an intravenous formulation of posaconazole was developed and FDA-approved in 2014 for patients who are unable to take oral medications.[39,44] The phase 1B trial results showed that the intravenous formulation of posaconazole was well tolerated in patients at high risk for IFIs.[44]

Posaconazole has a very broad spectrum of antifungal activity.[24,30] Like voriconazole, it is active against fluconazole-susceptible isolates of *Candida, Cryptococcus*, dimorphic fungi, and *Aspergillus*. In addition, posaconazole is active against many Zygomycetes. Among the azoles, only posaconazole and itraconazole have activity against these difficult-to-treat fungi. Posaconazole also has better efficacy than fluconazole for the prophylaxis of systemic fungal infections.[39,45] One major limitation of posaconazole is that it does not penetrate into the CSF well.[24] This raises concerns that it may not be suitable for treatment of invasive *Aspergillus* infections and disseminated candidiasis, both of which can cause CNS disease.

Isavuconazonium sulfate

Isavuconazonium sulfate is a water soluble pro-drug of the triazole isavuconazole that was approved by the FDA in 2015 for the treatment of invasive aspergillosis and invasive mucormycosis.[46] The pro-drug is rapidly metabolized into isavuconazole by plasma esterases after intravenous administration. The oral capsule (Table 1) formulation of isavuconazonium sulfate hydrolyzes and converts to the active form in the gut lumen.[47] Some studies have reported that the high bioavailability of the oral capsule is minimally affected by food intake.[48,49], but this requires more evidence to support its clinical relevance. Besides, initial studies suggest that blood levels are substantially more consistent than for voriconazole or posaconazole[24] Although the tissue distribution of isavuconazole has not yet been fully evaluated, it is highly protein bound and thus expected to have low levels in the CSF, although it may reach clinically useful concentrations in the brain parenchyma.[11] Active isavuconazole is not excreted in the urine. Because of the high water solubility of the isavuconazole prodrug (isavuconazonium sulfate) relative to voriconazole and posaconazole, isavuconazole does not require cyclodextrin, an agent with potential nephrotoxicity, to increase its solubility.[50] However, isavuconazole is a substrate and inhibitor of

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CYP3A4, so co-administration with a strong CYP3A4 inhibitor or inducer is a pharmacokinetic concern.[48]

Isavuconazole exhibits *in vitro* activity against azole-susceptible *Candida* species, *C. neoformans, Cryptococcus gattii*, dimorphic fungi, *Aspergillus*, and, importantly, many other molds including *Alternaria*, some Zygomycetes, and some species of *Scedosporium*.[11] Pfaller *et al.* showed that the vast majority of 21 *Candida* species were inhibited by isavuconazole with MIC $\leq$ 0.25 μg/mL, with the exception of *C. glabrata, C. krusei*, and *C. guilliermondii* with MIC $\geq$ 1 μg/mL[51]. Clinical trials of isavuconazonium sulfate for systemic candidiasis and other IFIs caused by *Aspergillus* and rare fungi are now complete, although many of the results have been released only as abstracts or FDA briefing documents.[11,51,52] Isavuconazonium sulfate was found to be non-inferior to voriconazole for the treatment of patients with proven or probable invasive fungal disease caused by filamentous fungi, including *Aspergillus*. Although it is not as active as voriconazole, isavuconazole has activity against some Zygomycetes.[11,24] The *in vitro* studies showed that isavuconazole exhibits potent antifungal activity against many Mucorales including *Mucor, Rhizomucor, Rhizopus*, and *Absidia*. However, the susceptibility of these Mucorales to isavuconazole varies largely.[49] In spite of that, the open-label clinical trial for licensing demonstrated that patients who received isavuconazole had similar mortality to patients who received amphotericin B or posaconazole.[49]

Therapeutic drug monitoring for triazole therapy

As one of the most prominent clinical difficulties in the use of itraconazole, voriconazole, and posaconazole is variability in plasma drug levels, many investigators have suggested that monitoring drug levels could optimize efficacy and/or decrease toxicity.[53] Unfortunately, the majority of studies investigating therapeutic drug monitoring (TDM) for triazoles have been retrospective descriptive studies that do not address the question of whether the use of TDM can improve patient outcomes. Furthermore, many of these studies rely on drug concentration measurements taken at random times with respect to the time since the most recent drug dose or the time since initiation of therapy.[32,54] Finally, the laboratory methods needed to accurately measure triazole concentrations typically require that samples be sent to specialized reference laboratories. Thus, results are often not available until 5 to 7 d after samples are obtained. Therefore, antifungal TDM is typically restricted to patients who require fairly long-term antifungal therapy.[53,55]

The relationship between drug concentration and therapeutic efficacy is clearest for itraconazole. After one to 2 weeks of therapy, patients with trough levels >0.5 to 1 μg/mL are more likely to have treatment success.[53,56] There is no strong correlation between itraconazole levels and adverse events. Although itraconazole use has decreased significantly since voriconazole and posaconazole became available, it remains the treatment of choice for infections such as coccidioidomycosis, and in these patients, itraconazole TDM is indicated.

Posaconazole drug levels are typically a function of absorption of the drug from the GI tract.[24,30]; the newer oral delayed release tablet formulation has substantially better, and more reliable, absorption than earlier formulations[39,40] A series of studies have shown a relationship between posaconazole levels and efficacy.[54,57,58] While these studies varied in their rigor with respect to TDM methods and definitions of efficacy, the body of data has led to recommendations of target steady-state trough posaconazole concentrations of > 700 ng/mL for prophylaxis and, provisionally, >1250 ng/mL for treatment of invasive fungal disease.[54,59] In a recent study, Cornely *et al.* found that 90% of adult patients taking the newer delayed release tablets for prophylaxis of fungal infections had steady-state trough levels >700 ng/mL and there was no correlation between drug levels and adverse events.[60] Thus, TDM is unlikely to provide clinical benefit to patients taking the delayed release formulation of posaconazole for prophylaxis of fungal infections. It is not yet clear whether there will be a role for TDM in patients taking posaconazole for treatment of invasive fungal disease.

In contrast to itraconazole and posaconazole, voriconazole levels are related more to variation in metabolism than absorption. Genetic polymorphisms in CYP2C19 are common and strongly affect voriconazole blood concentrations[30], and an increasing body of retrospective data suggests that there is a correlation between voriconazole levels and efficacy and toxicity with improved outcomes occurring in patients with trough voriconazole levels >1 mg/L[56]. A prospective, randomized, blinded study was recently performed comparing outcomes in patients who were managed with TDM and voriconazole dose adjustment to those without TDM.[61] This study showed improved clinical response in the TDM/dose adjustment group, with complete or partial response in 81% of the TDM group but only 57% of the control group. However, this study was a single-center study performed in Seoul, Korea. Given known variations in drug metabolism between Asian and non-Asian populations, results may differ in other patient populations. Interestingly, a recent meta-analysis of voriconazole TDM found that patients with voriconazole levels >1.0 mg/L were more likely to have a successful clinical response, but there was no difference in survival between patients who

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had therapeutic and subtherapeutic levels.[62] Taken together, the accumulating data suggest that TDM for voriconazole therapy may be clinically useful, but we do not currently have enough data to clearly define the clinical scenarios in which it would be most useful.[56,63] One special population for whom voriconazole TDM may be quite helpful is children with IFIs.[64] Typically, much less is known about the pharmacokinetics of drugs in pediatrics, but children often have increased drug clearance compare with adults. This makes them more vulnerable to sub-therapeutic dosing and thus they may be more likely to benefit from TDM.

Polyenes

The polyene antifungal amphotericin B was the first antifungal agent used for IFI treatment. With broad-spectrum fungicidal activity against yeasts and filamentous fungi, amphotericin B has been widely used clinically to treat systemic *Candida, Cryptococcus, Aspergillus*, and many other IFIs.[65] Despite its long use, the exact mechanisms of action of amphotericin B remain unclear.[66] In the most traditional model, amphotericin B kills fungal cells by forming pores in ergosterol-containing membranes. More recent studies have proposed a variety of other possible mechanisms. For example, amphotericin B directly binds to ergosterol and leads to electron transfer in the cell membrane, thus creating oxidative stress and reactive oxygen species.[67,68] Whatever the exact mechanism of action is, amphotericin B kills fungal cells with some specificity for fungal rather than mammalian cells. This specificity is related to the increased ergosterol content in fungal membranes in contrast to cholesterol, which is the major sterol in mammalian cell membranes. Nevertheless, toxicity toward cholesterol-containing cells occurs and leads to the significant adverse effects. Amphotericin B can cause serious nephrotoxicity as well as electrolyte abnormalities and severe infusion-related reactions such as hypomagnesium, chills, fever, and rigors.[66,69]

In view of this high toxicity but excellent efficacy, a drug structure or formulation modification was needed. Amphotericin B lipid complex (ABLC) and liposomal amphotericin B (L-AmB) are combinations of amphotericin B and lipids in a specific ratio that improve the safety profile.[70,71] (Table 1). Interestingly, different amphotericin B formulations possess distinct pharmacological properties and adverse effects. For example, Wade *et al.* found that patients who received ABLC had a higher risk of experiencing nephrotoxicity compared with those receiving L-AmB[72] Although the safety and toxicity of these new formulations are much improved compared with the old formulation, the toxicity of amphotericin B on the kidneys and infusion-related organs still remains a clinical concern.[73]

A landmark study recently reported the possibility of manufacturing less toxic amphotericin B derivatives.[74] The prototype of these derivatives is amphotericin B with a C'2 hydroxyl group deletion, which allows it to only bind to fungal ergosterol and not mammalian cholesterol.[75] *In vivo* toxicity and therapeutic experiments using a systemic candidiasis murine model demonstrated that amphotericin B methyl urea (AmBMU) was less toxic and more effective than the traditional deoxycholate amphotericin B formulation.[74] These results contribute to the exciting progress in antifungal drug development linked to the gold standard antifungal agent amphotericin B.

Resistance to polyene antifungals is still quite rare in the clinic, mostly because the fitness cost of developing modifications for survival is high.[76] Meanwhile, pathogens may be vulnerable and unable to evade the host immune system. Pathogens gain polyene resistance by decreasing ergosterol content in cell membranes and increasing catalase activity.[77] Meanwhile, mutation of *ERG3*, a gene involved in ergosterol biosynthesis in *C. albicans*, may lead to the accumulation of other sterols and thus reduce the affinity of amphotericin B to ergosterol in the fungal cell membrane. Utilization of other sterols instead of ergosterol and reduction of oxidative stress can change the physiology of pathogens and cause drug-resistant isolates.[78]

Echinocandins

The echinocandins are the newest class of antifungal agents and are currently widely used for the treatment of IFIs. Caspofungin, micafungin, and anidulafungin are echinocandin-class antifungals that have been approved for intravenous administration by the FDA and the European Medicines Agency[79] (Table 1). Echinocandins are cyclic lipopeptide molecules derived from natural products that inhibit fungal β-1,3 glucan synthase, a major enzyme complex functioning in cell wall synthesis.[79-81] Similar to polyenes and azoles that target fungal ergosterol and its biosynthesis pathway, echinocandins have a unique drug target that is only present in fungi but not in mammalian cells, and thus these agents are much less toxic to humans. Echinocandins have several additional merits, including fungicidal activity against *Candida* species[82], reduced emergence of drug-resistant isolates[79], and most importantly, an improved safety profile and fewer drug interactions.[82] Unfortunately, echinocandins also have a high molecular weight and are not stable in acid, so they are not

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amenable to oral use.[79] Oral glucan synthase inhibitors are now under development.

Although the echinocandins are generally very safe drugs, unexplained cardiac-associated adverse events such as arrhythmias and cardiac failure have occurred in some patients after the administration of caspofungin.[80] In addition, Fink *et al.* reported a fatal hemodynamic instability adverse event after anidulafungin administration[83], and in *ex vivo* testing, caspofungin and anidulafungin decreased left ventricular contractility.[80] Taken together, these data imply that echinocandins should be used cautiously in patients with preexisting cardiac dysfunction, though additional studies are required.

Echinocandins exhibit potent fungicidal activity against most *Candida* species.[31,79] In fact, in the new 2016 Infectious Diseases Society of America candidiasis guideline, echinocandins are the primary drugs of choice for invasive candidiasis.[31] In general, *Candida parapsilosis* isolates tend to have lower susceptibility to echinocandins *in vitro* but clinically the echinocandins are usually effective against this species.[24] Unfortunately, clinical reports of echinocandin-resistant *Candida* isolates are increasing.[84] Of particular concern is a group of *C. glabrata* isolates that are resistant to both azoles and echinocandins.[51] However, the vast majority of *Candida* isolates are currently highly susceptible to echinocandins.

Generally, echinocandins exhibit fungistatic activity against *Aspergillus* and are typically used only as alternative or second-line therapies against invasive aspergillosis.[24] Echinocandins are not active against *Cryptococcus*, dimorphic fungi, or Zygomycetes. Interestingly, echinocandins have antifungal activity against the cyst form but not the vegetative form of *Pneumocystis jirovecii*, a human fungal pathogen that causes pneumonia.[85] Because the vegetative form is a major component of disease, echinocandins are not used clinically to treat *Pneumocystis*.

Resistance to echinocandin antifungals is mostly due to mutations of *FKS*.[86] Fks is a subunit of glucan synthase and the drug target of echinocandins. Two conserved regions of *FKS*, Ser 645 and Phe 641, can mutate, leading to increased tolerance or resistance to antifungals.[87] In general, failure of echinocandin treatment for common *Candida*-causing candidiasis is rare, except for *C. glabrata*, a well-known multidrug-resistant species. A similar mechanism was implicated in the emerging echinocandin resistance in molds.[77]

Echinocandins have a unique structure and target a fungal-unique pathway, and are currently the safest antifungal drugs available. These agents are neither substrates nor inhibitors of CYP450, thus making clinical drug-drug interactions relatively rare.[23] Though caspofungin, micafungin, and anidulafungin possess similar antifungal activities, the differences in their backbone structures lead to distinct pharmacokinetics.[88] Caspofungin may affect the plasma concentration of cyclosporine A and tacrolimus.[88] However, Saner *et al.* demonstrated that co-administration of caspofungin with either of these 2 immunosuppressants in liver transplant patients resulted in an acceptable safety profile with no hepatotoxicity.[89] In addition, based on an open-label clinical trial in healthy adults, micafungin may increase exposure to amphotericin B about 30%; thus, it may not well tolerated during co-treatment in human host.[90] Overall, most of the drug-drug interactions between echinocandins and other drugs are not serious when compare with those associated with the azoles.

Antifungal agents in clinical trials

Several antifungal agents are currently being evaluated in clinical trials. We have selected 2 promising candidates and summarized their progress below.

VT-1161

VT-1161, a tetrazole developed by Viamet Pharmaceuticals, is a novel ergosterol synthesis inhibitor targeting fungal CYP51 (lanosterol 14 α-demethylase) that has been in phase 2 clinical trials for treatment of vaginal candidiasis since 2013.[91,92] Warrilow *et al.* demonstrated that VT-1161 tightly binds to *C. albicans* CYP51 and thus inhibits cellular function, and that it also weakly inhibits human enzymes such as CYP2C9, CYP2C19, and CYP3A4.[93] The lack of interference with human enzymes suggests that VT-1161 may potentially have fewer negative drug-drug interactions, thus overcoming a major issue of the triazoles.[34] In addition, VT-1161 retains high *in vitro* potency against several *C. albicans* isolates that are clinically fluconazole-resistant. In a murine model of vaginal candidiasis, Garvey *et al.* demonstrated that VT-1161 was equivalent to fluconazole for treatment of vaginitis due to fluconazole-susceptible *C. albicans* and significantly superior to fluconazole for the treatment of vaginal candidiasis due to fluconazole-resistant organisms.[94] These results suggest that VT-1161 has considerable potential to be an efficacious and safe antifungal agent.

SCY-078

Echinocandins have potent fungicidal activity against *Candida* species through the inhibition of the fungal enzyme β-1,3 glucan synthase.[95] Currently, echinocandins are only available in intravenous formulations.[82] SCY-078 (formerly MK-3118) is a potential candidate for an oral glucan synthase inhibitor that is currently in

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phase 2 clinical trials.[96] The mechanism of action of SCY-078 is similar to that of the echinocandins, but SCY-078 has a different chemical structure and possesses excellent oral bioavailability.[82,95,97,98] SCY-078 exhibits broad-spectrum antifungal activity against several *Candida* species and even some echinocandin-resistant isolates.[98] Moreover, it is also effective against some filamentous fungi, including *Aspergillus fumigatus, Paecilomyces variotii*, and *Scedosporium prolificans*.[95,98] The pharmacokinetics and pharmacodynamics of SCY-078 after oral treatment have been evaluated in a neutropenic murine model of disseminated candidiasis. The 1-log kill doses of SCY-078 were numerically lower than those of conventional intravenous echinocandins[97], indicating that SCY-078 is a promising antifungal agent. A clinical phase 1 study showed that SCY-078 was generally well tolerated. Adverse effects associated with SCY-078 included diarrhea, abdominal pain, and headache.[81]

Combination therapy

Due to the emergence of drug-resistant fungi and the limited efficacy of monotherapy, the therapeutic strategy of combining several current antifungal drugs with different mechanisms of action has often been considered. The only combination therapy that is supported by well designed, randomized clinical trials is the use of amphotericin B with flucytosine for the treatment of cryptococcal meningitis.[99,100] Because fungal infections typically have poor outcomes and treatment frequently results in adverse effects, clinicians are compelled in some cases to abandon conventional antifungal therapy for salvage therapy.[101] In the absence of effective monotherapy, combination therapies are frequently used as a "last ditch attempt" to treat potentially life-threatening IFIs. Individual case reports or case series describing success with combination therapy are common, but such reports are highly susceptible to publication bias and should be interpreted cautiously. In order to find the best options to improve outcomes and minimize risk, clinicians need to evaluate the *in vitro* and *in vivo* efficacy and drug interactions of antifungal drug combinations.

Combination therapy against candida

Invasive *Candida* infections can usually be treated with azoles, echinocandins, or amphotericin B monotherapy. As with other infections, case reports using combination therapy have been published[102-104], but there are no data to indicate that combination therapy is necessary for treatment of candidiasis.

Combination therapy against aspergillus

The clinical practice guidelines for the treatment of invasive aspergillosis recommend voriconazole over other antifungal drugs as a primary therapy, while amphotericin B, itraconazole, posaconazole, itraconazole, caspofungin, and micafungin serve as alternative therapies.[105] If patients are refractory to primary therapy or are predicted to fail monotherapy, clinicians may opt for combination therapy. In a recent large clinical study comparing voriconazole monotherapy versus combination therapy with voriconazole and anidulafungin, combination treatment did not significantly improve overall survival compare with monotherapy.[106] In this study, the primary endpoint was all-cause mortality at 6 weeks; 27.8% of patients on monotherapy and 19.5% of patients on combination therapy died, but this difference did not reach statistical significance. One tempting interpretation of these data are that combination therapy did have a benefit, but the study was underpowered. This must be balanced against the finding that other studies have also failed to demonstrate an improvement with combination therapy. Furthermore, most studies of combination therapy find an increase in adverse drug effects with combination therapy. Much of the available data on combination therapy comes from retrospective or non-comparative studies. For example, Raad *et al.* reported results from combination therapy of voriconazole and caspofungin vs. voriconazole alone based on a retrospective chart review. Combination therapy did not enhance the survival rate of patients compared with monotherapy, but adverse events were higher in the combination group.[107] Likewise, Lellek *et al.*, in an uncontrolled retrospective salvage therapy study, reported that patients with aspergillosis who failed to respond to primary therapy had a favorable response with combination therapy using posaconazole and caspofungin, but no comparison data for monotherapy were provided.[108] Although the precise use and success of combination therapy for aspergillosis remain uncertain, the potentially dire outcomes of invasive aspergillosis continue to drive consideration of combination treatment by clinicians at the bedside.

Combination therapy against cryptococcus

Treatment of cryptococcal meningitis is the only circumstance for which combination antifungal therapy is well supported with prospective randomized clinical trials. The fluorinated pyrimidine flucytosine (5-FC) is a seldom used antifungal drug that interferes with nucleic acid synthesis.[24] It is active against *Cryptococcus* and *Candida*, but it is not used as a monotherapy because

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drug resistance readily develops. It also causes significant bone marrow and liver toxicity.[109] Thus, the clinical use of flucytosine is typically limited to combination therapy for treatment of cryptococcal meningitis. Co-administration of amphotericin B and flucytosine is more efficacious than amphotericin B alone, and this fungicidal regimen is included in clinical practice guidelines for invasive *Cryptococcus* management.[100,110,111] Day *et al.* demonstrated that combination therapy of amphotericin B plus flucytosine for cryptococcal meningitis was more effective than amphotericin B alone or with fluconazole.[112] Judging by the decreased mortality and high rate of clearance of yeast in CSF, the combination of amphotericin B and flucytosine is an excellent therapeutic strategy against cryptococcosis and is the standard of care for induction therapy.[99,100,112] Nevertheless, treatment with amphotericin B and flucytosine requires a high level of supportive medical care not feasible in countries with limited medical resources, suggesting that an alternative approach must be developed for these areas.[113] Furthermore, flucytosine has significant toxicity and limited availability and high cost, even in the United States.

As fluconazole and amphotericin B are 2 major antifungal agents that can be obtained easily, the feasibility of combination therapy using these agents has been evaluated. A clinical trial performed by Loyse *et al.* on cryptococcal meningitis in HIV patients demonstrated that there was no significant difference in the early fungicidal activity of amphotericin B in combination with flucytosine, fluconazole, or voriconazole.[113] Thus, the fluconazole and amphotericin B combination provides another potential option for treating *Cryptococcus* infection if flucytosine is not available or not tolerated by the patient.[111,113,114]

Combination therapy against zygomycetes

Mucormycosis is an IFI that can be caused by any of the Zygomycetes, including *Mucor, Rhizopus, Rhizomucor*, and *Apophysomyces*. Due to extremely high mortality, management of mucormycosis has become a critical issue in the clinic. Currently, only amphotericin B, posaconazole, and isavuconazole have sufficient activity against these organisms to be used clinically.[49,115,116] As with treatment of invasive aspergillosis, ineffective monotherapy and serious side effects of amphotericin B have prompted clinicians to attempt alternative strategies, including combination therapy. Two retrospective analyses of combination therapy for treatment of mucormycosis infections have recently been published. Kyvernitakis *et al.* reviewed charts of 106 patients with hematologic malignancy and mucormycosis and found no difference in mortality 6 weeks after therapy between monotherapy and combination therapy.[117] In contrast, Reed *et al.* reviewed the charts of 41 patients with mucormycosis; 34% of these patients had malignancy, 10% had organ transplantation, and 83% had diabetes mellitus. In this group of patients, they found that treatment was successful 30 d after hospital discharge in 100% of patients given combination therapy vs. 45% of patients on monotherapy.[118] These data are particularly difficult to interpret when one considers that just 7 patients were given combination therapy, only one of whom had a malignancy. These reports illustrate the difficulties in interpretation of retrospective clinical data and highlight the quandary faced by clinicians caring for patients with mucormycosis.

Combination therapy against coccidioides

Coccidioides immitis and *Coccidioides posadasii* are the species that cause coccidioidomycosis, leading to symptoms such as pneumonia, fever, and skin nodules. In some individuals, infection progresses into a chronic disease.[119,120] The current practice guidelines advocate itraconazole, fluconazole, or amphotericin B alone as therapeutic regimens.[4] However, some cases are refractory to monotherapy. Few studies on combination therapy for *Coccidioides* have been reported. One case in 2006 described a patient with coccidioidomycosis who received caspofungin and fluconazole co-treatment with good efficacy instead of the recommended monotherapy with amphotericin B.[121] Levy *et al.* demonstrated several successful examples of combination therapy with voriconazole and caspofungin in pediatric patients with *Coccidioides* infection.[122] Although these case studies do not provide enough guidance on when to use combination therapy with this infection, refractory cases may warrant consideration of combination treatment.

Combination therapy as prophylaxis

Prophylaxis is important in high-risk patients, including immunocompromised, neutropenic, organ transplant, and chemotherapy patients. Currently, fluconazole, posaconazole, voriconazole, and micafungin have been proven to be effective prophylactic agents against IFIs in high-risk patients.[123-125] It is possible that combination prophylaxis would confer better protection from disease while decreasing the development of drug resistance. Krishna *et al.* demonstrated that posaconazole in combination with micafungin given to healthy volunteers was well tolerated and the pharmacokinetics of the 2 drugs were not affected.[126] Hiemenz *et al.* found that a combination of micafungin and fluconazole in immunocompromised bone marrow/stem cell transplant recipients

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was well tolerated for up to 4 weeks after transplant in a randomized, double-blinded dose escalation study.[127] Although the number of patients was low, a smaller percentage of patients in the combination prophylaxis group developed a suspected fungal infection. This evidence suggests the feasibility of successful combination prophylactic therapy with posaconazole and micafungin. More trials are needed to determine whether the possible benefits of combination prophylaxis outweigh the risks.

Conclusions

Over the past half-century, antifungal drugs have been developed to combat IFIs. However, IFIs are still associated with high morbidity and mortality, increased length of hospital stay, and high healthcare costs. This is partly due to the limited antifungal armamentarium, challenges in the timely diagnosis of pathogens, and adverse drug-drug interactions. Fortunately, newer formulations or antifungal agents (*e.g.*, isavuconazole) have entered the market (Table 1), providing clinicians with more options for the treatment of IFIs. In addition, combination therapy provides a potential strategy to increase the efficacy of 2 or more drugs, especially for drug-resistant fungal isolates, when fungicidal therapy is needed. Because each currently available antifungal drug has limitations in terms of the pharmacokinetics and pharmacodynamics profiles, spectrum activity, drug-drug interactions, and variability in absorption, TDM may be applied in patients receiving these antifungals. In the meantime, additional classes of antifungal drugs are needed to combat emerging fungal infections and drug-resistant isolates.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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ORCID

Ying-Lien Chen http://orcid.org/0000-0002-1966-470X

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