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Therapeutic alternatives for sporotrichosis induced by wild-type and non-wild-type *Sporothrix schenckii* through in vitro and in vivo assessment of enilconazole, isavuconazole, posaconazole, and terbinafine

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This study explores the effectiveness of various antifungal drugs in treating sporotrichosis caused by *Sporothrix schenckii*, especially in non-wild-type (non-WT) strains. The drugs tested include enilconazole (ENIL), isavuconazole (ISA), posaconazole (POS), terbinafine (TER), and itraconazole (ITC). The study involved in vitro and in vivo tests on 10 WT isolates and eight ITC non-WT isolates. Two isolates were assessed using time-kill assays, checkerboard assays, and *Galleria mellonella* infection models. In vitro studies have shown that all of these drugs were more effective than or equal to ITC against WT and non-WT isolates. No ITC resistance was observed with other azoles. All drugs inhibited fungal growth of WT and non-WT strains within 24 h at all incubations. ENIL and TER showed fungicidal effect against types at over 2x minimum inhibitory concentrations with no regrowth. POS was fungicidal against WT at high concentrations but not against non-WT. ISA was only fungicidal for non-WT. ITC did not exhibit any fungicidal activity. In checkerboard experiments, the combination of POS or ISA with TER showed enhanced activity against WT and non-WT strains, surpassing the combination of ITC with TER. In vivo model experiments demonstrated significantly reduced mortality rates with ENIL, POS, and TER against WT and with ENIL, ISA, POS, and TER against non-WT. The study concludes that monotherapy with ENIL, ISA, POS, and TER, and combinations of POS/TER or ISA/TER, show promise as effective antifungal treatments against *S. schenckii*, including ITC-non-WT isolates.

Keywords *Sporothrix schenckii.*, Sporotrichosis, Antifungals, Itraconazole nonwild-type, *Galleria mellonella* model

Sporotrichosis, often referred to as "rose gardener's disease" or "rose breeder's disease," is a subcutaneous mycosis caused by the *Sporothrix* species, a dimorphic saprophytic fungus[1]. The most common species observed in human and animal infections are *Sporothrix schenckii*, *S. globosa*, *S. brasiliensis*, and *S. luriei*. These species are grouped into the *Sporothrix* pathogenic clade based on molecular analysis. Infections typically occur through open wounds that come into contact with contaminated soil, plant debris, or infected animals. Zoonotic transmission can occur through scratches, bites, or penetrating skin lesions, with *S. schenckii* and *S. brasiliensis* known to have zoonotic transmission potential[2]. The geographical distribution of sporotrichosis reveals distinct patterns across various global regions. Brazil, particularly Rio de Janeiro, is one of the most significantly affected areas, experiencing hyperendemic outbreaks with substantial numbers of human and feline cases[1,3]. Other South

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American countries, such as Peru, Colombia, and Venezuela, also report notable disease prevalence[4]. In Asia, significant cases have been documented in China[5], India[6,7], and Japan[8], with increasing reports from Southeast Asian nations like Malaysia[9,10] and Thailand[11,12]. In Europe, particularly the United Kingdom, cases are sporadic and relatively rare, with occasional reports including isolated zoonotic transmissions from infected cats[13]. In Africa, hotspots are found in South Africa and Madagascar[14]. In North America, sporadic cases mainly occur in the U.S., especially in especially in southern and south-central state[15,16].

At present, antifungal medications are classified into five main classes: Allylamines, Azoles, Echinocandins, Polyenes, and Fluorinated Pyrimidine Analogs[17]. Reportedly, antifungal drugs from the Allylamines, Azoles, and Polyenes groups are frequently used as monotherapy or combination therapy to treat sporotrichosis in humans and animals[18,19]. Azole antifungals, divided into imidazole, triazole, and tetrazole groups, are the first-choice drugs for sporotrichosis. They disrupt fungal cell membranes by inhibiting lanosterol 14–α–demethylase, which converts lanosterol to ergosterol through Cytochrome P450 inhibition. This disruption increases cellular permeability and leaks cell contents. Among azoles, itraconazole is frequently used for *Sporothrix* infections. However, the growing incidence of drug resistance presents a notable therapeutic challenge. Terbinafine, an allylamine, has shown high in vitro efficacy against *Sporothrix* spp. and is recommended as a second-line therapy for human sporotrichosis[20]. This drug targets squalene epoxidase, a key enzyme in the early stages of ergosterol biosynthesis. By inhibiting this enzyme, terbinafine causes squalene accumulation in fungal cells, disrupting their function. Despite its promising effects, clinical evidence supporting terbinafine usage in sporotrichosis remains limited. Among Polyenes, Amphotericin B is pivotal in treating severe or resistant cases of sporotrichosis. Although effective, its use requires caution because of the associated side effects, including nephrotoxicity.

Evidence from in vitro and clinical trial studies indicates that drug combination therapy has increased efficacy over single-drug treatment. The combination of itraconazole with terbinafine is widely used to treat sporotrichosis in humans and cats[21]. In addition, in vitro studies have also demonstrated enhanced efficacy when itraconazole or fluconazole is used in conjunction with tacrolimus, particularly against *S. brasiliensis* and *S. schenckii* isolates[22]. Furthermore, local treatments such as amphotericin B intralesional injection and surgery are added to systemic therapy, they have shown promising results[23].

The emergence of drug resistance and adverse effects have prompted researchers to investigate newer alternative antifungal agents that promise enhanced efficacy and improved safety profiles. Examples of drugs currently under investigation for their efficacy in Sporothrix infection in human and animals include posaconazole and isavuconazole. Posaconazole (POS), a second-generation triazole, is a promising candidate for sporotrichosis treatment and has been used as an alternative to itraconazole to reduce hepatotoxic side effects. ISA, a second-generation triazole, is FDA-approved for first-line therapy of invasive Aspergillosis and mucormycosis[24]. Additionally, ENIL, an imidazole drug used only in veterinary medicine, is employed for treating Aspergillosis in cats via intranasal infusion and is primarily used as a topical therapy for dermatophytosis in animals. It is primarily considered as an alternative treatment for Sporothrix infections in animals.

sporotrichosis in cats was first reported in Thailand in 2017. Since then, the number of reported cases has been on the rise, especially in the Bangkok metropolitan region and southern Thailand[12,25]. Although sporotrichosis is potentially a zoonotic disease, reported human cases are sporadic[26,27]. Molecular epidemiology has revealed that the outbreak is associated with *S. schenckii*, a genetic lineage considered endemic only to Southeast Asia[25]. Recent reports suggest high levels of itraconazole non-WT isolates in this genotype, which are associated with subsequent treatment failure and recurrence[28]. Given these findings, there is an urgent need to investigate alternative antifungal drugs and treatment strategies. This study selected four antifungal drugs–enilconazole, isavuconazole, posaconazole, and terbinafine–as potential alternatives for treating sporotrichosis in wild-type (WT) and itraconazole non-WT clinical isolates of S. *schenckii* in Thailand. The in vitro antifungal activity of these drugs was assessed using broth microdilution, checkerboard, and time-kill assays. Additionally, the effectiveness of these antifungal drugs was determined using the *Galleria mellonella* infection model.

Results

Determination of MICs of selected antifungal drugs

The MICs of ENIL, ITC, ISA, POS, and TER against the mold phase of 18 clinical isolates and ATCC 58251 are detailed in Table 1 and 2. Among the 18 clinical isolates, 8 were classified as itraconazole non-WT and 10 as WT based on ECV ITC criteria (minimum inhibitory concentrations (MIC) > 2 mg/L)[29].

As observed from MIC ranges, all the 18 clinical isolates exhibited good activity against ENIL (MIC range < 0.06–4 mg/L) and TER (MIC range 0.25–4 mg/L) regardless of the ITC. Notably low MIC values for POS (MIC range 0.125–1 mg/L) were observed against ITC WT strains, while higher MIC values (MIC range 1–8 mg/L) were seen in ITC non-WT strains. ISA showed similar activity to POS against ITC non-WT strains. Notably, resistance to ITC was not observed with other azoles (POS and ISA) studied (Table 1).

Time–kill assays

For a more detailed analysis, we selected WT isolate VSMU21109 and ITC non-WT isolate VSMU21184 as representative strains. VSMU21109 was chosen due to its susceptibility profiles being similar to those of strain ATCC 58251, while VSMU21184 was randomly selected from a group of 7 isolates with high ITC MIC values (≥ 16 mg/L). Given that time–Kill assays, checkerboard assays, and in vivo experiments in *G. mellonella* are typically conducted using the yeast phase, we also performed the broth microdilution susceptibility test to determine the MICs of all tested drugs against the yeast phase of representative clinical isolates and ATCC 58251.

Figure 1 depicts the killing patterns of the representative WT and ITC non-WT strains when exposed to varying concentrations of the tested antifungal drugs. Due to solubility limitations, compounds ISA and ITC, which exhibited MIC values exceeding 16 mg/L, could not be effectively tested. Specifically, ISA could

Isolate number	MIC (mg/L)					Collection year	Location of sample collection	NCBI accessions number
	ITC	ENIL	ISA	POS	TER			
WT isolates ($n=10$)								
VSMU Deo	1	4	>8	0.5	4	2017	Bangkok	MG270181
VSMU Black	1	1	2	0.25	0.5	2018	Bangkok	PQ044604
VSMU TE	0.5	<0.06	2	0.25	0.25	2019	Bangkok	PQ044605
VSMU 21109	1	1	8	0.5	0.5	2021	Bangkok	PQ044606
VSMU 21170	0.25	2	8	0.5	1	2021	Nonthaburi	PQ044607
VSMU 21173	2	2	8	1	1	2021	Bangkok	PQ044608
VSMU 21198	1	2	8	0.125	1	2021	Nonthaburi	PQ044609
VSMU 21199	2	0.25	2	0.125	1	2021	Nonthaburi	PQ044610
VSMU 21200	0.5	1	8	0.125	2	2021	Nonthaburi	PQ044611
VSMU HN	0.5	1	4	0.25	0.5	2021	Bangkok	PQ044612
Range	0.25–2	<0.06–4	2–>8	0.125–1	0.25–4			
GM	0.81	0.93	4.92	0.29	0.87			
MIC_{50}	1	1	8	0.25	1			
MIC_{90}	2	2	8	0.5	2			
ITC Non-WT isolates ($n=8$)								
VSMU PP	>16	0.25	2	2	1	2017	Bangkok	MG270182
VSMU Bee	16	<0.06	1	1	0.25	2019	Bangkok	PQ044613
VSMU NN	4	2	8	8	1	2020	Bangkok	PQ044614
VSMU 21140	>16	<0.06	2	2	0.5	2021	Bangkok	PQ044615
VSMU 21164	>16	0.25	1	1	1	2021	Bangkok	PQ044616
VSMU 21184	>16	0.25	1	1	0.5	2021	Bangkok	PQ044617
VSMU 21185	>16	0.5	4	4	0.5	2021	Bangkok	PQ044618
VSMU 21221	>16	0.25	2	2	1	2021	Bangkok	PQ044619
Range	4–>16	<0.06–2	1–8	1–8	0.25–1			
GM	13.45	0.25	2	2	0.65			
MIC_{50}	*	*	*	*	*			
MIC_{90}	*	*	*	*	*			

Table 1. Comparison of MICs for five antifungal drugs against the mold phase of WT and ITC non-WT isolates. *ENIL* enilconazole, *GM* geometric mean, *ITC* itraconazole, *ISA* isavuconazole, MIC_{50} the lowest concentration of the tested antifungal at which 50% of theisolated were inhibited, MIC_{90} the lowest concentration of the tested antifungal at which90% of the isolated were inhibited, *POS* posaconazole, *TER* terbinafine, *WT*, wild-type, *non-WT* non-wild-type *MIC_{50} and MIC_{90} are unsuitable for datasets with fewer than 10 isolates.

Strain	MIC (mg/L)				
	ENIL mold yeast	ITC mold yeast	ISA mold yeast	POS mold yeast	TER mold yeast
ATCC 58251	1 1	1 1	8 8	0.25 0.25	0.5 0.5
VSMU 21109 (WT)	1 1	1 1	8 8	0.5 0.5	0.5 0.5
VSMU 21184 (ITC non-WT)	0.25 0.25	>16 >16	1 1	1 1	0.5 0.5

Table 2. MICs of selected antifungal drugs against representative WT and ITC-resistant strains in both mold and yeast phases. *ENIL* enilconazole, *ITC* itraconazole, *ISA* isavuconazole, *POS* posaconazole, *TER* terbinafine.

not be evaluated against WT strains, while ITC testing against non-WT strains was not feasible under these experimental conditions.

All tested drugs demonstrated killing activity against both strains at all tested concentrations after 24 h of incubation. Against the WT, ENIL, POS, and TER showed fungicidal effects at concentrations above 2x MIC, with no regrowth observed after 48–78 h. ENIL displayed rapid fungicidal activity within 48 h at 2 mg/L, followed by POS within 72 h at 1 mg/L, and finally TER within 96 h at 1 mg/L. No fungicidal activity was observed with ITC at any tested concentration over 96 h (Fig. 1a–d).

Against the non-WT strain, ENIL and TER showed the most effective fungicidal activity within 48 h at 0.5 mg/L and 1 mg/L, respectively. ISA exhibited fungicidal activity within 96 h at a 4xMIC concentration

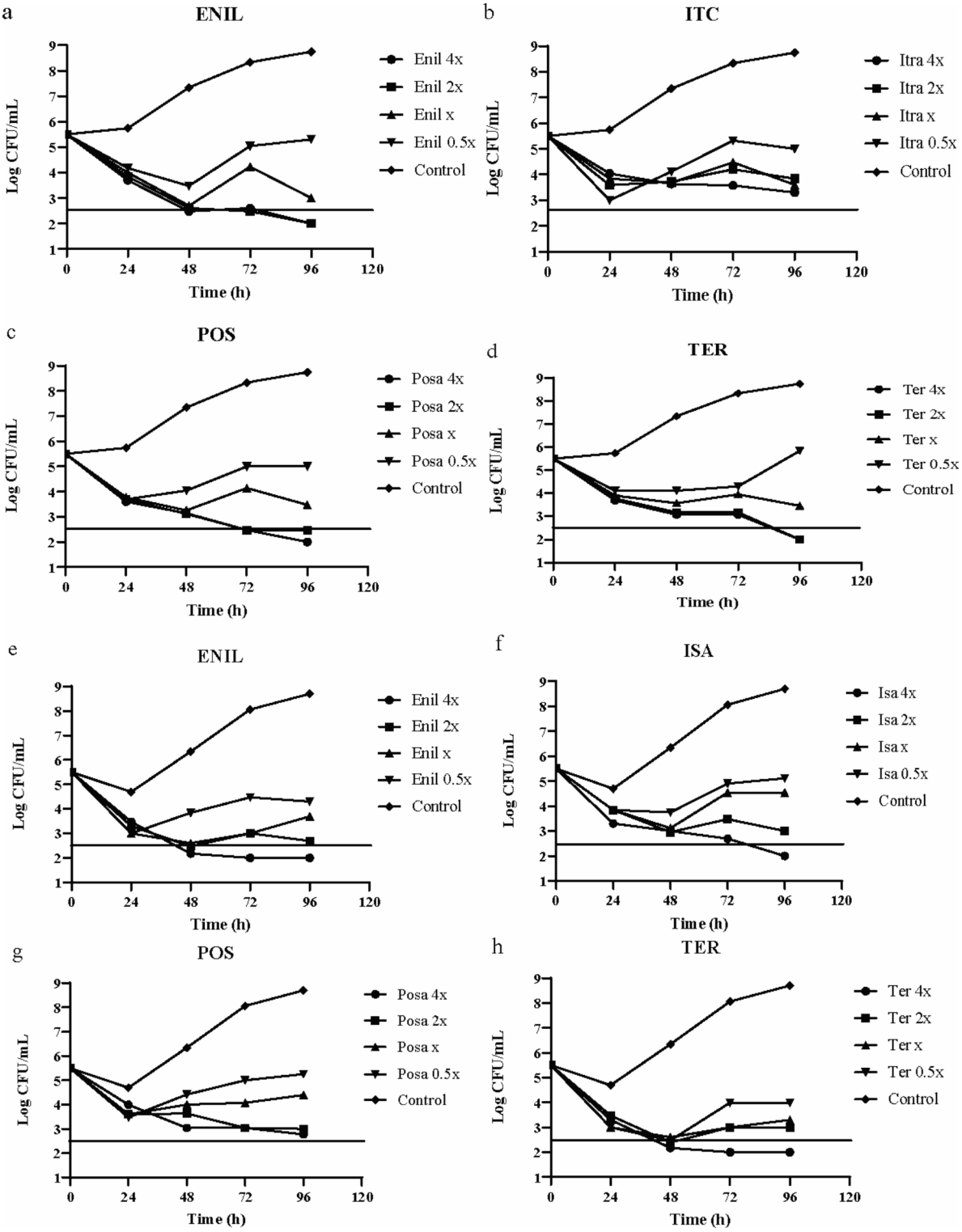

Fig. 1. Time-kill curves for selected antifungal drugs against WT and non-WT *Sporothrix schenckii* isolates at control, 0.5X, 1X, 2X, and 4XMIC. The figure presents time-kill curves for the WT after exposure to (**a**) enilconazole (ENIL), (**b**) itraconazole (ITC), (**c**) posaconazole (POS), and (**d**) terbinafine (TER), and for the non-WT after exposure to (**e**) enilconazole (ENIL), (**f**) isavuconazole (ISA), (**g**) posaconazole (POS), and (**h**) terbinafine (TER). The fungicidal line is indicated by a broken line. The time-kill curves presented here are from a single experiment, though experiments were performed in triplicate.

Isolates	Combination	FICI	Effect
Wild-type	TER + ISA	0.32	Synergy
	TER + ITC	0.7	Additive
	TER + POS	0.32	Synergy
Itraconazole non-wild-type	TER + ISA	0.2	Synergy
	TER + ITC	0.46	Synergy
	TER + POS	0.26	Synergy

Table 3. Results of the checkerboard assay for TER and triazole against wild-type and ITC non-wild-type strains of *Sporothrix schenckii*.

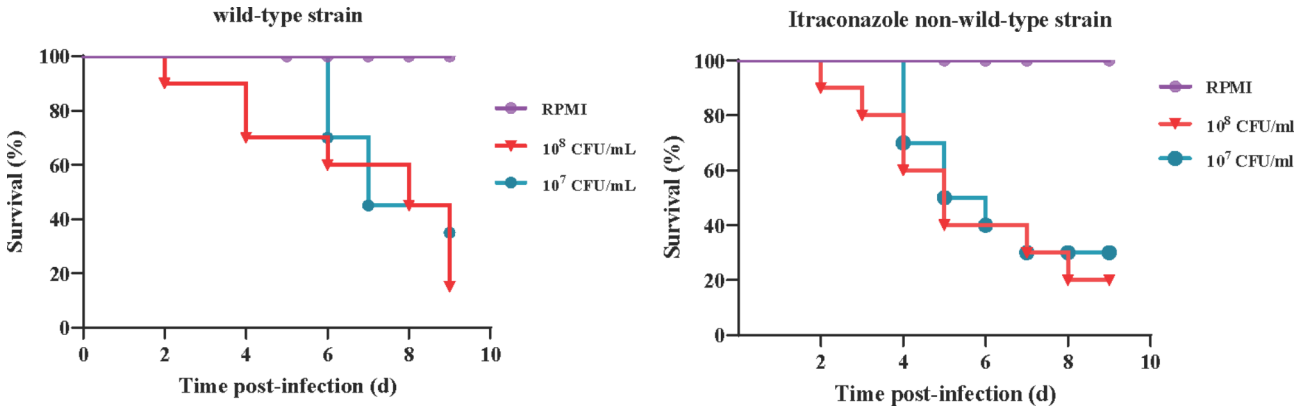

Fig. 2. Optimization of *Galleria mellonella* larvae as a model for clinical *S. schenckii* isolates. Dose-dependent mortality of *G. mellonella* larvae infected with two concentrations of wild-type or itraconazole non-wild-type, respectively. Survival was monitored every 24 h over a period of 9 days. Data are pooled from a minimum of two independent experiments.

(4 mg/L). POS showed a lower killing magnitude compared to the WT strain, and no fungicidal activity was observed (Fig. 1e–h).

Checkerboard assays

A synergistic effect was predominantly observed between TER and triazole drugs for the ITC non-WT strain. For this strain, the combination of TER with either ISA or POS proved equally effective, and both were more effective than the combination with ITC (Table 3).

Validation of *G. mellonella* as a model for *S. schenckii* infection

Prior to conducting the in vivo antifungal efficacy experiments, we performed two critical optimization studies. First, we assessed the safety of the drugs when administered in the *G. mellonella* model. Drugs and RPMI1640 medium were inoculated via the intrahemocoel route at the maximum effective dose. Nine days post-injection, no mortality was observed at the selected dosage (Data not shown). Concurrently, we determined the lethal dose of *S. schenckii* in the *G. mellonella* model. We infected the model with two doses (1×10^7 and 1×10^8 colony-forming units (CFU)/mL) of either WT or non-WT strains. With a high inoculum of 1×10^8 CFU/mL, the survival rates of *G. mellonella* observed until day 9 were 15% and 20% for the WT and non-WT groups, respectively (Fig. 2). These optimized parameters will be utilized in the antifungal efficacy experiments.

Antifungal drug efficacy in *G. mellonella* model

The antifungal efficacy in the *G. mellonella* was subsequently evaluated (Fig. 3). *G. mellonella* larvae treated with ENIL, ITC (or ISA for non-WT strain), POS, and TER exhibited significantly higher survival rates compared to untreated controls ($p<0.05$) for WT and non-WT strains at 1×10^8 CFU/mL. There were no significant differences among the antifungal treatments or between the low and high doses.

Discussion

In this study, we evaluated the effectiveness of alternative antifungal drugs, including ENIL, ISA, POS, and TER, compared to ITC against WT and itraconazole-resistant *S. schenckii* isolates from an endemic area in Thailand, using in vitro and in vivo methods. Our findings suggest that these alternative drugs may provide various treatment options, including single-agent regimens, combination regimens, and local treatments for controlling and minimizing *Sporothrix* infection, particularly in cases involving itraconazole-resistant isolates.

In this MIC study, we compared the efficacy of alternative drugs with that of ITC. For the WT category, our findings revealed that POS demonstrated the highest efficacy, followed by ENIL and TER. This is consistent

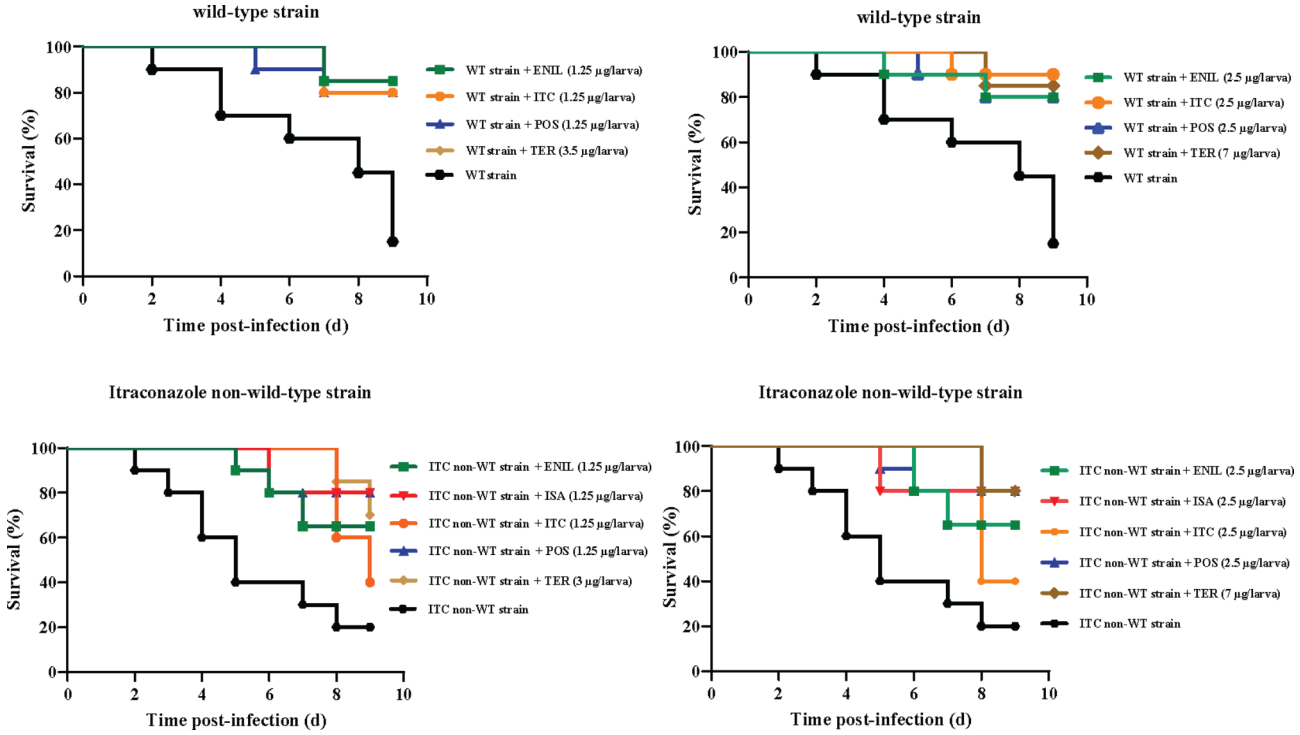

Fig. 3. Survival curves in Galleria mellonella. The figure presents Kaplan-Meier survival curves of G. mellonella larvae inoculated with 10^8 CFU/mL of the WT and non-WT. Following infection, larvae were treated with low or high doses of selected drugs. Control groups consisted of infected larvae treated with RPMI1640. Survival was monitored every 24 h over a period of 9 days. Data are pooled from a minimum of two independent experiments.

with previous studies that examined the MIC of POS and TER in a large number of isolates[29]. Interestingly, we observed variations in the activity of TER for *S. schenckii* and *S. bransileinsis*. The MIC_{90} value for TER with *S. schenckii* ranged from 0.5 to 1 mg/L[25]. In a comparative study of *S. brasiliensis* versus TER, more than 95% of *S. brasiliensis* had MICs to TER < 0.012 mg/L[29]. This suggests that *S. schenckii* has an intrinsically higher MIC to TER compared to *S. brasiliensis*. In contrast, we observed that ISA was not active against WT isolates, with a MIC_{90} of 8 mg/L, which aligns with recent studies[30]. For the ITC non-WT category, ENIL and TER showed the most in vitro activities. For POS, the MICs were higher than those of either agent for WT isolates, indicating reduced susceptibility to POS for this strain. However, they remained susceptible to POS based on POS ECV, suggesting no azole cross-resistance between POS and ITC among our isolates.

Clinical breakpoints or ECV for *S. schenckii* isolates are not well established. In this study, only the MIC of POS and ITC could be used to determine WT or non-WT characteristics[29]. This has significant implications for identifying appropriate treatment and monitoring susceptibility, suggesting that POS could be a good candidate against *S. schenckii*. For TER and ISA, no ECV has been established for *S. schenckii* due to insufficient numbers of isolates, suggesting caution is advised and further investigation into the establishment of ECV is warranted. It is important to note that non-WT does not necessarily imply resistance. Therefore, studying antifungal resistance mechanisms and their associated genes is crucial to determining the presence of true resistance.

Time–kill studies with fungal species have been used to evaluate the fungicidal effects of various drugs[31]. In this study, we used the yeast form of WT and non-WT strains to provide a comprehensive understanding of the drug effects. As expected, in the WT strain, ITC showed no fungicidal effect, similar to previous findings[32]. POS, ENIL, and TER were found to be remarkably fungicidal at concentrations greater than the MIC within 24, 48, and 72 h, respectively. Despite the limited number of in vitro studies, only TER in a previous study reported fungicidal activity against *S. schenckii* at 2xMIC within 24 h[33]. Conversely, the non-WT strain demonstrated that TER and ENIL maintained fungicidal activity similar to WT. However, these findings revealed that POS exhibited a lower killing magnitude. POS exhibited a lower killing magnitude than either agent for the WT strain, and no fungicidal activity was observed (Fig. 1c, g). These results strongly support the MIC study on reduced susceptibility to POS for this strain. This aligns with previous studies that tested azole drugs against *Candida* strains, resistant and nonresistant, showing comparable time–kill results among the drugs[34]. However, a limitation of this study is the use of only one representative strain per group, potentially hindering the generalizability of the results to a broader range of fungal strains. Further study of pharmacokinetic/pharmacodynamic (PK/PD) modeling is needed[35].

Combination antifungal treatment offers several benefits over monotherapy. It notably improves treatment efficacy, enhances effectiveness against drug-resistant cases, reduces necessary dosages, and minimizes drug toxicity[36]. A combination regime of amphotericin B (AMB) intravenously and azole orally is a recommended

option for sporotrichosis treatment[37]. Although AMB demonstrates efficacy against *Sporothrix* spp., its systemic administration is limited by adverse effects, particularly nephrotoxicity. Alternatively, TER offers an additional choice in combination treatments for sporotrichosis. Previous studies have demonstrated the effectiveness of combining TER with ITC[38,39]. Moreover, several cases of sporotrichosis in humans and cats were successfully treated with a combination of TER and ITC[21,40]. In this study, the results showed a synergistic effect between ISA or POS and TER in WT and non-WT strains, and POS/TER and ISA/TER were more effective than ITC/TER (Table 3). This study extends previous experiments, supporting the effectiveness of combining TER with ITC and providing enhanced activity of POS and ISA compared to ITC in the current combination drug treatment.

In a veterinary context, ensuring medication adherence in cats can pose a challenge for owners, often resulting in treatment failure[41]. A significant factor is the limited access to medication, particularly with extended oral administration schedules[42]. Consistent oral medication can be particularly difficult with cats, especially stray cats. POS and ISA emerge as promising alternatives due to their potential for intravenous administration[43,44]. The intravenous route reduces the risk of zoonotic transmission during medication administration compared to oral medications, as it eliminates the need to handle a cat's mouth[45].

Ulceration on the face and sinonasal region are common clinical manifestations of sporotrichosis in cats. An unconventional treatment strategy combining local and systemic antifungals has been employed. The use of AMB intralesionally, in conjunction with oral ITC, has been described for cases with cutaneous or lymphocutaneous sporotrichosis[23]. Given the in vitro and in vivo findings with an aerosol spray formulation, we propose ENIL as a topical drug candidate for sporotrichosis treatment. Furthermore, this drug also presents new potential applications via intranasal and nebulization routes[46].

Traditional antifungal drug development and pathogenesis research for *Sporothrix* spp. infection have relied on efficacy evaluation in mammalian models, such as mice and rats. However, these models can be costly and time-consuming[47]. The larva of the greater wax moth, *G. mellonella*, has emerged as a promising alternative model due to its low cost, rapid life cycle, and immune system similar to mammals[48]. It is known that the outcome of in vivo infections can be influenced by factors such as the doses of infection, the culture medium used, and the rearing and maintenance of *G. mellonella*[47,49,50]. In this study, we proposed a set of conditions for an in vivo study based on previous research[51,52]. With slight modifications, yeast growth on Brain Heart Infusion (BHI) agar, in-house rearing, and an inoculum dose of 1×10^8 CFU/mL are now well established in our lab. Our findings will be useful for future research directions that could explore the use of *G. mellonella* to evaluate a broader range of antifungal drugs and pathogenesis.

To our knowledge, the study of the virulence of WT and non-WT strains of *S. schenckii* using *G. mellonella* has not been determined. We conducted experiments to investigate the virulence of both strain types. When observing at 50% mortality, we noted a faster rate of fungal burden in the ITC non-WT compared to the WT within the *G. mellonella* model. This may suggest that the ITC non-WT strain is more virulent than the WT strain.

Our investigation revealed that the ITC treatment groups for the non-WT strain of *S. schenckii* exhibited the lowest survival rate (40%) compared with the alternative drugs. This observation strongly supports the notion that the non-WT strain has developed resistance to ITC. However, this observed difference did not achieve statistical significance ($p > 0.05$) within the confines of the current study. We are unable to conclusively identify the underlying reason for this observation. Further research with substantially larger and more diverse samples will be needed.

In conclusion, this study underscores the need for vigilant monitoring of feline sporotrichosis cases in Thailand, particularly those that are resistant or relapse following itraconazole (ITC) treatment. For managing sporotrichosis, it is advisable to consider alternative antifungal medications like ENIL, ISA, POS, or TER based on antifungal susceptibility testing outcomes. This is especially critical when dealing with strains that are not susceptible to ITC or when ITC may not be the most suitable choice. We recommend employing these alternative antifungal agents in scenarios where ITC resistance is evident or when ITC is not the optimal treatment. Further clinical research is necessary to explore the efficacy and safety of these alternative drug options in treating sporotrichosis.

Methods

Fungal strains and culture conditions

This study utilized *S. schenckii* ATCC 58251 and 18 clinical isolates of *S. schenckii.* ATCC 58251 was used as a quality control and reference strain. Among the 18 clinical isolates, 8 were classified as itraconazole non-WT and 10 as WT. All isolates were obtained from cats in Thailand and were selected from the Laboratory of Veterinary Mycology at Mahidol University (Salaya campus, Nakhon Pathom, Thailand). Each isolate was identified by PCR of ITS regions (Bangphoomi, unpublished). All isolates were tested for non-WT for itraconazole using a broth microdilution test according to CLSI itraconazole ECV, as previously described[29]. All isolates were stored in RPMI 1640 medium with MOPS (Invitrogen, USA) and 10% glycerol, and were kept at − 70 °C until use.

Before each experiment, the reference and clinical strains were cultured in either Sabouraud dextrose agar with chloramphenicol (Oxoid, UK) and incubated at 25 °C for 4–5 days for mold phase experiments, or in BHI agar (Himedia, India), and incubated at 37 °C for 5–7 days for yeast phase experiments. The isolates were examined microscopically with lactophenol blue stain and identified as *Sporothrix* spp. based on morphological identification.

Determination of MICs

The study determined the MICs using broth microdilution methods, following CLSI guidelines M27-A4 for yeasts[53] and M38-A3 for molds[54]. The tested drugs included enilconazole (ENIL), isavuconazole (ISA), itraconazole (ITC), posaconazole (POS), and terbinafine (TER) (Sigma Aldrich, USA). Final drug concentrations

ranged from 0.06 to 8 mg/L, except for itraconazole, which ranged from 0.06 to 16 mg/L. The MIC was defined as the lowest concentration achieving 100% inhibition of *Sporothrix* growth. Strains were categorized as non-WT if the MIC for ITC or POS exceeded > 2 mg/L. All tests were conducted in triplicate, with *Candida albicans* ATCC 90028, *Paecilomyces variotii* ATCC MYA-3630 and *Sporothrix schenckii* ATCC 58251 used as a quality control strain[55].

Time-kill assay

This assay assessed the time-dependent antifungal activity against WT and ITC non-WT strains of *Sporothrix schenckii*. A yeast suspension (10^6 CFU/mL) was cultivated at 0.5×, 1×, 2×, and 4× MIC of antifungals at 35 °C in a rotary shaker at 200 rpm. Samples were collected at 0, 12, 24, 48, 72, and 96 h, plated on BHI agar, and CFU were counted. Antifungal concentrations that reduced fungal cell counts by over 3 logs from the initial CFU/mL were considered fungicidal. This method was also performed in triplicate[56].

Checkerboard assay

The study also evaluated the synergistic effects of selected alternative azoles (ISA, POS, and ITC) combined with TER against the WT and ITC non-WT representative strains of *S. schenckii*. This was assessed using the broth microdilution checkerboard method[32]. The fractional inhibitory concentration (FIC) for each antifungal drug in combination was calculated by dividing the MIC of one antifungal in the mixture by its MIC when used alone. The combined effect of both antifungals was evaluated by calculating the FIC index (FICI), which is the sum of the FIC values of both antifungals. A FICI of 0.5 or less indicated synergistic interaction; a FICI greater than 0.5 but less than or equal to 4 suggested no significant interaction; and a FICI greater than 4 indicated antagonism. This method was also performed in triplicate.

Galleria mellonella infection assay

Healthy *Galleria mellonella* larvae (size 2–2.5 cm and average weight of 250 mg) were reared in-house starting from eggs, following the guidelines[49]. Groups of 20 randomly selected larvae were inoculated by intrahemocoel at the last left proleg of *G. mellonella* with Hamilton syringes. Ten microliters of WT and non-WT strains at 10^7 or 10^8 CFU/mL were injected. All groups were kept in Petri dishes at 37°C in the dark, and survival was checked daily. A cotton ball soaked with sterile distilled water was placed in the animal housing to prevent dehydration. Mortality was determined by a lack of irritability and significant body melanization. All experiments were repeated with two independent biological replications.

Antifungal drug efficacy in *G. mellonella* model

To evaluate the efficacy of antifungal drugs, yeast inocula were prepared from BHI agar and diluted to 10^8 CFU/mL in RPMI 1640. Groups of 20 randomly selected larvae were inoculated by intrahemocoel infection (10 µL of suspension) at the last left proleg of *G. mellonella*. Within 2 h of infection, 10 µL of an antifungal drug was injected into the last right proleg. Each antifungal dose was prepared in two increasing dosages, converted to reflect their relevance to the therapeutic doses administered to humans and animals, as per guidelines (measured in mg/kg). Control groups included: noninoculated larvae, larvae injected with RPMI 1640, larvae injected with antifungal drugs without inoculum, and larvae injected with only yeast suspension. All groups were kept in Petri dishes at 37°C in the dark, and survival was checked daily. All experiments were repeated with two independent biological replications.

Data analysis

Descriptive statistics, encompassing MIC, MIC_{50}, MIC_{90}, and geometric mean, were calculated for each drug. Survival analysis was conducted using Kaplan–Meier charts, and the data was analyzed using the log-rank (Mantel–Cox) method with the GraphPad Prism 8 program. Differences were deemed significant at a $^*p$-value of ≤ 0.05.

Data availability

The sequences have been deposited in GenBank under accession numbers MG270181-2 and PQ044604-19. The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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References

1. Barros, M. B., de Almeida Paes, R. & Schubach, A. O. Sporothrix schenckii and Sporotrichosis. *Clin. Microbiol. Rev.* **24**, 633–654. https://doi.org/10.1128/CMR.00007-11 (2011).
2. Rodrigues, A. M., Hagen, F. & de Camargo, Z. P. A spotlight on Sporothrix and Sporotrichosis. *Mycopathologia* **187**, 407–411. https://doi.org/10.1007/s11046-022-00642-9 (2022).
3. Gremiao, I. D. F., Oliveira, M. M. E., Monteiro de Miranda, L. H., Freitas, S. & Pereira, S. A. Geographic expansion of Sporotrichosis, Brazil. *Emerg. Infect. Dis.* **26**, 621–624. https://doi.org/10.3201/eid2603.190803 (2020).
4. Rodrigues, A. M. et al. The threat of emerging and re-emerging pathogenic Sporothrix species. *Mycopathologia* **185**, 813–842. https://doi.org/10.1007/s11046-020-00425-0 (2020).
5. Liu, T. T., Zhang, K. & Zhou, X. Molecular identification of Sporothrix clinical isolates in China. *J. Zhejiang Univ. Sci. B.* **15**, 100–108. https://doi.org/10.1631/jzus.B1300136 (2014).
6. Chakrabarti, A., Roy, S. K., Dhar, S. & Kumar, B. Sporotrichosis in north-west India. *Indian J. Med. Res.* **100**, 62–65 (1994).

7. Chakrabarti, A., Bonifaz, A., Gutierrez-Galhardo, M. C., Mochizuki, T. & Li, S. Global epidemiology of sporotrichosis. *Med. Mycol.* **53**, 3–14. https://doi.org/10.1093/mmy/myu062 (2015).
8. Suzuki, R. et al. Studies in phylogeny, development of rapid identification methods, antifungal susceptibility, and growth rates of clinical strains of Sporothrix schenckii Complex in Japan. *Med. Mycol. J.* **57**, E47–57. https://doi.org/10.3314/mmj.16-00005 (2016).
9. Han, H. S. & Kano, R. Feline sporotrichosis in Asia. *Braz J. Microbiol.* **52**, 125–134. https://doi.org/10.1007/s42770-020-00274-5 (2021).
10. Kamal Azam, N. K. et al. Molecular epidemiology of Sporothrix Schenkii isolates in Malaysia. *Med. Mycol.* **58**, 617–625. https://doi.org/10.1093/mmy/myz106 (2020).
11. Duangkaew, L., Yurayart, C., Limsivilai, O., Chen, C. & Kasorndorkbua, C. Cutaneous sporotrichosis in a stray cat from Thailand. *Med. Mycol. Case Rep.* **23**, 46–49. https://doi.org/10.1016/j.mmcr.2018.12.003 (2019).
12. Indoung, S. et al. Feline sporotrichosis caused by Sporothrix schenckii Sensu Stricto in Southern Thailand: phenotypic characterization, molecular identification, and antifungal susceptibility. *Med. Mycol.* **60** https://doi.org/10.1093/mmy/myac075 (2022).
13. Rachman, R., Ligaj, M., Chinthapalli, S. & Serafino Wani, R. Zoonotic acquisition of cutaneous Sporothrix braziliensis infection in the UK. *BMJ Case Rep.* **15** https://doi.org/10.1136/bcr-2021-248418 (2022).
14. Govender, N. P. et al. An outbreak of Lymphocutaneous Sporotrichosis among Mine-Workers in South Africa. *PLoS Negl. Trop. Dis.* **9**, e0004096. https://doi.org/10.1371/journal.pntd.0004096 (2015).
15. Zhang, Y. et al. Phylogeography and evolutionary patterns in Sporothrix spanning more than 14 000 human and animal case reports. *Persoonia* **35**, 1–20. https://doi.org/10.3767/003158515X687416 (2015).
16. Benedict, K. & Jackson, B. R. Sporotrichosis cases in commercial insurance data, United States, 2012–2018. *Emerg. Infect. Dis.* **26**, 2783–2785. https://doi.org/10.3201/eid2611.201693 (2020).
17. Bondaryk, M., Staniszewska, M., Zielinska, P. & Urbanczyk-Lipkowska, Z. Natural antimicrobial peptides as inspiration for design of a New Generation Antifungal compounds. *J. Fungi (Basel)*. **3** https://doi.org/10.3390/jof3030046 (2017).
18. Kauffman, C. A., Hajjeh, R. & Chapman, S. W. Practice guidelines for the management of patients with sporotrichosis. For the Mycoses Study Group. Infectious Diseases Society of America. *Clin. Infect. Dis.* **30**, 684–687. https://doi.org/10.1086/313751 (2000).
19. Alvarez, C. M., Oliveira, M. M. E. & Pires, R. H. Sporotrichosis. A review of a neglected disease in the last 50 years in Brazil. *Microorganisms* 10, (2022). https://doi.org/10.3390/microorganisms10112152
20. Marimon, R., Serena, C., Gene, J., Cano, J. & Guarro, J. In vitro antifungal susceptibilities of five species of sporothrix. *Antimicrob. Agents Chemother.* **52**, 732–734. https://doi.org/10.1128/AAC.01012-07 (2008).
21. Vettorato, R. et al. Sporotrichosis by Sporothrix schenckii senso stricto with itraconazole resistance and terbinafine sensitivity observed in vitro and in vivo: Case report. *Med. Mycol. Case Rep.* **19**, 18–20. https://doi.org/10.1016/j.mmcr.2017.10.001 (2018).
22. Borba-Santos, L. P. et al. Tacrolimus increases the effectiveness of itraconazole and fluconazole against Sporothrix spp. *Front. Microbiol.* **8**, 1759. https://doi.org/10.3389/fmicb.2017.01759 (2017).
23. Gremião, I. D. et al. Intralesional amphotericin B in a cat with refractory localised sporotrichosis. *J. Feline Med. Surg.* **11**, 720–723. https://doi.org/10.1016/j.jfms.2009.01.012 (2009).
24. Woerde, D. J., Wittenburg, L. A. & Dear, J. D. Pharmacokinetics of isavuconazole in healthy cats after oral and intravenous administration. *J. Vet. Intern. Med.* **36**, 1422–1429. https://doi.org/10.1111/jvim.16452 (2022).
25. Yingchanakiat, K. et al. Phenotypic and genotypic characterization and Antifungal susceptibility of Sporothrix schenckii Sensu Stricto isolated from a Feline Sporotrichosis Outbreak in Bangkok, Thailand. *J. Fungi (Basel)*. **9** https://doi.org/10.3390/jof9050590 (2023).
26. Chieosilapatham, P. et al. Sporothrix schenckii Sensu stricto related to zoonotic transmission in Thailand. *Med. Mycol. Case Rep.* **41**, 44–47. https://doi.org/10.1016/j.mmcr.2023.08.003 (2023).
27. Tovikkai, D. et al. Sporotrichosis: the case series in Thailand and literature review in Southeast Asia. *Med. Mycol. Case Rep.* **27**, 59–63. https://doi.org/10.1016/j.mmcr.2020.01.002 (2020).
28. Nakasu, C. C. T. et al. Feline sporotrichosis: a case series of itraconazole-resistant Sporothrix brasiliensis infection. *Braz J. Microbiol.* **52**, 163–171. https://doi.org/10.1007/s42770-020-00290-5 (2021).
29. Espinel-Ingroff, A. et al. International Study of MIC/MEC distributions for definition of epidemiological cutoff values for sporothrix species identified by molecular methods. *Antimicrob. Agents Chemother.* **61** https://doi.org/10.1128/AAC.01057-17 (2017). Multicenter.
30. Ribeiro, D. et al. Bimodal distribution of azole susceptibility in Sporothrix brasiliensis isolates in Brazil. *Antimicrob. Agents Chemother.* **68**, e0162023. https://doi.org/10.1128/aac.01620-23 (2024).
31. Ambrose, P. G. et al. Pharmacokinetics-pharmacodynamics of antimicrobial therapy: it's not just for mice anymore. *Clin. Infect. Dis.* **44**, 79–86. https://doi.org/10.1086/510079 (2007).
32. Borba-Santos, L. P. et al. Delta(24)-Sterol methyltransferase plays an important role in the growth and development of Sporothrix schenckii and Sporothrix brasiliensis. *Front. Microbiol.* **7**, 311. https://doi.org/10.3389/fmicb.2016.00311 (2016).
33. Almeida-Paes, R. et al. Melanins protect sporothrix brasiliensis and sporothrix schenckii from the antifungal effects of terbinafine. *PLoS ONE* **11**, e0152796. https://doi.org/10.1371/journal.pone.0152796 (2016).
34. Li, Y. et al. In vitro interaction between azoles and cyclosporin A against clinical isolates of Candida albicans determined by the chequerboard method and time-kill curves. *J. Antimicrob. Chemother.* **61**, 577–585. https://doi.org/10.1093/jac/dkm493 (2008).
35. Khan, D. D. et al. A mechanism-based pharmacokinetic/pharmacodynamic model allows prediction of antibiotic killing from MIC values for WT and mutants. *J. Antimicrob. Chemother.* **70**, 3051–3060. https://doi.org/10.1093/jac/dkv233 (2015).
36. Cuenca-Estrella, M. Combinations of antifungal agents in therapy–what value are they? *J. Antimicrob. Chemother.* **54**, 854–869. https://doi.org/10.1093/jac/dkh434 (2004).
37. Mario, D. N., Guarro, J., Santurio, J. M., Alves, S. H. & Capilla, J. In Vitro and in vivo efficacy of amphotericin B combined with posaconazole against experimental disseminated sporotrichosis. *Antimicrob. Agents Chemother.* **59**, 5018–5021. https://doi.org/10.1128/AAC.00052-15 (2015).
38. Scheid, L. A., Mario, D. A., Kubiça, T. F., Santurio, J. M. & Alves, S. H. In vitro activities of antifungal agents alone and in combination against fluconazole-susceptible and -resistant strains of Candida Dubliniensis. *Braz J. Infect. Dis.* **16**, 78–81. https://doi.org/10.1016/s1413-8670(12)70279-9 (2012).
39. Hassaan, Z., Mohamed, H. A. K., Eldahshan, R. M. & Elsaie, M. L. Comparison between the efficacy of terbinafine and itraconazole orally vs. the combination of the two drugs in treating recalcitrant dermatophytosis. *Sci. Rep.* **13**, 19037. https://doi.org/10.1038/s41598-023-46361-z (2023).
40. Guo, K., Wang, S., Wang, Z. & Zhang, L. Effective treatment using itraconazole combined with terbinafine in the treatment of nasal sporotrichosis: a case report. *Medicine* **98**, e17155. https://doi.org/10.1097/md.0000000000017155 (2019).
41. Gremião, I. D. F. et al. Advances and challenges in the management of feline sporotrichosis. *Rev. Iberoam Micol.* **39**, 61–67. https://doi.org/10.1016/j.riam.2022.05.002 (2022).
42. Taylor, S., Caney, S., Bessant, C. & Gunn-Moore, D. Online survey of owners' experiences of medicating their cats at home. *J. Feline Med. Surg.* **24**, 1283–1293. https://doi.org/10.1177/1098612x221083752 (2022).
43. Miceli, M. H. & Kauffman, C. A. Isavuconazole. A new broad-spectrum triazole antifungal agent. *Clin. Infect. Dis.* **61**, 1558–1565. https://doi.org/10.1093/cid/civ571 (2015).

44. Kersemaekers, W. M. et al. Pharmacokinetics and safety study of posaconazole intravenous solution administered peripherally to healthy subjects. *Antimicrob. Agents Chemother.* **59**, 1246–1251. https://doi.org/10.1128/aac.04223-14 (2015).
45. Gremiao, I. D. et al. Feline sporotrichosis: epidemiological and clinical aspects. *Med. Mycol.* **53**, 15–21. https://doi.org/10.1093/mmy/myu061 (2015).
46. Tamborini, A., Robertson, E., Talbot, J. J. & Barrs, V. R. Sinonasal aspergillosis in a British Shorthair cat in the UK. *JFMS Open. Rep.* **2**, 2055116916653775. https://doi.org/10.1177/2055116916653775 (2016).
47. Vargas-Macías, A. P., Gómez-Gaviria, M., García-Carnero, L. C. & Mora-Montes, H. M. Current models to study the Sporothrix-host Interaction. *Front. Fungal Biol.* **3**, 833111. https://doi.org/10.3389/ffunb.2022.833111 (2022).
48. Clavijo-Giraldo, D. M. et al. Analysis of Sporothrix schenckii Sensu Stricto and Sporothrix brasiliensis virulence in Galleria mellonella. *J. Microbiol. Methods*. **122**, 73–77. https://doi.org/10.1016/j.mimet.2016.01.014 (2016).
49. de Jong, A. W., van Veldhuizen, D., Groot, A. T. & Hagen, F. Standardized methods to rear high-quality Galleria mellonella larvae for the study of fungal pathogens. *Entomol. Exp. Appl.* **170**, 1073–1080. https://doi.org/10.1111/eea.13237 (2022).
50. Jemel, S., Guillot, J., Kallel, K., Botterel, F. & Dannaoui, E. Galleria mellonella for the evaluation of antifungal efficacy against medically important fungi, a narrative review. *Microorganisms* **8** https://doi.org/10.3390/microorganisms8030390 (2020).
51. Fernandes, G. F. et al. Characterization of virulence profile, protein secretion and immunogenicity of different Sporothrix schenckii sensu stricto isolates compared with S. Globosa and S. brasiliensis species. *Virulence* **4**, 241–249. https://doi.org/10.4161/viru.23112 (2013).
52. Lozoya-Perez, N. E. et al. Influences of the culturing media in the virulence and cell wall of Sporothrix schenckii, Sporothrix brasiliensis, and Sporothrix Globosa. *J. Fungi (Basel)*. **6** https://doi.org/10.3390/jof6040323 (2020).
53. CLSI. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast* 4th ed (CLSI standard M27, 2017).
54. CLSI. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi* 3rd ed (CLSI standard M38, 2017).
55. Bourgeois, N. et al. Antifungal susceptibility of 205 Candida spp. isolated primarily during invasive candidiasis and comparison of the Vitek 2 system with the CLSI broth microdilution and etest methods. *J. Clin. Microbiol.* **48**, 154–161. https://doi.org/10.1128/JCM.01096-09 (2010).
56. Klepser, M. E., Ernst, E. J., Lewis, R. E., Ernst, M. E. & Pfaller, M. A. Influence of test conditions on antifungal time-kill curve results: proposal for standardized methods. *Antimicrob. Agents Chemother.* **42**, 1207–1212. https://doi.org/10.1128/aac.42.5.1207 (1998).

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Author contributions

Conceptualization: Norasuthi Bangphoomi; Methodology: Norasuthi Bangphoomi; Formal analysis and investigation: Vasurom Aroonvuthiphong; Writing – original draft preparation: Vasurom Aroonvuthiphong; Writing – review and editing: Norasuthi Bangphoomi; Funding acquisition: Norasuthi Bangphoomi; Resources: Norasuthi Bangphoomi; Supervision: Norasuthi Bangphoomi.

Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

This study has obtained permission from the MUVS Application for Permission of Animal Care and Use (MUVS-ACU F01). The protocol has been approved with the number MUVF-2022-01-02.

Additional information

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