Synergistic activity of phenazines isolated from *Pseudomonas aeruginosa* in combination with azoles against *Candida* species

S. Nishanth Kumar, G. V. Nisha, A. Sudaresan, V. V. Venugopal, M. M. Sree Kumar, Ravi S. Lankalapalli and B. S. Dileep Kumar*

Agroprocessing and Natural Products Division, National Institute for Interdisciplinary Science and Technology (NIIST), Council of Scientific and Industrial Research (CSIR), Thiruvananthapuram 695 019, Kerala, India

*To whom correspondence should be addressed. E-mail: kumardileep@niist.res.in, micronishanth@rediffmail.com

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Abstract

Candidiasis infections are caused by yeasts from the genus *Candida*. The types of infection range from superficial to systemic. Treatment often requires antifungals such as the azoles; however, increased use of these drugs has led to the generation of yeasts with increased resistance to these drugs. Here, we describe the synergistic antifungal activity of three phenazines—phenazine-1-ol, phenazine-1-carboxylic acid, and phenazine-1-carboxamide. These phenazines were purified from *Pseudomonas aeruginosa* in combination with three clinically used azoles—fluconazole, itraconazole, and clotrimazole. The synergistic antifungal activities of phenazines and azoles were assessed using the checkerboard microdilution and time–kill methods. Study results show that the combined effects of phenazines and azoles were predominantly synergistic activity (fractional inhibitory concentration index < 0.5). The time–kill study, which included a combination of the minimum inhibitory concentration of phenazines and azoles, showed growth of *Candida* species that was completely attenuated after 0–6 h of treatment. These results, which suggest that the activity of phenazines and azoles may be beneficial, have potential implications in delaying the development of resistance, as the antifungal effect is achieved with lower concentrations of both agents (phenazines and azoles). The cytotoxicity of phenazines was also tested against a normal human cell line (foreskin normal fibroblast). No cytotoxicity was recorded at concentrations up to 200 µg/ml. The *in vitro* synergistic activity of phenazines and azoles against *Candida* species is reported here for the first time.

Key words: azoles, *Candida* species, phenazines, synergistic.

Introduction

The incidence of invasive fungal infections, particularly those caused by *Candida* species, has increased worldwide during the last 20 years, especially in immunosuppressed patients, including those with acquired immunodeficiency syndrome (AIDS) [1]. *Candida albicans* is the predominant causative organism of virtually all types of candidiasis [2];
however, today C. tropicalis, C. glabrata, C. krusei, and C. parapsilosis are involved in serious infections in patients [3–4]. In addition, C. albicans is a leading Candida species involved in bloodstream infections (candidemia), oral thrush, and vaginal yeast infections [5–6]. Candidemia often results in a high mortality rate (30%), particularly if appropriate antifungal drug treatments are delayed [7]. Azoles such as fluconazole (FLU) and ketoconazole are the most commonly to treat Candida infections [8]. However, with increasing usage of azoles against candidiasis, the number and variety of azole-resistant yeast strains have increased [9]. In addition, the introduction and use of these and other antifungal drugs have not kept pace with the increased incidence of drug resistance [10]. The use of natural compounds coadministered with conventional chemotherapeutics is an interesting strategy for overcoming drug-resistant fungi and, thereby, restoring their previous susceptibility.

Phenazines are natural products found in Pseudomonas, Streptomyces, and a few other bacterial genera from soil and marine habitats. They comprise a large family of colorful, nitrogen-containing tricyclic molecules with antibacterial, antitumor, and antiparasitic activities [11]. Phenazines isolated from Pseudomonas species (eg, P. aeruginosa, P. aureofaciens, P. fluorescens, and P. cepacia) are primarily simple hydroxyl- and carboxyl-substituted structures. Pyocyanin, phenazine-1-carboxylic, and phenazine-1-carboxamide are among the phenazines produced by pseudomonads, mainly rhizosphere isolates [12]. Interestingly, pseudomonads are known to produce 50 naturally occurring phenazine compounds [12,13]. Antifungal activity of phenazine compounds against the phytopathogenic fungi Rhizoctonia solani, Sclerotinia sclerotiorum, R. solani and Xanthomonas oryzae pv. oryzae, and Fusarium oxysporum has been reported [14–16]. Physiologically, phenazine inhibits and controls nucleic acid and protein synthesis [11]; therefore, the modes of action for phenazines may include interactions with DNA (intercalation or groove binding), topoisomerases, antioxidants, and charge-transferring molecules [11].

Here, we evaluate the in vitro synergistic effects of three phenazines isolated from P. aeruginosa in combination with azoles against Candida species. The present work highlights a promising role of phenazines in combination with the three most widely used azoles for anticandidal activity.

Materials and methods

Test compounds

The test phenazines—phenazine-1-ol (POL), phenazine-1-carboxylic acid (PCA), and phenazine-1-carboxamide (PCN; Fig. 1)—were isolated and purified from succinate broth that supported the growth of P. aeruginosa. Pseudomonas aeruginosa was grown on the succinate broth for 72 h at 28°C at 180 rpm in a rotary shaker. Supernatant was collected after the broth was centrifuged at 10,000 × g for 10 min. The crude was extracted using chloroform (3:1 v/v) in a separating funnel and concentrated to obtain dried crude extract. The extract was then purified by silica gel column chromatography (35 cm × 2 cm) using a hexane/ethyl acetate mobile phase combination with a gradient of 9:1, 4:1, 3:1, and 1:1 in order to yield the three phenazine compounds. The chemical structure of the compounds was established on the basis of spectral analyses. FLU, itraconazole (ITR), and clotrimazole (CLO; all from Sigma-Aldrich, MO, USA) were used as standard antifungal agents (Fig. 1).

Test organism

Candida albicans MTCC 277 and C. tropicalis MTCC 184, which were obtained from the Microbial Type Culture
Collection and Gene Bank (MTCC) Division, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India, were used in this study. The yeasts were subcultured in potato dextrose agar (PDA) and potato dextrose broth (PDB; Hi-media Laboratories Limited, Mumbai, India) at 37°C for 24–48 h to ensure viability and purity prior to testing.

Inoculum Preparation

Stock inocula of the Candida species were prepared by picking freshly grown colonies from 24-hour-old PDA cultures and suspending them in 5 ml of sterile saline (0.85%). Cell density was adjusted using a spectrophotometric method at 600 nm wavelength to achieve the turbidity equivalent 0.5 McFarland standard. The dilutions of yeast stock suspensions were adjusted to 1–5 × 10⁶ cells/ml.

Minimum inhibitory concentration and minimum fungicidal concentration

The macro broth dilution method with test tubes was used to determine the susceptibility of Candida species to phenazines and azoles. The phenazine andazole stock solutions were dissolved in dimethyl sulfoxide. The tubes containing PDB of varying phenazine (8000–1 µg/ml) and azole (250–1 µg/ml) concentrations were inoculated with actively growing Candida species and incubated at 37°C. The initial density of the Candida species (1 × 10⁶ colony forming units [CFU]/ml) was adjusted to obtain a final suspension of 1 × 10⁹ CFU/ml. After 24 h incubation, growth was monitored using a spectrophotometer (600 nm). The minimum inhibitory concentration (MIC) was defined as the lowest concentration required to totally inhibit the growth of fungi after 24 h (ie, 100% inhibition of growth compared with the control). From each tube that showed no visible growth, 100 µl was removed, serially diluted, and plated on PDA plates to obtain the minimum fungicidal concentration (MFC). MFC is the lowest concentration at which Candida species failed to grow in PDB and PDA inoculated with 100 µl of suspension (≥ 99.99% inhibition) [17]. The plates were then incubated at 37°C for 24 h, after which the colonies were counted.

Checkerboard microdilution assay

The drug interaction (phenazines with azoles) against Candida species was studied using the checkerboard microdilution assay in 96-well microtitre plates according to the modified methods of Chen et al. [18]. In brief, serial double dilutions of the test compounds were prepared as described here and expressed as micrograms per millilitre, that is, 0.03–2 (azoles) and 0.12–32 (phenazines). Serial dilutions of the phenazine–azole combination were mixed in PDB. The initial concentration of cell suspensions in the medium was 1 × 10⁹ CFU/ml. Plates were incubated at 37°C for 24 h, and the MIC was determined by measuring the optical density at 600 nm.

To assess the interaction of the phenazine–azole combination, data obtained spectrophotometrically were further analyzed using the fractional inhibitory concentration index (FICI). Drug interaction was defined as synergistic, indifferent, or antagonistic on the basis of the FICI. The FIC was calculated as follows: FIC of compound A (FICₐ) = (MIC of compound A in combination)/(MIC of compound A alone); FIC of compound B (FICₐ) = (MIC of compound B in combination)/(MIC of compound B alone). The sum of the FICIs for the two compounds in the combination was calculated as follows: FICₐ + FICₐ = FICI. The interaction between phenazines and azoles was considered to be synergistic at FICI ≤ 0.5, indifferent at 0.5–4 FICI, and antagonistic at FICI ≥ 4 [19]. The fractional fungicidal concentration (FFC) was calculated as described above by replacing the MICs with the MFCs.

Time–kill curve analysis

A time–kill curve (colony-forming units as a function of time) was evaluated in order to determine the rate and extent of Candida burden reduction when treated with phenazines and azoles individually and in combination. The experiments were conducted in PDB for 48 h. The concentrations used were based on the MICs of corresponding phenazines plus azoles alone (Table 1) and the ½ MIC of phenazines plus the MIC of azoles. An initial inoculum of approximately 1 × 10⁶ CFU/ml was taken for all experiments. Samples (0.1 ml) were collected at 0, 2, 4, 6, 8, 12, 24, and 48 h and serially diluted in normal saline and aliquoted onto PDA. These plates were then incubated at 37°C for 48 h, after which the colonies were counted. The broth without any agent was used as the control. The data were plotted as log CFU/ml vs. time (h) for each time point; tests were performed three times. Synergism was defined as a decrease in antifungal activity of ≥1 log₁₀ CFU/ml produced by the combination compared with the agent alone after 48 h. The lowest limit of quantification of time–kill assay was 0.5 log₁₀ CFU/ml (99.99% reduction).

Cytotoxicity test

The MTT [3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; Sigma-Aldrich, MO, USA] assay was used to determine the cytotoxicity of phenazines. A known cytotoxic drug, cisplatin, was used as the
Table 1. Synergistic effects of phenazines with azoles against *Candida* species.

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Agent</th>
<th>MIC/MFC (µg/ml)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Alone</td>
<td>Combination*</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>POL</td>
<td>64/125</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>FLU</td>
<td>1/1</td>
<td>0.12/0.12</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>64/125</td>
<td>16/16</td>
</tr>
<tr>
<td></td>
<td>ITR</td>
<td>8/16</td>
<td>4/8</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>64/125</td>
<td>4/8</td>
</tr>
<tr>
<td></td>
<td>CLO</td>
<td>1/2</td>
<td>0.06/0.12</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>POL</td>
<td>32/32</td>
<td>8/16</td>
</tr>
<tr>
<td></td>
<td>FLU</td>
<td>2/4</td>
<td>0.5/0.5</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>32/32</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>ITR</td>
<td>8/8</td>
<td>0.12/0.24</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>32/32</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>CLO</td>
<td>2/4</td>
<td>0.5/1</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>PCA</td>
<td>8/16</td>
<td>0.5/1</td>
</tr>
<tr>
<td></td>
<td>FLU</td>
<td>1/1</td>
<td>0.24/0.48</td>
</tr>
<tr>
<td></td>
<td>PCA</td>
<td>8/16</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>ITR</td>
<td>8/8</td>
<td>0.5/1</td>
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<tr>
<td></td>
<td>PCA</td>
<td>8/16</td>
<td>0.5/1</td>
</tr>
<tr>
<td></td>
<td>CLO</td>
<td>2/4</td>
<td>0.24/0.24</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>PCA</td>
<td>8/16</td>
<td>1/2</td>
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<td></td>
<td>FLU</td>
<td>2/4</td>
<td>0.12/0.24</td>
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<td></td>
<td>PCA</td>
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<td></td>
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<tr>
<td></td>
<td>CLO</td>
<td>2/4</td>
<td>0.06/0.12</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>PCN</td>
<td>2000/4000</td>
<td>32/64</td>
</tr>
<tr>
<td></td>
<td>CLO</td>
<td>1/1</td>
<td>2/4</td>
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<tr>
<td></td>
<td>PCN</td>
<td>2000/4000</td>
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<td>FLU</td>
<td>8/8</td>
<td>2/2</td>
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<tr>
<td></td>
<td>PCN</td>
<td>2000/4000</td>
<td>32/64</td>
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<tr>
<td></td>
<td>ITR</td>
<td>2/4</td>
<td>2/2</td>
</tr>
<tr>
<td><em>C. tropicalis</em></td>
<td>PCN</td>
<td>4000/4000</td>
<td>125/125</td>
</tr>
<tr>
<td></td>
<td>CLO</td>
<td>2/4</td>
<td>0.25/0.5</td>
</tr>
<tr>
<td></td>
<td>PCN</td>
<td>4000/4000</td>
<td>64/125</td>
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<td></td>
<td>FLU</td>
<td>8/8</td>
<td>2/4</td>
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<td></td>
<td>PCN</td>
<td>4000/4000</td>
<td>64/64</td>
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<tr>
<td></td>
<td>ITR</td>
<td>2/4</td>
<td>0.5/1</td>
</tr>
</tbody>
</table>

MICs/MFCs of phenazines and azoles alone were determined using the macro broth dilution method and MICs/MFCs of the combination were determined using the checkerboard assay. FICIs were obtained from the checkerboard assay. To evaluate the interaction between phenazines with azoles and *Candida* species, checkerboard experiments were performed. FICI/FFCI = (MIC/MFC of azoles in combination with phenazines divided by MIC/MFC of azoles alone) + (MIC/MFC of phenazines in combination with azoles divided by MIC/MFC of phenazines alone). An FICI/FFCI = 0.5 is indicative of synergism.

FFC, fractional fungicidal concentration; FFCI, fractional fungicidal concentration index; FICI, fractional inhibitory concentration index; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.

*The MIC and MFC of phenazines in combination with azole.

positive control and a foreskin (FS) normal fibroblast cell line was used in the studies. Briefly, cells (5 × 10^3 per well) were seeded in 0.2 ml of the medium (Dulbecco’s Modified Eagle’s medium [DMEM] with 10% phosphate-buffered saline [PBS]) in 96-well plates and treated with phenazines and cisplatin for 72 h. Cytotoxicity was measured by removing the media from the well, adding 25 µl of MTT solution (5 mg/ml in PBS) and 75 µl of DMEM to the wells (untreated and treated), and incubating the media. After 2 h, MTT lysis buffer was added to the wells (0.1 ml/well) and the media was incubated for another 4 h at 37°C. At the end of incubation, the optical densities at 570 nm were measured using a plate reader (Bio-Rad). The relative cell viability, as a percentage, was calculated as follows: (A_{570} of treated sample)/(A_{570} of untreated sample) × 100 [20].
Statistical analysis

All statistical analyses were performed with SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). For MIC, MBC, and FICI determinations, another test for the final result was performed if the results were different in both experiments. One-way analysis of variance–Duncan multiple range test was used for the time–kill assay. Data for time–kill analysis were presented as mean ± standard deviation. Statistical significance was defined as $P < 0.05$.

Results

Antifungal activity

The MICs and MFCs of phenazines and azoles when separated from each other are shown in Table 1. It is evident that the highest level of activity was found with PCA, ranging from 8 to 16 µg/ml, whereas POL was active in the range of 32 to 125 µg/ml and PCN at the much higher rate of 2000 to 4000 µg/ml. For azoles, activity ranged from 1 to 16 µg/ml, with the highest level of activity noted with FLU (1 to 4 µg/ml).

Checkerboard assay

The combined activities of phenazines with azoles from the in vitro checkerboard interactions against the Candida species are summarized in Table 1. FIC, FFC, FICI, FFCI, and interpretations of the activity of phenazines and azoles against the test Candida species were predominantly synergistic interactions. The combinations of POL plus ITR and PCN plus ITR were found to be indifferent. For these combinations, the MIC was reduced four-fold, but the final interpretation of data never indicated synergy. Antagonism was recorded for the combination PCN plus CLO against C. albicans.

For PCA and azoles, the maximum reduction in C. albicans occurred with PCA and FLU and was recorded within 2–4 h. This combination reduced 99.99% of C. albicans at 24 h ($P < 0.05$; Fig. 2C). Against C. tropicalis, maximum reduction occurred with PCA plus CLO and was recorded within 2–4 h, with 99.99% reduction of C. tropicalis at 48 h ($P < 0.05$; Fig. 2D).

When PCN and azoles were combined, maximum activity against C. albicans was found with PCN plus CLO, and maximum reduction of C. albicans growth was recorded within 6–12 h ($P < 0.05$; Fig. 2E). A similar result was obtained with C. tropicalis ($P < 0.05$; Fig. 2F). The time–kill assay that demonstrated the rate of killing showed that PCN in combination with azoles was more effective than either drug alone. Regrowth of Candida species treated with only phenazines was observed after 12 h ($P < 0.05$; Fig. 2). However, there was no development of colonies after treatment with phenazines and azoles, even after 48 h, except with POL plus ITR and PCA plus CLO against C. albicans. However, regrowth was never recorded with any combinations for C. tropicalis ($P < 0.05$; Fig. 2). The combination of 1/2 MIC for phenazines with the MIC for azoles had lower activity than the combined MICs for phenazines and azoles (Fig. 2).

Cytotoxicity test

The cytotoxic activity of phenazines and cisplatin was tested against the FS normal fibroblast cell line using the MTT assay. The results show that there was no significant cytotoxicity at levels up to 200 µg/ml, except with PCN (Fig. 3). At a 200-µg/ml concentration of phenazines, approximately 84% and 79% of cells were alive with POL and PCA, respectively (Fig. 3), whereas cisplatin recorded toxicity to FS (Fig. 3). These results clearly indicate that phenazines may be safe for normal human cells in lower concentrations, except for PCN.

Discussion

A number of microbial secondary metabolites are known to possess potent medicinal properties [21,22]. Many studies have shown improved efficacies of certain natural products when they are combined with clinically useful antibiotics, especially when used against C. albicans [23–25]. Kumar et al. [25] described the synergistic interaction of amphoterin B with three cyclic dipeptides isolated from Bacillus spp. against C. albicans. This finding has generated increased clinical interest in the use of natural products combined with antimicrobial agents to improve the spectrum of drug activity. Synergism of natural products...
Figure 2. Time–kill curve of phenazines and azoles alone and in combination against *Candida* species. Strains with a starting inoculum density of $10^6$ CFU/ml were used. At 0, 2, 4, 6, 8, 12, 24, and 48 h, aliquots were removed from each test tube in order to examine cell viability. The experiments were performed three times. Data are expressed as mean ± standard deviation. Column 1 shows *C. albicans* and column 2 shows *C. tropicalis*. (A, B) Phenazine-1-ol plus azoles against *C. albicans* and *C. tropicalis*. (C, D) Phenazine-1-carboxylic acid plus azoles against *C. albicans* and *C. tropicalis*. (E, F) Phenazine-1-carboxamide plus azoles against *C. albicans* and *C. tropicalis*. The $y$-axis represents logarithmic *Candida* survival.

CFU, colony-forming unit.
Figure 3. Cytotoxicity of phenazines on normal human cell line (foreskin normal fibroblast) conducted by MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. All measurements were take in replicates of three, and results are expressed as arithmetic mean ± standard error of the mean.

and antibiotics against pathogenic microorganisms is a major focus of research in medical microbiology and will hopefully lead to development of novel microbial pharmaceuticals.

Here, we investigated the in vitro anticandidal activity of three phenazines and three azoles alone and in combination through the use of the checkerboard assay in order to determine whether synergism, indifference, or antagonism would be the predominant response against two Candida species. The results clearly indicate that phenazines in combination with azoles predominantly result in synergistic effects (Table 1). Significant synergistic activity was recorded for POL and ITR against C. tropicalis (FICI = 0.08). Synergy resulted in significantly greater activity for two agents combined than that provided by the sum of each agent alone [26]. Currently no information regarding the anticandidal activity of phenazines in combination with azoles is available in literature. Therefore, we report, for the first time, the in vitro synergistic activity of phenazines and azoles against Candida species. Furthermore, we checked the toxicity of phenazines against a normal human cell line (FS) and found phenazines to be nontoxic to normal cells, thus indicating their therapeutic potential.

The phenazines used in our study were obtained from many Pseudomonas species [27,28]. The activity of phenazines on Candida has been described as primarily inhibition of filamentation, intercellular adherence, and biofilm development during interactions between C. albicans and P. aeruginosa through the action of P. aeruginosa-produced phenazines [29]. Inhibition of C. albicans biofilms by phenazines through the reactive oxygen species (ROS) mediated action, which enables phenazines to exchange electrons, for example, nicotinamide-adenine-dinucleotide (reduced) (NADH), Fe^{2+}/Fe^{3+}, or molecular oxygen, has been reported [30]. Azoles inhibit ergosterol, an essential component of the fungal plasma membrane, and the inhibition of its synthesis negatively influences membrane fluidity [30]. The efficacy of antifungal agents can be improved through the use of combination therapy, that is, the combination of agents with different antifungal mechanisms [31] or the combination of antifungal and nonantifungal agents [32,33]. In our study, antifungals were combined with phenazines; the numerous synergistic interactions noted during the study demonstrate that phenazines might be of use in combination therapy. In our study, the enhanced activity of phenazines and azoles may be due to the inhibition of ergosterol and ROS development. The correct mechanism of action for phenazines and azoles on yeast cells still needs to be elucidated.

Systems biologists have discovered that pathogenic fungi are composed of complex networking systems with very divergent signaling pathways [34,35]. Therefore, in order to treat the diseases associated with these pathogens, multicomponent therapies along the disease pathway may need to be manipulated simultaneously for an effective treatment. The drugs currently used to treat candidiasis have a single-dose target, which may lead to side effects and problems with drug resistance. If emphasis is placed on
multiple targets in a pathway by combining two drugs, high dosages of single drugs may not be necessary. However, the data presented here concerning the intrinsic antifungal activity of phenazines and azoles and their synergistic interactions in several combinations support the hypothesis that phenazines and azoles could represent a novel tool that may be valuable as an adjuvant for antifungal chemotherapy. In addition, when used in combination, activities occurred at very low concentrations of both agents. Thus, to permit full exploration of phenazines as new antimicrobial agents, it is important to investigate their interaction with the most commonly used antibiotics. In spite of the speculated mode of phenazine interaction, proof of clinical benefits is lacking.

To the best of our knowledge, this is the first study to report the synergistic effect of phenazines and azoles against Candida species. This study suggests that phenazines are not only a natural compound capable of inhibiting Candida growth, they also act synergistically with azoles in low doses. Our findings are encouraging in view of the increasing treatment failures and antibiotic resistance in Candida species and suggest a way of treating resistant Candida infections through a phenazine-azole combination approach.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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