Enhanced Antifungal Efficacy of Amphotericin B Against Resistant Candida albicans Through Combination with 3-Hydrazinoquinoxaline-2-Thiol

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ABSTRACT

Candida albicans is a commensal and opportunistic fungus capable of causing severe infections under specific circumstances, Candida albicans, for example, is a commensal organism that can cause serious infections under certain conditions. Fungal infections, particularly, among in immunocompromised individuals. C.abicans infections, have become a significant global health threat, with an estimated 1.7 to 2 million deaths annually. Despite advances in antifungal therapies, challenges remain in diagnosing and treating these infections effectively. This study aims to explore, for the first time, the potential synergistic effects of combining amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol in vitro against clinical strains of C. albicans, with the objective of reducing treatment duration and minimizing the toxicity of amphotericin BUp to our knowledge, this paper aims for the first time to explore the potential synergistic effects of combining amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol in vitro against clinical strains of Candida albicans. 22 of Clinical strains of Candida albicans were tested for antifungal synergy between amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol using broth microdilution and checkerboard assays. we've demonstrated that combining amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol shows promising potential to boost antifungal effectiveness against C.albicans. Specifically, We found strong synergy against 20 strains, with a Fractional Inhibitory Concentration Index (FICI) consistently below 0.5, and additive effects against 2 strains. We observed no antagonistic interactions. This combination significantly lowered the MIC for both agents, with amphotericin B reducing the MIC of 3-Hydrazinoquinoxaline-2-Thiol by 64-fold, and the reverse by 32-fold, which underscore the potential for not only increased antifungal efficacy but also reduced drug toxicity through combination therapy. In summary, the pairing of amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol presents a strong antifungal strategy against C. albicans. The consistent synergy and substantial MIC reductions highlight its promise in enhancing antifungal activity and addressing resistance, making it an area deserving of further research and clinical trials. For the first time we have showed that the combination of amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol shows promising potential in enhancing antifungal efficacy against Candida albicans infections, but further tests are needed. This study evaluated the synergistic effects of Amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol against 22 clinical Candida albicans strains. The combination demonstrated strong synergy in 20 cases, with a Fractional Inhibitory Concentration Index (FICI) consistently below 0.5, and additive effects in 2 cases. No antagonistic or indifferent interactions were observed. The combination reduced the MIC of both agents, with Amphotericin B lowering the MIC of 3-Hydrazinoquinoxaline-2-Thiol by 64-fold and vice versa by 32-fold. These findings highlight the potential for enhanced antifungal efficacy and reduced drug toxicity through combination therapy. In conclusion, combining amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol shows strong potential as an antifungal strategy against Candida albicans. The consistent synergy and significant MIC reductions highlight its promise in enhancing antifungal efficacy and overcoming resistance, warranting further research and clinical trials.

Keywords: Candida albicans, Fractional Inhibitory Concentration Index, Infections, Fungus

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INTRODUCTION

Fungal infections, predominantly impacting the skin, nails, and hair, are widespread and can considerably interfere with daily life, resulting in discomfort. In addition to these prevalent symptoms, tens of millions of individuals endure mucosal candidiasis, a condition frequently undervalued, which can lead to significant pain, discomfort, and severe health issues. While serious fungal diseases are often seen in immunocompromised individuals[1,2], they can also affect immunocompetent people, typically after occupational or environmental exposure, traumatic inoculation, or travel to endemic areas[2,3]. Invasive fungal infections carry high mortality rates, particularly when diagnosis is delayed or missed altogether[3,4].

Fungi can be found in two primary forms: yeasts and moulds. Yeasts are generally small, single-celled, and oval-shaped, while moulds grow as colonies made up of thread-like structures known as hyphae. Certain fungi exhibit dimorphism, meaning they can switch between yeast and mould forms based on environmental factors, such as temperature. Most fungi are widespread and thrive in natural environments without needing human or animal hosts. However, some species can become opportunistic pathogens, causing infections that range from superficial to systemic. Systemic infections usually occur through direct inhalation into the lungs or invasion of a wound.

Candida albicans, a yeast like organism, for instance, typically inhabits the gastrointestinal tract and skin but can enter the bloodstream under certain conditions, such as through medical devices like vascular catheters or during immunocompromised status like AIDS[5,6]. Certain fungi can cause disease in healthy individuals, but many species only become pathogenic when the host is weakened, such as in cases of compromised immune systems. The prevalence of such infections is rising due to the growing number of individuals with advanced HIV infections and advancements in modern medicine, like intensive chemotherapy and the use of immunosuppressive drugs, which can lead to increased vulnerability to fungal infections[7,8].

The increasing incidence of candida systemicfungal infections has grown into a major global health issue, impacting millions across the globe. Previously regarded as uncommon, these infections have now become serious threats to human health, particularly for those with weakened immune systems[9]. Fungal infections represent a substantial and frequently overlooked global health threat, impacting nearly a billion individuals across the world[10]. These The situation is further exacerbated by the fact that over 150 million individuals suffer from severe systemic candidaiasis infections, which primarily affect the skin, nails, and hair, are pervasive and can significantly disrupt daily life, leading to discomfort and stigma.

Beyond these common manifestations, tens of millions of people suffer from mucosal candidiasis, a condition that, while often underestimated, can cause considerable pain, discomfort, and serious health complications. The situation becomes even more alarming when considering the over 150 million people who are afflicted by severe fungal diseases[11,12]. These infections, which can target vital organs, Systemic candidiasis often lead to debilitating outcomes and can be life-threatening if not properly managed. The rise in severe fungal infections underscores the urgent need for increased awareness, better diagnostic tools, and more effective treatments to address this growing global health crisis[13].

The mortality rate of systemic fungal illnesses is estimated to range from 1.7 to 2 million fatalities annually Fungal diseases pose a serious global health challenge, leading to an estimated 1.7 to 2 million deaths each year[12]Click or tap here to enter text.. While serious fungal diseases are often seen in immunocompromised individuals[1,2], they can also affect immunocompetent people, typically after occupational or environmental exposure, traumatic inoculation, or travel to endemic areas[2,3]. Invasive fungal infections carry high mortality rates, particularly when diagnosis is delayed or missed altogether[3,4].

The management of systemic candidiasis, a common and pervasive fungal infection, faces considerable challenges, primarily due to two critical factors: the difficulty in achieving rapid and accurate diagnosis of the causative pathogen and the limited range of effective treatment options available[13,14]. Another issue in treating C. albicans is the increasing resistance to antifungal agents, particularly among azoles, which are commonly used as first line for treating Candida infections. Resistance to azoles, including fluconazole, has been seen, particularly in individuals with recurring infections or those who have received extended antifungal therapy[15]. This resistance frequently arises from mutations in the ERG11 gene, which encodes the target enzyme in the ergosterol biosynthesis pathway[16]. Evidence of cross-resistance among many kinds of antifungal agents complicates treatment options. For example, strains resistant to one azole may also demonstrate resistance to other antifungals.

Amphotericin B is a broad-spectrum antifungal drug effective against various fungal infections, including those in the Candida genus. Amphotericin B is typically designated for severe and systemic infections owing to its efficacy and possible adverse effects[17,18]. The primary indications for Amphotericin B are in immunocompromised patients, including those with HIV/AIDS, cancer, or persons undergoing organ transplantation, who are at elevated risk for systemic Candida infections, where conventional antifungals (such as fluconazole) prove ineffective or are poorly tolerated. Additionally, in instances where Candida infections demonstrate resistance to other antifungal agents such as azoles[17].

Although efficient, amphotericin B must be administered with caution due to its potential for severe adverse effects, such as nephrotoxicity and infusion-related responses. Progress in developing new antifungal agents has been sluggish, further narrowing the range of available treatments. This limited arsenal of effective therapies is increasingly worrisome given the growing issue of antifungal resistance[19]. Consequently, research is now being conducted on the use of amphotericin B in combination with other antifungal drugs or other synergizing compounds to improve efficacy, mitigate resistance, and reduce dosages.

Combination therapy uses numerous antifungals with different modes of action to boost treatment efficacy[20]. This method improves fungal infection treatment and reduces pathogen resistance by targeting the pathogen through numerous mechanisms[21,22]. Researchers can avoid the lengthy and expensive process of new medication development by finding and using the antifungal characteristics of medicines licensed for other indications[23]. This technique uses these medications' safety profiles to speed up the availability of potentially beneficial treatments[20, 24,25,26].

Recent studies have underscored the strong antifungal capabilities of quinoxaline derivatives, showing their efficacy in inhibiting a wide range of fungal pathogens[23,27]. For more than four decades, amphotericin B has served as the cornerstone of fungal infection therapy for its broadspectrum efficacy. Despite the challenges posed by its dose-limiting toxicity, it continues to be the preferred treatment for severe, disseminated fungal infections, largely owing to its broad-spectrum antifungal activity[28]. This research seeks to investigate the possible effects of 3-Hydrazinoquinoxaline-2-Thiol in conjunction with Amphotericin B in vitro against clinical strains of Candida albicans.

MATERIALS AND METHODS

Clinical Strains: Clinical strains of Candida albicans were obtained from patients diagnosed with candidiasis. These strains were isolated and identified using standard microbiological techniques. The isolates were stored at -80°C in cryovials containing 20% glycerol until further use.

Broth Microdilution Assay: The minimum inhibitory concentration (MIC) of amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol against Candida albicans was determined using the broth microdilution method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) protocol. Stock solutions of Amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. Then the inoculum was prepared by suspending Candida albicans colonies in sterile saline, adjusting to a turbidity equivalent to 0.5 McFarland standard. The assay was conducted in sterile 96-well microtiter plates, with each well containing 100 µL of the antifungal agent at twofold serial dilutions in Muller Hinton broth. The prepared inoculum was added to each well. Plates were then incubated at 37°C for 20 hours. The MIC was determined as the lowest concentration of the compound that completely inhibited visible growth.

Checkerboard Assay: The potential synergistic effect of amphotericin B combined with 3-Hydrazinoquinoxaline-2-Thiol was evaluated using the checkerboard microdilution method. A serial twofold dilutions of amphotericin B (50 µL) were combined with serial twofold dilutions of 3-Hydrazinoquinoxaline-2-Thiol (50 µL) in a 96-well plate. Each well contained a different combination of the two drugs. The inoculum of Candida albicans was prepared as described in the broth microdilution assay and added to the wells. The plates were incubated at 37°C for 20 hours. The fractional inhibitory concentration index (FICI) was calculated for each combination. Synergy was defined as an FICI ≤ 0.5, indifference as 0.5 < FICI ≤ 4, and antagonism as an FICI > 4.

Statistical Analysis: All experiments were performed in triplicate, and the data were analyzed using GraphPad Prism software (version 9.0). The results are expressed as mean ± standard deviation. Statistical significance between groups was determined using an unpaired t-test, with a p-value of < 0.05 considered statistically significant.

RESULTS

Minimum Inhibitory Concentrations (MICs) of 3-Hydrazinoquinoxaline-2-Thiol and Amphotericin B Against Candida albicans Clinical Strain

The MIC values of 3-Hydrazinoquinoxaline-2-Thiol against the clinical strain of C. albicans ranged from 8 to 16 µg/mL, indicating moderate antifungal activity. In comparison, the MIC values of the amphotericin B, varied between 2 to 8 µg/mL. According to the EUCAST breakpoint for Amphotericin B, these C. albicans strains are deemed resistant as they demonstrate a MIC exceeding 1 mg/ml.

Table 1. These results suggest that while 3-Hydrazinoquinoxaline-2-Thiol shows promising antifungal potential, amphotericin B remains more effective in inhibiting the growth of Candida albicans at lower MIC values.

Strain name	MIC of 3-Hydrazinoquinoxaline-2-Thiol	MIC Amphotericin B
C. albicans 1	8	4
C. albicans 2	16	4
C. albicans 3	8	8
C. albicans 4	16	4
C. albicans 5	16	4
C. albicans 6	16	2
C. albicans 7	8	4
C. albicans 8	8	4
C. albicans 10	16	4
C. albicans 11	8	4
C. albicans 12	16	2
C. albicans 13	8	4
C. albicans 14	16	8
C. albicans 15	8	8
C. albicans 16	16	4
C. albicans 17	16	2
C. albicans 18	16	8
C. albicans 19	16	4
C. albicans 20	16	4
C. albicans 21	8	8
C. albicans 22	16	4

Synergistic and Additive Interactions of 3-Hydrazinoquinoxaline-2-Thiol and Amphotericin B Against Candida albicans

In this study, the synergistic effects of amphotericin B combined with 3-Hydrazinoquinoxaline-2-Thiol were evaluated against 21 different clinical strains of Candida albicans to assess their potential in enhancing antifungal efficacy. The combination of these two agents demonstrated remarkable synergy across all the tested strains, with the Fractional Inhibitory Concentration Index (FICI) consistently recorded below 0.5. This result clearly indicates a strong synergistic interaction, suggesting that the combined use of these compounds could significantly (p value less than 0.0001) enhance the antifungal activity against Candida albicans. (Table 2).

Table 2. Synergistic and Additive Effects of 3-Hydrazinoquinoxaline-2-Thiol in Combination with Amphotericin B Against Candida albicans Clinical Strains. The table summarizes the interaction outcomes of 3-Hydrazinoquinoxaline-2-Thiol and Amphotericin B, showing the number of cases with synergistic, additive, indifferent, and antagonistic effects. Synergy was observed in 20 out of 22 cases, while 2 cases exhibited additive effects. No antagonistic or indifferent interactions were detected.

Number of strain	FICI	Percent
20	FICI ≤ 0.5	90.9 % S
2	FICI < 1	9.1 % A
0	0.5 < FICI ≤ 4	0 %
0	FICI > 4	0%

FICI, fractional inhibitory concentration index, S, Synergy, A, Additive In our study evaluating the interaction between 3-Hydrazinoquinoxaline-2-Thiol and Amphotericin B against clinical strains of C. albicans, we observed synergy in 20 out of 22 interactions. The synergistic effect between these compounds indicates a potential enhanced antimicrobial efficacy when used in combination. Additionally, 2 interactions showed an additive effect, where the combined agents contributed to the overall antimicrobial activity, but without synergy. Importantly, no antagonistic effects were noted in any of the interactions, suggesting that combining these compounds does not interfere with their respective activities. No indifferent interactions were observed, further emphasizing the consistency of the synergy or additive outcomes in this combination.

The synergy between amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol was further reflected in the ability of each compound to reduce the

MIC of the other by 8-fold. Specifically, the presence of Amphotericin B in the combination led to an 64-fold reduction in the MIC of 3-Hydrazinoquinoxaline-2-Thiol, thereby enhancing its effectiveness at much lower concentrations. Similarly, 3-Hydrazinoquinoxaline-2-Thiol also reduced the MIC of Amphotericin B by 32-fold, indicating that it significantly (p value less than 0.0001) potentiated the activity of Amphotericin B against the clinical strains of Candida albicans. (Figure 1)

The reduction in MIC values observed across all 20 clinical strains underscores the robustness of the synergistic interaction between amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol. Such a consistent enhancement of antifungal activity across a diverse set of strains suggests that this combination has a broad spectrum of efficacy against C. albicans, which is crucial given the variability in antifungal resistance observed among different clinical isolates.

The findings of this study hold significant implications for the treatment of C. albicans infections, particularly in the context of rising antifungal resistance.The ability to achieve such a pronounced reduction in MIC values with the combination therapy not only suggests a potent antifungal effect but also highlights the potential to reduce the dosage of each individual drug. This could be particularly beneficial in clinical settings where the dose-dependent toxicity of Amphotericin B is a major concern, as it may allow for effective treatment with lower, less toxic doses. Furthermore, the synergy observed between amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol could open new avenues for developing combination therapies that leverage the distinct mechanisms of action of these agents. By targeting different pathways within the fungal cell, this combination may reduce the likelihood of resistance development, thereby offering a more sustainable approach to managing fungal infections.

DISCUSSION

This is, to our knowledge, the inaugural investigation of the combination of Amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol in the treatment of Candida albicans infections. This innovative method demonstrates significant potential to improve antifungal effectiveness.

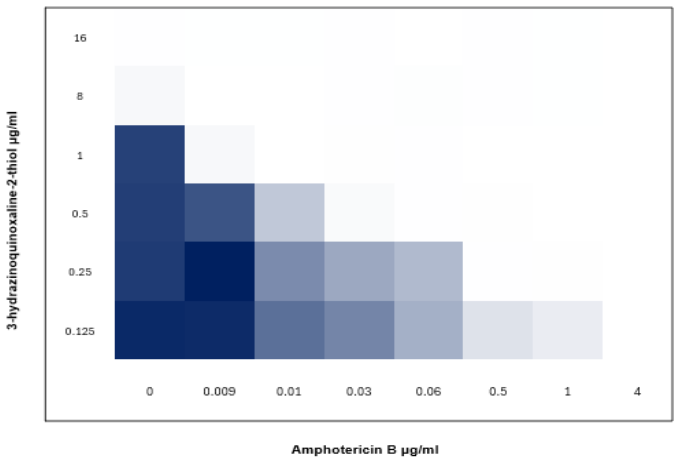

Figure 1. The synergistic interaction between Amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol significantly (p value less than 0.0001) reduced the MICs of both compounds against C. albicans clinical strains. Specifically, the presence of Amphotericin B led to a 64-fold reduction in the MIC of 3-Hydrazinoquinoxaline-2-Thiol, enhancing its antifungal efficacy at much lower concentrations. Conversely, 3-Hydrazinoquinoxaline-2-Thiol reduced the MIC of Amphotericin B by 32-fold, indicating its ability to potentiate the antifungal activity of Amphotericin B.

Amphotericin B is a recognized treatment for severe fungal infections; nevertheless, its application is frequently limited by dose-dependent toxicity, especially nephrotoxicity[30]. The combination with 3-Hydrazinoquinoxaline-2-Thiol may facilitate a synergistic antifungal effect, thereby diminishing the requisite dosage of Amphotericin B and alleviating its undesirable side effects[31].

The broth microdilution results for determining the MIC indicated that all 22 C. albicans isolates exhibit resistance to amphotericin B, as per the EUCAST clinical breakpoint of <1 mg/L. The minimum inhibitory concentration (MIC) for 3-Hydrazinoquinoxaline-2-Thiol varied from 8 to 16 mg/L. No clinical cutoff point has been previously established by CLSI or EUCAST; nonetheless, our prior investigation indicates equivalent in vitro activity to Amphotericin B and efficacy in vivo in murine models for treating the reference C. albicans strain ATCC 10231. In contrast to the reference strain previously examined, which exhibited MIC values of less than 1 µg/ml, the clinical strains in this study demonstrated elevated MIC levels ranging from 8 to 16 µg/ml, indicating greater resistance in the clinical strains compared to the reference strain utilized in our prior investigation regarding 3-Hydrazinoquinoxaline-2-Thiol[29].

The checkerboard assay revealed a synergistic effect between these two compounds against Candida albicans, as indicated by a FICI of $\leq$ 0.5 in several combinations. This synergism suggests that 3-Hydrazinoquinoxaline-2-Thiol may enhance the fungicidal activity of Amphotericin B, allowing for effective fungal suppression at lower drug concentrations.

Among the 22 C. albicans clinical isolates tested, only 2 showed an additive response to the combination of Amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol, while the majority exhibited synergistic interactions. This disparity in response may be attributed to the variability in the genetic and phenotypic characteristics of the isolates could influence their susceptibility to the combination therapy. Some strains might possess specific resistance mechanisms or metabolic adaptations that reduce the extent of synergy. In these cases, the antifungal agents may only work additively rather than synergistically, where the combined effect is equivalent to the sum of the individual effects, but not greater. Overall, while the combination therapy demonstrates broad synergy, the unique properties of a few isolates could lead to an additive rather than a synergistic effect, suggesting the need for further investigation into the molecular basis of these responses.

One of the key findings of this study is the potential mechanism underlying the observed synergy. Amphotericin B works by binding to ergosterol in fungal cell membranes, creating pores that lead to cell death[32]. In contrast, 3-Hydrazinoquinoxaline-2-Thiol may exert its antifungal effect through a different mechanism including generating ROS which led to cell damage. Additionally, inhibiting of DNA synthesis[20,33,34] The combination of these distinct mechanisms of action could account for the enhanced antifungal activity observed, as the two drugs may target different components of the fungal cell, leading to a more comprehensive assault on the pathogen.

In this study, we hypothesize that the enhanced efficacy observed with the combination of Amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol can be attributed to a multi-faceted mechanism. Amphotericin B's action on the cell membrane likely facilitates the passage of 3-Hydrazinoquinoxaline-2-Thiol into the fungal cell.

The cell wall and membrane alterations induced by Amphotericin B could also result in increased vulnerability of the fungal cells to oxidative stress, which may be exacerbated by the presence of

3-Hydrazinoquinoxaline-2-Thiol. Additionally, the disruption of membrane integrity might impair the fungus's ability to regulate its internal environment, further enhancing the cytotoxic effects of 3-Hydrazinoquinoxaline-2-Thiol.

The combination of these agents could therefore lead to a more effective eradication of Candida cells by simultaneously attacking multiple cellular targets and overcoming the defense mechanisms that these pathogens typically employ against single-agent treatments. This multi-target approach may also reduce the likelihood of resistance development[35], as the fungal cells would need to adapt to multiple simultaneous stresses.

The clinical implications of this study are significant, particularly in the context of rising antifungal resistance. Antifungal resistance, much like antibiotic resistance in bacterial infections, poses a significant global public health challenge, particularly among Candida species. According to the U.S. Centers for Disease Control and Prevention (CDC) 2019 Antibiotic Resistance Threat Report, drug-resistant Candida infections were responsible for over 34,000 cases and 1,700 deaths each year. Additionally, the report highlighted 323 cases of the emerging multidrug-resistant Candida auris, further emphasizing the growing concern surrounding antifungal resistance[36]. The synergy between Amphotericin B and 3-Hydrazinoquinoxaline-2-Thiol could provide an alternative treatment strategy for infections caused by resistant strains, offering a way to overcome the limitations of monotherapy. Furthermore, the ability to use lower doses of Amphotericin B in combination therapy could reduce the risk of toxicity, making the treatment more tolerable for patients, especially those who are critically ill or have compromised renal function.

This will bring in the future to assess the efficacy of this combination across different Candida specieses such as Candida auris, C. parapsilosis, C. krusei and Candida glabrata. Another important aspect to consider is the broader application of this combination therapy beyond Candida albicans. While this study focused on this specific pathogen, it is plausible that the combination could be effective against other fungal species, including those from the Aspergillus and Cryptococcus genera. Future studies should explore the efficacy of this combination against a wider range of fungi, as well as investigate the potential for resistance development when using this combination therapy over prolonged periods.

While the synergy observed between the combination of 3-Hydrazinoquinoxaline-2-Thiol and Amphotericin B is significant, a more detailed investigation into the dose-response relationship and pharmacokinetics of the combined therapy is necessary. Future studies will focus on examining the dose-dependency of Amphotericin B when used in combination with this compound, exploring how the presence of 3-Hydrazinoquinoxaline-2-Thiol influences the effectiveness of Amphotericin B at varying concentrations. Additionally, the pharmacokinetics and tissue distribution of the drugs, both individually and in combination, will be assessed to understand potential interactions or alterations in their metabolism when used together. These investigations will help establish optimal dosing strategies and provide deeper insights into the pharmacological dynamics of the combination therapy, enhancing its clinical applicability

This study primarily focuses on the antifungal effects of the combination on Candida albicans. However, it is important to note that the applicability of this combination to other fungal pathogens, particularly multi-drug-resistant species such as Candida auris, remains to be fully explored. Candida auris poses significant challenges in infection control and was not included in this study due to its serious nature and infection control restrictions. Additionally, the study also limited its scope to Candida albicans and included only limited cases of Aspergillus and Cryptococcus due to the rarity of these pathogens in the clinical isolates examined. Further investigation is required to assess the potential effectiveness of this combination against a broader range of fungal pathogens, particularly those with known drug resistance profiles.

CONCLUSION

In conclusion, the results of this study strongly support the potential of combining amphotericin B with 3-Hydrazinoquinoxaline-2-Thiol as a powerful antifungal strategy against Candida albicans. The consistent synergy observed across all tested strains, along with the significant reductions in MIC values, underscores the promise of this combination in enhancing antifungal efficacy and overcoming the challenges posed by antifungal resistance. Further research and clinical trials are warranted to fully explore the therapeutic potential and optimize the use of this combination in treating Candida albicans infections.

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Competing Interest: None

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