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Exploring the Potential of Antisense Technologies to Enhance Traditional Antifungal Treatments for *Candida albicans* Biofilms

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Abstract

Biofilm-related infections present critical challenges in the context of medical implantable devices, often necessitating the removal of these life-saving devices. Among these infections, *Candida albicans* biofilms are particularly prevalent and are often addressed with Caspofungin, a fungicidal echinocandin. However, prevailing antifungal treatments exhibit limited efficacy against biofilms due to their intricate structure and the emergence of antifungal resistance. The recent advancement of antisense therapy offers a promising avenue to augment the effectiveness of anti-biofilm treatments. Targeting enhanced filamentous growth protein (EFG1), a key transcription regulator in *C. albicans*, anti-EFG1 2′-OMethylRNA may increase membrane permeability while reducing antifungal properties. As such, there are potential therapeutic benefits in leveraging anti-EFG1 2′-OMethylRNA to amplify the fungicidal properties of echinocandins. Our study aims to assess the viability of cells subjected to individual and combined treatment modalities. Employing an *in vivo* benchtop biofilm model, we aim to investigate the efficacy of Caspofungin and anti-EFG1 2′-OMethylRNA, both independently and in combination. By evaluating their impact on biofilm growth, cellular viability, and gene and protein expressions (transcriptome), we seek to ascertain and explore the potential synergistic effects of this combinatorial therapeutic approach. Investigating the combination of antisense and Caspofungin therapy presents a promising direction toward the more effective treatment of biofilm-related infections on medical implantables. Furthermore, the results of this study may present promising opportunities for the future use of antisense technology in more efficacious treatment and novel therapeutic approaches to other illnesses.

Keywords: *Candida albicans*; fungal biofilms; biofilm infections; echinocandins; Caspofungin; EFG1; antisense oligomers; anti-EFG1 2′-OMethylRNA; antifungal treatment

Introduction

Candida albicans infections, capable of developing robust antifungal resistance properties, are opportunistic fungal infections that lead to upwards of 400,000 life-threatening cases per year worldwide [1]. In American hospital settings, up to 10% of bloodstream infections are caused by various strains of *Candida* [2], of which *C. albicans* is the most common [3]. For neutropenic patients, Candidemia can be a life-threatening infection [4]. The complexity of a *C. albicans* infection is exacerbated by the formation of a biofilm; a rigid matrix protecting the infection from the host. *C. albicans* biofilms often form on medical implantables, such as pacemakers or central venous catheters, and can necessitate the removal of these life-saving devices, also leading to further complications [5]. With the ubiquity of *Candida* and the increased incidence of hospital-acquired *C. albicans* infections, it is imperative to develop robust, effective treatment methods to combat *C. albicans* biofilms [5].

C. albicans biofilm formation begins with the adherence of *C. albicans* cells to a surface. For medical implantables, a rough surface yields high free surface energy and thus an energetically favourable condition for dispersion and subsequent biofilm formation [6]. As such, researchers are investigating coatings or topology modifications to alter the free surface energy and wettability of implantables [6-7]. After cell adherence, rapid proliferation ensues, with the production of filamentous hyphal and pseudohyphal cells forming the 3-dimensional extracellular matrix. With the development of the extracellular matrix, β-glucans and peptides are masked with an exterior layer of hyphal cells, allowing the infection to evade host recognition [8]. The biofilm provides a physical barrier, distinct from a cell wall, which is difficult for treatments to penetrate [9].

Current treatment options for *C. albicans* include azoles (e.g., Fluconazole), polyenes (e.g., Amphotericin B), and echinocandins (e.g., Caspofungin) [10]. Azoles prevent ergosterol production, weakening the membrane integrity of *C. albicans* cells [11]. Though azoles are effective treatments for superficial infections, they are sequestered by the biofilm matrix and cannot penetrate to reach the infection [11]. Moreover, azoles are substrates of efflux pumps and are removed if they penetrate the cell wall [12]. Polyenes, functioning similarly to azoles in targeting ergosterol production, are not substrates of efflux pumps and have demonstrated a greater ability to penetrate the biofilm [13]. That said, the window for administration of polyenes is short and the doses required for the treatment of *C. albicans* biofilms exceed the safe limits for the patient [13]. Echinocandins inhibit the synthesis of *C. albicans* structural wall components leading to cell rupture under osmotic pressure [14]. Echinocandins are not substrates of efflux pumps and are thought to be the most applicable treatment for mature biofilms [15-17], being mainly limited by their ability to penetrate the biofilm [18].

Caspofungin, a semi-synthetic echinocandin, presents substantial benefits as a primary treatment for *C. albicans* biofilms [14]. Due to a cyclic hexapeptide with modified N-linked acyl lipid side chains, Caspofungin readily attaches to fungal cell membranes and inhibits the synthesis of β-(1,3)-glucan [14]. This effect originates from the Caspofungin non-competitively binding to fungal kinase sensitive 1 protein (Fks1p), the subunit of a glycosyltransferase and inhibiting the enzyme's ability to catalyze β-(1,3)-glucan biosynthesis reactions. The absence of β-(1,3)-glucan yields structural abnormalities in the cell wall and can lead to cell death from osmotic pressure [19].

One of the best-studied genes in *C. albicans*, enhanced filamentous growth gene (*EFG1)*, encodes the enhanced filamentous growth protein (EFG1) that modulates hyphae growth and is responsible for a range of antifungal resistance properties [20-21]. Environmental cues such as nitrogen content, nutrient availability, pH, and carbon dioxide presence trigger signalling pathways for filamentation that EFG1 regulates. For example, peptidoglycan presence induces hyphal production through the cAMP/PKA pathway encoded by the catalytic subunits ternary complex protein kinase 1 and ternary complex protein kinase 2, TPK1 and TPK2 respectively, but is ultimately controlled by EFG1 [22]. This was concluded from the observation that overexpression of EFG1 in a *TPK2* deleted *C. albicans* mutant was shown to restore filamentation while for the opposite conditions, *EFG1* deletion and overexpression of TPK2, showed no observable effect [23]. Similar analyses have shown EFG1 to dominate RIM101, responsible for pH response, in the filamentation response to pH cues [24-25]. Also being a central regulator in the transcriptional response to cell wall damage, targeting EFG1 could reduce the antifungal resistance properties of *C. albicans* biofilm infections and improve current treatment options.

A potential solution to suppress EFG1 arises from the emergence of antisense therapy, demonstrated to treat conditions such as inflammatory bowel disease [26]. Antisense therapy involves synthesized antisense oligonucleotides (ASOs) that bind to complementary strands on messenger RNA (mRNA) [27]. The ASO blocks translation or signals additional mechanisms to cleave the mRNA, ultimately downregulating the targeted protein [27]. Recently, Araújo et al. have demonstrated that the ASO anti-EFG1 2′-OMethylRNA can inhibit the synthesis of EFG1 proteins, reducing filamentation, and thereby weakening the biofilm [28]. The oligomer binds to complementary mRNA transcribed from the *EFG1* gene and blocks the translation of EFG1 proteins [28]. The reduction in filamentation resulting from the antisense strand, in combination with Caspofungin, presents the potential for a novel approach to combat *C. albicans* biofilms. Overall, we hypothesize that a combinatorial therapeutic using anti-EFG1 2′-OMethylRNA and Caspofungin on *C. albicans* biofilms will decrease the survivability of *C. albicans*, in contrast to individual therapies, allowing for more effective treatment and improved clinical outcomes long-term.

Methods

To evaluate the individual and combinatorial performance of Caspofungin and anti-EFG1 2′-OMethylRNA on *C. albicans* biofilms, the four test conditions listed in Table 1 will be examined:

Table 1. Test conditions used throughout experimentation, including control groups (A), individual therapies (B and C), and the proposed combinatorial therapeutic (D)

Test Condition	Description
A1	Untreated control
A2	Vehicle control
A3	Antisense control
B	anti-EFG1 2′-OMethylRNA treated
C	Caspofungin treated
D	anti-EFG1 2′-OMethylRNA and Caspofungin treated

It should be acknowledged that (A) the control consists of three subgroups: a standard control, a vehicle control, and a control with a known non-related antisense strand. These control subgroups indicate whether the antisense strands are mRNA specific and only target their complementary mRNA strands.

The conditions will be studied using a benchtop *in vitro* static biofilm model with *C. albicans* grown using Granulated Media (Sigma-Aldrich, USA) on medical-grade silicone elastomers. Silicone elastomers are used to mimic biofilm growth on a medical implant and effectively support biofilm growth to enable subsequent experimentation. The biofilms will be grown according to the methods presented by Kuhn et al. [29]. Briefly, 0.15 M phosphate-buffered saline will be used to create a 10^7 cells/mL suspension of *C. albicans*. Then, 3 mL of the suspension will be added to each silicone elastomer disc. Caspofungin Acetate (GoldBio, US) will be purchased; anti-EFG1 2′-OMethylRNA, targeting the 5′-ACAATAACGGTATGCC-3′ sequence, will be chemically synthesized according to the solid-phase synthesis methods described by Araújo et al. [28, 30]. To summarize, after addition of the initial nucleoside to a support resin, a phosphoramidite-modified nucloeside is added. Then, acetonitrile, amongst other reagents, is applied to form the phosphodiester bond, linking the nucelotides. The sequence is repeated for all nucleotides and the final strand is purified by high-pressure liquid chromatography [28, 30]. Initial treatment using the antisense strand will deliver 40 nM of anti-EFG1 2′-OMethylRNA upon full growth of the biofilm as defined above. The sample will be incubated with the antisense oligomer for 6 hours, as found to be an optimal time for filamentation inhibition by Araújo et al. [30]. Subsequently, 0.5 µg/mL Caspofungin will be administered to each disc [31]. These are initial dosing quantities according to literature; future exploration should be conducted to iterate and optimize these parameters.

A series of three experimental components, listed in Figure 1, will be conducted to comprehensively understand the effects of the therapeutics in each of the test conditions. These will allow us to gain a strong understanding of the mechanistic characteristics, survivability, and effectiveness of each testing condition.

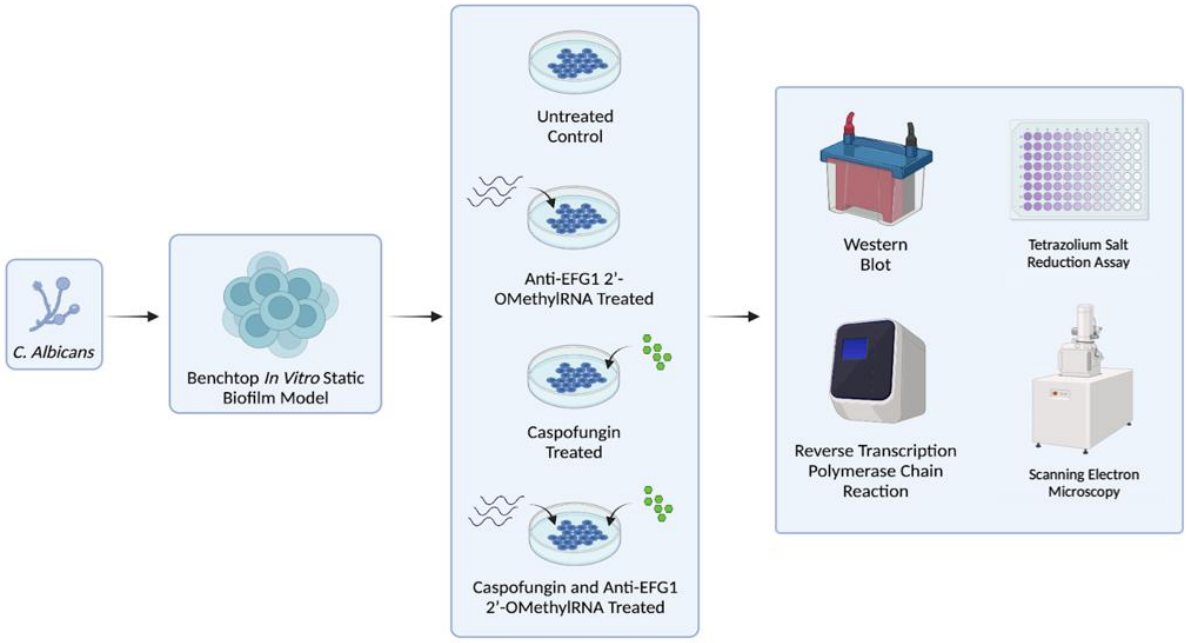

Figure 1. The proposed methodology to evaluate the combinatorial efficacy of Caspofungin with anti-EFG1 2′-OMethylRNA. Created with BioRender.com.

To validate anti-EFG1 2′-OMethylRNA activity, the transcriptome (specifically gene and protein expression) of EFG1 will be assessed using reverse transcription polymerase chain reaction (RT-PCR) in Experiment I and western blotting in Experiment II. This will allow a comprehensive understanding upstream and downstream of EFG1 translation, also verifying the effectiveness of the ASO. Additionally, this will elucidate the effect of each individual therapeutic approach and their combination to characterize potential challenges or problems. RT-PCR is used to quantify *EFG1* mRNA levels; western blotting will quantify EFG1 protein levels to assess the efficacy of translational inhibition from the antisense strand.

In Experiment III, the biofilm formation and survivability in each condition will be evaluated using a Tetrazolium Salt (XTT) Reduction Assay (Sigma-Aldrich, USA), where

mitochondrial enzymes of healthy cells convert the yellow XTT dye into the orange compound, formazan [32].

Our experimentation will assess 3-5 samples per condition to establish statistical significance using applicable statistical tests, such as ANOVA, where relevant. Throughout, the biofilm growth will be photographed and visualized using scanning electron microscopy to qualitatively observe the detailed surfaces of the fungi throughout the different stages of biofilm growth and treatment. With our biofilm culture, source, and environmental and growth conditions held constant (main controlled variables) prior to the different treatment conditions (manipulated variable), our analysis is inherently comparative, causing any confounding variables to be limited and considered systematic sources of error.

Results

Overall, we expect that a combinatorial therapeutic using anti-EFG1 2′-OMethylRNA and Caspofungin on *C. albicans* biofilms will decrease the survivability of *C. albicans* in contrast to individual therapies, supported by our anticipated results, presented in Figure 2.

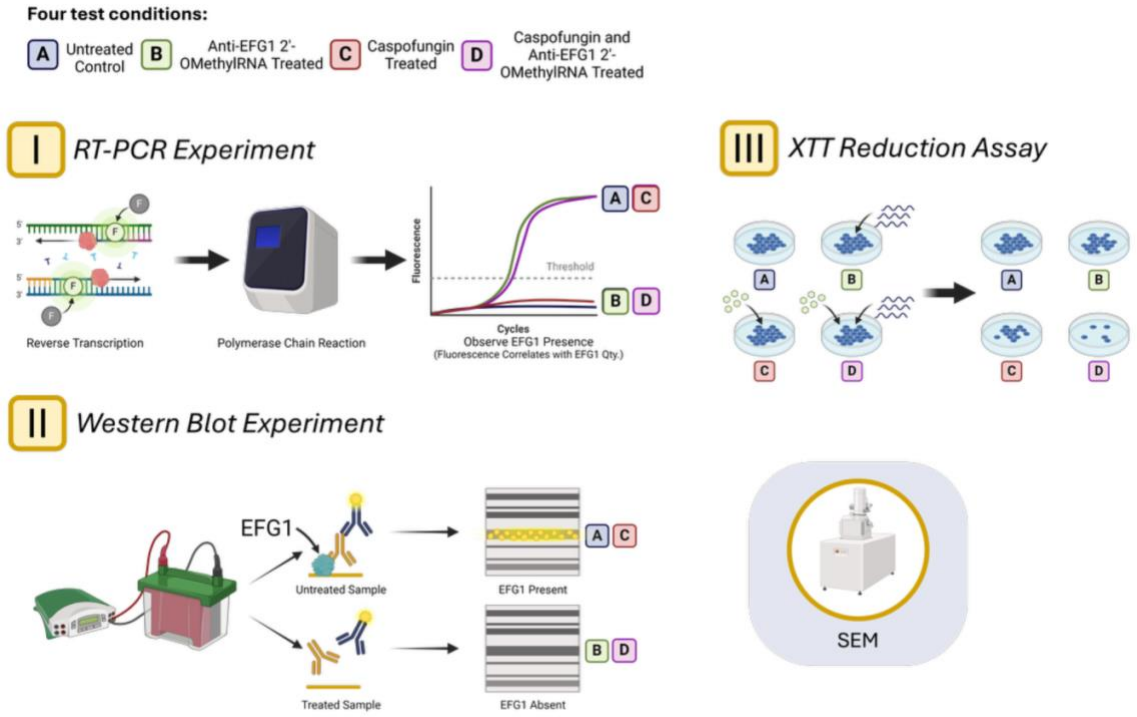

Figure 2. Main components of experimental methods, output, and predicted result for each of the four conditions. I) RT-PCR: Conditions A and C are expected to have regular *EFG1* mRNA levels, while conditions B and D are expected to have lowered expression due to the antisense/*EFG1* mRNA complex; II) western blot: Conditions A and C are expected to have higher EFG1 protein levels than conditions B and D; III) Tetrazolium Salt (XTT) Reduction Assay: Conditions B and D are expected to exhibit lower biofilm survivability than conditions A and C. Scanning Electron Microscopy (SEM) will be used on samples throughout for qualitative observation of fungal biofilm growth and morphology. Created with BioRender.com.

As shown in Figure 2, the outcomes for Condition A, the untreated control, and Condition C, Caspofungin treated, are expected to follow what has already been presented in the relevant literature for *C. albicans* biofilms. For Condition A, *EFG1* transcription and EFG1 synthesis are expected to result in a relatively high degree of antifungal resistance, as indicated by a high degree of filamentation and biofilm maturity through microscopy, and high biofilm survivability. For Condition C, *EFG1* transcription and EFG1 synthesis are expected to result in similar levels of antifungal resistance but moderately lower biofilm survivability is anticipated due to the Caspofungin treatment.

Again referring to Figure 2, the outcomes for Condition B, antisense treated, and Condition D, antisense and Caspofungin treated, are limited in their discussion in the existing literature and thus less evident. For Condition B, it is hypothesized that the EFG1 protein levels would be relatively low as the antisense therapy inhibits *EFG1* transcription. The complex formed from the antisense with the mRNA would also be expected to reduce *EFG1* mRNA expression during RT-PCR. As a result of the decreased EFG1 levels, antifungal resistance should be reduced, with qualitatively observed reduced filamentation. Condition B biofilm survivability is anticipated to be lower than Condition A since the absence of

EFG1 is expected to inhibit biofilm growth and modulation, weakening the biofilm overall. The degree to which it is lowered will remain to be seen. For Condition D, EFG1 expression and biofilm growth are anticipated to be inhibited similarly to Condition B. However, biofilm survivability is expected to decrease markedly compared to the other three conditions with enhanced Caspofungin penetration and reduced antifungal resistance from the biofilm, leading to more effective antifungal effects.

Discussion

Experiments I and II may support conclusions about the effectiveness of the antisense therapy in inhibiting *EFG1* expression and EFG1 synthesis. Since conditions A and C do not utilize targeted antisense therapy, they will provide a baseline for *EFG1* expression and EFG1 synthesis. The reduction in EFG1 synthesis as measured in Experiment II for conditions B and D is expected at approximately 60%, consistent with Araújo et al. [28]. If this is observed, the antisense therapy will have effectively inhibited EFG1 synthesis. Before assessing the relative reduction in EFG1 protein levels, the results from Experiment I will be interpreted to verify the effects of the antisense therapy on the mRNA levels.

The mRNA and protein levels of conditions B and D will permit analysis of any unforeseen effects of the combinatorial treatment. Though both conditions are antisense-treated, Condition D is additionally Caspofungin-treated and there may be either a positive, negative, or neutral effect of Caspofungin on antisense activity. For example, if Condition D is observed to have higher EFG1 protein levels than Condition B, it implies that Caspofungin may have an interaction effect and decrease antisense activity. Though this result is not expected based on previous literature, it has yet to be investigated experimentally and would lead to new research questions.

With the previous experiments elucidating the mechanism for the combinatorial treatment, Experiment III will permit analysis of the efficacy of the treatment. Though Araújo et al. showed a reduction in filamentation for antisense-treated *C. albicans* biofilms, the degree to which this reduction affects the efficacy of Caspofungin has yet to be explored [28]. This may be answered by comparing Condition C, representative of a current treatment option, to Condition D, the proposed combinatorial treatment. If Condition D displays lower biofilm survivability, it further supports that EFG1 is a primary regulator of the antifungal resistance properties of biofilms. More significantly, it validates the proposed hypothesis depicted in Figure 3 (lower) that inhibiting EFG1 sufficiently reduces the antifungal resistance properties of the biofilm to improve existing therapeutics. This is contrasted with the outcomes for the traditional treatment of Condition C, shown in Figure 3 (upper). While low biofilm survivability in Condition D will render the antisense and Caspofungin combinatorial therapy a potential treatment option, it may also motivate research looking at the combinatorial treatment of antisense with other echinocandins and therapeutics as well as optimizing the various parameters and approaches for a more effective treatment.

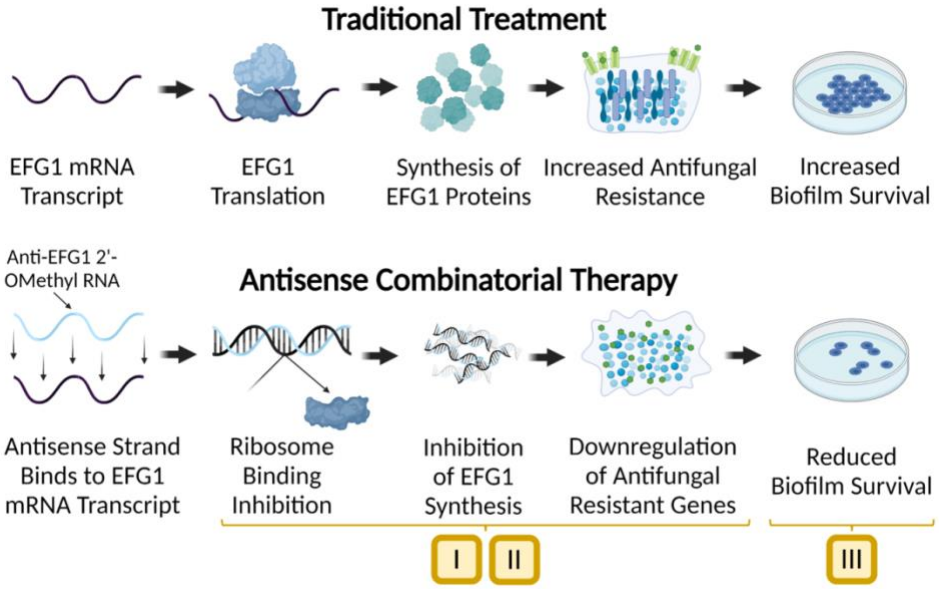

Figure 3. Traditional treatment (upper): theoretical path for *C. albicans* biofilm infections treated with traditional treatments such as Caspofungin. Antisense Combinatorial Therapy (lower): Hypothesized path for *C. albicans* biofilm infections treated with antisense therapy and Caspofungin. The antisense strand binds to the mRNA transcript to prevent ribosome binding and protein synthesis, consequently reducing gene expression and antifungal resistance properties; this is validated using Experiments I and II. Finally, there is an expected reduction in biofilm survivability as observed in Experiment III and qualitatively. Created with BioRender.com.

This investigation presents an initial exploration of the possibility of a combinatorial therapeutic approach using antisense therapy. As such, there are many directions for future research, including varying drug concentrations and timelines and considering different treatment combinations (e.g., azoles with antisense, with the possibility of antisense therapy reducing antifungal resistance and increasing azole effectiveness). Adding polyenes in safe concentrations alongside antisense therapy could yield advantageous therapeutic outcomes and is worth exploring. In addition, genomic analysis using next-generation sequencing may be conducted to gain a comprehensive outlook of the gene expressions and how they are affected by the different conditions. These results would be validated using RT-PCR for known adhesion-related, hypha-related, and biofilm-related genes, including RAS1, CPH2, and TEC1 [33]. Finally, testing biofilm survivability under environmental conditions known to affect EFG1 activity, such as pH, will be important to qualify the applicability of the combinatorial therapy and optimize the delivery. This further optimization and tuning of the therapeutic conditions and approach are required to eventually translate this potential combinatorial therapeutic approach into effective clinical applications.

Conclusions

In this study, we aim to determine the efficacy of a combinatorial therapeutic whereby anti-EFG1 2′-OMethylRNA is hypothesized to increase the susceptibility of *C. albicans* biofilms to the fungicidal agent Caspofungin and strengthen anti-biofilm effects. Given the novelty of the proposed therapeutic, this study is exploratory and aims to establish a comprehensive initial understanding and comparison of the therapeutic effects of antisense therapy Caspofungin, and their combinatorial treatment. As such, our methods assess each major component of the therapeutic effects, including survivability and qualitative changes in fungal form. The largest takeaways concerning therapeutic potential are derived from the XTT Reduction Assay, measuring survivability, since it implies therapeutic effectiveness of treating the biofilm. The other experiments evaluate the characteristics of the different treatment conditions, including the transcriptome, and challenge our hypothesis for the biological mode of action. Our study not only addresses the current problem of antifungal resistance development, but also offers a potential avenue for the development of targeted and effective antifungal therapies for biofilm-related infections, including those on medical implants. Moreover, the outcomes of this study may indicate the broader applicability of antisense therapy and its combinatorial potential with other therapeutic agents and approaches for various medical challenges.

List of Abbreviations Used

ASOs: antisense oligonucleotides
EFG1: enhanced filamentous growth protein 1
mRNA: messenger RNA
RT-PCR: reverse transcription polymerase chain reaction
XTT Reduction Assay: tetrazolium salt reduction assay

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study did not require participant consent due to the use of a proposed *in vitro*, bench-top-based model as opposed to participants. Ethics approval has not been required thus far due to this study being a research protocol and no experimental work occurring yet.

Authors' Contributions

SB: contributed substantially to the conceptualization and design of the study, drafted, revised, and finalized the manuscript for publication, contributed to designing the figures, and gave final approval of the version to be published.
JS: contributed substantially to the conceptualization and design of the study, drafted, revised, and contributed to the finalizing of the manuscript, and gave final approval of the version to be published.
CBH: contributed substantially to the conceptualization and design of the study, contributed to the drafting of the manuscript and creation of figures, and gave final approval of the version to be published.
TS: contributed substantially to the conceptualization and design of the study, contributed to the drafting of the manuscript, and gave final approval of the version to be published.

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