Summary of Ph.D. dissertation

Antifungal activity of rationally designed γ-core peptide derivatives of ascomycetous antifungal proteins

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1. INTRODUCTION

The prevalence of fungal infections in humans has risen alarmingly over recent decades, posing a public health challenge. Multiple factors drive this trend, such as the limited number of available antifungal treatments, increasing resistance to existing antifungal medications, the absence of new antifungal compounds with unique modes of action, and significant changes in fungal pathogen distribution linked to global climate change. The rise of drug-resistant and less susceptible fungal strains to conventional antifungals is a major factor driving the increasing incidence of fungal infections, highlighting the urgent need for novel antifungal drug development.

Considering these challenges, the World Health Organization coordinated efforts among researchers, healthcare providers, and policymakers to develop new antifungal therapies and optimize existing treatment protocols. Among the promising alternatives are antifungal peptide derivatives of ascomycetous antifungal proteins (AFPs), particularly those encompassing evolutionarily conserved γ-core motifs. The γ-core motif (GXC-X_{3-6}-C signature, where X represents any amino acid) is a conserved structural element found in numerous AFPs in both plants and animals that contributes significantly to their antifungal properties and structure stability. Such γ-core motif-containing antifungal peptides (γAFPs) demonstrated broad-spectrum antifungal efficacy and exhibited minimal toxicity toward mammalian cells. Despite this, their functional mechanisms and *in vivo* potential remain underexplored as standalone agents or in combination therapies. In our study, we designed 19 synthetic γAFPs based on conserved γ-core

motifs and tested their interactions with conventional antifungal agents, revealing synergistic effects that enhanced efficacy against pathogenic yeasts and filamentous fungi *in vitro* and *in vivo.*

2. OBJECTIVES

- To design a series of gamma core peptides (γAFPs) incorporating from Eurotiomycetes and evaluate their *in vitro* antifungal activity alone and in combination with conventional drugs against a panel of human and phytopathogenic fungi.
- To characterize the structure of the most effective γAFPs in the presence of conventional antifungal drugs, fungal cells, and conidia, and assess their toxicity and therapeutic efficacy in an animal model.

3. MATERIALS AND METHODS

In silico Analysis

- AFPs from Eurotiomycetes were retrieved from UniPro database, aligned using BioEdit, visualized with Jalview and analyzed for signal peptides using SignalP.
- Phylogenetic relationships were inferred in MEGA11 using a maximum likelihood approach with the WAG model, gamma rate variation, 1,000 bootstrap replicates, and plant defensins as an outgroup.
- Physicochemical properties were assessed by ExPASy ProtParam and the Antimicrobial Peptide Database tools, while tertiary structures were obtained from AlphaFold or predicted with PEP-

FOLD3, visualized in UCSF Chimera, and validated using Ramachandran plot analysis via MolProbity.

Peptide Synthesis

- γAFPs were synthesized by stepwise solid-phase peptide synthesis using Fmoc chemistry on a Liberty Blue synthesizer. AFPs were cleaved from the resin with a TFA-based mixture, precipitated, dissolved and freeze-dried.
- Semipreparative RP–HPLC was used to purify crude products, and verified by analytical RP–HPLC. The purified and lyophilized peptides were stored at −20°C.

Fungal Strains and Inoculum Preparation

- Freshly prepared mold conidia and mid-log phase yeast cells were cultured on PDA and YPD agar, respectively. Conidia were harvested from seven-day-old cultures, filtered, washed, and resuspended in spore buffer. Yeast mid-log phase cultures were generated by sequential growth in low-cationic medium with shaking.
- Before experiments, both conidia and yeast cells were adjusted to the required concentrations in LCM.

***In vitro* antifungal susceptibility tests**

- A broth microdilution susceptibility assay was conducted to evaluate the antifungal efficacy of synthetic γAFPs against four molds (*Aspergillus fumigatus* CBS 101355, *Botrytis cinerea* SZMC 21472, *Cladosporium herbarum* FSU 1148, *Fusarium subglutinans* CBS 747.97) and two yeasts (*Candida*

albicans SC5314, *Saccharomyces cerevisiae* SZMC 0644) in LCM.

- The same method was applied to determine the MICs of conventional antifungal drugs, including amphotericin B (AMB), fluconazole (FLC), micafungin (MFG), and terbinafine (TRB) against *C. albican*s SC5314 and *A. fumigatus* CBS 101355.

***In vitro* Interaction Between γAFPs and Antifungal Drugs**

- Broth microdilution checkerboard titration assay was used to assess interactions between γAFPs and antifungal drugs *against C. albicans* and *A. fumigatus*.
- Interaction ratios were calculated using the Abbott formula.

Electronic Circular Dichroism Spectroscopy

- Electronic circular dichroism (ECD) spectroscopy was used to analyze the secondary structures of γAFPs were analyzed spectroscopy using a Jasco J-815 spectropolarimeter.
- ECD was also used to determine conformational changes of potent γAFPs in the presence of fungal cells, antifungal drugs, and their combinations under the same conditions.

Fluorescence-Activated Cell Sorting

- Fluorescence-Activated Cell Sorting (FACS)was used to evaluate the antifungal activity of γAFPs, antifungal drugs, and their synergistic combinations against *C. albicans* cells and *A. fumigatus*.

Scanning Electron Microscopy

- In order to assess the morphological effects of γAFPs, antifungal drugs, and their synergistic combinations

on *C. albicans* and *A. fumigatus*, scanning electron microscopy (SEM) was used.

Hemolysis Assay

- The hemolytic potential of γAFPs, antifungal drugs, and their synergistic combinations was tested on sheep blood agar.

***Galleria mellonella* Toxicity Assay**

- The *in vivo* toxic effect of γAFPs, antifungal drugs, and their synergistic combinations was evaluated in *Galleria mellonella* larval model system.

***In vivo* Therapeutic Efficacy of γAFP-Antifungal Drug Combinations**

- The therapeutic effects of γAFPs, antifungal drugs, and their synergistic combinations were assessed on *G. mellonella* larval model systeme.

Statistical Analyses

- Data were interpreted by one-way ANOVA with Tukey's HSD for growth inhibition, Pearson's chi-squared test with Phi coefficient for FACS analysis, and log-rank plus Gehan–Breslow–Wilcoxon tests for *G. mellonella* survival. Significance was set at $p \leq 0.05$, and all analyses were performed using GraphPad Prism 7.

4. RESULTS AND DISCUSSIONS

AFP Selection for Peptide Design, and Physicochemical Properties of γAFPs

This study designed synthetic γ-core antifungal peptides (γAFPs) based on the γ-core motifs of ascomycetous antifungal proteins by extending the original γ-core motifs

with additional N- and C-terminal amino acids to enhance antifungal activity. These γAFPs showed wide variation in net charge and hydropathy across different antifungal protein groups. Several peptides exhibited high Boman index values (>2.5 kcal/mol), indicating strong membrane-binding potential and supporting a membrane-interaction. Their activity is influenced not just by charge or hydrophilicity; some positively charged peptides were inactive, while some neutral or negatively charged ones were effective. Overall, antifungal efficacy depends on a combination of several factors, including amino acid sequence and charge-hydrophobic balance.

Antifungal Activity of γAFPs

The antifungal activity of γAFPs was assessed *in vitro* against a panel of yeast and mould isolates. Although none of the γAFPs fully inhibited fungal growth at concentrations up to 200 μg/mL, several peptides demonstrated notable antifungal effects at the highest concentration tested, achieving inhibitory percentages (PI) ≥ 40%. The differences observed in γAFP activity across fungal species may be attributed to species-specific variations in plasma membrane lipid composition, which are known to modulate susceptibility to antifungal peptides.

Interaction Between γAFPs and Conventional Antifungal Drugs Against *C. albicans* and *A. fumigatus*

Checkerboard broth microdilution assay showed that AMB, MFG, and TRB inhibited *Candida albicans*, whereas FLC was ineffective, and none of the drugs fully inhibited *Aspergillus fumigatus*. AMB, MFG, and TRB were therefore

selected for combination studies with γAFPs. Most combinations were indifferent and non-antagonistic, but γAFP^{B6GXZ8} + TRB and γAFP^{A0A2J5HZT4} + FLC peptide-drug combinations showed synergy against *C. albicans* and *A. fumigatus*, respectively, achieving strong inhibition below individual MICs. A non-linear relationship between net charge and hydropathy was observed, with optimal hydrophilicity at a net charge of ~+4.2, indicating enhanced activity for moderately cationic peptides.

ECD Spectroscopy

ECD spectroscopic analyses revealed that none of the investigated γAFPs undergo significant structural rearrangements in the presence of fungal cells or conidia, antifungal agents, or their combinations. Consistent with previous studies, it is well-established that short, linear AFPs retain a disordered conformation in solution, even in the presence of fungal cells. Although adopting an ordered structure may enhance the stability and specificity of AFPs, it is not necessarily a prerequisite for antifungal activity, particularly in the case of peptides that exert their effects through direct membrane disruption rather than receptor-mediated mechanisms. This mode of action is characteristic of the modified peptides synthesized in this and in previous studies, which encompass the γ-core region.

Fungal Cell Killing Efficacy of γAFP + Antifungal Drug Combinations

FACS was utilized to quantify the cell-killing and membrane-disrupting capabilities of two potent γAFPs in combination with conventional antifungal agents (TRB and

FLC). These combinations were compared to the standalone application of each compound to elucidate the observed synergistic interactions. The γAFP^{B6GXZ8} + TRB combination demonstrated significantly higher cell-killing efficacy than either compound alone. The γAFP^{A0A2J5HZT4} + FLC combination also showed enhanced cell-killing activity compared to FLC alone. However, its efficacy was lower than that of the γAFP^{A0A2J5HZT4} peptide when applied individually. However, its efficacy was lower than that of the γAFP^{A0A2J5HZT4} peptide when applied individually

SEM Analysis

SEM analysis confirmed the antifungal effects of γAFPs, conventional drugs, and their synergistic combinations by revealing pronounced morphological damage. In *C. albicans,* γAFP^{B6GXZ8} and TRB each induced surface roughening, deformation, aggregation, and signs of membrane stress, while their combination produced combined features, including irregular surfaces and intercellular connections suggestive of biofilm-like growth. In *A. fumigatus,* untreated conidia remained smooth and intact, whereas γAFP^{A0A2J5HZT4} caused moderate surface damage and FLC alone showed minimal effects. In contrast, the γAFP^{A0A2J5HZT4} + FLC combination leading to severe structural disruption and conidial collapse, highlighting enhanced antifungal activity.

Hemolytic Activity and Toxicity of γAFP + Antifungal Drug Combinations

To evaluate the hemolytic activity and toxic effects, γAFP^{B6GXZ8} + TRB and γAFP^{A0A2J5HZT4} + FLC combinations were tested *in vitro* on sheep blood agar plates and *in vivo*

using *Galleria mellonella* larvae. None of the sole applications of γAFPs and antifungal drugs, nor their combinations, caused hemolysis or significantly reduced the survival of larvae. These findings support the conclusion that γAFPs and their combinations with antifungal drugs can be safely utilized for therapeutic purposes.

In vivo Therapeutic Potential of γAFP + Antifungal Drug Combination

In a *G. mellonella* infection model, *C. albicans* infection drastically reduced larval survival. Treatment with γAFP^{B6GXZ8} or TRB alone offered less protection, whereas their combination significantly improved and prolonged survival, demonstrating superior therapeutic efficacy over monotherapies.

In the case of *A. fumigatus*, infection markedly decreased survival. FLC alone was ineffective, while γAFP^{A0A2J5HZT4} moderately improved survival. The γAFP^{A0A2J5HZT4} + FLC combination further enhanced and prolonged survival compared with FLC alone, although it was not significantly better than γAFP^{A0A2J5HZT4} monotherapy. Overall, combination treatments provided greater *in vivo* benefit than single agents.

5. SUMMARY

- Not just positively charged peptides are antifungal active (as it was expected before), but the neutral ones can also have an antifungal effect.
- We suppose that the antifungal activity depends on the balance between the charge and the GRAVY, varies by fungal species, and is influenced by amino acid composition.

- The antifungal efficacy of γAFPs is influenced by the composition of amino acids within their sequences and physicochemical features, determining their structure and function.
- *In silico* modeling accelerates the identification and advancement of modesty AMPs with improved effectiveness and selectivity
- These findings demonstrate potential of γAFPs as antifungal agents and drug adjuvants, but further *in vivo* studies regarding animal models are needed for definitive validation.

6. LIST OF PUBLICATIONS

MTMT ID: 10090442

Cumulative IF:13.4

Publication Related to Thesis

Karemera JK, Váradi G, Bende G, Merber R, Dán K, Papp C, Farkas A, Maróti G, Tóth GK, Borics A, Galgóczy L. Screening the γ-core motif peptides of ascomycetous antifungal proteins for antifungal activity and potential therapeutic applicability. Probiotics Antimicrob Proteins. 2026. doi: 10.1007/s12602-025-10890-y. Epub ahead of print. IF_{2024}=4.4 (Q2)

Other Publications

Dán K, Zsindely N, Kele Z, Laczi K, **Karemera JK**, Papp C, Farkas A, Maróti G, Borics A, Bodai L, Galgóczy L. Beyond plasma membrane disruption: novel antifungal mechanism of

Neosartorya (*Aspergillus*) *fischeri* antifungal protein 2 in *Candida albicans*. Int J Biol Macromol. 2025;327:146558. doi:10.1016/j.ijbiomac.2025.146558. IF_{2024}=8.5 (D1)

Emmanuel M, Parfait C, Callixte Y, **Karemera J**. Contribution of medical wards contamination to wound infection among patients attending Ruhengeri Referral Hospital. Int J Microbiol. 2021;7838763.doi.org/10.1155/2021/7838763. IF_{2021}=0.4 (Q4)

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7. DECLARATION

We hereby certify that we are familiar with the Ph.D. thesis of Karemera K. John. With regard to the results obtained in our jointly published work, we confirm that the candidate made a substantial and independent contribution. We further declare that the principal publications forming the scientific basis of this dissertation have not been used to obtain any Ph.D. degree in the past and will not be submitted as part of any other Ph.D. dissertation in the future.

Szeged, January 26, 2026

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