The series of a new fluconazole-based conjugates with cell penetrating and antimicrobial peptides

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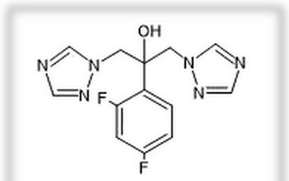
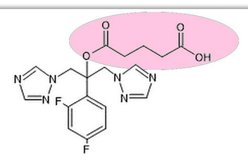
Drug



FLC

Fluconazole (**FLC**) is a **drug** used to treat a number of fungal infections (particularly *Candida* infections). It inhibits the lanosterol 14α-demethylase and decreases the level of ergosterol required for the integrity of fungal cell membranes. FLC causes the accumulation of the ergosterol precursors (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol) inside the cell, resulting in the altered structure and functioning of the cell membranes [1]. Fluconazole was the first triazole antifungal drug introduced in the medicinal practice.

Linker



FLCpOH

In all studied conjugates, **glutaric acid** served as a **linker,** forming covalent bonds between hydroxyl group of FLC and N-terminal amino group of the peptide (ester and amide moieties, respectively).

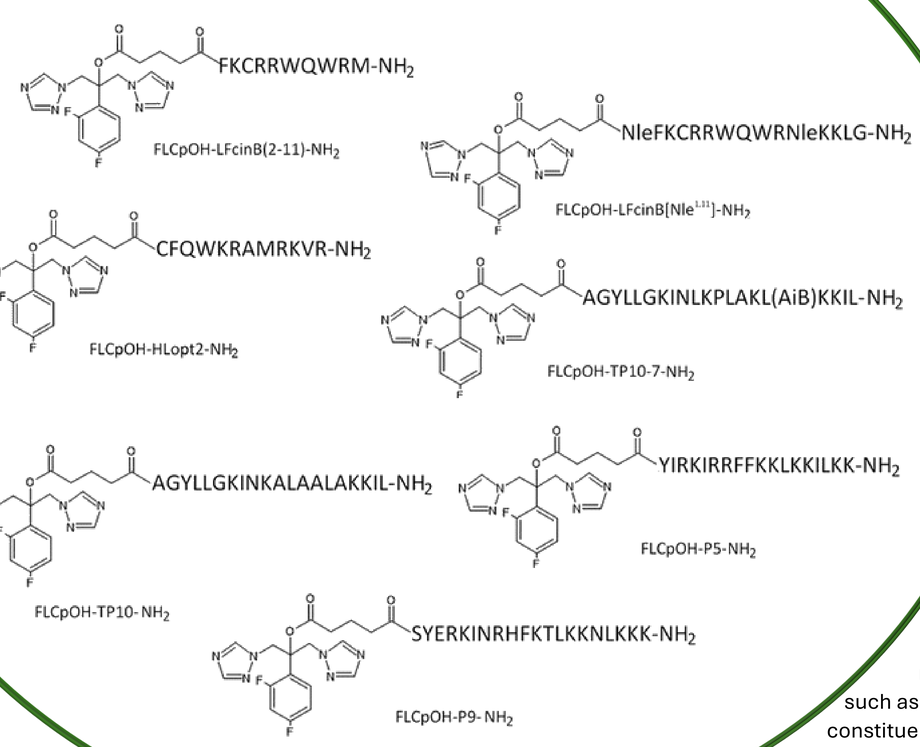
Peptide

Selected CPP and AMP peptides:

LFcinB(2-11)-NH_2: FKCRRWQWRM-NH_2
LFcinB[$Nle^{1,11}$]-NH_2: NleFKCRRWQWRNleKKLG-NH_2
HLopt2-NH_2 : CFQWKRAMRKVR-NH_2
TP10-NH_2 : AGYLLGKINLKALAALAKKIL-NH_2
TP10-7-NH_2 : AGYLLGKINLKPLAKL(AiB)KKIL-NH_2
P5-NH_2 : YIRKIRRFFKKLKKILKK-NH_2
P9-NH_2 : SYERKINRHFKTLKKNLKKK-NH_2

- CPPs serving as carrier peptides and are considered the fundamental part of an extensively developed concept of a drug delivery system; TP-10 is one of the most promising peptides in this family. These peptides effectively penetrate cell membrane.
- Modified fragments of bovine lactoferrin (LFcin) and human lactoferrin (HLopt2) are members of the AMP family, peptides that constitute an important segment of the body's natural immunity against microorganisms.
- P5 and P9 are AMPs, which are designed based on *Artisicluthys nobilia* interferon-I, inhibit Methicillin-resistant Staphylococcus aureus (MRSA) and show low cytotoxicity.

Peptide Fluconazole-Based Conjugates



Introduction

Many pathogenic bacteria and fungi strains have become resistant to the most of commercially available antibiotics as the result of their common use in medical practice. The fungal infections have a major impact on quality of life and also exhibit high levels of mortality. That is why effective and safe therapeutic treatment of fungal infections is a major challenge for modern medicine.

Infections of Candida spp. etiology are frequently treated with azole drugs. Among azoles, the most widely used in the clinical practice remains fluconazole (FLC). However, fluconazole usage has developed some negative consequences, among which the most important are: (i) selection of fungal pathogens that exhibit lower susceptibility to fluconazole activity, including some non-albicans Candida spp. (e.g., *C. krusei*, *C.glabrata*); (ii) development of resistance against this agent among species that are naturally sensitive, e.g., *C. albicans*.

Promising results in treatment of dangerous, systemic *Candida* infections demonstrate the advantages of combined therapies carried out with combinations of at least two chemical molecules with different antimicrobial activity that in the end display a different mechanism of action than the parent compounds.

The aim of the work

In this study we proposed modification of fluconazole's (and other azoles') structure by covalently linking it to other substances-peptides with different structures and biological activities.

Herein we report the chemical synthesis and biological activity of conjugates of fluconazole with (i) cell-penetrating peptides (CPP), namely TP10-NH_2 and TP10-7-NH_2, or (ii) antimicrobial peptides (AMP), such as LFcinB(2-11)-NH_2, LFcinB[Nle1,11]-NH_2, HLopt2-NH_2, P5 and P9. Both constituents of produced conjugates (FLC and peptides) display different modes of antifungal activity and affect different molecular targets within fungal cells.

The major foreseen advantages of conjugates of FLC and selected CPPs or AMPs include improved antifungal (hopefully fungicidal) efficiency and increased drug delivery. Moreover, according to our previous research [2], we would like to check if synthesized fluconazole-based conjugates (especially those with P5 and P9) have also anti-cancer properties.

Table 1. MIC and MFC Values (in M) Determined for Compounds, As Well As for Two Control Antifungal Agents (AmB and FLC) against Various Yeast Strains [5].

Yeast Strains	MIC* and MFC** [μM]	Compounds							
		FLCpOH	FLCpOH-TP10-NH_2	FLCpOH-TP10-7-NH_2	FLCpOH-LFacinB[$Nle^{1,11}$]-NH_2	FLCpOH-LFacinB(2-11)-NH_2	FLCpOH-HLopt2-NH_2	Fluconazole (FLC)	Amphotericin B (AmpB)
Candida albicans Gu4	MIC_{50}	>250	45	23	125	>250	>250	-	<0.1
	MIC_{90}	>250	62	31	250	>250	>250	31	<0.1
	MFC	-	62	31	-	-	-	-	<0.1
Candida albicans Gu5	MIC_{50}	>250	62	31	>250	>250	>250	-	<0.1
	MIC_{90}	>250	125	62	>250	>250	>250	>250	<0.1
	MFC	-	62	62	-	-	-	-	<0.1
Candida albicans 48	MIC_{50}	>250	45	15	250	250	250	-	<0.1
	MIC_{90}	>250	62	31	>250	>250	>250	>250	<0.1
	MFC	-	250	125	-	-	-	-	<0.1
Candida albicans 138	MIC_{50}	>250	45	23	23	190	>250	-	<0.1
	MIC_{90}	>250	62	31	31	250	>250	>250	<0.1
	MFC	-	250	125	-	-	-	-	<0.1
Candida albicans 190	MIC_{50}	>250	45	23	90	190	250	-	<0.1
	MIC_{90}	>250	62	31	125	250	>250	>250	<0.1
	MFC	-	250	125	-	-	-	-	<0.1
Candida albicans 247	MIC_{50}	>250	45	23	45	190	>250	-	<0.1
	MIC_{90}	>250	62	31	62	250	>250	>250	<0.1
	MFC	-	250	125	-	-	-	-	<0.1
Candida albicans 574	MIC_{50}	>250	45	23	45	190	250	-	<0.1
	MIC_{90}	>250	62	31	62	250	>250	>250	<0.1
	MFC	-	-	250	-	-	-	-	<0.1
Candida albicans 604	MIC_{50}	>250	45	23	45	190	>250	-	<0.1
	MIC_{90}	>250	62	31	90	250	>250	>250	<0.1
	MFC	-	250	250	-	-	-	-	<0.1

* MIC—minimum inhibitory concentration; ** MFC—minimum fungicidal concentration.

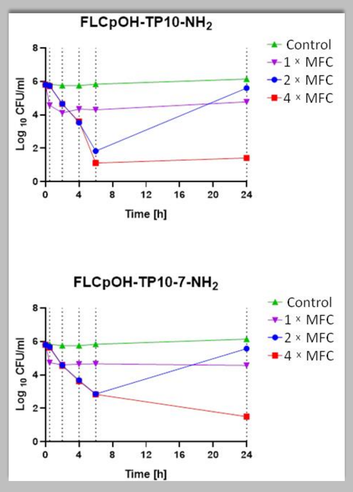


Figure 1. Time-kill determinations for *C. albicans* strain after treatment with FLCpOH-TP-NH_2 and FLCpOH-TP10-7-NH_2 alone at 1 x MFC, 2 x MFC and 4 x MFC. The x-axis represents the killing time, and the y-axis represents the logarithmic *C. albicans* SC5314 survival [5].

Table 2. In vitro cytotoxicity (IC_{50} and IC_{90} SEM (M)) of the FLC conjugates and their constituents toward Hs27 and HUVEC cells [5].

Compound	Hs27		HUVEC	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
FLC	>100	>100	>100	>100
FLCpOH	>100	>100	>100	>100
FLCpOH-TP10-NH_2	12.00 ± 0.93	28.81 ± 0.87	14.45 ± 1.13	34.30 ± 1.00
FLCpOH-TP10-7-NH_2	13.07 ± 0.35	29.78 ± 0.29	15.16 ± 1.26	34.03 ± 1.90
FLCpOH-LFacinB[$Nle^{1,11}$]-NH_2	>100	>100	75.58 ± 1.73	>100
FLCpOH-LFacinB(2-11)-NH_2	>100	>100	>100	>100
FLCpOH-HLopt2-NH_2	>100	>100	>100	>100

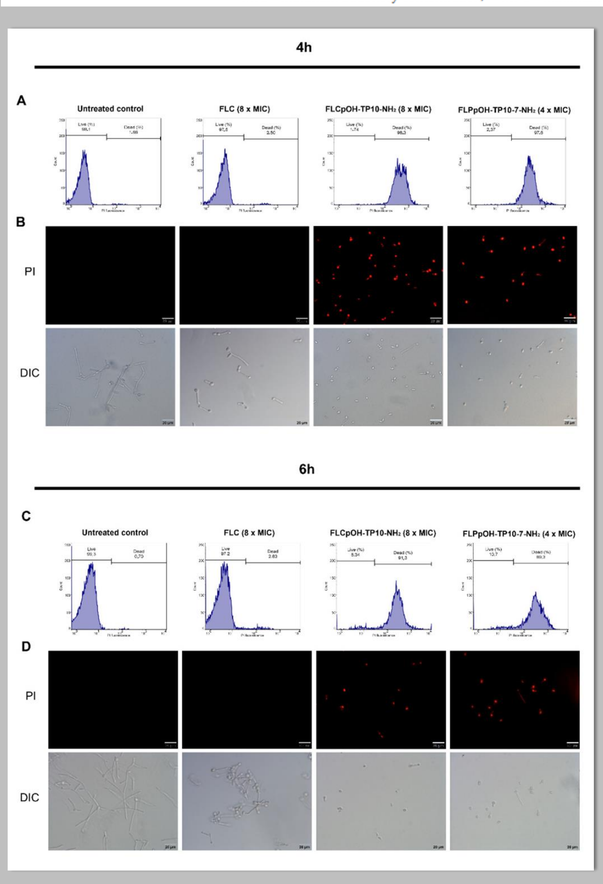


Figure 2. (**A**,**C**) Flow-cytometric analysis of membrane permeabilization assay by PI uptake. Cells were treated with FLC and FLC conjugates and stained with PI. After the completion of treatment and staining process, the cellular fluorescence was then analyzed via flow cytometry. (**B**,**D**) Fluorescence microscopy analysis of membrane permeabilization assay by PI uptake in treated as well as untreated yeast cells. The results of selected images are chosen as the best representatives of one of three different experiments with two replicates; similar results were observed each time. DIC—differentia interference contrast microscopy, PI—fluorescence microscopy. Scale bars correspond to 20 m [5].

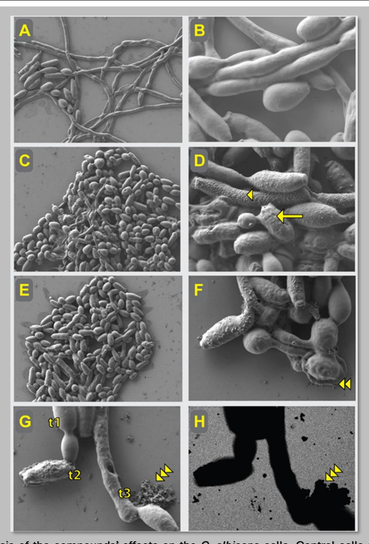


Figure 3. SEM analysis of the compounds' effects on the *C. albicans* cells. Control cells (**A**,**B**) demonstrating normal appearance of *C. albicans*, with long hyphae stage well-represented (A) and uniformly smooth surface (**B**). Compound FLC-TP10-7-NH_2, (**C**,**D**), in contrast, resulted in reduction of hyphae numbers (**C**), roughed cells surface (arrow) in large part of cell population, granularities (arrowhead), perforations and increased debris (**D**). Compound FLCpOH-TP10-NH_2, (**E**–**H**) demonstrated signs of stronger toxicity against *C. albicans*, lack of hyphae (**E**), with intense roughing of the cel surface and membrane disintegration or spreading (double arrowheads) (**F**), frequent perforations and granularities (**G**), membrane blebbing and more debris. (**G**,**H**) Different stages of deleterious effects of the compound, from mild (t1), through roughing (t2) to perforation and fragmentation of cells (t3). Chemical contrast imaging (**H**), correlated pixel-to-pixel with topography imaging. (**G**) Improved identification of blebbing and fragmentation of *C. albicans* cells (triple arrowheads). Gray appearance of the substrate in (**H**) stems from silicon polished crystal and facilitates detection of multiple organic fragments with dark appearance in (**H**), by chemical contrast mapping of non-coated sample. (**A**–**G**)—Everhart–Thornley detector, (**H**)—EsB detector imaging. (**A**,**C**,**E**)—magnification 5000 (field of view 60 m), (**B**,**D**,**G**,**H**)—magnification 20,000 (field of view 15 m).

Conclusions

- In reported conjugates, FLC was linked to the peptide by its hydroxyl group.
- Coupling of TP10-NH_2, analogues of LFcinB and HLopt2 to the FLC triazole ring yielding a practically inactive conjugates [3,4].
- Two of synthesized compounds, namely **FLCpOH-TP10-NH_2** and **FLCpOH-TP10-7-NH_2**, exhibit high activity against reference strains and fluconazole-resistant clinical isolates of *C. albicans*, including strains overproducing drug transporters.
- Moreover, both of them demonstrate higher fungicidal effects compared to fluconazole.
  - The MFC values against *C.albicans* reference strains were the same or only twice higher compared to MIC_{90}.
- The high fungicidal activity of both conjugates was also confirmed with the kill-time assay.
- Analysis performed with fluorescence and scanning electron microscopy as well as flow cytometry indicated the cell membrane as a molecular target of synthesized conjugates. In this respect, TP peptides behave like AMPs, disrupting cell membranes.
- Two compounds produced by us and reported herein appear to be potential candidates for novel antifungal agents. Some of the produced conjugates show promising activity and are good candidates for obtaining new effective antifungals.

Methods

Synthesis of analogue of FLC- FLCpOH

A solution of fluconazole (306 mg, 1 mmol), glutaric anhydride (1369 mg, 12 mmol) and DMAP (28,6 mg, 0,234 mmol) in 11 mL of DMF was heated at 85 °C for approximately 20 h under the reflux. After distillation of the solvent under reduced pressure, the resulting oil was dissolved in 40 ml of the solvent mixture water:acetonitril (4:1 v/v) and solid residue was filtered off. The crude product was purified on a preparative HPLC and the final product was confirmed by HPLC, MALDI-TOF and NMR.

Synthesis of Fluconazole-Based Conjugates

The mixture of purified FLCpOH, HOBt, and DIC (in molar ratio 1:1:1) was dissolved in 6 mL of DMF, added to SPPS vessel with peptidyl resins with free N-terminal amino groups. The coupling efficiency was monitored after 2 h using the chloranil test. In case of a positive test result, acylation was repeated using the mixture of purified FLCpOH, TBTU, HOBt and DIPEA (in molar ratio 1:1:1:2) dissolved in 6 mL of DMF. Usually, two repeats was required to complete the coupling. The conjugates were cleaved from the resin. The crude products were purified and analyzed the yields were 10–46%.

Time-Kill Assay

A starting inoculum was prepared by inoculating a tube of sterile water with growth from 24 h YPD agar plates (*C. albicans* SC5314), and OD660 was adjusted to about 0.1 (around 106 cells per 1 mL). The suspension was then diluted 10-fold with RPMI 1640 medium, and FLCpOH-TP10-NH_2 and FLCpOH-TP10-7-NH_2 were added at concentrations corresponding to 1 x MFC, 2 x MFC, and 4 x MFC. A negative control (growth medium without drug or organism) was also included at each time of testing. Tubes were incubated at 37 °C on an orbital shaker and vortexed before removing a sample for the determination of colony counts. After the appropriate time of incubation (0.5, 2, 4, 6, and 24 h), 1 mL of each suspension was centrifuged (2 min, 9170 g), and the pellet was re-suspended in 1 mL of PBS pH 7.4. Ten-fold serial dilutions with PBS were prepared, and 100 L of each was inoculated on YPD agar plates in duplicate. Plates were incubated for 24 h at 37 °C. Colony forming units in the range of 30–300 were counted (on the plates containing from 30 to 300 colonies) and the number of yeast cells in 1 mL (CFU/mL) was calculated.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

Antifungal activity of the tested compounds against yeast cells was determined in 96- well plates as described in the CLSI document M27-A3, with minor modifications (CLSI, 2008). Suspensions of the microorganisms were prepared by taking one loop of pure culture into sterile water and adjusting optical density to 0.1 at 660 nm wavelength and further 50-fold dilution in RPMI 1640 medium, resulting in cell concentration of approximately 2 x 104 CFU/mL. Then, 100 L of cells were added to the wells of a 96-well microtiter plate that contained 16–500 M tested compounds and 16–500 M of FLC or 0.2–62 M AmB. The plates were incubated for 24 h at 37 °C. Each test was performed in triplicate. The final concentration of DMSO was ensured to be around 1% in all experiments. For determination of minimum fungicidal concentrations (MFC), small aliquots of suspensions (around 10 L) from each well were transferred using the pipette to YPD agar plates without inhibitors and incubated for 24 h at 37 °C.

Cytotoxicity Assay

Hs27 and HUVEC mammalian cell lines were cultured as mentioned above. Once cell lines were about 80% confluent, cells were counted by a hemacytometer (Hausser Scientific, Horsham, PA, USA) and plated in 96-well microtiter plates at concentrations of 4500 cells per well for Hs27 and 2000 cells per well for HUVEC. Cells were allowed to attach overnight. Drugs were serial dissolved (100–1.5 M) in cell culture medium and added to wells in 100 L, in triplicates. The final concentration of DMSO was ensured to be around 1% in all experiments. Cells were incubated with studied compounds for 72 h at 37 °C and 5% CO_2. After incubation, 20 L of MTT solution in PBS (4 mg/mL) was added to all wells and incubated further for 3 h at 37 °C. Formed formazan crystals were dissolved in 150 L DMSO, and absorbance was measured using an Asys UVM340 multi-well plate reader at λ = 540 nm. Cytotoxicity was determined compared to drug-free control. All experiments were performed in biological triplicates.

Membrane Permeabilization Assay Using Propidium Iodide Staining

Suspensions of *C. albicans* SC5314 were prepared by taking one loop of pure culture growth from 24 h YPD agar plates into RPMI 1640 medium and adjusting optical density to 0.1 at 660 nm wavelength. Then, FLCpOH-TP10-NH_2 and FLCpOH-TP10-7-NH_2 were added at concentrations corresponding to 8 x MIC and 4 x MIC. FLC (8 x MIC) was used as a negative control. The cell suspensions were then treated for 0.5, 2, 4, 6, and 24 h at 37 °C with continuous agitation (180 rpm). The cells were then centrifuged and resuspended in 200 L of PBS buffer (pH 7.4). Subsequently, cells were treated with propidium iodide (1 mg/mL, final concentration) and incubated for 30 min at room temperature in the dark. Cellular fluorescence was visualized using lens 20, using Olympus Fluorescence Microscope BX60 (Olympus, Tokyo, Japan). The images were also post-processed utilizing cell Sens program. The fluorescence intensity emitted by DNA-bound propidium iodide was measured using a flow cytometer (Merck Millipore guava easyCyte 8, Darmstadt, Germany). Data were obtained from at least two independent experiments.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy of yeast was performed at a low accelerating voltage of the primary beam with or without the coating of the samples. *C. albicans* fungi suspension fixed with 2.5% glutaraldehyde was applied onto a silicon chip and allowed to adhere for 30 min. The samples were dehydrated in a series of methanol solutions (25–50–75–100–100%) in one-hour steps at 4 °C. Samples underwent critical point drying with methanol exchanged for liquid CO_2 in an automatized approach, (CPD300 AUTO, Leica Microsystems, Vienna, Austria) and were imaged with cross-beam scanning electron microscope equipped with Schottky field-emission cathode (Auriga 60, Carl Zeiss, Oberkochen, Germany) at 0.8 kV accelerating voltage; thus, the imaging was performed within a mode referred to as low-voltage, field-emission scanning electron microscopy (LV-FESEM) of non-labeled, critical point-dried sample. This process was implemented with the low-voltage field-emission scanning electron microscopy (LV-FESEM) mode and by applying the low-energy loss electron principle for generating the highly resolved chemical contrast. Images were acquired with the Everhart–Thornley electron detector (SE2 secondary electrons) and correlated with the energy-selective back-scattered electron detector (EsB), directly from the sample surfaces, with no coating or contrasting applied; thus, chemical cell-endogenous components were directly mapped. Polished silicon crystal used as a substrate for deposition of *C. albicans* allowed for chemical mapping of the sample's features and for identifying of the disintegrated pathogen cells in a correlative mode, with topography, contours and chemical aspects analyzed with pixel-to-pixel precision of correlation.

References

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