

Influence of New *N*-Methylquinolinyl Porphyrins on Different *Candida albicans* Development Forms

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The activity of new N-methylquinolinyl porphyrins against different morphological forms of Candida albicans (unattached, free-dividing cells, as well as structures formed in the process of biofilm maturation) was characterized. Modification of porphyrin molecules by N-methyl-6-quinolinyl increases the fungicidal effect on the yeast cells, in contrast to N-methyl-7-quinolinyl derivatives that are effective against hyphal structures. Candida albicans biofilm cells are more resistant to low concentrations of the studied compounds however the significant inhibition of the microbial community is in the presence of 1 μM.

Keywords: Quinolinyl porphyrins, *Candida albicans*, biofilm, hyphae, antifungal activity.

Влияние новых *N*-метилхинолинилпорфиринов на различные формы развития *Candida albicans*

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Для новых N-метилхинолинилпорфиринов проведено определение антифунгальной активности в отношении разных морфологических форм Candida albicans – неприкрепленных, свободно делящихся клеток, а также структур, образующихся в процессе созревания биопленки. Модификация молекул порфиринов N-метил-6-хинолинилом способствовала повышению фунгицидного влияния на дрожжеподобные клетки, в отличие от N-метил-7-хинолинилпроизводных, эффективных в отношении гифальных структур. Клетки Candida albicans при формировании биопленки приобретали резистентность к низким концентрациям исследуемых соединений, однако, в присутствии 1 мкМ наблюдалось существенное ингибирование развития микробного сообщества.

Ключевые слова: Хинолинилпорфирины, *Candida albicans*, биопленка, гифы, противогрибковая активность.

Introduction

The excessive and often groundless antibiotic use limits their effectiveness because of the very rapid microbial resistance development.^[1] Therefore, the search and development of new treatments of infections, including those pathogens that are characterized by increasing resistance, is important direction of microbiological and biotechnological research.

Photodynamic therapy (PDT) is a real alternative that is effective on bacteria (including resistant strains), fungi, viruses and protozoa.^[2] The basis of this method is photosensitizers, including porphyrins – heterocyclic macrocycles derived from four pyrrole-like subunits interconnected by their α -carbon atoms *via* methine bridges.^[3] These molecules are characterized by the ability to absorb certain wavelength light and generate reactive oxygen species, toxic to target cells.^[4] Also porphyrins have not only photodynamic but dark activities that allow microbial growth inhibiting in the absence of pre-activated light exposure.^[3] Among the porphyrin compounds quinoliny derivatives have a number of advantages. First of all, there is a large amount of quinoliny-containing substances with antimicrobial properties. Therefore, the introduction of this fragment into porphyrin molecules leads to an expansion of the activity spectrum as compared to other substituted and unsubstituted tetrapyrroles.^[2] In addition, the quinoliny substituent provides water solubility due to quaternization of the *N*-atom, which greatly facilitates the study of the biological activity of such compounds.^[5]

Recently, antimicrobial PDT does great progress towards the treatment of the oral cavity infections, the infected wound healing and acne treatment.^[6] Thus, the first product Periowave™ Ondine, based on a porphyrin photosensitizer, appeared in 2005, and is widely applied to dental oral pathogen treat.^[7] Another photosensitizer XF-73 that has been clinically tested started to apply for the *Staphylococcus aureus* antibiotic-resistant strain destruction.^[8]

Candida albicans are opportunistic microorganisms that usually colonizes the human epithelial integuments.^[9] A weakened immune system, host microbiota disruption or serious co-infection may contribute to the candidiasis development during which microorganisms form biofilm on large damaged tissues and organ surfaces.^[10] In this existence form *C. albicans* is more resistant to antifungal substances. There have been several reports on the use of photosensitizers and light to kill this fungus species.^[11,12] However, there has been much less systematic study on the types of physicochemical properties necessary in a photosensitizer in order to make it effective in mediating to kill such microorganisms.^[13]

The aim was to determine the sensitivity of different morphological forms of *Candida albicans* to a number of synthetic quinoliny-porphyrins.

Experimental

The effects of *cis*-[5,10-di(*n*-propyl)-15,20-di(*N*-methyl-7-quinoliny)]; 5,10-di(*n*-propyl)-15,20-di(*N*-methyl-6-quinoliny)]- and *trans*-[5,15-di(*n*-propyl)-10,20-di(*N*-methyl-7-quinoliny)]; 5,15-di(*n*-propyl)-10,20-di(*N*-methyl-6-quinoliny)]-porphyrin tosylates were studied.

Synthesis

Synthesis of compounds was carried out by mixed aldehyde condensation of mixture quinoline-6-carbaldehyde (or its isomer quinoline-7-carbaldehyde) and *n*-butyraldehyde with pyrrole.^[5] The NMR spectra were detected on a spectrophotometer DPX-300 (Brucker) with an operating frequency 300.13 MHz (internal standard – TMS, solvent – CDCl₃), FAB mass spectra – on the VC 7070 EQ device. The preliminary sterilization of the studied compound stock solutions in DMSO was carried out by autoclaving at 0.5 atm.

Microorganisms

Candida albicans ATCC 18804 was used as a test strain obtained from the Microbial Culture Collection of Microbiology, Virology and Biotechnology Department (I.I. Mechnikov Odessa National University). The previous preparation and cultivation of the strain was carried out using Sabouraud Dextrose Agar by the standard method according to ^[14,15]. To obtain the microbial suspension the cells were washed with sterile saline and standardized to 0.5 units McFarland standard.

Porphyrin Inactivation of *Candida albicans*

There were used not only liquid Sabouraud Dextrose medium but Spider nutrient one that contained mannitol for the morphogenesis change stimulation and the specific *C. albicans* cell form generation, in particular hyphae.^[16] The 0.05 ml of microorganism suspension was put in sterile 48-well plates with 1.0 ml one of the liquid medium variant (Sabouraud Dextrose or Spider media) and the studied porphyrin solutions. The final concentration of compounds accounted for 0.01, 0.1 or 1 μ M.

The microorganism cultivation was implemented during 24-72 hrs at 37 °C after which the nutrient fluid containing a «suspension» *C. albicans* culture were carefully selected out of wells. The cell number determination in liquid medium was carried out using μ Quant BioTek spectrophotometer at 540 nm and converting the absorbance values in CFU/ml (colony formation units/ml) about the appropriate standard curve.

Porphyrin Influence on Fungal Biofilm

The saline washed wells were filled with 1.0 ml of 96 % ethanol for *C. albicans* biofilm fixation. Ethanol was picked out in 10 min and plates were left to dry in air. For biofilm cell staining 0.1 % crystal violet aqueous solution was put in the wells and left for 10 min. Then dye solution removed, the plates were washed with distilled water and dried in air.^[17] The biofilm visualization was carried out by means of light microscopy, photos were made using Webcam «Led HD Trust Fuli1 1080p» camera.

For quantitative analysis of biofilm formation intensity the dye, that has been absorbed by the *C. albicans* cells, was extracted and the optical density of the obtained solution was determined. Aqueous solution of dodecyl sulfate and sodium hydroxide was used for cell lysis. This procedure was carried out for 60 min. The results were determined by means of μ Quant BioTek spectrophotometer at 592 nm.^[18]

The *C. albicans* cells that have been grown in similar conditions without studied compound adding was used as the culture control. To obtain reliable results in all experiments 5 repeats were estimated. The comparative analysis of the data was carried out by Student's *t*-criteria; a reliable difference of values was at $p \leq 0.05$.

Results and Discussion

The studied substance solutions had spectra in which the position of the absorption bands and their relative inten-

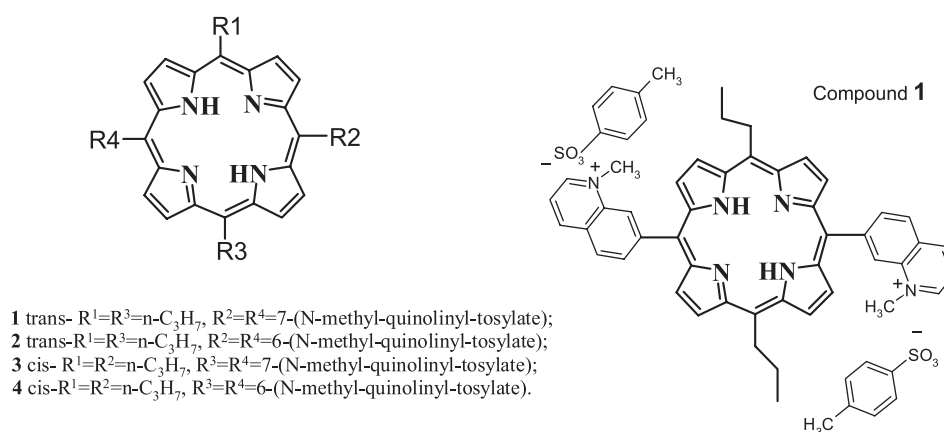


Figure 1. The studied synthetic quinolinyl-porphyrin structures.

sities are identical with the published data.^[5] In the presence of culture medium components the spectra have not changed, indicating about the interaction absence of culture medium substances with the studied porphyrins.

It was detected that some of the studied derivatives (Figure 1) significantly influenced on the biomass growth intensity during exploring the synthesized porphyrin impact on *C. albicans* suspension growth.

Firstly, it was observed that the presence of *N*-methyl-6-quinolinyl in the porphyrin molecules increased the level of antifungal activity in comparing with *N*-methyl-7-quinolinyl substituted ones (Table 1). Almost for all variants of the compounds **2** and **4** (in Sabouraud medium) the *C. albicans* inhibition level was on 15 % to 50 % higher than for the compounds **1** and **3**, respectively.

The antimycotic activity of quinolinyl-porphyrins was not directly dependent on the compound concentration. For the greater number of them the most effective of the stud-

ied concentration range proved to 1 μM. For example, 1 μM of the *cis*-isomer of *N*-methyl-7-quinolinyl-compound induced the *C. albicans* «suspension» cell number increasing in Sabouraud medium that is not exceeding 30 % compared with the control, while other corresponding values – from 35 % to 61 %.

The spatial features of the studied porphyrin structures induced time-dependent differences of their antimicrobial activity. In the case of the *trans*-isomer adding in Sabouraud medium the *C. albicans* biomass inhibition during 24–72 hrs were becoming more expressed. However, for *cis*-isomers the constant antifungal effects were observed throughout the whole incubation period.

For *C. albicans* cultured in the Spider nutrient medium spatial compound configuration also led to differences in the antifungal activity, but inverse (Table 2). The *trans*-isomers showed a permanent activity against test-microorganism, which over time practically was not chang-

Table 1. The studied quinolinyl-porphyrins influence on *C. albicans* suspension cell number in the Sabouraud nutrient medium ($\cdot 10^9$ CFU/ml).

Compound	Concentration, μM			
	0	0.01	0.1	1
24 hrs				
1		21.0	19.6	40.6
2	37.5	28.9	29.0	21.8
3		13.3	13.1	8.6
4		14.1	8.9	11.8
48 hrs				
1		67.4	39.5	11.6
2	44.9	16.5	28.7	7.8
3		25.9	18.1	12.3
4		17.9	24.2	15.8
72 hrs				
1		33.0	18.4	14.1
2	65.6	10.7	15.8	21.8
3		39.8	48.7	20.8
4		15.0	21.1	15.8

Table 2. The studied quinolinyl-porphyrins influence on *C. albicans* suspension cell number in the Spider nutrient medium ($\cdot 10^9$ CFU/ml).

Compound	Concentration, μM			
	0	0.01	0.1	1
24 hrs				
1		19.4	10.7	23.9
2	35.4	21.3	21.6	12.1
3		15.4	18.0	17.4
4		17.9	15.0	19.9
48 hrs				
1		21.8	18.5	18.2
2	54.0	0.0	14.8	24.5
3		5.1	5.4	20.4
4		0.0	34.4	16.3
72 hrs				
1		34.3	19.4	17.6
2	68.7	23.5	31.2	11.1
3		10.0	24.6	28.7
4		126.8	0.0	19.1

ing. But the impact of *cis*-porphyrin derivatives increased on the third day compared with the cultivation beginning.

Unlike the previous cultivation variants there is the *N*-methyl-7-quinoliny-compounds characterized by a higher inhibition activity than *N*-methyl-6-quinoliny-porphyrins in the Spider medium. The *C. albicans* cells that were growing in this medium have not found a well-defined dependence on the compound content. But it was detected that both *N*-methyl-6-quinoliny-porphyrin isomers were most effective at a concentration of 1 μM , whereas the *N*-methyl-

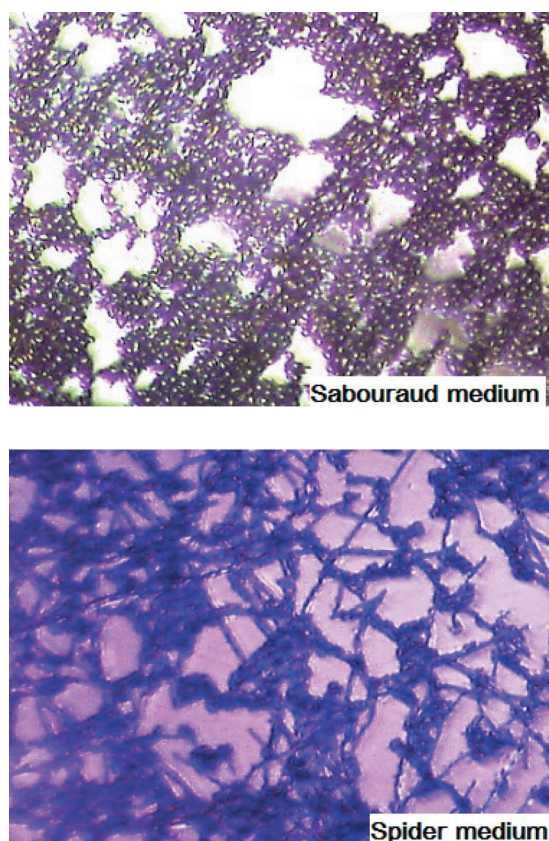


Figure 2. The characteristics of *C. albicans* biofilm (0.1 % crystal violet cell staining, photos by Webcam «Led HD Trust Fuli1 1080p» camera, 8.3 MP (3840×2160)).

7-quinoliny-porphyrins caused a significant inhibition effect in smaller concentrations.

A distinctive characteristic of *C. albicans* biofilm is a set of morphological forms. *In vivo* *C. albicans* is polymorphic microorganism that can exist as either true yeasts with oval cells or as unshared hyphae and elongated cells with constrictions, so-called pseudohyphae.^[18] It is detected that *C. albicans* hyphae have greater invasive potential than the yeast forms. On the other hand the smaller sized cells are characterized by the easier spread in human organism than fungal hyphae.^[19]

During microorganism cultivation in the Sabouraud and Spider media there were determined differences between *C. albicans* morphological forms that have produced a biofilm on the polystyrene plate well surfaces (Figure 2). Thus, in the first medium the most cells had an oval shape, were actively dividing and eventually generated a continuous biofilm layer. In the case of *C. albicans* growth in Spider medium cells elongated, were practically not separating from each other during the division that resulted to the formation of pseudohyphae filaments.

Similarly to the study *C. albicans* suspension culture the used quinoliny-derivative porphyrins had a great influence on the biofilm forming process (Figure 3). Generally, the test strain that consisted from the cells grown at the phase interface was less sensitive to the action of the compounds than their «suspension» analogues. In the case of Sabouraud medium the maximum reduction of cell biofilm number reached 40 % compared to 60 % in suspension culture.

Comparing the porphyrin concentration effectiveness it was detected that higher values, in particular 1 μM , are more active. But the dependence of *C. albicans* cell number inhibition on the cultivation period during the biofilm formation was not fixed.

As in the case with *C. albicans* suspension culture, *N*-methyl-6-quinoliny-porphyrins were characterized by more active antifungal effect on attached cells in comparison to *N*-methyl-7-quinoliny-derivatives.

Concerning to spatial characteristics of the studied compounds, the differences of impact between *cis*- and *trans*- isomers in biofilm formation in nutrient medium Sabouraud was not determined. A similar characteristic

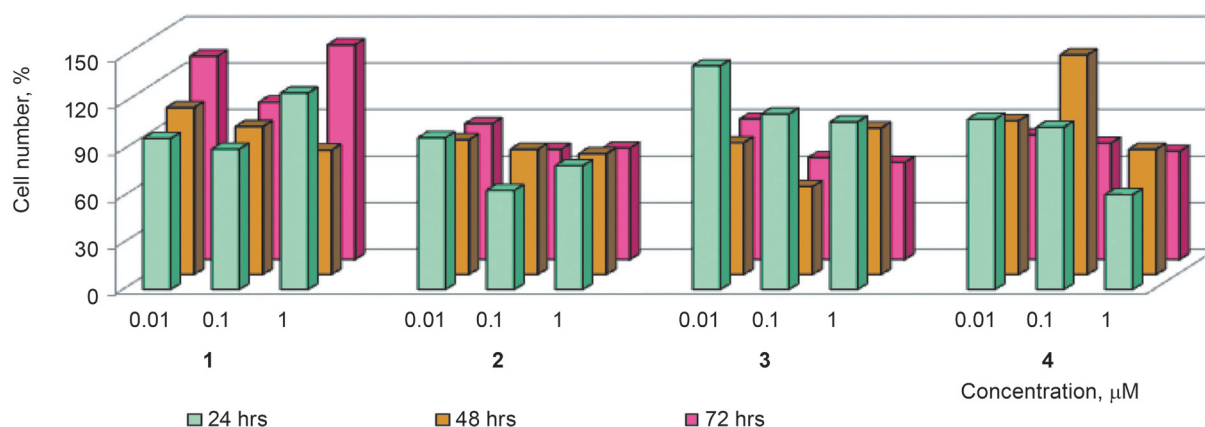


Figure 3. The influence of the *N*-methyl-quinoliny-porphyrins on *C. albicans* biofilm formation in Sabouraud medium in comparison with the control value (microorganism growth without of compound adding, 100 %).

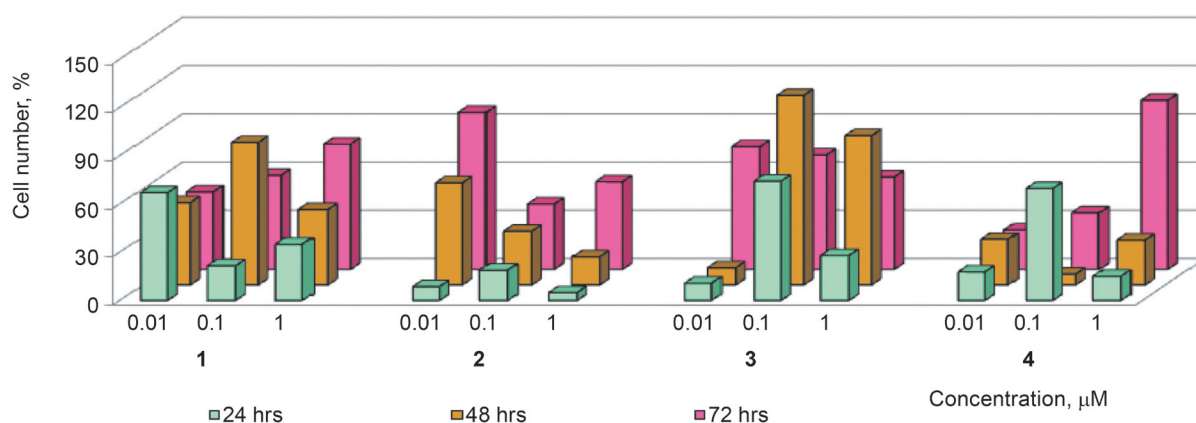


Figure 4. The influence of the *N*-methyl-quinolinyl-porphyrins on *C. albicans* biofilm formation in Spider medium in comparison with the control value (microorganism growth without of compound adding, 100 %).

was also noted for the development of *C. albicans* «surface» cells in Spider nutrient medium.

At the same time, generally, the hyphae and pseudohyphae that formed in the biofilm in Spider nutrient medium had a greater sensitivity to the compounds than *C. albicans* yeast forms in the case of Sabouraud cultivation (Figure 4).

At first, a high antifungal activity level of the smallest selected concentration – 0.01 μM indicates about this. For example, almost 2-fold attached cell number reduction was observed compared to controls for the whole cultivation period in the presence of 0.01 μM compound 1. Secondly, at certain stages the presence of 0.01 μM quinolinyl-derivatives significantly inhibited biofilm formation process that conformed only 10–64 % of the reference values.

Conclusions

It has been widely noted that *C. albicans*, like other yeasts, is slightly more difficult to kill by porphyrins than bacterial cells. This has been attributed to the presence of a nuclear membrane in the yeasts, the greater cell size and the reduced number of targets per unit volume of cell.^[17]

Results of experimental investigations have demonstrated conclusively that different *C. albicans* development forms can be effectively killed by the studied porphyrin action. But as the studies have shown, the cells developing in the thickness of the nutrient medium were more sensitive to the porphyrin effects. Apparently, this is because the main principle of the compound action is primarily associated with their impact on the microbial cell walls by changing the charge.^[14] A suspension *C. albicans* culture is more "available" targets for their influence.

In vivo, both in the environment and during the course of infection, organisms frequently exist in adherent, organized communities termed biofilms rather than as the independent entities usually seen during planktonic growth.^[15] Of particular importance clinically, biofilm populations are more resistant to antibiotic concentrations that are effective against the same population if the biofilm is dispersed.^[16]

In this work there was determined whether biofilm development rendered *C. albicans* more resistant to the studied porphyrin influence. Under the experimental conditions used, the sensitivity of *C. albicans* to the studied porphyrins did not diminish during the course of mature biofilm formation.

Growth conditions and cell morphology had a dramatic effect on the susceptibility of *C. albicans* to the studied porphyrins. *C. albicans* grown as a filamentous form in the Spider medium were much more sensitive than yeast-like forms grown in the Sabouraud nutrient medium.

These observations suggest that the cells require particular environmental conditions to take up porphyrins. One possible explanation is that the porphyrins are actively transported into the cell through an uptake mechanism that is induced by growth under more nutritionally restrictive conditions.^[6] Another possible mechanism is that changes in the composition of the cell wall occur under different physiological conditions that allow for more efficient passive entry of the compound into the cell.^[3] Since the studied porphyrins are fairly lipid-soluble molecules, the extent of cell wall hydrophobicity may be an important determinant of uptake in these assays. The hydrophobic properties of the cell wall have been shown to be strongly influenced by growth conditions and may contribute to the patterns we observed. The surface properties and composition of *C. albicans* are complex and dynamic, however, and relative hydrophobicity is one of many potential factors that may influence uptake.^[12]

So, therapy with porphyrins has the potential to evolve into a useful treatment for difficult to eradicate fungal infections of accessible regions of the body. For example, the prospect of eradicating oral thrush in an AIDS patient, or denture stomatitis in an elderly nursing home resident in a single session, or a once-off curative treatment for *Candida* mycosis, is a scenario that would be attractive to both patients and health service providers.^[17,20] However, this will not become a clinical reality until pharmaceutical companies and grant-awarding bodies devote considerable resources to the development of both photosensitisers specifically designed for antifungal treatment and drug delivery systems that allow such agents to be efficiently delivered to their sites of action.

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