KnE Energy & Physics

PhysBioSymp17
The 2nd International Symposium on
Physics, Engineering and Technologies for Biomedicine
Volume 2018

Knowledge E
Engaging minds

Conference Paper

Potential of Antifungal Drugs as Photosensitizers

Mikulich A.V.[1], Tretyakova A.I.[1], Knukshto V.N.[1], Plavskaya L.G.[1], Leusenka I.A.[1], Ananich T.S.[1], Plavskii V.Yu.[1], and Ulaschik V.S.[2]

[1]B.I. Stepanov Institute of Physics of NASB, 68-2 Nezavisimosti Ave., Minsk, Belarus
[2]Institute of Physiology of NASB, 28 Akademichnaya Str., Minsk, Belarus

Abstract

In the present work, using commercially available formulation of polyene antifungal antibiotic amphotericin B, it is shown that its antifungal activity could be enhanced in combination with optical radiation corresponding to the absorption band of amphotericin B due to photodynamic effect. It is assumed that the radical processes dominate in the mechanism of photodynamic action in the presence of amphotericin B.

Keywords: Amphotericin B, antimicrobial/antifungal photodynamic therapy (aPDT), lactate dehydrogenase (LDH), singlet oxygen, laser, LEDs

Corresponding Author:
Mikulich A.V.
a.mikulich@ifanbel.bas-net.by

Received: 17 January 2018
Accepted: 25 March 2018
Published: 17 April 2018

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the PhysBioSymp17 Conference Committee.

1. Introduction

Microbial infections along with cardiovascular and cancer diseases continue to be the most challenging problems for healthcare professionals [1, 2]. In the recent time, the incidence of fungal infections is increasing in a growing manner. Mostly, this is due to the increase in the number the immunocompromised patients (e.g. cancer, HIV/AIDS). Besides, the intensive usage of numerous chemotherapeutic drugs to treat fungal infections has led to the appearance of new resistant forms of fungi. In this connection, new therapeutic approaches are developing continuously to overcome this problem. One of them is antimicrobial/antifungal photodynamic therapy which utilizes the combination of special drug (photosensitizer), light and molecular oxygen to eliminate the pathogenic species. It has been demonstrated in vitro and in vivo that aPDT could be very effective against a wide range of pathogens – bacteria, viruses and fungi [3 – 8]. However, one of the limiting factors here is the almost total absence of clinically approved antimicrobial/antifungal photosensitizers. The most encouraging way to solve this problem could be the application already approved for clinical use antimicrobial/antifungal chemotherapeutic drugs as photosensitizers.

In this paper, using commercially available formulation of polyene antifungal antibiotic amphotericin B, we demonstrate in molecular, cellular and animal models that in

the presence of optical radiation corresponding to the absorption spectrum of antibiotic it can be used as efficient photosensitizer and its antifungal efficiency can be significantly enhanced.

2. Materials and methods

2.1. Chemicals

Commercially available formulation of polyene antifungal antibiotic amphotericin B (Fungizone) was used throughout the experiments. Lactate dehydrogenase (LDH) from bovine heart, nicotinamide adenine dinucleotide reduced (NADH), sodium pyruvate, sodium azide (NaN_3), nicotinamide adenine dinucleotide (NAD^+), L-cystine, 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT); solvents - deuterium oxide (D_2O), 2-propanol-d_8 were purchased from Sigma-Aldrich (Germany).

2.2. Objects and assays

2.2.1. Enzymatic assay

LDH activity was monitored spectrophotometrically by measuring NADH oxidation (decrease in NADH absorbance at 340 nm) in the presence of pyruvate, drug and laser radiation with λ = 405 nm (fluence rate - 70 mW/cm^2). Initial concentrations of reagents were 1.5×10^{-4} M NADH, 1.9×10^{-4} M pyruvate, 3×10^{-9} M LDH in 0.05 M PBS pH 7.3. The reagents have been chosen in such a way that absorbance at 340 nm was linear function of time during first two minutes of reaction. The reaction was started by the addition of non-irradiated (control) or irradiated (experiment) in the presence of amphotericin B solution of LDH to spectrophotometry cuvette (l = 10 mm) with binary mixture of NADH-pyruvate.

To evaluate photosensitizing effect of the drug, the following formula was used:

$$\gamma = (\Delta D_s/\Delta D_k)\cdot 100\%, \quad (1)$$

where ΔD_s and ΔD_k - variation of absorbance at λ = 340 nm during the first minute for experiment and control reactions, respectively.

2.2.2. Cell culture, photosensitization and survival analysis

Buffalo green monkey kidney (BGM) cells were obtained from the Republican Research and Practical Center for Epidemiology and Microbiology (Minsk, Belarus). Cells have grown as adherent monolayers in Petri dishes (Greiner Bio-One, USA) in Dulbecco's modified eagle medium at 37°C in a humidified atmosphere with 5% CO_2.

Afterwards the cells were incubated for 2 hours with amphotericin B. Then the Petri dishes with cells were irradiated by LEDs in a bottom-up configuration with emission peak at λ = 395 nm. At the bottom of Petri dishes the fluence rate was 20 mW/cm^2. Immediately after the irradiation the cells placed back into the incubator for 24 h.

To determine the surviving cells after photosensitization with amphotericin B colorimetric method based on the 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) reduction to formazan was used [9]. Mitochondrial dehydrogenases only viable cells are capable of reducing MTT to formazan. The survival cell index was calculated using spectrophotometric method (absorbance at 570 nm).

2.2.3. Evaluation of photosensitizing effect *in vivo*

40 adult Wistar rats weighing approximately 200 to 300 g were used in study. The rats were maintained in standard day-night cycle conditions. The rats had access to water and food.

Contact dermatitis was induced by double topical application of 0.1 ml of 5-% ethanol solution of 2.4-dinitrochlorobenzene (DNCB) on depilated skin areas of rats. The first group (Group I) is intact animals. The second group (Group II) is untreated animals with contact dermatitis. The third group (Group III) received topical application amphotericin B on areas with dermatitis. Group IV was treated by optical radiation of LEDs with emission peak at λ = 405 nm (fluence rate – 100 mW/cm^2) in addition to topical application of amphotericin B.

Microscopic analysis of scrapings and their seeding on fluid thioglycollate medium and on solid media (Sabouraud, Czapek agars) to determine the presence of fungi was carried out.

2.2.4. Spectrophotometric analysis

Absorption spectra of amphotericin B were obtained using spectrophotometer Specord M40 UV/VIS (Carl Zeiss, Germany).

2.2.5. Quantum yield of singlet oxygen measurement

Quantum yield of singlet oxygen generation γ_Δ by amphotericin B was determined by a relative method using a phenalenone in 2-propanol-d_8, the latter being a standard [10] with γ_Δ = 0.97±0.03. For excitation of samples, LED source emitting at λ = 405 nm with output power of 500 mW was used.

3. Results and Discussion

The chemical structure of amphotericin B is shown in Figure 1.

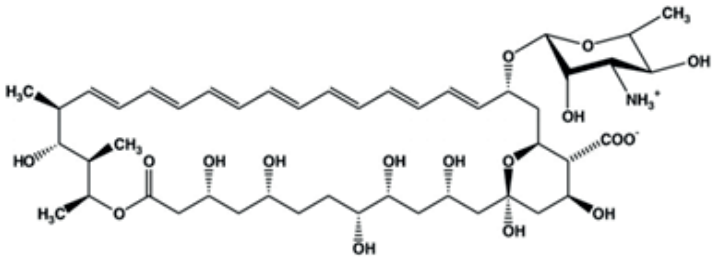


Figure 1: Chemical structure of amphotericin B.

Optical properties of amphotericin B are fully determined by its structure. Molecule of amphotericin B is a macrolactone ring – the chromophore with a system of seven conjugated double bonds and hydrophilic subunit containing seven hydroxyl groups [11]. The presence of hydrophobic and hydrophilic molecular groups in the amphotericin B explains its poor solubility in apolar and highly polar solvents, for example in water [12].

Figure 2 shows the absorption spectra of concentrated and diluted aqueous solutions of amphotericin B at pH 6.0.

As follows from the Figure 2, curve 1, the concentrated solution of amphotericin B exhibits the presence of mixture of forms: the maximum in absorption spectrum of the drug at λ_{max} = 408 nm corresponds to the monomers while intense peak at λ_{max} = 324 nm is caused by dimers. The results obtained (Figure 2, curve 2) show that strong dilution of amphotericin B (0.06 μM) promotes monomerization of the drug. The confirmation of that is the disappearance of absorption band at λ_{max} = 324 nm. It should be noted that due to very low value of optical density at such dilution absorption spectra were registered in spectrophotometric cuvettes with optical path of 50 mm.

As mentioned earlier, for assessment of the photosensitizing activity of amphotericin B the key enzyme of glycolysis – lactate dehydrogenase - was used. The choice of enzymes as a model is explained by that fact that the enzymes are characterized

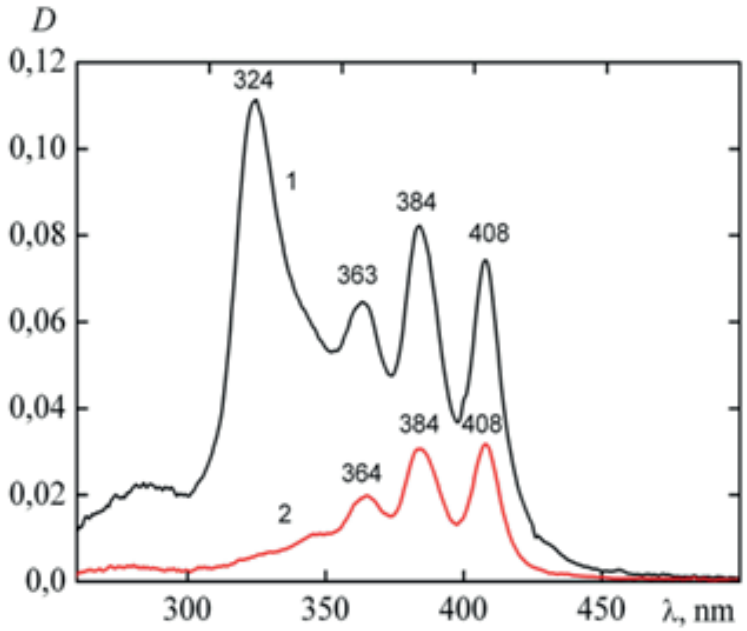


Figure 2: Absorption spectra of concentrated (C = 2 μM) (1) and diluted (C = 0.06 μM) (2) aqueous solutions (pH 6.0) of amphotericin B: 1 – cuvette, l = 10 mm; 2 – cuvette, l = 50 mm.

by the largest, among other molecules, quantum yield of photodamage. Lactate dehydrogenase catalyzes the reversible reaction: L-lactate + NAD^+ in equilibrium pyruvate + NADH. This reaction is one of the main metabolism pathways and key reaction in the cycle of glycolysis – splitting glucose to low molecular weight substrates. It should be noted thaPersonNamet glycolysis system of the cells works on so called «conveyor» principle consisting of set of interrelated biochemical reactions occurring in the presence of natural catalysts – enzymes. The disruption of one of the catalysts leads to disruption in work of the entire system and, as a consequence, to cellular death.

Enzymatic activity of LDH upon exposure to optical radiation with λ_{max} = 405 nm in the presence of amphotericin B is shown in Table 1.

Table 1: Enzymatic activity of LDH (in % to the control) upon exposure to laser radiation with λ = 405 nm (70 mW/cm^2) in the presence of amphotericin B.

Irradiation time, min	W/o additives	10^{-2} M NaN_3	4×10^{-5} M NAD^+	10^{-4} M L-cystine	D_2O
0	100 %	100 %	100 %	100 %	100 %
5	55%	97%	70%	89%	98%

As follows from Table 1, after exposure of LDH solutions to laser radiation with λ = 405 nm for 5 minutes in the presence of amphotericin B enzymatic activity is decreased significantly. We assume that the mechanism of such photoinactivation is

determined mainly by radical processes. This is supported by a pronounced decrease in the effect of photoinactivation of enzyme when adding cystine or NAD^+ to mixture under irradiation. It is known that cystine and NAD^+ have a high affinity to the electron and therefore inhibit reactions related to the solvated electrons such as formation of superoxide and hydroxyl radicals upon their (electrons) interaction with molecular oxygen. Besides, in the case of the predominant participation of singlet oxygen in biochemical reactions one would expect a sharp increase in LDH photoinactivation with H_2O replaced by D_2O due to increase of the lifetime of singlet oxygen by at least an order of magnitude. The anomalous deuteration effect of solvent on quantum yield of antibiotic fluorescence also points out to the prevalence of radical processes sensitized by amphotericin B in aqueous media. Practically full quenching of the antibiotic fluorescence registered in channel $S_1 \rightarrow S_0$ at excitation of amphotericin B in channel $S_0 \rightarrow S_2$ upon change of H_2O to D_2O is observed.

In this respect, the observed decrease in the effect of LDH photoinactivation sensitized by amphotericin B upon irradiation of solutions in the presence of sodium azide can be also explained by quenching of excited states of antibiotic by sodium azide.

Another evidence that the radical processes contribute mainly to the mechanism of photosensitizing action of antibiotic is a higher value of quantum yield of enzyme photoinactivation in thepresence of amphotericin B compared to the quantum yield of singlet oxygen generation. It was found that quantum yield of singlet oxygen generation by amphotericin B upon excitation to optical radiation with λ = 405 nm in 2-propanol-d_8 – γ_Δ = 0.04±0.008.

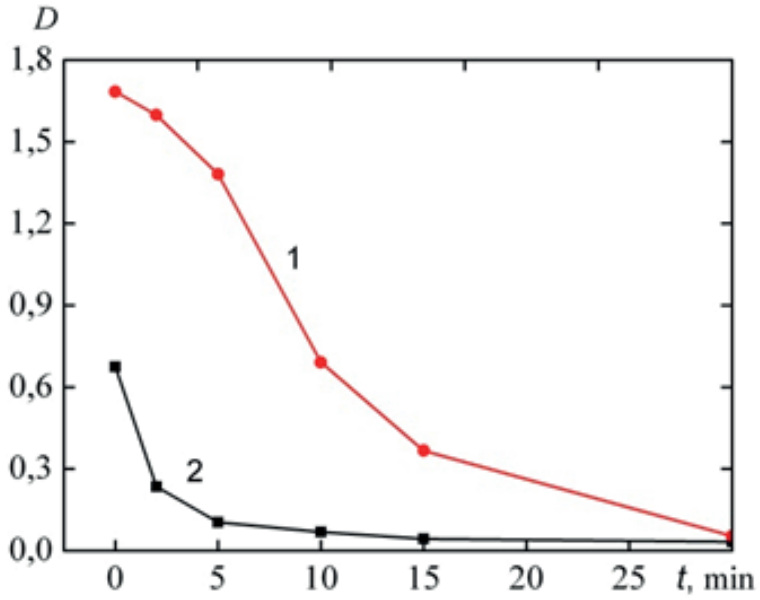
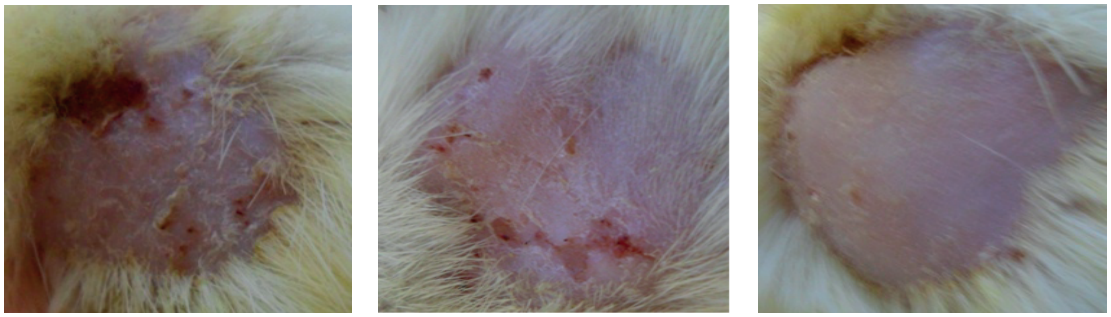


Figure 3: Cellular survival assessed by MTT – test upon irradiation of cellular monolayer in absence (1) and presence (2) of amphotericin B (λ = 395 nm, P = 20 mW/cm^2).

Potential of amphotericin B to act as photosensitizer has been also demonstrated in BGM cell line (Figure 3).

Figure 3 shows that preincubation of cells with amphotericin B followed by irradiation with λ = 395 nm produced a significant level of cytotoxicity as indicated by MTT-test. As follows from Figure 3, cell survival is also influenced by the dose of acting radiation and decreases upon increase of aforementioned parameter.

The ability of optical radiation (LEDs with emission peak at λ = 405 nm, fluence rate - P = 100 mW/cm^2) corresponding to the absorption band of amphotericin B to enhance its fungicidal action is demonstrated when modelling contact dermatitis on depilated areas of the skin of rats. After 3 procedures of combined action of amphotericin B and optical radiation (irradiation time – t = 5 min) the skin of the rats turned back to its normal state. Area of skin inflammation of experimental model of dermatitis before (a), after treatment with amphotericin B alone (b) and after photodynamic therapy with amphotericin B (c) and mycology in epidermal microflora of rats are shown in Figure 4 and Table 2, respectively.



a b c

Figure 4: Area of skin inflammation of experimental model of dermatitis before (a), after treatment with amphotericin B alone (b) and after photodynamic therapy with amphotericin B (c).

Table 2: Mycology in epidermal microflora of rats with dermatitis in the presence of amphotericin B and optical radiation.

Microorganism, CFU/cm^2	Group I (intact animals)	Group II (untreated animals)	Group III (amphotericin B)	Group IV (amphotericin B+optical radiation)
Candida albicans	Single colonies	250	100	50
Penicillium spp.	-	10^4	-	-
Rhizopus spp.	-	-	-	-

Mycological analysis of the skin of intact animals (Group I) did not show the presence of dermatophytes, fungi Candida albicans were detected as single colonies. In a group of animals with contact dermatitis (Group II) the appearance of fungi with hemolytic

activity Penicillium spp. with 10^4 CFU/cm^2 and Candida albicans with 250 CFU/cm^2 was detected on the third day. The application of amphotericin B alone decreased the number of colonies two-fold – only Candida albicans with of 100 CFU/cm^2 were detected (Group III). The highest activity against fungi was registered for combined action amphotericin B and optical radiation. As in the previous case, only fungi Candida albicans with of 50 CFU/cm^2 were detected (Group IV).

4. Conclusions

The study showed that amphotericin B can be used as photosensitizer and its fungicidal action can be significantly enhanced in combination with optical radiation corresponding to the absorption band of the drug.

References

[1] Nii-Trebi NI, "Emerging and Neglected Infectious Diseases: Insights, Advances, and Challenges," BioMed Research International, vol. 2017, Article ID 5245021, 15 pages, 2017.

[2] C. Lee Ventola, "The Antibiotic Resistance Crisis. Part 1: Causes and Threats," Pharmacology&Therapeutics, vol. 40, no. 4, pp. 277 - 283, 2015.

[3] Ludmila M. Baltazar, Anjana Ray, Daniel A. Santos, Patrícia S. Cisalpino, Adam J. Friedman and Joshua D. Nosanchuk, "Antimicrobial photodynamic therapy: an effective alternative approach to control fungal infections," Frontiers in Microbiology, vol. 6, Article 202, 11 pages, 2015.

[4] T. Dai, H. Ying-Ying, and M.R. Hamblin, "Photodynamic therapy for localized infections - state of the art," Photodiagnosis and Photodynamic Therapy, vol. 6, no. 3-4, pp. 170 - 188, 2009.

[5] G. Jori, C. Fabris, M. Soncin, S. Ferro, O. Coppellotti, D. Dei, L. Fantetti, G. Chiti, and G. Roncucci, "Photodynamic therapy in the treatment of microbial infections: basic principles and perspective applications," Lasers in Surgery and Medicine, vol. 38, no. 5, pp. 468 – 481, 2006.

[6] T. Maisch, "Anti-microbial photodynamic therapy: useful in the future?," Lasers in Medical Science, vol. 22, no. 2, pp. 83 – 91, 2007.

[7] M. Bartolomeu, S. Rocha, Â. Cunha, M. G. P. M. S. Neves, Maria A. F. Faustino and A. Almeida, "Effect of Photodynamic Therapy on the Virulence Factors of Staphylococcus aureus, " Frontiers in Microbiology, vol. 7, Article 267, 11 pages, 2016.

[8] "Photodynamic Inactivation of Microbial Pathogens: Medical and Environmental Applications (Comprehensive Series in Photochemical & Photobiological Sciences)," Edited by Michael R Hamblin, and Giulio Jori, 434 p., 2011.

[9] Tim Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," Journal of Immunological Methods, vol. 65, no. 1–2, pp. 55–63, 1983.

[10] David García Fresnadillo, and Sylvie Lacombe, "Reference Photosensitizers for the Production of Singlet Oxygen," in a Book "Singlet Oxygen: Applications in Biosciences and Nanosciences," vol. 1, p. 105 - 143, 2016.

[11] Katarzyna N. Jarzembska, Daniel Kamiński, Anna A. Hoser, Maura Malińska, Bogusław Senczyna, Krzysztof Woźniak, and Mariusz Gagoś, "Controlled crystallization, structure, and molecular properties of iodoacetylamphotericin B," Crystal Growth&Design, vol. 12, no. 5, pp. 2336–2345, 2012.

[12] D. M. Kaminski, "Recent progress in the study of the interactions of amphotericin B with cholesterol and ergosterol in lipid environments," European Biophysics Journal, vol. 43, pp. 453 – 467, 2014.