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EPR studies of *Cladosporium cladosporioides* complexes with amphotericin B

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Abstract. Free radicals in *Cladosporium cladosporioides* cultured with amphotericin B were spectroscopically studied. The effect of drug concentrations on free radicals in the fungal melanin biopolymer was examined. It was pointed out that the concentrations of free radicals in the complexes of *Cladosporium cladosporioides* mycelium with amphotericin B depend on the drug concentrations. Eumelanin dominates in the fungal studied samples. The slow spin-lattice relaxation processes exist in the tested samples. Free radicals take a part in the formation of *Cladosporium cladosporioides* mycelium complexes with amphotericin B.

Key words: amphotericin B • *Cladosporium cladosporioides* • electron paramagnetic resonance (EPR) spectroscopy • free radicals • melanin

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Introduction

Electron paramagnetic resonance (EPR) studies indicate that a high amount of *o*-semiquinone free radicals exist in melanins [8, 9, 12, 13, 15, 17–19, 21]. *o*-Semiquinone free radicals play an important role in melanin complexing by drugs [2, 7, 11, 16, 24]. The free radicals concentrations change after bounding drugs to melanin.

EPR spectroscopy was used earlier by us to examine different *Cladosporium cladosporioides* samples [4, 5, 10, 14, 26]. Pigmented soil fungi *Cladosporium cladosporioides* contain melanin and EPR lines of this polymer dominate in their spectra [5, 10, 15]. The amount and properties of free radicals in the fungal melanin depend on pH [14] and the contents of dia- and paramagnetic metal ions in the environment [4, 5, 10, 25]. The complexes of *Cladosporium cladosporioides* with flucytosine – the antifungal drug were tested by us [26]. In this work free radicals in *Cladosporium cladosporioides* mycelium with amphotericin B – the other typical antifungal drug were studied by EPR spectroscopy. The aim of this study is to determine the influence of the concentration of amphotericin B on *o*-semiquinone free radicals in *Cladosporium cladosporioides* mycelium. Complexes of *Cladosporium cladosporioides* with amphotericin B were not examined by the EPR method earlier.

Amphotericin B is the antifungal drug [1, 27], which is often used intravenously for systemic fungal infections (e.g., in critically ill, comorbidly infected or immunocompromised patients). Two amphotericins: amphotericin A and amphotericin B are known, but only B is used clini-

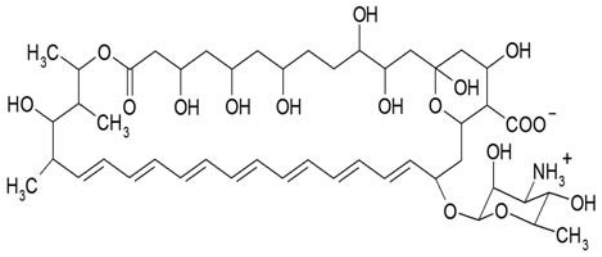

Fig. 1. Chemical structure of amphotericin B [27].

cally [27]. The chemical structure of amphotericin B is shown in Fig. 1 [27]. Amphotericin B is an amphoteric substance containing a primary amino group in the sugar moiety, mycosamine, and a carboxyl group attached to the macrolide ring. It is a heptaene macrolide containing seven conjugated double bonds [1].

Electron paramagnetic resonance spectroscopy was used in this study of the melanin complexes with amphotericin B, because our earlier examination of complexes of melanins with different drugs proved that these type of paramagnetic centres play an important role during formation of complexes [2, 6, 7, 11, 15, 16, 24]. The free radicals concentrations change during formation of melanin-drug complexes. Chemical spectrophotometrical analysis of melanin-drug complexes confirmed such effects [24]. The effect of amphotericin B on the parameters of the EPR spectra of melanin, microwave saturation of the spectra were tested.

Experimental details

The pigmented soil fungi *Cladosporium cladosporioides* complexes with amphotericin B were studied. *Cladosporium cladosporioides* pigmented soil fungi were cultured in the standard medium containing glucose (20 g), yeast extract (10 g), peptone (10 g) and bidistilled water (ad 1 dm^3). The media were adjusted to pH 7 by adding sodium hydroxide solution. Fungi cultures were incubated at a temperature of 26–28°C at ambient laboratory conditions. After 14 days the fungi were filtered washed with bidistilled water and dried to constant weight.

Binding of amphotericin B to mycelium *Cladosporium cladosporioides* was studied as follows: 5 mg of mycelium were placed in plastic test-tubes, where drug solutions were added to a final volume of 5 ml. Amphotericin B was dissolved in 5% glucose to the final concentrations 1.8, 4.5 and 8.1 μg/cm^3. Control samples contained 5 mg of mycelium *Cladosporium cladosporioides* and 5 ml of 5% glucose without drug. All samples were incubated for 90 min at room temperature. The suspensions were filtered after incubation and dried to constant weight.

The EPR spectra of the examined natural samples were compared to those for the model eumelanin – DOPA-melanin. DOPA-melanin was synthetized by the Binn's method [3].

The spectra were recorded by the use of EPR spectrometer produced by Radiopan (Poznań). Magnetic modulation was 100 kHz. Microwave frequency from X-band (9.3 GHz) was measured by MCM 101 recorder of Eprad (Poznań). The first-derivative EPR spectra were measured in the wide range of microwave power from 2.2 to 70 mW. Amplitudes (A), integral intensities (I) and linewidth (ΔB_{pp}) of the spectra were analyzed.

g-Factor was obtained from the resonance condition according to the formula [22, 23]

$$g = \frac{h\nu}{\mu_B B_r} \tag{1}$$

where: h – Planck constant, ν – microwave frequency, μ_B – Bohr magneton, B_r – resonance magnetic induction.

Free radical concentration (N) in the samples was determined as follow [23]

$$N = n_u \frac{I}{I_u} \frac{W_u A_u}{W A m} \tag{2}$$

where: n_u – number of paramagnetic centres in ultramarine (the reference), W, W_u – receiver gains for sample and ultramarine, A, A_u – amplitudes of ruby signal for the sample and ultramarine, I, I_u – integral intensities for the sample and ultramarine, m – mass of the sample. Free radicals concentration was measured at a low microwave power of 2.2 mW to avoid the effect of microwave saturation on the result. A ruby crystal was permanently placed in the resonance cavity and it was used as the secondary reference during measurements of the concentration.

Continuous microwave saturation of EPR lines was applied to the examination of spin-lattice relaxation processes [22, 23].

Results and discussion

o-Semiquinone free radicals were found in *Cladosporium cladosporioides* mycelium and the mycelium with amphotericin B. EPR spectra of the studied samples are shown in Figs. 2a–2d. Their shape was compared to this of DOPA-melanin spectrum (Fig. 2e). The spectra in Fig. 2 are measured with a low microwave power of 2.2 mW without microwave saturation. Similar shapes are observed for all the tested fungal EPR spectra (Figs. 2a–2d) and the EPR spectrum of the model eumelanin – DOPA-melanin (Fig. 2e). The lines of eumelanin (Fig. 2e) dominates in the EPR spectra of *Cladosporium cladosporioides* mycelium (Fig. 2a) and complexes with amphotericin B (Figs. 2b–2d).

The shapes of the fungi and the complexes with amphotericin B change with microwave power. The exemplary EPR spectra of *Cladosporium cladosporioides* complexes with amphotericin B recorded at different microwave powers are presented in Fig. 3. The component line of pheomelanin appears at the higher microwave powers, but its fraction in the total spectrum is very low. The free radicals of eumelanin are the main components of the paramagnetic centres system of the analyzed fungal samples. Similar results about the main type of melanin in *Cladosporium cladosporioides* were obtained by us earlier [4, 5, 10, 15]. Eumelanin also mainly exists in *Cladosporium cladosporioides* complexes with flucytosine [26].

The parameters of the EPR spectra of *Cladosporium cladosporioides* mycelium, the mycelium *Cladosporium cladosporioides* with amphotericin B, and the reference – DOPA-melanin are compared in Table 1. Linewidths

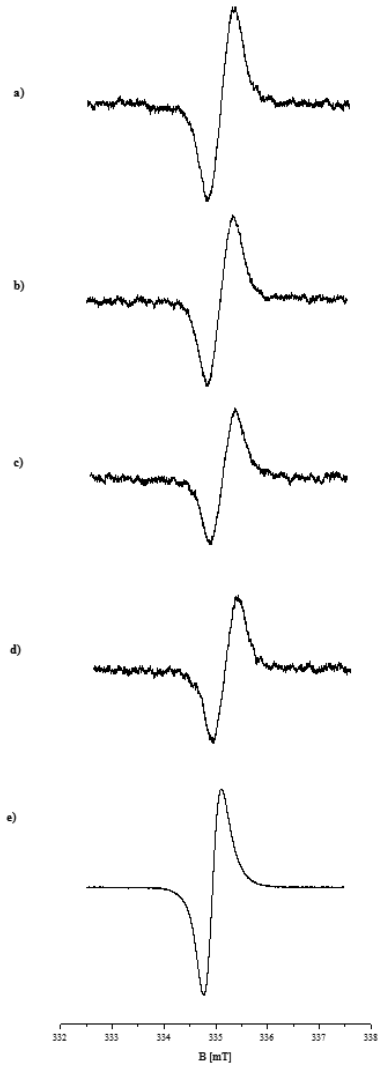

Fig. 2. EPR spectra of *Cladosporium cladosporioides* mycelium (a) and the mycelium with amphotericin B for concentrations of the drug: 1.8 μg/cm^3 (b), 4.5 μg/cm^3 (c) and 8.1 μg/cm^3 (d), respectively. EPR spectrum of DOPA-melanin (e). The microwave power was 2.2 mW.

(ΔB_{pp}) of the EPR spectra and *g*-factors are shown. The broad EPR lines with linewidths (ΔB_{pp}) in the range of 0.34–0.49 mT were measured for the studied samples (Table 1). *g*-Values in the range of 2.0039–2.0058 (Table 1) are typical of *o*-semiquinone free radicals [6, 20, 21]. Similar broad EPR lines were observed for *Cladosporium cladosporioides* complexes with flucytosine [26].

Free radicals concentrations (*N*) in the mentioned above samples are also presented in Table 1. The high free radicals concentrations ($\sim 10^{19}$ spin/g) characterize the tested samples. The concentrations in the *Cladosporium cladosporioides* mycelium and the mycelium with amphotericin B are lower than the concentrations in model DOPA-melanin (Table 1). The binding of amphotericin B by *Cladosporium cladosporioides* mycelium decreases free radicals concentrations in the samples (Table 1). This effect indicates that unpaired electrons of *o*-semiquinone free radicals are paired by amphotericin B molecules binding to *Cladosporium cladosporioides* mycelium.

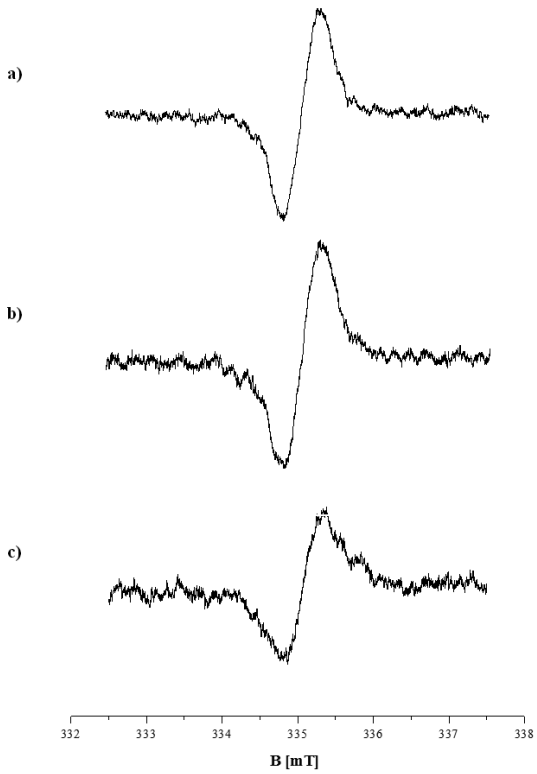

Fig. 3. EPR spectra of *Cladosporium cladosporioides* complexes with amphotericin B measured at 2.2 mW (a), 14 mW (b) and 70 mW (c). The concentration of amphotericin B is 8.1 μg/cm^3.

The amplitude dependence on the concentration of amphotericin B is not a proportional correlation, because of the complex structure of the tested samples and the number of site of binding of amphotericin in its chemical units. The binding of amphotericin B via free radicals to the tested melanin biopolymer decreases the free radicals concentration in the sample. The higher amounts of this drug decreases higher the free radicals concentration. But this effect is not observed at the too high amphotericin B concentration, when most of the possible binding sites are used to form these complexes.

Table 1. Free radicals concentrations (*N*), *g*-factor and linewidths (ΔB_{pp}) of EPR spectra of DOPA-melanin, *Cladosporium cladosporioides* mycelium and the mycelium with amphotericin B. The data are obtained from the EPR spectra measured at a microwave power of 2.2 mW

Sample	$N \times 10^{19}$ ($\pm 0.1 \times 10^{19}$ spin/g)	*g* (± 0.0002)	ΔB_{pp} (± 0.02 mT)
DOPA-melanin	9.7	2.0039	0.34
Cladosporium cladosporioides mycelium	5.4	2.0058	0.49
Cladosporium cladosporioides mycelium – amphotericin B (1.8 μg/cm^3)	5.2	2.0054	0.49
Cladosporium cladosporioides mycelium – amphotericin B (4.5 μg/cm^3)	4.4	2.0053	0.49
Cladosporium cladosporioides mycelium – amphotericin B (8.1 μg/cm^3)	4.7	2.0053	0.49

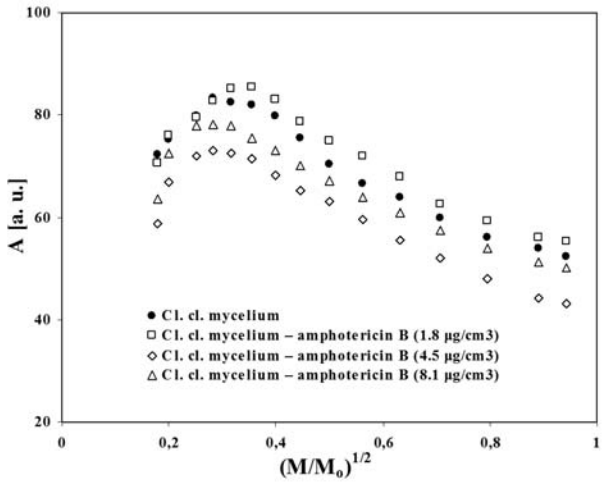

Fig. 4. Influence of microwave power (M) on amplitude (A [± 0.1]) of EPR spectra of *Cladosporium cladosporioides* mycelium and *Cladosporium cladosporioides* mycelium with amphotericin B. The amphotericin B concentrations are: 1.8, 4.5 and 8.1 μg/cm^3. M_0 is the total microwave power produced by klystron and its value is 70 mW.

Such an effect was observed for complexes of melanin with amphotericin B for the drug concentration of 8.1 μg/cm^3. The free radicals concentration in the complex increases, refering to the complex for the drug concentration of 4.5 μg/cm^3. The mechanism of binding of drug to melanin is not only via free radicals, but also by other chemical reactions [2].

The linewidths do not depend on the amphotericin B concentration in the samples (Table 1). The distances between free radicals in the tested melanin biopolymer may be probable responsible for such effect. The binding of drugs to melanin was confirmed by a lot of works [2, 6, 7, 10, 11, 15, 16, 24, 26].

The EPR spectra of *Cladosporium cladosporioides* mycelium and the mycelium with amphotericin B recorded at different microwave powers (2.2–70 mW) were analyzed. The changes of amplitudes (A) and linewidths (ΔB_{pp}) with increasing microwave power (M) are presented in Figs. 4 and 5, respectively. The slow spin-lattice relaxation processes exist in all the tested samples. EPR lines of *Cladosporium cladosporioides* and *Cladosporium cladosporioides* complexes with amphotericin B begin saturate at low microwave powers (Fig. 4). Homogeneous broadening [22, 23] of the measured EPR lines are observed (Figs. 4–5). The linewidth (ΔB_{pp}) increases with increasing microwave power (Fig. 5). Similar effects of microwave power on the parameters of the EPR spectra of *Cladosporium cladosporioides* complexes with flucytosine were observed [26].

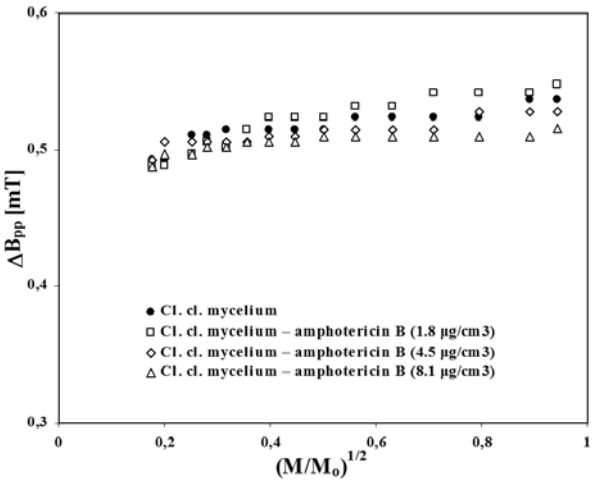

Fig. 5. Influence of microwave power (M) on linewidth (ΔB_{pp}) of EPR spectra of *Cladosporium cladosporioides* mycelium and *Cladosporium cladosporioides* mycelium with amphotericin B. The amphotericin B concentrations are: 1.8, 4.5 and 8.1 μg/cm^3. M_0 is the total microwave power produced by klystron and its value is 70 mW.

In this work the role of *o*-semiquinone free radicals of *Cladosporium cladosporioides* mycelium in binding of amphotericin B was proved. The binding of drugs to melanin via free radicals was observed by us earlier [2, 6, 7, 11, 15, 16, 24, 26]. Similar to flucytosine [26], mainly EPR component of the eumelanin exists in the resonance absorption curve of *Cladosporium cladosporioides* mycelium with amphotericin B. Only the low amount of pheomelanin exists in the tested complexes.

The model of binding between *o*-semiquinone free radicals and amphotericin B is difficult to present. The tested EPR spectra are single broad line without hyperfine structure. These studies confirmed the role of free radicals in the formation of complexes between melanin and this drug. The model of binding sites sold be tested in the future by chemical methods.

EPR spectra of melanin and its complexes with drugs are dependent on temperature [11]. The influence of temperature on the integral intensities of EPR spectra of melanin complexes with drugs indicted that two types of paramagnetic centres exist in melanins, mainly *o*-semiquinone free radicals with spin $S = 1/2$ and additionally biradicals with spin $S = 1$. The role of biradicals in the formation of melanin complexes with drugs is lower than the role of free radicals. The influence of the measuring temperature on the EPR spectra of *Cladosporium cladosporioides* melanin – amphotericin B complexes were not tested, but it is expected that similar correlations are observed as in others melanin samples [11]. Probably the role of biradicals in the interactions of *Cladosporium cladosporioides* melanin with amphotericin B is lower than the role of *o*-semiquinone free radicals. The temperature tests should be performed in the future to resolve this question.

Concentrations of free radicals and the parameters of the spectra of the examined samples depend on the amount of amphotericin B in the sample. The complexation of melanin in *Cladosporium cladosporioides* by amphotericin B may cause prolongated interactions of this drug with the whole sample. Free radicals of the examined melanin biopolymer play an important role in interactions of amphotericin B with the biological systems.

Conclusions

The performed EPR studies of *Cladosporium cladosporioides* complexed with amphotericin B indicate that:

1. Mainly eumelanin exists in *Cladosporium cladosporioides* mycelium with amphotericin B.
2. *o*-Semiquinone free radicals take a part in *Cladosporium cladosporioides* mycelium – amphotericin B complex formation.

3. Free radicals concentrations depend on the amount of amphotericin B introduced to the tested fungal samples.
4. Slow spin-lattice relaxation processes characterize the examined samples containing amphotericin B.

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