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EXPERIMENTAL ARTICLES

The Photodynamic Inactivation of the Yeast *Candida guilliermondii* in the Presence of Photodithazine

M. G. Strakhovskaya*, [1], N. S. Belenikina*, E. V. Ivanova*, Yu. K. Chemeris*, and E. F. Stranadko**

**Department of Biophysics, Faculty of Biology, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*

***State Research Center for Laser Medicine, ul. Studencheskaya 40, Moscow, Russia*

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Abstract—Photodithazine, a glucosamine salt of chlorin e_6, is highly effective in sensitization of *Candida guilliermondii* cells to visible light. The sensitizing effect of photodithazine was found to be related to free or cell surface–bound molecules of this dye. Sodium azide (a singlet oxygen quencher) and propyl gallate (an inhibitor of lipid peroxidation) protected yeast cells from the photodithazine-enhanced photoinactivation.

Key words: yeasts, photosensitized inactivation, visible light, chlorin e_6.

Yeasts, which are unicellular eukaryotic microorganisms, are widely used as model systems for studying the mechanism of photodynamic chemotherapy at the cellular level [5, 6, 9–11, 22, 23]. The recent attention to this problem has been stimulated by the ever-increasing resistance of pathogenic fungi and yeasts to drugs and the need for alternative therapeutic methods [12, 16], such as photodynamic antimicrobial chemotherapy [20]. The last method is based on the phenomenon of the inactivation of microbial cells by active oxygen species generated by the photoexcited molecules of sensitizers. With photochemotherapy, as opposed to conventional chemotherapy, these is no problem of developing cell resistance because of a large number of potential targets for oxidative destruction (such as proteins, enzymes, and unsaturated lipids). The investigation of the photosensitization of *Candida* species, about 10% of which are pathogenic or opportunistic [18], is of particular interest.

Some photosensitizers, including phenothiazines [14] and porphyrins [3, 5, 6, 9, 15, 21–23], possess high fungicidal activity. Earlier, we devised a method for the endogenous photosensitization of yeasts by inducing the biosynthesis and intracellular accumulation of protoporphyrin IX [1, 2, 19].

The aim of the present work was to evaluate the photosensitizion efficiency of photodithazine, a glucosamine salt of chlorin e_6, and to study its effect on the inactivation of *Candida guilliermondii* cells by visible light.

[1]Corresponding author. E-mail: marstr@biophys.msu.ru

MATERIALS AND METHODS

Strain and growth conditions. Experiments were carried out with a diploid strain of the yeast *Candida guilliermondii* obtained from the Laboratory of Selection and Physiology of Microorganisms, State Research Institute of Protein Biosynthesis, Moscow, Russia. Growth conditions and the induction of the biosynthesis of protoporphyrin IX with 0.4 mM 5-aminolevulic acids and 0.2 mM 2,2'-dipyridyl are described earlier [1].

Illumination experiments. A suspension containing 10^6 yeast cells per ml was placed in a temperature-controlled cuvette (22°C) 1 cm in diameter and irradiated with visible light of wavelengths longer than 380 nm from a DRSh-1000 high-pressure mercury lamp equipped with a BS-8 light filter and a heat-absorbing water-filled trap. The distance to the suspension from the lamp was 15 cm. The suspension was illuminated with stirring at a fluence rate of 50 W/m^2, which was measured with a high-sensitivity Hilger FT 16.1/622 thermocouple calibrated using an F116/1 galvanometer.

Yeast survival. The survival rate of yeast cells was determined as described earlier [1].

The photosensitivity of yeast cells was defined as the reciprocal ($1/D_{37}$) of the illumination fluence (in kJ/m^2) that provided for the 37% cell survival rate (D_{37}). The photosensitivity of cells has the dimension m^2/kJ.

Photodynamic inactivation. Immediately after the illumination of a suspension containing 2×10^7 cells/ml with a certain fluence of visible light, its aliquot was transferred to the temperature-controlled (22°C) measuring cell of a Clark-type oxygen electrode, and the rate of oxygen consumption was measured using an

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LP-7 polarograph (Czech Republic). The same suspension was used to prepare serial dilutions for the determination of cell survival.

Protoporphyrin IX was extracted from yeast cells by shaking a separatory funnel containing 100 mg of wet cells suspended in 9 ml of a chloroform–methanol (2 : 1) mixture at 4°C for 30 min. After the separation of phases, the lower chloroform phase with the extracted protoporphyrin IX was collected, and its concentration was determined spectrophotometrically from the absorbance at 405 nm. The molar extinction coefficient of protoporphyrin IX (189 mM^{-1} cm^{-1}) was determined from a calibration curve. The concentration of protoporphyrin IX was calculated per the cell number and per the cell volume. The number of cells used for the extraction of protoporphyrin IX was determined microscopically using a Goryaev counting chamber. The mean volume of one cell was calculated by assuming that its mean dimensions are $8.5 \times 6 \times 6$ μm.

Porphyrin spectra were recorded in a Hitachi-557 spectrophotometer (Japan).

Reagents. Chlorin e_6 and its glucosamine salt photodithazine were obtained from the ZAO Veta (Moscow, Russia). All other reagents were purchased from Sigma (United States).

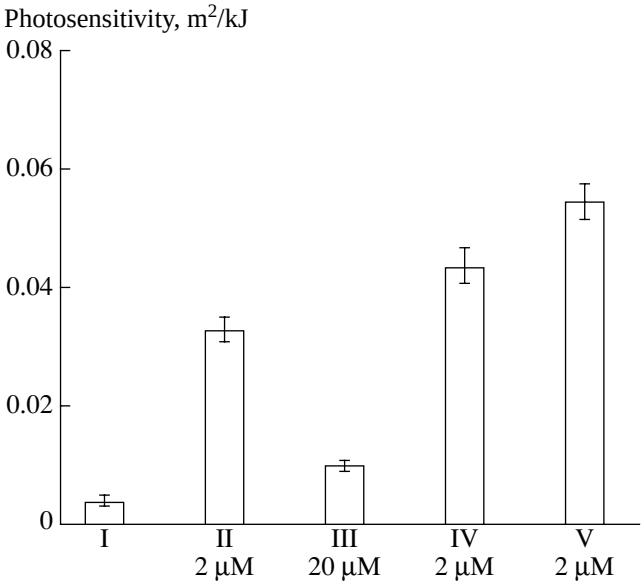

Fig. 1. The photosensitivity of (I) control *C. guilliermondii* cells, (II) cells with the induced synthesis of protoporphyrin IX, as well as cells incubated with (III) exogenous protoporphyrin IX, (IV) chlorin e_6, and (V) photodithazine added at concentrations of 20, 2, and 2 μM, respectively.

RESULTS AND DISCUSSION

Figure 1 shows the effects of photodithazine, chlorin e_6, and endogenous or exogenous protoporphyrin IX (PP IX) on the sensitivity of *C. guilliermondii* cells to the inactivating action of visible light. The induction of the synthesis of PP IX in *C. guilliermondii* cells by the previously developed method [1], which allows the intracellular concentration of PP IX to reach about 2 μM, led to an 8-fold increase in the cell photosensitivity. At the same time, even at a considerably higher concentration (20 μM), the exogenously added PP IX almost did not enhance the cell photosensitivity. According to Ricchelli [17], the high photosensitizing activity of endogenous PP IX is due to its occurrence in cells in a monomeric form, which is characterized by a high quantum yield of singlet oxygen generation. The 37% survival rate of *C. guilliermondii* in the presence of 2 μM chlorin e_6 was observed under illumination at fluence of 22.8 kJ/m^2, i.e., at an order-of-magnitude lower fluence than in the absence of chlorin e_6. The photosensitization efficiency of 2 μM photodithazine was 25% higher than that of 2 μM chlorin e_6, which may be due to a better solubility of photodithazine in water and, hence, to a lower degree of aggregation of photodithazine molecules in aqueous solutions. When the concentration of photodithazine was raised to 20 μM (i.e., by ten times), its photosensitization efficiency increased by more than 25 times (Fig. 2).

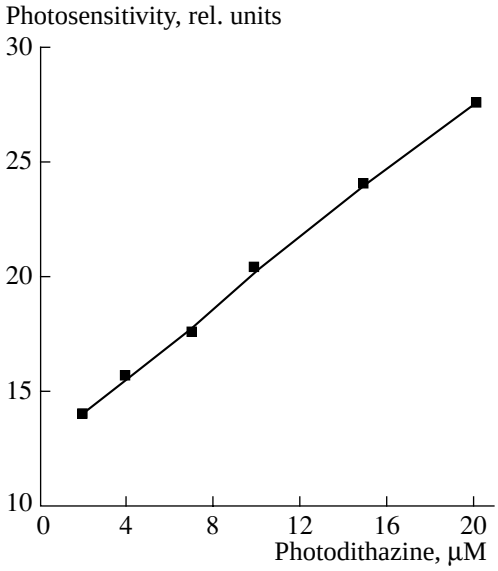

Fig. 2. The dependence of the photosensitivity of *C. guilliermondii* cells on the concentration of photodithazine in the preincubation medium. The preincubation time was 20 min. The photosensitivity of the control cells (0.004 m^2/kJ), which were not preincubated with photodithazine, was taken as unit photosensitivity.

Experiments with the varied time of cell preincubation with photodithazine showed effective yeast photosensitization (30% cell survival) even after short (15 s) incubation with the dye (Fig. 3, curve *1*). The photosensitizing effect increased with an increase in the preincubation time to 10 min. In this case, the survival rate of unilluminated photodithazine-treated cells changed

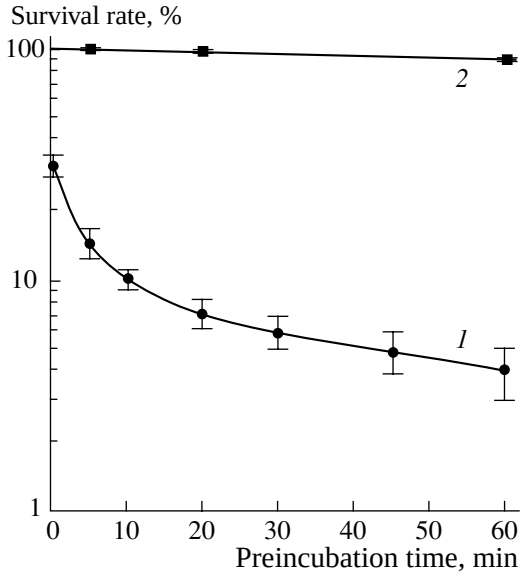

Fig. 3. The dependence of the survival rate of *C. guilliermondii* cells, either (*1*) illuminated for 10 min at an intensity of 30 kJ/m^2 or (*2*) unilluminated on the time of preincubation with 7 μM photodithazine.

insignificantly (Fig. 3, curve *2*). Increasing the preincubation time to 1 h decreased the survival rate of both illuminated and unilluminated cells by the same value (about 6%). These data suggest that the photosensitization of yeast cells is induced by free or surface-bound photodithazine molecules. This suggestion is confirmed by the fact that the single washing of yeast cells preincubated with photodithazine for 20 min considerably diminished the inactivating effect of illumination on the cells (Fig. 4).

It is known that the rate of oxygen consumption by a suspension of yeast cells is proportional to the concentration of viable cells (i.e., cells with the colony-forming ability) in this suspension [13]. Therefore, respiration measurements may serve as an alternative method for evaluating cell viability. In fact, the illumination of the control yeast cells led to a correlated decrease in their respiration and viability (Fig. 5, curves *1* and *2*). The illumination of yeast cells with the 2,2'-dipyridyl-induced synthesis of protoporphyrin IX led to a decrease in their respiration, which appeared to be more sensitive to photosensitization than viability (Fig. 5, curves *3* and *4*). This is in line with the earlier observation that the mitochondria of yeast cells grown in the presence of 2,2'-dipyridyl accumulate PP IX in considerable amounts [2]. Conversely, the colony-forming ability of yeast cells preincubated with photodithazine was more sensitive to inactivation with visible light than their respiration (Fig. 5, curves *5* and *6*). These data suggest a reduced contribution of mitochondrial photodamage to photoinduced lethal effects of photodithazine on yeast cells and confirm the above suggestion that the photosensitization efficiency of

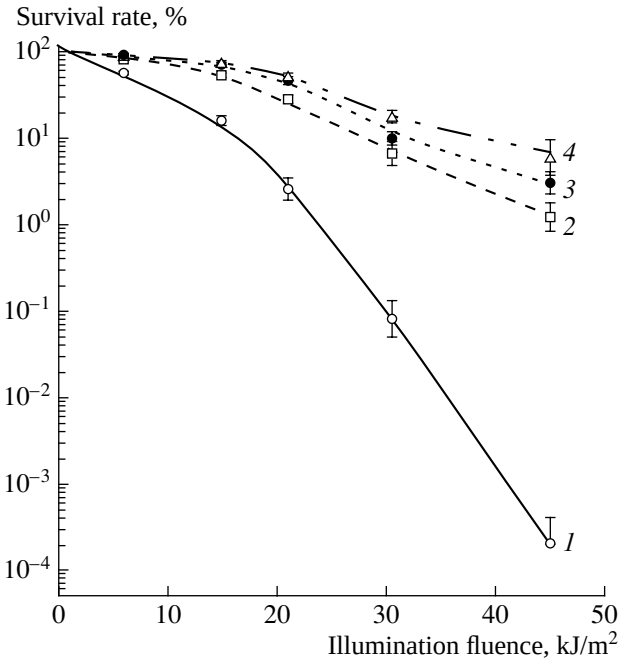

Fig. 4. The survival–illumination fluence curves of *C. guilliermondii* cells which were (*1*) preincubated with 20 μM photodithazine for 20 min, (*2*) washed singly from photodithazine before illumination, (*3*) preincubated with 20 μM photodithazine and 5 mM sodium azide for 20 min, and (*4*) preincubated with 20 μM photodithazine and 1 mM propyl gallate for 20 min.

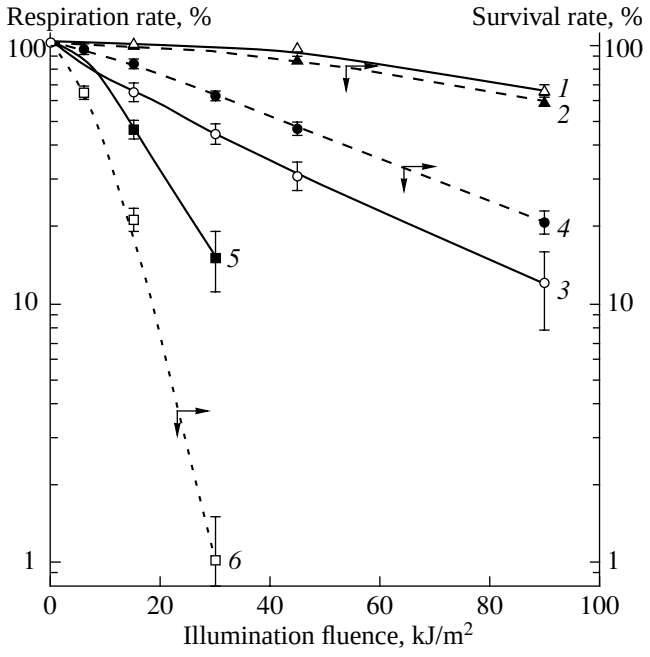

Fig. 5. (*1, 3, 5*) The survival–illumination intensity and (*2, 4, 6*) the respiration–illumination intensity curves of (*1, 2*) control *C. guilliermondii* cells, (*3, 4*) cells with the induced synthesis of protoporphyrin IX, and (*5, 6*) cells preincubated with 20 μM photodithazine for 20 min. The respiration and survival rates of each type of cells before illumination were taken as 100%.

photodithazine is due to the free or cell surface–bound molecules of this dye. It can be suggested that, like hematoporphyrin [3, 9], photodithazine sensitizes yeast cells through its action on plasma membranes.

Experiments with sodium azide (a singlet oxygen quencher [7]) and propyl gallate (an antioxidant inhibiting lipid peroxidation [8]) showed that both compounds protected yeast cells from the photosensitizing action of photodithazine (Fig. 4). The protective effect of sodium azide indicates that singlet oxygen considerably contributes to the phototoxicity of photodithazine (i.e., to the photodynamic effect). The singlet oxygen oxidizes ergosterol (an important component of yeast plasma membranes [4]) with the formation of polar products, which enhance the permeability of the membranes and thus may be responsible for most (up to 90%) of the photodynamic decrease in the colony-forming ability of yeast cells [9]. The singlet oxygen also interacts with unsaturated membrane lipids, inducing their peroxidation [12]. The protective effect of propyl gallate on the photoinactivated yeast cells suggests that the photodithazine-enhanced lethal effects of visible light are related to lipid peroxidation processes.

In spite of the fact that the comparison of the photosensitization efficiencies of porphyrins on yeast cells evaluated by various authors cannot be adequately compared because of the use of different light sources with different spectral characteristics [3, 5, 9, 12, 14, 23], we believe that photodithazine is a promising photosensitizer with high antifungal activity.

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