ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 4, pp. 473-479. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 4, pp. 581-589.

Cyanines as Efficient Photosensitizers in Photodynamic Reaction: Photophysical Properties and *in vitro* Photodynamic Activity

J. Kulbacka[1]*, A. Pola[2], D. Mosiadz[2], A. Choromanska[1], P. Nowak[3], M. Kotulska[4], M. Majkowski[5], A. Hryniewicz-Jankowska[5], L. Purzyc[1], and J. Saczko[1]

[1]Medical University, Department of Medical Biochemistry, Chalubinskiego 10, 50-368 Wroclaw, Poland; fax: +4871-784-0085; E-mail: jkulbacka@gmail.com
[2]Medical University, Department of Biophysics, Chalubinskiego 10, 50-368 Wroclaw, Poland
[3]Wroclaw University of Technology, Institute of Physical and Theoretical Chemistry, Wybrzeze Wyspianskiego 27, 50-370 Wroclaw, Poland
[4]Wroclaw University of Technology, Institute of Biomedical Engineering and Instrumentation, Wybrzeze Wyspianskiego 27, office D-1/115, Wroclaw, Poland
[5]Wroclaw University, Institute of Biochemistry and Molecular Biology, Laboratory of Cytobiochemistry, Tamka 2, 50-137 Wroclaw, Poland

Received October 28, 2010
Revision received November 26, 2010

Abstract—The purpose of the present study was to explore the potential application of cyanines in photodynamic treatment. The photophysical features of four cyanines (KF570, HM118, FBF-749, and ER-139) were investigated by elemental and spectral analyses. Two malignant cell lines (MCF-7/WT and MCF-7/DOX) were used to test the potential for use in the photodynamic therapy. The cytotoxic effects of these dyes were determined by the MTT assay after 4 and 24 h of incubation with the cyanine. KF570 and HM118 were irradiated with red light (630-nm filter) and FBF-749 and ER-139 with green light (435-nm filter). The results showed that the cyanine HM118 demonstrated a major phototoxic effect. It was also noted that the efficiency of photodynamic therapy was higher in the doxorubicin-resistant cell line (MCF-7/DOX).

DOI: 10.1134/S0006297911040110

Key words: breast adenocarcinoma cells, cyanines, photodynamic therapy, cytotoxicity

Photodynamic therapy (PDT) is a promising technique for the treatment of neoplastic and nonmalignant diseases (severe acne, actinic keratosis, rosacea). PDT involves the combination of two agents, a photosensitizer and the proper light, which, when combined, initiate cancer cell destruction [1-4]. Mainly porphyrins and their derivatives, chlorins, and core-modified porphyrins are currently applied in PDT. The success of any new drug as a photosensitizer for PDT depends on its photophysical and photochemical properties [5]. Thus Photofrin® is generally used in the treatment of a variety of cancers and in other applications. However, a major disadvantage of Photofrin is that it is a mixture [6]. Authors suggest that the different Photofrin components can affect different parts of the diseased tissue and bind to different cellular components (e.g. cell membranes, mitochondria, proteins, and other organelles) [6]. Cyanines appear to have interesting properties, and they have been studied as potential PDT dyes during the last decade. They are a complex group of dyes containing numerous subgroups featuring cationic and anionic compounds [7-10].

Although not every cyanine has a place in the range of the "phototherapeutic window" (600-1000 nm), they can be applied in superficial malignant changes, in combination with laparoscopy, or in PDT in combination with electroporation. Cyanine dyes have received less attention, remaining, to the best of our knowledge, to be investigated with regard to their application in PDT. Some authors introduced indocyanine green to photodynamic practice [11, 12]. Santos et al. also proposed squarylium cyanine dyes as potential photosensitizers [5]. Another cyanine dye, MC540, was used for the

Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PC, phosphatidylcholine; PDT, photodynamic therapy.

* To whom correspondence should be addressed.

selective purging of ocular leukemia, lymphoma, and neuroblastoma cells [13, 14]. According to the above investigations, cyanines seem to have interesting properties. Depending on the experimental conditions and cell sensitivity, the cytotoxic molecular species induced by PDT may trigger cell apoptosis or provoke necrosis [2, 15].

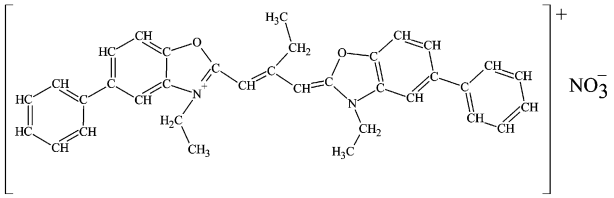
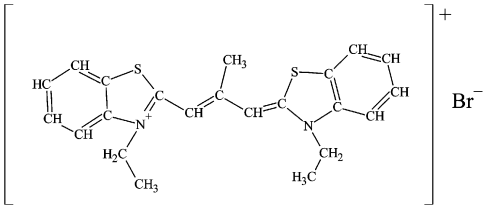
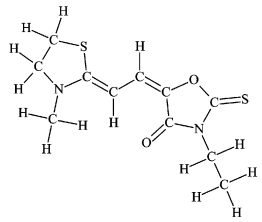
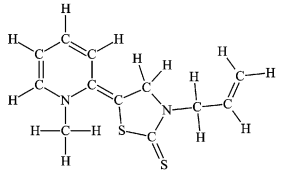
The main goal of this study was the identification of a potentially potent photosensitizer from the group of cyanines. These compounds were chosen from the group of previous tested dyes in potential application in photographic process. These four dyes indicated light-sensitive properties. The current study verifies potential photodynamic treatment with cyanines.

MATERIALS AND METHODS

Cyanine dyes. Two carbocyanines (KF570 and HM118), a merocyanine (FBF-749), and a pyridin-thiazolidine (ER-139) were investigated. The structures and full names are presented in Table 1. All these compounds were synthesized by P. Nowak at the Institute of Physical and Theoretical Chemistry, Wroclaw University of Technology.

Cell culture. The studies were performed on doxorubicin-sensitive (MCF-7/WT) and doxorubicin-resistant (MCF-7/DOX) human breast adenocarcinoma cell lines. The cell lines were a kind gift from the Department of Tumor Biology, Comprehensive Cancer Center, Maria

Table 1. Chemical structure of studied cyanines

Name		Structure formula	FW*
3,3′-Diethyl-5,5′-diphenyl-9-ethyloxacarbocyanine	KF570		575.65
3,3′-Diethyl-9-methylthiacarbocyanine	HM118		460.47
(5E)-3-ethyl-5-[(2Z)-2-(3-methyl-1,3-thiazolidin-2-ylidene)ethylidene]-2-thioxo-1,3-oxazolidin-4-one	FBF-749		270.37
(5Z)-5-(1-methylpyridin-2(1H)-ylidene)-3-(prop-2-en-1-yl)-1,3-thiazolidine-2-thione	ER-139		250.38

* FW, formula weight.

Sklodowska-Curie Memorial Institute in Gliwice. The cells were grown in DMEM (Sigma, USA) with addition of 10% fetal bovine serum (Biowhittaker) and supplemented with antibiotics (penicillin/streptomycin; Sigma). The cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. For the experiments the cells were removed by trypsinization (0.025% trypsin and 0.02% EDTA; Sigma) and washed with PBS.

Biophysical studies. Aqueous solutions of the investigated compounds in micromolar concentrations were prepared from stock solutions (2 mM) of these compounds in DMSO (Sigma). Liposomes were prepared by sonication of an aqueous suspension of lipid (phosphatidylcholine from bovine brain). Absorption and fluorescence spectra were recorded using Perkin Elmer Lambda Bio 20 and LS 50B spectrophotometers, respectively.

Cytotoxicity assay. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) assay was used to test mitochondrial metabolic function. Cells were seeded into 96-well microculture plates at $1 \cdot 10^4$ cells/well and allowed to attach overnight. After incubation with various concentrations of the cyanines, the assay was performed according to the manufacturer's protocol. The absorbance was measured using a multiwell scanning spectrophotometer at 570 nm (Multiskan MS microplate reader). The result was expressed as the percentage of viable cells relative to untreated control cells. The cytotoxicity test was performed after 24 h of incubation with cyanines.

Photosensitization *in vitro* study. The phototoxic effect of the four dyes was determined after 4 and 24 h of incubation with 1 μM concentration of applied compounds. Then the cultures were irradiated and culture medium was exchanged. KF570 was irradiated with 630 nm; HM118 was irradiated with 630 and 530-550-nm and FBF-749 and ER-139 with 435 nm. PDT dose was 6 J/cm^2. After 24 h post irradiation the MTT assay was performed.

Confocal microscopy: cyanine localization. Microcultures were trypsinized from the culture dishes and grown on cover glasses. The cells were incubated with the compounds (0.5 μM) for 2 h. Then the cells were washed in PBS, fixed in 4% buffered formalin (Polysciences, Inc.) and washed in PBS. The cells were examined under a confocal scanning laser microscope (Carl Zeiss GmbH, Germany). The dyes' emission was observed using a 575-754 nm excitation filter for KF570, 530-754 nm for HM118, 475-565 nm for FBF-749, and 420-565 nm for ER-139.

Statistical analysis. Statistical analysis was performed using Statistica v.8.0 commercial software. The significance of differences between mean values of different groups of cells was assessed by Student's *t*-test. Values of $p \leq 0.05$ were taken to imply statistical significance.

RESULTS

Biophysical studies. Two absorption bands can be observed in the absorption spectra of aqueous solutions of the two carbocyanines (KF570 and HM118) and the merocyanine (FBF-749). The absorption bands, which were located at shorter and at longer wavelengths, corresponded to dimer (H-aggregates) and monomer forms of these compounds, respectively (Table 2). For the carbocyanines (KF570 and HM118), the ratio of dimer and monomer absorption intensities (A_D/A_M) increased with increasing dye concentration (Fig. 1). However, in the case of the merocyanine (FBF-749) the A_D/A_M ratio was independent of dye concentration. The spectrum of the fourth compound (pyridin-thiazolidine (ER-139) in aqueous solutions exhibited only one absorption band.

The presence of phosphatidylcholine (PC) liposomes in the system had an influence on the absorption spectra of the carbocyanine dyes (KF570 and HM118). Increasing PC liposome concentration caused a decrease in the dimer absorption band intensity and a simultaneous increase in the monomer absorption band intensity. Moreover, a shift of both absorption bands to longer wavelength with increasing PC liposome concentration can be observed as a result of the decreasing polarity of the environment of the incorporated carbocyanine molecules in the liposome bilayer. In the fluorescence spectra

Table 2. Spectroscopic parameters for investigated cyanines

Cyanine	Dimer absorption maximum (λ_{max}), nm	Monomer absorption maximum (λ_{max}), nm	Excitation wavelength (λ), nm	Fluorescence maximum (λ_{max}), nm	Stokes shift, nm
KF570	472	502	473	518	16
HM118	505	540	520	564	24
FBF-749	444	466	450	484	18
ER-139	–	436	420	491	55

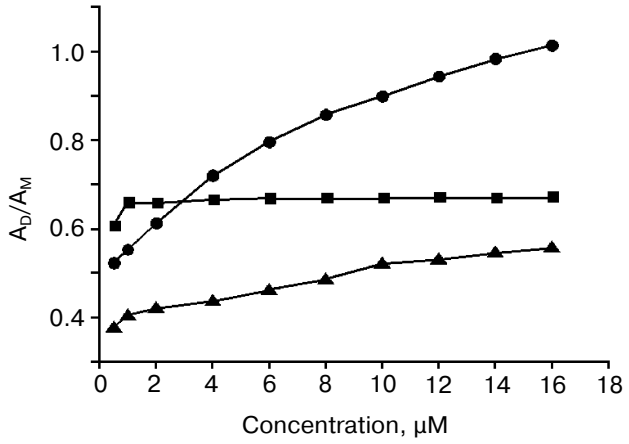


Fig. 1. Plot of the ratio of dimer and monomer absorption intensities (A_D/A_M) vs. dye concentration: KF570 (circles), HM118 (triangles), and FBF-749 (squares).

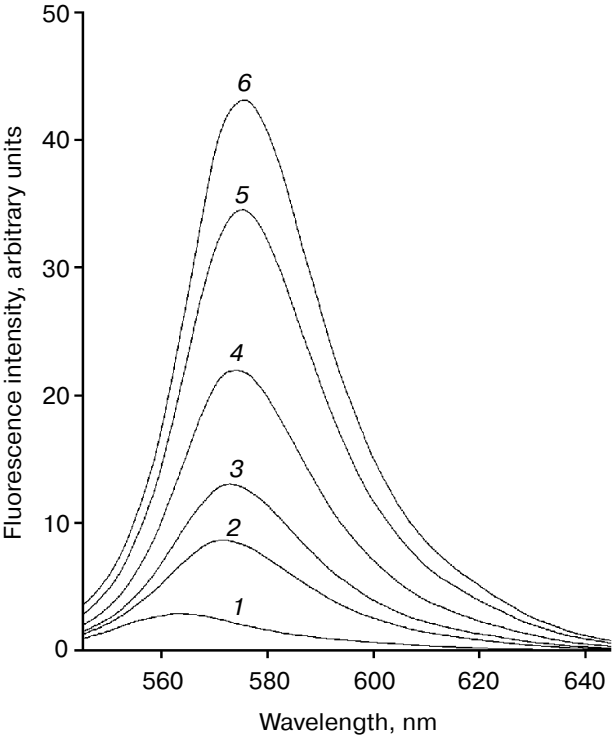


Fig. 2. Emission spectra of cyanine HM118 with the presence of PC liposomes. PC concentration: 0 (*1*), 50 (*2*), 100 (*3*), 200 (*4*), 400 (*5*), 600 (*6*) μM. λ_{ex} = 520 nm.

for these compounds (Figs. 2 and 3), an increase in monomer emission band intensity and simultaneous shift of the emission maximum to longer wavelength with increasing PC liposome concentration can be observed (for cyanine HM118 λ_{max} = 564 nm without PC, λ_{max} = 576 nm with PC (600 μM); for cyanine KF570 λ_{max} = 518 nm without PC and λ_{max} = 531 nm with PC (600 μM)). The ratio of monomer emission intensities F/F_0 (F the monomer emission intensity in the presence of lipid, F_0 the monomer emission intensity without lipid) versus lipid concentration is shown in Fig. 4. For the carbocyanines (KF570 and HM118) the F/F_0 ratio increased with increasing lipid concentration. In the case of the mero-

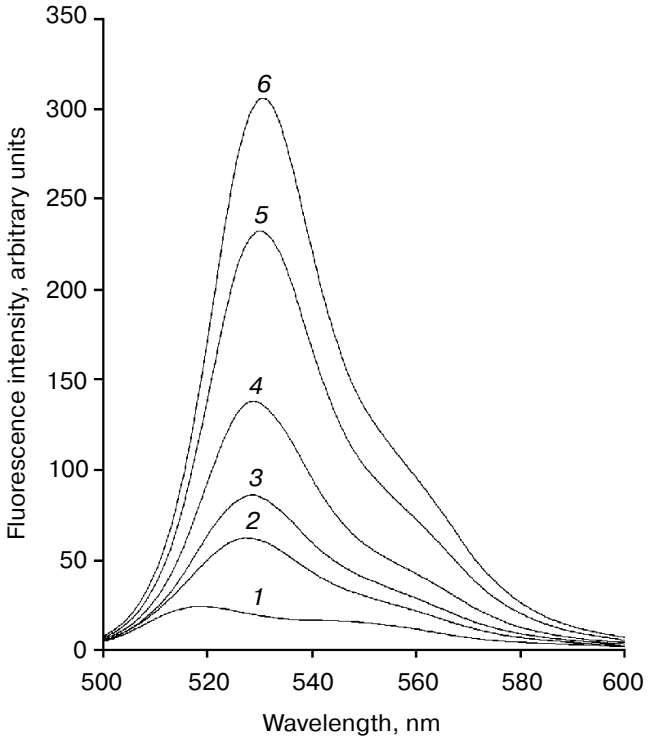


Fig. 3. Emission spectra of cyanine KF570 with the presence of PC liposomes. PC concentration: 0 (*1*), 50 (*2*), 100 (*3*), 200 (*4*), 400 (*5*), 600 (*6*) μM. λ_{ex} = 473 nm.

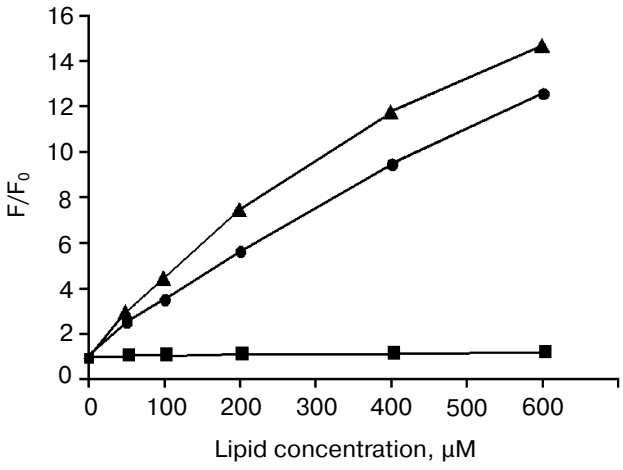


Fig. 4. Plot of the ratio of F/F_0 vs. PC liposome concentration: KF570 (circles), HM118 (triangles), and FBF-749 (squares).

cyanine (FBF-749), the presence of PC liposomes did not influence the F/F_0 ratio.

Cytotoxicity and photocytotoxicity assay. The results of cyanine cytotoxicity are presented in Fig. 5. All the examined compounds were nontoxic to the cell lines, especially in the case of MCF-7/DOX, and at all concentrations. Because of this, we settled on one concentration of 1 μM for the photodynamic reaction. The effect of PDT with cyanines is presented in Fig. 6, a and b. We obtained the highest effectiveness of the therapy in the case of the cyanine HM118. Based on our previous study with Photofrin, we also decided for irradiation in the range of the "optical window" (630 nm) [16-22]. Similarly to porphyrin-based sensitizers, this compound also has very weak absorbance in this range. As presented in Fig. 6, this compound was more active 24 h after therapy for both wavelengths. However, this compound was more active after 630-nm sensitization and the cell viability reached a value of 15.7% for MCF-7/DOX (p = 0.000002), and 23.3% for MCF-7/WT (p = 0.000014). Surprisingly, HM118 was more efficient as a photosensitizer in MCF-7/DOX cells when irradiated in the "optical window".

Intracellular distribution of cyanines. The localization of the investigated cyanines is presented in Fig. 7. We found stronger distribution of HM118, FBF-749, and ER-139 in the sensitive cell line. HM118 accumulated mainly in the cytoplasm and nuclei and FBF-749 and ER-139 appeared generally in the cytoplasm, which suggests the primary target of PDT. KF570 showed very weak fluorescence in both cell lines. In the resistant cell line, all the investigated dyes accumulated with low intensity.

DISCUSSION

Photosensitizers from the group of cyanines have been developed as potential PDT agents during the last decade [7, 14, 23-26]. In the present study we demonstrated the photo-dependent effects of four cyanine dyes. The investigated carbocyanines (KF570 and HM118) as well as the merocyanine (FBF-749) in aqueous solution appeared in two forms: as dimers (H-aggregate) and as monomers. Increase in carbocyanine concentration caused a shift in the dimer–monomer balance to the benefit of the dimer form; in the case of merocyanine the dimer–monomer balance was not dependent on the dye concentration. However, PC liposomes exhibited a deaggregative property only in the case of the carbocyanines. Pyridin-thiazolidine (ER-139) in aqueous solution appeared only in one form (probably a monomer) regardless of concentration and practically did not fluoresce. Wilk et al. loaded cyanines (IR-768, IR-780, and IR-783) in biodegradable nanocapsules and obtained better characteristics, being more potent [27]. Other authors investigated the effects of photodynamic therapy with indocya-

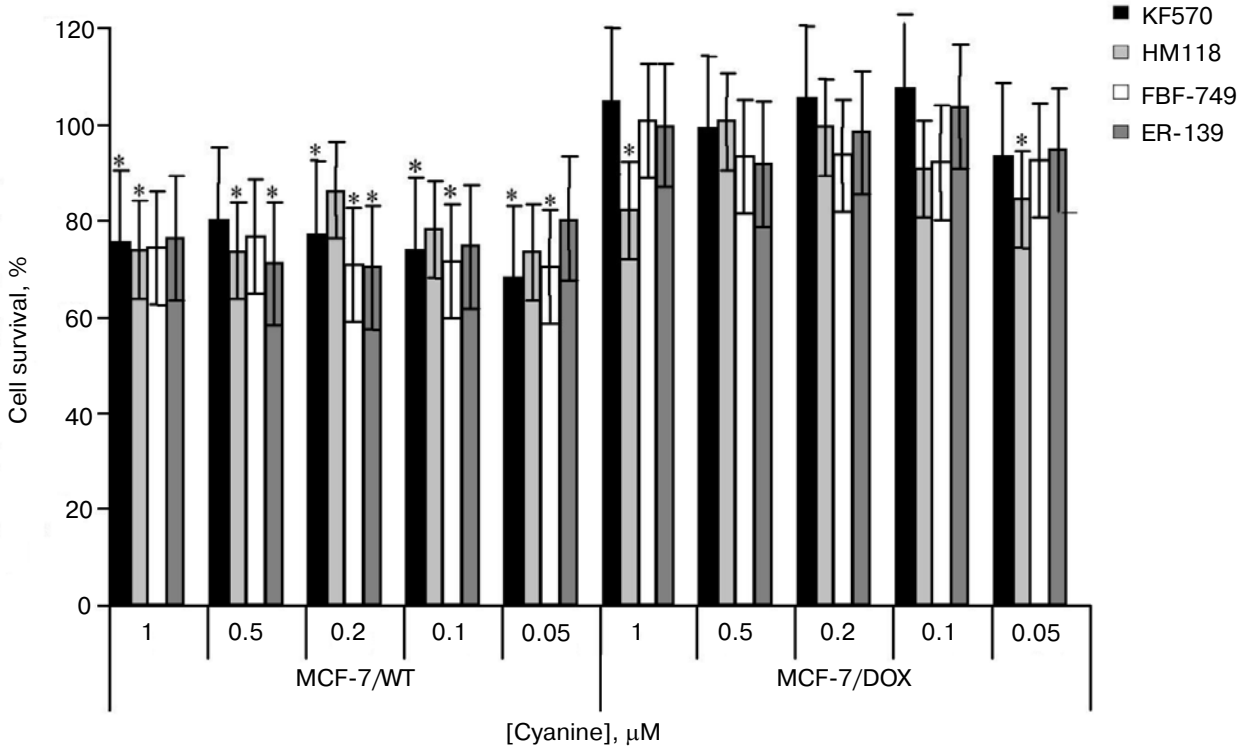


Fig. 5. Cytotoxicity of KF570, HM118, FBF-749, and ER-139 after 24 h. The MTT assay was performed for concentrations of 1, 0.5, 0.2, 0.1, and 0.05 μM. Results are expressed as means ± SD from three independent experiments; * $p < 0.05$.

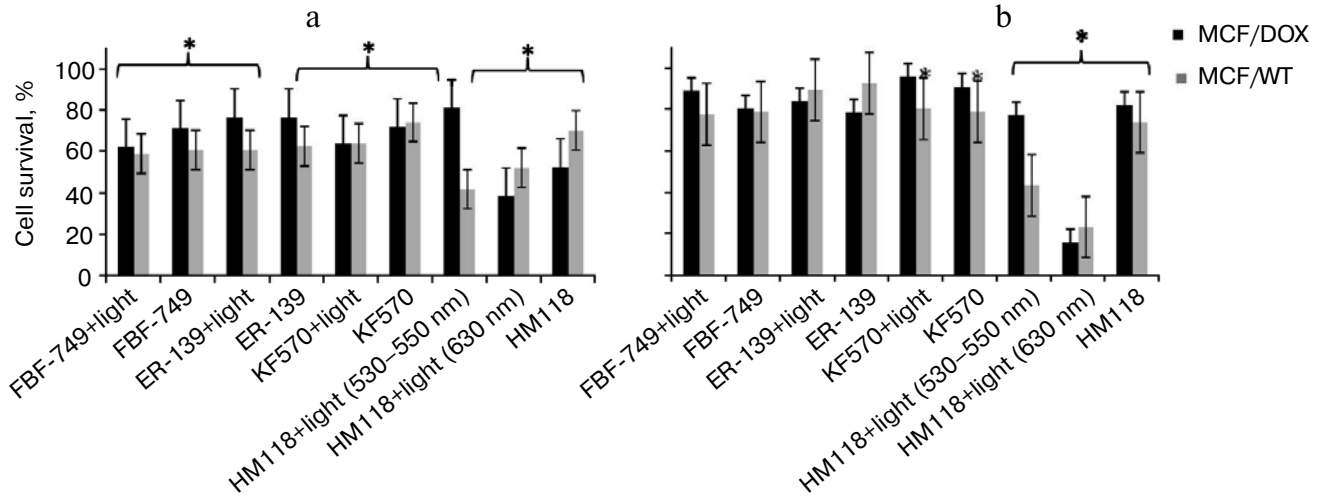


Fig. 6. Photocytotoxicity efficiency after the photodynamic reaction. Photodynamic reaction was determined after 4 h (a) and 24 h (b) of incubation with cyanines. Results are expressed as means ± SD from three independent experiments; * $p < 0.05$.

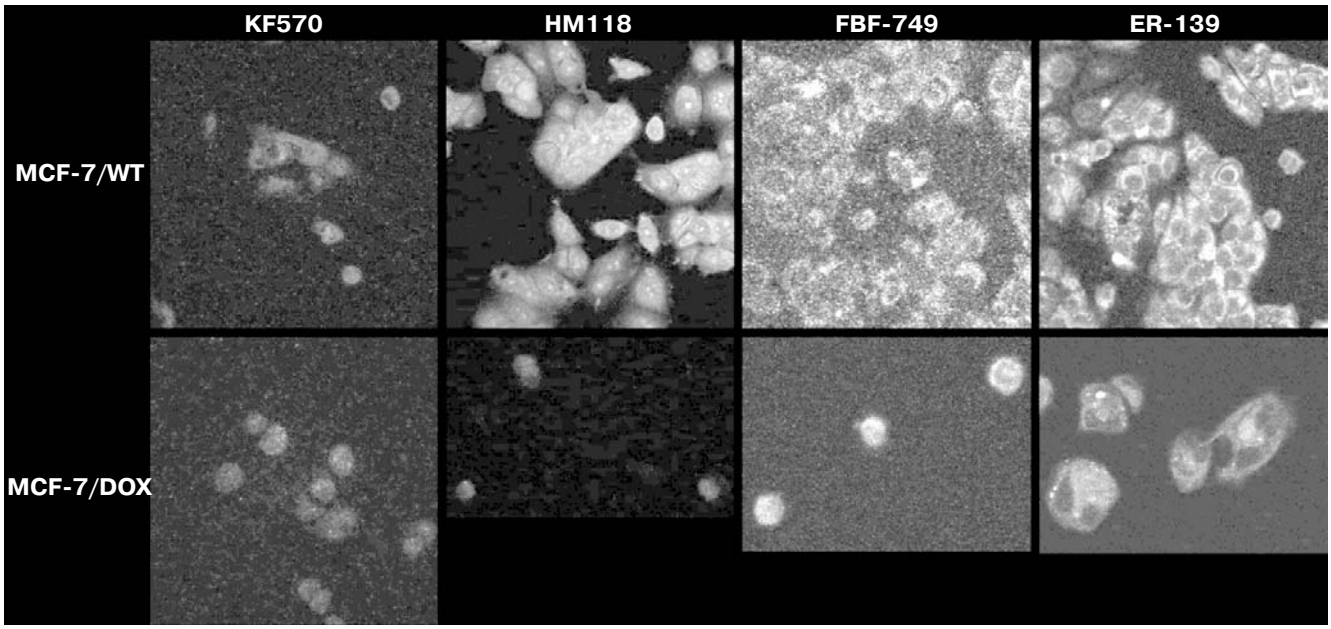


Fig. 7. Intracellular distribution of the cyanines in MCF-7/WT (upper panel) and MCF-7/DOX cells (lower panel).

nine combined with low-dose chemotherapy (cisplatin) on MCF7 cells. Their findings imply that low doses of cisplatin may be even more effective than the currently used doses if appropriately combined with PDT. PDT with indocyanine caused damage to Bcl-2 and the up-regulation of Bax [28]. Nowak-Sliwinska et al. tested Photofrin II, Verteporfin, and Merocyanine 540 on S91/I3 cell line. MC540 turned out to be the least effective photosensitizer against S91 melanoma cells *in vitro* [14]. However Lydaki et al. presented results of MC540 in a selective killing of leukemic cells Reh-6 and HL-6014 [29]. Marchal et al. performed therapy with Foscan on MCF-7 cells. These authors observed an increase in caspases-7 and -6 activation, which was strongly associated with the expression of GRP78. They also suggested that Foscan accumulation in the ER improves the photoactivation of the caspase-7 apoptotic pathway, which is weakly related to mitochondrial damage [30].

In the current photodynamic study, HM118 demonstrated a major phototoxic effect. The efficiency of PDT was stronger in the doxorubicin-resistant cell line (MCF-7/DOX) than in MCF-7/WT cells. We observed stronger

intracellular distribution of HM118, FBF-749, and ER-139 in MCF-7/WT cells. KF570 was taken up very weakly in both cell lines. However, we detected weaker dye distribution of all the examined cyanines in the resistant cells. Alexandratou et al. applied zinc phthalocyanine (ZnPc) and observed primary and intermediate ROS action with subcellular localization. They investigated the early mechanism of the cellular response to oxidative stress in HFFF2 cells. The ZnPc localized to mitochondria after a longer incubation time. These authors suggest that the fluorescence of phthalocyanines may indicate the sites of direct photodamage [31].

The major intention of the present study was to compare the efficiencies of the photodynamic reaction using cyanines against two human breast adenocarcinoma cell lines. The concentration of the applied dye, the light dose, and the mechanism of the photosensitizer action determine the level of biological damage. Summarizing our study, cyanines have good potential as photosensitizing agents in biological systems, particularly in systems involved in multidrug resistance. However, this basic research requires further investigation into phototherapeutic applications.

This study was supported by Polish Ministry Funds (NR R05 043 03) and by Statutory Funds of Wroclaw Medical University supported by the Polish Ministry of Science and Higher Education.

REFERENCES

1. Szokalska, A., Makowski, M., Nowis, D., et al. (2009) *Cancer Res.*, **69**, 4235-4243.
2. Almeida, R. D., Mandas, B. J., Carvalho, A. P., and Durate, C. B. (2004) *Biochim. Biophys. Acta*, **1704**, 59-86.
3. Robertson, C. A., Hawkins, E. D., and Abrahamse, H. (2009) *J. Photochem. Photobiol. B*, **96**, 1-8.
4. Saczko, J., Daczewska, M., Chwilkowska, A., et al. (2004) *Prog. Med. Res.* (online), **2**, poz. 31.
5. Santos, P. F., Reis, L. V., Almeida, P., et al. (2003) *J. Photochem. Photobiol. A: Chem.*, **160**, 159-161.
6. Samaroo, D., Vinodu, M., Chen, X., and Drain, C. M. (2007) *J. Comb. Chem.*, **9**, 998-1011.
7. Delaey, E., van Laar, F., de Vos, D., Kamuhabwa, A., Jacobs, P., and de Witte, P. (2000) *J. Photochem. Photobiol. B*, **55**, 27-36.
8. Ishihara, M., and Fujisawa, S. (2007) *In vivo*, **21**, 163-173.
9. Ishii, K., Takayanagi, A., Shimizu, S., Abe, H., Sogawa, K., and Kobayashi, N. (2005) *Free Rad. Biol. Med.*, **38**, 920-927.
10. Kaestner, L., Cesson, M., Kassab, K., et al. (2003) *Photochem. Photobiol. Sci.*, **2**, 660-667.
11. Fickweiler, S., Szeimies, R. M., Baumler, W., et al. (1997) *J. Photochem. Photobiol. B*, **38**, 178-183.
12. Abels, C., Karrer, S., Baumler, W., et al. (1998) *Br. J. Cancer*, **77**, 1021-1024.
13. Diwu, Z., and Lown, J. W. (1994) *Pharmacol. Ther.*, **63**, 1-35.
14. Nowak-Sliwinska, P., Karocki, A., Elas, M., et al. (2006) *Biochem. Biophys. Res. Commun.*, **349**, 549-555.
15. Nowis, D., Makowski, M., Stoklosa, T., et al. (2005) *Acta Biochim. Pol.*, **52**, 339-352.
16. Kulbacka, J., Boehm, D., Chwilkowska, A., et al. (2004) *Adv. Clin. Exp. Med.*, **13**, 897-901.
17. Saczko, J., Kulbacka, J., Chwilkowska, A., et al. (2004) *Proc. 40th Symp. of the Polish Histochemical and Cytochemical Society*, Bialystok, Poland, 16-18 September 2004, *Ann. Acad. Med. Bialost.*, **49** (Suppl. 1), 82-84.
18. Saczko, J., Kulbacka, J., Chwilkowska, A., et al. (2007) *Acta Biomed. Eng.*, **2**, 247-256.
19. Saczko, J., Kulbacka, J., Chwilkowska, A., et al. (2007) *Folia Histochem. Cytobiol.*, **45**, 93-97.
20. Saczko, J., Chwilkowska, A., Kulbacka, J., et al. (2008) *Folia Biol.* (Praha), **54**, 24-29.
21. Saczko, J., Skrzypek, W., Chwilkowska, A., et al. (2009) *Exp. Oncol.*, **31**, 195-199.
22. Kulbacka, J., Chwilkowska, A., Bar, J., et al. (2010) *Exp. Biol. Med.*, **235**, 98-110.
23. Kassab, K. (2002) *J. Photochem. Photobiol. B*, **68**, 15-22.
24. Sharma, M., Bansal, H., and Gupta, P. K. (2002) *Indian J. Exp. Biol.*, **40**, 252-257.
25. Taquet, J. P., Frochot, C., Manneville, V., and Barberi-Heyob, M. (2007) *Curr. Med. Chem.*, **14**, 1673-1687.
26. Skrivanova, K., Skorpikova, J., Svihalek, J., et al. (2006) *J. Photochem. Photobiol. B*, **85**, 150-154.
27. Wilk, K. A., Zielinska, K., Pietkiewicz, J., and Saczko, J. (2009) *Chem. Eng. Trans.*, 10-13 May 17(2); *ICheaP-9 The Ninth Int. Conf. on Chemical and Process Engineering*, Rome (Italy), pp. 987-992.
28. Crescenzi, E., Varriale, L., Iovino, M., et al. (2004) *Mol. Cancer Ther.*, **3**, 537-544.
29. Lydaki, E., Dimitriou, H., Papazoglou, Th., et al. (1996) *J. Photochem. Photobiol. B*, **32**, 27-32.
30. Marchal, S., Francois, A., Dumas, D., et al. (2007) *Brit. J. Cancer*, **96**, 944-951.
31. Alexandratou, E., Yova, D., and Loukas, S. (2005) *Free Rad. Biol. Med.*, **39**, 1119-1127.