EKSPERIMENTINIAI TYRIMAI

Hypericin as novel and promising photodynamic therapy tool: studies on intracellular accumulation capacity and growth inhibition efficiency

Živilė Lukšienė, Peter A. M. de Witte[1]
Institute of Material Science and Applied Research, Vilnius University, Lithuania,
[1]Laboratorium voor Farmaceutische Biologie en Fitofarmacologie, K. U. Leuven, Belgium

Key words: *photodynamic therapy, hypericin, Ehrlich ascites tumor.*

Summary. *In this study we set out to investigate the photodynamic efficacy of hypericin (Hyp), three other photosensitizers and 5-aminolevulinic acid-induced protoporphyrin IX in their ability to block the growth of rather aggressive tumor – Ehrlich ascites tumor in mice. Hypericin was found to exhibit the highest tumor growth inhibiting activity in treating Ehrlich ascites tumor by photodynamic therapy. The different photosensitizers were ranked as follows: Hypericin > hematoporphyrin dimethyl ether > Photofrin II > meso-tetra-(para-sulfophenyl) porphin > 5-aminolevulinic acid. Of importance, just after Hyp-based photodynamic therapy 75% of mice survived a 4-month period, and no recurrence of tumor within this period was detected in 25% of the treated mice. The clear cut correlation observed between tumor dye concentration in the tumor cells and efficiency of photodynamic therapy, supports the idea that the intracellular accumulation of the photosensitizer is one of the most important factors in determining the benefit of photodynamic therapy. Hence, the accumulation of the photosensitizer in the tumor cells should be considered as one of the prognostic factors for the determination of the therapeutic outcome.*

Introduction

Because of the promising clinical results obtained with photodynamic therapy (PDT), more and more new photosensitizers with improved chemico-physical properties continue to be developed. Hypericin (Hyp) is a photoactive natural pigment present in *Hypericum perforatum*, a plant widely distributed throughout the world (1). The photophysical and photochemical properties of hypericin, as well as its photobiological activity, have been intensively investigated during the last few years. It has been convincingly shown that hypericin exhibits high singlet oxygen generation and is endowed with a high fluorescence yield. Several lines of evidence indicate that the compound binds strongly to plasma proteins, such as albumin or lipoproteins (2). It is furthermore important to note that hypericin is devoid of toxic or genotoxic effects *in vitro* or *in vivo* (3–4). Being amphiphilic, the compound localizes in the membrane structures of the cell and organelles, and therefore these sites represent principal targets of photoactivated hypericin (5–7).

From the numerous studies describing the *in vitro* photodynamic action of hypericin on different human and murine cancer cell lines, it can easily be concluded that the compound possesses a powerful photocytotoxic effect (8–12). Moreover, there is a growing body of evidence suggesting that hypericin exerts *in vivo* significant antitumor activity, although its efficacy seems to depend on the histological origin of tumor (13–18). Of interest, so far no reports have been published describing the photodependent antitumor efficacy of hypericin in comparison with the PDT efficacy of other commonly studied photosensitizers. Hence, we found of special importance to compare the PDT effects of hypericin with the effects of other well-known first- and second-generation photosensitizers. Attention has also been paid to a possible correlation between the intracellular dye accumulation and tumor growth inhibition.

Correspondence to Ž. Lukšienė, Institute of Material Science and Applied Research, Vilnius University, Saulėtekio 9, 2040 Vilnius, Lithuania. E-mail: z.luksiene@post.skynet.lt

Materials and methods

Chemicals. The stock solution of hematoporphyrin dimethyl ether (HPde) (a gift of Prof. G. V. Ponomarev, Russia) was prepared in physiological saline solution (2.5×10^{-3} M) and stored in the dark below 10°C. 5-Aminolevulinic acid (ALA) was kindly provided by PhotoCure (Oslo, Norway). Stock solutions (5 ml) were prepared and sterilized the same day as they were used. Meso-tetra-(para-sulfophenyl)porphin ($TPPS_4$) (a gift of Prof. J. Moan, Norway) was prepared in physiological saline solution (2.5×10^{-3} M) and stored in the dark below 10°C.

Hypericin was synthesized and purified with silica and Sephadex LH-20 column chromatography as described previously (19). A stock solution of hypericin (5 mg/ml) was prepared in DMSO (2×10^{-2} M) and stored at –20°C in the dark. The stock solution was further diluted in RPMI-1640 medium. Photofrin II (PII) (Porphyrin Products, USA) was prepared as stock solution (2.5×10^{-3} M) physiological saline and kept in the dark below 10°C.

Tumor model and light irradiation. The experiments were carried out using the BALB/c mice strain. Ehrlich ascites tumor (EAT) was transplanted into female mice aged 6–7 weeks and weighting approximately 21 g as briefly summarized. After tumor dissection from a donor mouse, Ehrlich ascites tumor cells (0.8×10^6) were inoculated intraperitoneally (i. p.) to healthy mice using a 25 G needle. On 7th day after tumor inoculation, corresponding to the tumor exponential growth phase, the photosensitizer was injected i. p. at the dose of 40 mg/kg body weight. This drug concentration corresponded to the optimal concentration for this type of tumor as analyzed previously (20). After 3 h incubation Ehrlich ascites tumor cells were removed from the intraperitoneum and adjusted *ex vivo* in the dark to a homogeneous cell suspension with an optical density of 0.6 at λ=590 nm, corresponding to 3.7×10^6 cells/ml. Irradiation of the cells was performed in 2 mm cuvettes using a light source as described below. After treatment 0.2 ml of irradiated cell suspension (0.8×10^6 cells) was inoculated in healthy mice i. p. and the tumor growth was measured for 15 days. Every group consisted of 8 mice. The control mice group was inoculated with untreated EAT cell suspension. All experiments were done in dark and repeated 3 times. All animals were under general anesthesia (ketamine hydrochloride, i. p.) during all experiments. Animals were kept according to requirements for the use of Laboratory Animals in Scientific Experiments in Lithuania (1999).

Light source. The light source used for irradiation of Ehrlich ascites tumor cell suspension consisted of a tungsten lamp (500 W) optical system for light focusing and optical filter for UV and infrared light elimination (370 nm<λ<680 nm). Light intensity at the position of the cells was 50 mW/cm^2 and the irradiation time 90 s.

Tumor growth determination. Relative Ehrlich ascites tumor growth was measured every day up to the 15th day of its growth according to equation: $S=(S_1-S_0)/S_0$, where S_1: final weight of mouse with tumor, S_0: initial weight of intact mouse, and S: relative tumor growth (20). Data of relative tumor growth afterwards were used to calculate tumor growth inhibition in percents. $I\%=S_0\%-S_1\%$, where I% is growth inhibition in percents S_0%: growth inhibition in percents in control, not treated group, S_n% – growth inhibition in percents in treated by photodynamic therapy group.

Measurements of intracellular concentration of photosensitizer. Tumor cells were collected from the mice 3 h after treatment with the photosensitizer. Tumor cells were suspended in phosphate-buffer solution (PBS) to an optical density of 0.6 (3.7×10^6 cells/ml). The fluorescence spectra of the suspension were measured with a spectrofluorimeter СФР-1 (Moscow, Russia) (22). The sample was excited through the interference filter with λexc=405 nm and an epiobjective. The fluorescence was registered from the front surface of the sample. The constructional features of the device made it possible to measure the fluorescence of a thin layer (less than 1 mm) of the solution without spectrum distortions due to the effects of the intrinsic filter and light scattering. The fluorescence was excited with the radiation of a mercury lamp through an interference filter with λexc=405 nm, and was registered at λ=600–680 nm with an emission slit of 10 nm. The measurements were made at room temperature.

An EAT suspension, treated in the same manner without photosensitizer was taken as control. Standard curves were produced by adding known amounts of the photosensitizer. Protein concentration was determined by Bradford method.

Statistical evaluations. Every experimental group consisted of 6–8 mice. All experiments were repeated at least three times. Every point is average of 25 mice. Averaged values, standard deviations, p-significance were calculated using Excel programme.

Results

First of all, cytotoxicity of all photosensitizers per se, without activation by light, was evaluated. No significant changes in EAT cell proliferation was observed after incubation of cells with ALA, $TPPS_4$, or PII. Whilst proliferation of EAT cells was diminished up

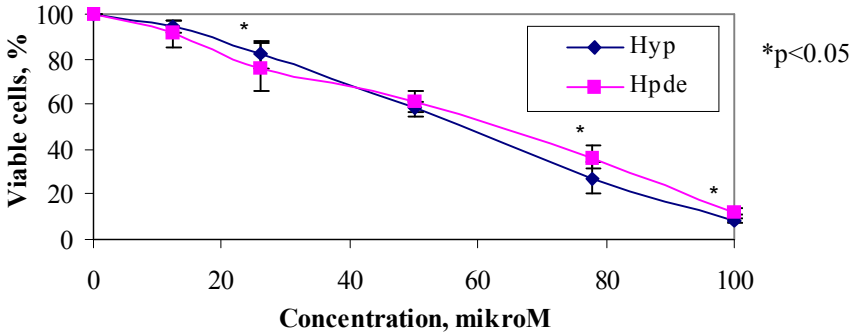

Fig. 1. **Cytotoxic effects of hypericin and hematoporphyrin dimethyl ether on EAT proliferation 48 hours after incubation (40 mg body weight, i. p. injection, 3-hour incubation)**

to zero, when incubation with increasing concentrations (0–100 μM) of Hyp or HPde was performed (Fig. 1).

EAT growth inhibition after Hyp-based photosensitization was used as one of the primary parameters to evaluate PDT efficacy. Mice tumor growth was monitored for 15 days. Control group consisted of mice inoculated with untreated EAT cells which usually died following 25 days after EAT inoculation. Other groups of mice were treated by different photosensitizers and light. The used drug concentration in all cases, except for ALA, was 40 mg/kg body weight, as this dose was found to be optimal for the ascites tumor model system (19). The incubation time selected was 3 h, as i. p. injection of the photosensitizer results in a much faster deliver of the drug to the tumor as compared to i. v. injection.

The results depicted in Fig. 2, which were obtained comparing the PDT effects on EAT growth of Hyp and other well-known photosensitizers including PII, ALA-induced PpIX, Hpde and $TPPS_4$, show that there is a significant difference in the tumor growth inhibition efficacy among these drugs. For instance, ALA, which is a clinically established agent, is absolutely ineffective. While similar results are obtained with $TPPS_4$, treatment with either PII or HPde leads to a significant tumor growth inhibition 15 days after PDT. Remarkably, PDT with Hyp is by far the most efficient in inhibition of tumor growth when compared to all photosensitizers used in this investigation.

The differential antitumoral activities of ALA-induced PpIX, PII, $TPPS_4$, HPde and Hyp, observed in the EAT model system, prompted us to examine the tumor accumulation potential of these drugs, since a possible correlation between photosensitizer intracellular concentration and phototherapeutic efficiency can be assumed. Due to the fact that all photosensitizers exhibit fluorescence, we used a fluorimetric technique to measure their cellular accumulation. The use of epifluorimeter was considered as the most advantageous in comparison with fluorescence measurements of chemically extracted photosensitizers, because it gives the opportunity to evaluate the intracellular concentration of any photosensitizer in intact cells.

From Fig. 3, it is evident, that the relative intracellular concentration of the different first- and second-generation photosensitizers differs dramatically. It is remarkable that well-known photosensitizers as PII, ALA-induced PpIX or $TPPS_4$ exhibit very poor accumulation in EAT cells under these experimental conditions. By contrast, HPde and especially Hyp showed a very high accumulation potential in these cells.

In order to better investigate the photocytotoxic po-

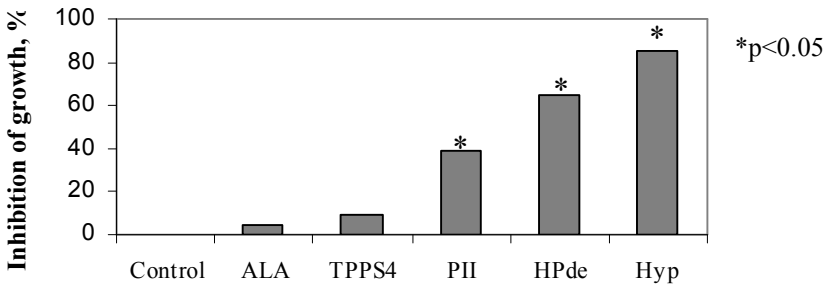

Fig. 2. **Growth inhibition after photodynamic therapy measured 15 days after treatment using different photosensitizers (ALA-PpIX, $TPPS_4$, PII, HPde, Hyp)**
Experimental conditions as described in previous Fig.1.

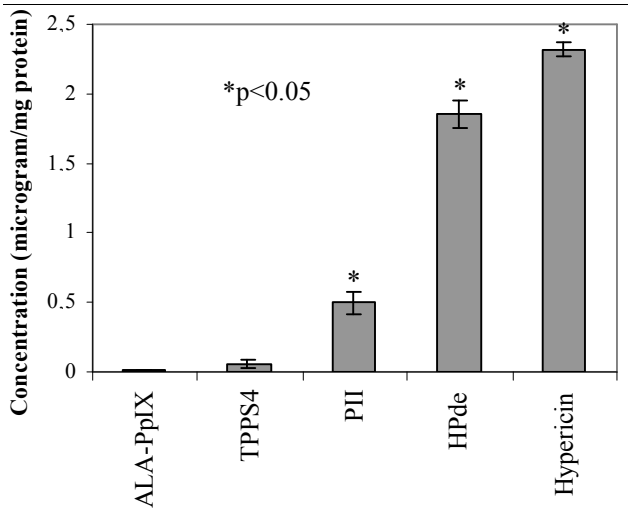

Fig. 3. **The intracellular concentration of different photosensitizers in Ehrlich ascites tumor cells (40 mg/kg i. p. injection, 3-hour incubation)**
Measured by fluorescence technique.

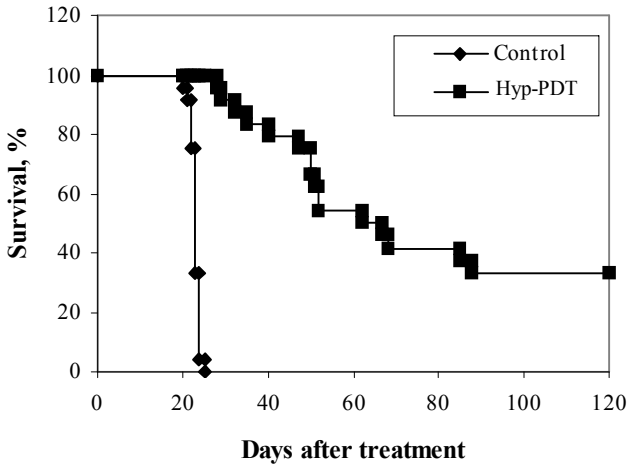

Fig. 4. **Survival of mice, bearing EAT**
♦ – control group; ■ – Hyp-PDT treated group (40 mg/kg hypericin, i. p. injection, 3-hour incubation).

tential of Hyp, as well as to ascertain its antitumoral efficiency and therapeutic outcome, we evaluated the survival of mice treated with Hyp-based PDT. Due to some ethical problems other photosensitizers showing less promising features for the treatment of EAT, were not included in this experiment. Thus, irradiated EAT cell suspension was injected into one group of mice (8 mice). Other group of mice (8 mice) was injected with not irradiated EAT cells served as control group. Survival of mice, calculating every day the number of alive mice was performed for 4 months. Data shown in Fig. 4, indicate that the survival time of 75% tumor-bearing mice after Hyp-based PDT was prolonged for 4 months and more.

Of note, about 25% of the Hyp-PDT treated animals were cured whereas mice of control group usually survived no longer than 25 days. In 25 % of the survived mice no signs of EAT were observed, tumors were virtually absent and no recurrence was detected as well.

Discussion

Recent *in vivo* and *in vitro* studies on the antitumor activity of hypericin (8–18) have suggested that hypericin might be a potential photosensitizer for photodynamic treatment of cancer. But, in order to evaluate the real antitumor activity of compound, it is necessary to compare it with other well known sensitizers.

First of all, dark toxicity or cytotoxicity of all photosensitizers without activation of light was evaluated. According to the data obtained, ALA, $TPPS_4$, PII were absolutely non cytotoxic. Maybe, this is due to very low accumulation of these photosensitizers in EAT cells. On the contrary, Hyp and HPde exhibited rather strong cytotoxicity per se, without activation of light. This fact might be explained in such way: Hyp is well-known proteinkinase C inhibitor and inductor of cyt_c release from mitochondria, whereas HPde as dicarboxylic porphyrin is ligand of mitochondrial benzodiazepine receptors, responsible for regulation of proliferation.

One of the major outcome of this comparative study, where the antitumor activity of different photosensitizers (i. e. ALA-induced PpIX, PII, $TPPS_4$, HPde, Hyp) against EAT-bearing mice is analyzed, is that the different drug-cell interactions exhibited by the different drugs dramatically influence their photodynamic efficacy. This infers that the choice of the most suitable, effective photosensitizer for every type of tumor is of critical importance. For instance, neither ALA-PDT nor $TPPS_4$-PDT caused a significant EAT growth inhibition, whereas PII-PDT and HPde had a moderate effect (Fig. 1). Interestingly, Hyp-PDT exerted a clear and remarkable Ehrlich ascites tumor growth inhibition.

Taking into account the differential photocytotoxicities induced by the photosensitizers used, we then examined whether there was some correlation between photosensitizer induced tumor growth inhibition and intracellular drug concentration. Interestingly, we found out that there exists a rather clear-cut correlation between the capability of the photosensitizer to accumulate in EAT cells and the elicited tumor growth inhibition following photodynamic treatment. It is anticipated the capacity of hypericin to bind strongly to plasma pro-

teins, such as albumin or lipoproteins (2) favors its high accumulation potential in the EAT cells. Moreover amphiphilic nature of compound results in the preference localization of it in functionally important membrane structures of the cell and organelles, and therefore these sites represent principal targets of photoactivated hypericin (5–7).

Thus, the therapeutic benefit is partly based on the sensitizer ability to accumulate in the tumor cells. It is more or less accepted that the main factors, which have general impact on the therapeutic efficiency of PDT are the sensitizer intracellular accumulation, light energy and oxygenation of tumor. Our data support the idea, that knowledge of the intracellular photosensitizer concentration in tumor tissue might be considered as a prognostic factor for determination of the therapeutic outcome. Moreover, the "sensitizer dose" is essential for evaluating the optimal treatment time, thus maximizing the therapeutic effect of PDT while minimizing toxicity.

In conclusion, it is evident that Hyp is a potent and very effective photosensitizer in the EAT model, when compared to other commonly used compounds including ALA, PII, $TPPS_4$ and HPde. The superior antitumoral effect of Hyp is likely due to its highest intracellular accumulation potential. The overall findings of this study strongly support the idea that hypericin should be considered as a very effective photosensitizer for treating those tumors in which this molecule seems to easily accumulate.

Acknowledgements. Authors would like to thank prof. G. Maksimov and Dr. S. Jurkonienė (Institute of Botany, Lithuania) for help in the interpretation of fluorescence spectra, L. Rutkovskienė for technical assistance and participation in all experiments. This study was financially supported by the Lithuanian State Science and Studies Foundation (Grant No. K-024).

Hipericinas – naujas fotodinaminėje terapijoje naudojamas fotosensibilizatorius: gebos kauptis navike ir augimo inhibicijos tyrimai

Živilė Lukšienė, Peter A. M. de Witte[1]

Vilniaus universiteto Medžiagotyros ir taikomųjų mokslų institutas,

[1]K. U. Leuveno katalikiškojo universiteto Biologinės Farmacijos ir Filofarmakologijos laboratorija, Belgija

Raktažodžiai: fotodinaminė terapija, hipericinas, Erlicho ascitinis navikas.

Santrauka. Straipsnyje analizuojamas hipericino fotodinaminis veiksmingumas ir jis lygintas su kitais fotosensibilizatoriais: 5-aminolevulininės rūgšties indukuotu protoporfirinu IX, meso-tetra-(para-sulfofenil) porfinu, fotofrinu ir hematoporfirino dimetilo eteriu pagal gebėjimą blokuoti agresyvaus pelių Erlicho ascitinio naviko augimą. Hipericino geba stabdyti šio naviko augimą buvo didžiausia: hipericinas > hematoporfirino dimetilo eteris > fotofrinas > meso-tetra-(para-sulfofenil) porfinas > 5-aminolevulininės rūgšties indukuotas protoporfirinas IX. Tik po hipericino medijuotos fotodinaminės terapijos 75 proc. pelių išgyveno ilgiau kaip keturis mėnesius, o visiškai pasveiko net 25 proc. pelių. Akivaizdi koreliacija pastebėta tarp fotosensibilizatoriaus gebos kauptis naviko ląstelėse ir fotodinaminės terapijos veiksmingumo. Tai patvirtina teiginį, kad fotosensibilizatoriaus geba kauptis naviko ląstelėse yra vienas svarbiausių faktorių, lemiančių jo veiksmingumą. Taigi fotosensibilizatoriaus kaupimasis navike galėtų būti vienas prognostinių faktorių vertinant gydymo rezultatus.

Adresas susirašinėjimui: Ž. Lukšienė, Vilniaus universiteto Medžiagotyros ir taikomųjų mokslų institutas, Saulėtekio 9, 2040 Vilnius. El. paštas: z.luksiene@post.skynet.lt

References

1. Diwu Z. Novel therapeutic and diagnostic applications of hypocrellins and hypericins. Photochem Photobiol 1995; 61(6):529-39.
2. Lavie G, Mazur Y, Lavie D, Meruelo D. The chemical and biological properties of hypericin – a compound with a broad spectrum of biological activities. Med Res Rev 1995;15:111-9.
3. Meruelo D, Lavie S, Lavie D. Therapeutic agents with dramatic antiretroviral activity and little toxicity at effective doses: aromatic polycyclic diones hypericin and pseudohypericin. Proc Natl Acad Sci (USA) 1988;85:5230-4.
4. Okpanyi SN, Lidzba H, Scholl BS, Miltenburger HS. Genotoxicity of a standardized Hypericum extract. Drug Res 1990;40:851-5.
5. Thomas C, Pardini RS. Oxygen dependence of hypericin-induced phototoxicity to EMT6 mouse mammary carcinoma

cells. Photochem Photobiol 1992;55:831-7.
6. Hadjur C, Richard MJ, Parat MO, Jardon P, Favier A. Photodynamic effects of hypericin of lipid peroxidation and antioxidant status in melanoma cells. Photochem Photobiol 1996;64:375-81.
7. English DS, Doyle RT, Petrich JW, Haydon PS. Subcellular distributions and excited-state processes of hypericin in neurons. Photochem Photobiol 1999;69:301-5.
8. Andreoni A, Colasanti A, Colasanti P, Mastrocinque M, Riccio P, Roberti G. Laser photosensitization of cells by hypericin. Photochem Photobiol 1994;59:529-33.
9. Richter A, Davies DE. Effects of anthralin and hypericin on growth factor signalling and cell proliferation *in vitro*. Biochem Pharmacol 1995;50:2039-45.
10. Yu H, Wolford ST, Kegode R, Zhao W, Osweiler GD. Hypericin-induced phototoxicity in cultured fibroblasts and swine erythrocytes. Photochem Photobiol 1996;64:168-73.
11. Johnson SAS, Pardini RS. Antioxidant enzyme response to hypericin in EMT6 mouse mammary carcinoma cells. Free Rad Biol Med 1998;24:817-26.
12. Kamuhabwa AR, Agostinis PA, D'Hallewin MA, Kasran A, de Witte PAM. Photodynamic activity of hypericin in human urinary bladder carcinoma cells. Anticancer Res 2000;20: 2579-84.
13. Chen B, de Witte PA. Photodynamic therapy efficacy and tissue distribution of hypericin in a mouse P388 lymphoma tumor model. Cancer Letters 2000;150:111-7.
14. Thomas C, Macgill RS, Weill P, Pardini RS. The *in vitro* and *in vivo* photoinduced antineoplastic activity of hypericin. Proc A Assoc Cancer Res 1992;33:500.
15. Vandenbogaerde AL, Geboes KR, Cuveele JF, Agostinis PM, Merlevede WJ, de Witte PA. Antitumor activity of photosensitized hypericin on A431 cell xenografts. Anticancer Res 1996;16:1619-25.
16. Zupko I, Kamuhabwa AR, D'Hallewin M-A, Baert L, de Witte PA. *In vivo* photodynamic activity of hypericin in transitional cell carcinoma bladder tumors. Int J Oncology 2001; 18:1099-105.
17. Chen B, Xu Y, Roskams T, Delaey E, Agostinis P, Vandenheede JR, de Witte PA. Efficacy of antitumoral photodynamic therapy with hypericin: relationship between biodistribution and photodynamic effects in the RIF-1 mouse tumor model. Int J Cancer 2001;93:275-82.
18. Xie X, Hudson JB, Guns ES. Tumor – specific and photodependent cytotoxicity of hypericin in the human LNCaP prostate tumor model. Photochem Photobiol 2001;74:221-5.
19. Vandenbogaerde AL, Delaey EM, Vantieghem AM, Himpens BE, Merlevede WJ, de Witte PA. Cytotoxicity and antiproliferative effect of hypericin and derivatives after photosensitization. Photochem Photobiol 1998;67:119-25.
20. Luksiene Z, Juodka B. Porphyrins as radiosensitizers in ^{60}Co irradiated EAT cells: possibility to combine ^{60}Co irradiation with PDT. In: Dougherty T, editor. Proc. Optical Methods for Tumour Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy. Washington; 1993. p. 67-75.
21. Luksiene Z, Kalvelyte A, Supino R. On the combination of photodynamic therapy with ionizing radiation. Photochem Photobiol 1999;52:35-42.
22. Luksienė Z, Rutkovskienė L, Jurkoniene S, Maksimov G, Griciūte L. Evaluation of the photobiological efficiency of $TPPS_4$ in two different types of mice tumors. Acta Medica Lithuanica 2002;9:32-6.

Received 25 March 2003, accepted 15 April 2003
Straipsnis gautas 2003 03 25, priimtas 2003 04 15