Hypericin-based Photodynamic Therapy: I. Comparative Antitumor Activity and Uptake Studies in Ehrlich Ascite Tumor

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Hypericin was found to exhibit the highest antitumoral activity in treating EAT by PDT: Hyp > Hpde > PII > $TPPS_4$ > ALA. Moreover, 75% of mice after Hyp-based PDT survived for 4 months, if compared with control group, and no recurrence of tumor within this period was detected in 25% of mice. The data obtained suggested the idea that intracellular photosensitizer accumulation is one of the most important factors in determing the therapeutic benefit of PDT, because it was in clear correlation with intracellular dye concentration and could be described as follows: Hyp > Hpde > PII > $TPPS_4$ > ALA. Besides, the data obtained suggest that intracellular photosensitizer concentration might be a prognostic factor for determination of therapeutic outcome.

Key words: photodynamic therapy (PDT), hypericin, Ehrlich ascite carcinoma

INTRODUCTION

Because of promising clinical results obtained with photodynamic therapy, more and more photosensitizers with more suitable chemico-physical properties continue to be developed. Hypericin is a natural photoactive pigment which mostly presents in *Hypericum perforatum*, a plant widely distributed throughout the world (1). Its photophysical and photochemical properties, as well as photobiological activity have been intensively investigated during the last few years. It has been shown convincingly that hypericin has a comparatively high singlet oxygen generation and 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 important to note that hypericin has never exhibited toxic or genotoxic effects *in vitro* or *in vivo* (3, 4). Mostly because the compound is very lipophilic, the membrane structures in the cell are the principal target of photoactivated hypericin (5–7).

Reviewing in short the numerous studies on hypericin *in vitro*, it could be summarized that hypericin exerts a really powerful phototoxicity on different cell lines (8–12). Moreover, there is a growing body of evidence suggesting that hypericin exhibits in some cases a significant antitumor activity, but it varies and depends on the histological origin of tumor (13–18).

So far no reports have been published reflecting the comparative antitumor efficiency of hypericin. Of special importance would be investigation of hypericin's antitumor activity in comparison with other well-known first- and second-generation photosensitizers, using one tumor model. So, the aim of this study was to put in one line (to examine) the antitumor activity of different photosensitizers (PII, ALA, $TPPS_4$, HPde) and to compare it with that of hypericin. Attention will also be paid to a possible correlation between intracellular dye accumulation and tumor growth inhibition.

MATERIALS AND METHODS

Chemicals. The stock solution of hematoporphyrin dimethyl ether (a gift from Prof. G. V. Ponomarev, Russia) was prepared in physiological saline (2.5×10^{-3} M) and was stored in the dark below 10 °C.

5-aminolevulinic acid (ALA) and 5-aminolevulinic acid hexyl ester (ALA-HE) were kindly provided by PhotoCure (Oslo, Norway). ALA-HE was dissolved in 0.5 ml ethanol (stock solution, 12×10^{-3} M) and further diluted in serum-free culture medium (RPMI 1640, Life Technologies, Inc.) with the final concentration of ethanol less than 1%. The stock solutions (5 ml) were made and sterilized the same day as they were used.

Meso-tetra-(para-sulfophenyl)porphyrin ($TPPS_4$) (a gift from Prof. J. Moan, Norway) was prepared

in physiological saline (2.5×10^{-3} M) and was stored in the dark below 10 °C.

Hypericin (Hyp) (a gift from Prof. P. de Witte, Belgium) was dissolved in DMSO ($2x10^{-2}$M) and then prepared in RPMI-1640 medium.

Photofrin II (PII) (Porphyrin Products, USA) was prepared as stock solution (2.5×10^{-3} M) and was kept in the dark below 10 °C.

Tumors and experimental apparatus. The experiments were carried out using the BALB/c mice strain. Ehrlich ascite carcinoma was transplanted into female mice aged 6–7 weeks and weighing approximately 21 g. The implantation procedure can be summarized as follows: tumor is dissected from a donor mouse and E. ascites tumor cells (0.8×10^6) are inoculated intraperitoneally (i. p.) using a 25 G needle to healthy mice.

On the 7th day after tumor inoculation in its exponential growth phase the photosensitizer was injected i. p. (40 mg/kg body weight as an optimal concentration for this type of tumor which had been evaluated before) (19). After 3 h of incubation Ehrlich ascite tumor cells were excluded from the intraperitoneum and prepared *ex vivo* in the dark as a homogeneous cell suspension with optical density (at λ = 590 nm) OD = 0.6 (3.7×10^6 cells/ml). Irradiation of cells was performed in 2 mm cuvettes. After treatment 0.2 ml of irradiated cell suspension (0.8×10^6 cells) was inoculated in healthy mice i. p. and tumor growth was measured for 15 and more days. Each group consisted of 8 mice. The control mice group was inoculated with untreated EAT cell suspension. All experiments were done in the dark and repeated 3 times.

Irradiation sources. The light source used for irradiation of Ehrlich ascite carcinoma 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. The irradiation time was 90 s.

Tumor growth determination. Relative Ehrlich ascite tumor growth was measured every day up to day 15 of its growth according to the equation:

$$S = (S_1 - S_0)/S_0,$$

where S_1 – final weight of mouse with tumor,

S_0 – initial weight of intact mouse,

S – relative tumor growth.

Moreover, Ehrlich ascite tumor growth was measured in two other ways:

1) absolute tumor volume growth during 15 days;

2) tumor cell number during 15 days.

The correlation between absolute tumor weight and relative tumor growth was found very strong (r = 0.98) (20). In order to simplify the experimental protocol we usually measured just relative tumor growth.

Measurements of intracellular concentration of photosensitizer. Ehrlich ascite was collected from the mice 3 h after treatment with the photosensitizer. Tumor cells were suspended in phosphate-buffer solution (PBS) to an optical density OD = 0.6 (3.7 mln/ml). The fluorescent spectra of the suspension were measured with a unique СФР-1 spectrofluorimeter (Moscow, Russia) (21), the sample being excited through an interference filter with λ_{exc} = 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 by the radiation of a mercury lamp through an interference filter with λ_{exc} = 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 a known amount of the photosensitizer.

Protein quantitation determination. The quantitation of protein was determined by the Bradford method.

Statistical evaluations. Each experimental group consisted of 6–8 mice. All experiments were repeated at least three times. Averaged values and standart deviations were calculated.

All animals were kept according to requirements for the use of Laboratory Animals in Scientific Experiments in Lithuania (1999).

RESULTS

1. Antitumoral effects of PDT with different first and second generation photosensitizers

EAT growth delay after hypericin-based photosensitization was used as one of the parameters to evaluate PDT efficiency. Mice tumor growth was observed for 15 and more days. The control group consisted of mice that were inoculated with untreated EAT cells and usually died following 25 days after tumor inoculation procedure. The drug concentration in all cases was 40 mg/kg body weight as optimal for ascite tumor (19). The incubation time was 3 h, because i. p. injection of photosensitizer

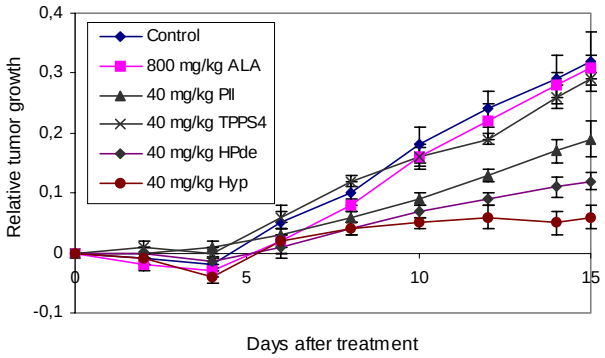

Fig. 1. Relative EAT growth after PDT with different photosensitizers

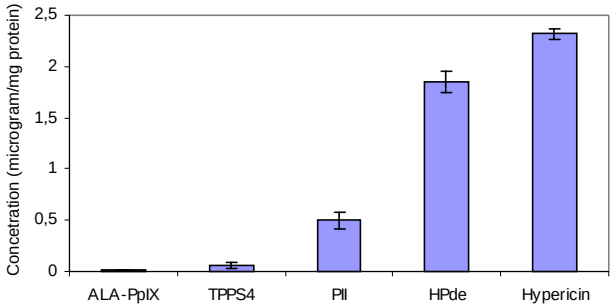

Fig. 2. Intracellular concentration of different photosensitizers in Ehrlich ascite tumor cells (40 mg/kg i.p.3 h incubation)

gives a chance to deliver the drug much faster in comparison with i. v. injection. Neither Hyp nor light alone caused any delay of tumor growth under such conditions. Hence, the results obtained are depicted in Fig. 1.

The data, however, obtained with different well-known photosensitizers, including PII, ALA, HPde, $TPPS_4$, suggested that there is a great difference in the antitumoral efficiency of these drugs. For instance, ALA, being a clinically established agent, is absolutely ineffective in delaying the tumor growth. Similar results were obtained with $TPPS_4$, while PII and HPde exhibited a much more significant growth inhibition following 15 days after PDT treatment. Surprisingly, Hyp exerted the highest antitumor activity, if compared with all the photosensitizers under investigation.

2. **Intracellular concentration measurements of different photosensitizers in EAT**

The broad spectrum of different antitumoral activities found in EAT cells with the aid of ALA, PII, $TPPS_4$, HPde and Hyp, prompted us to examine the accumulation potential of these drugs. It seems reasonable to find a possible correlation between the photosensitizer accumulation potential and phototherapeutic efficiency. Due to the fact that all photosensitizers exhibit fluorescence, we used a fluorimetric technique to measure their cellular accumulation. The use of epi-fluorimeter was considered more advantageous in comparison with fluorescence measurements of chemically-extracted photosensitizers, because it gives the possibility to evaluate the intracellular concentration of any photosensitizer in intact cells. Thus, the data obtained are presented in Fig. 2.

It is evident, that different first- and second-generation photosensitizers exhibit significant differences in the relative amount normalized on protein amount in EAT cells. Most interesting is the fact that such well-known photosensitizers as PII, PpIX (when ALA as a precursor is used) or $TPPS_4$ exhibit a very poor accumulation under these experimental conditions. On the contrary, HPde and most of all Hyp showed a very high and notable accumulation potential in these cells.

3. **Survival of mice after hypericin-based PDT**

In order to clear up the phototoxic potential of hypericin, to ascertain its antitumor efficiency and therapeutic outcome, the survival of mice treated with hypericin-based PDT was observed. Due to some ethical problems, other photosensitizers that seemed much less effective and not promising for treating EAT were not used in this experiment. Data presented in Fig. 3 indicate that the survival time of 75% of tumor-bearing mice after hypericin-based PDT was prolonged for 4 months and more. Surprisingly, about 25% of the treated animals were cured, whereas mice from control group usually survived no longer that 25 days. In 25% of survived mice no signs of EAT were observed – tumors were impalpable within the whole life and no recurrence was observed.

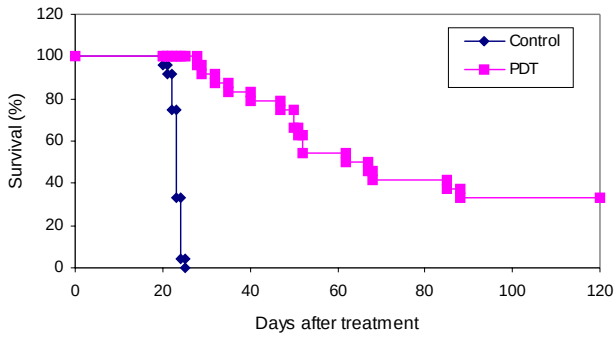

Fig. 3. Survival of mice bearing EAT and treated with Hyp-based PDT

DISCUSSION

The recent results of hypericin antitumor activity, clearly observed measuring EAT growth delay after PDT treatment, indicate that hypericin might be a

potential photosensitizer for the photodynamic treatment of cancer. Moreover, a comparison of the antitumor activity of different photosensitizers (ALA, PII, $TPPS_4$, HPde, Hyp) clearly showed that there was a deep specifity in drug–cell interaction in the case of every sensitizer. For instance, neither ALA-PDT nor PII-PDT showed a notable efficiency in treating EAT, whereas $TPPS_4$, HPde and especially Hyp were found to exert a clear and remarkable tumor growth inhibition. This means that finding a suitable, effective photosensitizer for every type of tumor is of critical importance.

Taking into account differential phototoxicities of PII, ALA, $TPPS_4$, HPde and Hyp, we have then examinated if there was some correlation between the photosensitizer-induced tumor growth delay and the intracellular drug concentration. Surprisingly, we have observed a clear correlation between photosensitizer accumulation in EAT cells and tumor growth inhibition, which followed photodynamic treatment. It is more or less accepted that the main factors which have a general impact on the therapeutic efficiency of PDT are sensitizer intracellular accumulation, light energy and oxygenation of tumor. Our data supported the idea that knowledge of the intracellular photosensitizer concentration in tumor tissue might be a prognostic factor for determination of the therapeutic outcome. Moreover, "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 hypericin extracted from *Hypericum perforatum* is a potent and very effective photosensitizer in EAT model, if compared with ALA, PII, $TPPS_4$, HPde. The subsequent evaluation of intracellular concentrations of these photosensitizers showed a clear correlation with antitumoral activity and therapeutic outcome. Thus, the therapeutic benefit of therapy is partly based on sensitizer's ability to accumulate in the tumor cells. Hypericin, exhibiting the highest intracellular accumulation potential, was the most effective antitumor agent indicating that the survival of mice bearing EAT is directly related to this capability of the sensitizer. The overall findings of this study strongly support the idea that hypericin could be a very effective photosensitizer for treating various tumors in which it accumulates.

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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: 111–19.
3. Maruelo 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: 831–37.
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: 817–826.
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 Lett 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. Lukšienė Z, Juodka B. Porphyrins as radiosensitizers in ^{60}Co irradiated EAT cells: possibility to combine ^{60}Co irradiation with PDT. In: Dougherty T, ed. Proc Optical Methods for Tumour Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy. Washington; 1993: 67–75.
20. Lukšienė Z, Kalvelytė A, Supino R. On the combination of photodynamic therapy with ionizing radiation. Photochem Photobiol 1999; 52: 35–42.
21. Lukšienė Z, Rutkovskienė L, Jurkonienė S, Maksimov G, Griciūtė L. Evaluation of the photobiological efficiency of $TPPS_4$ in two different types of mice tumors. Acta Medica Lithuanica 2002; 9(1): 32–6.

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HYPERICINO MEDIJUOTA FOTODINAMINĖ TERAPIJA: PALYGINAMIEJI PRIEŠNAVIKINIO AKTYVUMO IR AKUMULIACIJOS EAN LĄSTELĖSE TYRIMAI

S a n t r a u k a

Nustatyta, kad hypericinas pasižymi didžiausiu antinavikiniu aktyvumu po fotodinaminio poveikio EAN, lyginant su kitais fotosensibilizatoriais: Hyp > Hpde > PII > > $TPPS_4$ > ALA. Be to, 75% pelių, paveiktų hypericino medijuota FDT, išgyveno daugiau negu 4 mėnesius, 25% paveiktų pelių visiškai pasveiko. Gauti rezultatai patvirtina, kad viduląstelinė sensibilizatoriaus akumuliacija yra vienas iš esminių veiksnių, nulemiančių FDT efektyvumą, kadangi pastebėta visiška koreliacija tarp viduląstelinės akumuliacijos ir antinavikinio efektyvumo.

Be to, manome, kad viduląstelinė fotosensibilizatoriaus akumuliacija naviko ląstelėje galėtų būti prognozinis terapinio efektyvumo markeris.