Exp Oncol 2006
28, 4, 299–302

Experimental ONCOLOGY

PHOTODYNAMIC EFFICACY OF TOPICAL APPLICATION OF CHLORIN e6 — POLYVINYL PYRROLIDONE COMPLEX IN TUMOR-BEARING RATS

*Y.P. Istomin[1,] *, T.P. Laptsevich[1], S.A. Bizyuk[2], E.N. Alexandrova[1]*
[1]N.N. Alexandrov Research Institute of Oncology and Medical Radiology, Ministry of Health, Minsk 223040, Belarus
[2]Institute of Molecular and Atomic Physics NAS of Belarus, Minsk 220072, Belarus

Aim: **To evaluate the antitumor efficacy of photodynamic therapy with ointment form of chlorin e6 — polyvinyl pyrrolidone complex.** ***Methods:*** **2 or 5% chlorin e6 ointment was applied on the surface of rat SM-1 tumor for 15 min — 5 h, and then tumors were scored for photosensitizer accumulation and tissue damage induced by laser irradiation.** ***Results:*** **Selectivity of chlorin e6 accumulation in tumor tissues considerably increases when photosensitizer was applied topically compared with intravenous administration. Antitumor efficacy of photodynamic therapy using topical application of chlorin e6 — polyvinyl pyrrolidone complex is just as high as upon intravenous administration of the preparation.** ***Conclusion:*** **The level of tissue accumulation of chlorin e6 — polyvinyl pyrrolidone complex administered in ointment form allows to carry out fluorescence diagnosis and PDT of superficially localized malignant tumors.**
Key Words: **photodynamic therapy, photosensitizers, chlorin e6, rat sarcoma M-1.**

Photodynamic therapy (PDT) is based on the ability of specific substances, the so-called photosensitizers (PS) to accumulate selectively in tumor tissue being injected i. v. and to be photoactivated with light of specific wavelength to generate singlet oxygen and free radicals, which cause destruction of tumor cells [1, 2].

The main disadvantage of the most photosensitizers recently used for PDT in clinics is prolonged skin photosensitivity. During 3–6 months following treatment with Photoheme or Photosense (Russia) and during 1 week following treatment with Photolon (Belarus), patients should not be exposed to direct sunlight etc. to prevent phototoxic reactions such as hyperemia, edema and burn of open sites of a body.

Creation of new PSs and appropriate lasers is not the only way to enhance the efficacy of PDT. New methods of local application of PS are being developed enabling to reduce the side effects of treatment and to lower its cost. So, it has been shown that local application of the mono-L aspartyl chlorin e6 or 5-aminolevulinic acid, a precursor of endogenic PS protoporphyrin IX, promotes considerably higher accumulation of PS in tumor tissue, in comparison with normal tissues [3, 4]. The PDT using local application of 20% cream of 5-aminolevulimic acid for superficial malignant skin tumors has been carried out [5].

The photosensitizer of the second generation — chlorin e6 was reported to have significant efficacy in killing cancer cells *in vitro* and *in vivo*. PDT with chlorin e6 — polyvinyl pyrrolidone complex (Photolon) administrated intravenously is a highly effective method for treatment of the patients with skin cancer and intradermal metastases of melanoma and breast cancer. Objective effect of treatment in such patients has been achieved in 100% of cases [6].

The aim of this study was to develop a technique and to investigate antitumor efficiency of PDT using Photolon in the form of 2 or 5% ointment. The investigation tasks were: to develop a technique of topical application of Photolon ointment in tumor-bearing rats; to study the accumulation of 2 and 5% Photolon ointment in normal and tumor tissues of laboratory animals; to evaluate the efficacy of PDT with different concentrations of Photolon ointment applied for different periods — from 15 min to 5 h.

MATERIALS AND METHODS

Experimental animals and tumors. Eighty white randomly bred rats obtained from the vivarium of the N.N. Alexandrov Research Institute of Oncology and Medical Radiology (Minsk, Belarus) were used. The animals received a standard diet and had permanent access to water. SM-1 experimental rat fibrosarcoma was obtained from the tumor strains collection of the N.N. Blokhin Cancer Research Centre of the Russian Academy of Medical Sciences (Moscow, Russia) and was passed by serial transplantation. For experiments tumor homogenate was implanted subcutaneously into the inguinal region by the injection of 0.5 ml of 10% tumor cell suspension in the Hanks' solution. Before the treatment the animals were anesthetized with droperidol (5 mg/kg) and fentanil (0.05 mg/kg) and restrained. All manipulations were carried out according to the international and scientific ethic standards of the quality of planning and carrying out of animal investigations, specifically, according to "Methodic instructions for carrying out preclinical investigations of pharmacokinetics of pharmacologic substances and drugs" presented in the "Guide on experimental (preclinical) study of new pharmacologic substances" // Health Ministry of Russian Federation,

Received: October 17, 2006.
*Correspondence: E-mail: istomin06@mail.ru
Abbreviations used: PDT – photodynamic therapy; PS – photosensitizer.

State Pharmacologic Committee of Russian Federation, Moscow, 2000.

Photosensitizer. Chlorin e6 conjugated with polyvinyl pyrrolidone (Photolon, produced by Scientific Pharmaceutical Center of RUE "Belmedpreparaty", Minsk, Belarus) was used as PS, as an injection form for i. v. injections (control) and as application form for external application (2 and 5% "Photolon" ointment).

Fluorescence diagnosis. Animals were taken for experiment 6–7 days after the tumor inoculation, when the tumors reached the size of 2 cm. The procedures were carried out in a room with light at the low level. Before the investigation the hair and the skin on the top of the tumor (1 cm^2) were removed. Then 0.3 ml of 2 or 5% Photolon ointment was applied on the surface of the tumor and covered with a polyethylene film and a foil to protect from light and drying. After the Photolon ointment application for 15 min–5 h, the tumor surface was rinsed carefully with warm soap water solution and "Septocide-Sinergy" antiseptic. For comparison an injection form of Photolon was administered into the tail vein at the dose of 2.5 mg/kg of body weight of the animal.

The accumulation dynamics of PS in tumor and normal tissues (the skin of the contralateral thigh) was recorded by the method of the lifetime computerized fluorescence spectrophotometry using laser-fibrous spectroanalyzer "LESA-6" with helium-neon "LHN 633-25" laser (BioSpec, Moscow, Russia). For monitoring in real time, every time interval after injection or topical application of the preparation, the distal (diagnostic) end of the catheter was placed over the tumor or the skin of the contralateral thigh to obtain the comparative spectrum of the diffuse reflection and the fluorescence of the preparation. The method provides PS level monitoring under selected conditions of the pharmacokinetic experiment, specifically, guarantees the required follow-up duration, and corresponds to general standards of selectivity, precision and reproduction.

Photodynamic therapy. Photoirradiation of tumors was carried out 3–4 h after i. v. Photolon injection at the dose of 2/5 mg/kg. It was the time of maximum accumulation of the preparation according to the data of spectral-fluorescence investigation.

The SM-1-bearing animals treated with 2 or 5% of Photolon ointment were distributed into 16 groups, 5 rats per group in dependence on the time of ointment application. Photoirradiation was carried out in 15, 30, 45 min, 1, 2, 3, 4 and 5 h after application of 2 or 5% Photolon ointment on the SM-1 surface using semiconductor diode laser "LD 680-2000" (670 nm, BioSpec, Moscow) at the dose of 100 J/cm^2. Power density was 0.51 W/cm^2, the output — 0.4 W, the light spot diameter — 1 cm, irradiation time — 3.27 s.

Antitumor efficacy. Antitumor efficacy of PDT with the injection or application form of Photolon was evaluated 24 h after the treatment by quantitive estimation of necrosis area of SM-1 tumor by a vital staining of tumor bearing animals with 0.6% Evans' blue solution. The animals were sacrificed by use of chloroform; the tumors were removed and fixed in a 10% formalin solution. After fixation, 20–35 transversal sections were made in the largest diameter of the tumor. The sections were registered with a photocamera connected with a computer. To calculate the necrosis area, the method and the program of color tints computer scanning of histotopographic tumor sections have been developed in which the algorithm of recognition of the stained (viable) tumor areas has been fulfilled. Necrotic tumor areas due to direct cytotoxic effect or structural-functional disorders of microcirculation remained unstained (Fig. 1). The main advantage of a designed technique was the direct determination of a size of tumor necrosis that allowed estimate the efficacy of PDT. The automated approach for detection of necrotic zones allows increase the processing rate of the results, as well as to raise calculation accuracy.

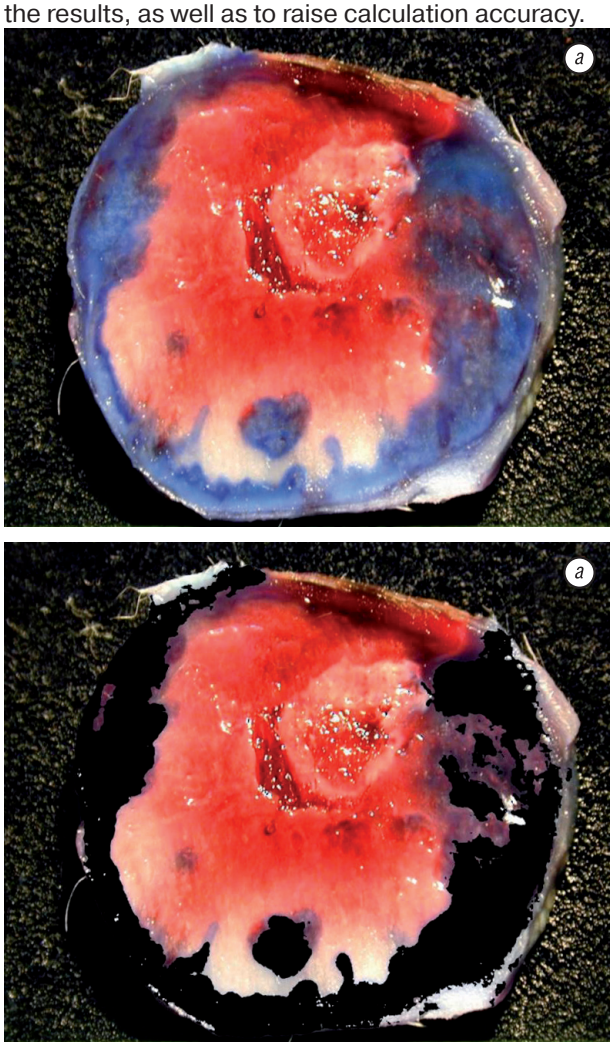

Fig. 1. Histotopographic tumor section before (*a*) and after (*b*) the processing using the method of computer scanning of color tints

Statistical analysis. The values obtained were processed using standard statistical methods of Origin Stat 6.1, Microsoft Excel 2003 programs. The significance level has been determined as 0.05. The data are presented as average values ± standard average error.

RESULTS AND DISCUSSION

Fig. 2 presents the data on Photolon accumulation in normal and tumor tissues of rats depending on the

time after i. v. injection of PS at the dose of 2.5 mg/kg. Photolon accumulation increased in a time-dependent manner and achieved its maximal value at 3–4 h post injection; then the level of a signal decreased. The calculation of a selectivity ratio (contrast), characterizing the ratio of Photolon fluorescence in tumor and normal tissues has shown that the greatest contrast (1.90–2.08) of Photolon accumulation in the tumor tissue of rats was scored at 3–6 h post injection (Table 1).

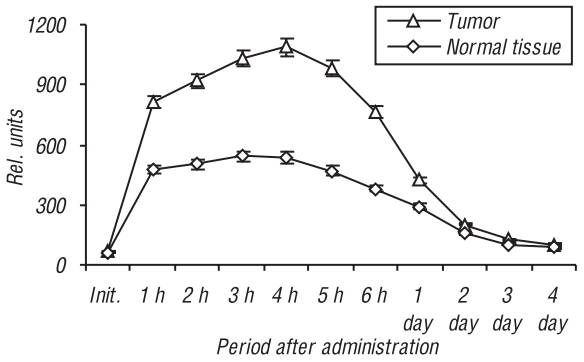

Fig. 2. Photolon accumulation dynamics in rat tissue after i. v. injection of PS at the dose of 2.5 mg/kg

The study of the ointment form of PS has shown that Photolon fluorescence in tumor tissues is dozens times higher than that in normal tissues (fluorescence intensity in normal tissues was not higher than 100 relative units), and fluorescence intensity in tumor directly depended on the period of application and PS concentration in Photolon ointment. Maximal fluorescence in tumor tissue was registered after a 3 h ointment application and yielded 3800 and 5500 relative units for 2 and was 5% ointment respectively (Fig. 3).

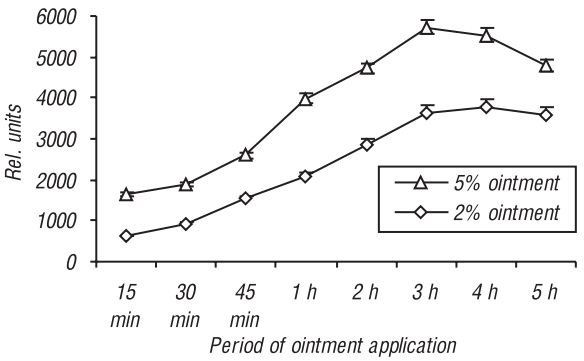

Fig. 3. Photolon accumulation dynamics in SM-1 tumor of rats following application of 2 and 5% Photolon ointment

Thus, comparative spectral-fluorescence study of accumulation levels of the ointment and injection form of Byelorussian Photolon photosensitizer in rat SM-1 tumors has shown that selectivity of its accumulation in tumor tissue considerably increases when it is applied locally.

Antitumor efficacy of PDT using topical and i. v. application of Photolon was compared. The data obtained have shown that photoirradiation of rat SM-1 tumor at the dose of 100 J/cm^2 3 h after i. v. injection of Photolon at the dose of 2.5 mg/kg led to necrosis of 60.6% of tumor tissue (Table 2).

Table 2. Necrosis area in histotopographic sections of SM-1 tumor of rats after i. v. injection of Photolon at the dose of 2.5 mg/kg and photoirradiation at the dose of 100 J/cm^2

Time interval	Number of sections	Tumor square, cm^2	Necrosis square	
			cm^2	%
3 h	30	3.59 ± 0.13	2.15 ± 0.09	60.60 ± 2.53

Table 3 and 4 show the data of necrosis area in 170 histotopographic sections of SM-1 tumors after application of 2% ointment, and in 232 sections of SM-1 tumors after application of 5% ointment for 15 min to 5 h followed by photoirradiation at the dose of 100 J/cm^2. The study of antitumor effect revealed the direct relation between duration of ointment application and the size of necrotic area in histotopographic tumor sections. Significant antitumor efficacy of PDT was achieved upon application of 2 or 5% Photolon ointment for 3 h (necrotic area in histotopographic sections of animal tumors were 53.6 and 77.8% respectively). Thus, the study has shown that the antitumor efficacy of PDT using topical application of Photolon is just as high as in the case of intravenous injection of the drug.

Table 3. Necrosis area in histotopographic sections of SM-1 tumor of rats after 2% Photolon ointment application and photoirradiation at the dose of 100 J/cm^2

Time interval	Number of sections	Tumor square, cm^2	Necrosis square	
			cm^2	%
15 min	20	2.26 ± 0.17	0.31 ± 0.03	13.30 ± 3.13
30 min	20	2.87 ± 0.12	0.56 ± 0.03	19.70 ± 1.09
45 min	20	2.17 ± 0.20	0.56 ± 0.05	26.80 ± 1.51
1 h	20	3.25 ± 0.20	1.07 ± 0.09	33.85 ± 2.60
2 h	25	2.35 ± 0.19	0.96 ± 0.08	41.88 ± 1.81
3 h	25	3.34 ± 0.21	1.76 ± 0.09	53.64 ± 1.31
4 h	20	2.48 ± 0.09	0.94 ± 0.07	37.70 ± 2.32
5 h	20	2.68 ± 0.15	0.74 ± 0.05	27.60 ± 0.97

Table 4. Necrosis area in histotopographic sections of SM-1 tumor of rats after 5% Photolon ointment application and photoirradiation at the dose of 100 J/cm^2

Time interval, h	Number of sections	Tumor square, cm^2	Necrosis square	
			cm^2	%
15 min	25	1.56 ± 0.07	0.48 ± 0.04	31.92 ± 2.25
30 min	30	2.52 ± 0.12	1.13 ± 0.30	32.16 ± 1.66
45 min	28	3.02 ± 0.11	1.62 ± 0.12	52.14 ± 2.44
1 h	35	1.72 ± 0.05	0.97 ± 0.05	58.02 ± 1.97
2 h	28	3.03 ± 0.11	1.88 ± 0.09	62.21 ± 2.05
3 h	30	4.11 ± 0.10	3.18 ± 0.09	77.80 ± 1.17
4 h	31	2.37 ± 0.21	1.33 ± 0.08	60.02 ± 2.21
5 h	25	2.51 ± 0.14	1.60 ± 0.11	61.76 ± 2.49

SM-1 experimental rat fibrosarcoma was used as a model to investigate the efficacy of topical application of chlorin e6 photosensitizer Photolon. In our investigation, it was detected that the selectivity of PS accumulation in tumor tissue using the ointment form of Photolon was significantly higher than using the injection of Photolon. Tumor damage after PDT with use of Photolon ointment was found to be just as high as upon intravenous injection of Photolon.

The results obtained indicate that the level of PS accumulation in tumor tissue using topical application of chlorin e6 photosensitizer Photolon enables to carry

Table 1. Photolon accumulation contrast in SM-1 tumor of rats after i. v. injection of 2.5 mg/kg

Time	1 h	2 h	3 h	4 h	5 h	6 h	1 day	2 day	3 day	4 day
Tumor	814.9 ± 40	918.0 ± 49	1030.5 ± 44	1086.7 ± 46	980.9 ± 35	760.7 ± 46	423.6 ± 14	202.0 ± 13	128.0 ± 1	101.1 ± 0,9
Normal tissues	475.5 ± 12	502.5 ± 12	541.5 ± 11	534.5 ± 11	469.8 ± 6	381.4 ± 16	288.7 ± 13	155.4 ± 4	103.9 ± 5	86.4 ± 4.2
Contrast	1.71	1.82	1.90	2.03	2.08	1.99	1.46	1.30	1.24	1.17

out fluorescence diagnosis and PDT of superficially localized malignant tumors. We can assume that clinical application of Photolon ointment for photodynamic treatment of tumors of topical localization will allow avoid systemic side effects of the therapy, simplify essentially the PDT technique, decrease significantly the amount of PS required for the treatment and to receive appreciable economic benefit in comparison with conventional methods of treatment.

REFERENCES

1. **Kutsenok VV, Gamaleya NF.** Photodynamic therapy 0f malignant tumors. Onkologiya 2003; **5**: 69–72 (in Russian).

2. **Hasan T, Ortel B, Moor A, Brian W.** Photodynamic therapy of cancer. Cancer Medicine 2003; **6**: 605–22.

3. **Wong TW, Aizawa K, Sheyhedin I, Wushur C, Kato H.** Pilot study of topical delivery of mono-L-aspartyl chlorin e6 (NPe6): implication of topical NPe6-photodynamic therapy. J Pharmacol Sci 2003; **93**: 136–42.

4. **Manivasager V, Wan Sia Heng P, Hao J, Zheng W, Chee Soo K, Olivo M.** Macro-microscopic fluorescence imaging of human NPS xenografts in a murine model using topical vs intravenous administration of 5-aminolevulinic acid. Int J Oncol 2002; **21**: 1003–7.

5. **Lui H, Salasche S, Kollias N, Flotte T, Mclean D, Anderson RR.** Photodynamic therapy of nonmelanoma skin cancer with topical aminolevulinic acid; a clinical and histological study. Arch Dermatol 1995; **131**: 737–8.

6. **Stranadko YF, Ryabov MV.** Photodynamic therapy of skin cancer with preparation "Fotolon": expierence in application and optimization of parameters. Laser Medicine 2006; **10**: 4–10 (in Russian).

ИССЛЕДОВАНИЕ ФОТОДИНАМИЧЕСКОЙ ЭФФЕКТИВНОСТИ МЕСТНОГО ПРИМЕНЕНИЯ КОМПЛЕКСА ХЛОРИН е6 — ПОЛИВИНИЛПИРРОЛИДОН В ЭКСПЕРИМЕНТЕ НА КРЫСАХ-НОСИТЕЛЯХ ОПУХОЛЕЙ

Цель: **исследовать противоопухолевую эффективность фотодинамической терапии с аппликационной формой комплекса хлорин е6 — поливинилпирролидон.** ***Методы:*** **мазь, содержащую 2 или 5% хлорина е6, наносили на поверхность опухоли Са М-1 крыс на 15 мин–5 ч, а затем определяли накопление фотосенсибилизатора и повреждение опухоли лазерным излучением.** ***Результаты:*** **при местном введении фотосенсибилизатора избирательность накопления хлорина е6 в опухолевых тканях значительно возрастала, по сравнению с внутривенным введением. По противоопухолевой эффективности фотодинамической терапии местное применение комплекса хлорин е6 — поливинилпирролидон не уступало внутривенному введению препарата.** ***Выводы:*** **уровень накопления фотосенсибилизатора в тканях крыс при использовании мазевой формы комплекса хлорин е6 — поливинилпирролидон позволяет проводить как флуоресцентную диагностику, так и фотодинамическую терапию поверхностно расположенных злокачественных опухолей.**

Ключевые слова: **фотодинамическая терапия, фотосенсибилизаторы, хлорин е6, саркома М-1 крыс.**

Copyright © Experimental Oncology, 2006