

EXPERIMENTAL STUDY OF ANTIANGIOGENIC AND PHOTODYNAMIC THERAPIES COMBINATION FOR TREATMENT OF PERITONEAL CARCINOMATOSIS: PRELIMINARY RESULTS

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Aim: To create adequate orthotopic model of peritoneal carcinomatosis in rats using a transplantable rat tumor M-1 sarcoma, to assess the early tumor response after intraperitoneal photodynamic and/or antiangiogenic therapy for peritoneal carcinomatosis. **Methods:** In 14–18 days after intraperitoneal inoculation, eighteen tumor-bearing animals were divided into three groups and undergone intraperitoneal photodynamic therapy and/or antiangiogenic therapy. Assessment of the tumor posttreatment changes was performed using a method of vital staining with Evans blue, MRI-monitoring and morphologic investigation. **Results:** Percentage of necrosis in disseminated tumors of animals undergone combination therapy significantly higher then after each of the methods alone and achieved 89.46% vs 41.47% after antiangiogenic therapy and 69.73% after photodynamic therapy. Contrast-enhanced MRI showed entirely necrotic tumor nodes in rats undergone the combination therapy. Morphologic study confirmed that tumor response after combination therapy was characterized by maximal spread of necrotic and inflammatory changes in tumor. **Conclusion:** Preliminary results demonstrate enhance of the treatment outcome after combination of antiangiogenic and intraperitoneal photodynamic therapies for peritoneal carcinomatosis in rats. **Key Words:** orthotopic model, peritoneal carcinomatosis, antiangiogenic therapy, intraperitoneal photodynamic therapy.

Peritoneal carcinomatosis is a pattern of disease spread in gastrointestinal malignancies, ovarian cancers and sarcomas. It has always been regarded as a terminal condition. Important natural history studies established a 3-month median survival and 6-month overall survival in this group of patients [1]. Nowadays standard strategy for management of peritoneal carcinomatosis includes aggressive surgery combined with hyperthermic intraperitoneal chemotherapy [2]. Though the results of treatment for the patients are improved, the problem of peritoneal carcinomatosis is still unresolved [3, 4].

Photodynamic therapy (PDT) is a modality based on cytotoxic effect of radical oxygen species through activation of the photosensitizer by light of the appropriate wavelength at the treatment site. The antitumor effects of PDT result from direct tumor cell kill, leading to necrosis, damage to the vasculature, leading to the ischemia, and activation of nonspecific inflammation. Clinical trial of Photofrin-mediated intraperitoneal PDT (i. p. PDT) for patients with peritoneal carcinomatosis has shown feasibility of this method [5]. On the other hand, stimulation of pro-angiogenic factors release following PDT results to increase of VEGF, the most powerful factor of neoangiogenesis that leads to restore of the microvasculature and reperfusion of tumor. The pro-angiogenic response may help the malignant cells survival and further spread upon peritoneal surface and to attenuate therapeutic action of PDT. On base of understanding of the pathways controlling cellular functions, host/cancer interactions and mechanisms of cancer cell survival, photodynamic therapy has been combined with antiangiogenic therapy

and demonstrated improved antitumor effect for prostate and pancreatic cancer in experimental studies [6–8].

Tumor host microenvironment is known to influence tumor cell gene expression, features of tumor growth, angiogenesis, metastases, drug delivery and sensitivity to therapeutic agents [9]. The microenvironment of the peritoneum and characteristics of the tumor contribute to the unique conditions for dissemination, distribution of photosensitizer and response to the photodynamic and/or antiangiogenic therapy. At the present time in preclinical trials of new therapeutic agents orthotopic models are considered to be adequate for correct evaluation of their anticancer efficacy [10, 11].

A sensitive and non-invasive assessment of tumor response to angiogenic therapy and PDT would allow evaluating the effectiveness of the treatment, to predict outcomes and to make recommendation on treatment scheme modification. Magnetic resonance imaging (MRI) can noninvasively provide a wealth of information regarding tumor morphology, metabolism and pathology, thereby allowing the assessment of the response to treatment by changes induced on these parameters. Several MRI techniques have been employed for the detection of response to therapy in various types of cancer in both clinical and preclinical models [12]. Recently, dynamic contrast-enhanced magnetic resonance imaging has emerged as a useful technique for noninvasive imaging of tumor vasculature. This technique yields parameters related to tissue perfusion (T2w method) and permeability (T1w method) [13]. Diffusion-weighted magnetic resonance imaging (DWI) is also a powerful tool for the assessment of damage induced in tumors by cytotoxic therapies when changes in cell vitality are often associated with significant changes in water diffusion [12]. Preclinical work has shown that DWI is able to discriminate between nonperfused but viable and nonperfused, nonviable (necrotic) tissues [14].

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Abbreviations used: DWI – diffusion-weighted imaging; MRI – magnetic resonance imaging; PDT – photodynamic therapy; i. p. PDT – intraperitoneal PDT; Sa M-1 – M-1 sarcoma.

The purposes of this pilot preclinical work were to create adequate orthotopic model of peritoneal carcinomatosis in rats, to determine the early posttreatment morphologic changes in the intraperitoneal disseminated tumor, to assess the early tumor response after i.p. PDT and/or antiangiogenic therapy for peritoneal carcinomatosis using method of vital staining with Evans blue and MRI-monitoring.

MATERIAL AND METHODS

Animals and tumor model. The pilot study was carried out on eighteen white randomly bred rats obtained from the vivarium of the N.N. Alexandrov National Cancer Center of Belarus (Minsk, Belarus). The animals were kept on the usual daily diet.

M-1 sarcoma (SaM-1), a transplantable rat tumor, was used in the experiments. The strain was obtained from the Oncological Research Centre of the Russian Academy of Medical Sciences and was passed by serial transplantation. Tumor inoculation for experiments was as follow. Tumor nodes were isolated and homogenized, then Hank's solution was added to yield 10% suspension. Inoculation was carried out via laparotomy performed under general anesthesia (droperidol 5 mg and fentanyl 0.05 mg per 100 g of body mass). The abdomen was shaved and cleaned with 70% alcohol. The laparotomy was performed using a lower middle incision of 2 cm. The tumor cell suspension was injected under the capsule of the peritoneal surface in the left lower side of the abdomen. The experiments were performed 14–18 days after tumor transplantation.

All experiments were conducted in the compliance with regulation of international scientific ethic standart of the quality of planning and carrying out of animal investigations, specifically, according to "Methodology instruction for carrying out preclinical investigation of pharmacokinetics of pharmacologic substances and drug" presented in the "Guide on experimental (preclinical) study of new pharmacologic substances" // Health Ministry of Russian Federation, State Pharmacologic Committee of Russian Federation, Moscow, 2000.

Intraperitoneal PDT and/or antiangiogenic therapy. Chlorin e6 conjugated with polyvinyl pyrrolidone (Fotolon) photosensitizer (RUE "Belmedpreparaty", Minsk, Belarus) was injected in tail vein in standard dose 2.5 mg/kg.

Bevacizumab (Avastin) anti-vascular endothelial growth factor antibody (Genentech, Inc.; South San Francisco, CA) was injected in tail vein in dose 15 mg/kg.

Treatment was carried out in 18 days after inoculation when primary tumor reached diameter of 10–15 mm and peritoneal dissemination developed. All tumor-bearing animals were divided into three groups and undergone i. p. PDT (n = 5), Avastin treatment (n = 5) or i. p. PDT + Avastin treatment (n = 5). Three untreated animals were used as control.

Intraperitoneal PDT included Fotolon injection and illumination of abdominal cavity at laparotomy under general anesthesia (droperidol 5 mg and fentanyl 0.05 mg per 100 g of body mass). The abdomen was shaved and cleaned with 70% alcohol. Illumination regimen: power density of 0.03 W/cm², a total light dose of

5 J/cm² for the bowel by one field with diameter 5 cm and with a boost treatment up to 100 J/cm² from a point with diameter 1.5–2 cm for sites of gross disease. We used semiconductor diode laser "LD 680–2000" (670 nm, BioSpec, Moscow) with flat-cut optical fiber.

Avastin was administered intravenously both as single injection and synchronously with Fotolon injection in the combination with i. p. PDT.

Tumor response evaluation. For assessment of the early posttreatment response the post-PDT (n = 3), post-Avastin (n = 3), post-PDT + Avastin (n = 3) and two control rats were undergone to investigation by the method of vital staining with Evans blue in 4 days after therapy. For this purpose 0.6% solution of the dye was i.v. injected at 1 ml per 100 g of body mass. The animals were sacrificed in 2 h after injection; the tumor nodes were resected, fixed for 1 h in 10% formalin and frozen. Transverse tumor sections 1–1.5 mm in thickness were made. Necrotic tumor areas due to direct effect or structural-functional disorders of microcirculation remained unstained. Percentage of the necrotic unstained parts of the tumor were estimated using program ImageJ (NIH, Bethesda, USA).

In the remaining post-PDT (n = 2), post-Avastin (n = 2), post-PDT + Avastin (n = 2) and one control rat, tumor posttreatment changes were evaluated noninvasively by means of MRI and correlated with morphologic data. MRI scans were performed under general anesthesia. Baseline MRI was performed before treatment and consisted of coronal and transversal T2w and native T1w images followed by T1w images after intravenous injection of Gadolinium (Omniscan, GE Healthcare). Control MRI was carried out in 4 days after treatment following the same protocol. Animals were euthanized using chloroform immediately after MRI.

Morphological method of posttreatment changes evaluation. The treated and control animals were euthanized using chloroform after the last MRI scans at 4 days post-PDT (n = 2), post-Avastin (n = 2), post-PDT + Avastin (n = 2). At necropsy, the tumors were removed, photographed and fixed in 10% neutral buffered formalin for over 24 h. H&E staining of parallel sections of tumor nodes was done using standard protocol of histologic investigation.

RESULTS

Biological and morphologic characteristics of intraperitoneal sarcoma M-1 growth. Intraperitoneal transplantation of 0.25–0.3 ml 10% tumor cell suspension results in moderate percentage (85%) of tumor yields with latency of 8 days. Primary tumors were indicated by palpation in nine days. Intraperitoneal sarcoma M-1 developed peritoneal carcinomatosis without fail in 14–16 days after transplantation (Fig. 1). Firstly disseminates spread locoregionally, then seeding expanded over whole abdominal cavity. Ascitis was observed with advanced dissemination. Rats died in 20–22 days after inoculation due to widespread of tumor mass in abdomen (Table 1).

Table 1. Biological characteristics of intraperitoneal sarcoma M-1 growth

Experimental model	Inoculation, %	Latency, days ± SD	Carcinomatosis development, %	Period of carcinomatosis development, days	Mean life span, days ± SD
Sa M-1	85	8.6 ± 1.9	100	14–16	20.7 ± 1.9

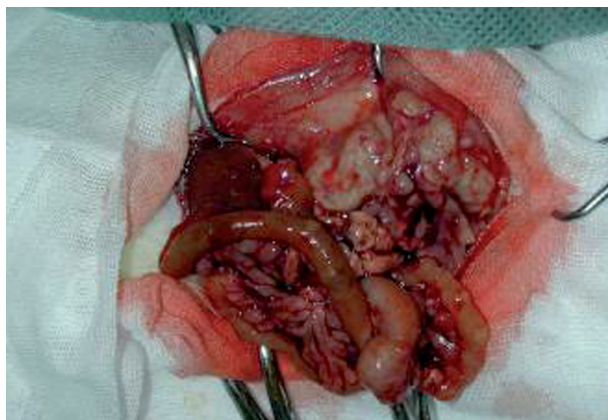


Fig. 1. Orthotopic model of peritoneal carcinomatosis in rats

Microscopically, tumor was characterized by nodular growth pattern with pseudocapsule formation and small central areas of necrosis, cytologically typified by round tumor cells with abundant eosinophilic cytoplasm, vesicular nuclei and numerous mitotic figures. Capillaries proliferation was seen at the periphery of tumor lesions and was accompanied by weak inflammatory reaction.

Earlypost-treatment changes. Earlypost-treatment necrotic changes after intraperitoneal PDT and/or antiangiogenic therapy were assessed using method of vital staining with Evans blue. Table 2 shows the data of necrosis area in 77 histotopographic sections of SM-1 after antiangiogenic therapy, i. p. PDT, combination and untreated control.

Table 2. Necrosis area in histotopographic sections of intraperitoneal SM-1 tumor of rats after treatment

Groups	Number of animals	Number of sections	Tumor square $\pm$ SD, cm ²	Necrosis square $\pm$ SD	
				cm ²	%
Avastin	3	18	1.53 $\pm$ 0.46	0.62 $\pm$ 0.44	41.47 $\pm$ 11.11*
i. p. PDT	3	21	0.83 $\pm$ 0.52	0.62 $\pm$ 0.23	69.73 $\pm$ 12.67**
i. p. PDT + Avastin	3	20	0.58 $\pm$ 0.55	0.51 $\pm$ 0.5	89.46 $\pm$ 7.21***
Control	2	18	2.1 $\pm$ 0.45	0.67 $\pm$ 0.21	32.5 $\pm$ 7.54

*i. p. PDT vs Avastin, $p = 0.000$; **i. p. PDT + Avastin vs i. p. PDT, $p = 0.000$;

***i. p. PDT + Avastin vs Avastin, $p = 0.000$.

Percentage of necrosis in groups of animals undergone Avastin, i. p. PDT and i. p. PDT + Avastin was 41.47% (Fig. 2, a), 69.73% and 89.46% (Fig. 2, b) respectively. Spontaneous necrosis area in control animals spread up to 32.5% (Fig. 2, c).

MRI scan. Baseline MRI scans showed disseminated intraperitoneal tumors as heterogenous nodes with defined margins. On follow-up MRI scans, control and treated tumors could be defined with areas of varying amount of necrosis clearly seen in all cases. Contrast-enhanced images of control (Fig. 3, a), post-PDT (Fig. 3, b) and

post-Avastin rats demonstrated tumor nodes with central non-enhancing area of necrosis and viable enhancing tumor tissue on the periphery. In post-PDT + Avastin rats totally necrotic tumor nodes were revealed (Fig. 3, c).

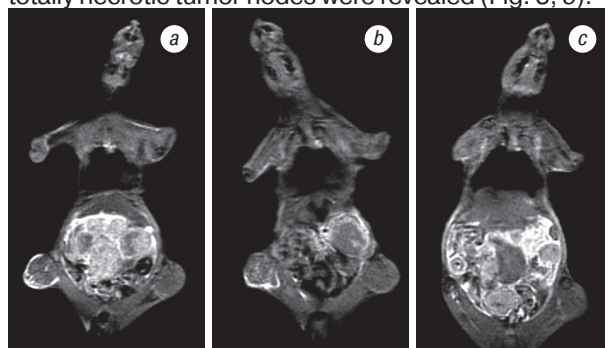


Fig. 3. Contrast-enhanced MRI scans (a — control untreated tumor; b — post-PDT tumor; c — post-PDT + Avastin tumor)

Histological findings. Microscopically, the most extensive changes were seen in the post-PDT + Avastin tumor tissue, they were characterized by the areas of confluent necrosis accompanied by capillaries proliferation at the margins and numerous eosinophils in the infiltrate (Fig. 4, a). The post-PDT + Avastin tumor necrosis was subtotal and occupied 90% of tumor tissue (Fig. 4, b). The PDT alone induced necrosis that was less prominent and extended approximately 75% of tumor tissue (data are not shown). Sparse inflammatory infiltrate contained few eosinophils. The Avastin alone led to tumor necrosis that was seen in less than 50% of lesion and was associated with hardly defined inflammation (data are not shown). Unlike tumor necrosis caused by therapy, spontaneous necrosis in the control tumor was weak and represented less than 30% of tissue (Fig. 4, c).

DISCUSSION

A numerous studies emphasize the importance of using orthotopic tumor model in preclinical evaluation of cancer treatment [9–11]. Created model of peritoneal carcinomatosis in rats using intraperitoneal transplantation of sarcoma M-1 cells suspension is considered to be reproducible and adequate for correct evaluation of anticancer treatment efficacy.

Since the abdomen is a very complicated area with lots of loops of bowel, it is very difficult to get a homogenous dose of light to all surfaces. Moreover, accumulation of photosensitizer in small disseminates may be insufficient due to undeveloped microvasculature. There are conditions for suboptimal PDT (either not enough light or not enough photosensitizer or both), that can lead to regrowth

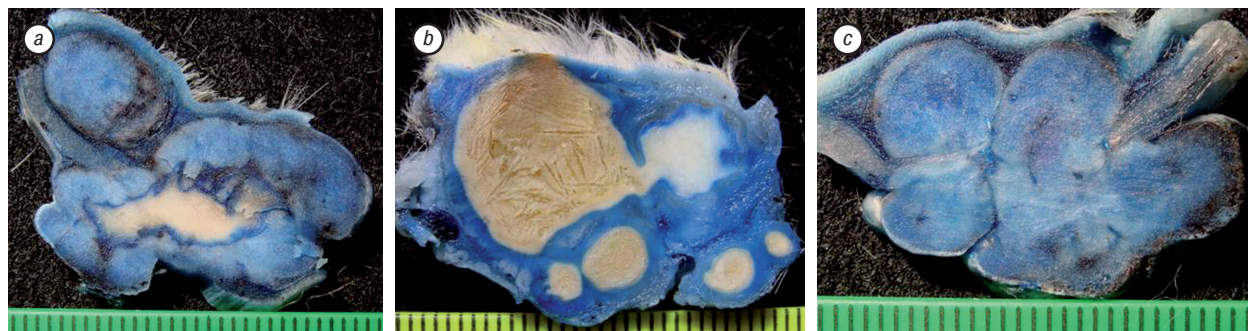


Fig. 2. Histotopographic sections of SM-1 after vital staining with Evans blue (a — post-Avastin tumor; b — post-PDT + Avastin tumor; c — control untreated tumor)

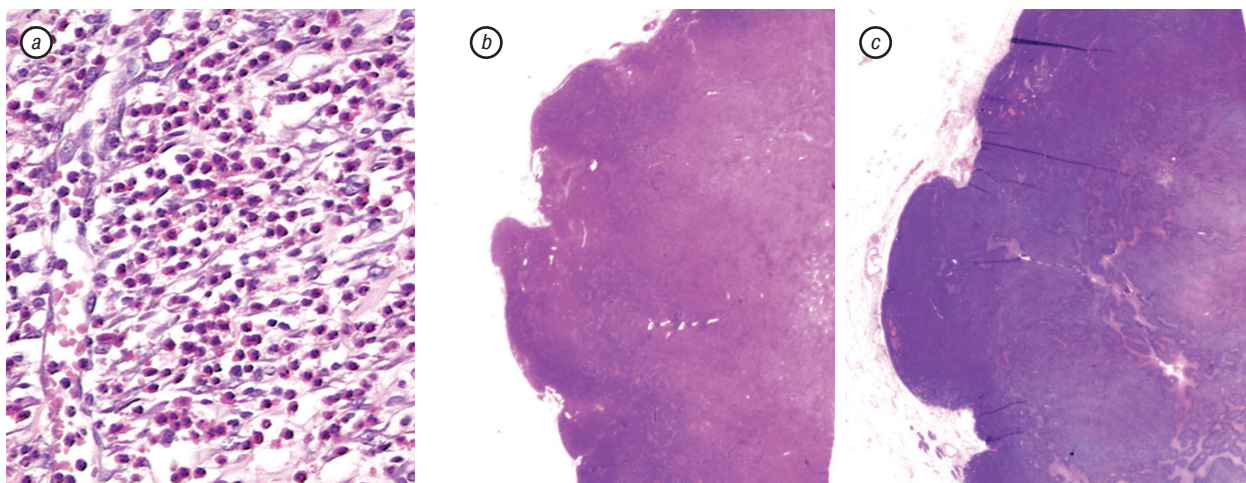


Fig. 4. *a* — eosinophils in the infiltrate and capillaries proliferation at periphery of tumor node; *b* — tumor after PDT + Avastin; *c* — control untreated tumor

and further dissemination. Subcurative PDT-induced increase of VEGF secretion accompanied by an increase of metastases was proved in orthotopic model of prostate cancer [6]. Combination of antiangiogenic therapy with PDT is logical for leveling down proangiogenic effect of PDT and preventing of renovation of tumor vasculature and malignant cells survival. The combination is showing promise for enhancing the treatment outcome in solid tumors. We used intraperitoneal PDT with Fotolon and antiangiogenic therapy with Avastin to determine efficacy of their combination for intraperitoneal disseminated tumors in rats.

As indicated in Table 2, neither Avastin alone nor i. p. PDT alone was able to lead to the significant tumor necrosis. But percentage of necrosis in tumor nodes reached up to 90% when these two suboptimal treatments were combined.

For many anticancer therapies to non-invasively accurately monitor and quantify early tumor response and to determine the effectiveness of treatment regimen is desirable. On contrast-enhanced T1w scans 4 days after PDT or Avastin alone we noted a rim of viable tumor around central necrotic area. This finding could be result of either suboptimal PDT or restoring of tumor vasculature or both and creation of conditions for survival of tumor cells with sublethal injuries and further growth. Synchronous application of Avastin and photosensitizer injections resulted in timely realization of antiangiogenic effect and attenuation of proangiogenic effect of PDT.

Morphologic study confirmed that tumor response after combination i. p. PDT + Avastin was characterized by maximal degree of necrotic and inflammatory changes in tumor.

In summary, in this report we presented some preliminary results demonstrating that the combination of PDT and antiangiogenic therapy enhanced tumor cells death and had significantly higher efficacy in the treatment of peritoneal carcinomatosis of rats comparing with each-method alone. Non-invasive MRI-monitoring helps to optimize sequence and regimen of treatment combination and to assess early posttreatment response.

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