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Investigation of the Effect of Photosensitizer Tiosense on the Tumor Model mel Kor-TurboRFP Expressed Red Fluorescent Protein

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Abstract—The work is devoted to the study of the action of photosensitizer Thiosens on the fluorescent tumor model mel Kor-TurboRFP. It was shown that cell line mel Kor-TurboRFP is sensitive to the phototoxic action of Thiosens *in vitro*. That allows to use of fluorescent tumor mel Kor-TurboRFP to study the photodynamic action of Thiosens *in vivo*. Non-invasive monitoring of fluorescence of tumor mel Kor-TurboRFP sensitized by Thiosens allows independent control of the dynamics of the three-dimensional distribution of the fluorescent tumor and the photosensitizer. This makes it possible to optimize the PDT approach, allowing the most optimal way to choose the time to start of tumor irradiation, irradiation time and the irradiated area patterns.

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INTRODUCTION

Fluorescent diagnostics and photodynamic therapy are based on application of photosensitizers which are deemed to be developing methods for diagnostics and treatment of cancer diseases. Photosensitizers are selectively accumulated in tumor and have anticancer effect upon irradiation with light of appropriate wavelength.

Efficiency of photodynamic damage of biological tissue is mainly determined by the level of photosensitizers's accumulation, its localization in tissue and photochemical activity. One of the problems of photodynamic therapy is increasing of selectivity of accumulation of photosensitizers in tumor. To reduce side-effects caused by photosensitizers' accumulation in some healthy organs and tissues (in liver, spleen, kidneys and in skin) it is necessary investigate the dynamics of photosensitizers' accumulation in tumor and healthy tissue and for each preparation to determine the optimal time for performance of photodynamic therapy. To do this different methods may be applied including noninvasive, for example, spectral fluorescence, spectroscopy of diffusive reflection, etc.

With the help of the tumor models established on the base of the tumor cells expressing colored fluorescent proteins (FP), the progress of the primary tumors and metastasis is possible to monitor in real-time mode. Such models make it possible to visualize very early events in tumor response to drug therapy [1] which is shown in experiments with use of both known drugs and new substances on the stage of preclinical and clinical studies. Application of FP-labeled tumors opens additional opportunities for study of mechanism of photosensitizers' effect and photodynamic therapy efficiency.

There are several works known where different aspects of photodynamic therapy were studied both *in vitro* and *in vivo* with application of FP-expressing tumor cells. Thus, in work [2] on cells of mouse adenocarcinoma CT-26, mitochondria which express CFP, intracellular localization of sulfoderivatives of tetraphenylporphins and phototoxic effect of these photosensitizers on cells have been studied [2]. In work [3] photodynamic therapy on the tumor model expressing EGFP protein has been performed [3]. By means of non-invasive fluorescent visualization the tumor progress was observed and by fluorescence of conjugate of fluorophore with albumin injected after photodynamic therapy the vascular response was obtained [3].

For further investigations on the basis of the human melanoma cell line mel Kor [4] we established

fluorescent cell line mel Kor-TurboRFP expressing red fluorescent TurboRFP protein. The cell line was characterized as per rate of *in vitro* and *in vivo* growth, morphology and immunophenotyping [5]. By using this cell line dynamic of accumulation and distribution of Thiosens infrared liposomal photosensitizer as well as its photodynamic effect on tumor cells and tumor have been studied.

EXPERIMENTAL

Materials. *Cell line of the fluorescent melanoma mel Kor-TurboRFP*. Fluorescent cell line mel Kor-TurboRFP was obtained from cell line of human melanoma mel Kor by liposomal transfection of cells with pTurbo-RFP-C plasmid ("Evrogen," Russia) [6] with the following selection with the help of geneticin antibiotic and cloning (receiving of individual clones of fluorescent cells) with the use of cloning cylinders. Red fluorescent protein TurboRFP is characterized by hyper-bright fluorescence with the maxima of excitation and emission at 553 and 574 nm respectively [6].

Photosensitizer Thiosens on the base of substance of tetra-3-phenyl-thiophthalocyanine of aluminum hydroxide (State Scientific Center "NIOPIK," Russia) in liposomal medicine form (Institute of Experimental Diagnostics and Therapy of Tumors of Blokhin Cancer Research Center of the Russian Academy of Medical Sciences).

Other materials: culture medium DMEM, solution trypsin-EDTA, buffer solution DPBS, mixture of antibiotics penicillin-streptomycin ("Paneco," Russia), fetal calf serum ("Bioclot," Germany).

Fluorescein diacetate ("Invitrogen," UK), 0.5% solution of trypan blue ("Flow Lab.," UK).

Equipment. *Inverted fluorescent microscope* Nikon Eclipse TE2000-U ("Nikon," Japan) for registration of fluorescent cell images *in vitro*.

Fluorescent analytic system UVP iBox with Biolight source light (UVP, USA) and *diffuse fluorescence tomograph* DFT-3 (Institute of Applied Physics of Russian Academy of Sciences, Nizhni Novgorod) for noninvasive production of fluorescent images of animals *in vivo*.

Laser LFT-01-730-BIOSPEK ("Biospek," Russia) with wavelength of light 732 nm for cells irradiation of the cells and tumors.

Fluorimeter Fluoroscan II ("Labsystems," Finland) for fluorescence registration upon detection of cytotoxic effect of photosensitizers on cells monolayers in wells of 96-well plate.

Investigation methods. The experiments were peformed on cell culture mel Kor-TurboRFP (fluorescent human melanoma). The cells were grown in CO_2-incubator (5% CO_2) at 37°C in cultural flasks (surface square 75 cm^2) on DMEM cultural medium (pH 7.2) containing 10% of fetal calf serum and antibiotics (penicillin and streptomycin). The cells were maintained in logarithmic phase of growth by reseeding in 2–3 days in ratio of 1 : 3 or 1 : 4.

Preparation of cells' monolayers. Cells mel Kor-TurboRFP being in logarithmic phase of growth were seeded onto 96-wells cultural plates excluding outside vertical and horizontal lines in complete growth medium (DMEM containing 10% of fetal calf serum, penicillin, 100 units per mL and streptomycin, 100 μg/mL). The amount of cells necessary for preparation of 70–80% confluent monolayer was taken by the moment of the investigation of antitumor effect.

For this purpose the cells were grown in cultural flasks then treated with trypsin-EDTA solution, washed off with complete growth medium followed by centrifugation (1000 rpm for 5 min), then the cells were re-suspended in the complete growth medium until single-cell suspension was obtained. Concentration of the cells in suspension was determined by color staining with trypan blue with the help of haematocytometer [7]. Then the cells were seeded in 60 inner wells of 96-well cultural microplate, 10000 cells in 100 μL of complete growth medium per well and incubated for 36–48 hours until obtaining of 70–80% monolayer of confluence.

Determination of cytotoxicity of the photosensitizer. In black-out conditions the growth medium was exchanged for complete growth medium containing photosensitizer in various concentrations and the cells were kept incubated in CO_2-incubator within different time periods. After incubation the medium containing photosensitizer was removed, the cells were washed off with DMEM without serum, the complete growth medium was added and then the cells were irradiated with laser light under given irradiation power density for different intervals of time and were left for a day. The cells to which the complete growth medium without photosensitizer was added were used as a negative control.

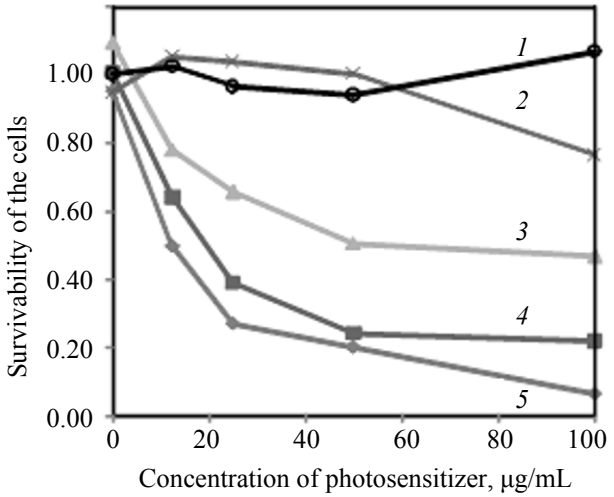

Fig. 1. Dependence of phototoxic effect of Thiosens on its concentration and time of irradiation. Experimental conditions: duration of incubation 20 h, irradiation with laser light with wavelength of 732 nm, "power density of irradiation 115 mW/cm^2. Survivability of the cells was determined by fluorescence of TurboRFP: (*1*) without irradiation; (*2*–*5*) irradiation for 1, 2, 4, 6 min respectively.

The plate with cells was placed on the platform with round diaphragm which restricted the area of irradiation by one well.

To determine dark toxicity of photosensitizer the plates after washing of and replacement of medium were left for a day in CO_2-incubator and then survivability of the cells was determined.

Determination of survivability of cells by intrinsic fluorescence of fluorescent cells. For cells of Chinese hamster's ovaries (CHO) stably expressing green fluorescent protein (GFP), the procedure of estimation of their survivability dependent on composition of medium was earlier proposed. This procedure includes cultivation of cells into the wells of cultural 96-wells microplate and estimation of the cells amount according to fluorescence of GFP protein using microplate fluorimeter [8]. In the present work survivability of the cells after phototoxic effect was estimated according to fluorescence of cells expressing red fluorescent protein.

To do this day after the irradiation the cells were washed off from medium by buffer solution DPBS (200 μL per well) and added 100 μL of the same buffer solution in to each well of the plate followed by measurement of fluorescence on Labsystems Fluoroscan II fluorimeter To excite fluorescence the interference filter with wavelength of spectral maximum 544 nm was used; interference filter with wavelength of the maximum 607 nm was applied for registration. The software which provides interface of the instrument with PC allows data to be obtained in ASCII codes. The obtained data were processed with Microsoft Excel program.

The number of survived cells was determined by using a calibration curve, which is plotted as a dependence of the fluorescent signal (with the exception of the fluorescent signal from the wells "control without cells") on the amount of seeded cells. Upon determination of this dependence the cultural microplate was used where the cells were seeded simultaneously with seeding of cells into the above mentioned plates but as follows: in, the 2nd row the same amount of cells as in plates for determination of antitumor activity; the 3rd to 11th rows contain serial 1.5 or 2-fold dilutions of the cell amounts. The 12th row in all plates was used as control (without cells).

According to the intensity of fluorescent cells' signal in plates intended for determination of anticancer activity taking into account graduated curve the number of survived cells was estimated assuming that initial amount of the cells was the same in all wells of the plate.

Experimental animals and tumor models. Animals used in these experiments were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Pushino branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of Russian Academy of Sciences). Animal studies were conducted in accordance with "Guidelines for experimental (preclinical) studies of new pharmacological substances," issued by the Ministry of Health of Russian Federation, Department of Quality Control, Effectivenes and Safety of Remedies, Scientific Center of Examination and the State Control of Medical Products, Pharmacological State Committee of the Russian Federation (Moscow, 2000).

The efficiency of photodynamic therapy with liposomal Thiosens was determined on fluorescent model of melanoma mel Kor-TurboRFP tumor. Suspension of tumor cells was inoculated subcutaneously into the back of Balb/C-Nu mice (breeded by Blokhin Russian Cancer Research Center of the Russian Academy of Medical Sciences). The inoculating dose was 10^7 tumor cells in 0.2 mL. The tumor cells were inoculated subcutaneously on back of the mice in such a way to exclude the tumor localization in projection of significantly blood-filled organs, such as liver. It

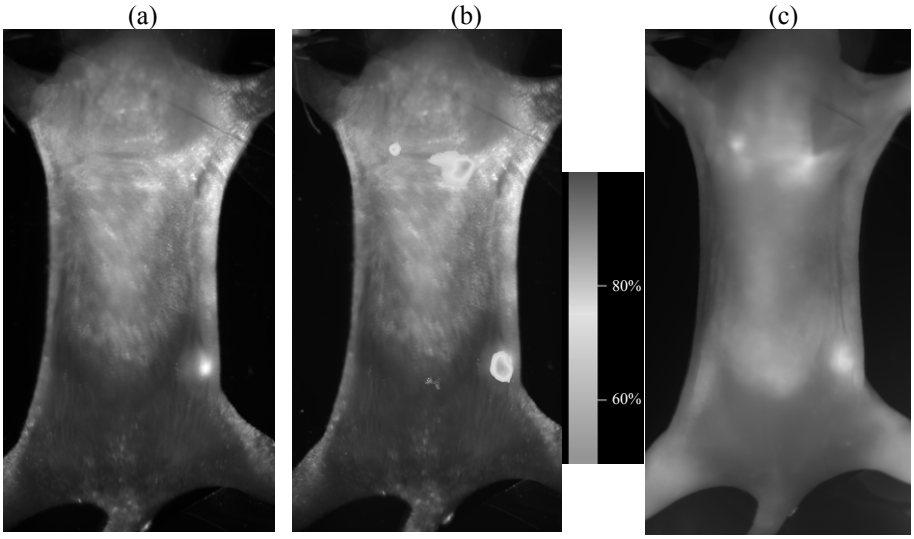

Fig. 2. Fluorescent images of mouse with the tumor after intravenous injection of liposomal Thiosens (6 mg per kg of body weight). TurboRFP: fluorescence excitation 502–547 nm, fluorescence emission 570–640 nm, Thiosens: fluorescence excitation 600–645 nm, fluorescence emission > 700 nm. Superposition of fluorescent images of the tumor and Thiosens was obtained and color-encoded by means of UVP software. (a) Fluorescence of the tumor, (b) superposition of fluorescent images of the tumor and Thiosens, and (c) fluorescence of Thiosens.

allowed to exclude the influence of signal from accumulated photosensitizer in such organs especially during the first hours after injection upon estimation of the accumulation selectivity in tumor compared to the surrounding healthy tissues and avoid damaging of vital organs when tumor is irradiated

RESULTS AND DISCUSSION

Cell line mel Kor-TurboRFP is sensitive to phototoxic effect of Thiosens *in vitro* (Fig. 1) upon prolonged incubation. It allows to use fluoresced tumor mel Kor-TurboRFP for investigation of photodynamic effect of Thiosens *in vivo*.

In order to choose optimal period for irradiation of tumors sensitized with Thiosens, dynamics of its accumulation in normal and tumor tissues was studied at mice of BalbC/Nu line inoculated with fluorescent melanoma mel-TurboRFP.

After injection of Thiosens to the mice with the help of automatic system UVP iBox the fluorescent pictures of fluorescent tumor and photosensitizer distribution were obtained (Fig. 2). Intensity of fluorescence of photosensitizer in the tumor and normal tissue was calculated with the help of program ImageJ 1.42q (NIH, USA). Contents of Thiosens in the tumor achieves its maximum in 24 h after its intravenous injection; at that time index of selectivity is equal to 3. During several following days index of selectivity increases but at the same time content of the photosensitizer in the tumor reduces (Fig. 3).

Based on the data on dynamics of Thiosens accumulation in tumor, it was suggested to irradiate the tumor in 24 h after Thiosens injection when content of photosensitizer in tumor is relatively high and selectivity of its accumulation in tumor is near to maximum compared to the surrounding healthy tissues.

Upon investigation of Thiosens distribution in organs and tissues of mice fluorescence of the photo-

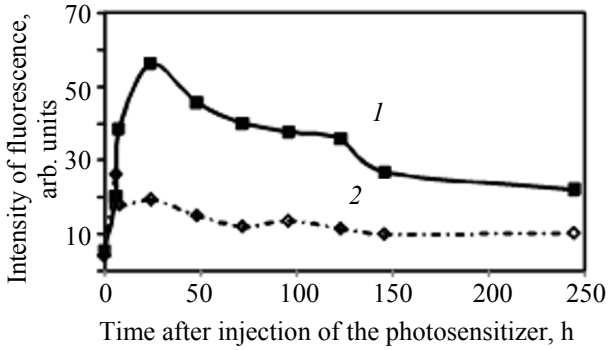

Fig. 3. Dynamics of accumulation of Thiosens in tumor mel Kor-TurboRFP (*1*) and normal tissue (*2*) of mouse.

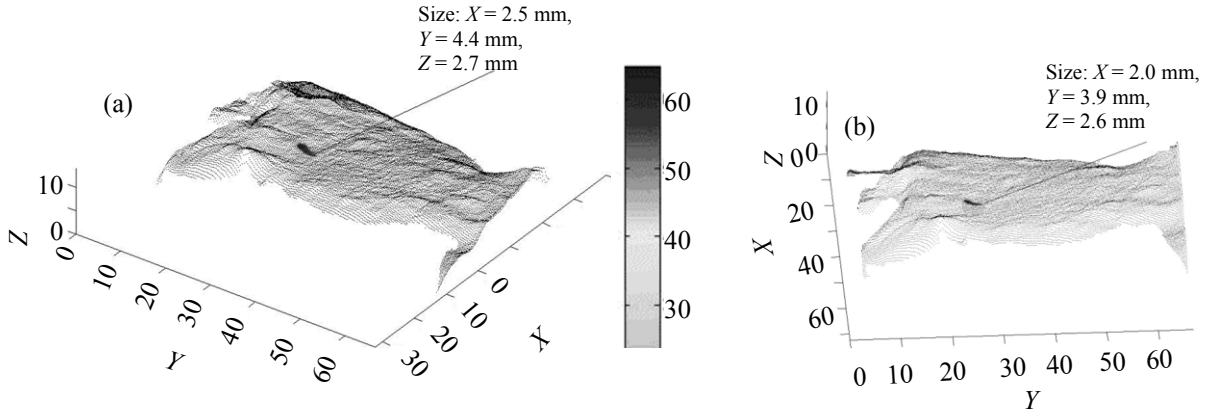

Fig. 4. Reconstructed color-coded 3D models of the tumor before and after photodynamic therapy (PDT): (a) 8th day of the tumor progress, before PDT and (b) 11th day of the tumor progress, after PDT .

sensitizer in the nearest to the tumor lymph node, inguinal was also observed.

When photodynamic therapy was performed liposomal dispersion of Thiosens was injected once intravenously in dose of 4 mg per kg of animal body weight. In one day after injection the tumor was irradiated with laser light with wavelength of 732 nm for 20 min, power density of irradiation was 260 mW/cm^2. Then fluorescent of photosensitizer and TurboRFP protein was monitored. After 20 min of irradiation, there was observed reduction of intensity of the photosensitizer fluorescence on the irradiated area up to background meanings that probably evidences either on change of optical properties of tumor and surrounding tissues, e. g. due to edema or photosensitizer photobleaching (reduction of fluorescence intensity).

Dimensions of tumor on different stages of photodynamic therapy obtained according to the results of 3D-reconstruction

Experimental group	Day after tumor inoculation		
	8 day (before PDT)	11th day (3 day after PDT)	18th day (10 day after PDT)
	Tumor volume, mm^3		
Photodynamic therapy	14.9	10.1	7.7
Control	15.8	20.0	33.4

For precise definition of the tumor volume a 3D-reconstruction of the fluorescent tumor was accomplished according to the data of fluorescence diffuse tomography on fluorescent of TurboRFP protein. This method provides information on emission of fluorescence in different projections obtained by change of relative position of source of fluorescence excitation and detector of fluorophore emission. Compared to planar fluorescence imaging, fluorescence diffuse tomography provides more correct information about fluorescent area of tissue, and specifically it allows to localize this area and investigate a lifetime dynamics of its dimensions change [9]. Fluorescence diffuse tomography allowed controlling the results of photodynamic therapy with Thiosens of the tumor at different time after irradiation (Fig. 4 and table) .

CONCLUSIONS

Noninvasive florescent monitoring of mel Kor-TurboRFP tumor sensitized with Thiosens allows independently controlling in dynamics 3D-distribution of the fluorescence of tumor and photosensitizer that provides opportunity to optimize tactics of photodynamic therapy and choose efficiently time interval for beginning of irradiation after injection of sensitizer and determine duration of irradiation and irradiated area patterns as well.

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