PRIMARY BIOLOGICAL SCREENING OF PHOTOSENSITIZERS FOR PHOTODYNAMIC ACTIVITY ON A NONTUMOR MODEL IN VIVO

V. E. Normanskii and N. T. Raikhlin

UDC 615.831.4.015.21:615.263].038

KEY WORDS: photosensitizers; screening; laser

Development of a method of screening photosensitizers for photodynamic activity is a cardinal problem in the creation of an effective therapeutic agent for photodynamic therapy (PDT).

An objective screening method for biological monitoring of photosensitizer quality is essential at nearly all stages of processing of the therapeutic preparation: initially to select a biologically active substance for further trials, and later for operative quality control of the photosensitizer in different experimental batches during the elaboration of methods of production for use in large laboratories and on an experimental production scale, sterilization techniques, estimates of shelf life, and so on.

In this paper we suggest a new practical approach to the evaluation of photodynamic activity (PDA) of photosensitizers (PS).

EXPERIMENTAL METHOD

The in vivo screening method we developed is as follows: healthy noninbred male laboratory mice weighing 18-20 g were sensitized by intravenous injection of a photosensitizer. Irradiation was carried out 24 h later: after secure fixation of the sensitized animals and removal of hair from the anterior abdominal wall, the region of the liver was irradiated under visual control without laparotomy. Next day, only standard results were recorded on the surface of the liver: one focus of necrosis, with a regularly circular shape. Morphometric measurements of the area of the foci of necrosis were carried out with the aid of standard measuring systems and a binocular loupe.

The adequacy of this method of screening of photosensitizers on the basis of their PDA in order to select the optimal PS was assessed in experiments to study PDT of a transplantable tumor model in vivo. In this case the main parameter for evaluation of the antitumor effect of PS was the number of tumor-bearing animals in which no tumors could be detected either visually or by palpation one month after PDT.

As the experimental tumor model a solid form of transplantable Ehrlich's ascites carinoma on the 6th day after subcutaneous transplantation of ascites suspension of tumor cells, numbering $5 \cdot 10^6$, was used.

Two classes of compounds were used as PS: first generation PS – commercial therapeutic preparations, HpD derivatives – Photosan-2 (Ps-2), Photosan-3 (Ps-3) (obtained from Germany), Photofrin-2 (Pf-2) (Canada), and MPD (from China); second generation PS – three samples of sulfated aluminum phthalocyanine ($AlPcS_x$): with predominance of the monosulfated component ($AlPcS_1$); with predominance of tri- and disulfated component ($AlPcS_{2-3}$); and finally, tetrasulfated aluminum phthalocyanine ($AlPcS_4$). For comparative screening of photosensitizers on the basis of their PDA they were used in the same dose, namely 10 mg/kg. For PDT, because of the great difference in their PDA, these preparations were used in different dosages: $AlPcS_{2-3}$ in a dose of 10 mg/kg, the rest (Pf-2, $AlPcS_1$, $AlPcS_4$ – in a dose of 25 mg/kg). A tunable argon injection dye laser, manufactured by "Coherent" (USA), model D-2000, was used as the laser source. The spectral range 630 ± 5 nm was used for comparative analysis of the PDA and antitumor efficacy of PS that are HpD derivatives, and 670 ± 5 nm for $AlPcS_x$.

Research Institute of Organic Semifinished Products and Dyes, Oncologic Scientific Center, Russian Academy of Medical Sciences, Moscow. (Presented by Academician of the Russian Academy of Medical Sciences Yu. N. Solov'ev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 114, No. 12, pp. 646-648, December, 1992. Original article submitted March 18, 1992.

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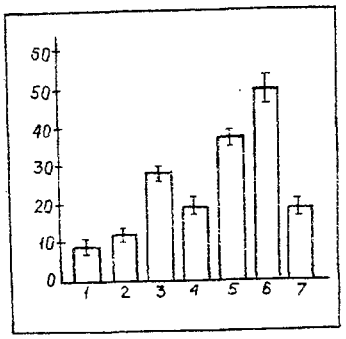

Fig. 1. Comparative analysis of photosensitizers on nontumor model in vivo, based on area of focus of necrosis, in mm^2: 1) Photosan-1, 2) Photosan-3, 3) Photofrin-2, 4) $AlPcS_4$, 5) $AlPcS_1$, 5) $AlPcS_{2-3}$, 7) HpD.

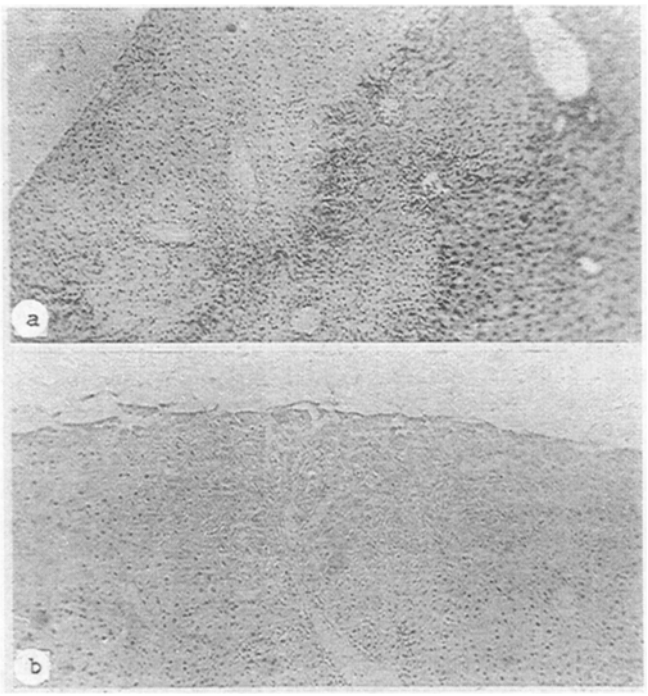

Fig. 2. Histologic characteristics of foci of photodynamic liver tissue necrosis one day after laser irradiation: a) predominance of karyopycnosis in focus of photodynamic liver tissue necrosis in animal sensitized with HpD. 100×. Stained with hematoxylin and eosin; b) zones of karyorrhexis in focus of photodynamic liver tissue necrosis in animal sensitized with $AlPcS_{2-3}$. Magnification 100. Stained with hematoxylin and eosin.

Irradiation of the sensitized animals for PS screening on the basis of their PDA was carried out by means of a collimated laser beam 2 mm in diameter. with a power of 20 mW, and in a total dose of 144 J/cm^2.

The test object for the morphologic investigation consisted of modified areas of the liver parenchyma, on the day after irradiation. Autopsy material for histologic investigation was fixed in 5% neutral formalin and embedded in paraffin wax in the usual way; histologic sections were stained with hematoxylin and eosin. Material for electron

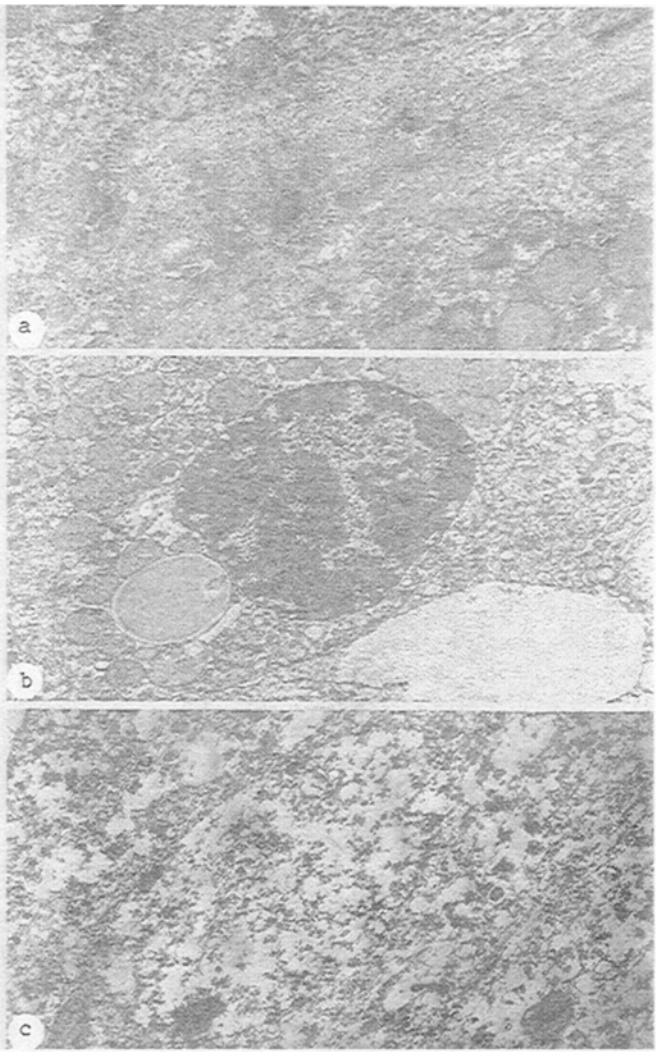

Fig. 3. Characteristics of foci of photodynamic necrosis of liver 1 day after laser irradiation: a) necrosis of Kupffer cell. 12,000×; b) vacuolation of hepatocyte cytoplasm with destruction of mitochondrial cristae and with karyopycnosis in animals sensitized with HpD. 12,000×; c) lysis of cytoplasmic organelles and karyolysis of hepatocyte in animal sensitized with $ASPcS_{2-3}$. 20,000×.

microscopy was fixed in 2% glutaraldehyde in cacodilate buffer, pH 7.4, and embedded in Epon-812. Ultramicrotomy was carried out on the "LKB-3" Ultrotome (Sweden). The investigation was carried out on an IEM-100C electron microscope.

EXPERIMENTAL RESULTS

Combined action on the liver of the sensitized animals led to the appearance of a focus of necrosis on the liver surface 24 h after irradiation. Macroscopically this focus consisted of clearly outlined pale spots against the

background of intact liver tissue, and on section, the standard foci of necrosis as a rule were shaped like a truncated cone.

After morphometric investigation and statistical analysis of the results of comparative analysis cf PDA, the series of PS described above can be arranged in the following order (Fig. 1): $AlPcS_{2-3}$ > $AlPcS_1$ > Pf-2 > $AlPcS_4$ ≥ HpD > Ps-3 > Ps-2. Control experiments involving PDT, using Pf-2 and AlPcS derivatives on a model of transplantable Ehrlich's carcinoma revealed a directly proportional relationship between PDA cf the PS and their antitumor efficacy: $AlPcS_{2-3}$ = 90, $AlPcS_1$ = 60, $AlPcS_4$ = 25, and Pf-2 = 50%.

Histologic investigation of foci of necrosis revealed the zonal character of photodynamic damage, and four zones were identified successively on the liver surface: with karyorrhexis, with karyopycnosis, with necrolytic changes, and a zone of paranecrosis.

It must be pointed out that the dimensions of the zones of liver damage depend on the class of PS. In the case when HpD derivatives were used, the main of the bulk of necrosis was occupied by a zone of necrosis with karyopycnosis (Fig. 2a). Conversely, when second generation PS were used, based on $AlPcS_x$, the main bulk of photodynamic necrosis consisted of a zone of necrosis with karyorrhexis (Fig. 2b).

Ultrastructural analysis of foci of photodynamic necrosis showed that necrosis of the Kupffer cells and endothelial cells of the liver was common for all PS (Fig. 3a).

Changes in the hepatocytes when HpD derivatives were used were mainly detected in the cytoplasm: intracellular edema and vacuolation were accompanied by condensation of the mitochondrial matrix and destruction of cristae; the appearance of single lipid drops in the cytoplasmic matrix also was typical. Against this background an increase in optical density of the masses of heterochromatin was observed in the hepatocyte nuclei (Fig. 3b). When PS based on $AlPcS_x$ were used necrolytic changes were marked in the hepatocytes: the cell boundaries were undefined, outlines of the nuclei could be identified with difficulty, and the cytoplasmic organelles were completely destroyed in connection with total lysis of membrane structures (Fig. 3).

The results of the morphologic investigation thus revealed a stronger cytolytic and nucleotropic action of PS of the $AlPcS_x$ group than of HpD derivatives on the parenchymatous cells.

Laboratory methods of screening PS on different models in vitro, described in the literature [1-4, 10-12] presuppose precise quantitative characteristics of the PDA of the preparations concerned, but they have certain deficiencies, the most important of which is a significant difference in the mechanism of sensitized photodynamic cell damage in vitro and in vivo. For example, the photodynamic activity of PS based on HpD in vitro is due mainly to the monomeric fraction of the preparation, whereas in vivo PDA is determined by the oligomeric component of HpD [7, 9].

In another known method of screening on transplantable tumors in vivo [5] both quantitative and qualitative assessment of PDA of the photosensitizers is difficult, due to the unique manner of growth cf the transplanted tumors. Foci of hypoxic necrosis and paranecrosis are known to be well defined in transplantable tumors measuring more than 0.5 cm in diameter [6], and since PDA is extraordinarily sensitive to hypoxia [8] it will be evident that the objective evaluation of PDA of the photosensitizers on large tumors is difficult. Meanwhile assessment of PDA of modern powerful synthetic PS by this method (measurement of the depth of photodynamic necrosis) on tumors under 0.5 cm in diameter is impossible.

The suggested method of PS screening on a nontumor model in vivo, besides being free from the above-mentioned defects, can provide not only accurate quantitative, but also qualitative characteristics of the PDA of photosensitizers belonging to different classes.

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