

EKSPERIMENTINIS TYRIMAS

Experimental evidence on possibility to radiosensitize aggressive tumors by porphyrins

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Key words: radiosensitization, photosensitization, porphyrins, aggressive tumors.

Summary. Two murine experimental tumor models of different aggressiveness (murine Ehrlich ascitic carcinoma and MH-22A hepatoma) were used to investigate the radiosensitization by porphyrins. Data obtained clearly indicate that hematoporphyrin dimethyl ether, photofrin and hematoporphyrin derivative exert some radiosensitizing properties which are in clear correlation with purity of the compound. Of interest to note, that just aggressive Ehrlich ascitic carcinoma was radiosensitized to γ -radiation, whereas no signs of radiosensitization were observed in MH-22A hepatoma tumor. Data obtained support the idea, that dicarboxylic porphyrins, being ligands of peripheral benzodiazepine receptors (responsible for proliferation and highly expressed in aggressive tumors) might induce several sublethal injuries in the cell which further work in concert with ionizing radiation producing synergistic interaction of two antiproliferative factors.

Introduction

In order to maximize therapeutic outcome and to reduce side effects, modern cancer treatment usually is a combination of different modalities. Photodynamic therapy (PDT) step by step begins to emerge as a promising alternative therapy for the treatment of early and localized tumors, in which adequate local surgery is difficult (1). Unfortunately, deeper tumors cannot be treated by this method, because light, used for photodynamic treatment, isn't able to penetrate into the tissue more than 0.8 cm. In this context new approach to enhance the effectiveness and expansion of PDT is necessary. One of them may be the combination of PDT with radiotherapy. From theoretical point of view, this combination might be very effective, if porphyrins would act for two destructive "jobs": photosensitization and radiosensitization.

As early as 1955, S. Schwartz has treated patients with a combination of radiotherapy and hematoporphyrin derivative (HPD). The results showed an improved local tumor control, especially in squamous cell carcinoma, rhabdomyosarcoma, fibrosarcoma and carcinoid tumors. So far only a few very short reports have been published reflecting this problem (2, 3).

Further J. Moan (4) carried out experiments with

NHIK 3025 cells. Under aerobic conditions hematoporphyrin (HP) in concentration range 0.5–0.7 mM had no influence on cell growth. Results obtained by D. A. Bellnier (5) confirmed the previous study of J. Moan – there was no change in radiosensitivity of Chinese hamster ovary fibroblasts when HPD was present.

In contrary, W. Zhang (6) has showed significant radiosensitization of human malignant glioma cells to ionizing radiation (2–6 Gy) by well known photosensitizer hypericin. In addition, the efficacy of radiotherapy prior to surgery (40%) for the treatment of maxillofacial tumors has been determined with and without sensitization with HPD (7). Further H. Kostron (8) described the interaction of HPD, light and ionizing radiation in a rat glioma. ^{60}Co irradiation produced a significant tumor growth inhibition, which was increased in the presence of HPD (5–20 mg/kg). It was directly related to the concentration of HPD. Light exposure 30 min prior to ^{60}Co irradiation produced the largest growth inhibition. Moreover D. Y. Chen demonstrated the sensitizing effect of HPD to radiotherapy in the treatment of S180 tumors, transplanted into mice (9). The inhibitory effect on the tumors after radiotherapy alone was 21%, while that

after a combination of HPD and radiotherapy was 50%.

Such controversial results might be explained by the fact that HPD is complex mixture of different porphyrins. Moreover tumors and malignant cells used for investigations are extremely different from biological point of view. Taking this into account, we examined the radiosensitizing properties of more purified hematoporphyrin dimethyl ether (HPde) on Ehrlich ascitic tumor model and obtained remarkable decrease of cell viability after combined treatment (radiosensitization) (10).

Moreover, it has recently been shown that PII can radiosensitize human bladder cancer (RT4) and glioblastoma cells (U-373MG), but not adenocarcinoma (HT-29) (11). It becomes obvious, that cell type, as well as porphyrin used, might modify the radiosensitization efficiency.

In order to shed more light upon the capacity of porphyrin-type photosensitizers to work as radiosensitizers, in order to understand, is the radiosensitization phenomenon specific or universal for all malignancies, we investigated series of well known porphyrin-type photosensitizers on two murine tumors of different aggressiveness.

Material and methods

Object

Due to the main idea to investigate radiosensitization by porphyrins in the experimental tumors of different aggressiveness, 2 types of tumors were selected. The best representative of aggressive tumor might be Ehrlich ascitic tumor (EAT), the less aggressive – hepatoma MH-22A

The experiments were carried out using the BALB/c and DBA/C_{57BLACK} mice strains. Ehrlich ascitic carcinoma and correspondingly MH-22A hepatoma were used as transplanted tumors.

Chemicals

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

5-aminolevulinic acid (ALA) (Sigma, Chemical Co.) 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 final concentration of ethanol less than 1%. The stock solutions (5 ml) were made and sterilized the same day as they were used.

Photofrin (PII) and HPD were purchased from Sigma Chemical Co, prepared as stock solution as well and stored in the dark below 10°C. The dark toxicities

of ALA, PII, HPD, HPde were evaluated in animal experiments as insignificant, which did not influence tumor growth.

Source of ionizing radiation

γ -irradiation was performed by ^{60}Co irradiation at a dose rate from 1.0 to 1.62 Gy/min. The dose given to tumors was 0–15 Gy (11).

Experimental design

EAT was transplanted into female mice aged 6–7 weeks and weighting approximately 21 g. The implantation procedure is summarized as follows: tumor was extracted from a donor mice and Ehrlich ascitic tumor cells (0.8×10^6) were inoculated i. p. to healthy mice using a 26 G needle.

On the 7th day after EAT tumor inoculation (exponential growth phase) sensitizer was injected i. p. at the concentration 20 mg/kg body weight. The selected sensitizer concentration was adopted for ascitic tumor, extrapolated from data *in vitro* (1.2×10^{-5} M) and corresponds to clinically accepted sensitizer concentration to treat solid tumors (pg/cell). Following 3 hours after injection of photosensitizer irradiation procedure was performed. Afterwards tumor growth was observed and measured for 15 and days.

The hepatoma A22 implantation procedure could be summarized as follows. Tumor mass was separated from connective tissues, rinsed in physiological saline (0.9% NaCl) and cut into small pieces, passed through 26-gauge needle and then implanted into right leg of the recipient mouse (0.3 ml tumor cell suspension). The tumors grew to a volume of about 200 mm³ within 12 days after inoculation and at this size were used for the experiments (12).

Of importance to note, that subcutaneously inoculated hepatoma grew at a less aggressive rate than EAT and reached volume of $0.7 \times 0.7 \times 0.5$ mm on 12th day after transplantation. No spontaneous remission of tumor or detectable formation of necrotic areas was observed at this time, hence 12-day transplantation interval was generally adopted for all pharmacokinetic and irradiation experiments. Sensitizer in this case was injected i. p. and the used concentration (20 mg/kg) was adopted for solid tumor and corresponded to the clinically accepted. All irradiation experimental design was the same as for EAT bearing mice. The rate of hepatoma tumor growth was measured with a caliper at every second day and afterwards these data were used to calculate tumor growth inhibition.

Every experimental group consisted of 6–8 mice. Control mice group was with not treated tumors having the same size and inoculated the same day.

During all experiments animals were kept under

mild anesthesia (ketamine hydrochloride, i. p.).

Moreover, all animals were kept according to requirements for the use of Laboratory Animals Experiments in Lithuania (1999).

Tumor growth determination

Relative Ehrlich ascites tumor growth was measured every day up to 15th day of its growth according to equation:

$$S = (S_1 - S_0) / S_0, \text{ where}$$

S_1 – final weight of mouse with tumor,

S_0 – initial weight of the mouse,

S – relative tumor growth.

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

1. Evaluating absolute tumor volume (cm³) growth during 15 days.
2. Determining tumor cells number (mln) during 15 days.

The correlation between absolute tumor weight and relative tumor growth was found very strong ($r^2=0.98$). In order to simplify the experimental protocol we usually measured only relative tumor growth (13).

The volume of MH-22A hepatoma was measured *in vivo* and calculated:

$$V = 1/2(4\pi/3) * (l/2) * (w/2) * h$$

l – is the longer perpendicular axis,

w – is the shorter perpendicular axis,

h – is the high of half-ellipsoidal tumor (14).

Measurements of intracellular concentration of photosensitizer in tumors

Ehrlich ascitic tumor cells were collected from the mice 3 hours after injection of photosensitizer. They were suspended in phosphate-buffer solution (PBS) to an optical density at $\lambda=590$ nm OD=0.6 (usually reflects the density of cells 3.7 mln./ml). The fluorescence of the suspension was measured with epifluorescence spectrophotometer SFR-1 (Russia) at $\lambda=600$ –680 nm. An EAT suspension treated in the same manner without photosensitizer was taken as control. The standard curves were produced by adding known amount of investigated photosensitizer (15).

MH-22A hepatoma tumor was separated from the mice 24 hours after treatment with photosensitizer and tumor cells were suspended in PBS to an OD ($\lambda=590$ nm) = 0.8 (usually reflects the density of cells 3.7 mln./ml). Fluorescence spectra were performed in the same manner and amount of sensitizer was counted to protein amount.

Statistical evaluations

500 experimental animals (mice) were used for overall investigations.

Every experimental group consisted of 6–9 mice

selected of the same age, sex and weight, with the identical size of tumor. All experiments were repeated at least three times (one mean is reflecting average from 27–42 mice.). All statistical evaluations necessary for publication of data (average values, standard deviations, standard errors, p-significance) were calculated using special program (Excel). The differences of comparative quantities were estimated as reliable at $p \leq 0.05$.

Results

The studies were performed using two distinct tumor models, namely Ehrlich ascitic carcinoma, which was cytologically verified (in the State Center of Pathology) to be very aggressive and MH-22A hepatoma, which was verified as not aggressive tumor.

The tumor bearing mice were injected with different photosensitizers at the therapeutic dose (20 mg/kg body weight). Data, published before (12) indicate, that used concentration results in intracellularly accumulated HPde about 2 μ g/mg protein and is start-point for effective photosensitization. At 3 hours after i. p. injection of photosensitizer the mice were irradiated with 2 Gy. The timing for irradiation of mice was selected on the basis of the known pharmacokinetic properties of the drugs in order to achieve maximal concentration of porphyrin in the tumor cells.

Control group of mice bearing tumors was not treated by sensitizer neither irradiated. With the aim to evaluate dark toxicity (toxicity per se) of the drugs the next 3 groups were treated by all used photosensitizers (HPde, PII, HPD). The last 3 groups of mice were treated by all used photosensitizers and irradiated with 2 Gy. All investigated photosensitizers had no effect on the rate of tumor growth in the absence of subsequent irradiation. Just combination of photosensitizer and radiation appears to exert an effect on the rate of tumor growth (Fig. 1)

None of the evaluated porphyrins showed comparable with HPde radiosensitizing effect (~90%). It appears from the plots shown in Fig 1. that the irradiation of EAT in the absence of photosensitizer results in some but not total tumor growth inhibition (30%).

Based upon these results we performed other series of experiments to investigate the dependence of radiosensitization on irradiation dose in the presence of the most effective photosensitizer HPde (0–15 Gy). Data obtained reveal, that there is significant difference between tumor growth when mice were just irradiated (0–15 Gy) or pretreated with HPde and afterwards irradiated (Fig. 2). Therefore it confirms, that HPde at these experimental conditions is sensitizing

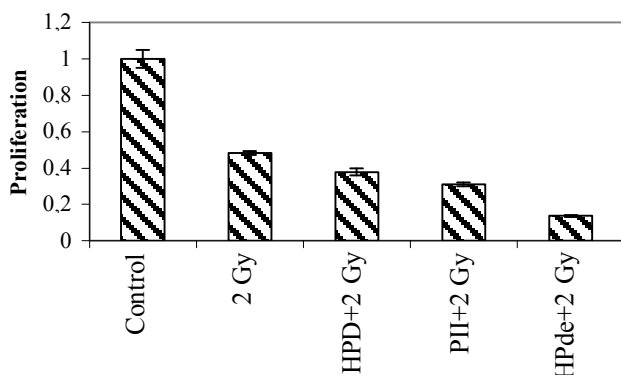


Fig. 1. Ehrlich ascitic tumor response (growth inhibition) to different treatments

Control – not treated tumor growth, 2 Gy – growth of just irradiated (2 Gy) tumors; HPD+2 Gy, PII+2 Gy – tumor growth of tumors pretreated with different photosensitizers (20 mg/kg, i. p. 3 h incubation) and irradiated afterwards with 2 Gy.

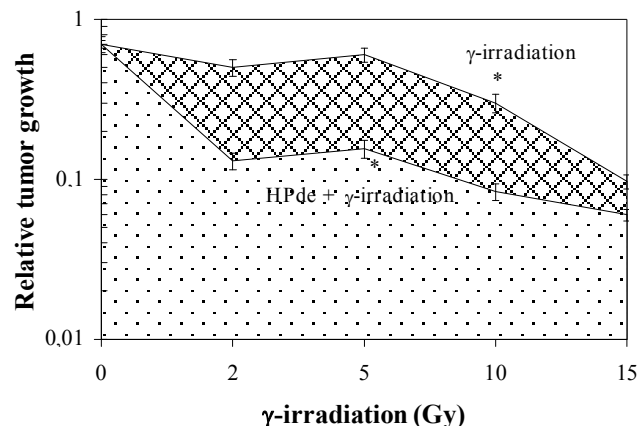


Fig. 2. Ehrlich ascitic tumor growth inhibition as function of irradiation dose, when tumor was

● – just irradiated, ■ – pretreated with HPde (20 mg/kg) and afterwards irradiated.

EAT cells to γ -radiation, reducing significantly the shoulder of survival curve of just irradiated cells.

It was of interest to quantify the HPde radiosensitization degree in EAT cells and compare it with that of accepted radiosensitizers. Cell proliferation was chosen as the end-point of the effect. This criterion does not allow the registration of the initial radiation induced events without any major interference by subsequent repair process, but reflects better the real situation *in vivo*. Thus, calculations of sensitizer enhancement ratios (SER) were made according to formula, described in detail by M. Imamura (16). Fig. 3 shows the results calculated from the experiments performed before. The data indicate that SER is really dependent on the concentration of drug. In our case SER for EAT cells treated with HPde varies from 1 till 2.7.

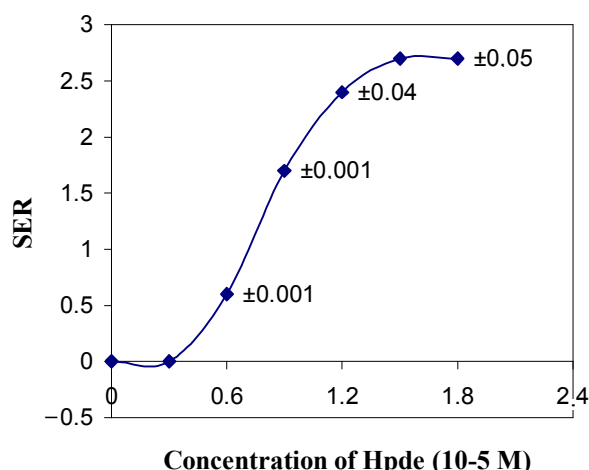


Fig. 3. Sensitizer enhancement ratio (SER) of HPde as function of used concentration in Ehrlich ascitic tumor

The other selected tumor for investigation of radiosensitization was not aggressive murine hepatoma MH-22A. The reason for such choice was the fact, that dicarboxylic hematoporphyrins (HPde, PII, HPD) might be ligands of peripheral benzodiazepine receptors responsible for proliferation. The expression of these receptors usually is about 15 times higher in aggressive tumors if compared with not aggressive ones (15) and might influence the response of tumor to radiosensitization.

Thus, we attempted to determine the radiosensitizing properties of the described before porphyrin-type photosensitizers (PII, HPde, ALA) in less aggressive murine MH-22A hepatoma.

Results, presented in Fig. 4 convincingly reveal that no appreciable difference between 2 Gy irradiated and drug-pretreated and irradiated MH-22A tumor

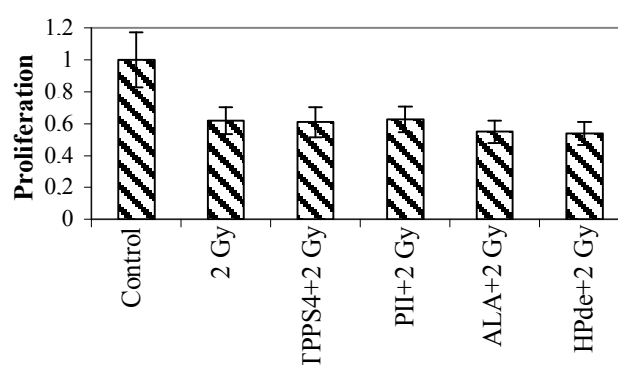


Fig. 4. MH-22A hepatoma tumor response (growth inhibition) to different treatments

Control – not treated tumor growth, 2 Gy – growth of just irradiated by 2 Gy tumors; PII+2 Gy, ALA+2 Gy, HPde+2 Gy – growth of pretreated with different photosensitizers (20 mg/kg, i. p. 3 h incubation) tumors and afterwards irradiated with 2 Gy.

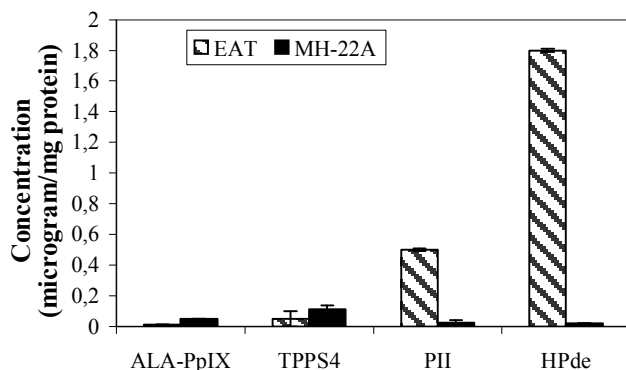


Fig. 5. Intracellular concentration of several photosensitizers detected in Ehrlich ascitic tumor (▨) and MH-22A hepatoma tumor (■) (20 mg/kg, i. p. 3 h incubation).

were observed. Moreover, no one used porphyrin-type photosensitizer – HPde, PII, ALA – exerted radiosensitizing properties. On these bases it appears of particular interest to evaluate the intracellular concentration of these photosensitizers in EAT as well as in MH-22A hepatoma.

Data, presented in Fig. 5 indicate, that PpIX, PII or HPde accumulation in MH-22A hepatoma tumor is extremely low and incomparable with that in EAT. For instance, accumulation of PII and HPde in EAT cells is 0.6–1.7 microgr/mg protein, whereas the same photosensitizers haven't been found in MH-22A hepatoma at all. The most interesting is the fact that radiosensitization was obtained just in cases, when accumulation of photosensitizer was more than 0.5 microgr/mg protein. Thus, from data obtained it is possible to draw a conclusion as to at what experimental conditions photosensitizer can act as radiosensitizer.

Discussion

It looks like, that a detailed understanding of the mechanisms involved in EAT radiosensitization by porphyrin-type photosensitizers is hampered, at least in part, by the heterogeneous chemical composition of some substances (HPD, PII). It is therefore unlikely that the radiosensitizing effects are due to a direct interaction between the porphyrin and irradiation. Nevertheless of particular interest is the finding that the choice of photosensitizer is of critical importance. It is worldwide accepted that the physico-chemical properties of every photosensitizer are different and determine their accumulation capacity in tumor. So far, it is necessary to point out, that nobody from received authors tried to examine porphyrin accu-

mulation potential in the investigated object.

The other question arises, what might be explanation of observed phenomenon, that other less purified porphyrin-type photosensitizers exert lower radiosensitizing properties? It looks like, that the more purified form of sensitizer was used, the higher radiosensitization was reached (for instance HPde). In addition, investigation of intracellular concentration, of photosensitizers, accumulated in EAT cells indicates, that there is remarkable difference in accumulating potential of PII and HPde (Fig. 5). Maybe our data can support the idea, that the quantity of the porphyrin-type drug accumulated in the cell might determine the efficiency of radiosensitization. In any case, the radiosensitizing efficiency of HPde observed in EAT cells, is evident.

Thus, the results demonstrate, that HPde can really act as an effective radiosensitizing agent, if applied under appropriate conditions. Further observations demonstrate that the radiosensitizing activity of HPde under optimal conditions in aggressive tumors approached a factor of 3. This means, that 5 Gy radiation combined with HPde exerts the same effect on EAT as a 15 Gy irradiation by itself. Moreover, data obtained are in line with published results and confirm the idea, that HPde behaves similarly to its less purified analogue PII or HPD (1).

Finally, the most effective photosensitizer HPde was compared by SER with misonidazole. Experiments performed by other authors with well-known hypoxic cell radiosensitizer misonidazole illustrated that SER for this well known drug is dependent on used concentration as well and might vary from 1 to 2.4 (17). By no means, SER value for sensitizer-treated cells depends strongly on end-point chosen (for instance Ak-2123 $\mu\text{mol dm}^{-3}$ SER might vary from 2.0 till 1.75, depending on end-point). In the 1970's worldwide clinical trial was performed using 2-nitroimidazole as radiosensitizer. With the exception of head and neck tumors, the result was disappointing due to its neurotoxicity.

Our results clearly indicate, that in EAT, MH-22A as well as in WiDr cells (18) radiosensitizing potential of HP, HPde, PpIX, PII strongly depends on intracellular drug concentration.

Quantifying, just more than 0,5 $\mu\text{g/mg}$ protein intracellularly accumulated PpIX or HPde could produce sensitization of tumor cells to ionizing radiation. This idea is strongly supported by experimental data, obtained on MH-22A tumor model, where extremely low HPde and PII intracellular concentrations were obtained.

ned and consequently no significant radiosensitization was observed. In other words, the same photosensitizer HPde was very effective radiosensitizer in EAT cells, where accumulation is very high (pg/cell, 2 microgr/mg protein) and absolutely ineffective in MH-22A tumor, where no signs of its uptake were detected.

Consequently, further combination of PDT with ionizing radiation seems reasonable, in the cases of aggressive tumors. It means, that combining PDT with ionizing radiation, when tumors are pretreated with HPde we are combining two destructive processes – photosensitization and radiosensitization. Eventually this combination is producing synergistic effect on therapeutic outcome (16). But, if drug concentration is low (as in the case of MH-22A tumor), the combination of two treatments reflects just additive interaction.

In conclusion, it must be emphasized, that this study is a clear demonstration of the possibilities to use some porphyrin-type photosensitizers as radiosensitizers. Nevertheless, only under certain – well-defined conditions – having high accumulation potential

and just in the aggressive tumor – dicarboxylic porphyrin-type photosensitizers can work as radiosensitizers. Moreover, combination of photosensitization with radiosensitization allows damaging deeper tumor levels as well as to reduce the dose of the photosensitizer without any negative effect on therapeutic outcome. Eventually, there is no doubt that the possibility to use the same chemical compound as both a photo- and radio- sensitizer offers unique possibility for a concerted action of the two cancer treatment modalities for better control of tumor growth.

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Agresyviai augančių navikų radiosensibilizacija porfirinais

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Raktažodžiai: radiosensibilizacija, fotosensibilizacija, agresyviai augantys navikai.

Santrauka. Radiosensibilizacinių porfirinų savybių tyrimui panaudoti du skirtingo laipsnio pelių navikų modeliai. Tyrimo duomenys rodo, kad hematoporfirino dimetilo eteris, fotofrinas, hematoporfirino derivatas gali žymiai radiosensibilizuoti Ehrlichio ascito naviko ląsteles. Taigi radiosensibilizacijos laipsnis tiesiogiai susijęs su junginio cheminiu grynumu. Svarbu pažymėti, kad tik Ehrlichio ascito naviko atveju (agresyviai augančio naviko tipas) pavyko užfiksuoti radiosensibilizaciją γ -spinduliams. Apskaičiuota, kad hematoporfirino radiosensibilizacijos koeficientas siekė 2,7 agresyviai augančiame navike. Be to, pastebėta, kad radiosensibilizacijos laipsnis priklauso nuo junginio cheminio vienalytiškumo: hematoporfirinas > fotofrinas > hematoporfirino derivatas. Visiškai jokios radiosensibilizacijos nerasta esant MH-22A hepatomai. Ištirta hipotezė, kad porfirinai, būdami periferinių benzodiazepininių receptorių, atsakingų už proliferaciją agresyviai augančioje navikinėje ląstelėje ligandais, sukelia daug subletalų pokyčių, kurie kartu su jonizuojančia spinduliuote lemia dviejų antiproliferacinių faktorių sinerginę sąveiką.

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