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THE EFFECT OF METRONIDAZOLE ON THE CHLORIN e_6 PHOTOSENSITIZED DEATH OF EHRLICH CARCINOMA CELLS

The hypoxic tumor cells, i. e. neoplastic cells developing by a reduced oxygen content, are characterized by a high resistance not only to traditional chemo- and radiotherapy, but also to such a relatively new treatment as photodynamic therapy (PDT) [1, 2]. To overcome this drawback it is recommended to carry out preliminary hyperoxygenation, change the regime of neoplasm tissue photoirradiation [3], and use fluorocarbonic emulsion [4]. Besides, attempts have been made to enhance the photosensitivity of tumor cells by combining PDT with the administration of nitroimidazole compounds widely used as radiosensitizers of the hypoxic cells in tumor radiotherapy. In this case, as a rule, a haematoporphyrin derivative (HpD) serves as a photosensitizer (PS). However, the results obtained are not uniform. So, Hirsch et al. [5] and Gonzalez et al. [6] established a considerable increase in the effectiveness of PDT in combination with misonidazole (MISO). Mitchell et al. [7], on the contrary, observing the high resistance of the hypoxic tumor cells to PDT, came to the conclusion that the use of such a nitroimidazole radiosensitizer as SR 2508 was ineffective. At the same time, a search for more effective PS is in progress. In particular, a chlorin compound characterized by higher spectral indices, a more rapid removal from an organism, and less pronounced negative side-effects [8], may serve as an alternative to HpD PS. Having this in mind, the present work was aimed at assessing the dependence of the phototoxicity of chlorin e_6 on the medium's oxygen content and finding out ways of increasing the effectiveness of metronidazole (MZ) in the photosensitized death of tumor cells. In its widest range of concentration (0.25—10 mM) and under normal aeration conditions, this preparation does not affect the survival of mammalian cells. At the same time, MZ may cause selective death of hypoxic cells in both the culture tissue and monolayer (by nitrogen flow) [9]. MZ is well endured by an organism and has been widely applied in the treatment of urogenital trichomoniases for many years already. It is easily taken up, quickly absorbed and accumulated in the blood that ensures its effective access to the tumor focus.

Materials and Methods

Chlorin e_6 trimethyl ester (E6) was prepared on the basis of pheophytin *a* extracted from nettle leaves according to the method of Lötjönen and Hynninen [10]. HpD was synthesized from haematoporphyrin (Hp) applying the original method of Lipson et al. [11] modified by Kes-

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sel et al. [12]. In particular, 100 mg of Hp IX dihydrochloride (Aldrich) was acetylated in a 2 ml mixture of glacial acetic and concentrated sulphuric acids (19:1 by vol.). The mixture was stirred at room temperature in dark for 60 min. Then 15 ml of 5% sodium acetate was added to the mixture. The precipipate of Hp acetates formed was removed by centrifugation and washed twice with distilled water. The suspension was lyophilized and a brown powder was obtained. Thereafter the Hp acetates were subjected to hydrolysis. The lyophilized preparation was mixed with 0.1 N NaOH (20 mg of Hp acetates per 1 ml of 0.1 N NaOH) and stirred at 22 °C for 60 min. Then the solution's pH was brought to 7.2 by adding 0.1 N HCl. By the addition of 0.9% NaCl, the HpD concentration was diluted to 5 mg/ml. The preparation was stored at —20 °C in dark.

The Ehrlich ascites carcinoma (EAC) was transplanted on 25—30 g mongrel female mice weekly, $3 \cdot 10^7$ cells per animal. To carry out the experiments *in vitro*, a 6—8 day tumor was withdrawn from the mouse abdominal cavity, 4 volumes of a 0.9% NaCl solution were added, and the cell suspension obtained was centrifuged at 4° C for 6 min. The cells precipitated were resuspended in 0.9% NaCl and stored over melting ice until the beginning of the experiment. The cell suspension was diluted with a physiological solution to a concentration of $5 \cdot 10^6$ per ml, sodium phosphate buffer (pH 7.4) was added up to a concentration of 10 mM. In case of need, glucose solution was added. Immediately after adding the PS dissolved in 0.9% NaCl (HpD) or in a minimal volume (5—10 μl) of dimethyl sulphoxide (E6), the cells were irradiated with light at 665 and 630 nm for E6 and HpD, respectively. The irradiation was carried out in quartz cuvettes (layer thickness 2—3 cm) by magnetic stirring at 20 °C, a 1 kW xenon lamp instrumented with a focusing optical system serving as the radiation source.

The anaerobic conditions were generated by passing nitrogen through the cell suspension in the sealed cuvette. The share of the damaged cells in the suspension was determined by staining them with 0.05% trypan blue (Sigma, USA) in the phosphate-salt buffer (pH 7.2). After 2—5 min 150—200 cells were examined in the Goryaev chamber and the proportion of trypan blue stained cells was determined.

The experiments *in vivo* were carried out with 25 g white mongrel female mice who were injected i/p with 0.25 ml of EAC. Five days after neoplasm inoculation the animals were subjected to phototherapy. At the same time, the E6 dissolved in dimethyl sulphoxide was injected i/p at a concentration of 5 mg/kg 15 min prior to irradiation. The same applies to the MZ (2.5 mM/kg; Sigma) dissolved in 0.9% NaCl. The irradiation was carried out with the light (665 nm) isolated from the spectrum of a 1 kW xenon lamp. The flux of light was focused into the stain (D. 2.5 cm) by means of an optical system and directed into the abdominal region. At the intensity of irradiation of 400 mW, the light exposure time was 15 min (energetic exposure 75 J/cm^2). The development of EAC both before and after phototherapy was observed by a change in the weight of tumor-bearing animals.

Results and Discussion

It can be seen (Fig. 1) that the reduced oxygen content (irradiation of cells in the nitrogen atmosphere) led to a considerable decrease in the rate of E6 photosensitized injure of carcinoma cells. The case is similar to that with HpD from a phenomenological point of view. Thus, it was shown that at 1% oxygen content, the quantum yield of HpD-photo-

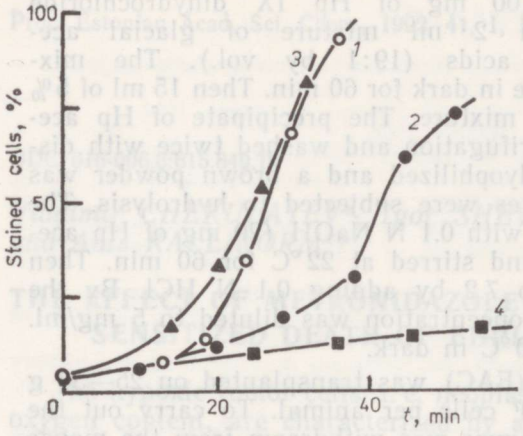


Fig. 1. The influence of metronidazole (MZ) on E6-photosensitized injure of EAC cells ($5 \cdot 10^6$ cells/ml) irradiated at 665 nm, intensity 200 mW: *1* — E6 (2 μM) in the presence of air oxygen; *2* — E6 (2 μM) in the nitrogen atmosphere; *3* — E6 (2 μM) + MZ (4 mM) in the nitrogen atmosphere; *4* — irradiation of the cells suspension in the nitrogen atmosphere without PS, but in the presence of 4 mM MZ. Each point is the average of three experiments (SD<5%).

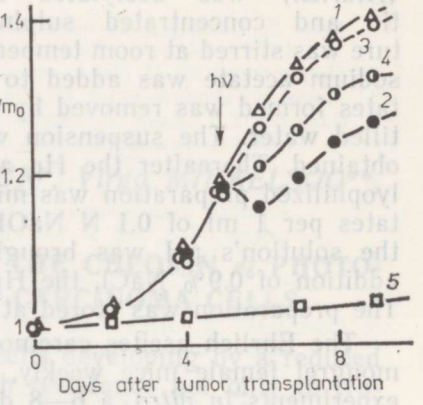


Fig. 2. The change in the weights of tumor-bearing mice before and after PDT: *1* — control animals with EAC; *2* — E6 (5 mg/kg) + MZ (2.5 mM/kg) + irradiation; *3* — E6 + MZ without irradiation; *4* — E6 + photoirradiation; *5* — the non-tumor-bearing mice. In each experiment 15-animal groups were used (SD< <5%).

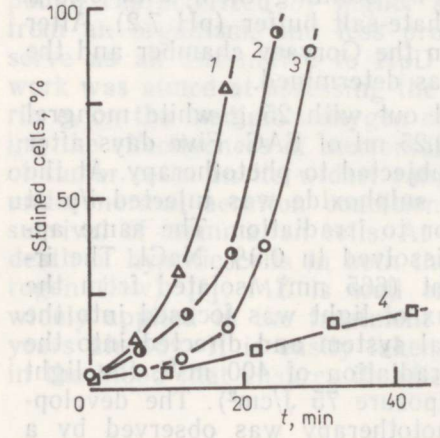


Fig. 3. E6-photosensitized injure (2 μM) of EAC cells in the presence of MZ: *1* — 5 mM, *2* — 2.5 mM, *3* — without MZ; *4* — photoirradiation in the presence of MZ (5 mM) without PS. Irradiation in the presence of air oxygen, 665 nm, intensity 200 mW.

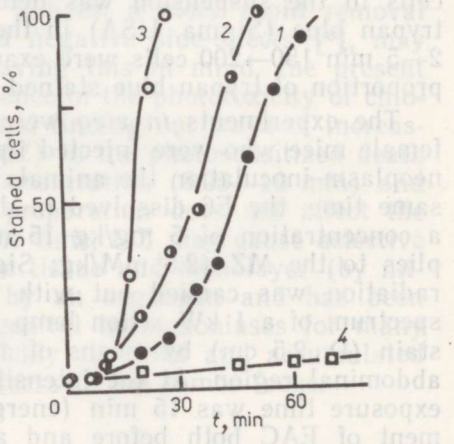


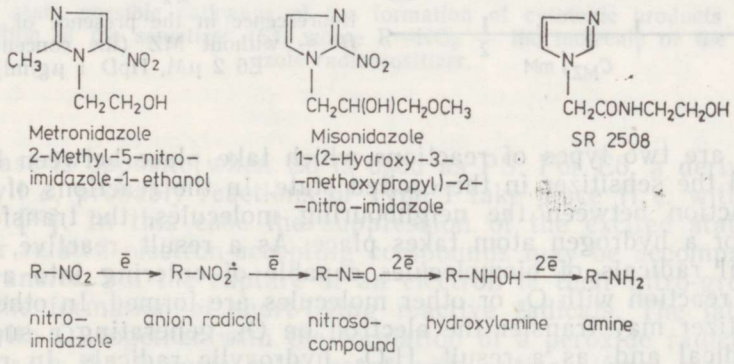
Fig. 4. HpD-photosensitized damage (1 μg/ml) of EAC cells in the presence of MZ: *1* — 2.5 mM, *2* — 1.25 mM; *3* — without MZ; *4* — photoirradiation in the presence of MZ (2.5 mM) without PS. Irradiation in the presence of air oxygen, 630 nm, intensity 125 mW.

sensitized inactivation of the cells of carcinoma NHIK 3025 decreased by 50% as compared with that in conventional air atmosphere [12]. Thus, as in the case of HpD, also for E6 the intensity of the photodamage of tumor cells seems to be strongly dependent on the oxygen content.

Under hypoxic conditions the addition of MZ (to a concentration of 4 mM) resulted in an almost two-fold increase in the E6-photosensitized destruction of EAC cells (Fig. 1). But on irradiation of tumor cells under the anoxic conditions and in the presence of MZ the proportion of the damaged cells did not increase considerably. The potentiating effect of MZ on the photosensitized death of EAC cells was also observed *in vivo*. Thus (Fig. 2), a combined introduction of E6 and MZ into an organism and photoirradiation suppressed the development of carcinoma cells more strongly.

When EAC was irradiated under the aerobic conditions, MZ revealed quite unexpected effects. Thus (Fig. 3), MZ (in mM concentrations) was able to promote considerably the E6 photosensitized injure of EAC cells. The effect was the opposite when HpD was used as PS (Fig. 4). As the effect of MZ is mostly determined by the nature of PS, then it does not seem to be related to its direct toxic effect on the tumor cell. Moreover, this suggests that an increase in the phototoxicity of E6 is caused mainly by the interaction between MZ and the excited molecules of PS.

The mechanism of the cytotoxic effect of MZ and the related nitroimidazole compounds on the hypoxic cells has been insufficiently studied. However, it is supposed that under anoxic conditions these compounds are capable of an intracellular reduction with the formation of active intermediate metabolites which are obviously able to covalently bind to certain macromolecules of the cell [13] (Scheme 1).



Scheme 1. Reduction of the nitro group of radiosensitizers (R—NO_2) in the hypoxic condition.

In this connection, the combination of hypoxia with measures taken to increase the level of reduced equivalents in the cell must lead to an increase in the amount of active nitroimidazole metabolites and, as a result, to an increased cytotoxicity. Thus, Ling and Sutherland [14] observed a strong modulating effect of glucose on the cytotoxicity of MISO (an analog of MZ) with recpect to the EMT6/Ro cells cultivated by a reduced oxygen content. In particular, an increase in the glucose concentration in the culture medium contributed both to the cytotoxicity of MISO and its binding to the acid-insoluble fraction. In the authors' opinion this is connected with the activation of a hexose monophosphate pathway of glucose balance and an increase in the NADPH

level in the cell. Under our conditions, however, hyperglycemia (up to 8 mM) did not increase the cytotoxicity in the cells of EAC photosensitized by E6 in the presence of MZ. This may be explained by the fact that in case of a combined use of MZ and E6 active cytotoxic products are probably formed as a result of the interaction of the excited PS molecule with MZ.

Thus, MZ contributes to E6-photosensitized death of EAC cells under both the aerobic and hypoxic conditions. The case was opposite when HpD was used as PS, MZ revealing a well-pronounced inhibiting effect. What accounts for these different, PS-dependent effects of MZ? In order to elucidate this phenomenon, the effect of MZ on the intensity of the fluorescence of the above PS was studied. It turned out (Fig. 5) that an increase in the MZ concentration in the mixture with PS leads to a decrease in the quantum yield of its fluorescence with both E6 and HpD used. In the latter case, this effect was more pronounced. Why does the quenching of PS fluorescence by MZ lead to an increase in the photodamage of EAC cells but, in case of HpD, to noticeable protective effect?

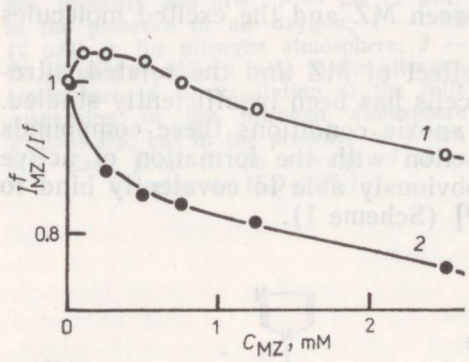
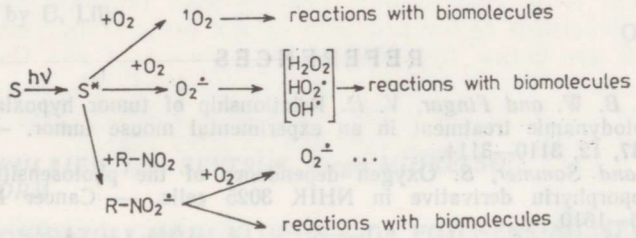


Fig. 5. The influence of MZ on the intensity of the fluorescence of E6 (*1*) or HpD (*2*) in a 3 : 2 mixture of acetonitrile-water, where I^f_{MZ} — the intensity of PS fluorescence in the presence of MZ, and I^f — without MZ (the concentration of E6 2 μM, HpD 1 μg/ml).

There are two types of reactions which take place between biomolecules and the sensitizer in the excited state. In the reactions of Type 1, by interaction between the neighbouring molecules, the transfer of an electron or a hydrogen atom takes place. As a result, reactive, charged or neutral radicals of biomolecules capable of entering into a further chemical reaction with O_2 or other molecules are formed. In other cases, the sensitizer may transfer the electron on O_2, generating a superoxide anion radical and, as a result, H_2O_2, hydroxylic radicals. In reactions of Type 2, PS, being in a triplet excited state, interacts with the O_2 molecule and transfers it into a singlet excited state. The type of the reaction (1 or 2) according to which the photosensitized reaction proceeds is determined by the nature of PS and the substrate, as well as by the relative concentration of O_2 and the substrate. Results of the experiments *in vitro* and *in vivo* carried out in different laboratories demonstrate that the main cytotoxic effect is most probably achieved on account of reactions of Type 2 [15, 16] when using HpD as PS, although some authors [17] have succeeded in establishing the formation of free radicals by photoirradiation of Hp solutions when using the methods of EPR and spin trapping. This view is also indirectly confirmed by our results. Indeed, the suppression of the fluorescence of HpD observed on the addition of MZ may give evidence of a noticeable decrease in the yield of PS molecules which are in a triplet excited state. Also, some electron acceptors, including aromatic nitro-compounds, are capable of

a direct deactivation of triplet-excited porphyrin molecules [18]. This must be connected with a decrease in the formation of highly reactive molecules of singlet oxygen and, as a result, lead to a decrease in the efficiency of the photosensitized damage of tumor cells. This may be observed when using HpD as PS (Fig. 4). The data obtained may account for the ineffectiveness of the administration of nitroimidazole sensitizers into the organism prior to PDT. This was observed by Winther et al. [19] on the basis of photofrin-II retinoblastoma-like tumor when MISO was used in combination with PDT. Moreover, performing PDT with HpD, Gonzalez et al. [6] established that the growth of a transplanted tumor was more effectively suppressed by injecting MISO not prior to photoirradiation but 30 min after it. It is known that due to the damage of the tumor vascular system, PDT is a powerful hypoxic factor, leading to a considerable decrease in the partial pressure of oxygen in the neoplastic tissue [5, 20, 21]. This fact and our experimental data show that it is more advantageous to inject nitroimidazoles not prior to tumor irradiation but after it. Otherwise, the effect may be negative, especially when HpD is used as PS.



Scheme 2. Main possible pathways of the formation of cytotoxic products under the photoexcitation of the sensitizer (S), where R—NO_2 — the molecule of the nitroimidazole radiosensitizer.

The case is opposite when E6 is used as PS. For E6, a derivative of chlorophyll *a*, probably reactions of Type 1 take place (i. e. with charge transfer) [22]. In this case the suppression of the excited state of E6 by MZ or related electron-accepting compounds may be accompanied by charge transfer, but the capture of an electron of their nitro-group may lead to the formation of short-living reactive radicals. The latter may oxidize with O_2 together with the formation of a peroxide radical either directly or in the hypoxic cells, react with critical (from the point of view of cell survival) biomolecules, contributing to an increase in cytotoxicity by photosensibilization of neoplastic cells (Scheme 2). It should be pointed out that although the electron-accepting properties of nitroimidazoles are considerably less pronounced than those of oxygen, their charged (or neutral) radicals most probably contribute to cytotoxicity more than those of oxygen ($OH^{\cdot}$, $OH_2^{\cdot}$, $O_2^{\cdot-}$) or even $^1O_2$, as the potentiating effect of the former is revealed even under the aerobic conditions (Fig. 3).

The views presented in this work require further experimental verification. However, on their basis the following conclusions can be made:

1. The expediency of a combination of nitroimidazole sensitizers of hypoxic cells, in particular, MZ, with PDT based on the use of chlorin e_6 has been established. At the same time, unlike HpD, the potentiating effect of MZ may be revealed even under the aerobic conditions;

2. The potentiating effect of MZ is obviously caused by its direct interaction with the excited molecule of E6 that leads to the formation of cytotoxic nitroimidazole radicals;

3. When E6 is used as PS, the stimulating effect of MZ is most probably the sum of a direct cytotoxic effect of nitroimidazole on the hypoxic tumor cells (as a result of its metabolic activation) on the one hand, and a lethal effect of the MZ radicals formed by its interaction with the excited molecule of PS on the other. An increase in the effectiveness of PDT resulting from the use of HpD is, in our opinion, caused only by a direct cytotoxic influence of nitroimidazoles on the hypoxic fraction of neoplasm cells;

4. As some nitroimidazole sensitizers of hypoxic cells are capable of suppressing the excited state of HpD (that may lead to a considerable decrease in the formation of singlet oxygen), then it is advantageous to inject them into the organism only after the phototherapeutic seance.

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METRONIDAZOLI MÕJU KLORIIN-e_6-GA FOTOSENSIBILISEERITUD EHRLICHI ASTSIITSE KARTSINOOMI RAKKUDE HÄVIMISELE

Pahaloomulise kasvaja koldes esineb regionaalne hüpoksia, mis võib põhjustada kasvaja fotodünaamilise teraapia (FDT) efektiivsuse languse. FDT, mis võib halvata kasvaja veresoonkonna, kutsub omakorda esile veelgi suurema hapniku partsiaalrõhu languse neoplasma kudedes. Võttes arvesse neid fakte, on otstarbekas kasutada FDT-d kombinatsioonis radiosensibilisaatoritega — preparaatidega, mis on valikuliselt toksilised just hüpoksiliste rakkude suhtes.

On leitud, et kloriin-e_6 (E6) manulusel ja hapniku puudulikkuse tingimustes (rakke kiiritati lämmastiku atmosfääris) langes kasvaja rakkude fotovigastuste intensiivsus. Metronidazoli (MZ) manulusel nii *in vitro* kui ka *in vivo* täheldati märgatavat E6-ga fotosensibiliseeritud Ehrlichi kartsinoomi rakkude hävimise suurenemist. Kuid erinevalt hematoporfüriini derivaadist MZ ei suurendanud E6 fotodünaamilise mõju efektiivsust mitte ainult hüpoksia, vaid ka aeroobsetes tingimustes. Arvatakse, et E6 fototoksilisuse suurenemise fenomenis mängib tähtsat osa MZ interaktsioon fotosensibilisaatori ergastatud molekulidega. Sealjuures tekivad tsütotoksilised nitroimidasoolsed radikaalid tüüp 1 fotokeemiliste protsesside tõttu.

Владимир ЧЕКУЛАЕВ, Игорь ШЕВЧУК, Вирго МИХКЕЛЬСОО, Антс КАЛЛИКОРМ

ВЛИЯНИЕ МЕТРОНИДАЗОЛА НА ФОТОСЕНСИБИЛИЗИРОВАННУЮ ХЛОРИНОМ e_6 ГИБЕЛЬ КЛЕТОК АСЦИТНОЙ КАРЦИНОМЫ ЭРЛИХА

Установлено, что в присутствии хлорина e_6 пониженное содержание кислорода (облучение клеток в атмосфере азота) приводит к подавлению интенсивности фотоповреждения клеток неоплазмы. Однако при введении метронидазола как in vitro, так и in vivo наблюдается значительное ускорение фотосенсибилизированной хлорином e_6 гибели клеток карциномы Эрлиха. Причем, в отличие от производного гематопорфирина, метронидазол повышает эффективность фотодинамического воздействия не только в гипоксичных, но и в аэробных условиях. Предполагается, что в феномене усиления фототоксичности хлорина e_6 важную роль играет взаимодействие метронидазола с возбужденными молекулами фотосенсибилизатора. При этом в результате фотохимических процессов первого типа происходит образование цитотоксичных нитроимидазольных радикалов.