studia biophysica Volume 138 (1990) Number 3, p. 219 - 228

PHOTODYNAMIC ACTIVITY OF HPD-PHOTOPRODUCTS

K. KOENIG[1], A. FELSMANN[1], W. DIETEL[1], M. BOSCHMANN[2],

1 Department of Physics, Friedrich Schiller University Jena, GDR
2 Institute of Biochemistry, Medical Academy Magdeburg, GDR

HpD, the photosensitizer mostly used in the photochemotherapy, changes its fluorescence and absorption properties during light exposure. Some new absorption bands in the red spectral range, especially around 640 nm, occur as a result of the formation of stable photoproducts. The red spectal region is important for photochemotherapy because of high tissue transmission. As shown by cell devitalization experiments these formed photoproducts have also a photodynamic activity but less than HpD. HpD incubated cells show the greatest lethal rate in the spectral range 630 nm-660 nm during 630 nm exposure whilst the photoproduct incubated cells show the greatest rate at about 640 nm - 650 nm due to its absorption behaviour. The formation of the photoproducts during light exposure can be prevented by the additional administration of metronidazole. In this case the cytotoxic effects are determined by type I photo-oxidation processes.

Introduction

Hematoporphyrin derivative (HpD), a mixture of different porphyrins and their chemically and physically bounded aggregates, is the photosensitizer mostly used in the photodynamic therapy of tumors /6,9,10/.

The absorption spectrum of HpD consists of a broad band (Soret band) in the UV/violet range and four smaller absorption bands in the visible range. The absorption coefficient decreases with higher wavelength and is less than $2 \times 10^{-3} M^{-1} cm^{-1}$ (molecular weight for HP-monomer ≈ 600 g) in the red range. However, the red/NIR spectral region is important for therapy because of the higher light penetration in tissue.

In order to increase the therapeutic effect, other photosensitizers with a stronger absorption in the red/NIR spectral range are investigated, e.g. phthalocyanines /17/, Methylene Blue /12/, phaeophorbide a /21/ or chlorine e_6 /7,8/.

Recently it has been found that HpD changes its fluorescence and absorption properties during strong light exposure mainly in the UV/violet spectral range /11,15,19,22/. Beside a bleaching process, some new absorption bands in the red spectral range occur as a result of the formation of a stable

photoproduct, especially at about 635-640 nm in aqueous solutions /5/, as also has been described by ROTOMSKIENE et al./22/. Further, the formation of a fluorescent and photochemically unstable photoproduct in vivo and in solution can be observed /5/ (in cells and tumors the absorption and fluorescence spectrum is about 15-20 nm red-shifted due to a different environmental micropolarity, e.g. /2/).

The aim of this paper is to report on the photodynamic activity of an irradiated HpD-solution containing a high contents of the formed photoproduct.

Materials and Methods

Chemicals: The HpD used was kindly provided by Dr. Lotz, ENT clinic of Halle University, GDR. The effectivity of this drug was tested as described elsewhere /13,18/. A 10^{-4}M HpD-solution (diluted with PBS, pH 7.4) was irradiated by the 488 nm radiation of an argon-ion laser (type ILA 190, Carl Zeiss Jena, dose: 200 J) in order to form the photoproducts. FIG. 1 shows the fluorescence spectra of HpD in dependence on the irradiation time. Note the increase of an additional fluorescence band around 640 nm due to the formation of the fluorescent photoproduct(s). There are changes in the absorption spectra especially in the red spectral range, FIG.2. The absorption in this range increases and a new absorption maximum is formed at about 640 nm. The final concentration of the unirradiated (fresh) HpD in the cell suspension was 10^{-5}M.

The nitroimidazole used was metronidazole(Efloran, KRKA Jugoslavia).

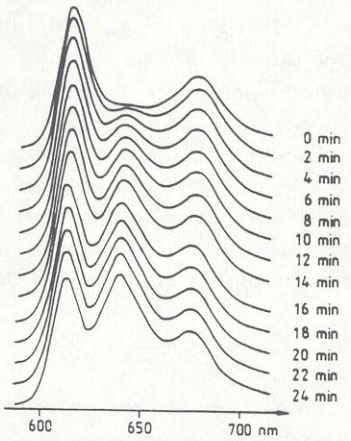

FIG. 1
Fluorescence spectrum of 10^{-4}M HpD-PBS-solution, pH 7.4 dependent on irradiation time (488 nm, 100 mW).
Note the formation of the fluorescence peak around 640 nm

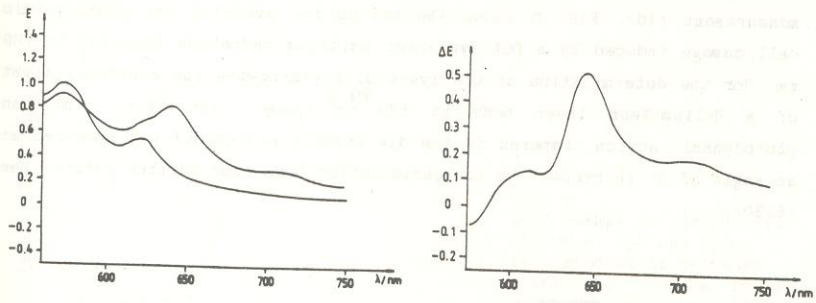

FIG. 2a Absorption spectrum of a fresh unirradiated (lower curve) and irradiated HpD-PBS solution (10^{-4}M), a new absorption maximum appears at 640 nm (photopoduct formation)
FIG. 2b Difference spectrum of the irradiated and unirradiated solution from FIG 2a. Three additional maxima in the red range occur during the photoproduct formation

Cells: Human erythrocytes were obtained from a hospital blood bank. The blood cells were diluted in a PBS solution (pH 7.4) up to a cell concentration of 10^6/ml. An Ehrlich ascites cell suspension obtained from the Centre for Experiments on Laboratory Animals at Jena was used with the same concentration. Hepatocytes were isolated as described by BERRY and FRIEND /3/ with the modifications reported by SEGLEN /23/. Male, 24 hour-starved Wistar rats were used as liver donors. Hepatocytes (1.6×10^7/ml) were stored in KREBS-RINGER bicarbonate medium, pH 7.4 at 0°C. The photodynamic action on the cells after incubation of HpD and HpD-photoproduct (incubation time 15 min) was examined by irradiation at 514 nm and at 600-670 nm (DCM dye-laser). The kinetics of lysis of erythrocytes was measured by means of small angle light scattering. The cell damage of the rat cells was determined by trypan blue staining in a final concentration of 0.25% and the damage of the Ehrlich ascites cells was controlled by measurement of the density and volume of the Ehrlich-Ascites Carcinoma (EAC) formed after i.p. injection, and by measurement of the growth of the solid form of this tumor after s.c. injection of these treated cells to male ICRmice. During the illumination the cell suspension was continuously stirred.

Experimental set-up: An optical multichannel analyzer (OVA 284, Centre of Scientific Instruments, Berlin) was used for absorption and fluorescence measurement /16/. FIG. 3 shows the set-up for studying the photodynamic cell damage induced by a DCM-dye laser emitting radiation from 590 to 720 nm. For the determination of the lysis of erythrocytes the scattered light of a Helium-Neon laser beam at 633 nm (power less than 1 mW, no photodynamic action compared to the dye-laser irradiation) was measured at an angle of 3° in respect to the undiffracted beam (for further details see /8,20/).

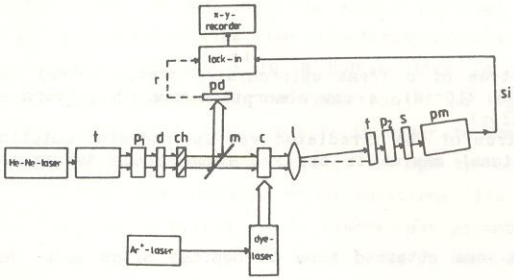

FIG. 3
Experimental set-up for the small angle light scattering on erythrocytes

t – telescope
P_1P_2-polarizers
d – diaphragm
ch – chopper
m – mirror
c – cuvette with scattering medium
l – lens
f – filter
s – slit
pm – photomultiplier
pd – photodiode
si – signal
r – reference signal

Results

In FIG. 4 the rate of damaged liver cells incubated with HpD and HpD-photoptoduct is shown after irradiation at 630 nm, the wavelength mostly used in the HpD photochemotherapy. The cells containing HpD are destroyed by light doses less than 4 J whereas the liver cells incubated with the photoproducts are significantly less damaged. In order to examine the behaviour in dependence on the wavelength especially in the region of the absorption maximum of the photoproduct around 640-650 nm, the

photohemolysis was determined by the sensitive method of small angle scattering.

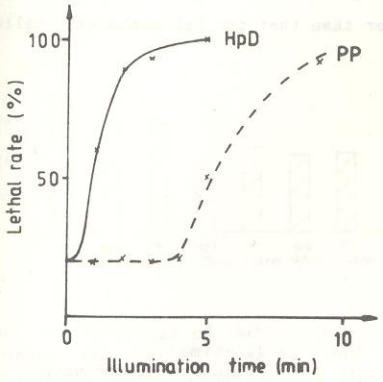

FIG. 4
Cell damage by photodynamic action of HpD and HpD-photoproduct on isolated liver cells (630 nm, 15 mW) dependent on illumination time

FIG. 5 shows the scattering signal from erythrocytes in HpD-PBS solution in dependence on the irradiation time. During the irradiation the membrane of the erythrocytes is damaged and the scattering signal goes down. Approximately 50 % of the cells undergo hemolysis at the time T_{50} where the scattering intensity is half of its initial value.

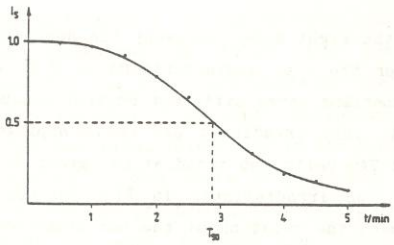

FIG. 5
Time dependent scattering signal of HpD incubated erythrocytes during irradiation (15 mW, 630 nm)

In the HpD-incubated cell suspension the greatest photodynamic activity in the spectral range from 630 nm to 660 nm occurs during 630 nm irradiation

(see Fig. 6). However, the photodynamic activity of the photoproduct containing samples is lower (compare the T_{50}-time in Fig. 6). A minimum of the photodynamic activity of the photoproduct occurs around 640 - 650 nm due to the absorption behaviour. In this spectral region the photodynamic activity is equal to or slightly higher than that for HpD containing cells.

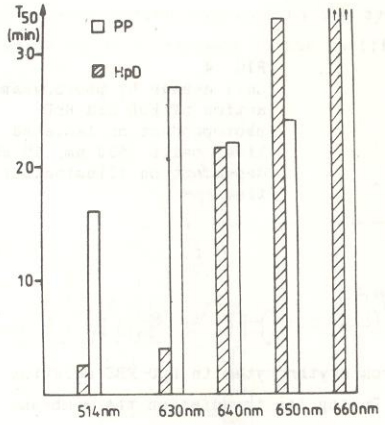

FIG. 6
T_{50}-time dependent on the wavelength
HpD: unirradiated solution
PP: photoproduct (HpD irradiated)

In order to verify the results with red light exposure on tumor cells, EAC cells obtained from mice were incubated with HpD and HpD enriched photoproduct, irradiated with red light and i.p. as well as s.c. reinjected.

In FIG. 7a the four columns on the right side represent the densities of the EAC-suspensions one week after the i.p. administration of 0.25 ml of the irradiated EAC cells. In comparison three different control groups are shown on the left side (group 1: only irradiated EAC cells applied, no drug/ group 2: only HpD-incubated EAC cells, no irradiation/ group 3: only photoproduct-incubated EAC cells, no irradiation). In FIG. 7b the four columns on the right side represent the relation of the wet tumor weights of the solid Ehrlich-Ascites-carcinoma growing one week after s.c. injection of irradiated EAC cells incubated with HpD and HpD enriched

photoproduct. In agreement with the results above no higher phototoxic rate of the photoproduct containing cells could be observed.

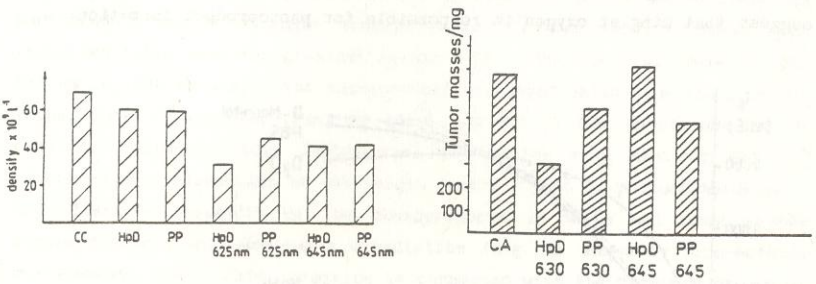

FIG. 7a Densities of the EAC-cell suspension after i.p. administration of treated EAC cells to mice (HpD and HpD-photoproduct incubated and irradiated, four columns on the right side and three control groups on the left side)
FIG. 7b Tumor weights of the Ehrlich-Ascites-carcinoma growing after s.c. injection of treated EAC cells to mice (four columns on the right side, CA: control animals with HpD administration but without any irradiation) 10 animals each group. Relative standard deviation 25-35%
For both cases (Fig. 7a,7b): incubation time for the EAC cells 30 min, light exposure 30 mW, 15 min, wavelength as indicated. The HpD-photoproduct (PP) was obtained by irradiation of an HpD-PBS solution with a mercury-lamp (50 mW/cm², 1 h)

So it can be concluded that the formation of the photoproduct reduces the efficacy of the photochemotherapy in spite of the stronger absorption of the photoproduct in the red spectral range.

Former investigations showed /4,5/ that in aqueous HpD solution under irradiation the HpD-fluorescence is bleached and stable nonfluorescent as well as instable fluorescent photoproducts are formed. The formation of both of them takes place only in the presence of oxygen and is mediated via the excited triplet state of porphyrin. Fig. 8 presents the formation of the fluorescent photoproduct(s) during irradiation in the case of different solvents as well as for the addition of different quenchers. The photoproduct formation is strong in the case of PBS but significantly reduced by addition of NaN_3, a singulet oxygen quencher. The quenching of the triplet state of HpD by metronidazole inhibits the photoproduct formation (as also shown in Fig.9). On the other hand D_2O accelerates the

formation (and the subsequent photoproduct destruction) because of the longer lifetime of singulet oxygen in this medium. Radical scavengers (e.g. the OH^- scavenger D-mannitol) have no remarkable effects. These results suggest that singlet oxygen is responsible for photoproduct formation.

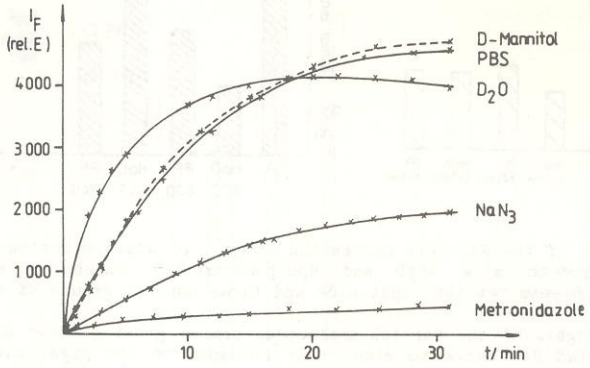

FIG.8 Formation the fluorescent HpD-photoproduct (monitored at 640 nm) during irradiation in the case of different solvents (10^{-4}M HpD in PBS and D_2O) and in the presence of D-mannitol (10^{-4}M), NaN_3 (10^{-2}M) and metronidazole (0.16 ml of 0,5 % metronidazole in 1 ml HpD-PBS solution)

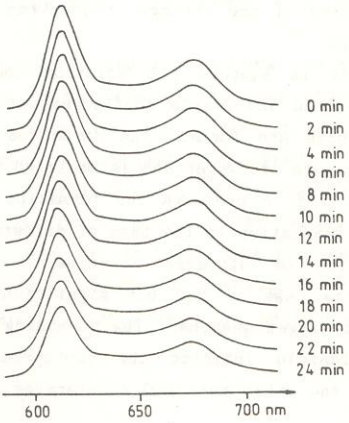

FIG. 9
Fluorescence spectra of HpD in PBS (10^{-4}M, 1 ml) and additional use of 0.1 ml metronidazole (0.5%). conditions are the same as in FIG. 1

Discussion

As shown in these experiments with different cell types the photodynamic activity of the HpD-photoproduct is not so high as that of HpD although the photoproduct has a stronger absorption in the red spectral range. HpD incubated cells show the greatest lethal rate in the spectral range 630 nm-660 nm at 630 nm whilst the photoproduct incubated cells show the greatest lethal rate (lowest T_{50}-time) at about 640-650 nm due to its absorption behaviour. However, this lethal rate is smaller than that of the HpD incubated cell suspension at wavelength commonly used in photochemotherapy. The effective formation of the photoproducts in cells and tumor tissue occurs during short-wavelength irradiation (e.g. as shown by fluorescence measurements /15/). This formation is connected with the presence of oxygen /5/. This fact can be of interest for the measurement of the oxygen status of tumor tissue. In photochemotherapy the formation of the photoproduct can be prevented. The additional injection of metronidazole inhibits its formation. Nitroimidazoles and HpD form chemically bound complexes which are photodynamically active by type-I photooxidation /1/. Especially in the case of hypoxic tumor areas, photochemotherapy is then possible and effective /14/.

Acknowledgement

The authors are grateful to Dr. S.N. Cherenkevich and Dr. T. Zorina from Institute of Physics, Belorussian University Minsk (USSR), for helpful discussions.

References

/1/ BAZIN M., SANTUS R., Photochem. Photobiol. 3(1986)235
/2/ BELTRAMINI M., FIREY P.A., RICCHELLI, F., RODGERS M.A.J., JORI G., Biochem. 26(1987)6852
/3/ BERRY M.N., FRIEND D.S., J. CELL. Biol. 43(1969)506
/4/ DIETEL W., KÖNIG K., Second International Conference on Laser Scattering Spectroscopy of Biological Objects, Pecs (1988)
/5/ DIETEL W., KÖNIG K., ZENKEVICH E., Lasers in the life sciences 3(1990)197,
DIETEL W., KÖNIG K., Third Congress of the European Society for Photobiology, Budapest (1989)
/6/ DOIRON D.R., GOMER C.J. Porphyrin localization and treatment of tumors

Liss, NY (1983)
/7/ GURINOVICH G.P., ZHAKOV I.G., KOSTEVICH G.A., KOCHUBEJEV G.A., SARZHEVSKAJA M.V., FROLOV A., Vestnik Akademii Medizinskie Nauk SSSR, 12(1987)13 (in russian)
/8/ GURINOVICH G.P., ZORINA T.E., ZORIN VP., SARZHEVSKAJA M.V., CHERENKEVICH S.N., Biophysica XXXIII 2(1989)314 (in russian)
/9/ HAYATA Y., DOUGHERTY T.J., Lasers and HpD in cancer. Igaku-Shoin, Tokio, NY (1983)
/10/ KESSEL D., Int. J. Radiat. Biol. 6(1986)901
/11/ KINOSHITA S., SEKI T., KUSHIDA T., Photochem. Photobiol. B 2(1988)195
/12/ KÖNIG K., BOCKHORN V., DIETEL W., SCHUBERT H., J. Cancer Res. Clin. Oncol. 113(1986)301
/13/ KÖNIG K., BOCKHORN V., KRAUSE U., DIETEL W., SCHUBERT H., LOTZ P., LÖBE L.P., Arch Geschwulstforsch. 1(1989)1
/14/ KÖNIG K., BOCKHORN V., Radiobiol. Radiother. 30(1989)535
/15/ KÖNIG K., DIETEL W., Arch Geschwulstforsch. 60(1990)1
/16/ KÖNIG K., Prom. A, Jena (1989)
/17/ LIER VAN J.E., ALI H., ROSSEAU J., in: /6/, pp 315
/18/ LÖBE L.P., KRAUSE U., LOTZ P., KÖNIG K., DIETEL W., BARTH J. Paper 141 on the International Conference on photodynamic therapy, London (1988)
/19/ MOAN J., KESSEL D., Photochem. Photobiol. B 1(1988)429
/20/ RAVEY J.C., J. Colloid Sci. 2(1984)435
/21/ RÖDER B., Studia biophysica 114(1986)183
/22/ ROTOMSKIENE J., KAPOCIUTE R., ROTOMSKIS R., JONUSAUSKAS G., SZITO T., NIZHNIK A. Photochem. Photobiol. B 2(1988)373
/23/ SEGLEN P.D., Meth. Cell. Biol. 13(1976)29

Received on 27. 2. 1990, in final version on 28. 9. 1990

Dr. K. KÖNIG, Department of Physics, Friedrich Schiller University Jena, Max-Wien-Platz 1, O-6900 Jena, FRG