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Preliminary Studies on a More Effective Phototoxic Agent Than Hematoporphyrin[1,2]

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ABSTRACT: Phototoxicity of benzoporphyrin derivative (BPD) has been tested in vitro and compared with that of hematoporphyrin (HP). After 1-hour activation with visible light, BPD was 10 times more cytotoxic than HP toward human adherent cell lines: A549 lung cancer, Calu-1 lung carcinoma, and CCD-19Lu normal lung, killing 100% of cells at the concentration of 70 ng/ml. Under the same conditions, BPD was 10-70 times more cytotoxic than HP toward nonadherent cells and cell lines. Tested were human leukemia cell lines HL60, K562, and KG1, normal human lymphocytes, and mouse mastocytoma cell line P815. The concentrations required to kill 100% of cells varied between 10 and 500 ng BPD/ml and between 0.2 and 10 mug HP/ml. The difference between the nonadherent cell lines in respect to their sensitivity to phototoxicity of both BPD and HP seemed to be related to the cell sizes, with the smallest cells being the most vulnerable. The most attractive characteristic of BPD in addition to its powerful phototoxicity is its maximum absorption around 700 nm, which is in the range of wavelengths penetrating tissues the best. This characteristic alone could make BPD a drug of choice in cancer photodynamic therapy when the safety of its use is ensured. Preliminary tests in vivo have shown that DBA/2J mice can tolerate a single ip injection of 20-60 mug BPD as well as the same dose of HP. The biodistribution and toxicity studies of BPD are under way in our laboratory. -- JNCI 1987; 79:1327-1332.

TEXT:

For over a decade porphyrins have been used in cancer photodynamic therapy in both animal models and human clinical trials [n1]. Extensive studies have been done on the photobiology of porphyrins, and especially on the chemical and biologic properties of HPD, which is presently used in cancer therapy [n2,n3]. The exact mechanism by which porphyrins cause cellular damage and death is not clearly understood at present. In general it is believed that damage to cells is caused at various loci by singlet oxygen and possibly other toxic oxygen species such as superoxide and hydroxyl radicals produced by porphyrins upon stimulation with light.

Since light is an essential factor in porphyrin cytotoxicity, the delivery of light to the porphyrin-sensitized cells is of vital importance. Although various types of light sources are available at present and the output of light can be very precisely controlled [n4], the ability of light to penetrate the tissue conditions the amount and type of light actually delivered to the sensitized cells. The research on light penetration in tissues [n5] led to the conclusion that in order to obtain a maximum photochemical reaction a photosensitizer should have maximum absorption in the range of either 700-800 or 950-1,100 nm, where tissue attenuation is the least and light penetration is the best. HP has maximum absorption at around 400-nm wavelength at which most of the light is scattered and absorbed by tissues, and it absorbs only very poorly in the region of the better penetrating wavelengths. Hence an intensive search is in progress for better

photosensitizers.

We have synthesized a photoreactive compound that may be potentially useful in cancer photodynamic therapy since upon light activation it is more cytotoxic than HP and has a maximum absorption at around 700 nm. In the present paper we report the results of in vitro tests comparing its cytotoxicity toward several cancer cell lines with that of HP.

MATERIALS AND METHODS

Photoreactive agents. -- HP IX was purchased from Aldrich Chemical Company, Inc., and used in the experiments without any modifications. The BPD was synthesized as described in detail elsewhere [n6]. In brief: protoporphyrin prepared from HP (ICN Nutritional Biochemicals, Inc.) [n7] was reacted with dimethyl acetylenedicarboxylate giving Diels-Alder adducts, which were subsequently rearranged with 1,5-diazabicyclo [5.4.0] undec-5-ene. The resulting diastereomeric mixture of methyl esters (text-fig. 1A) was hydrolysed with 25% hydrochloric acid at room temperature for 5 hours in the dark, then frozen in liquid nitrogen, and dried under high vacuum. The isolated BPD (text-fig. 1B) was used without further purification.

Both porphyrins were stored dissolved in DMSO at -20 degrees C. Working stock solutions were prepared by diluting DMSO stocks tenfold with PBS and were kept at 4 degrees C for no longer than a month. Further dilutions were made immediately before each experiment with the use of culture media. At all times the reagents were protected from light.

The absorption spectra of BPD, HP, and Photofrin II (commercially available dihematoporphyrin ether; Photofrin Medical, Inc.) in PBS were obtained by means of a Hewlett Packard UV/Visible Spectrophotometer model 8452A (text-fig. 2). To fit them all on one graph, the concentrations of the porphyrins were adjusted and the absorption spectra at the wavelength shorter than 470 nm (Soret band) were cut off. The extinction coefficients are 13,000 M<-1> cm<-1> at 692 nm and 3,000 M<-1>cm<-1> at 623 nm for BPD and HP, respectively.

Cells and cell lines. -- Cells grown as monolayers: Human cell lines A549 lung cancer, Calu-1 lung carcinoma, and CCD-19Lu normal lung were obtained from the American Type Culture Collection. These lines were maintained in EMEM containing nonessential amino acids in Eagle's balanced salt solution with L-glutamine and supplemented with 10% FCS at 37 degrees C and in 10% CO[2].

Cells grown in suspension: Three human leukemia cell lines K562 (myelo-monocytic), KG1 (myeloid), and HL60 (pro-myelocytic) and one mouse cell line P815 (mastocytoma from DBA/2J mice) were maintained in our laboratory. All except HL60 were cultured in DMEM. HL60 cells were cultured in RPMI-1640 medium. The media were supplemented with 10% FCS, and the cells were incubated in a humidified incubator at 37 degrees C in 10% CO[2]. The cells were in the logarithmic phase of growth when tested in cytotoxicity assays. For the determination of cell size, the cell diameters were measured under the microscope by means of a micrometer.

Normal peripheral blood lymphocytes were obtained from healthy volunteers in our laboratory by density gradient centrifugation of heparinized blood samples over Ficoll-Hypaque as described previously [n8].

Cytotoxicity assay. -- In preparation for the cytotoxicity assay, cells grown as monolayers were lifted up with the use of 0.25% trypsin solution (GIBCO) and subcultured in microslides (Miles Scientific) containing eight chambers each, at a concentration of 10<5> cells per chamber. They were allowed to grow to confluence, usually for 3 days. Immediately before the treatment with porphyrins, they were washed three times with PBS, and then various concentrations of either of the porphyrins were added in 200 mul of EMEM per chamber. The concentrations tested ranged from 7 to 200 ng BPD/ml and from 70 to 2,000 ng HP/ml and were aimed to cover the full range of cellular response between 100% killing and 100% survival of the cells.

Cells grown in suspension and lymphocytes were washed three times with DMEM, resuspended in DMEM without serum at a concentration of 1.6-2.3X10<6> cells per ml, and distributed in 0.4-ml aliquots into 12X75-mm plastic tubes. The cells were then treated with porphyrins added in 100 mul DMEM per tube. Individual cell types were tested with at least four to five different concentrations of either of the porphyrins, and the range of concentrations was adjusted according to the cell sensitivity. Each set of concentrations included a control receiving 100 mul of medium alone.

The experiments were performed in dim light to protect the reagents from premature activation and the treated cells from uncontrolled exposure to light. Dilutions of the porphyrins were prepared immediately before adding them to the cells. Individual experiments were set up to test each cell line with both porphyrins and expose them to light in both direct and affinity tests.

Exposure to light. -- Direct test: Cells in medium containing porphyrins were immediately exposed to the light source for 1 hour. After the exposure the cells in microchambers were washed three times with PBS, and 300 mul of EMEM containing 10% FCS was added to each chamber for incubation at 37 degrees C in 10% CO[2]. The cells in tubes were washed twice with DMEM (2 ml/tube), resuspended in 0.5 ml of DMEM containing 10% FCS per tube, and dispensed into 96-well culture plates.

Affinity test: Cells in medium containing porphyrins were incubated at 37 degrees C for 1 hour in the dark. Then the cells in microchambers were washed three times with PBS and exposed to the light source for 1 hour in 200 mul of EMEM per chamber. The cells in tubes were washed twice with DMEM (2 ml/tube), resuspended in 0.5 ml of DMEM per tube, and exposed to light for 1 hour. After exposure to light, the microchambers were washed once with PBS and filled with 300 mul of EMEM containing 10% FCS and incubated at 37 degrees C in 10% CO[2]. The cells in tubes were spun down and resuspended in 0.5 ml of DMEM containing 10% FCS and dispensed into 96-well culture plates.

Determination of cell survival. -- After 18 hours of incubation, the survival of cells in microchambers was determined by a differential viability stain with the use of ethidine bromide and acridine orange [n9]. The cells were stained and examined by means of a fluorescence microscope. Cell counts were done in five central areas of each chamber, and percent viability was calculated.

The survival of cells from lines grown in suspension was determined by their ability to divide and incorporate [<3>H]thymidine. Variously treated cells were tested in quadruplicates or quintuplicates with the use of 96-well culture plates (Falcon #3072). Each well contained 0.10-0.15X10<6> cells in 200 mul DMEM or RPMI-1640 (HL60 only) supplemented with 10% FCS and 2 muCi [<3>H]thymidine. The plates were incubated at 37 degrees C in 10% CO[2] for 18 hours. After the incubation, 50 mul of 25% trichloroacetic acid and 20 mul of cold thymidine (15 mg/ml in distilled H[2]O) were added to each well, and the plates were harvested by means of a MASH harvester. The amount of incorporated radiolabeled thymidine was measured in counts per minute by means of a Packard Tri-Carb 4450 scintillation counter.

The survival of lymphocytes was determined by their ability to incorporate radiolabeled thymidine following mitogenic stimulation. The tested cells were first incubated for 24 hours in DMEM containing 10% FCS and 0.5% phytohemagglutinin (Difco) in a volume of 150 mul per well. At 24 hours 2 muCi of [<3>H]thymidine in 50 mul of complete culture medium was added to each well, and the incubation was continued for a further 24 hours. At this time the cells were harvested, and the amount of radioactivity incorporated was determined as described above.

Light source. -- A light source was a bank of four fluorescent tubes (General Electric F20T12-Cool White). The spectrum of light generated ranged from 300 to 750 nm. The intensity of light as measured by YSI-Kettering model 65 radiometer was 1.5 mW/cm<2>. According to the spectral output chart supplied by the manufacturer [n10], the relative intensity of light at 400 nm was at least twice that at 700 nm. The temperature at the level of exposed samples, that is, at the 11-cm distance from the lamp, did not exceed 25 degrees C at any time.

Relative toxicity of porphyrins in mice. -- A preliminary test was done to determine whether animals would tolerate

BPD as well as HP. Twenty-four mature female DBA/2J mice, each weighing about 25 g, were divided into 6 groups, 4 mice per group. Each group received a different dose of either HP or BPD in 200 mul of saline in a single ip injection. The doses were as follows: 60 or 20 mug HP per mouse and 60, 20, 6, or 2 mug BPD per mouse. Following the injection, the mice were first kept for 4 hours in the dark and then exposed to the light source (the same as used for in vittro experiments) for 2 hours. They were then kept in the routine animal laboratory conditions in which there is intermittent 12 hours of light and 12 hours of dark. The animals were observed daily. Two mice per group were sacrificed on day 12, and the remaining 2 per group were sacrificed on day 28. At the time of sacrifice, their internal organs were examined macroscopically.

RESULTS

In our experimental conditions, BPD proved to be more cytotoxic than HP toward all cell types tested. The results obtained with the cells grown as monolayers and representing either solid tumors (A549, Calu-1) or a normal lung tissue (CCD-19Lu) are shown in text-figure 3. BPD was cytotoxic to all 3 cell lines in concentrations about 10 ng/ml or greater, whereas HP was cytotoxic in concentrations of about 100 ng/ml or greater. The total range of cellular response from 100% cell survival to 100% cell killing was obtained within 7-70 ng BPD/ml and 70-700 ng HP/ml. Neither BPD nor HP exhibited any selective toxicity toward tumor cells in vitro. Both were as cytotoxic to the lung cancer lines as to the normal lung cell line. Both porphyrins were cytotoxic to the cells in the affinity test almost as much, if not more, as in the direct test.

Similarly, BPD was more cytotoxic than HP toward all types of nonadherent cells tested. The results are presented in text-figure 4. The LD[50]'s of BPD and HP were approximated from the graphs and are presented in table 1. BPD was about 10-20 times more potent than HP in the direct test and 10-70 times more potent in the affinity test.

The cell lines differed in respect to their sensitivity to BPD and HP, and their sensitivity appeared to be related to the cell size. LD[50] doses determined for each cell line were proportional to the average cell size as expressed by a radius to the third power (text-fig. 5).

Single ip injections of 2-60 mug of BPD or 20-60 mug of HP per mouse did not have any visible effect on the mice. The animals appeared to be normal. There was no sign of skin itchiness. There was no visible change in the appearance of their skin on the exposed parts such as ears and foot pads. All internal organs examined, with special attention given to spleen, kidney, liver, gallbladder and thymus, appeared to be normal. There was no visible change of peritoneum at the site of injection.

DISCUSSION

In the conditions of our experiments, BPD proved to be a very powerful phototoxic agent. Its toxicity was solely due to the activation by light. The treatment of cells with BPD without subsequent exposure to light did not affect the cell viability (preliminary experiments, results not shown). In comparison with HP, BPD was 10 times more cytotoxic to cells grown in monolayers, thus representing either solid tumors or normal tissue, and 10-20 times more cytotoxic to free-floating cancer and normal cells.

HPD has been shown to be considerably more active in vivo than standard preparations of HP due to undefined factors that might include aggregation, or water of lipid solubility that lead to higher levels of accumulation in tumor tissue. However, it has also been reported that in vitro, HPD is only about twice as effective as HP in photosensitizing human NHIK 3025 cells [n11]. Obviously, when biodistribution studies are done with BPD, comparisons will have to be made with both HP and HPD, but for the purposes of this report we believe that the comparison with HP alone is

valid.

Adherence to cells is the first step in the process of uptake and accumulation of HP in cancer cells. The results of affinity tests have shown that BPD adheres to cancer cells at least as well as HP, or, as in the case of P815, KG1, and K562 cells, even better than HP. The ratios of LD[50] doses of HP to BPD in these cell lines were much higher in the affinity than in the direct test (table 1), indicating that in proportion to concentration more BPD than HP molecules adhered to the cell mem- membrane and possibly penetrated it faster than HP, causing more extensive damage to the cells. Similarity between LD[50] doses of BPD for P815 cells determined in affinity and direct tests suggests that the cell membrane is a possible site of toxic action (since the incubation time did not seem to make much difference).
TABLE 1. -- Concentration of BPD and HP required to kill 50% of cells LD[50] in direct and affinity tests<a>

The relationship between the cell size and sensitivity to phototoxic effects of BPD and HP (text-fig. 5), if not purely coincidental, supports further the suggestion that, at least in vitro, the cell membrane is the site of action. Since the cells are of a sphere shape, their volume increases in proportion to their radius to third power while their surface increases in proportion to their radius to second power. Thus small cells have greater surface in proportion to their volume than large cells, which apparently makes them more vulnerable. It is interesting, though difficult to comment upon, that the relationship between the cell size and sensitivity to BPD, but not to HP, differs between direct and affinity tests. As a result, large cells are more sensitive to BPD in the affinity test than in the direct test while small cells (HL60, lymphocytes) are more sensitive to BPD in the direct rather than the affinity test.

Both BPD and HP were cytotoxic to normal cells in the same range of concentrations as to cancer cells. It has been previously reported [n12,n13] that in vitro neoplastic and normal cells do not differ in their uptake of porphyrins. In vivo cancer seem to accumulate porphyrins more than normal cells, though the mechanism is not yet clear. We do not know at present if BPD can be taken in and retained by cancer cells like HPD. However, the danger of killing normal cells during cancer therapy could be circumvented by the use of the drug conjugated to the antibody against cancer antigen. This allows more precise targeting of the drug on cancer cells as well as reducing the effective concentration of the drug. We have previously reported results with HP-antibody conjugates where the conjugates reduced the growth of mouse tumors[n14] or killed tumor cell lines[n15] in concentrations at which HP alone was ineffective.

The main advantage of BPD is that its maximum absorption is around 700 nm. This means, that in contrast to HP, BPD can more readily absorb the light that penetrates tissues. Comparing the extinction coefficients in 600- to 700-nm wavelength range, BPD is about four times more efficient than HP in absorbing the light. This alone could make BPD more suitable than HP for cancer photodynamic therapy. The relative intensity of light produced by the light source used in our experiments favored HP rather than BPD. Thus it is possible that BPD is even more cytotoxic in comparison with HP than our experiments have shown.

There were no apparent signs of toxicity observed in mice following ip injections of either BPD or HP. The doses given were equivalent to in vitro 0.11-3.4 mug BPD/ml and 1.14-3.4 mug HP/ml, if we assume that the animal body consists of 70% fluid. Since this range covers the killing range of concentrations of BPD in vitro, it is likely to be suitable for experiments in vivo. At present it is not known if the biodistribution of BPD is the same as that of HP[n16,n17], and the experiments with radio-labeled BPD are under way.

In conclusion, BPD appears to be superior to HP as a photosensitizing agent because of its high cytotoxicity and ability to absorb light better than HP at the wavelength that best penetrates tissue. It is of importance that the parent compound of BPD is inexpensive and the synthesis of BPD is relatively simple. Thus BPD could be easily obtained for use in therapeutics. However, further tests are presently being conducted in our laboratory to ensure its safe use as a therapeutic agent.

ABBREVIATIONS USED: BPD=benzoporphyrin derivative; DMEM=Dulbecco's modified Eagle medium; DMSO=dimethyl sulfoxide; EMEM=Eagle's minimum essential medium; FCS=fetal calf serum; HP=hematoporphyrin; HPD=hematoporphyrin derivative; LD[50]=lethal dose killing 50% of cells; PBS=phosphate-buffered saline.

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GRAPHIC: TEXT-FIGURE 1. -- Structures of the methyl ester (A) and BPD (B); TEXT-FIGURE 2. -- The absorption spectra of BPD, HP, and dihematoporphyrin ether (Photofrin II) in the wavelength range from 470 to 726 nm. For more information, see text.; TEXT-FIGURE 3. -- Cytotoxicity of BPD and HP toward various cell lines grown as monolayers, after exposure to light in direct and affinity tests (for details, see text).; TEXT-FIGURE 4. -- Cytotoxicity of BPD and HP toward normal lymphocytes and various tumor cell lines grown in suspension, after exposure to light in direct and affinity tests (for details, see text).; TEXT-FIGURE 5. -- Relationship between the sensitivity of various cell types grown in suspension to BPD or HP expressed as LD[50] and the cell size expressed as a radius to third power. (SYMBOL ILLEGIBLE) direct test; (SYMBOL ILLEGIBLE) affinity test.