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Hematoporphyrin derivative and anthracyclines mutually inhibit cellular uptake and toxicity

R. M. Böhmer, K. Hoffmann, and G. Morstyn

Ludwig Institute for Cancer Research, Tumor Biology Branch, P.O. The Royal Melbourne Hospital, Victoria 3050, Australia

Summary. Hematoporphyrin derivative (HpD) is a fluorescent photosensitizer which localizes selectively in tumors and is used in photoirradiation therapy of various types of cancer. Photoirradiation is a local form of treatment. There may be advantages in combining it with systemic chemotherapy, and we therefore studied the interaction of HpD and various cytotoxic drugs [daunorubicin (DR), adriamycin (AD), mitozantrone (MI), *cis*-platinum (CP) and 5-fluorouracil (FU)] with respect to cellular uptake and toxicity. Cultured cells were incubated with HpD and the cytotoxic drugs, either simultaneously or sequentially. The amount of DR, AD and HpD taken up by cells was quantitated by measuring cellular fluorescence and by determining the specific cytotoxic action of the drugs (light-induced cell kill for HpD and loss of proliferative potential for the cytotoxic drugs). The presence of 100 mg/l HpD during drug incubation caused 30-, 12- and 6-fold reductions in the cytotoxicity of DR, AD and MI respectively. HpD-associated fluorescence and photosensitization were reduced upon co-incubation of cells with HpD and AD. When cells were incubated with HpD and AD sequentially, fluorescence was equal to the sum of fluorescence values achieved by treatment with HpD and AD alone, and the cytotoxicity was not reduced. This suggests that the effects were due to an interaction of HpD with the drugs outside the cells, resulting in a reduction of free drug available for cellular uptake. CP and FU did not interact with HpD. The results suggest that protocols which combine photoirradiation therapy and chemotherapy with anthracyclines or similar compounds need to take this interaction into account.

Introduction

Photoirradiation therapy is a form of local treatment for cancers of many different histologic types including squamous call carcinomas of the lung and esophagus, transitional carcinomas of the bladder and adenocarcinomas of the stomach [1–8]. Patients are administered hematoporphyrin derivative (HpD), which is a photosensitizer that selectively localizes in tumors and sensitizes the tumors to killing by light of the appropriate wavelength. This local treatment of cancer could be combined with a systemic form of therapy such as chemotherapy [1].

However, HpD is a complex multicomponent mixture of porphyrins [2] and its interaction with chemotherapeutic drugs is not well investigated. In the present study we have analyzed the interactions of HpD and several anticancer drugs: adriamycin (AD, doxorubicin), daunorubicin (DR), *cis*-platinum (CP), 5-fluorouracil (FU) and mitozantrone (MI). These are drugs that might be combined with photoirradiation in cancer therapy.

The interactions were analyzed with respect to cytotoxicity and cellular uptake, using flow cytometry to measure drug fluorescence as well as the time to light-induced cell death.

Materials and methods

Cell culture. The experiments were carried out using the murine myelomonocytic cell line WEHI 3B D+ (provided by Dr. D. Metcalf, Walter and Eliza Hall Institute, Melbourne). The cells grow as a suspension culture in DME medium supplemented with 10% fetal calf serum (FCS). The population doubling time is 10 h. Assays on clonal survival were made using the Chinese hamster lung fibroblast cell line V79 (supplied by J. B. Mitchell, National Cancer Institute, Bethesda, MD, USA). The cells grow as monolayers in DME medium with 10% FCS.

Drugs. Hematoporphyrin derivative was prepared by the method described by Forbes et al. [9] using hematoporphyrin hydrochloride purchased from Sigma Chemical Co., St. Louis, MO, USA.

Sources of cytotoxic drugs:

Cerubidin (daunorubicin): May & Baker, Vic, Australia;
Adriamycin (doxorubicin): Farmitalia, Milan, Italy;
Novantrone (mitozantrone): Lederle, N.S.W., Australia;
Cis-platinum, 5-fluorouracil:
David Bull Laboratories, Vic., Australia.

Flow cytometrical quantitation of cellular fluorescence. The desired combination of HpD and drugs was prepared in 5 ml of growth medium, and 5×10^4 cells were added. The

Offprint requests to: R. M. Böhmer, Ludwig Institute for Cancer Research, P. O. Royal Melbourne Hospital, Victoria 3050, Australia

suspension was then incubated for 1 h at 37° C, centrifuged, resuspended in 10 ml of growth medium (containing 10% FCS), and stored at room temperature for 1 h to allow loosely cell-associated fluorescent material to dissociate into the medium. The cell-associated fluorescence of HpD, DR and AD was quantitated using a flow cytometer (Ortho System 50, Ortho Diagnostic Systems, Westwood, MA, USA). The fluorescence was excited with the 514-nm line of an argon ion laser, and the fluorescence signals were recorded at 577 nm (DR) and beyond 620 nm (AD and HpD). Fluorescent plastic beads (1.76 μm, coumarin, Polysciences, Warrington, PA) were introduced into the cell suspensions for fluorescence normalization and as a numerical reference for cell counting. Biparametrical distributions of low-angle light scatter versus fluorescence as well as low-angle versus right-angle light scatter were recorded. The mean fluorescence values of the cell populations were determined in units of bead fluorescence, and the fluorescence of control cells not treated with drug was subtracted.

Quantitation of HpD-induced phototoxicity. Flasks containing a suspension in growth medium of the cell populations subjected to drug treatment were placed onto a screen which was evenly illuminated with cool white light in the range of 450–650 nm, the intensity of which was ca. 60 nW/cm [10]. At 5-min intervals during the illumination aliquots of the suspensions were harvested without interruption of the light exposure, a known number of plastic beads were added, and a biparametric histogram of low-angle versus right-angle light scatter was recorded. In such histograms, dead cells (as defined by propidium iodide staining) appear in a discrete cluster with higher right-angle and lower low-angle light scatter values. Using the beads as a numerical reference, the number of vital cells per ml of suspension could thus be determined.

Estimation of drug cytotoxicity in suspension cultures. The cell populations were treated with the drugs for 1 h at 37° C, washed, and suspended at 2.5×10^4 cells/ml in growth medium and incubated under standard growth conditions for 8 days. At this time and later, the flasks contained either a completely overgrown population with more than 10^6 cells/ml or approximately the starting cell numbers. No intermediate values were found at 8 days and later. Thus, the threshold of drug dosage leading to complete growth inhibition could be determined within the concentration increments used. The overgrown populations could also be readily identified because they were cloudy with an acidic (yellow) medium.

Estimation of growth inhibition in adherent cell cultures. Per 25 cm^2 culture flask, 200 cells were seeded and allowed to adhere for 4 h. The medium was then replaced by medium containing the drugs, and the cells were incubated for 1 h at 37° C. The medium was then removed carefully, and the cells were incubated in fresh medium for 10 min at room temperature in the dark. In the case of sequential drug incubation, the above was then repeated for the second drug. The medium was replaced again by fresh medium, and the cells were incubated at 37° C for 10 days. Colonies were counted after hematoxylin staining.

Results

Fluorescence study of the uptake of adriamycin and daunorubicin in the presence of HpD

A decrease in the cellular DR and AD fluorescence was observed after co-incubation with HpD. Cells were incubated for 1 h simultaneously with 40 mg/l DR (Fig. 1A) or 40 mg/l AD (Fig. 1B) and various concentrations of HpD (0–300 mg/l), then washed and their fluorescence analyzed. DR fluorescence was measured at 577 nm, a wavelength at which HpD fluorescence was found to be negligible. Figure 1A shows a reduction of DR fluorescence with increasing HpD concentration. Since cell-bound AD has no fluorescence at 577 nm, AD fluorescence had to be measured together with HpD fluorescence at >620 nm (Fig. 1B). The combined fluorescence of HpD and AD decreased with increasing HpD concentrations up to a certain concentration of HpD, followed by a sharp rise of fluorescence at higher HpD concentrations. The descending part of the curve presumably represents the decreasing AD fluorescence, while the ascending part represents the increase in HpD fluorescence. A comparison was made between normal (10%) and low (0.1%) serum concentrations during incubation, since we had found previously that HpD binding to cells is increased at low serum concentrations [10]. No dependence of the fluorescence reduction on serum concentration was observed for DR (Fig. 1A). Figure 1B shows little dependence of fluorescence on

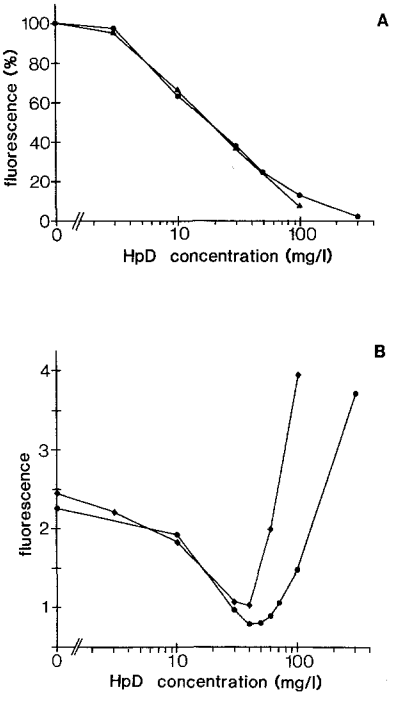

Fig. 1. A. Cellular fluorescence at 577 nm after incubating WEHI 3B cells with 40 mg/l daunorubicin for 1 h at 37° C, together with various concentrations of hematoporphyrin derivative. Fluorescence values shown as a percentage of the values measured for incubation with daunorubicin alone. Incubation in medium with 10% (●) or 0.2% (▲) fetal calf serum. **B.** Cellular fluorescence above 620 nm after incubating WEHI 3B cells with 40 mg/l adriamycin together with various concentrations of HpD in medium with 10% (●) or 0.2% (◆) fetal calf serum. Fluorescence given in arbitrary units

serum concentration up to ca. 30 mg/l HpD, and a steeper rise of fluorescence at higher HpD concentrations in low serum concentration, confirming the interpretation that the rise is due to HpD uptake.

Reduction of HpD phototoxicity by AD

Cells were incubated for 1 h with 100 mg/l HpD together with 0, 10, 20, 40 and 80 mg/l AD, washed twice for 30 min, then illuminated with white light, and the number of vital cells was monitored with the flow cytometer as a function of time during illumination. The decrease in the proportion of vital (see Materials and Methods) cells from 100% to 0% occurred within a narrow time interval (ca. 10% of the time when the first cells die), so that the time of population death $t_{1/2}$ (50% dead) was easy to determine. $T_{1/2}$ shifted to longer times at increased concentrations of AD during the incubation with HpD (Fig. 2).

At concentrations of AD above ca. 80 mg/l, HpD precipitated and sedimented at unit gravity.

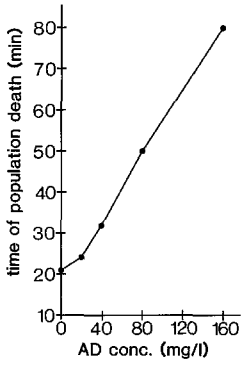

Fig. 2. Photosensitivity after incubation with 100 mg/l HpD together with various concentrations of adriamycin. Incubation in medium with 10% fetal calf serum. *Ordinate:* time of population death during illumination, determined flow-cytometrically as described in the text

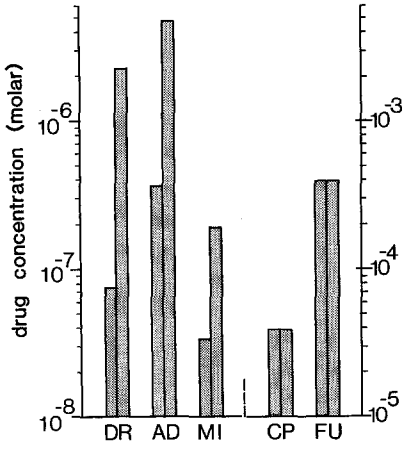

Fig. 3. Molar concentrations of the various drugs (indicated in the figure) leading to a complete inhibition of culture growth (1 h incubation for AD, DR and MI, 2 h incubation for CP, FU), as determined 8 days after the incubation. Comparison of incubation with the drugs alone (*left bars*) and of co-incubation with 100 mg/l HpD (*right bars*). Concentration scale for DR, AD and MI indicated at *left ordinate*; concentration scale for CP and FU indicated at *right ordinate*

Effects of HpD/AD precipitation on cellular toxicity

Precipitation of HpD (at 100 mg/l HpD) by AD (and DR) occurred at and above AD/DR concentrations of ca. 80 mg/l, i.e. when the molar AD/DR concentration exceeded that of HpD. After sedimentation of the precipitate by centrifugation the supernatant was clear of HpD, as judged by the colour, and it did not retain any HpD measurable with the sensitivity of the phototoxicity assay (data not shown). When the precipitate was redissolved in the original volume of medium without AD/DR it could not be sedimented again, but the solution did not regain phototoxicity. Preliminary assays (data not shown) on clonal survival revealed that the cytotoxicity (effective concentration) of DR in the supernatant as well as in the redissolved precipitate was strongly reduced (five- to tenfold). The cytotoxicity of the redissolved precipitate did not increase after prolonged storage (24 h), indicating the existence of a strongly bonded complex.

Reduction of drug toxicity by HpD

The change of effective drug concentration by co-incubation with HpD was assayed by a simple technique determining the concentration at which cell growth is completely inhibited in a culture (see *Materials and methods*). WEHI 3B cells were incubated for 1 h at 37° C with 100 mg/l HpD and a wide range of drug concentrations. To determine the specificity of the interaction between HpD and the anthracyclines DR and AD, we studied the structurally unrelated cytotoxic drugs *cis*-platinum (CP) and 5-fluorouracil (FU), as well as the drug mitozantrone (MI), which has the anthracene-dione portion of the anthracyclines (Fig. 3). Co-incubation of DR, AD and MI with HpD shifted the thresholds for complete growth arrest by the factors of 30, 12 and 6 respectively, whereas CP and FU toxicity were not affected by HpD. Note the separate concentration axis drawn for CP and FU, which were required at much higher molar concentrations to achieve cell kill.

The dependence of protection from DR toxicity on HpD concentration was investigated by varying both the HpD and DR concentrations, using the same assay as for Fig. 3. Table 1 shows at which combinations of concentrations of HpD and DR proliferation occurred (cell numbers $> 10^6$/ml, code +) and at which no proliferation occurred (cell numbers $< 3 \times 10^4$/ml, code −). Lower HpD concentrations were required for protection at lower DR concentrations.

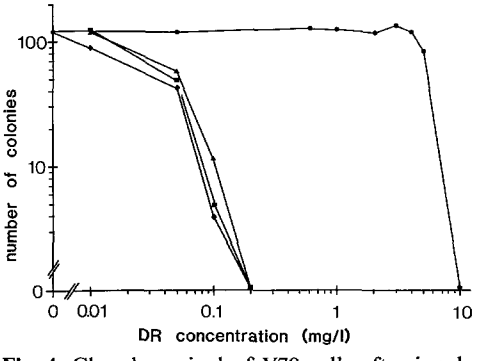

Fig. 4. Clonal survival of V79 cells after incubation for 1 h with various concentrations of daunorubicin alone (◆) and with daunorubicin plus 100 mg/l of HpD, either simultaneously (●) or sequentially with HpD before (▲) or after (■) daunorubicin

Table 1. Combinations of DR and HpD concentrations (incubation for 1 h at 37°C) leading to a complete inhibition of culture growth, as determined 8 days later

HpD (mg/l)	Daunorubicin (mg/l)				
	0.1	0.5	1.0	1.5	2.0
10	+	−	−	−	−
20	+	+	−	−	−
40	+	+	−	−	−
60	+	+	+	−	−
80	+	+	+	+	−
100	+	+	+	+	−
300	+	+	+	+	+

+, Overgrown cultures ($>10^6$/ml); −, flasks containing original cell numbers (ca. 2.5×10^4/ml)

Table 2. Comparison of simultaneous and sequential incubation with HpD and AD for successive 1-h periods with one washing step between incubations. Fluorescence values were determined 1 h after the last incubation and are given in arbitrary units. $T_{1/2}$ (time in minutes when 50% of the cells were dead) was determined 2 h after the last incubation as described in the text.

Incubation	Fluorescence	$t_{1/2}$
HpD alone	0.75	50
AD alone	2.5	>120
AD then HpD	3.5	50
HpD then AD	3.2	60
HpD and AD together	0.78	>120

HpD light sensitization and fluorescence after sequential exposure to HpD and AD

We incubated cells with HpD and AD successively instead of simultaneously in order to determine whether the mutual reduction of fluorescence and toxicity of the drugs was due to a reduced drug uptake based on drug interaction outside the cells, or whether it was due to an interaction within the cells. Table 2 shows fluorescence and phototoxicity data comparing sequential with simultanous incubation. Following sequential incubation, the cellular fluorescence was roughly equal to the sum of fluorescence values obtained by incubating with HpD and AD individually, and the phototoxicity from HpD was not reduced by AD.

Clonal survival after simultaneous and sequential exposure to HpD and DR

V79 cells seeded at 200 cells per flask were incubated with various concentrations of DR and 100 mg/l HpD for 1 h at 37° C either simultaneously or sequentially, with HpD incubation either before or after DR incubation. Figure 4 shows the number of colonies per flask as a function of DR concentration. It is apparent that HpD had no protective effect when cells were incubated with HpD before or after exposure to DR. Simultaneous incubation with DR and HpD resulted in an approximately 70-times higher concentration of DR required to cause cell kill.

Discussion

The major findings of this study were that co-incubation of the anthracyclines AD, DR and MI with HpD resulted in decreased effectiveness of both the HpD and the cytotoxic drugs. This was shown by a reduction in the growth-inhibiting activity of the drugs and by the reduction of porphyrin-induced photosensitization of the cells. It was related to a reduced uptake of fluorescent material. From the correlation between the reduction in fluorescence and toxicity, as well as from the additivity of drug fluorescence values and the lack of a reduction in toxicity after sequential incubation, we conclude that the observed changes were not based on a drug interaction inside the cell. The basis for this phenomenon seems to be a mutual inhibition of cellular drug uptake. Further studies are required to explain this mutual inhibition of uptake. It could be caused either by a complexing of porphyrins and drugs in solution, hindering cellular incorporation, or by a mutual blocking of the cellular uptake mechanism. The approximately constant molar ratio for complete growth inhibition shown in Table 1 and the precipitation of HpD at high AD concentrations indicate a binding of the drugs to HpD in solution. The preliminary studies with the precipitated material further indicate that the binding is irreversible. We had shown previously that HpD uptake into cells is strongly increased at low serum or albumin concentrations [10], presumably because of an increased amount of free HpD. However, we found no effect of serum on the interaction between HpD and the anthracyclines, suggesting that the binding of HpD to the drugs and to serum proteins occurs at independent sites. Drugs of a different structure (CP, FU) did not produce the effect, whereas MI toxicity was affected by HpD. This drug shares with AD and DR the anthraquinone portion; thus, the similar interaction with the porphyrin suggests that this portion of the molecule is involved in the interaction. Studies to characterize the chemical interaction between HpD and the drugs are in progress.

Our results are of clinical importance whenever photoirradiation therapy is combined with systemic chemotherapy using adriamycin, daunorubicin, mitozantrone and maybe other similar drugs. Our own observations (unpublished data) show that porphyrins persist in the blood for up to 3 weeks after administration. The peak plasma levels of daunorubicin and adriamycin achieved in patients are on the order of 1 mg/l [11]. Although the decrease in plasma levels is rapid [11], the initial concentrations are well above the concentrations shown to be affected in our toxicity studies. The concentrations achieved in the local environment of tumor cells are not known. The very basis of HpD therapy is the selective accumulation and retention of HpD in tumor tissue. Therefore, HpD can be expected to be present in tumor tissue in concentrations which can effect protection against drug toxicity. Furthermore, our data suggest that the HpD-drug interaction occurs through a stong and irreversible binding, so that the interaction is determined by the molar ratio of HpD and cytotoxic drugs rather than by absolute concentrations. Therefore, we assume that an interaction between HpD and the cytotoxic drugs occurs in the plasma and in the tissue. The anthracycline/HpD precipitate described is presently under chemical analysis. However, it is difficult to determine the nature of the binding between the two materials since HpD is a complex mixture of many porphyrins.

Preliminary studies using a rat glioma model showed that the redissolved HpD/DR precipitate retained its tumor-localizing property, although with altered kinetics of

uptake and retention as compared with HpD alone (data not shown). Therefore, studies are now underway to test whether the complexed material leads to improvements in photoirradiation therapy and whether it can be used to transport the cytotoxic drugs into tumor tissue by means of the tumor selectivity of HpD.

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