

# Pharmacological Modulation of Photodynamic Therapy with Hematoporphyrin Derivative and Light

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## ABSTRACT

The interactions between photodynamic therapy (PDT) with hematoporphyrin derivative (HPD) and treatment with cytotoxic drugs have been examined using both an *in vitro* tissue culture assay and an *in vivo* transplantable mouse tumor assay.

Adriamycin (0.5–4.0 mg/kg) administered with HPD and at the time of irradiation potentiated the photodynamic effect, doubling the duration of tumor control. Adriamycin administered after PDT was not as effective. Methotrexate (0.2 mg/kg) also potentiated the tumor response to PDT. The other cytotoxic agents tested, cyclophosphamide, thiotepa, vincristine, and 5-fluorouracil, did not result in significant increases in tumor responses at the doses tested.

In contrast to the effects observed *in vivo*, Adriamycin inhibited the photodynamic destruction of Raji or Lewis lung carcinoma cells *in vitro*, in part by reducing the uptake of HPD. Methotrexate had no effect on either the uptake of HPD or the efficacy of photodynamic destruction of Raji cells *in vitro*. The discrepancy between the *in vitro* and *in vivo* results implies that the interaction between PDT and other pharmacological agents cannot be assessed *in vitro*.

## INTRODUCTION

PDT<sup>2</sup> with HPD uses the selective retention of HPD in malignant tumors (1, 2) and activation of the drug by red light (approximately, 630 nm). PDT with HPD has been used for the treatment of a range of tumors including bronchial carcinoma, breast carcinoma metastatic to the chest wall, melanoma, glioma, bladder tumors, basal cell carcinoma, and a number of other tumors (1, 3–7), with encouraging results, particularly in the treatment of small localized tumors where complete eradication can be achieved. The major side effect of the treatment is cutaneous photosensitivity which may last 4–6 weeks after HPD infusion.

Activation of HPD leads to generation of toxic oxygen species, particularly singlet oxygen (8, 9), and hydroxyl radicals (10, 11). Tumor necrosis may result either from direct cytotoxic effects of the toxic oxygen species on the tumor cells, or, indirectly, from vascular damage leading to infarction (12). It is logical to consider the use of PDT in combination with other chemotherapeutic agents, to enhance effective regimes and by reducing doses of cytotoxic drugs and HPD, to lessen the side effects. Studies were therefore undertaken, both *in vitro* and with transplantable tumors, to examine the effect of PDT in combination with six drugs used frequently for cancer chemotherapy.

## MATERIALS AND METHODS

**Mouse Tumor Assay.** Lewis lung carcinoma cells were maintained in culture in RPMI 1640 supplemented with 10% fetal calf serum (Flow Laboratories), 2.3 mM NaHCO<sub>3</sub>, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 0.16 µg/ml gentamicin. The cells were

harvested by treatment with 0.01% trypsin, and 10<sup>6</sup> cells were injected s.c. into the back of C57BL mice. Fresh cultures were established every 3 months from stock frozen in liquid nitrogen. Animals were treated when transplanted tumors were 5–7 mm in diameter.

The efficacy of PDT was assessed as previously described (13, 14). Mice in groups of 10 were given HPD (30 mg/kg i.p.). Twenty-four h later, the mice were anesthetized with Sagatal (May and Baker Aust. Pty. Ltd.), the fur over the tumor shaved, and the tumors irradiated with 225 J/cm<sup>2</sup> red light (610–680 nm) delivered from a tungsten halogen lamp fitted with the appropriate long and short pass filters (15). Mice were examined daily for palpable tumor and the time for 50% of tumors to recur was measured (TC<sub>50</sub>). Cytotoxic drugs were administered i.p. in doses given below, both concurrently with HPD and at the time of irradiation. Alternatively, cytotoxic drugs were given i.p. 24 and 48 h after irradiation.

**Drugs.** HPD was prepared in the pharmacy of The Queen Elizabeth Hospital by a previously published protocol (3). Cytotoxic drugs obtained as described below were reconstituted and stored according to the manufacturer's instructions. All dilutions were prepared in sterile saline immediately before administration. Two doses of each drug were used, representing the upper and lower ends of the therapeutic range. The drugs used were: methotrexate, David Bull Laboratories Pty., Ltd.; doxorubicin HCl (Adriamycin), Farmitalia Carlo Erba S.p.A., Milan; cyclophosphamide (Endoxan-Asta), Bristol; 5-fluorouracil, Roche; vincristine sulfate (Oncovin), Eli Lilly (Australia) & Co; Thiotepa, Lederle.

**Skin Photosensitivity.** Mice were given HPD and cytotoxic drugs i.p. as above. Twenty-four h later, the left footpad was irradiated (225 J/cm<sup>2</sup>) and the presence of erythema and edema was noted 24 h after irradiation (13, 14).

**Fluorescence Detection of HPD Uptake.** Mice were given HPD and the higher doses of cytotoxic drugs i.p. as described below. Twenty-four h later, the mice were sacrificed, and fluorescence intensity of frozen sections of tumors was examined under a Zeiss fluorescence microscope with excitation wavelength of 420–490 nm (13, 14). Fluorescence intensity of HPD in Raji cells (16) or Lewis lung carcinoma cells was assessed after incubation for 1 h with HPD (25 µg/ml) and graded doses of Adriamycin.

***In Vitro* Photocytotoxic Activity.** *In vitro* photocytotoxic activity of HPD was measured as previously described (16). <sup>51</sup>Cr-labeled Raji cells or Lewis lung carcinoma cells were suspended in RPMI 1640 (Flow Laboratories) without serum and incubated for 1 h at 37°C with HPD (25 µg/ml) and Adriamycin (0–200 µg/ml) or methotrexate (0–200 µg/ml). The cells were washed once by centrifuging for 5 min at 200 × g in RPMI 1640 without serum, resuspended in calcium- and magnesium-free Dulbecco's phosphate-buffered saline and irradiated for 0–20 min with red light from a quartz halogen lamp (8 mW/cm<sup>2</sup>, 600–650 nm). The percentage of <sup>51</sup>Cr release was determined immediately after irradiation as previously described (16). Background percentage of <sup>51</sup>Cr, determined from cells incubated with HPD but not exposed to light, was subtracted from all experimental values.

## RESULTS

### Treatment of Transplantable Tumors in Mice

**Treatment with HPD and Light Only.** The tumors of mice treated with 30 mg/kg HPD and 225 J/cm<sup>2</sup> light without cytotoxic drugs responded with a TC<sub>50</sub> of 3.6 ± 0.47 (SD) days (three experiments). HPD or light alone did not affect the rate of tumor growth. No cures were observed, and all the tumors had recurred within approximately 2 weeks after PDT.

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<sup>2</sup> The abbreviations used are: PDT, photodynamic therapy; HPD, hematoporphyrin derivative; TC<sub>50</sub>, median time to recurrence.

**Effect of Adriamycin on PDT.** The influence on tumor response of administration of two doses of Adriamycin at the time of HPD injection and at the time of irradiation is illustrated in Fig. 1. There was a dose dependent increase in the  $TC_{50}$  from 3.6 to a maximum of 8 days with 3 mg/kg Adriamycin, indicating that Adriamycin caused potentiation of the photodynamic effect. A single dose of Adriamycin (3 mg/kg) at the time of irradiation gave a  $TC_{50}$  of 5 days (data not shown). Adriamycin alone (3.0 mg/kg), Adriamycin plus HPD, or Adriamycin plus light all had no macroscopic effect on tumor growth. However, one or two mice died in each group at the higher doses, indicating toxic levels had been reached. Higher doses of Adriamycin were not tested.

The effect of administration of Adriamycin after an effective PDT treatment was also examined. Mice were treated with HPD (30 mg/kg) and light (225 J/cm<sup>2</sup>). Twenty-four h after irradiation, 3.0 mg/kg Adriamycin was administered i.p. In two experiments, this treatment resulted in a  $TC_{50}$  of 5 days, suggesting a slight potentiation of the photodynamic effect. Thus Adriamycin administered before PDT was much more effective.

**Effect of Other Cytotoxic Drugs.** The effect of administration of five different cytotoxic drugs on the response of tumors to

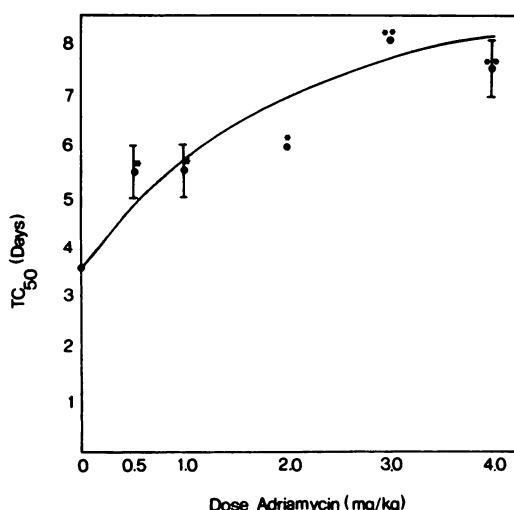


Fig. 1. Relationship between  $TC_{50}$  and dose of Adriamycin. Groups of 10 mice with Lewis lung carcinoma were given HPD (30 mg/kg) and Adriamycin i.p. Twenty-four h later, mice were given a second dose of Adriamycin and the tumors treated with 225 J/cm<sup>2</sup> red light. Points, mean  $\pm$  SD (bars) of two experiments.

Table 1 Influence of cytotoxic drugs on  $TC_{50}$

Groups of 10 mice with Lewis lung carcinoma were given HPD (30 mg/kg) and cytotoxic drugs i.p. Twenty-four h later, mice were given a second dose of cytotoxics, and the tumors were irradiated with 225 J/cm<sup>2</sup> red light. Values are mean  $\pm$  SD of two experiments.

Drug	Dose (mg/kg)	$TC_{50}$ (days)
Cyclophosphamide	5	4.5 $\pm$ 0.5
	10	5.0 $\pm$ 1.0
Methotrexate	0.1	3.5 $\pm$ 0.5
	0.2	6.0 $\pm$ 0
5-Fluorouracil	12	3.5 $\pm$ 0.5
	50	4.0 $\pm$ 0
Thiotepa	0.2	5.0 $\pm$ 0
	0.4	4.5 $\pm$ 0.5
Vincristine	0.001	5.0 $\pm$ 0
	0.025	4.5 $\pm$ 0.5
No cytotoxic drugs	0	3.6 $\pm$ 0.5

PDT is shown in Table 1. Each result represents the mean of two experiments. Slight increases in  $TC_{50}$  observed with cyclophosphamide, vincristine, and thiotepa may not be significant. 5-Fluorouracil had no effect at the doses tested. Methotrexate at 0.2 mg/kg resulted in a 2-fold increase in  $TC_{50}$  to 6 days. Drug alone, drug plus HPD, or drug plus light all had no visible effect on tumor growth. At the higher drug doses, it was common for at least one mouse in each group to die.

**Fluorescence Uptake Studies.** Fluorescence of HPD was more intense in tumors from mice receiving cytotoxic drugs concurrently with HPD than in mice receiving HPD alone. The most intense HPD fluorescence was seen following administration of Adriamycin or methotrexate, the drugs that resulted in the greatest potentiation of PDT. There was no increase in fluorescence intensity of HPD in tumors after concurrent injection of HPD and 5-fluorouracil. This drug did not alter the  $TC_{50}$ .

**Effect of Cytotoxic Drugs on Cutaneous Photosensitivity.** There was considerable erythema and edema in the footpad of most mice after treatment with HPD and light only. The method did not permit detection of a significant difference between this group and those also treated with cytotoxic drugs.

#### Effect of Cytotoxic Drugs on Photodynamic Destruction of Cells *in Vitro*

Since Adriamycin and methotrexate increased the uptake of HPD into Lewis lung tumors in mice, the phenomenon was studied *in vitro* for evidence distinguishing a direct effect of Adriamycin or methotrexate on the tumor cells from an indirect effect which could be mediated, for example, through tumor vasculature. The effect of Adriamycin on <sup>51</sup>Cr release after irradiation of HPD-treated Raji cells is shown in Fig. 2. In the absence of Adriamycin, release of <sup>51</sup>Cr increased with time of irradiation until maximum <sup>51</sup>Cr release (65–75%) was reached. This corresponded with 100% of the cells being nonviable (16). Adriamycin caused a concentration-dependent inhibition in the rate of <sup>51</sup>Cr release (Fig. 3A). Similar inhibition of photodynamic damage with Adriamycin was also observed with Lewis lung carcinoma cells (Fig. 3B).

The effect of methotrexate on the efficacy of photodynamic destruction of HPD-sensitized Raji cells is illustrated in Fig.

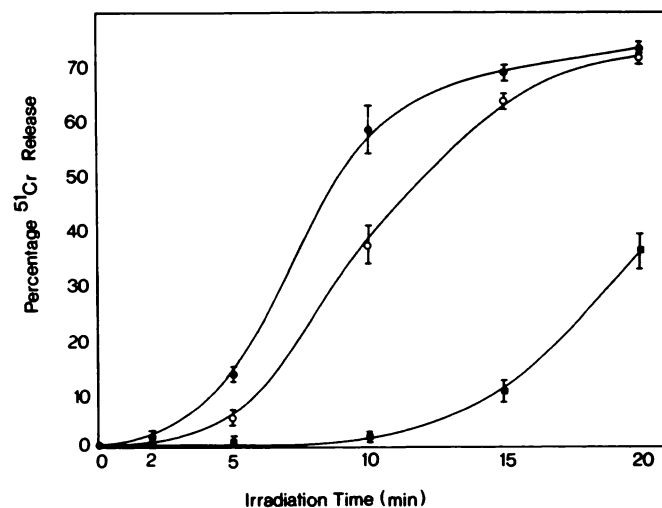


Fig. 2. Influence of Adriamycin on <sup>51</sup>Cr release from HPD-sensitized Raji cells. <sup>51</sup>Cr-labeled cells were incubated for 1 h with 25 µg/ml HPD and 0–200 µg/ml Adriamycin, washed, and irradiated. Background percentage <sup>51</sup>Cr representing cells incubated in HPD but not irradiated was subtracted from all experimental values. Points, mean  $\pm$  SD (bars) of triplicate tubes. ●, HPD, 25 µg/ml; ○, HPD, 25 µg/ml plus Adriamycin, 20 µg/ml; ■, HPD, 25 µg/ml plus Adriamycin, 200 µg/ml.

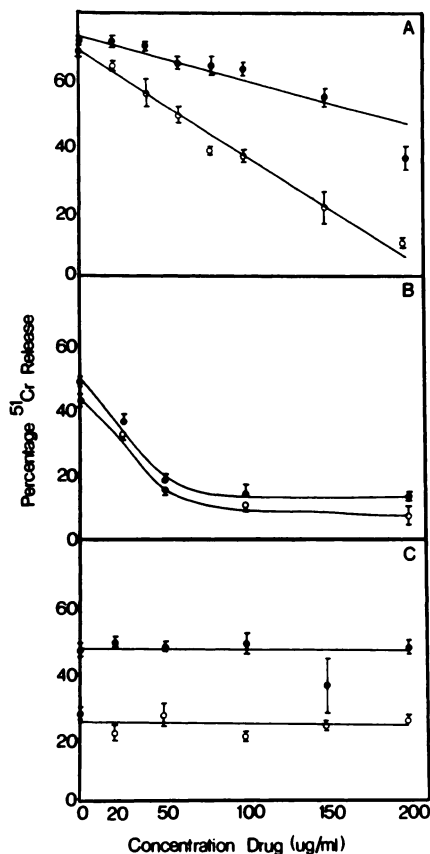


Fig. 3. *A*, relationship between percentage  $^{51}\text{Cr}$  release and concentration of Adriamycin. Cells were incubated in HPD plus Adriamycin, then irradiated. ●, 15-min irradiation; ○, 20-min irradiation. *B*, influence of Adriamycin on  $^{51}\text{Cr}$  release from HPD-sensitized Lewis lung carcinoma cells.  $^{51}\text{Cr}$ -labeled cells were incubated for 1 h in 25  $\mu\text{g}/\text{ml}$  HPD and 0–200  $\mu\text{g}/\text{ml}$  Adriamycin, washed, and irradiated. ●, 10-min irradiation; ○, 15-min irradiation. *C*, influence of methotrexate on  $^{51}\text{Cr}$  release from HPD-sensitized Raji cells.  $^{51}\text{Cr}$ -labeled Raji cells were incubated for 1 h with 25  $\mu\text{g}/\text{ml}$  HPD and 0–200  $\mu\text{g}/\text{ml}$  methotrexate, washed, and irradiated. ●, 2-min irradiation; ○, 5-min irradiation. Bars, SD.

3C. In contrast to the potentiation of PDT observed *in vivo*, methotrexate had no effect on the efficacy of  $^{51}\text{Cr}$  release. Higher concentrations of methotrexate or Adriamycin could not be tested because 200  $\mu\text{g}/\text{ml}$  alone resulted in slightly elevated  $^{51}\text{Cr}$  release.

Although assessment of intracellular content of porphyrins by fluorescence is imprecise because the red fluorescence of HPD is similar to the orange-yellow fluorescence of Adriamycin, it could clearly be seen that the red HPD fluorescence decreased with increasing doses of Adriamycin and was absent in the cells incubated with 200  $\mu\text{g}/\text{ml}$  Adriamycin. Inhibition of uptake of HPD by Adriamycin was also observed with Lewis lung carcinoma cells. In contrast, increasing concentrations of methotrexate had no effect on the intensity of HPD fluorescence in Raji cells. The influence of cytotoxic drugs on HPD uptake corresponded with the alteration in the efficacy of photodynamic destruction of cells.

## DISCUSSION

There are few reports of interactions between PDT and cytotoxic drugs used for cancer chemotherapy. We have described a transplantable mouse tumor model (13) for investigating interactions between PDT and cytotoxic drugs (17) and have described synergy between PDT and glucocorticoids (14) when these are administered after irradiation, but not before. Cytotoxic drugs chosen in the present study represent some of

the main classes of compounds in common clinical use. Doses used corresponded approximately to the therapeutic range for human tumors.

The Lewis lung carcinoma appears to be relatively resistant to chemotherapy (18–20). It is sensitive to Adriamycin at high doses (5–20 mg/kg) (21–23) which are very toxic to mice. We observed no direct therapeutic effect at 4 mg/kg.

Gillio and Cortese (24) demonstrated increased mortality when mice given Adriamycin in addition to HPD were given irradiation to the whole body. Tumor response was not examined. Dougherty (25) reported that patients treated with Adriamycin and given PDT to cutaneous tumors showed severe reactions even in uninvolved skin. Creekmore and Zaharko (26) showed that actinomycin D or L-phenylalanine mustard were synergistic with hematoporphyrin and light in inactivating L1210 cells.

Lewis lung carcinoma cells have been shown to take up Adriamycin *in vitro* by passive diffusion and accumulate it in the mitochondria, microsomes, and nuclei (21–23, 27). While Adriamycin enhanced uptake of HPD in Lewis lung tumors *in vivo* and enhanced the response to photodynamic therapy, this drug inhibited uptake *in vitro* into both Raji and Lewis lung carcinoma cells, resulting in reduced susceptibility to photodynamic damage. This apparent contradiction is not readily explained and suggests that *in vitro* studies are of limited usefulness. It could be speculated that the inhibitory effect *in vitro* results from inhibition of uptake. Since Adriamycin increases hydrophilic glycoproteins in the membrane (28), uptake of HPD, being very hydrophobic (13, 29, 30), may be inhibited. In contrast to Adriamycin, methotrexate had no effect *in vitro* either on HPD uptake or the efficacy of photodynamic destruction of Raji cells.

Short (1 h) incubations in HPD result in uptake of relatively inactive porphyrins that are readily washed out of the cells, but the most photoactive component of HPD is taken up more slowly and is tightly bound to cells (31). Recent studies have suggested that longer (21 h) incubations with HPD may be a more appropriate model of HPD uptake. The influence of cytotoxic drugs on uptake of HPD during longer incubation could not be examined since longer incubation with either Adriamycin or methotrexate was toxic to Raji cells.

Treatment with Adriamycin alone did not cause any reduction of Lewis lung tumor. When, however, treatment with Adriamycin was combined with PDT, the effect of PDT was enhanced. It is difficult to propose a satisfactory hypothesis to account for this enhancement. As well as the possibility that Adriamycin exerts its enhancing effect on PDT by increasing the uptake of HPD, Adriamycin may act at a number of points in the metabolism of tumor cells, adding to the metabolic damage sufficiently to result in death of cells sublethally damaged by PDT (32). Additive effects could be expected, because Adriamycin (21, 22, 27) and HPD (33) both accumulate in the mitochondria, and HPD (8, 9) and Adriamycin (27, 34) have both been postulated to inactivate cells by radical-mediated damage. Since vascular damage leading to infarction appears to be very important in tumor destruction by PDT (12), another consideration is an effect of cytotoxic drugs on capillaries in PDT-treated tumors, inhibiting their recovery from damage caused by PDT (32).

Of the other drugs tested, methotrexate was also effective in potentiating the tumor response. It was also associated with increased intensity of HPD fluorescence within the tumor. The qualitative nature of the assay available for fluorescence uptake of HPD in cells and tumors must be acknowledged. Although

Lewis lung carcinoma has been reported to be sensitive to 20–300 mg/kg cyclophosphamide (17, 20) resulting in cure of early tumors and good effect as adjuvant chemotherapy after surgery (18), cyclophosphamide was not synergistic with PDT. Similarly, although 300 mg/kg 5-fluorouracil caused regression of Lewis lung carcinoma (20), lower doses (12–50 mg/kg) were not synergistic with PDT.

Reduction of cutaneous photosensitivity would greatly enhance the usefulness of this treatment. Unfortunately no evidence was obtained to indicate that the cytotoxic drugs had any influence on this side effect. This may be due to unsatisfactory methods for detecting small or moderate differences in photosensitivity.

We conclude that the interactions between PDT and cytotoxic drugs merit further investigation. The lack of correlation between *in vitro* and *in vivo* effects of Adriamycin and methotrexate indicate that the interactions between PDT and other pharmacological agents must be carried out in the intact animal or in humans. The role of PDT in cancer treatment may be as one of a combination of available therapies. PDT should be considered as being similar to most cytotoxic agents, which when used alone may not have the desired effectiveness, but when used in combination with others may lead to tumor control with tolerable side effects.

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