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Synergy between Preactivated Photofrin-II and Tamoxifen in Killing Retrofibroma, Pseudomyxoma and Breast Cancer Cells

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Exposure of photoactive compounds to light prior to their use in biological systems (preactivation) results in the generation of tumour cell specific metastable cytotoxic species that are no longer dependent on the light energy. Thus, preactivation renders the photoactive compounds suitable for systemic use. We have examined the *in vitro* effect of preactivated photofrin-II and tamoxifen in retroperitoneal fibroma, pseudomyxoma and male breast carcinoma cell lines. These cells were found to be non-responsive to tamoxifen and were negative for oestrogen receptors. Incubation of these cells with 0.5 μg/ml preactivated photofrin-II and tamoxifen ($< 10^{-6}$ mol/l) resulted in a significantly enhanced ($P < 0.001$) inhibition of DNA synthesis compared with either agent alone. This synergistic effect between tamoxifen and preactivated photofrin-II was determined by multiple drug effect analysis. Treatment of cells with preactivated photofrin-II did not cause the increased expression of oestrogen receptors. These observations suggest that a combination of antihormonal drugs with preactivated compounds may be of clinical value.
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INTRODUCTION

TAMOXIFEN IS one of a number of non-steroidal compounds known for its anti-oestrogenic and antifertility properties in laboratory animals [1]. The antitumour activity of tamoxifen was reported in a preliminary report [2]. However, clinical experience has now established tamoxifen as the antihormonal agent of choice. In recent years, it has been used successfully in the treatment of oestrogen positive breast cancer [3, 4], but the mechanism of its antitumour activity is not clear. It has been reported that tamoxifen mediates its antitumour activity via oestrogen [5–7] through one of its reactive metabolites, hydroxytamoxifen, which is formed *in vivo* [8]. The effect of tamoxifen in oestrogen receptor positive cell lines is mediated via oestrogen receptors (ER) requiring low concentrations of tamoxifen, while the antitumour effects observed in oestrogen receptor negative cell lines are thought to be non-receptor-mediated non-specific cytotoxic mechanisms [5, 9–14]. Tamoxifen is a tumoristatic agent but not a tumoricidal agent, since if therapy is stopped, regrowth of tumour occurs [15].

Recently, three cell lines were established in our laboratory from tumour biopsy specimens obtained from patients with retroperitoneal fibrosis, pseudomyxoma and male breast carcinoma. The case histories and tamoxifen-mediated clinical

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responses of these patients will be reported elsewhere. *In vitro*, these cell lines were unresponsive to tamoxifen treatment at doses of less than 1 μmol/l. In this paper, synergistic effects of tamoxifen and preactivated photofrin-II on these cell lines are reported. Photofrin-II is the active component in the haematoporphyrin derivative mixture which is used in the photodynamic therapy of cancer. Preactivation is a novel process which renders the photoactive compounds suitable for systemic use without the necessity of further illumination [16, 17].

MATERIALS AND METHODS

Materials

[6,7-^{3}H(N)] oestradiol (2.38 T Bq/mmol) was purchased from New England Nuclear (Boston) and dimethylformamide Baker analysed reagent from J.T. Baker (Phillipsburg, New Jersey). Tamoxifen citrate, Norit A-activated charcoal and dextran T-70 were obtained from Sigma. Photofrin-II was obtained from Quadra Logic Technologies (British Columbia). Tissue culture media and fetal bovine serum (FBS) were obtained from Gibco. Culture dishes and plates were obtained from Falcon (Becton Dickinson) and Costar (Cambridge, Massachusetts), respectively.

Cell lines and growth conditions

Five human cell lines were used in this study. MCF-7 (human breast carcinoma, oestrogen positive) and BT-20 (human breast carcinoma, oestrogen negative) cell lines were obtained from the American Type Culture Collection (Rockville, Maryland). Both cell lines were grown in OPTI-MEM (Gibco) supplemented with 4% FBS. Male breast carcinoma, pseudomyxoma and retrofibroma cell lines were established in our laboratory from tumour biopsy specimens obtained from patients during surgical procedures at Baylor University Medical Center. Briefly, tissues were cut in small pieces (1–2 mm) followed by enzyme treatment (pronase or trypsin EDTA) for 10 minutes at 37°C. The cells were then washed in maintenance medium and plated in 75 cm^2 flasks. Generally, after 7–10 days, when cell growth had initiated, an aliquot of cell-free SV-40 virus was added. These cells have continued to grow for 18 months. The doubling times for male breast carcinoma, pseudomyxoma and retrofibroma were approximately 65, 60 and 74 hours, respectively. These cell lines were not cloned. Morphological examination shows stellate-shaped retrofibroma cells with extended cytoplasmic processes containing blunt dispersed chromatin and cytoplasm filled with basophilic inclusions. Male breast carcinoma cells had prominent nuclei and multiple nucleoli with fewer cytoplasmic inclusions. These cells were fibrocytic with very long extended processes. Pseudomyxoma cells contained marked fibrillar, stellate-shaped components with extended processes and hyperchromatic dispersion. These cell lines were routinely maintained as monolayers in OPTI-MEM medium (Gibco) supplemented with 10% FBS and in a humidified atmosphere of 95% air : 5% CO_2 at 37°C.

Effect of tamoxifen on the growth of cell lines

For each experiment, the cells were removed from the flasks with trypsin-EDTA solution, washed, resuspended in growth medium and plated in 96-well plates (5000 cells/200 μl/well) for overnight incubation before drug treatment. A stock solution of tamoxifen (0.1 mol/l) was prepared in absolute alcohol and stored at −20°C. Twelve different dilutions (5×10^{-5} to 1×10^{-10} mol/l) of the stock in growth media were used for assays. Cells were grown in the presence of tamoxifen for 1 day before ^{3}H-thymidine (14.8 kBq/well) was added. After 24-hour and 5-day incubations, cells were harvested using a PHD cell harvester. ^{3}H-thymidine incorporation of each sample was determined by using a scintillation counter (Beckman LS1701). Results are expressed as percent inhibition of DNA synthesis.

Oestrogen receptors from whole cells

Binding capacity of the whole cell was determined using the method of Katzenellenbogen *et al.* [18] with the following modifications. Briefly, cells were incubated overnight in growth medium with FBS which had been treated with charcoal-dextran slurry to remove any endogenous oestrogen. Monolayers were then collected with 1 mmol/l EDTA in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (pH 7.4). Samples were washed twice with PBS and homogenised in cold Tris-EDTA-azide (TEA) buffer (0.01 mol/l Tris-HCl-0.0015 mol/l EDTA-0.003 mol/l [0.02%] sodium azide [pH 7.4 at 25°C]) with a precooled Douce homogeniser. The homogenates were centrifuged at 800 *g* for 15 minutes. The pellets were rehomogenised and the supernatant was added to the previous preparation.

The radioactivity lost in the final pellet was determined as follows. All experiments were carried out in polypropylene test tubes. Various amounts of protein were used in each tube with [^{3}H]oestradiol and incubated at 4°C for 20–24 hours. Tubes were then washed twice in 300 μl TEA buffer by centrifugation at 800 *g* for 15 minutes, and supernatant was carefully collected using a micropipette. Radioactivities of both pellet and supernatant were determined. The pellet was found to contain approximately 3% per 10 μg protein of the total radioactivity and was not used in final calculations of the total number of ER sites on MCF-7 cells.

In preliminary studies, the amount of protein needed for receptor assay was determined using various dilutions of cell homogenate in TEA buffer. Non-specific binding was also determined by incubating 10 μg of cell homogenate protein with saturating quantities of oestradiol (2×10^{-4} to 2×10^{-7}, 11 different concentrations) for 1 hour before [^{3}H]oestradiol was added. For the Scatchard analysis, 10 μg of protein in 50 μl TEA buffer and five concentrations of [^{3}H]oestradiol solution from 1.1×10^{-8} to 4.3×10^{-11} mol/l were used [19, 20].

Effect of tamoxifen and preactivated photofrin-II on the growth of cultured cell lines

Combinations of tamoxifen and preactivated photofrin-II were added to cells from each cell line for the determination of their effect on cell growth. The process of preactivation has been described earlier [16]. Briefly, a solution of photofrin-II (1 mg/ml) was irradiated with fluorescent light (bank of eight light bulbs, Phillips, cool white, 20W for 1 hour at a vertical distance of 10 cm from the top of petri plate to the bottom of the bulb). After preactivation, cells were incubated with preactivated photofrin-II (0.5 or 1 μg/ml) overnight. The next day the medium in each well was replaced with tamoxifen or a mixture of tamoxifen plus preactivated photofrin-II for another overnight incubation. Inhibition of cell growth was determined as described above. In separate experiments, incubation of cells was also carried out for 5 days after the treatment with tamoxifen plus preactivated photofrin-II.

Effect of preactivated photofrin-II on oestrogen receptor production of cells in vitro

To determine whether the effect of preactivated photofrin-II was receptor-mediated or not, cells were first treated with

preactivated photofrin-II for 24 hours and receptor assays were performed as described above.

Data analysis

The results are presented as the arithmetic mean (S.D.) for each control and experimental group. Differences among the mean of groups were determined using Student's two tailed t test, and P values ≤ 0.05 were considered to be significant. The determination of the synergistic effect was analysed by dose-effect relationships according to the method of Chou and Talaly [21]. The following median-effect equation derived by Chou was used [22, 23]:

$$f_a/f_u = (D/D_m)m \quad \text{(A)}$$

where D is dose, D_m is median-effect dose that produces 50% effect (e.g. ED_{50}, IC_{50}, etc.), f_a is the fraction affected, f_u is the fraction unaffected, and m is the Hill-type coefficient signifying the degree of sigmoid shape of the dose-effect curve. The logarithmic form of equation A gives the basis for the median-effect plot [23, 24], $x = \log(D)$ vs. $y = \log f_a/f_u$, which gives slope (m) and the x-intercept ($\log D_m$). The D_m can be calculated from the antilog of ($-y$-intercept/m). The synergism, summation and antagonism of drug effects were quantitatively analysed by the multiple-drug effect analysis developed by Chou and Talaly [24– 26]. The interaction of effects of these two drugs is quantitatively determined by the combination index (CI) [25, 26]:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} + \frac{\alpha(D)_1\,(D)_2}{(D_x)_1\,(D_x)_2} \quad \text{(B)}$$

where D_x is the dose that is required to produce X% effect. The relationship between D_x and X% can be determined by the median-effect plot parameters m, D_m and equation A. When $\alpha = 0$ and $\alpha = 1$, the effects of two drugs are mutually exclusive and mutually non-exclusive, respectively. This analysis generates combination effects; when CI = 1 summation is indicated; when CI < 1 synergism is indicated; when CI > 1 antagonism is indicated. In equation B if the regression lines of the median-effect plot for each drug alone are not parallel exclusivity of the drug cannot be clearly determined. Under these conditions, it is suggested that the CI value be determined using mutually exclusive ($\alpha = 0$) and non-exclusive ($\alpha = 1$) conditions.

RESULTS

Oestrogen binding site concentration

For oestrogen receptor assays, MCF-7 and BT-20 cells were used as positive and negative controls, respectively. Figure 1 shows the results of a typical experiment in which MCF-7 cell homogenate was incubated with increasing concentrations of ^{3}H-labeled oestradiol. The data plotted in the form of a Scatchard plot shows K_d values of 2.6 nmol/l and oestrogen receptor site binding concentration of 236 fmol/mg protein. All other cell types tested (BT-20, male breast carcinoma, pseudomyxoma and retrofibroma) showed only background levels of ER receptor. These findings correspond to the receptor-mediated response of these cells to tamoxifen, as only MCF-7 cells showed inhibition of growth at concentrations $< 10^{-6}$ mol/l.

Synergistic effect of tamoxifen and preactivated photofrin-II on cell growth

Cell growth was not affected *in vitro* by tamoxifen alone except at doses higher than 10^{-6} mol/l, which is considered to be a non-receptor-mediated effect [27]. However, all cell lines were

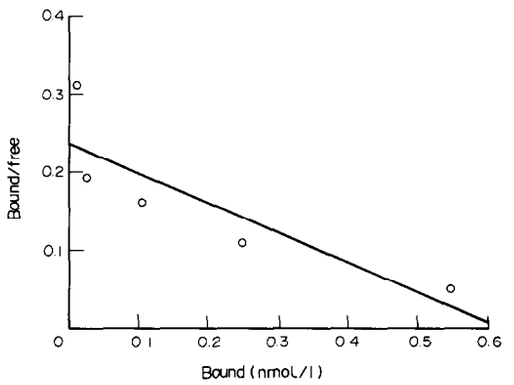

Fig. 1. Determination of the presence of oestrogen binding site concentration on MCF-7 breast cancer cell line. Cell supernatant was incubated with increasing (4.3×10^{-11} to 1.1×10^{-8} mol/l) concentrations of [^{3}H]oestradiol. Scatchard plot analysis. K_d = 2.6 nmol/l, 236 fmol/mg protein.

affected by the combination of tamoxifen and preactivated photofrin-II. Data show (Fig. 2) that tamoxifen (5×10^{-7} mol/l) alone or preactivated photofrin-II (0.5 μg/ml) alone resulted in a minimal inhibition (8% and 5%, respectively) of cell growth in male breast carcinoma cells. However, a combination of tamoxifen and preactivated photofrin-II resulted in a significantly greater ($P < 0.001$) inhibition (61%) of cell growth (Fig. 2). When the concentration of preactivated photofrin-II was increased to 1 μg/ml, the observed synergistic effect was reduced. Combination indexes calculated from Chou's equations for all cell lines were less than one, which indicates the synergistic effect of preactivated photofrin-II. The mechanism of this inhibition is not clear. Similar results were obtained in retrofibroma and pseudomyxoma cells (Figs 3 and 4). The sequence of the addition of preactivated photofrin-II and tamoxifen was not important, as identical results were obtained when preactivated photofrin-II and tamoxifen were added before, after, or simultaneously. After overnight treatment of BT-20 and MCF-7 cells by preactivated photofrin-II, the addition of tamoxifen or tamoxifen plus preactivated photofrin-II produced identical results, indicating that additional preactivated photofrin-II did not enhance the inhibitory effect. It should be noted that incubation of cells for 5 days after the treatment with tamoxifen and preactivated photofrin-II did not result in a significantly

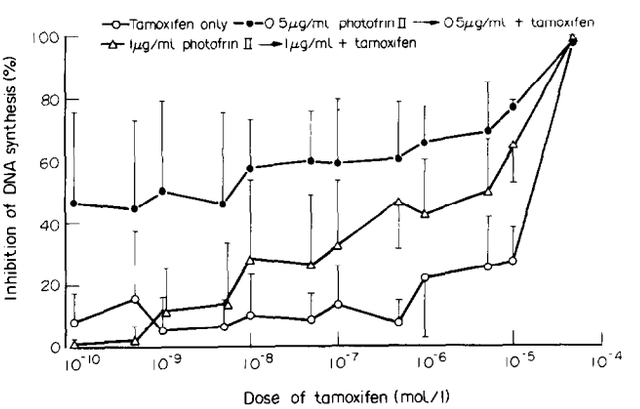

Fig. 2. Effect of preactivated photofrin-II and tamoxifen on male breast carcinoma cells. Inhibition of DNA synthesis was determined by ^{3}H-thymidine incorporation. Mean (S.D.) of three separate experiments.

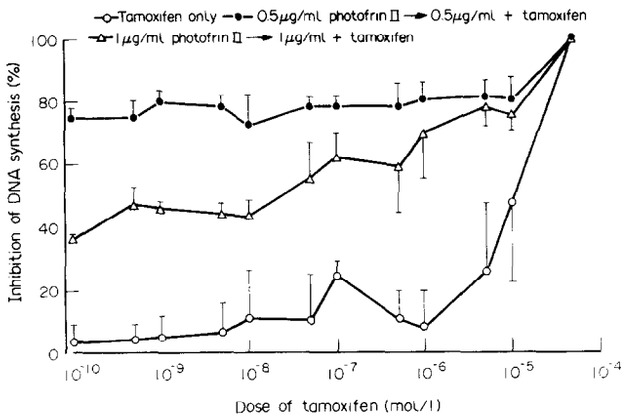

Fig. 3. Effect of preactivated photofrin-II and tamoxifen on retrofibroma cells. Inhibition of DNA synthesis was determined by ^{3}H-thymidine incorporation. Mean (S.D.) of three separate experiments.

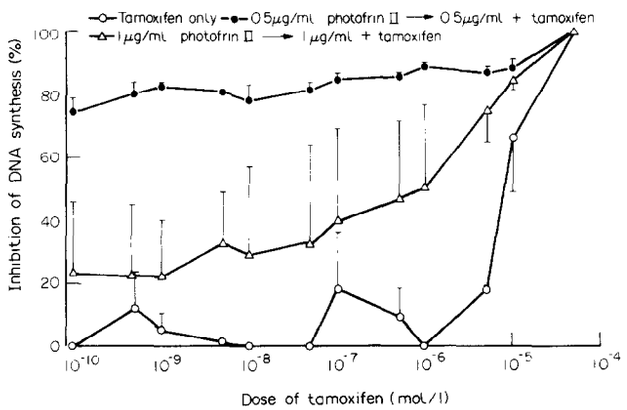

Fig. 4. Effect of preactivated photofrin-II and tamoxifen on pseudomyxoma cells. Mean (S.D.) of three separate experiments.

altered toxicity. Treatment of BT-20 but not MCF-7 cells by preactivated photofrin-II and tamoxifen ($< 10^{-6}$ mol/l) resulted in a significant ($P < 0.001$) enhancement of the cytotoxicity by combined agents as compared to either agent alone (Figs 5 and 6). The doses of preactivated photofrin-II and tamoxifen required for maximum effect were 0.5 μg/ml and 10^{-8} mol/l, respectively, for BT-20 cells. These results show that the combination of preactivated photofrin-II and tamoxifen

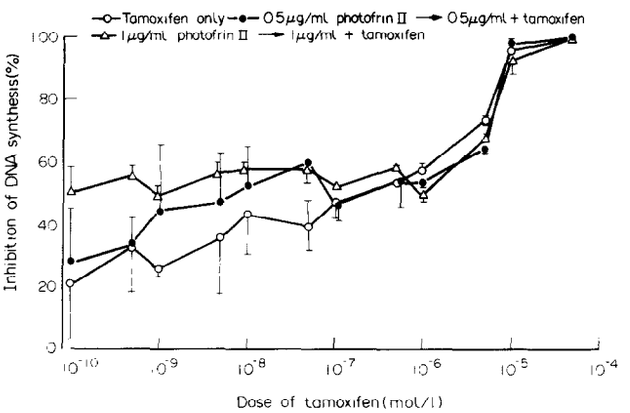

Fig. 5. Effect of preactivated photofrin-II and tamoxifen on MCF-7 cells. Mean (S.D.) of three separate experiments.

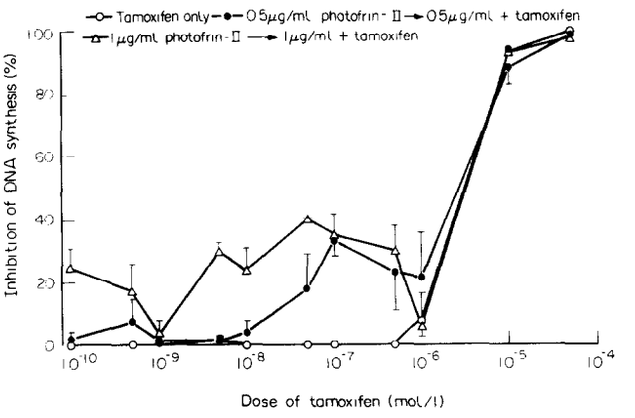

Fig. 6. Effect of preactivated photofrin-II and tamoxifen on BT-20 cells. Mean (S.D.) of three separate experiments.

was significantly more effective than either agent alone. In order to determine whether the preactivated photofrin-II treatment caused the increased expression of ER-receptors, oestrogen assays were performed in preactivated photofrin-II-treated cells. After overnight incubation, ER- receptors remained at undetectable levels (data not shown). Non-activated photofrin-II alone or in combination with tamoxifen did not significantly inhibit cell growth (Fig. 7, Table 1).

DISCUSSION

Often, surgery has been the only effective treatment for retroperitoneal fibrosis, a disorder first described in 1948 [27, 28]. Fibrosis, pseudomyxoma and male breast carcinoma are relatively uncommon disorders, but they can be lethal. In some cases, they are responsive to antihormonal therapy [29]. In this study, we have shown that the cells from male breast carcinoma, retrofibroma and pseudomyxoma are refractory to the *in vitro* tamoxifen treatment at a dose of < 1 μmol/l. The oestrogen-binding sites on these cells were undetectable by the [^{3}H]oestradiol binding assay. However, 236 fmol/mg protein of receptor were detected on MCF-7 cells by using the same assay, suggesting that the observed effects at higher doses are non-receptor-mediated and non-specific. It therefore appears that the response of oestrogen-positive tumours to tamoxifen can be explained in terms of a receptor-mediated event [9–14, 30]. In breast cancer patients the plasma levels of tamoxifen have been reported to be 100 to 400 ng/ml ($2.5 \times 10^{-7} - 10^{-6}$ mol/l), while actual tissue

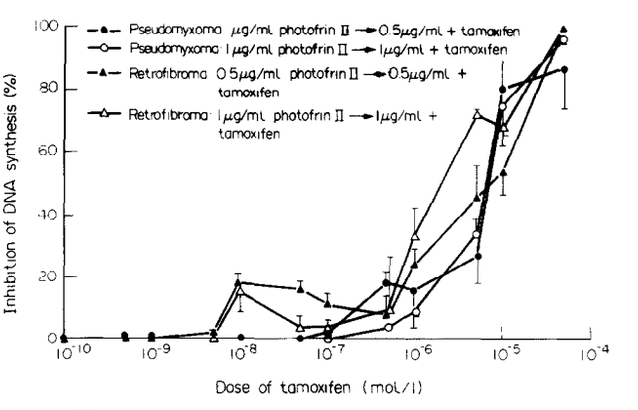

Fig. 7. Effect of non-activated photofrin-II and tamoxifen on the inhibition of growth in pseudomyxoma and retrofibroma cells. Mean (S.D.) of three separate experiments.

Table 1. Effect of photofrin-II and preactivated photofrin-II on the inhibition of cell growth

	Photofrin-II (μg/ml)		Preactivated photofrin-II (μg/ml)	
Cell line	0.5	1	0.5	1
Pseudomyxoma	1 (0.4)	0 (0.0)	14 (15.1)	4 (3.5)
Retrofibroma	8 (0.1)	10 (2.9)	10 (7.1)	11 (4.9)
Male breast carcinoma	0 (0.0)	0 (0.0)	5 (6.6)	2 (3.3)
MCF-7	3 (2.1)	1 (2.3)	1 (1.4)	2 (1.1)
BT-20	0 (0.0)	4 (1.7)	2 (1.0)	1 (1.5)

Percent inhibition, mean (S.D.).

levels may be higher [31] due to concentrative effects of antioestrogen binding protein [32]. At this time, the function of the antioestrogen binding protein is not clearly understood. The functional and structural differences of the receptors on the new cell lines reported here also are not known and are the subjects of our current investigation.

In an effort to develop more effective treatment for the tamoxifen refractory tumours, the effect of preactivated photoactive compounds was investigated. Preactivation is a process that allows the systemic use of photoactive compounds without further dependence on light [16, 17]. In recent studies in this laboratory, it was determined that preactivated photoactive compounds retain their tumour cell-specific cytotoxicity while normal cells and tissue (*in vitro* and *in vivo*) remain virtually unaffected [16]. In preliminary experiments, a combination of preactivated merocyanine 540 (a membrane potential sensitive photoactive compound) and taxoxifen was found to be ineffective in retarding the growth of tumour cells reported in this paper. However, a combination of preactivated photofrin-II and tamoxifen was effective in their cell growth inhibitory effect. Preactivated photofrin-II treated cells were significantly more responsive to subsequent tamoxifen treatment, even at very low concentrations. This increased response to tamoxifen was not due to the increased expression of oestrogen receptors caused by preactivated photofrin-II treatment because the receptor levels remained virtually unchanged. However, in oestrogen receptor-positive cell line MCF-7 this synergy was not observed, suggesting that preactivated photofrin-II may act as a primer only in receptor-negative cells, rendering them more responsive to tamoxifen. Experiments aimed at resolving some of the issues of the mechanism(s) involved and the efficacy of this protocol *in vivo* models are underway. Treatment of cells with non-activated photofrin-II resulted in a small increase in sensitivity of cells to tamoxifen, probably due to its non-specific activation by room light during manipulations. This increase in sensitivity, however, was significantly ($P < 0.001$) less than the preactivated photofrin-II. Results presented here may stimulate research in the combined use of tumour cell selective preactivated compounds and antihormonal drugs.

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Comparative Study of Two Human Melanoma Cell Lines with Different Sensitivities to Mustine and Cisplatin

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The cytotoxic effects of the bifunctional DNA-reactive drugs cisplatin, mustine and melphalan were studied in two human melanoma cell lines, RPMI 8322 and A 375. A 375 cells were 3.0 times more sensitive to cisplatin and 1.5 times more sensitive to mustine than RPMI 8322 cells. In contrast, A 375 cells were less sensitive to melphalan than RPMI 8322 cells. The increased sensitivity of A 375 cells was parallelled by an increased induction of DNA interstrand crosslinks following exposure to cisplatin and mustine. After cisplatin exposure A 375 cells also showed higher levels of platinum-DNA intrastrand adducts than RPMI 8322 cells. The increased effect of cisplatin in A 375 cells was not due to an increased drug accumulation in these cells. The higher sensitivity of A 375 cells to cisplatin may be related to lower intranuclear levels of glutathione, compared to RPMI 8322 cell nuclei, while the sensitivity to mustine may depend on lower overall levels of glutathione than in RPMI 8322 cells.
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INTRODUCTION

CIS-DIAMMINEDICHLOROPLATINUM(II) (cisplatin) is an important chemotherapeutic drug, which has shown clinical activity in several different tumour types [1]. Malignant melanoma, however, is generally resistant to cisplatin therapy. Only 10% of patients with disseminated melanoma obtain objective tumour responses following treatment with cisplatin as a single agent [2].

There is considerable evidence that the cytotoxic effects of cisplatin are caused by reactions with DNA [3]. It has been demonstrated that cisplatin induces DNA intrastrand crosslinks, DNA interstrand crosslinks and DNA–protein crosslinks [4, 5]. It is not known which of these lesions are responsible for the cytotoxic effect of cisplatin.

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During recent years several studies on the mechanisms of resistance of tumour cells to cisplatin have been performed. Most investigators have compared sensitive parent cell lines to daughter cell lines which had been made resistant *in vitro* by exposure to high levels of cisplatin. Several mechanisms contributing to such *in vitro*-induced resistance have been described. These include: reduced intracellular accumulation of the drug [6–12]; increased levels of glutathione [7, 13–16] or increased glutathione transferase (GST) activity [6], which may play a role in inactivating cisplatin; reduced induction of DNA lesions by cisplatin [6, 17]; and increased DNA repair [8, 14, 18]. In addition, increased levels of metallothionein have been demonstrated in cell lines with increased resistance to cadmium, which were crossresistant to cisplatin [19]. Binding of cisplatin to metallothionein was also demonstrated in this study. While these studies have demonstrated several biochemical mechanisms for induced cellular resistance to cisplatin, it remains to be established whether these are important for the intrinsic resistance of human tumours such as malignant melanoma.

Fewer studies have been performed on human cells with inherent differences in cisplatin sensitivity [20, 25]. Such studies may be more relevant for demonstrating which mechanisms