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Acknowledgements—Dr G.J. Peters (Department of Oncology, Free University Hospital, Amsterdam, The Netherlands) is acknowledged for useful comments on the manuscript. We thank H. Dekker, U. Holwerda, S. Mülder and D. Bets (Department of Oncology, Free University Hospital, Amsterdam, The Netherlands) for valuable technical assistance. This work was supported by the Netherlands Cancer Foundation (grant no. IKA 85-06).

Eur J Cancer, Vol. 28A, No. 8/9, pp. 1452–1458, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00

MTT Assays Allow Quick and Reliable Measurement of the Response of Human Tumour Cells to Photodynamic Therapy

Jean-Louis Merlin, Samir Azzi, Dominique Lignon, Carole Ramacci, Nadia Zeghari and François Guillemin

MCF-7 and HT-29 cell lines were selected as a reliable model to examine the possible parameters affecting the sensitivity of tumour cells to photodynamic therapy (PDT) using a dye-laser at 630 nm. The chemical composition of haematoporphyrin derivative (HPD) was determined by high-performance liquid chromatography (HPLC) analysis and was in agreement with reported values. MTT assays were performed to assess the time-dependency of PDT and the influence of the output power and light fluence. The results showed a maximal cytotoxicity 48 h after photoirradiation. The output power (1 or 2 W) did not significantly affect the cytoxicity when the fluence was constant (20 J/cm²). However, an increase in fluence (10–40 J/cm²) led to a significant enhancement of cytotoxicity until maximal values were reached (30–40 J/cm²). A further increase in fluence (50 J/cm²) proved to induce a fall-off in cytotoxicity related to the intense photobleaching of HPD.
Eur J Cancer, Vol. 28A, No. 8/9, pp. 1452–1458, 1992.

INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) is based upon the specific properties of a compound such as haematoporphyrin derivative (HPD) to be retained in tumour tissue longer than in normal tissues such as skin, lung, brain, muscle [1]. When the sensitised tissue is exposed to an argon-dye laser generated red light (630 nm), the sensitiser is activated photochemically, thus permitting a selective destruction of the tumour. The resulting cytotoxicity has been reported to be induced by the generation of oxygen singlets [2], damaging mainly plasma membranes [3], mitochondria [4], cytoplasmic or nuclear organelles and enzymes [5, 6], in addition to the surrounding vessels [7]. Although it is well stated that the transport of HPD involves binding to serum proteins, the nature of the actual mechanism responsible for the selective tumoral biodistribution of HPD remains unclear [8] but has been demonstrated to be related to the pH-mediated generation of ionic species in tumoral sites. However, at the cellular level, it has been demonstrated that HPD accumulation involves passive diffusion facilitated by the low tumour pH [9].

Since PDT was first introduced for the treatment of malignant tumours, more than 4000 patients have undergone this therapy [10, 11]. HPD has dominated the biomedical studies since its discovery in 1961 [12] and is now the only photosensitiser permitted for use in clinical trials mainly for the treatment of tumours of the upper aerodigestive tract [13, 14], gastrointestinal [15] or disseminated intraperitoneal [16] neoplasms. New developments are being investigated for the treatment of brain tumours [17] and attempts to use topic administration of HPD [18] have been reported. The main advantage of HPD lies in the lack of severe toxicity, especially on nonirradiated areas, while its major drawback is its chemical composition, comprising a mixture of several porphyrin-derivated compounds including oligomeric species [19]. For this reason and despite numerous studies, the relationship between the chemical composition and the photodynamic activity of HPD remains unclear, the activity

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Revised 12 Dec. 1991; accepted 29 Jan. 1992.

being attributed to various components including dihaematoporphyrin ethers, esters or oligomeric compounds [1, 20, 21].

Therefore, the relationship between dose and cytotoxic effect should be defined in well-stated conditions and comparisons between the different HPD solutions established with regard to their respective chemical compositions since any difference could drastically modify the biological activity [22].

This study was developed to establish a reliable experimental model using a procedure based on colorimetric measurements proving less time-consuming than conventional colony-forming assays. The MTT assay would allow an easy-to-manage and rapid evaluation of parameters influencing cell and tumour response to PDT. The respective influence of the laser light power used for photoirradiation (output power), its total fluence (total energy per surface unit) and time dependency of the cytotoxicity were investigated.

MATERIALS AND METHODS

Cell lines and culture conditions

MCF-7 and HT-29, human breast and colon adenocarcinoma cell lines, respectively, were studied. MCF-7 cell line was examined because of its ability to form three-dimensional models such as spheroids which is planned as a future extension of our experimental study. HT-29 cells were grown according to the recommendations of our research group (French INSERM network of scientists "Cancer Photochemotherapy"), since they could easily be xenografted into athymic mice and used as an *in vivo* model.

Both cell lines were maintained in Dulbecco's modified Eagles medium (Intermed, Noisy-le-Grand), supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma), penicillin and streptomycin at 37°C in a 5% CO_2 atmosphere. Serum-containing medium was prefered in order to mimic the *in vivo* biological conditions since HPD is known to be transported bound to serum proteins.

The cultures proved to be mycoplasma-free by the EORTC Clonogenic Assay Screening Study Group test procedure (Dr L. Suardet, Institut Suisse de Recherches Experimentales sur le Cancer, Lausanne).

Exponentially growing cells were harvested by enzymatic disaggregation (trypsin–EDTA) and assayed for cytotoxicity.

Haematoporphyrin derivative

HPD was purchased from the Queen Elizabeth Hospital, Woodville, Australia, in 5 mg/ml solution for human use and stored at 0°C in the absence of light. Aliquots were diluted in physiological saline to produce *in vitro* concentrations ranging from 1 to 200 μg/ml and were used within 24 h.

High-performance liquid chromatography (HPLC)

The HPD solution was characterised by HPLC analysis using a Vista LC 5500 system (Varian) on a C18 Novapak column (160 × 4.6 mm; Waters, St Quentin en Yvelines). Linear gradient elution was performed from a mixture made of 5 mmol/l tetrabutylammonium phosphate : acetonitrile : methanol (45:45:10 v/v/v) to acetonitrile : methanol (90:10 v/v) at a flow rate of 1 ml/min. The ultraviolet detection was carried out at 405 nm as reported [21].

HPD-uptake kinetics

Monolayered cell cultures plated in 25 cm^2 culture flasks (2×10^6 cells per flask) were incubated in 5–100 μg/ml HPD solutions at 37°C. One flask was used for each concentration and each time of incubation. The cells were incubated with HPD then harvested by trypsination, washed twice and resuspended in phosphate buffered saline (PBS) before being assayed for intracellular fluorescence in an Orthocyte flow cytometer (Ortho Diagnostics) using excitation at 488 nm. The fluorescence was detected above 600 nm as described elsewhere [23]. Cells were first identified and selected using forward angle light scattering (FALS) and then analysed for red fluorescence emitted by intracellular HPD. Logarithmic amplification was applied to the signals in order to obtain the whole range of HPD concentrations (5–100 μg/ml) on the same scale. Each analysis was performed on a minimum of 10^4 cells. Red fluorescence histograms were analysed and the values of the median peak channel (N) were converted into relative fluorescence intensities (F) using the equation $F = 10^{N/100}$. Therefore, in the case of logarithmic amplification, a 2-fold increase in the fluorescence channel number represents a 10-fold increase in fluorescence.

Photoirradiation

Cells in 96-well dishes (2×10^4 cells per well) were incubated with HPD for 3 h, then washed twice with PBS to remove non-internalised HPD, and resuspended in phenol red-free medium, since it was shown to interfere with PDT on MCF-7 cells [24]. The dishes were then immediately photoirradiated at 630 nm using a Spectra Physics 375B argon-pumped dye laser (Spectra Physics, Les Ulis). The standardisation experiments were first performed with a total fluence of 20 J/cm^2 and an output power of 1 W. The effect of both parameters on cytotoxicity was then evaluated using output powers of 1 and 2 W and fluences from 10 to 50 J/cm^2. Light spots of 22 cm in diameter were used for simultaneous irradiation of two microwell dishes. The spot homogeneity was adjusted by fastening the optical fibre and controlled photoelectrically. All dishes were kept covered during the photoirradiation in order to maintain the sterility of the culture. The temperature was controlled by immersion of a NiCr/NiAl thermal probe in the culture medium through the cover of the multiwell dishes exposed to light.

Cytotoxicity assays

MTT assays were carried out according to a procedure based on Tada *et al.* [25]. MTT, 3-[(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was dissolved in PBS, filter sterilised and stored at 4°C. After photoirradiation, the dishes were incubated at 37°C for 0–72 h. 50 μl of a 0.5% MTT solution was added to each well and incubated for 3 h at 37°C to allow MTT metabolisation.

The crystals formed were dissolved within 15–30 min, by adding 100 μl per well of 25% SDS solution and vigorously pipetted to ensure homogeneity of solution prior to scanning. The absorbances at 540 nm were measured on a Multiskan MCC 340 plate reader (Flow). The results were compared with the control wells and expressed with respect to control values.

RESULTS

Chemical characterisation of HPD

The reversed phase HPLC analysis of HPD revealed a mixture of monomeric porphyrins: haematoporphyrin IX (Hp), 2-hydroxyethylvinyldeuteroporphyrin isomers (HVD1, HVD2), protoporphyrin (Pp) and a hydrophobic fraction containing dihaematoporphyrin ether ester (DHE) and oligomeric compounds. The retention times (Fig. 1) were found to be 3.3 min for Hp, 7.1 min for HVD1, 8.0 min for HVD2 and 10.0 min for Pp. DHE and oligomeric compounds eluted between 10 and

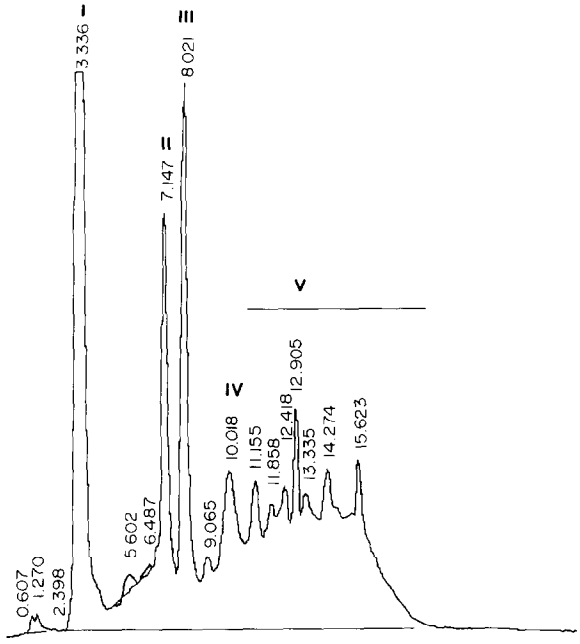

Fig. 1. Reversed phase HPLC analysis of HPD. Analysis was performed on a C18 Novapak column (160 × 4.6 mm) using linear gradient elution at 1 ml/min and detection at 405 nm. Retention times were 3.3 min for Hp (I), 7.1 min for HVD1 (II), 8.0 min for HVD2 (III), 10.0 min for Pp (IV), DHE (V) was eluted between 10 and 20 min.

20 min. The respective amounts of each compounds were calculated: 31% for Hp, 8.7% for HVD1, 10.4% for HVD2, 7.7% for Pp and 38.6% for DHE and oligomeric compounds.

HPD-uptake kinetics

The uptake kinetics of HPD were determined using MCF-7 and HT-29 cells incubated in 5, 10, 20, 50 and 100 μg/ml HPD solutions for 1, 3, 24 and 48 h.

The results (Fig. 2) expressed as relative fluorescence intensity showed that the concentration of intracellular HPD rose with

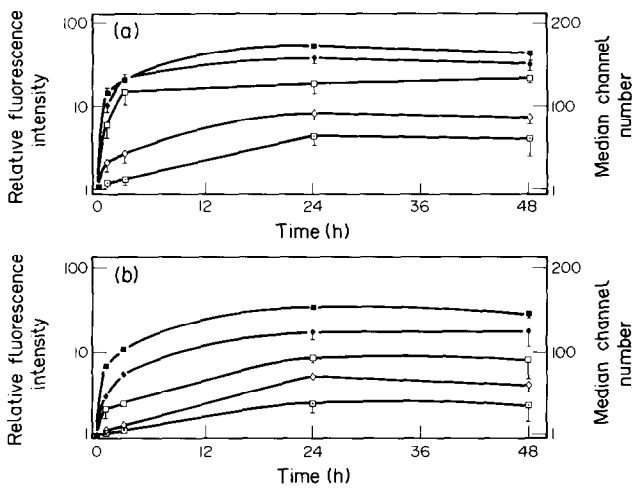

Fig. 2. HPD uptake kinetics. Cells were incubated in 5–100 μg/ml HPD solutions then trypsinised and analysed by flow cytometry with excitation at 488 nm and detection above 600 nm. Results are mean relative fluorescence intensities deduced from median peak values of duplicated red-fluorescence histograms (S.D.). □ 5 μg/ml, ◇ 10 μg/ml, □ 20 μg/ml, ◆ 50 μg/ml, ■ 100 μg/ml, (a)MCF-7, (b) HT-29 cells.

extracellular concentration and time of incubation. Maximal values were attained at about 24 h with no further significant increase in HPD accumulation up to 48 h.

A 3-h incubation was selected for all the following experiments in order to shorten the total duration of the assay since similar results were obtained using 24- and 48-h time points.

HPD-uptake kinetics were established on both cell lines and showed the same general pattern although MCF-7 cells (Fig. 2a) appeared to incorporate approximately twice as much HPD as HT-29 cells (Fig. 2b).

Thermal effect of PDT

The exposure to a 20 J/cm^2 light did not generate any noticeable temperature fluctuations. As the photoirradiation was performed in a 25°C heated room, a slight decrease in temperature tended to stabilise the medium temperature near 25°C in 60 min. This slight drop in temperature did not affect the growth of HT-29 or MCF-7 cells in unirradiated control dishes (data not shown). No thermal effect appeared thereafter to be associated with PDT under our experimental conditions using 630 nm light, large spot irradiation and the cytotoxic effects assessed in the following experiments resulted only from the photodynamic activity.

Cytotoxicity assays

Before performing cytotoxicity assays, we examined the linearity of the MTT assay with increasing number of cells plated between 10^4 and 10^6 cells/ml and found quite satisfactory results for both cell lines ($r > 0.995$). Blank measurements were performed on wells containing no cell and no HPD. Other possibilities, no cell with various HPD concentrations (100 or 200μg/ml) were examined and were found not to induce any significant differences.

The HPD solution did not appear cytotoxic in either MCF-7 or HT-29 cells lines when the cells were not exposed to light. Similarly, light exposure alone (20 J/cm^2) did not induce any significant cytotoxicity in either cell line (data not shown).

MTT assays, performed 0, 24, 48, 72 h after the PDT (20 J/cm^2)(Fig. 3) on cells plated into multiwell dishes, indicated that the effects of PDT were time-dependent. Maximal cell killing was not apparent immediately after the exposure to light and appeared to increase with time up to a maximal value 48 h after photoirradiation. A longer interval of time (72 h) brought on an apparent regrowth since the surviving cells probably began to divide.

The two cell lines showed a similar pattern of time-delayed cytotoxicity. According to the HPD uptake results obtained by flow cytometry analyses, HT-29 cells (Fig. 3b) appeared to be slightly more sensitive to PDT than MCF-7 cells (Fig. 3a) since no significant difference could be found between these two cell lines in terms of cytotoxicity to HPD, while HT-29 cells internalised less HPD than MCF-7 cells. All the following experiments were carried out by MTT assays performed 48 h after PDT.

Influence of the output power

MTT assays were performed on cells receiving the same total fluence of 20 J/cm^2 but at different rates: 1 W during 134 min and 2 W during 67 min; no significant differences were detected between the two series of experiments (Fig. 4).

Light exposure alone (thermal effect) did not appear to affect the growth rate of cells receiving only photoirradiation as these were comparable to the growth rates of control cells (data not

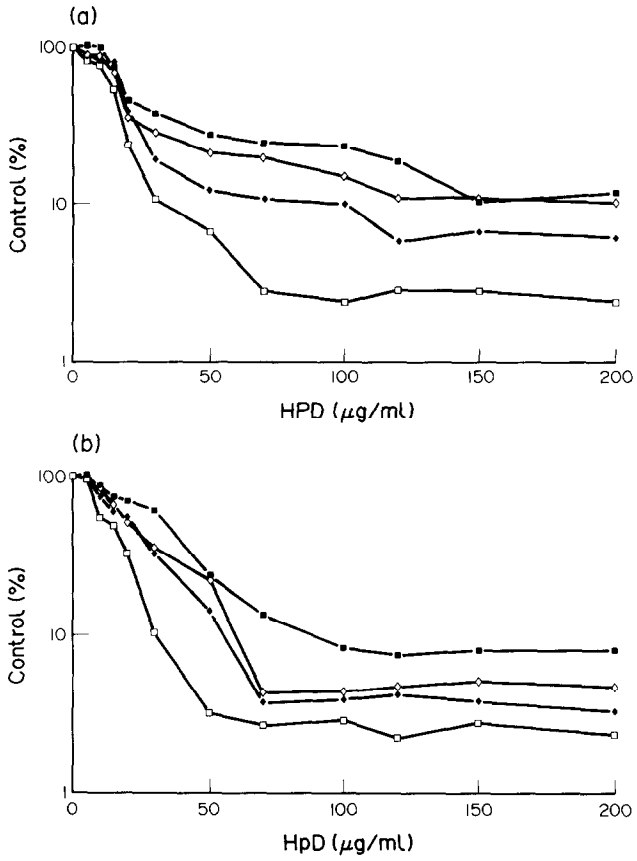

Fig. 3. Time delayed cytotoxicity of PDT. MTT assays were performed immediately (■), or 24 (◆), 48 (□) or 72 h (◇) after exposure of the cells to 630 nm light. Results are mean values of three triplicated assays. S.D. were always less than 10% of the values. (a) MCF-7, (b) HT-29 cells.

shown). These results were consistent for both MCF-7 (Fig. 4a) and HT-29 (Fig. 4b) cell lines. A 2 W photoirradiation was selected for the following experiments in order to shorten the duration of light exposure.

Influence of the total fluence

Variation of the total light fluence from 10 to 50 J/cm^2 was achieved by increasing the overall duration of exposure of a 2 W light spot from 34 to 168 min. Increasing the total fluence (Fig. 5) caused a proportional enhancement of the cytotoxicity from 10 to 40 J/cm^2. A 50 J/cm^2 light fluence did not appear to induce any further increase in cytotoxicity. At the highest HPD concentrations, a fall-off in cytotoxicity was noted for both cell lines and seemed to be correlated to the fluence value. Plotting the results as % control vs. fluence for HPD concentration of 20, 50, 100, 150 and 200 μg/ml (Fig. 6) clearly demonstrated that an increase in both fluence and HPD extracellular concentration resulted in a decrease in cytotoxicity. Between 30 and 40 J/cm^2, the pattern of cytotoxicity was thoroughly modified; at 50 J/cm^2, the results appeared conflicting since the highest HPD concentrations produced the lowest cytotoxicity.

From these results, the rate of the loss of photodynamic activity induced by photo-irradiation at high fluences was estimated. For 50, 100, 150 and 200 μg/ml HPD concentrations, iso-effects lower concentrations leading to the same cytotoxicity were deduced from the dose–response curves of each cell line at 10, 20, 30, 40, and 50 Jcm^2 (Table 1). From these values, we calculated the apparent rates of loss of photodynamic activity, i.e. the percentage of HPD unable to generate any photodynamic effect when exposed to light. These values were plotted against the fluence values for each HPD concentration (Fig. 7). This phenomenon appeared to be quite proportional to both the fluence and the HPD concentration and to reach high values (60–90%) for cells exposed to 50 J/cm^2.

DISCUSSION

The importance of an *in vitro* biological model for PDT using cultured cells is summarised in the following two points. Firstly, analysis and interpretation may be used for the development of mechanistic models. Secondly, they can be a simple way of evaluating photosensitisers from different origins and testing new photosensitisers. Many studies have already reported the survival rates of tumour cells to photosensitisers on models related to PDT using conventional clonogenic assays as a means of determining the overall survival rates of cells exposed to cytotoxic agents. However, these experiments are quite often time consuming since 7–21 days are needed to allow for colony formation [11, 26–28]. To our knowledge, other methods such as MTT assays to determine time-dependent cytotoxicity were not employed, although they could shorten the total duration of the experiment and yield more numerous results since most of the steps are automated. The results presented in this paper demonstrate that photodynamic survival curves obtained with MCF-7 or HT-29 cell lines and HPD using MTT assays are

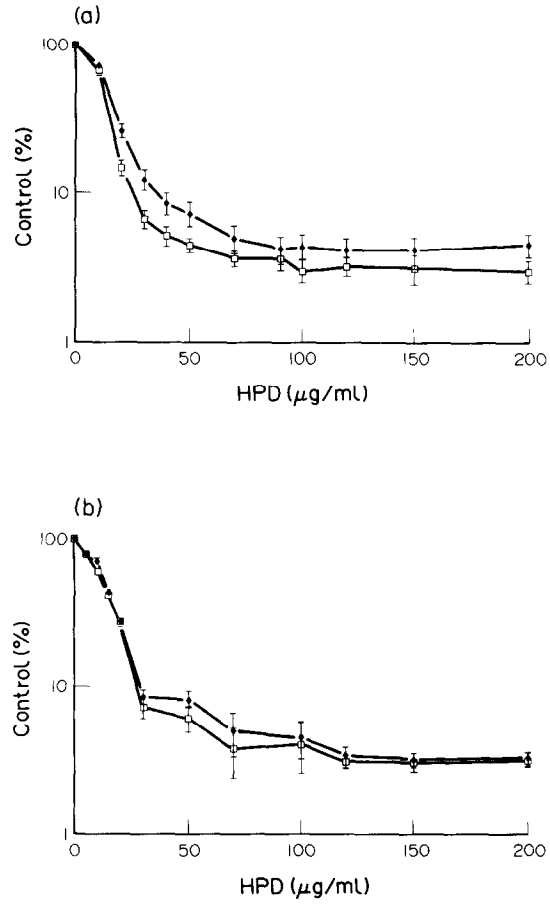

Fig. 4. Influence of the output power. Cells were exposed to a 20 J/cm^2 constant light fluence delivered by means of 1 (□) or 2 (◆) W laser light. Results are mean values of three triplicated assays (S.D.). (a) MCF-7, (b) HT-29 cells.

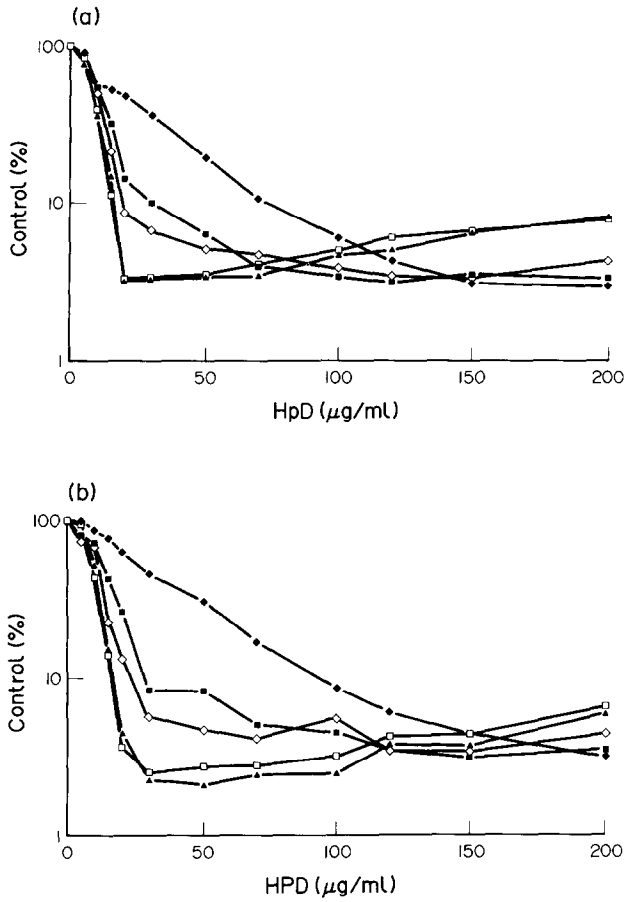

Fig. 5. Influence of the total light fluence. Using 2 W light, cells were exposed to fluences from 10 to 50 J/cm². Results are mean values of three triplicated assays. S.D. always less than 15% of the values were omitted from the graph. ◆ 10 J/cm², ■ 20 J/cm², ◇ 30 J/cm², ▲ 40 J/cm². □ 50 J/cm². (a) MCF-7, (b) HT-29 cells.

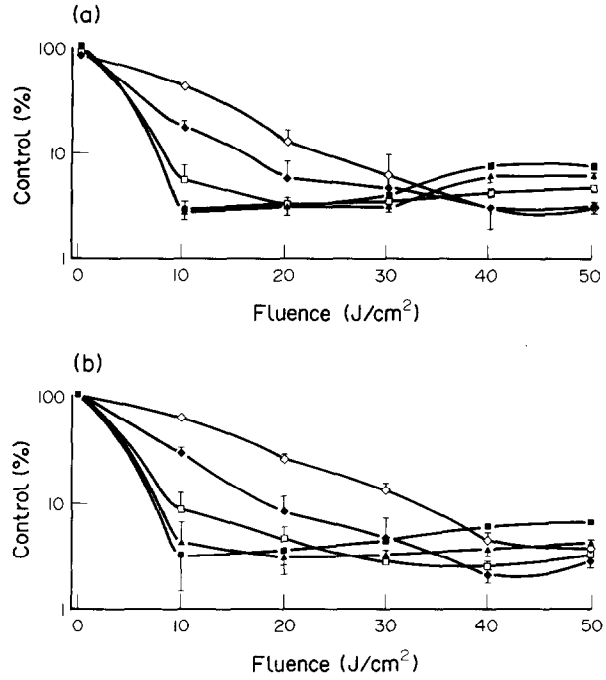

Fig. 6. Spontaneous decrease of the cytotoxicity. Decreases in cytotoxicity were observed in relation with the fluence value. This effect was particularly intense for cells exposed to high HPD concentrations and to high fluences (40 and 50 J/cm²). Results are mean values of three triplicated assays. S.D. always less than 15% of the values were omitted from the graph. ◇ 20 μg/ml, ◆ 50 μg/ml, □ 100 μg/ml, ▲ 150 μg/ml, ■ 200 μg/ml. (a) MCF-7, (b) HT-29 cells.

quite reproducible and yield results within 48 h. The general patterns of cytotoxicity obtained using MTT assays were consistent with those determined using conventional colony-forming assays on both MCF-7 (24) and HT-29 cells (data not shown). Although the variations of the IC_{50} and IC_{90} values were found between the two procedures, the values obtained with the MTT being approximately 1.5-fold higher, the difference remained in the range of the ratios previously reported [29] with anticancer agents.

Before concluding or comparing our results with previously published data, two important points have to be discussed concerning the chemical composition of the HPD solution employed and the level of interference due to thermal effects in our experimental system, since laser-generated light could increase the temperature of the culture media and potentiate the effects of PDT as already reported [30–32].

The composition of our HPD solution was found to correlate with the values reported by Kessel [1, 21]: Hp 31%, HVD 19%, Pp 8% and DHE 39% for our solution against 35, 20, 5 and 35% reported by Kessel. Under our experimental conditions using large spot for photoirradiation no significant increase in temperature was noted, suggesting that the observed cytotoxic effects were due to PDT.

The results reported in this paper show that the cellular cytotoxicity of PDT is mainly due to time-delayed effects appearing maximal 48 h after photoirradiation, further supporting the comparison between X-ray irradiation cytotoxic effects and PDT [23]. The antitumour effects of PDT were found to be closely related to the total fluence up to 40 J/cm² where maximal values were attained. This effect may be linked to the occurrence of photobleaching that was clearly demonstrated by fluorescence studies, reporting high fluorescence decays comparable to the relative decrease in cytotoxicity we calculated from iso-effects HPD concentrations. Using 630 nm light, values of 50% photobleaching were reported to occur at 10 J/cm² [33] and 63% at 28 J/cm² [34] as well as 80% at 2.3 J/cm² using 404

Table 1. For each fluence value, the lowest HPD concentration leading to the same cytotoxicity (isoeffect concentration) as 50, 100, 150 and 200 μg/ml were deduced from the dose–response curve of each cell line

HPD (μg/ml)		Fluence (J/cm²)				
		10	20	30	40	50
MCF-7 cells	50	—	—	—	—	20(60)
	100	—	—	—	30(60)	20(80)
	150	—	—	100(35)	30(80)	20(85)
	200	—	100(50)	70(65)	20(90)	20(90)
HT-29 cells	50	—	—	—	—	20(60)
	100	—	—	—	30(70)	20(80)
	150	—	—	70(55)	30(80)	20(85)
	200	—	120(40)	50(75)	30(85)	30(85)

Numbers in brackets express the relative quantity (%) of HPD, which appears to loose its photodynamic activity when exposed to high fluences.

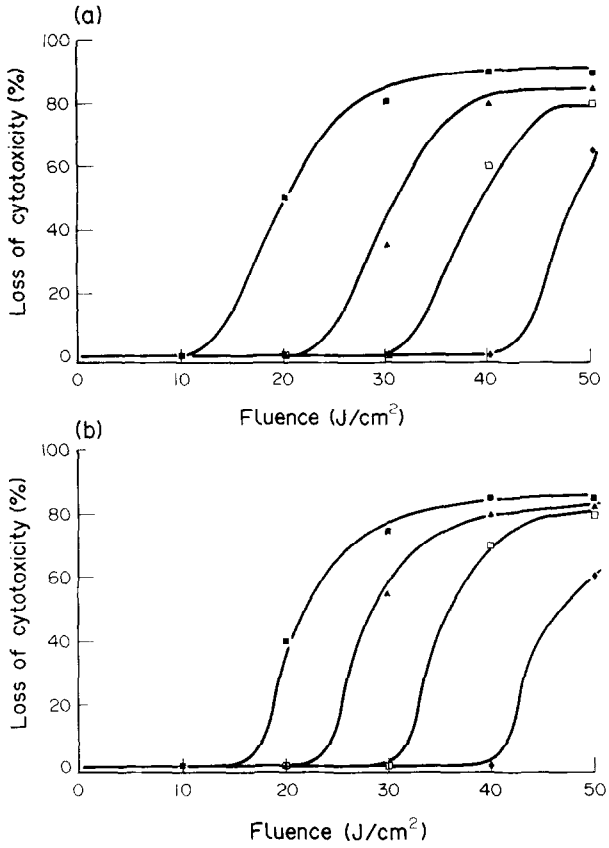

Fig. 7. Evaluation of the relative loss of cytotoxicity. Each point was calculated as ratios of isoeffect HPD low and high concentrations obtained with each fluence: 50(◆), 100 (□), 150 (▲) and 200 (■) μg/ml. (a) MCF-7, (b) HT-29 cells.

nm light [35] on human tumour cells. This particular finding may prove very useful in clinical applications since overdosing light above this limit will only generate time consumption and raise the incidence of side-effects without enhancing the response. On the other hand, underdosing light could drastically affect the efficiency of PDT treatments since the antitumour effect could be lessened if no bleaching is generated (under the limit) but increased if the total fluence is set above the limit of photobleaching. This is of main importance in view of the major research interests in PDT arising from the fractionation of photoirradiation [36], which should not be undertaken without taking into account this time dependency and the photobleaching phenomenon.

Throughout this study, MCF-7 and HT-29 cell lines appeared quite suitable to develop an experimental two-dimensional model for PDT.

The results reported in this paper emphasise the importance of both photosensitiser and light dosimetries in PDT. The pharmacological parameters of HPD, together with the optical characteristics of tissues are to be precisely determined in order to optimise both doses of HPD and light. In this particular case, and as far as dosimetry experiments are concerned, the development of three-dimensional models (*in vitro* or *in vivo*) is required to allow the extrapolation of the results to human clinical applications [37]. Such models for light dosimetry in human tumours are under current investigation.

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Acknowledgements—The authors are grateful to Mrs F. Pauly and Mrs D Chanel for their expert technical and secretarial assistance and to Dr S. Shellard (ICRF in London) for kindly editing this paper. This study was supported by grants from the French "Ligue Nationale contre le Cancer" and performed within the French INSERM Network of Scientists "Cancer Photochemotherapy". Experimental data were partly presented at the Clonogenic Assay Screening Study Group meeting during the 1991 EORTC Joint Meeting in Vienna.

Eur J Cancer, Vol. 28A, No. 8/9, pp. 1458–1462, 1992.
Printed in Great Britain

0964–1947/92 $5.00 + 0.00
Pergamon Press Ltd

Production of Chromogranin A and B Derived Peptides in Human Small Cell Lung Carcinoma Cell Lines

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Production of chromogranin (Cg)A and B derived peptides [pancreastatin (PST), GAWK, CCB] was studied using human lung carcinoma derived cell lines. PST-like immunoreactivity (LI) was detected in the culture medium in 3 of 6 small cell lung carcinoma (SCLC) cell lines, while GAWK- and CCB-LIs were detected in 5 of 6 and all the 6 SCLC cell lines, respectively. CCB-LI was produced in large amounts in SCLC cell lines as compared to PST- and GAWK-LIs. In non-SCLC cell lines, on the other hand, PST- and GAWK-LIs were not detected. CCB-LI was detected in 1 of 7 non-SCLC cell lines, but not detected in the remainder. PST, GAWK and CCB-LIs, secreted by these cell lines, consisted of several peaks, and these peaks were different among cell lines. This suggests that processing of CgA and B is different in the cell lines. Production of CgA and B derived peptides seems to be a characteristic feature of SCLC, and among them, CCB LI may be a useful marker for SCLC.
Eur J Cancer, Vol. 28A, No. 8/9, pp. 1458–1462, 1992.

INTRODUCTION

A CHROMOGRANIN (Cg) and secretogranin (Sg) family [CgA, CgB (SgI), SgII (CgC)] shares characteristic biochemical features and is distributed in secretory granules of neuroendocrine (NE) tissues of several species [1]. The primary structures of these proteins were recently described, and the aminoacid sequences deduced from cDNAs reveal several sites of paired or more adjacent basic aminoacids, which are potential proteolytic cleavage sites in the processing of precursor proteins [2–4]. Thus, a Cg/Sg family is considered to be a precursor for biologically active peptides. Pancreastatin was initially isolated from porcine pancreas [5], and its sequence has been shown to be located in the CgA molecule [6]. Similarly, GAWK and CCB were initially isolated from human pituitary glands [7, 8]. These names were derived from the first four aminoacids of the initially isolated fragment of a GAWK molecule [Gly(G)-Ala(A)-Trp(W)-Lys(K)] and the abbreviation of *C*-terminal region of chromogranin *B*, respectively. The aminoacid sequences of GAWK and CCB are entirely homologous to human CgB 420–493 and 597–653, respectively [8]. Therefore, these peptides could be originated from CgA and CgB through processing.

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Revised 11 Feb. 1992; accepted 12 Feb 1992.