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Original Paper

Efficacy and Mechanism of Aluminium Phthalocyanine and its Sulphonated Derivatives Mediated Photodynamic Therapy on Murine Tumours

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The efficacy of photodynamic therapy (PDT) mediated by aluminium phthalocyanine (AlPc) and its mono- and disulphonated derivatives ($AlPcS_1$ and $AlPcS_2$, respectively) on murine EMT-6 tumour were compared *in vivo*. AlPc (0.25 μmol/kg) PDT resulted in no tumour recurrence in all treated mice. In contrast, PDT with $AlPcS_1$ (2 μmol/kg) and $AlPcS_2$ (1 μmol/kg) only produced tumour cure in 75% and 86% of mice, respectively. Immediately after AlPc-PDT, tumour cells were found to be viable as determined by *in vitro* clonogenicity, but progressive cell death occurred thereafter. In contrast, $AlPcS_1$ and $AlPcS_2$ PDT produced substantial cell death (approximately 35% and 70%, respectively, of entire tumour) immediately after phototherapy, and yet further loss of tumour cell viability continued after PDT. In all cases, few vascular effects were observed at 0 h post-PDT, as indicated by the retention of ^{99m}Tc-MIBI in the tumour. However, the reduction of blood flow in tumours progressed with time, such that blood flow in tumours fell to approximately 25% of the control level by 24 h after both AlPc and $AlPcS_1$ PDT. With $AlPcS_2$, there was only an approximate 50% fall in tumour blood flow by 24 h. These results demonstrate a greater PDT efficiency with AlPc on tumour destruction, which is an indirect mechanism involving damage of tumour vasculature, whereas $AlPcS_2$ has a greater effect on direct tumour cytotoxicity and $AlPcS_1$ exerts both direct and indirect modes of action against tumours. © **1997 Elsevier Science Ltd.**

Key words: EMT-6 tumour, blood flow, clonogenicity, Cremophor, photodynamic therapy, phthalocyanine

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INTRODUCTION

THE METALLOPHTHALOCYANINES (Pc) have drawn considerable attention in recent years for their potential use as second-generation photosensitisers for photodynamic therapy (PDT) of neoplastic diseases [1, 2]. The major advantage of Pc over the Photofrin II (PII), which is currently the most widely used preparation for clinical PDT, is their strong absorption of red light at approximately 675 nm (extinction coefficient $2.5 \times 10^5\ M^{-1}cm^{-1}$) which allows light penetration into tissues to almost twice the depth of that achieved using 630 nm light with PII-PDT [3]. In addition, Pcs have low absorption of light in other parts of the solar spectrum, thus lowering the risk of skin photoreaction that often occurs in patients after PII-PDT. In earlier studies, water-soluble sulphonated derivatives [4–6] were used for demonstrating Pc photoefficacy in the destruction of *in vivo* tumours. More recently numerous lipophilic Pc derivatives [7, 8] have proven to be effective PDT agents *in vivo*, but the success of these agents relies upon their incorporation into carrier vehicles or their formulation in specific reagents, such as liposomes and oil emulsions, that circumvent the problem of aggregation in biological fluids.

Aluminium phthalocyanine (AlPc), which is chemically stable and is relatively easy to synthesise in large quantities, yields a single isomeric product which has been shown previously by us [9] and by others [10] to be highly effective in photoinactivating cultured cells. However, while the water-soluble sulphonated derivatives of AlPc have attracted

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considerable interest for *in vivo* use, especially the disulphonated derivative ($AlPcS_2$) [11–14] which is now being tested clinically, no further studies on the *in vivo* evaluation of the non-sulphonated AlPc have been pursued. Recently it has been shown [15] that an emulsified AlPc formulated in Cremophor, when administered *in vivo*, appeared to be retained preferentially by gliosarcoma relative to the brain tissue and was able to induce necrosis in this tumour model. These observations prompted us to investigate further the *in vivo* potential of this dye as well as to re-evaluate the *in vivo* efficacy of its water-insoluble monosulphonated derivative ($AlPcS_1$), which was previously found to be the most potent sulphonated species *in vitro* but was totally devoid of tumoricidal activity *in vivo* [13].

In the present study we have characterised the PDT efficacy and mechanism of Cremophor formulated AlPc and $AlPcS_1$ in tumour eradication and have compared them to the more hydrophilic derivative $AlPcS_2$ in phosphate-buffered saline (PBS). Tumour responses following *in vivo* PDT were assessed using a murine EMT-6 tumour model. The kinetics of tumour cell survival after PDT were determined by assaying for their clonogenic ability *in vitro* and the changes in tumour blood flow were monitored by the retention of ^{99m}Tc-MIBI in the tumour.

MATERIALS AND METHODS

Photosensitiser preparation

AlPc featuring a chloride group as axial ligand was purchased from Eastman Kodak (Rochester, New York, U.S.A.). $AlPcS_1$ and $AlPcS_2$ were synthesised using a condensation method and were purified as previously described [16]. Both dyes feature a hydroxyl group as axial ligand and $AlPcS_2$ bears sulphonate groups on adjacent benzene rings.

AlPc and $AlPcS_1$ were formulated in an emulsion containing 10% Cremophor EL (Sigma, Aldrich Canada, Mississauga, Ontario, Canada) and 3% 1,2-propanediol (Sigma) in PBS following sonication and Millipore filtration. The stock solutions were quantified by spectrophotometry and were kept in the dark at room temperature to avoid possible aggregation.

The stock solution of $AlPcS_2$ was prepared by dissolving the dye in PBS followed by filtration and was then stored in the dark below 8°C.

Tumour model

EMT-6 cells, tumour cells of a murine mammary sarcoma originally induced in Balb/c mice [6], were cultured routinely in Waymouth's medium (Gibco, Burlington, Ontario, Canada) supplemented with 15% fetal bovine serum. Balb/c mice (6–7 weeks old) were purchased from Charles River Inc. (St. Constant, Quebec, Canada). Prior to tumour cell implantation, hair around both legs of the mice was removed with depilatory cream and 2.5×10^5 trypsinised EMT-6 cells suspended in 0.05 ml medium were then intradermally injected into the hind thighs of both legs. All experiments conducted on mice followed the regulations set out by the Canadian Council on Animal Care and the in-house ethics committee.

In vivo *PDT and tumour responses*

7–10 days after tumour inoculation when the tumour size reached 3–6 mm diameter, mice were injected via the lateral tail vein with various concentrations of Pcs. Phototherapy of tumours (one tumour per mouse, the unirradiated contralateral tumour served as control) was conducted 24–30 h after dye administration. Red light for PDT was generated from a 1000 W Xenon lamp (Hanovia, J.B. Atlas Company Ltd., Ontario, Canada) coupled with a 10 cm water filter to eliminate a possible hyperthermic effect on the tumour and LL650 and LS700 (Corion Corporation, Holliston, Massachusetts, U.S.A.) filters that allow light transmission in the range of 650–700 nm. The incident fluence rate delivered at the level of the tumour surface over a 8 mm diameter spot was 200 mW/cm^2 for a total fluence of 400 J/cm^2 as measured by a radio meter. Under these conditions, an increase of tumour temperature of approximately 2°C was recorded, as monitored by means of a microthermocouple inserted at the base of the tumour. Mice were examined daily for 20 days following PDT in order to assess gross tissue effect and tumour recurrence.

Assessment of tumour cell viability

In situ tumour cell viability following PDT was assessed using an *in vitro* colony-forming assay as described previously [17] with slight modification. At different time intervals after PDT, mice were sacrificed by cervical dislocation. The light irradiated tumours as well as the unirradiated contralateral tumours (controls) were excised, isolated from the surrounding normal tissues, weighed and minced with a scalpel. The minced tissues were then disaggregated in 5 ml Waymouth's medium containing 0.05% collagenase (Sigma Type I), 0.015% pronase (CalBiochem, San Diego, California, U.S.A.) and 0.01% DNase (Boehringer Mannheim Canada, Laval, Quebec, Canada) under continuous stirring for 1.5 h at 37°C. Single cells were washed and resuspended in 5 ml of complete growth medium. 2–200 µl of cell suspensions were added to 60 mm Petri dishes (four dishes per tumour) containing 3 ml growth medium. Cells were allowed to grow for 7–9 days before being fixed in methanol and stained with 0.5% crystal violet. Numbers of colonies (> 50 cells) per dish were counted. Total numbers of clonogenic cells per gram of PDT treated tumour tissue were calculated and expressed as the percentage survival relative to the controls (unirradiated tumours). Each data point was obtained from 3–5 independent experiments.

Assessment of vascular perfusion

Changes in tumour vascular perfusion were monitored using a modified ^{99m}Tc-MIBI (technetium-99m-hexakis-2-methoxy-isobutyl isonitrile) retention technique [18]. The cationic ^{99m}Tc–MIBI complex was prepared in saline from a commercially supplied kit (Cardiolite®, Du Pont Pharma, Montreal, Quebec, Canada) to yield an activity of 500 µCi/ml. At various times after PDT, ^{99m}Tc-MIBI (10 ml/kg b.w.) was injected via the tail vein into the mice. Mice were killed 2 min after injection. Both light irradiated and the unirradiated tumours of the same mice were excised, weighed and counted for radioactivity (cpm/g tissue). Any reduction of radioactivity detected in PDT-treated tumour relative to the untreated tumour indicates a decrease of vascular perfusion and reflects the occurrence of vascular damage in the tumour. Data were collected from three separate experiments conducted on different days.

RESULTS

Pc-mediated PDT efficacy in eradication of tumours

Tumour responses to Pc-mediated PDT are summarised in Table 1. At the administered dose of 0.25 µmol/kg, AlPc PDT provoked a massive destruction of the treated tumours,

Table 1. Gross tissue effects following Pc-mediated PDT on EMT-6 tumours

Pc	Administered dose (μmol/kg)	No. of mice tested	Skin oedema around tumour area*	% Of tumour response†	% Of tumour cure‡
AlPc	0.25	8	+	100	100
	0.5	4	+	100	100
$AlPcS_1$	2	8	+++	100	75
$AlPcS_2$	1	7	+++	100	86

*Assessed 24 h after PDT; + = slightly swollen around tumour area, +++ = severely swollen extended to the pelvic region. †Assessed 48 h after PDT for tumour flattening and tissue necrosis. ‡No recurrent tumour at 20 days post-PDT.

resulting in no tumour recurrence in all treated mice for 20 days after PDT. At the same dye dose, PDT with both sulphonated Pc derivatives did not result in any tumour cure and had little effect on reducing tumour burden (data not shown). Only at the doses of 1 μmol/kg for $AlPcS_2$ and 2 μmol/kg for $AlPcS_1$, was there a significant effect on tumour growth which resulted in tumour regression in most of the treated mice. Development of oedema usually occurred in the PDT treated areas with all Pcs used at the doses that resulted in substantial tumour necrosis. Although it subsided within 48 h, oedema was considerably more severe when induced by $AlPcS_{1-2}$ PDT than by AlPc PDT as evident by the extensive swollen skin on the irradiated leg extending to the pelvic region.

Effect of Pc-mediated PDT on tumour cell survival

The kinetics of *in situ* tumour cell survival following PDT with Pc, as determined by the *in vitro* colony-forming ability of the dissociated tumour cells, are presented in Figure 1. The PDT administered doses used for comparing the effects are 0.25 μmol/kg for AlPc, 2 μmol/kg for $AlPcS_1$ and 1 μmol/kg for $AlPcS_2$, as at these doses they inflict similar levels of gross tumour damage. With AlPc PDT, no apparent loss of tumour cell viability was detected until several hours after light irradiation, thereafter cell death progressed rapidly. A similar trend in progressive tumour cell death was also observed with $AlPcS_1$, but differing in that a fraction of the tumour cell population (35% of the entire tumour) was already found non-viable as soon as photoirradiation was completed. In comparison, a much larger fraction of cell death (>70%) occurred immediately after light treatment with $AlPcS_2$ PDT and viable tumour cell fell to approximately 3% of the control by 24 h after PDT.

Changes in tumour vascular perfusion following Pc-mediated PDT

PDT-mediated effects on tumour blood flow, as monitored by the retention of ^{99m}Tc-MIBI in tumours, are presented in Figure 2. As compared with those tumours receiving light alone but no Pc administration, Pc-mediated PDT had little effect on tumour blood flow immediately after photoirradiation. Thereafter, progressive reduction in tumour blood flow was seen in all Pc-mediated PDT. However, this appeared to occur more rapidly and more severely with AlPc PDT treated tumours in the first 3 h following PDT, such that blood flow in the tumours was reduced to 35% of the control level followed by a slower decline to less than 25% by 24 h. In comparison, blood flow in $AlPcS_1$ PDT treated tumours decreased more gradually but reached the same level as AlPc

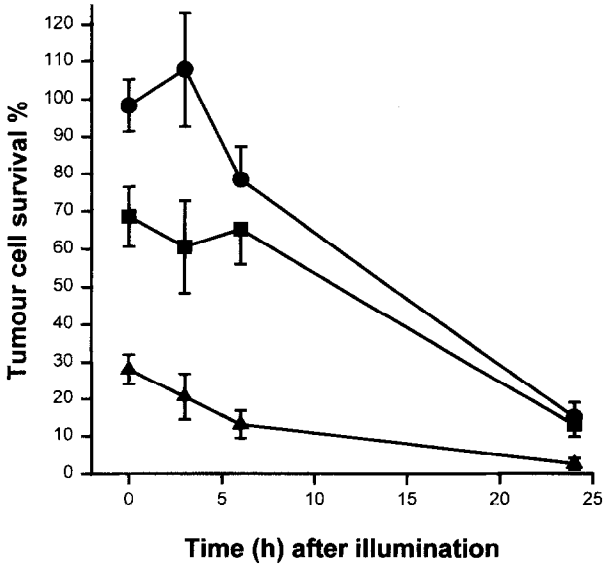

Figure 1. Tumour cell survival *in vitro* as a function of time remaining *in situ* following Pc-mediated PDT. AlPc (0.25 μmol/kg administered dose)(●), $AlPcS_1$ (2 μmol/kg)(■), $AlPcS_2$ (1 μmol/kg)(▲). Total clonogenic cells per gram of tumour obtained from unirradiated tumours are expressed as 100% survival. Individual data point represents the mean of 5–16 tumours and the error bars show the standard error.

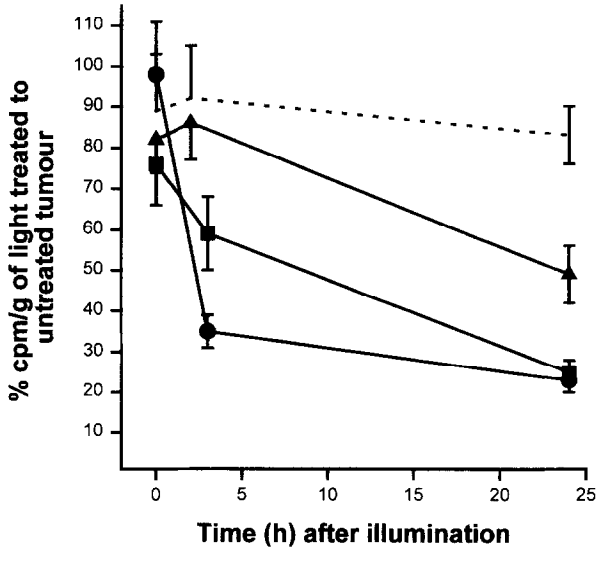

Figure 2. Kinetics of blood flow in tumours as a function of time following PDT relative to the blood flow obtained from the light unirradiated tumours of the same mice. Control (light alone, 650–700 nm, - - - -), AlPc-PDT (0.25 μmol/kg b.w, ●), $AlPcS_1$-PDT (2 μmol/kg, ■) and $AlPcS_2$-PDT (1 μmol/kg, ▲). Each data point represents the mean and standard error (bar) of 5–10 tumours.

PDT treated tumours by 24 h post-PDT, while blood flow in tumours treated with $AlPcS_2$ PDT remained at approximately 50% of the control level.

DISCUSSION

The present investigation aimed to compare the photoefficacy of AlPc and two of its sulphonated derivatives, $AlPcS_{1-2}$, in eradication of solid tumour and to characterise the mechanistic differences of these agents in inflicting tumour necrosis.

AlPc was found previously to be highly potent in photoinactivating cultured cells [9, 10]. We have here demonstrated that Cremophor formulated AlPc possesses a striking ability in mediating PDT tumour destruction *in vivo* even as compared with $AlPcS_2$, the most promising species among the differently sulphonated analogues for clinical PDT. In contrast, $AlPcS_1$, which has previously been found to be the most effective sulphonated analogue *in vitro*, but is totally inactive *in vivo* when delivered in alcohol [13] showed in this study, when delivered in Cremophor, an increased *in vivo* activity against tumour, albeit far less effective compared with AlPc and $AlPcS_2$.

By 24 h post-PDT, cell death induced by Pc-mediated PDT was less than $2 \log_{10}$. The shape of the survival curves (Figure 1) and, particularly for AlPc PDT, the complete tumour regression in all treated tumours (Table 1), indicate that loss of tumour cell viability continued and that early cell survival is not necessarily predictive of tumour regression. However, what is clear from these cell survival studies (Figure 1) is the profound diversity in mechanism of antitumour action among these dyes. With AlPc PDT, reduction in tumour cell clonogenicity only occurred several hours after the completion of light treatment. This delayed cell death effect is consistent with that due to tumour anoxia [17, 19] and thus suggests that an indirect mode of tumour cell killing is the major mechanism involved in AlPc-mediated PDT [17, 19, 20]. In contrast, $AlPcS_2$ PDT resulted in >70% tumour cell death at 0 h after PDT, and such an early effect on cell viability cannot be explained by an indirect mode of action and can only be attributed to the consequence of direct tumour cell phototoxicity. Cell viability declined further as a function of time following $AlPcS_2$ PDT, suggesting that an additional action via an indirect mechanism contributes to tumour cell death. While both direct and indirect effects on tumour cell viability occurred with $AlPcS_1$ PDT, the observed cell loss was predominantly the result of the indirect mode of killing.

Delayed cell death has been observed following PDT with various sensitisers including PII [19, 20] and a mixture of sulphonated AlPc derivatives [17]. Such an effect is thought to be the consequence of oxygen and nutrient deprivation in the tumour induced by PDT damage to tumour vasculature [21]. Our results on tumour retention of ^{99m}Tc-MIBI (Figure 2) show that PDT with all Pcs tested did have an adverse effect on tumour blood flow. This effect was considerably more acute and severe with AlPc and $AlPcS_1$ as compared to $AlPcS_2$. This varying extent of tumour vascular shutdown induced by these Pcs parallels the observed progressive reduction in tumour cell viability after PDT, suggesting that the delayed tumour cell death is the consequence of PDT damage to tumour vasculature.

It has been observed that, by 24 h after administration, $AlPcS_2$ accumulates preferentially in the neoplastic cells and in the interstitium of the tumour [22, 23] but not in the vascular stroma [24]. These observations may explain the increased direct tumour cell photocytotoxicity and lesser adverse effect on the vascular perfusion seen with $AlPcS_2$ PDT. $AlPcS_1$ was also found to be selectively localised in tumour cells [22, 23], but this does not seem to be consistent with the minimal direct cell killing it produced. However, this previous observation was made where the dye was delivered in alcohol, that in which $AlPcS_1$ is completely inactive *in vivo* [13]. It is likely that the dye formulation may affect the localisation pattern in the tumour and may modulate *in vivo* PDT potency. Cremophor induces plasma lipoprotein degradation and this causes a shift in the binding of a hydrophobic sensitiser, ketochlorin, from the albumin to degraded lipoprotein as well as to low-density lipoprotein [25]. Such changes in the binding properties of the sensitiser were associated with its longer persistence in plasma and tissues, and with an increased PDT efficacy. The latter likely reflects augmented vascular photosensitisation which is consistent with the indirect PDT effects observed here with AlPc and $AlPcS_1$. In contrast, another study [26] on a less hydrophobic photosensitiser, mesoporphyrin, showed that Cremophor has a rather limited effect on inducing changes in the distribution of this sensitiser among plasma proteins. Therefore, the substantial differences in PDT efficacy that we observed between AlPc and $AlPcS_1$ may in part be explained by the differences in their hydrophobicity. $AlPcS_1$ is considerably less hydrophobic than AlPc which may render it less sensitive to the effect of Cremophor, consequently reducing its PDT efficiency.

In summary, this study indicates the importance in both choice of the sensitisers and their formulation for optimising PDT efficacy. The results demonstrated marked differences in PDT efficiency and diversity in the mechanism of antitumour action among non-sulphonated and sulphonated Pcs. In comparison, the Cremophor formulated AlPc is the most effective PDT agent in eradication of tumours, while exerting relatively minor effects against normal tissues. This high photoefficacy with simplicity in preparation that results in a single isomeric product warrants further studies for its clinical usefulness.

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