Hypericin-photodynamic therapy (PDT) using an alternative treatment regime suitable for multi-fraction PDT

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Abstract

Photodynamic therapy (PDT) outcome depends on the conditions under which it is carried out. Maintaining the tumour tissue oxygen level is important for PDT efficacy and using a low fluence rate can improve outcome. In this work we studied the response of human nasopharyngeal carcinoma tumours in murine models to hypericin-PDT carried out under low fluence and fluence rate. A drug-light interval (DLI) of 1 h or 6 h was used for 1 h-PDT and 6 h-PDT, respectively. Evan's blue test was used to assess necrosis and TUNEL staining for apoptosis. Nuclear microscopy was used to quantify elemental concentrations in tumours. Serum vascular endothelial growth factor (VEGF) levels were also determined.

TUNEL results showed that 6 h-PDT induced significantly more apoptosis compared to 1 h-PDT ($p < 0.01$). This was supported by nuclear microscopy showing an increase in calcium and a decrease in zinc levels (both known triggers of apoptosis) in 6 h-PDT tumours compared to non-PDT tumours ($p < 0.05$). These results further imply a zinc-mediated pathway in hypericin-PDT induced apoptosis. 6 h-PDT also resulted in a significant increase in copper concentrations compared to non-PDT tumours ($p < 0.05$). Serum VEGF levels measured after 6 h-PDT were lower than those obtained after 1 h-PDT.

Overall tumour response to hypericin-PDT under low fluence and fluence rate and using a 6 h DLI showed increased apoptosis and lower serum VEGF levels. This treatment regime is suitable for the alternative approach of multi-fraction PDT in which the tumour can be exposed to multiple PDT fractions for complete tumour response. This alternative approach might yield improved outcome.
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1. Introduction

Photodynamic therapy (PDT) is an alternative cancer treatment modality in which a photosensitising drug is administered, followed by exposure to light of a specific wavelength to kill tumour cells via the production of reactive oxygen species (ROS) [1]. PDT is being developed for certain cancers including nasopharyngeal carcinoma (NPC). The outcome of PDT depends on the conditions under which it is carried out. For example, although hypericin-PDT is known to induce both apoptosis and necrosis, higher drug and light doses appear to induce more necrotic cell death [2–5]. Other factors affecting the outcome of PDT include the interval between drug administration and light delivery (drug-light interval, DLI), localisation of the photosensitiser, photosensitiser concentration and cell type [2,5–8].

One important consideration is the rate at which light is delivered during PDT treatment, or the fluence rate. Since PDT works via the generation of ROS, maintaining sufficient oxygen levels in the tissue during PDT is important

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for its efficacy [9,10]. This puts a limit to the fluence rate that should be used. It was recently reported that oxygen-conserving low fluence rate PDT yielded 70–80% tumour cures compared to only 10–15% with oxygen-depleting high fluence rate PDT [10].

It has been shown that PDT resulted in lowered serum VEGF levels in a fibrosarcoma mouse model and that this correlated with a prolonged survival rate compared to controls [11]. It was therefore proposed that PDT could work by lowering serum VEGF levels and inhibiting the ability of tumour tissue to form new vessels [11]. Thus serum VEGF levels could be another factor affecting PDT outcome.

In this work, we used murine models bearing xenograft tumours of human NPC to study tumour response to hypericin-PDT carried out under low fluence and fluence rate conditions. Based on earlier work by our research group, two DLI, 1 h and 6 h, were chosen to coincide with the highest drug concentration in the serum and tumour, respectively [12]. While PDT with a single low dose fraction is not expected to give good curative outcome, it can be repeated up to a targeted dose. In this alternative approach of multi-fraction PDT, the tumour can be exposed to multiple low dose PDT fractions to achieve complete tumour response.

2. Materials and methods

2.1. NPC xenograft models

The cell line used, NPC/HK1, was kindly provided by Prof K.M. Hui of the National Cancer Centre, Singapore. A cell suspension of 1.5×10^4 cells/ μl was prepared in Hank's balanced salt solution (Gibco BRL, USA) and a volume of 100 μl was injected sub-cutaneously into the flanks of 6–8 week-old male Balb/c nude mice. The mice were kept for 10–14 days for the tumours to grow to sizes between 100–300 mm^3 as estimated by using the formula, volume = $(\pi/6 \times d1 \times d2 \times d3)$ where $d1$, $d2$ and $d3$ are tumour dimensions in 3 orthogonal directions.

2.2. Photodynamic therapy

Hypericin (Molecular Probes Inc, USA), prepared in dimethyl sulfoxide and phosphate buffered solution, was injected intravenously into the tail veins of the animals at a dose of 2 or 5 mg/kg body weight. The animals were kept under conditions of subdued lighting until PDT. A broadband halogen light source (Zeiss KL1500) was fitted with a customised 560–640 nm band-pass filter. After a DLI of 1 h or 6 h for 1 h-PDT and 6 h-PDT, respectively, the animals were anaesthetised for survival procedures. Thereafter the tumours were exposed to light for a dose of 30 J/cm^2 delivered at a fluence rate of 25 mW/cm^2 as measured using a power meter (Coherent, USA). After PDT, the animals were kept under conditions of subdued lighting.

2.3. Evans blue method

The depths of PDT-induced necrosis was assessed at 48 h post-PDT using the Evans blue method [13] after 1 h-PDT ($n = 5$) and 6 h-PDT ($n = 6$). Forty two hours after PDT, 400 μl of 1% Evans blue dye (Merck, Germany) was injected intraperitoneally into the mice. Six hours later, the animals were sacrificed by carbon dioxide and the tumours excised. Vertical sections were cut and examined under a Stemi 2000C stereomicroscope (Carl-Zeiss, Germany) to determine the depths of necrosis. Areas stained with the Evans blue dye showed tissue with blood supply still intact while unstained areas were taken to be necrotic.

2.4. TUNEL staining

Apoptosis was assessed at 24 h post-PDT using the DNA fragmentation detection kit, TdT-FragEL™ (Oncogene Research Products, USA). Using a drug dose of 5 mg/kg, PDT-induced apoptosis was assessed in tumours that have been subjected to 1 h-PDT ($n = 4$) and 6 h-PDT ($n = 5$). Freshly cut cryo-sections were processed using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) technique according to the manufacturer's instructions. The TUNEL-stained sections were then examined under light microscopy to determine the apoptotic indices. The apoptotic index is defined as the number of apoptotic bodies per 100 nuclei counted in the area where most apoptosis was observed. Adjacent sections were used for haematoxylin and eosin (H&E) staining.

2.5. Nuclear microscopy

Using the same cryofixed samples as for TUNEL staining, 10 μm sections of unstained tumour tissue were picked up on freshly prepared sub-micron pioloform film and freeze-dried for quantitative elemental analysis using nuclear microscopy. The nuclear microscope at the Centre for Ion Beam Applications, National University of Singapore, is a state-of-the-art instrument and is particularly suited for quantitative elemental analysis of biological specimens [14,15]. As previously described, a 2.1 MeV proton beam with a lateral resolution of 2 μm was used to carry out off-axis scanning transmission ion microscopy (for sample imaging), Rutherford backscattering spectrometry (RBS) and particle induced X-ray emission (PIXE) [16]. PIXE, in conjunction with RBS, offers simultaneous multi-elemental analysis at the parts per million (ppm) level. The total elemental concentrations of the major elements, phosphorus (P), sulphur (S), chlorine (Cl), potassium (K) and calcium (Ca), and trace elements, copper (Cu) and zinc (Zn) in tumours that have been subjected to 1 h-PDT ($n = 4$) and 6 h-PDT ($n = 4$) are presented. The results were compared to values obtained from non-PDT tumours ($n = 5$) from mice that were not subjected to PDT.

2.6. Serum VEGF levels

Blood serum samples were collected at 24 h post-PDT to assess the serum VEGF levels using the mouse VEGF Duo Set ELISA Development System kit (R&D Systems) according to the manufacturer's instructions. Preliminary results for mice subjected to 1 h-PDT ($n = 4$) and 6 h-PDT ($n = 4$) are presented in relative units as mean optical density readings.

2.7. Statistical analysis

All test parameters are presented as the mean ± SE (standard error of the mean). The error bars presented in bar charts are the standard errors of the means. Statistical analysis was carried out using unpaired, 2-tailed Student's *t*-test to determine the significance of the differences in the mean values of all test parameters. For nuclear microscopy, 1 h-PDT and 6 h-PDT results were individually tested against values from non-PDT tumours. A *p* value of <0.05 was considered to be significant.

3. Results

3.1. Assessment of necrosis using Evans blue method

The Evan's blue method was used to assess the depths of PDT-induced necrosis in tumours at 48 h post-PDT. Fig. 1(a) shows an image of a vertical section through a tumour subjected to 1 h-PDT. The scale bar represents 1 mm in the tumour section. The white necrotic area, unstained by the Evans blue dye, indicate a depth of necrosis of about 4.5 mm from the skin, while the stained surrounding tissue, which appears dark in this greyscale image, still has intact blood supply. Fig. 1(b) shows an image of a section through a dark control tumour that was not subjected to PDT. The entire tumour volume was stained, indicating an absence of PDT-induced necrosis. The mean depths of necrosis (presented as depths in millimetres from the skin) found in tumours subjected to 1 h-PDT (hollow bar) and 6 h-PDT (solid bar) are presented in the bar chart in Fig. 2. The error bars presented in the chart are the standard errors of the mean. 1 h-PDT is seen to induce more necrosis than 6 h-PDT. An unpaired, 2-tailed Student's *t*-test was used to determine the significance of the difference in the mean depths of necrosis induced by 1 h-PDT and 6 h-PDT. 1 h-PDT induced more necrosis than 6 h-PDT at the $p = 0.07$ level.

3.2. Assessment of apoptosis using TUNEL method

TUNEL immunohistochemical staining of cryofixed tumour tissue sections was carried out to assess PDT-induced apoptosis at 24 h post-PDT. Fig. 3 shows (a) a TUNEL-stained section of a tumour subjected to 6 h-PDT, and (b) an H&E-stained section from the same tumour tissue, both viewed through a 40× objective lens under a light
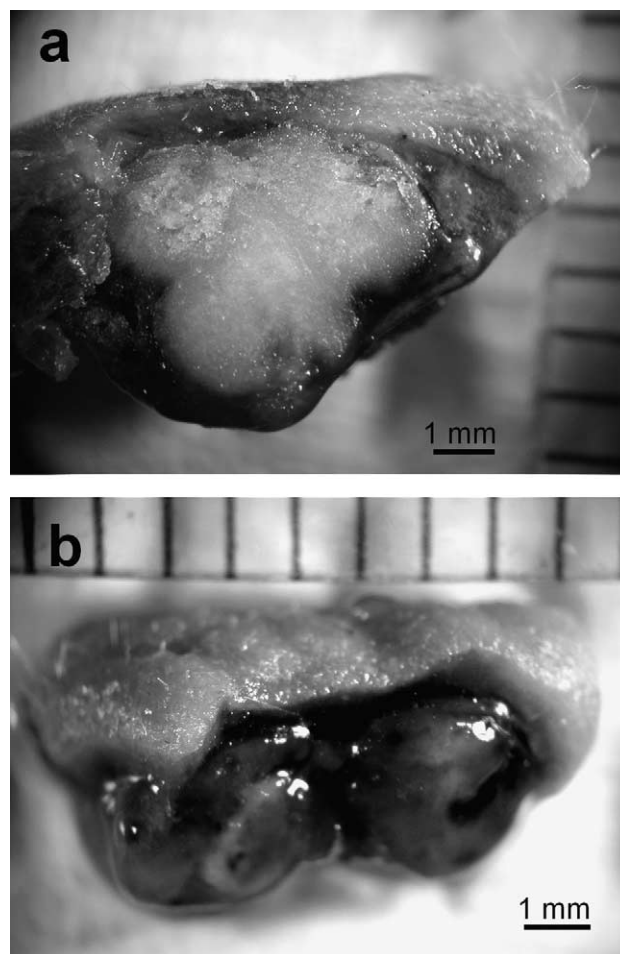

Fig. 1. (a) This image shows a vertical section through a tumour subjected to 1 h-PDT and processed using the Evan's blue method to assess PDT-induced necrosis at 48 h post-PDT. The scale bar represents 1 mm in the tumour section. The white necrotic area, unstained by the Evan's blue dye, indicate a depth of necrosis of about 4.5 mm from the skin while the stained surrounding tissue, which appears darker in this greyscale image, still has intact blood supply. (b) This image shows a section through a dark control tumour that was not subjected to PDT. The entire tumour volume is stained blue, indicating an absence of PDT-induced necrosis.

microscope. The scale bars in both images represent 10 μm in the tissue sections. As can be observed in Fig. 3(a), the apoptotic nuclei appear as dark condensed bodies in contrast to non-apoptotic nuclei. The TUNEL stained sections were examined under light microscopy to determine the apoptotic indices. The apoptotic index is defined as the number of apoptotic bodies per 100 nuclei counted in the area where most apoptosis was observed. Statistical significance of the difference in the mean apoptotic indices in 1 h- and 6 h-PDT tumours was assessed using an unpaired, 2-tailed Student's *t*-test. The mean apoptotic indices of tumours subjected to 1 h-PDT ($n = 4$) and 6 h-PDT ($n = 5$) are presented in the bar chart in Fig. 4. The results show that 6 h-PDT induced significantly more apoptosis (27.1 ± 3.3) in tumour tissue than 1 h-PDT (8.0 ± 1.6). This increased apoptosis was significant at the $p = 0.01$ level.
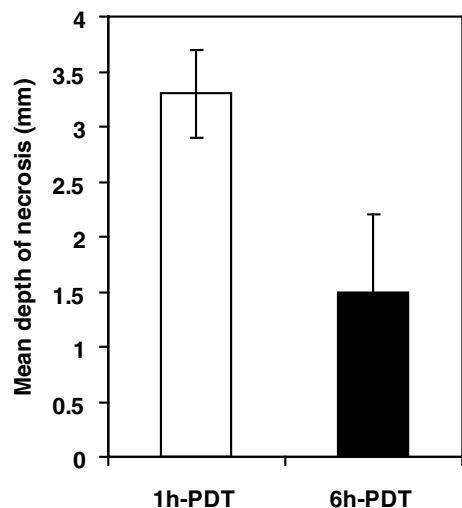
Fig. 2. This bar chart shows the mean depths of necrosis (presented as depths in millimetres from the skin) observed in tumours subjected to 1 h-PDT (hollow bar; $n = 5$) and 6 h-PDT (solid bar; $n = 6$). The error bars presented are the standard errors of the means. A Student's t-test was used to determine the significance of the difference in the mean depths of necrosis. 1 h-PDT resulted in slightly more necrosis than 6 h-PDT ($p = 0.07$).

3.3. Multi-elemental analysis using nuclear microscopy

The total elemental concentrations of both major and trace biological elements were quantitatively determined using nuclear microscopy. Fig. 5 shows the mean elemental concentrations of biological elements (in parts per million, ppm) in tumour tissue. The significance of the differences between the mean concentrations of elements in tumours subjected to 1 h- and 6 h-PDT were individually tested against concentrations in tumours that have not been subjected to PDT (non-PDT tumours) using Student's t-tests. Mean concentrations in PDT-tumours that are significantly different from non-PDT values at the $p = 0.05$ level are indicated with asterisks.

Fig. 5(a) shows the mean concentrations of the major elements phosphorus (P), sulphur (S), chlorine (Cl) and potassium (K) in tumours subjected to 1 h-PDT (hollow bars), 6 h-PDT (solid bars) and non-PDT tumours (grey bars). We observe that there is a significant decrease in the concentrations of P, S and K in both 1 h- and 6 h-PDT tumours compared to non-PDT tumours ($p < 0.05$). There were no observable changes in chlorine concentrations between both 1 h- and 6 h-PDT tumours and non-PDT tumours within errors. Fig. 5(b) shows the mean calcium (Ca) concentrations in tumours subjected to 1 h-PDT and 6 h-PDT compared to values in non-PDT tumours. We observe that there is a significant ($p < 0.05$) 2-fold increase in Ca concentrations in 6 h-PDT tumours (1050 ± 200 ppm) compared to non-PDT tumours (560 ± 100 ppm). Fig. 5(c) and (d) show the results for copper (Cu) and zinc (Zn), respectively. There is a significant increase in Cu concentrations in 6 h-PDT tumours (13.3 ± 0.9 ppm) compared to non-PDT tumours
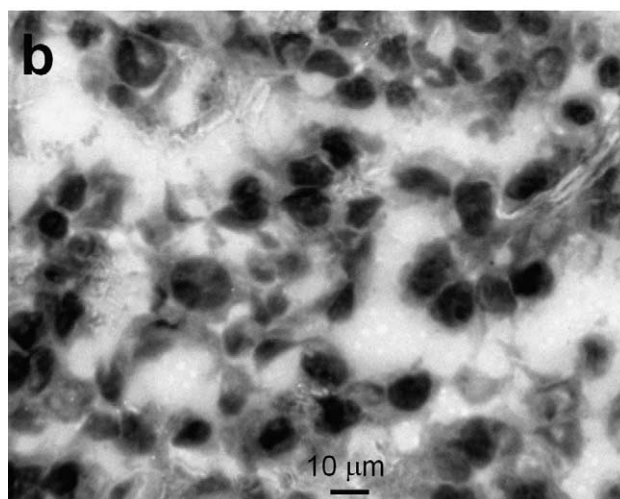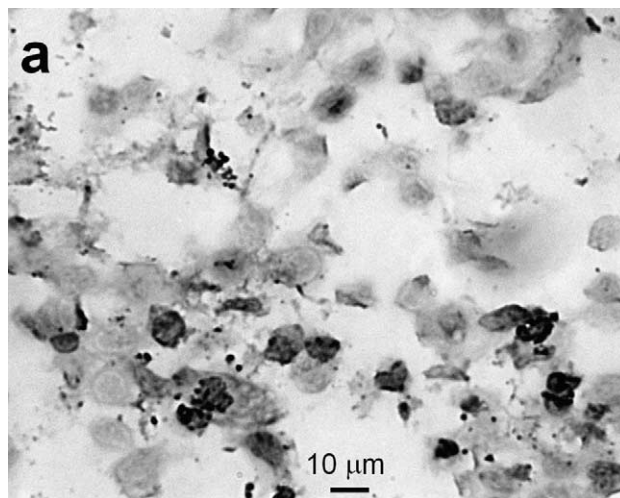

Fig. 3. (a) This image shows a TUNEL-stained section of a tumour that was subjected to 6 h-PDT and viewed through a 40× objective lens under a light microscope. The apoptotic nuclei appear as dark condensed bodies in contrast to non-apoptotic nuclei. (b) This image shows an H&E-stained section from the same tissue, also viewed through a 40× objective. The scale bars in both images represent 10 μm in the tissue sections.

(7.4 ± 1.1 ppm). Zinc concentrations are significantly decreased in 6 h-PDT tumours (72 ± 14 ppm) compared to values in non-PDT tumours (96 ± 4 ppm).

3.4. Assessment of VEGF levels

The blood serum VEGF levels were assessed at 24 h post-PDT. Preliminary results are presented in relative units as mean optical density readings. Fig. 6 shows the relative serum VEGF levels (mean optical density readings) obtained from mice that were subjected to 1 h- and 6 h-PDT. The standard errors of the means are presented as error bars. 6 h-PDT is seen to result in lower serum VEGF levels relative to those obtained in mice subjected to 1 h-PDT. An unpaired, 2-tailed Student's t-test was used to determine the significance of the difference between the 1 h- and 6 h-PDT values. The relative serum VEGF level
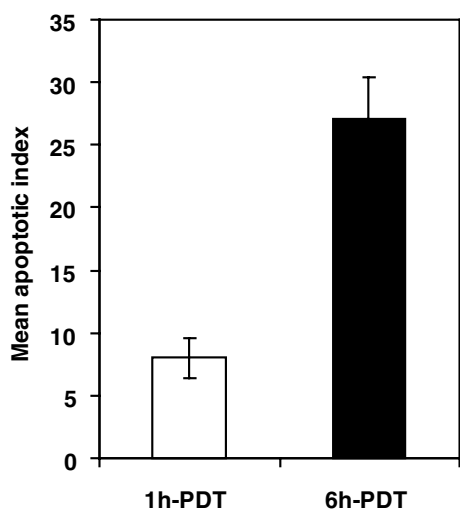
Fig. 4. This chart shows the mean apoptotic indices in TUNEL-stained tissue sections of tumours subjected to 1 h- and 6 h-PDT. The apoptotic index is defined as the number of apoptotic bodies per 100 nuclei counted in the area where most apoptosis was observed. Statistical significance of the difference in the mean apoptotic indices in 1 h- and 6 h-PDT tumours was assessed using a Student's *t*-test. The results show that 6 h-PDT induced significantly more apoptosis (27.1 ± 3.3) in tumour tissue than 1 h-PDT (8.0 ± 1.6). This increased apoptosis was significant at the $p = 0.01$ level.

in mice subjected to 6 h-PDT was significantly lower than the values in mice subjected to 1 h-PDT ($p < 0.01$).

4. Discussion

Tumour response to PDT depends on the conditions under which the treatment is carried out. One important consideration is the light fluence rate used during PDT treatment. Since PDT works via the generation of reactive oxygen species (ROS), maintaining the oxygen level in tumour tissue is important for the efficacy of PDT [9,10]. A treatment regime in which oxygen is depleted too rapidly leads to tissue hypoxia and reduced PDT efficacy [9,10]. On the other hand, using a lower fluence rate can improve PDT response. For example, in previous work with an RIF mouse tumour model, it was reported that a fluence rate of $30 \, mW/cm^2$ improved response to Photofrin-PDT compared to PDT at carried out at $150 \, mW/cm^2$ [9]. In this work, we investigated the response of xenograft human nasopharyngeal carcinoma (NPC) tumours in murine models to hypericin-PDT carried out under a low fluence rate of $25 \, mW/cm^2$. PDT is being developed as an alternative treatment modality for several cancers including NPC. Xenograft models enable us to study the response of human NPC tumours to PDT in animal models in a preclinical setting and to optimise treatment parameters prior to clinical trials. Results of this study will be relevant to future clinical applications of PDT as a treatment modality for human NPC.

Another factor affecting PDT outcome is the time interval between photosensitiser administration and exposure to light, or the drug-light interval (DLI). Based on earlier work reported by our research group [12], two DLI, 1 h and 6 h, were chosen. At 1 h after administration of hypericin, the drug concentration in the serum was the highest and more PDT induced vascular damage is expected [12]. At 6 h, the drug concentration in circulation was low but the uptake of hypericin in tumour tissue was highest, with more direct cell killing expected [12]. In other reports of work carried out on a RIF-1 mouse tumour model, hypericin concentration was highest at 0.5 h after drug administration, resulting in vascular damage, while tumour drug concentration was the highest after 6 h [7,8]. It has been proposed that vascular damage, which is more effectively achieved using a short DLI, is an important mode of hypericin-photodynamic killing in both the RIF-1 and P388 mouse tumour models [7,8,17]. It was further reported that apoptosis was the result of vascular damage and that more apoptosis was obtained through this pathway [8]. On the other hand, vascular collapse and tissue hypoxia may limit the photodynamic effect, and thus the efficacy of PDT, as discussed above.

In this work, PDT was carried out under both low fluence ($30 \, J/cm^2$) and fluence rate ($25 \, mW/cm^2$) conditions. Under these conditions, PDT using a longer DLI of 6 h was found to induce significantly more apoptosis compared to PDT using a short DLI of 1 h. This outcome is opposite to what has been reported for high dose hypericin-PDT carried out at a higher fluence rate, in which a 0.5 h DLI was found to induce more apoptosis than a 6 h DLI [8]. However, it is in agreement with recent data that low fluence rate PDT induced higher levels of apoptosis than high fluence rate PDT [10]. Thus, our results show that under low fluence and low fluence rate conditions, a 6 h DLI resulted in more apoptosis than a 1 h DLI in hypericin-PDT.

TUNEL data showing increased apoptosis in tumours subjected to 6 h-PDT compared to tumours subjected to 1 h-PDT was supported by quantitative elemental analysis using nuclear microscopy. Analysis of the intra-tumoural total elemental concentrations shows that 6 h-PDT results in a significant 2-fold increase in calcium (Ca) levels compared to non-PDT tumours. The increase in Ca levels is in agreement with the report that an increase in intracellular Ca^{2+} concentration plays a important role in photodynamically induced cell killing by hypocrellin-A, a group of photosensitiser in the same family of compounds as hypericin [18]. Ca^{2+} overload is a known trigger of apoptosis [19] and Ca^{2+} has been implicated in PDT-induced apoptosis [20,21]. Thus our results confirm the involvement of calcium in hypericin-PDT cell killing under a low fluence rate.

The observation of increased apoptosis is further supported by a significant decrease in intra-tumoural zinc (Zn) levels in 6 h-PDT tumours compared to non-PDT tumours. A decrease in Zn^{2+} level is another known trigger of apoptosis, and this has been further linked to caspase-3 activation leading to apoptosis [22–24]. Taken together,
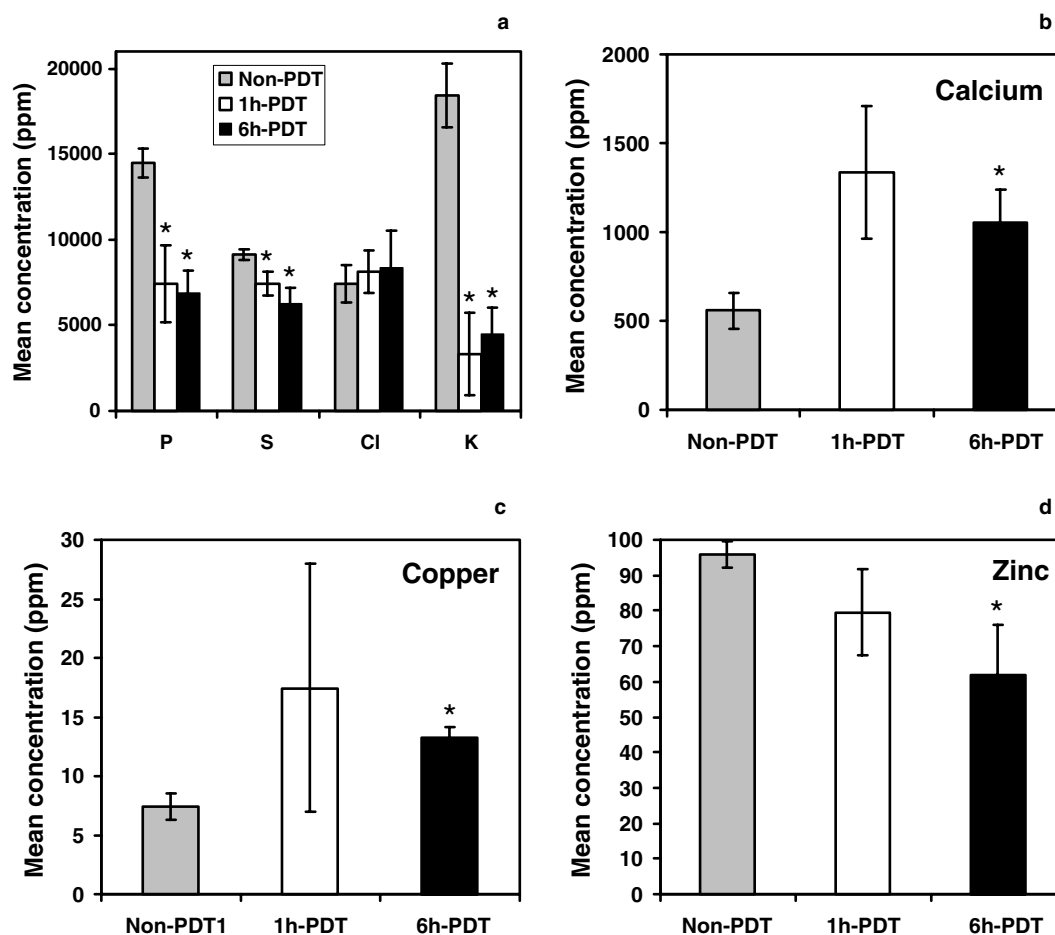
Fig. 5. (a) This chart shows the mean elemental concentrations (parts per million, ppm) of the major biological elements phosphorus (P), sulphur (S), chlorine (Cl) and potassium (K) in tumours subjected to 1 h-PDT (hollow bars), 6 h-PDT (solid bars) and non-PDT tumours (grey bars). 1 h- and 6 h-PDT values that are significantly different from non-PDT values at the $p = 0.05$ level are indicated with asterisks. There is a significant decrease in the P, S and K concentrations in both 1 h- and 6 h-PDT tumours compared to non-PDT tumours while there were no observable changes in the chlorine concentrations within errors. (b) This chart shows the mean calcium (Ca) concentrations in tumour tissue subjected to 1 h-PDT and 6 h-PDT compared to values in non-PDT tumours. There is a significant 2-fold increase in Ca concentrations in 6 h-PDT tumours compared to non-PDT tumours ($p < 0.05$). Charts (c) and (d) show the results for the trace elements copper (Cu) and zinc (Zn), respectively. There is an increase in Cu concentrations and a decrease in Zn levels in 6 h-PDT compared to non-PDT tumours, both significant at the $p < 0.05$ levels. The increase in Ca and decrease in Zn levels, both known triggers of apoptosis, lend support to TUNEL data showing increased apoptosis resulting from 6 h-PDT compared to 1 h-PDT.

our nuclear microscopy results support the observation that 6 h-PDT results in more apoptosis than 1 h-PDT and further imply the involvement of a zinc-mediated caspase-3 pathway in hypericin-PDT-induced apoptosis. The latter is in agreement with earlier reports of the involvement of caspases in hypericin-PDT induced apoptosis [2,3,5].

Nuclear microscopy results also show a significant decrease in phosphorus (P), sulphur (S) and potassium (K) concentrations in both 1 h- and 6 h-PDT tumours compared to non-PDT tumours. Since this is observed in 1 h- and 6 h-PDT tumours alike, the general loss of the major biological elements P, S and K is probably a non-specific hallmark of both apoptotic and necrotic death in photodynamic cell killing. Copper (Cu) concentrations in 6 h-PDT tumours were significantly higher than those in non-PDT tumours. The increased copper levels in 6 h-PDT tumours

may imply the involvement of this trace transition metal in hypericin-PDT cell killing. Since Cu is known to catalyse ROS production in biological systems [25], it is possible that Cu can catalyse the production of ROS necessary in photodynamic cell killing.

Angiogenesis of new tumours blood vessels following PDT treatment can lead to re-growth of tumours and compromise long-term tumour control. In a study using a mouse fibrosarcoma model, it was shown that PDT resulted in lowered serum VEGF levels and that this correlated with a prolonged survival rate compared to controls [11]. Therefore PDT could work by lowering serum VEGF levels and inhibiting the formation of new tumour vasculature and tumour re-growth. Our study shows that under conditions of low light fluence and fluence rate conditions, 6 h-PDT resulted in a lower serum level of VEGF compared to 1 h-PDT and may thus inhibit the formation of
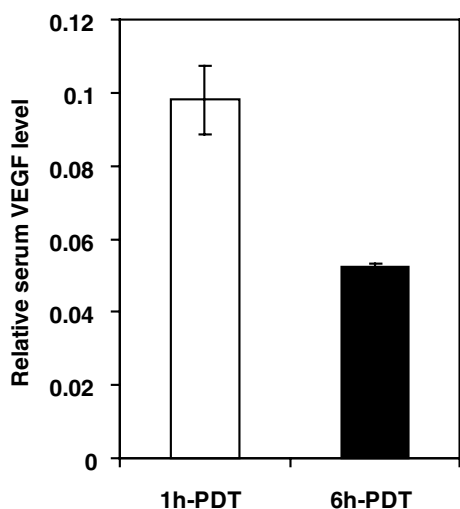
Fig. 6. This bar chart shows the mean relative serum VEGF levels (presented as mean optical density readings) from mice that were subjected to 1 h- and 6 h-PDT. 6 h-PDT is seen to result in lower serum VEGF levels relative to those obtained in mice subjected to 1 h-PDT. A Student's t-test was used to determine the significance of the difference between the 1 h- and the 6 h-PDT values. The mean relative serum VEGF level in mice subjected to 6 h-PDT was significantly lower than the values in mice subjected to 1 h-PDT ($p < 0.01$).

new tumour vasculature. Thus PDT using a long drug-light interval of 6 h and carried out under these conditions might yield improved outcome in terms of inhibiting tumour re-growth and achieving better tumour control in the long term.

PDT is often carried out using a single high dose fraction. A more recent approach is that of metronomic-PDT, in which both the light and drug are administered at low doses over a longer treatment period [26]. An alternative approach is that of multi-fraction PDT in which we can administer multiple low dose fractions up to a targeted PDT dose. While a single low dose PDT session is not expected to lead to good curative outcome, treatment can be repeated to expose the tumour to multiple fractions for complete tumour response. For this alternative approach, a long drug-light interval that results in less severe vascular damage is particularly appropriate. This will ensure a sufficient supply of oxygen to the tumour tissue, especially during PDT fractions subsequent to the first. Indeed, it has been reported that low levels of pre-existing tumour hypoxia, induced by Photofrin-PDT using a fluence rate of 30 mW/cm^2, do not limit photodynamic therapy efficacy in an RIF mouse model [27]. Thus hypericin-PDT can be repeated in a multi-fraction treatment regime using a long drug-light interval.

Overall our results show that hypericin-PDT using a 6 h drug-light interval and carried out under low fluence and fluence rate conditions has the desirable characteristics of inducing more apoptosis and lowering serum VEGF levels. This treatment regime is suitable for the alternative approach of multi-fraction PDT and might yield improved response.

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