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## Comparing the efficacy of photodynamic and sonodynamic therapy in non-melanoma and melanoma skin cancer

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### ABSTRACT

Sonodynamic therapy (SDT) involves the activation of a non-toxic sensitizer drug using low-intensity ultrasound to produce cytotoxic reactive oxygen species (ROS). Given the low tissue attenuation of ultrasound, SDT provides a significant benefit over the more established photodynamic therapy (PDT) as it enables activation of sensitizers at a greater depth within human tissue. In this manuscript, we compare the efficacy of aminolevulinic acid (ALA) mediated PDT and SDT in a squamous cell carcinoma (A431) cell line as well as the ability of these treatments to reduce the size of A431 ectopic tumours in mice. Similarly, the relative cytotoxic ability of Rose Bengal mediated PDT and SDT was investigated in a B16-melanoma cell line and also in a B16 ectopic tumour model. The results reveal no statistically significant difference in efficacy between ALA mediated PDT or SDT in the non-melanoma model while Rose Bengal mediated SDT was significantly more efficacious than PDT in the melanoma model. This difference in efficacy was, at least in part, attributed to the dark pigmentation of the melanoma cells that effectively filtered the excitation light preventing it from activating the sensitizer while the use of ultrasound circumvented this problem. These results suggest SDT may provide a better outcome than PDT when treating highly pigmented cancerous skin lesions.

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### 1. Introduction

Photodynamic therapy (PDT) is currently approved as a first line treatment for non-melanoma skin cancer and has been trialled in the treatment of other cancers such as head and neck, oesophageal, bladder and prostate.<sup>1,2</sup> PDT requires the presence of three distinct components before a cytotoxic effect can be observed; (i) a sensitizing drug, (ii) light of an appropriate wavelength and (iii) molecular oxygen. The combination of all three components result in the generation of singlet oxygen and other reactive oxygen species (ROS), that when generated in sufficient quantities, result in cell death.<sup>3</sup> The attraction of PDT over other cancer therapies is that generation of the cytotoxic species can be controlled by careful positioning of the illumination source. This targeting is further enhanced when treating skin cancer as the sensitizer prodrug aminolevulinic acid (ALA) is converted to the active sensitizer protoporphyrin IX (PpIX) more effectively by cancer cells when com-

pared to non-cancer cells. PpIX is naturally generated during the cellular heme cycle and is meticulously controlled to prevent its natural accumulation.<sup>4</sup> This negative feedback system is thought to be modified in cancer tissues due to enzymatic defects that lead to an increase in protoporphyrinogen IX oxidase and/or reduced activity of ferrochelatase.<sup>5</sup> Another advantage of PDT is the excellent cosmetic outcome observed post treatment when compared to surgery or topical chemotherapy treatment (i.e., 5-fluorouracil).<sup>6,7</sup> However, the limited penetration of light through mammalian tissue has restricted the use of PDT to the treatment of superficial lesions and reduces its effectiveness in treating more deeply-seated or highly pigmented lesions.<sup>8</sup> Indeed, while PDT is routinely used to treat superficial basal cell carcinoma (BCC), it is not indicated for the treatment of malignant melanoma.<sup>9</sup> The dark pigmentation associated with melanotic lesions acts as a filter for the light used to activate the majority of conventional sensitizers that absorb in the visible range of the electromagnetic spectrum.<sup>10</sup> To compound matters further, melanin has natural anti-oxidant properties thereby acting as a scavenger for ROS generated during PDT.<sup>11,12</sup> The development of near-infrared (NIR) absorbing sensitizers that possess absorption maxima in a region where melanin

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does not absorb have produced impressive results in treating melanoma, suggesting the anti-oxidant effect of melanin may be overcome when using PDT.<sup>13–16</sup>

Sonodynamic therapy (SDT) has recently emerged as an alternative to PDT and uses low intensity ultrasound instead of light to activate the sensitiser. This interaction of the sensitiser with an acoustic field generates ROS that result in cytotoxic effects similar to those observed in PDT.<sup>17,18</sup> The major benefit of using SDT instead of PDT is that ultrasound is clinically approved as a safe and effective imaging modality and unlike light can achieve penetration depths in soft tissue in the region of tens of centimetres.<sup>19</sup> Therefore, SDT offers the potential of treating more deeply seated solid tumours than currently possible using PDT.

In this manuscript, we compare the effectiveness of PDT and SDT at treating non-melanoma and melanoma skin cancer in pre-clinical models. Specifically, we determine the effect of ALA mediated PDT/SDT in treating A431 squamous cell carcinoma and Rose Bengal mediated PDT/SDT in treating B16-F10-Luc2 melanoma cells both *in vitro* and *in vivo*. The resulting efficacy of these treatments is then discussed.

## 2. Experimental

### 2.1. Materials and reagents

ALA and Rose Bengal were purchased from Aldrich at the highest grade possible. Metvix cream was purchased from Galderma. All other chemicals were purchased from commercial sources at the highest possible purity and used as received. Human epidermoid carcinoma A431 cell line was obtained from ATCC and the B16-F10-Luc2 cell line was purchased from PerkinElmer. SCID and athymic nude mice were obtained from Harlan Laboratories. In these studies all animals were treated humanely and in accordance with licensed procedures under the UK Animals (Scientific Procedures) Act 1986.

### 2.2. ALA mediated PDT and SDT treatment of A431 cells *in vitro*

A431 cells, maintained in DMEM medium supplemented with 10% (v/v) foetal bovine serum at 37 °C in a humidified 5% (v/v) CO<sub>2</sub> atmosphere were seeded into wells of a 96-well tissue culture plate at a concentration of  $5 \times 10^3$  cells per well and incubated overnight. The medium was then removed and replaced with 100  $\mu$ L of ALA solution in PBS at concentrations of 1 or 2  $\mu$ M. The cells were then incubated for a further 4 h at 37 °C in a humidified 5% (v/v) CO<sub>2</sub> atmosphere, the ALA solution removed, the wells washed with PBS and treated with either red light using a Phototherapeutics Paterson BL1000A lamp (630 nm  $\pm$  15 nm, total dose = 5 J/cm<sup>2</sup>) or ultrasound (Sonidel SP 100 sonoprotator, frequency = 1 MHz, a power density = 3.0 W cm<sup>-2</sup>, 50% duty cycle (pulse repetition frequency = 100 Hz, total dose = 90 J/cm<sup>2</sup>) from the underside of the plate. The wells were washed with PBS and 100  $\mu$ L aliquots of DMEM medium supplemented with 10% (v/v) foetal bovine serum were dispensed into each well. The plates were then incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37 °C for 21 h and cell viability then determined using an MTT assay.<sup>20</sup> A similar procedure was repeated using ALA at the same concentrations without stimulus. Results were compared against control experiments where cells were treated with only the vehicle (i.e., PBS).

### 2.3. DPBF assay of PpIX and Rose Bengal

An EtOH/H<sub>2</sub>O (50:50 v/v) (10 mL) solution was prepared containing PpIX or Rose Bengal (0.5  $\mu$ M) and 1,3-diphenylisobenzofuran (DPBF) (20  $\mu$ M). The solutions were then irradiated for 30 min

with white light (Fenix LD01 LED, 50 mW output) or ultrasound (Sonidel SP 100 sonoprotator, frequency = 1 MHz, a power density = 3.0 W cm<sup>-2</sup>, 50% duty cycle, pulse repetition frequency = 100 Hz). Aliquots were taken at specific time points and the absorbance at 410 nm recorded using a Cary 50 UV-Vis spectrometer. Control experiments in the absence of drug (i.e., DPBF + stimulus) were also performed for comparative purposes.

### 2.4. PDT and SDT treatment of human xenograft A431 tumours in SCID mice using topical Metvix cream

A431 cells were maintained in DMEM medium supplemented with 10% foetal bovine serum. Cells were cultured at 37 °C in a humidified 5% (v/v) CO<sub>2</sub> atmosphere. The cells ( $1 \times 10^6$ ) were re-suspended in 100  $\mu$ L of Matrigel and implanted into the rear dorsum of female SCID mice. Tumour formation occurred approximately 2 weeks after implantation and tumour measurements were taken daily using calipers. Once the tumours had reached an average volume of 205 mm<sup>3</sup>, calculated from the geometric mean diameter using the equation, tumour volume =  $4\pi R^3/3$ , animals were randomly distributed into 3 groups ( $n = 3$ ) which included (i) no stimulus group (control), (ii) a PDT treated group and (iii) a SDT treated group. Following induction of anaesthesia (intraperitoneal injection of Hypnorm/Hypnovel), a 1 mm thick layer of Metvix cream was topically applied to the tumour and 5–10 mm of the surrounding skin.<sup>21</sup> The residual cream was then removed 4 h later, and the tumours treated with (i) no stimulus (ii) red-light (total dose: 40 J/cm<sup>2</sup>) or ultrasound (at a frequency of 1 MHz using 3.5 W/cm<sup>2</sup> and a duty cycle of 30% – total dose: 220 J/cm<sup>2</sup>). After treatment, animals were allowed to recover from anaesthesia and tumour volume and body weight were recorded on a daily basis for 7 days.

### 2.5. PDT and SDT treatment of RIF-1, HeLa and B16 melanoma cells using Rose Bengal

B16-F10-Luc2 melanoma and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Foetal Bovine Serum (FBS) while RIF-1 cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640) supplemented with 10% (v/v) FBS. All cell lines were incubated at 37 °C in a humidified 5% (v/v) CO<sub>2</sub> atmosphere. Cells were seeded at a concentration of  $5 \times 10^3$  cells per well in 96-well tissue culture plate for 24 h. The medium was then removed from each well and replaced with 100  $\mu$ L of Rose Bengal at a concentration of 0.5  $\mu$ M. The cells were incubated for 3 h at 37 °C in a humidified 5% (v/v) CO<sub>2</sub> atmosphere. Individual wells were washed with PBS to remove excess Rose Bengal before treatment with either white light (parameters as in Section 2.3: 30 s, total dose: 11.4 J/cm<sup>2</sup> or 60 s, total dose 22.8 J/cm<sup>2</sup>) or ultrasound (parameters as in Section 2.2, 30 s, total dose: 45 J/cm<sup>2</sup> or 60 s, total dose: 90 J/cm<sup>2</sup>) for 30 or 60 s. Wells were washed again with PBS and 100  $\mu$ L aliquots of the respective medium supplemented with 10% (v/v) foetal bovine serum were dispensed into each well. Plates were then incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37 °C for 21 h. The cell viability was then determined using a MTT assay. A similar procedure was repeated using RB at the same concentration in the absence of light or ultrasound and for stimulus alone. Results were compared against control experiments where cells were treated with only the vehicle (i.e., PBS).

### 2.6. PDT and SDT treatment of ectopic B16 melanoma tumours in nude mice using Rose Bengal

B16-F10-Luc2 cells ( $3 \times 10^5$ ) maintained as described in Section 2.4, were re-suspended in 100  $\mu$ L of PBS and implanted into

the rear dorsum of male nude mice. Tumour formation occurred approximately 10 days after implantation and tumour measurements were taken every other day using calipers. Once the tumours had reached an average volume of 246 mm<sup>3</sup>, calculated from the geometric mean diameter using the equation: tumour volume =  $4\pi R^3/3$ , animals were randomly distributed into two treatment groups, PDT and SDT ( $n = 3$ ). Following induction of anaesthesia (intraperitoneal injection of Hypnorm/Hypnovel), a 100  $\mu$ L aliquot of PBS containing 100  $\mu$ M RB was injected directly into each tumour and animals were treated with either with white light (parameters as in Section 2.4; total dose: 68.4 J/cm<sup>-2</sup>) or ultrasound (parameters as in Section 2.4, total dose: 220 J/cm<sup>-2</sup>). After treatment animals were allowed to recover from anaesthesia and tumour volume was monitored up to 6 days after the procedure. Where applicable, treatment was also repeated on days 2 and 4 after the daily measurement was taken. The % increase in tumour volume was calculated employing the pre-treatment measurements for each group.

### 2.7. In vitro PDT/SDT treatment through a skin barrier

The procedure outlined in Section 2.4 was repeated using RIF-1 cells with the following modification: after incubation with Rose Bengal for 3 h, individual wells were washed with PBS to remove excess RB and sections of guinea pig skin ranging from 1.7, 3.4, 5.1 or 6.4 mm thick, prepared by repeatedly doubling the skin over, were fixed to the underside of the wells before being treated with either white light or ultrasound. Following determination of cell viability using a MTT assay the results were compared against the cytotoxicity observed when no skin barrier was applied between the stimulus and the plate.

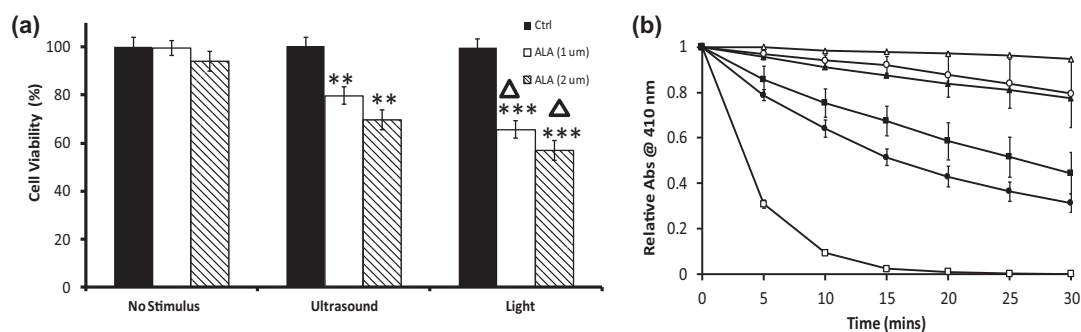
## 3. Results & discussion

The initial focus of this study was to evaluate the efficacy of ALA mediated SDT as a treatment for non-melanoma skin cancer when compared to conventional ALA mediated PDT treatment. To enable this comparison, A431 squamous cell carcinoma cells were seeded in a 96 well plate and incubated with ALA (1 or 2  $\mu$ M) for 4 h followed by treatment with either red light (630 nm  $\pm$  15 nm) or ultrasound. The cell viability was determined 24 h later using a MTT assay. The results (Fig. 1a) show that both PDT and SDT treatments produced statistically significant reductions in cell viability at each ALA concentration tested compared to the ALA only control (i.e., no stimulus). Indeed, at the higher concentration of ALA (2  $\mu$ M), reductions in cell viability of 31% ( $p < 0.01$ ) and 43% ( $p < 0.001$ ) were observed for SDT and PDT respectively. Further-

more, neither stimulus alone (i.e., light or ultrasound) caused any negative effect on cell viability. These results suggest that after conversion of ALA to the active sensitiser PpIX by the cellular heme pathway, application of light or ultrasound generates ROS resulting in the observed cytotoxic effect.

The improvement in efficacy observed for PDT compared to SDT in these experiments most likely results from PpIX being a more efficient photosensitiser than sonosensitiser. To determine if this was the case, we utilised the chromogenic ROS probe 1,3-diphenylisobenzofuran (DPBF). DPBF displays a strong absorbance band at 410 nm in its native furan form but is bleached to the corresponding diketone upon interaction with ROS. By measuring the reduction in the absorbance of the furan form at 410 nm an indication of ROS production is possible.<sup>19</sup> We treated solutions containing PpIX and DPBF with light or ultrasound and measured the amount of ROS generated in each case. Figure 1b shows a plot of % reduction in DPBF absorbance against time following irradiation with light or ultrasound and confirms PpIX to be a much more efficient photosensitiser than a sonosensitiser. Indeed, after 30 min irradiation with light, a 60% reduction in DPBF absorbance (relative to light only control) was observed while the corresponding reduction for ultrasound (relative to ultrasound only control) was only 17%. This variation in ROS production by PpIX and Rose Bengal is in keeping with their singlet oxygen quantum yields with Rose Bengal being a much better singlet oxygen generator upon light irradiation than porphyrins (Refs.<sup>22,23</sup>). While the mechanism for ROS production in PDT is well understood, the mechanism for the generation of ROS in SDT is less clear. One suggestion is that the process of ultrasound inertial cavitation, which involves the formation, oscillation and collapse of gas filled bubbles in samples irradiated with ultrasound is responsible for initiating the generation of ROS in SDT.<sup>19</sup> When this cavitation phenomenon becomes dominated by inertial forces, the bubbles collapse violently leading to sonoluminescence emission.<sup>19</sup> This luminescence emission may subsequently excite the nearby sonosensitiser by an energy transfer process resulting in the generation of ROS by the very same mechanism as in PDT. Another possible explanation is that sonosensitiser drugs in the vicinity of collapsing bubbles experience such high local temperatures that ROS are generated through pyrolysis reactions. The results shown in Figure 1b reveal a similar pattern in the amount of ROS generated for PpIX and Rose Bengal when irradiated with light and ultrasound suggesting a sonoluminescence mechanism may be involved.

Having determined the effect of PDT and SDT treatment on A431 cells in vitro, the next step was to evaluate the effect of these treatments in vivo. Ectopic A431 tumours were established on the dorsum of SCID mice and once the tumours had reached an average



**Figure 1.** (a) Plot showing % viability for A431 cells treated with ALA (1 or 2  $\mu$ M) and light or ultrasound. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  for ultrasound or light treatment of ALA compared to ALA alone at the appropriate concentration. Error bars represent SEM where  $n = 4$ . <sup>^</sup> $p < 0.05$  for light versus ultrasound treatment of ALA at the appropriate concentration. (b) Plot of relative absorbance at 410 nm for solutions of DPBF treated with (i) ultrasound only (open triangles); (ii) light only (filled triangles); (iii) PpIX and light (filled circles); (iv) PpIX and ultrasound (open circles); (v) Rose Bengal and light (open squares) and (vi) Rose Bengal + ultrasound (filled squares). Error bars represent  $\pm$  SEM where  $n = 3$ .

volume of 205 mm<sup>3</sup>, topical methyl aminolevulinatate was administered to each tumour in the form of Metvix cream. Four hours later, residual cream was removed and the animals were separated into three groups. One group was exposed to red light (630 nm ± 15 nm), one group to ultrasound and one group received no stimulus. The tumour volume was recorded each day for a period of one week. The results are shown in Figure 2a and reveal an almost identical growth profile for animals treated using PDT or SDT, with tumours being 22% and 11% greater respectively at day 7 when compared to their pre-treatment volume. In contrast, tumours in the Metvix only group grew by 90% over the same time period. Indeed, while PDT proved more effective in the 2D cell culture experiments mentioned above, no statistically significant difference was observed between PDT or SDT treatment in vivo. This suggests that while PpIX is a more potent photosensitiser than it is a sonosensitiser, the ability to activate it at a greater depth using ultrasound in a 3D tumour environment, compensates for its reduced ROS production when compared to light activation, resulting in a similar if not slightly better tumour reduction effect for SDT compared to PDT, particularly at the later time points. Analysis of the average body weight for animals in each of the groups showed no noticeable reductions over the course of the experiment suggesting either PDT or SDT treatment did not produce any acute adverse effects (Fig. 2b).

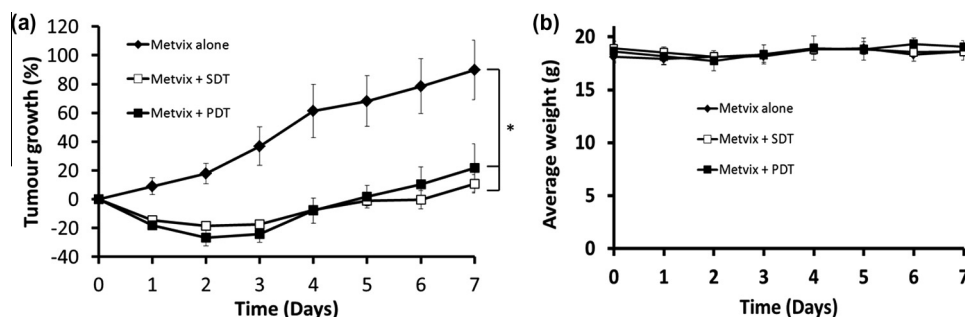
The second part of this study investigated the relative efficacy of PDT and SDT for the treatment of melanoma skin cancer. Luciferase expressing B16 melanoma cells were selected as a target as these cells form extremely aggressive highly pigmented tumours.<sup>24</sup> We were interested in determining if the pigmentation present in these cells would inhibit PDT treatment and if SDT could provide any benefit given the ability of low intensity ultrasound to penetrate deep into mammalian tissue regardless of its pigmentation. To test this hypothesis, we first evaluated the efficacy of Rose Bengal mediated PDT or SDT treatment on two amelanotic cancerous cell lines (HeLa & RIF-1) as well as the melanin producing B16 cells. Rose Bengal was chosen as a sensitiser in these experiments due to its superior singlet oxygen quantum yield when compared to PpIX.<sup>22,23</sup> In addition, Rose Bengal is also a more potent sonosensitiser than PpIX as evidenced by the DPBF assay results shown in Figure 1b. The three cell lines were incubated with Rose Bengal for 3 h at a concentration of 0.5 μM and exposed to either white light or ultrasound for treatment periods of 30 or 60 s. The cell viability was measured 24 h later using a MTT assay and the results are shown in Figure 3.

Both PDT and SDT treatment of the RIF-1 and HeLa cells produced similar reductions in cell viability (~50%) which was statistically significant relative to the untreated control ( $p < 0.001$ ). However, in the B16 melanoma cells, SDT provided a significantly improved reduction in viability compared to PDT for both the 30

and 60 s irradiation times. Specifically, 30 s SDT treatment resulted in a 25% reduction in cell viability while the same duration of PDT treatment produced a mere 7% reduction. Similarly, 60 s SDT treatment reduced viability by 40% while for PDT a reduction of only 18% was observed. This statistically significant improvement in efficacy ( $p < 0.001$ ) for SDT when compared to PDT is more clearly observed in the line graph of cell viability against irradiation time (Fig. 3d) where the poor efficacy of PDT in the B16 cells is clearly differentiated from PDT treatment of the two non-melanotic cell lines. Furthermore, SDT treatment results in comparable reductions in cell viability in all three cell lines and is also similar to the reductions observed for PDT treatment of the two amelanotic cell lines. These results support the conclusion that the melanin present in B16 melanoma cells effectively filters the excitation light destined for the sensitiser thereby inhibiting PDT efficacy while SDT treatment remains relatively unaffected.

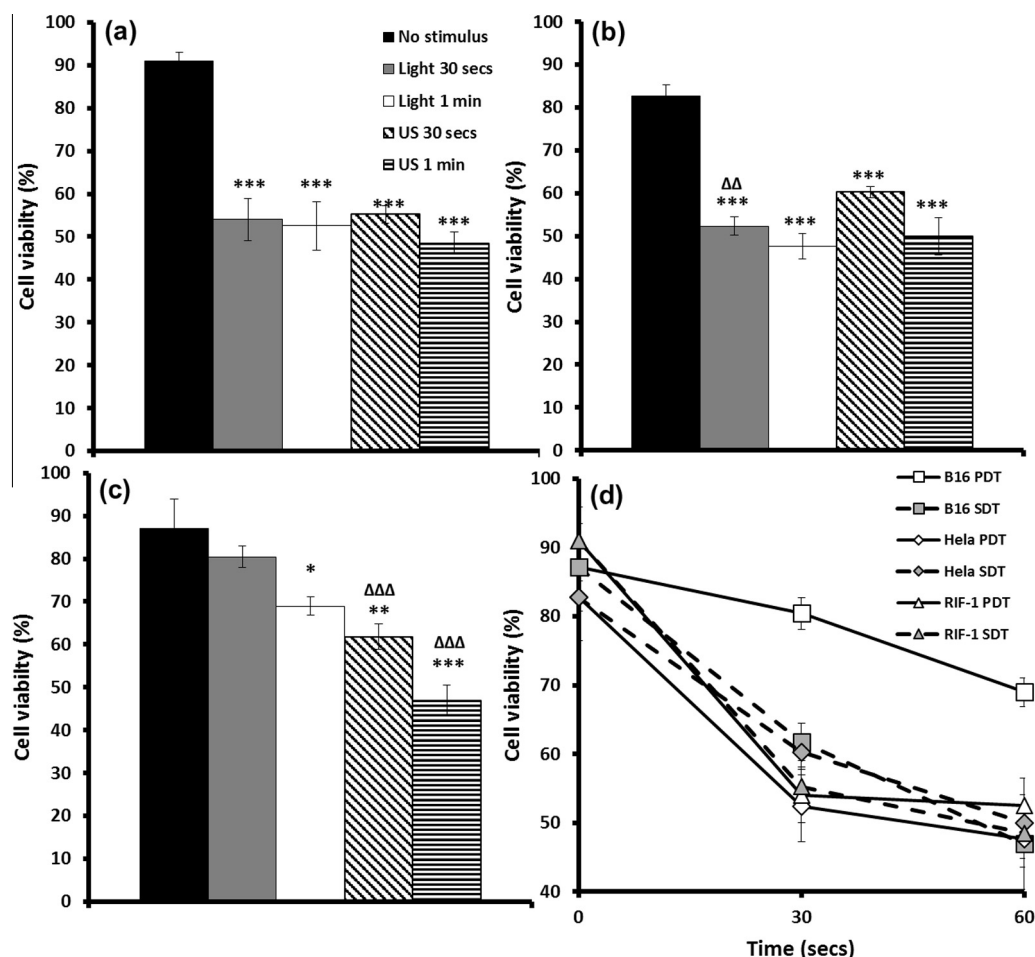
While the above results suggest that the efficacy of SDT is unaffected by tissue pigmentation, these experiments were performed in a 2D cell culture environment and provide no evidence on the ability of ultrasound to activate sensitiser at depth within human tissue. To investigate this, an in vitro model was established where a guinea-pig skin barrier was inserted between the applied stimulus (i.e., light or ultrasound) and the cells, with the thickness of the skin barrier (0–6.8 mm) being gradually increased between experiments. The resulting efficacy of PDT and SDT treatments was measured after each experiment and the results expressed as the % reduction in cytotoxicity as a function of skin thickness. As can be observed in Figure 4a, the efficacy of PDT treatment was inhibited significantly more than SDT with increasing skin thickness. For example, when the skin barrier was 3.4 mm, the cytotoxicity of PDT treatment reduced by 47% (i.e., compared to no skin barrier) while cytotoxicity of SDT treatment reduced by only 6%. Similarly, when the skin barrier was increased to 6.8 mm, PDT efficacy reduced by 82%, while the SDT efficacy reduced by only 29%. These results confirm that low intensity ultrasound penetrates mammalian tissue much more effectively than white light, and, as a consequence, is able to activate Rose Bengal more effectively leading to an improved therapeutic response.

To determine if the in vitro effects observed above would also be apparent in vivo, ectopic B16 melanoma tumours were established in athymic nude mice. These tumours are extremely aggressive and possess a high degree of pigmentation (Fig. 4b). Once the tumours reached an average volume of 246 mm<sup>3</sup> the mice were separated into two groups, with one group receiving PDT treatment and the second group SDT treatment. This pre-treatment tumour volume is much higher than typically reported for other in vivo experiments using this tumour model,<sup>15,25,26</sup> and was selected intentionally to challenge the ability of SDT to produce cytotoxic effects in such a large and highly pigmented lesion. The

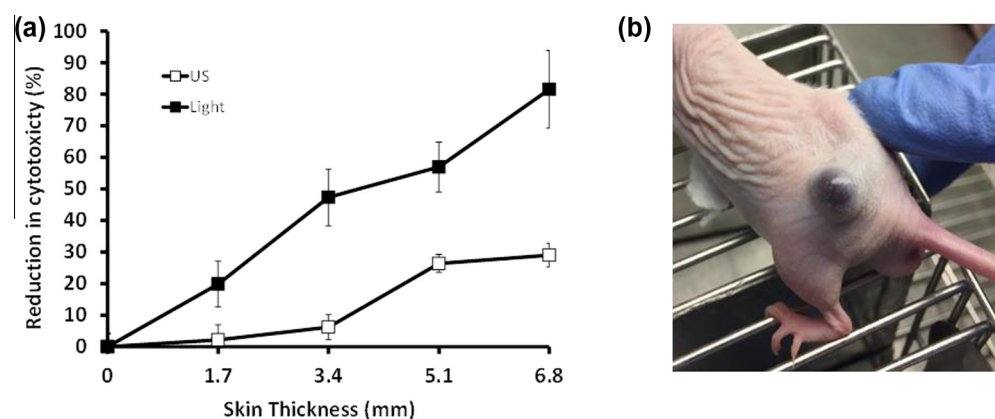


**Figure 2.** (a) Plot of % tumour growth against time for ectopic A431 tumours treated with (i) no treatment (diamonds); (ii) metvix + light (filled squares) and (iii) metvix + ultrasound (open squares). Error bars represent SEM where  $n = 3$ .  $p < 0.05$ , (ii) compared to (i) and (iii) compared to (i). (b) Plot of average body weight for mice in groups (i)–(iii) over the course of the experiment.





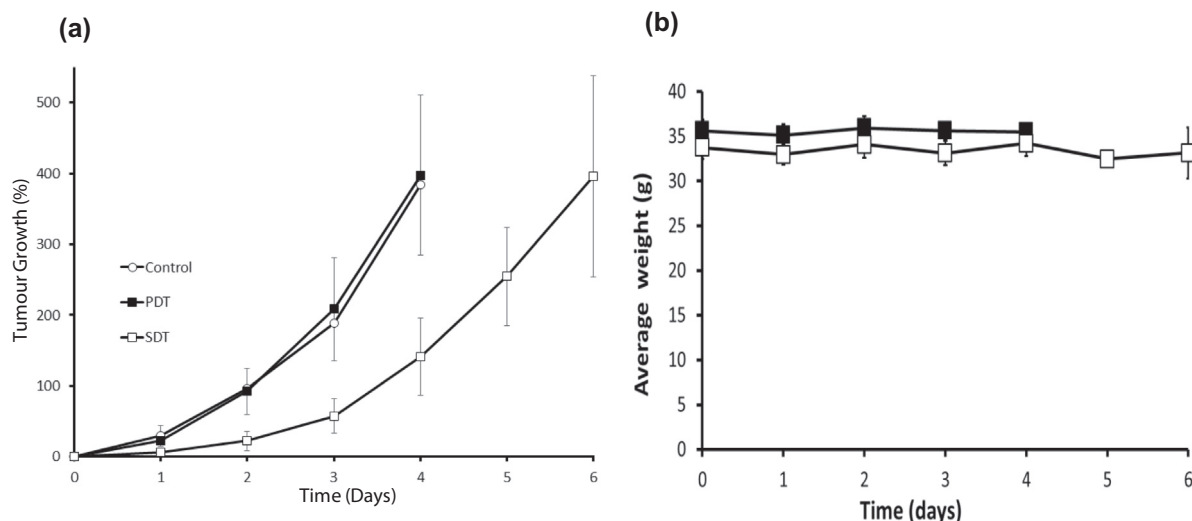
**Figure 3.** Plot of cell viability for (a) RIF-1, (b) HeLa and (c) B16 melanoma cells treated with  $0.5 \mu\text{M}$  Rose Bengal alone or in combination with light (30 or 60 s) or ultrasound (30 or 60 s). (d) Line graph combining data from (a) to (c). Error bars represent SEM where  $n = 4$ . \* $p < 0.05$ , \*\* $p < 0.05$ , \*\*\* $p < 0.001$  for PDT or SDT treatments compared to no stimulus (i.e., Rose Bengal alone).  $\Delta\Delta\Delta p < 0.001$  for SDT treatment, compared to PDT treatment, at the appropriate irradiation time.



**Figure 4.** (a) Plot showing % reduction in cell viability for RIF-1 cells treated with  $0.5 \mu\text{M}$  Rose Bengal and exposed to either light or ultrasound delivered through guinea-pig skin of various thickness. (b) Representative image of B16-F10-Luc2 ectopic tumours used in this study. Error bars represent SEM where  $n = 3$ .

tumour volume was recorded each day following treatment until the tumours reached the maximum permissible volume. The results, shown in Figure 5a, reveal a substantial reduction in tumour growth for the SDT treated mice compared to the PDT treated mice ( $p < 0.05$ ). Indeed, four days following treatment, tumours in the PDT group were approximately 400% larger than their pre-treatment size while tumours in the SDT group had grown by only

120%. Due to the size of the tumours in the PDT group no further measurements could be taken after day 4 and the animals were euthanised. However, for the SDT treated animals, tumours were within the maximum permissible limits for up to six days following treatment reaching the same 400% increase in tumour growth observed for the PDT group at day 4. Again, no noticeable reduction in average body weight was observed for either group of animals



**Figure 5.** (a) Plot showing % tumour growth plotted as a function of time for ectopic B16-F10-Luc2 tumours treated with Rose Bengal and light (filled squares) or ultrasound (open squares). Untreated control group shown as solid squares. (b) Plot of average body weight for mice in the PDT and SDT groups over the course of the experiment. Mice in the PDT group were euthanised on day 4 as their tumour volume was approaching the maximum permissible value. Error bars represent SEM where  $n = 3$ .

over the course of this experiment. These results complement those from the *in vitro* studies and highlight the benefit of SDT when compared to PDT in the treatment of highly pigmented lesions.

In conclusion, we have demonstrated that ALA mediated PDT and SDT provide similar therapeutic outcomes using the A431 pre-clinical human squamous cell carcinoma tumour model in mice. In addition, when treating a preclinical model for pigmented melanoma *in vitro* and *in vivo*, SDT was found to be superior to PDT and the data suggest that this results from the ability of ultrasound to circumvent light penetration challenges presented by pigmentation in such lesions. We believe the data presented in this study suggest that SDT could play a significant role in the treatment of lesions that are currently beyond the therapeutic capabilities of PDT.

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#### References and notes

- Dougherty, T. J.; Grindley, G. B.; Fiel, R.; Weishaupt, K. R.; Boyle, D. J. *Natl. Cancer Inst.* **1975**, *55*, 115.
- Triesscheijn, M.; Baas, P.; Schellens, J. H.; Stewart, F. A. *Oncologist* **2006**, *9*, 1034.
- Castano, P.; Mroz, P.; Hamblin, M. R. *Rev. Cancer* **2006**, *6*, 535.
- Fotinos, N.; Campo, M. A.; Popowycz, F.; Gurny, R.; Lange, N. *Photochem. Photobiol.* **2006**, *82*, 994.
- Chen, J. Y.; Mak, N. Q.; Cheung, N. H.; Leung, R. N.; Peng, Q. *Acta Pharmacol. Sin.* **2001**, *22*, 163.
- Morton, C. A. *Arch. Dermatol.* **2004**, *140*, 116.
- Lehmann, P. *Br. J. Dermatol.* **2007**, *156*, 793.
- Wainwright, M. *Color. Technol.* **2010**, *126*, 115.
- National Cancer Institute—Photodynamic Therapy for cancer <http://www.cancer.gov/about-cancer/treatment/types/surgery/photodynamic-fact-sheet#q4> (accessed 25th September 2005).
- Ma, L. W.; Nielsen, K. P.; Iani, V.; Moan, J. J. *Environ. Pathol. Toxicol. Oncol.* **2007**, *26*, 165.
- Sharma, K. V.; Bowers, N.; Davids, L. M. *Cell Biol. Int.* **2011**, *35*, 939.
- Hadjur, C.; Richard, M. J.; Parat, M. O.; Jardon, P.; Favier, A. *Photochem. Photobiol.* **1996**, *64*, 375.
- Busetti, A.; Soncin, M.; Jori, G.; Rodgers, M. A. *J. Br. J. Cancer* **1999**, *79*, 821.
- Dąbrowski, J. M.; Urbanska, K.; Arnaut, L. G.; Pereira, M. M.; Abreu, A. R.; Simões, S.; Stochel, G. *Med. Chem.* **2011**, *6*, 465.
- Mroz, P.; Huang, Y. Y.; Szokalska, A.; Zhiyentayev, T.; Janjua, S.; Nifli, A. P.; Sherwood, M. E.; Ruzi, C.; Borbas, K. E.; Fan, D.; Krayner, M.; Balasubramanian, T.; Yang, E.; Kee, H. L.; Kirmaier, C.; Diers, J. R.; Bocian, D. F.; Holten, D.; Lindsey, J. S.; Hamblin, M. R. *FASEB J.* **2010**, *24*, 3160.
- Biolo, R.; Jori, G.; Soncin, M.; Rihter, B.; Kenney, M. E.; Rodgers, M. A. *J. Photochem. Photobiol.* **1996**, *63*, 224.
- Costley, D.; McEwan, C.; Fowley, C.; McHale, A. P.; Atchison, J.; Nomikou, N.; Callan, J. F. *Int. J. Hyperthermia* **2015**, *31*, 107.
- Hu, Z.; Fan, H.; Lv, G.; Zhou, Q.; Yang, B.; Zheng, J.; Cao, W. *J. Dermatol. Sci.* **2015**, *79*, 155.
- Nomikou, N.; Fowley, C.; Byrne, N. M.; McCaughan, B.; McHale, A. P.; Callan, J. F. *Chem. Commun.* **2012**, 8332.
- McHale, A. P.; McHale, L. *Cancer Lett.* **1988**, *41*, 315.
- PDT Conditions Were Chosen to Mimic as Close as Possible Those Used in the Clinic for the Treatment of BCC* Joint Formulary Committee. British National Formulary, 69th ed.; BMJ Group and Pharmaceutical Press: London, 2015.
- Fernandez, J. M.; Bilgin, M. D.; Grossweiner, L. I. *J. Photochem. Photobiol., B* **1997**, *37*, 131.
- Gandin, E.; Lion, Y.; Van de Vorst, A. *Photochem. Photobiol.* **1983**, *37*, 271.
- Overwijk, W. W.; Restifo, N. P. *Curr. Protoc. Immunol.* **2001**(39), 20.1.1.
- Beack, Songeun; Kong, Won Ho; Jung, Ho Sang; Do, In Hwan; Han, Seulgi; Kim, Hyemin; Kim, Ki Su; Yun, Seok Hyun; Hahn, Sei Kwang *Acta Biomater.* **2015**. <http://dx.doi.org/10.1016/j.actbio.2015.08.027>. in press.
- Wang, S.; Hu, Z.; Wang, X.; Gu, C.; Gao, Z.; Cao, W.; Zheng, J. *Ultrasound Med. Biol.* **2014**, *40*, 2125.