

Assessing the photodynamic therapeutic effects of 5-aminolevulinic acid on human colon cancer cells using light-emitting diodes

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Abstract. The minimal invasiveness and high selectivity of 5-aminolevulinic acid (5-ALA)-based photodynamic therapy (PDT; 5-ALA-PDT) renders it a viable therapeutic option for the treatment of various types of cancer. Compared with conventional lasers, light-emitting diodes (LEDs) are an inexpensive and convenient low-energy light source option. Nevertheless, the scope of LEDs in the 5-ALA-PDT of colorectal cancer (CRC) has yet to be fully determined. Thus, the aim of the present study was to assess the efficacy of LEDs in the 5-ALA-PDT of colon cancer *in vitro* by evaluating cytotoxic activity. 5-ALA-treated human CRC cells (SW480) were irradiated with LEDs of varying wavelengths: Red (630 nm), green (515 nm), blue (456 nm) and violet (399 nm). An MTS assay was conducted to determine cell viability. Additionally, a concentration-response experiment was conducted with the most therapeutic wavelength (violet) to examine the 5-ALA pharmacodynamics *in vitro*. The results revealed that only violet light in 5-ALA-PDT produced antitumour activity; this combination alone produced a drug concentration- and energy-related decrease in cell viability. The decrease in viability was partially reversed by 3-methyladenine, but not by Z-VAD(OMe)-FMK, suggesting that 5-ALA induced the autophagy, but not the apoptosis of SW480 cells. The nature of the multi-well plates used markedly affected the effectiveness of PDT. Black-walled plates appeared to protect approximately

25% of cells from the effects of PDT. By contrast, clear plates permitted light access to the wells, even when protected from direct PDT treatment. On the whole, the findings of the present study indicate that the use of LEDs in 5-ALA-PDT *in vitro* induce the fluence-dependent tumour cell death of SW480 cells. The choice of multi-well plates greatly affects the results obtained *in vitro*. The antitumour effect was high with violet light. Hence, the use of LEDs in 5-ALA-PDT may prove to be an effective potential treatment for CRC.

Introduction

Currently, colorectal cancer (CRC) is considered to be the third most frequent type of cancer and the second most frequent cause of cancer-related mortality worldwide (1). While treatments and recovery rates continue to improve (2,3), there is current evidence to indicate a new wave of early-onset CRC, described as being diagnosed in patients <50 years of age (4). The primary treatment modality in CRC is surgical resection, frequently accompanied by chemotherapy as an adjuvant (5). Recurrence is a common issue, with an estimated 30-50% of patients suffering from recurrence within the first 5 years following surgery (3). Unresectable stage II or III tumours could possibly decrease in size with radiation therapy, making them eligible for surgical resection. Regrettably, however, radiation therapy has several side-effects (6). Furthermore, there is a trend in that the efficacy of current CRC therapies is decreasing due to the development of resistance, which has been found to occur in almost all patients with CRC (7). Hence, the necessity for novel CRC treatment modalities is undeniable.

A promising treatment modality used in controlling several types of cancers is photodynamic therapy (PDT) (8,9). Depending upon the location of the targeted tissue, it involves the topical or intravenous delivery of a photosensitiser: A molecule that accumulates in target cells (and/or tissue). The light of a specific wavelength can activate the photosensitiser. The absorbance of photons excites the photosensitiser to a higher energy state, causing it to react with molecular oxygen and produce free reactive oxygen species that induce cell death (10). The major advantage of PDT, in contrast to conventional radio- and chemotherapy, is its selectivity in cell

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Abbreviations: 5-ALA, 5-aminolevulinic acid; CRC, colorectal cancer; DMSO, dimethyl sulfoxide; LED, light-emitting diode; PBS, phosphate-buffered saline; PDT, photodynamic therapy; PpIX, protoporphyrin IX

Key words: 5-aminolevulinic acid, 96-well plates, cell proliferation, colorectal cancer, photodynamic therapy

destruction, which minimises the harm inflicted to adjacent healthy tissue (11,12).

Protoporphyrin IX (PpIX) is a safe, innate photosensitiser produced in the mitochondria from 5-aminolevulinic acid (5-ALA) (11-13). It has been demonstrated that following treatment with 5-ALA, PpIX accumulates in malignant cells, rendering it a good candidate for photodynamic therapy (13). Despite its long history, the use of 5-ALA-based PDT (5-ALA-PDT) *in vitro* is proving to be problematic. The high cost and energy output of lasers cause limitations to their use. While such lasers are used to treat skin, thermal damage can occur (14).

The present study addresses a few points from the literature regarding 5-ALA-PDT and identifies novel concerns and issues regarding its use. The main aim of the present study was to assess the efficacy of light-emitting diodes (LEDs) as a light source in the 5-ALA-PDT treatment of the SW480 colorectal cancer cell line.

Materials and methods

Cells and cell culture. The SW480 human colorectal cell line (a kind gift from Dr G.B. Willars, University of Leicester, UK) was grown in high-glucose DMEM (D5796, Sigma-Aldrich; Merck KGaA) enhanced with 10% foetal calf serum (F7524, Sigma-Aldrich; Merck KGaA), 100 µg/ml streptomycin and 100 U/ml penicillin (F4333, Sigma-Aldrich; Merck KGaA). Mycoplasma testing was performed prior to the use of these cells in the experiments described below.

Cells were grown and tested using two different types of 96-well multi-plates: Black-walled cell plates (3603, Costar; Corning, Inc.); or clear plates (655180, Greiner; Sigma-Aldrich; Merck KGaA). Each plate was seeded with SW480 cells (20,000 cells/well) or medium alone. Phosphate-buffered saline (PBS; pH 7.4) was seeded in between wells to limit the edge effect (15). Cells were incubated in a humidified incubator containing 5% CO₂/95% air at 37°C for 24 h.

Preparation of 5-ALA. 5-ALA (A3785, Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide (DMSO) (100 mM). Each 100 µl was diluted in 880 µl PBS and pH adjusted to 7.4 by the addition of 20 µl of 1.5 M Tris (pH 8.8), yielding a 10-mM solution of 5-ALA. Further dilutions were performed in PBS.

Photodynamic therapy light sources. Cultured cell plates were exposed to four different LED lights: Red (630 nm, 2.1 mW/cm²), green (515 nm, 1 mW/cm²) and blue (456 nm, 1 mW/cm²) using a 30 W U'King multi-LED stage lamp purchased from Shenzhenshi Yimosi, as well as violet (399 nm, 3 mW/cm²) using a MICTUNING 18x3 W LED stage lamp purchased from amazon.co.uk. Light intensity was determined using a pyroelectric photo-radiometer. The time required for exposure was calculated using 1 mW/cm² x seconds exposure = mJ/cm².

Photodynamic activation. Following 24 h of culture, the medium was supplemented with 10 µl of 10% DMSO in PBS (vehicle) or 5-ALA (1.25 mM-10 mM; final concentration, 125 µM-1 mM). Following a set time, the medium containing 5-ALA or PBS was removed and replaced with fresh PBS. The cell plates were placed on a heating block set at 37°C and

irradiated with a 0-4 J/cm² light of the set wavelength. In a previous study, a single plate was used for several time points of light irradiation. Wells were protected from irradiation by covering them with aluminium foil (16).

Determination of the mechanisms of cell death. The mechanisms through which 5-ALA induces cell death were investigated using 30 µM Z-VAD(OMe)-FMK (ab120487, Abcam, prepared as a 10-mM stock solution in DMSO and diluted in PBS) or 3 mM 3-methyladenine (ab120841, Abcam, prepared as a 30 mM working solution in PBS). Each reagent was added immediately prior to the addition of 250 µM 5-ALA. Each reagent was also present during the light irradiation step and the subsequent 48-h post-irradiation incubation period.

Assessment of cell viability by MTS assay. At 48 h post-irradiation, cell viability was assessed by MTS assay, as previously described (17). The absorbance (490 nm) of the samples was determined immediately and 2 h after the addition of the reagent (10 µl MTS reagent; 2 mg/ml MTS containing 0.04 mg/ml PMS). The absorbance at time zero was subtracted from the absorbance following 2 h of incubation in a humidified incubator at 37°C to obtain the signal reading. To determine changes in viability, pairwise comparisons were made between cells receiving 5-ALA and those receiving 10% DMSO in PBS for each light exposure time point. This was done to remove any bias from light-induced cell damage at each wavelength (18). Each experiment was repeated at least four times in triplicate.

Curve fitting. Curve fitting, where appropriate, was performed using GraphPad Prism software (v.9.0.2; GraphPad Software, Inc.). The results are presented as the mean values and standard error of the mean.

Statistical analysis. Paired data were analysed and compared using one-way ANOVA and Dunnett's post hoc test or a Bonferroni post hoc test to correct for multiple analyses using GraphPad Prism (v.9.0.2; GraphPad Software, Inc.).

Results

Efficacy of LEDs in the 5-ALA-PDT of CRC *in vitro*. Initially, the MTS assay was used to assess the efficacy of 5-ALA-mediated PDT on SW480 cells grown in black-walled, clear bottomed 96-well plates. The cells were treated with 5-ALA (1 mM) for 4 h before the medium was removed and replaced with PBS. The cells were then exposed to light of different wavelengths (red, 630 nm; green, 515 nm; blue, 456 nm; and violet, 399 nm) for increasing amounts of time (and hence, increasing fluence at these set wavelengths). In addition, some wells were treated with 5-ALA and not exposed to light.

The combination of 5-ALA (1 mM) with red (3.78 J/cm²), green (3.60 J/cm²), or blue (3.60 J/cm²) light did not significantly affect the viability of the SW480 cells when compared with the 'no light' control (P>0.35, one-way ANOVA, multiple paired comparisons, Dunnett's post hoc analysis) (Fig. 1). By contrast, the 5-ALA (1 mM)-treated cells exposed to violet light exhibited a rapid loss of viability. A fluence of 0.18 J/cm² was sufficient to significantly decrease cell viability (P=0.014). Higher fluences caused a greater reduction in cell viability (P<0.005 in all cases).

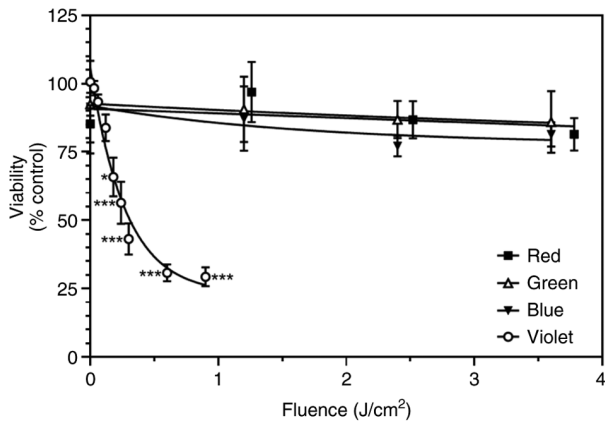


Figure 1. SW480 cell viability was measured by MTS assay with the increasing light fluence (J/cm²) of different wavelengths: Red (630 nm), green (515 nm), blue (456 nm) and violet (399 nm) following treatment with 1 mM 5-ALA for 4 h. Data are presented as the mean ± SEM of four (red, green, blue) or nine (violet) independent experiments. 5-ALA, 5-aminolevulinic acid. *P=0.014 and ***P<0.005 when compared with the zero-hour control.

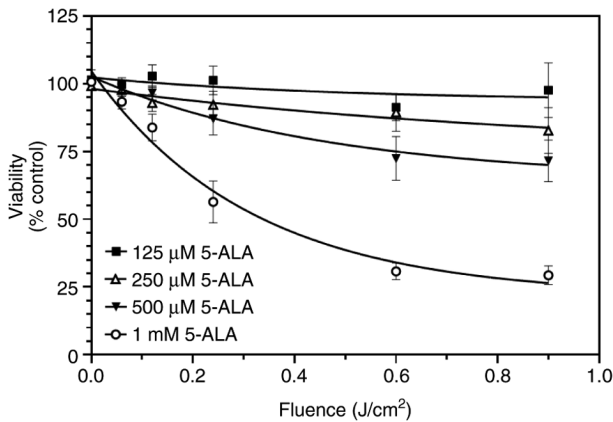


Figure 2. SW480 cells were incubated with increasing concentrations of 5-ALA (125 µM-1 mM) and then exposed to violet light (0.06-0.9 J/cm²). Data are presented as the mean ± SEM from six (125-500 µM) and nine (1 mM) independent experiments. 5-ALA, 5-aminolevulinic acid.

The maximum decrease in cell viability appeared to plateau at approximately 75% (Fig. 1). The fluence required to reduce cell viability to 50% was 0.31 J/cm² in the presence of 1 mM 5-ALA.

5-ALA concentration-response curve. The concentration-dependent effects of 5-ALA on SW480 cell viability were then assessed. This was determined using 5-ALA (125 µM-1 mM) and fluences of violet light (0.06-0.9 J/cm²). The loss of viability was concentration-dependent: Treatment with 1 mM 5-ALA led to a greater decrease in viability at all time points, whereas 125 µM appeared to have a minimal effect, if any (Fig. 2). Fitting these curves to a single exponential decay revealed plateau values of 94, 64, 44 and 22% of time 0 control for 125, 250, 500 µM and 1 mM 5-ALA, respectively (Fig. 2). The fluence required to reduce cell viability to 50% was 0.34 J/cm² in the presence of 1 mM 5-ALA.

5-ALA treatment time-course. The time required for 5-ALA to exert its optimal effects on SW480 cell viability was then assessed. This was determined using 5-ALA (125 µM-1 mM)

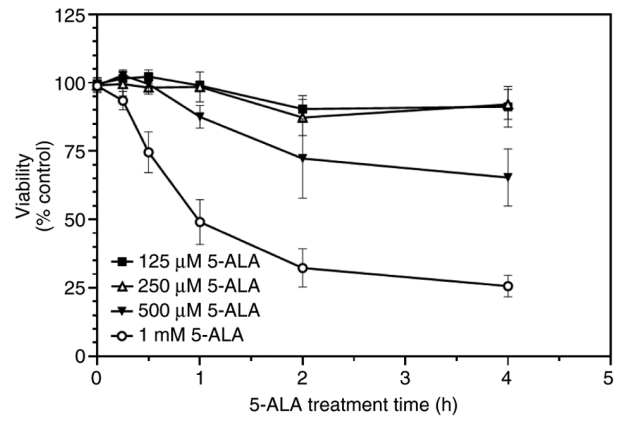


Figure 3. SW480 cells were incubated with increasing concentrations of 5-ALA (125 µM-1 mM) for 15 min to 4 h and then exposed to violet light (0.9 J/cm²). Data are presented as the mean ± SEM from six (125-500 µM) and 10 (1 mM) independent experiments. 5-ALA, 5-aminolevulinic acid.

and a single fluence of violet light (0.9 J/cm²); the 5-ALA treatment times ranged from 15 min to 4 h. The loss of viability was time-dependent: Treatment with 1 mM 5-ALA led to a greater decrease at all time points, whereas 125 and 250 µM appeared to have a minimal effect, if any (Fig. 3). There appeared to be a delay in 5-ALA exerting an effect (Fig. 3).

It was noted that the loss of viability reached a maximum of ~75% in each analysis. Thus, it was considered whether the black walls of the 96-well plates were reducing the effective fluence to a proportion of the cell population. This may occur if the well sides were casting a shadow on some cells close to the edges of each well. These experiments were repeated using clear plates.

Initially, individual wells not intended to be irradiated were covered with aluminium foil to achieve time-course data. After a fixed time, the foil was moved to expose further wells to obtain the time-course. Three observations were made: Firstly, the viability of cells was reduced by 100% (in the presence of 500 µM or 1 mM 5-ALA), indicating that all cells were exposed to violet light; secondly, the decline in viability required much less time than with black-walled plates; thirdly, wells covered with foil throughout exposure (0 time) exhibited a marked, concentration-dependent decrease in viability compared with their paired untreated controls (Fig. 4). This identified that light was reflecting/refracting and exposing all cells to light from above.

Therefore, these experiments were repeated using individual clear plates for each time point. Using separate plates, it was found that 5-ALA alone, or light alone, did not affect the growth of SW480 cells. In addition, two further observations were made: Under these conditions, viability was maximally decreased by 100%; the fluence required to reduce cell viability to 50% was 0.08 J/cm² in the presence of 1 mM 5-ALA (Fig. 5). These observations were in marked contrast to those obtained using black-walled plates (Fig. 2). Firstly, the combination of light plus 5-ALA was able to reduce viability by 100%. Secondly, this reduction was more rapid than that observed in experiments using black-walled plates (Fig. 2). In the presence of 1 mM 5-ALA, the fluence required to reduce cell viability to 50% was 0.08 J/cm², in contrast to 0.34 J/cm² when using the black-walled plates.

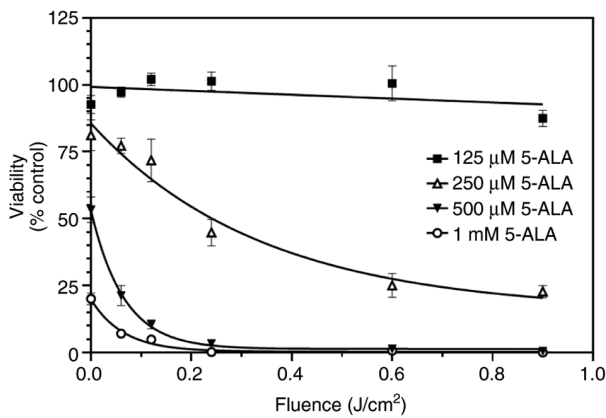


Figure 4. SW480 cells were grown in clear plates and incubated with increasing concentrations of 5-ALA (125 μ M-1 mM) for 4 h and then exposed to violet light (0.06-0.9 J/cm²). Foil was used to prevent light exposure to the wells until needed. Data are presented as the mean \pm SEM from four independent experiments. 5-ALA, 5-aminolevulinic acid.

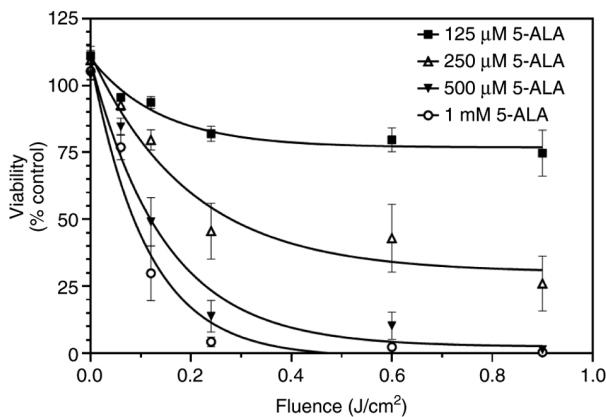


Figure 5. SW480 cells were grown in clear plates and incubated with increasing concentrations of 5-ALA (125 μ M-1 mM) for 4 h and then exposed to violet light (0.06-0.9 J/cm²). Individual plates were used for each time point, and wells were not covered with foil at any time. Data are presented as the mean \pm SEM from eight independent experiments. 5-ALA, 5-aminolevulinic acid.

Mechanism of action of 5-ALA. Finally, the present study sought to determine the death mechanisms of the SW480 cells. The addition of Z-VAD(OMe)-FMK (30 μ M) did not attenuate the effects of 5-ALA ($P > 0.999$, one-way ANOVA, multiple comparisons, Bonferroni post hoc analysis), whereas 3-methyladenine (3 mM) partially reversed these effects ($P = 0.0074$) (Fig. 6). Neither reagent significantly affected the control values ($P > 0.999$). This suggested that 5-ALA induced cell death via autophagy and not apoptosis.

Discussion

Generally, lasers are the prevailing light sources utilised in 5-ALA-PDT. However, over the years, several studies have reported the efficacy of LEDs in 5-ALA-PDT (8,9). The present study demonstrated the efficacy of LEDs as a potential source of light in the 5-ALA-PDT of human CRC cells *in vitro*. Compared to lasers, LEDs are easy to use, cost-effective and convenient. The low wattage also results in a low-temperature

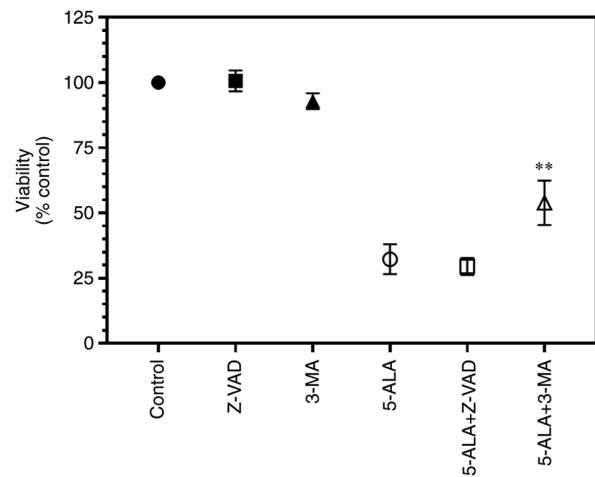


Figure 6. SW480 cells were grown in clear plates and incubated with 5-ALA (250 μ M) for 4 h in the presence of Z-VAD (30 μ M) or 3-MA (3 mM) and then exposed to violet light (0.9 J/cm²). Data are presented as the mean \pm SEM from eight independent experiments. ** $P = 0.0074$ when compared with the cells treated with 5-ALA alone. 5-ALA, 5-aminolevulinic acid; Z-VAD, Z-VAD(OMe)-FMK; 3-MA, 3-methyladenine.

device. The present study additionally preferred the use of LEDs as a light source in as they are safer than lasers, and do not require a sophisticated experiment set up (8,9), which opens up the clinical possibility of using 5-ALA-PDT in third world countries that lack the resources for laser set ups. Additionally, LEDs possess the ability to irradiate a large area, which is another advantage over lasers. LEDs can be arranged in into a planner array to increase the irradiated area even further. These arrays can be costly (>\$1,000) (19), or low in price (<\$25, the present study). Such advantages may render LEDs as a popular light modality in the 5-ALA-PDT of patients with CRC.

The present study found that violet light (399 nm) was highly effective. This is in agreement with the study by Gederaas *et al* (20), who found that while SW480 cells accumulated less PpIX than other cell lines, treatment with 1 J/cm² caused ~50% cell death. Violet light has also been shown to be the most effective at creating reactive oxygen species, reducing cell viability and driving necrosis in a range of gastric cancer cell lines, albeit not with the selectivity demonstrated herein (21). The results of the present study, as shown in Fig. 5, demonstrated that in the presence of 1 mM 5-ALA, only 0.08 J/cm² was required to lead to a 50% reduction in viability. Differences in light sources could explain the differences in results; the LED in the present study emitted a narrow band of light, whereas the lamp used in the study by Gederaas *et al* (20) spanned 340-440 nm. These fluences were much lower than those published in the literature. Previous research using gastric cell lines only yielded results with 1 mM 5-ALA and 3 J/cm² light fluence, although almost 100% of the cells had died under these conditions (21). However, the wavelength used herein was also different from that in the majority of studies (8,14,22-25).

In the present study, red, green and blue LEDs failed to produce significant cell death with fluences of ~3.5 J/cm². Previously, other researchers used red light, albeit at fluences higher than used herein: Human gingival Ca9-22 cells treated with 1 mM 5-ALA required 4.3 J/cm² red light (635 nm) to achieve 50% cell death (22); SW480 cells treated with 5-ALA

(500 μ M-1.5 mM) required 60 J/cm² red light to cause a reduction in viability (23); KLN205 lung cancer cells treated with 1.2 mM 5-ALA and 10 J/cm² red light (630 nm) reduced cell viability to 42%; concentrations as high as 5 mM did not cause further death (24). Blue light (405 nm) has been reported to cause an ~20-fold greater photobleaching of PpIX than red light (635 nm) following 5-ALA treatment; this has been associated with the ability of PDT to induce cell death (25). In the absorption spectrum of PpIX, compared with the 630-635 nm absorbance peak, the Soret peak (400-500 nm) is relatively 20-30-fold greater (8). While red light is used clinically with 5-ALA, the energy required to activate the PpIX does cause local heat damage (14).

The observations of the present study that violet light was a far superior light source for activating PpIX may offset the greater penetration of red light (650 nm, 4-5 mm) over violet light (395 nm, <1 mm) (26). In addition, the lower required fluence of violet light reduces pain and erythema when performing 5-ALA-PDT on skin (14). As previously demonstrated, HT-29 human CRC cells, both *in vitro* and subcutaneously inoculated *in vivo*, were more efficiently killed using blue light (456 nm) than using white (broad-band) or red (635 nm) light (8). By contrast, data modelling light travelling through blood and tissue has demonstrated that violet light (405 nm) is quenched more readily than red light (633 nm), and is less suitable for most diagnostic uses, despite the fluorescence signal of PpIX requiring a 50-100-fold higher fluence of red light than violet light (27). Despite this lack of penetration by violet-blue light, it has been successfully used in identifying tumour location during surgery (28).

As the present study demonstrated violet light as being the most therapeutic, the 5-ALA concentration-response association was examined under violet irradiation. The consequent analysis successfully demonstrated a 5-ALA concentration-dependent decrease in the viability of SW480 cells. Previous studies have demonstrated that the effects of 5-ALA can be reversed with Z-VAD(OMe)-FMK, indicating apoptosis in some conditions (29). Others have demonstrated that 5-ALA causes cell death through autophagy (30,31), whereas some researchers claim that neither pathway is involved (32). The present study observed that the death of SW480 cells was partially reversed by 3-methyladenine, whereas Z-VAD(OMe)-FMK did not exert an effect. This suggests that autophagy, rather than apoptosis was the main route of cell death in the system used herein.

More importantly, the present study demonstrated that the type of multi-well plate used in these studies markedly affects the results obtained. It is common practice that black-walled multi-plates are used (19,33). The present study demonstrated that this may restrict light exposure to all the cells in all the wells. The results identified that the maximal reduction was ~75%. It was suggested that some of the cells around the periphery of each well may be protected from light as the wall side may cast a shadow. By contrast, clear plates also introduce challenges: Merely covering wells with aluminium foil that are not planned for light exposure (16) is insufficient, as the cells in these wells do receive sufficient light exposure to cause PDT-mediated cell death. We advise the use of clear plates, with individual plates being used for each time point. While costly, this will lead to an improved quality of data.

In conclusion, the present study demonstrates that the use of LEDs in 5-ALA-PDT induces the death of SW480 CRC cells

in vitro, with violet LEDs producing the optimal antitumour effect. The use of black-walled multi-plates may lead to disappointing results and may underestimate the effects of PDT in *in vitro* assays.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

STS and FZH designed and supervised the study. ETS, HAOM, UJA, FA and STS performed the experiments and collected and analysed the data. ETS drafted the manuscript. ETS and STS confirm the authenticity of the raw data. All authors amended the manuscript and have read and approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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