Evaluation of Ala-Mediated Sonodynamic, Photodynamic, and Sonophotodynamic Therapies in Early-Stage Murine Melanoma

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***Abstract*—Cutaneous melanoma is an aggressive form of skin cancer. This study evaluates 5-aminolevulinic acid (ALA)-mediated photodynamic (PDT), sonodynamic (SDT), and sonophotodynamic (SPDT) therapies in a murine model of early-stage melanoma, using a conical waveguide for ultrasound delivery. Tumor growth was monitored by high-resolution ultrasound imaging, and tumors were later excised for histological analysis (H&E and Ki67). SDT achieved the greatest tumor growth inhibition (87±11%), while SPDT (79±18%) showed no additional benefit over SDT alone. These results underscore the potential of ALA-mediated SDT for melanoma treatment and demonstrate the effectiveness of the waveguide in focusing ultrasound energy on small regions.**

***Index Terms*—sonodynamic, photodynamic, sonophotodynamic, 5-aminolevulinic acid, melanoma**

I. Introduction

Cutaneous melanoma is an aggressive form of skin cancer characterized by high invasiveness and metastatic potential. According to the American Joint Committee on Cancer (AJCC), melanoma is classified into stages 0–IV, with early-stage melanoma corresponding to Stage 0 (thickness $<$1 mm), Stage I ($\leq$2 mm), and Stage II ($>$2 mm). [1] The standard treatment is surgical excision; however, surgery may be suboptimal or excessively invasive in certain cases. [2], [3] This has motivated the search for alternative, less invasive strategies, including photodynamic (PDT), sonodynamic (SDT), and sonophotodynamic therapy (SPDT).

PDT relies on the activation of a photosensitizer (PS) by low-intensity light at a specific wavelength in the presence of molecular oxygen, generating reactive oxygen species (ROS) that trigger targeted cell death. [4] While PDT is effective for superficial non-melanoma skin cancers, its performance in melanoma is limited by melanin absorption, which restricts light penetration in tissue. [5]

SDT involves the activation of sonosensitizers (SS) by low-intensity ultrasound in an oxygenated environment, inducing both sonomechanical (e.g., permeabilization of biological structures) and sonochemical (e.g., ROS production) effects. These processes are largely driven by acoustic cavitation in liquid media exposed to acoustic fields. [6], [7] Unlike light, ultrasound penetrates deeply into tissues and is unaffected by melanin, making SDT promising for treating pigmented and deep-seated tumors. [8], [9] Preclinical studies have used diverse setups for ultrasound delivery, including water tanks, tissue layers, tubes or bags filled with degassed water, and waveguides such as aluminum cones. [10], [11] The absence of standardization highlights the need for a clinically feasible, efficient, and simplified ultrasound delivery system.

SPDT combines PDT and SDT, exploiting the synergistic effects of light and ultrasound for enhanced tumor control in deeper tissues. Preclinical studies have reported its efficacy in multiple cancer models [12] and its potential as an antimicrobial approach [13]. Clinical investigations have explored its use against internal and metastatic tumors. [14], [15] However, to date, no *in vivo* studies have evaluated SPDT in melanoma.

5-aminolevulinic acid (ALA), a precursor of protoporphyrin IX (PpIX), preferentially accumulates in tumor cells through enzymatic conversion. ALA is clinically approved for dynamic therapies (Europe, 2001; United States, 1999) and has been successfully applied in *in vivo* melanoma models using PDT and SDT. [11], [16], [17]

The present study investigates the cytotoxic effects of ALA-mediated PDT, SDT, and SPDT in a murine model of early-stage cutaneous melanoma.

II. Materials and methods

A. Equipments

The LINCE device probe (MMOptics Ltda., São Paulo, Brazil), emitting at 630 ± 10 nm, was used for light irradiation. The probe tip had a diameter of 3.3 cm, and a black fabric mask was applied around the tumor to shield adjacent normal tissues. [18] Ultrasound irradiation was performed using the Sonidel SP100 system (Sonidel Limited, Dublin, Ireland) equipped with a large-area probe (Effective radiating area (ERA): 5 cm^2) coupled to a custom conical aluminum waveguide. In a previous study, acoustic measurements demonstrated that the waveguide reduced the ERA from 5 cm^2 to 0.8 cm^2, enabling targeted treatment of early-stage tumors. [19]

B. Tumor model and ALA administration

B16-F10 melanoma cells (1×10^6 per animal) were intradermally implanted into the right flank of Nu/J athymic mice (20–25 g, 6–8 weeks old). ALA (EMI Pharma, São Carlos, SP, Brazil) was prepared at 100 mg/mL in sterile water, adjusted to pH 5.0–6.5 with 1 N sodium hydroxide, and administered intraperitoneally at 200 mg/kg. Treatments were initiated three hours post-injection. [20] All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Texas A&M University (protocol 2023-0137).

C. Experimental groups

When tumors reached 1–1.5 mm in thickness, mice were randomly assigned to five groups (n=4 per group): control, ultrasound, PDT, SDT, and SPDT. Control mice received no treatment. The ultrasound group received only ultrasound irradiation. In the SDT group, mice received ALA followed by ultrasound (2 W/cm², 100 Hz, 50%, 30 min). In the PDT group, mice received ALA followed by light irradiation (630 nm, 100 mW/cm², 20 min, 120 J/cm²). In the SPDT group, mice received ALA, followed by ultrasound irradiation for 30 min and subsequent light irradiation for 20 min A thin layer of coupling gel was applied between the waveguide and the tumor. Treatments were performed in two sessions separated by 24 h, and tumor size was measured daily.

Tumor measurements and histological analysis

Tumor thickness and volume were assessed by high-resolution ultrasound imaging (Vevo 3100, FUJIFILM VisualSonics, USA) using the MX550D transducer (40 MHz). 3D tumor images were acquired and analyzed using Vevo LAB software to determine tumor volume. Three days after the second treatment session (endpoint), mice were euthanized and tumors were collected for histological analysis. At the endpoint, the tumor growth inhibition ratio was calculated (TGI% = (1-(mean normalized volume of the treated group/mean normalized volume of the control group)x100%)).

Tissue sections (5 μm) were stained with H&E and immunostained for Ki67 (1:500, GA626; Dako–Agilent) as a proliferation marker. WARP-Red chromogen (alkaline phosphatase system) was used to distinguish positive staining from melanin. Two sections per tumor were digitized (Aperio ImageScope®, Leica, Canada) and analyzed with HALO® software (Indica Labs) to quantify cell count and Ki67 expression. Immunostaining was considered negative when $<10\%$ of tumor cells were positive.

Data analysis

Experiments were performed on four independent occasions. The data were presented as a mean $\pm$ standard deviation. Statistical analysis was performed using Student's t test (*p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.001, n.s. = not significant).

III. Results and Discussion

Ultrasonography revealed a marked reduction in tumor volume at the endpoint in the PDT, SDT, and SPDT groups compared to controls (Figure 1). Additionally, damage to the stratum corneum and epidermis overlying the tumor, skin swelling around the tumor, and structural alterations in the tumor were observed, particularly in the SDT and SPDT groups. The TGI% was -22$\pm$65% (n.s. *vs.* control) in the ultrasound group, 87$\pm$11% (***p *vs.* control; *p *vs*. PDT; n.s. *vs.* SPDT) in the SDT group, 53$\pm$17% (***p *vs.* control; *p *vs.* SPDT) in the PDT group, and 79$\pm$18% (***p *vs.* control) in the SPDT group.

SDT induced greater tumor inhibition than PDT, whereas SPDT did not produce a statistically significant improvement over either therapy alone. The lack of synergy in SPDT may result from oxygen consumption during ultrasound irradiation, reducing ROS generation during the subsequent PDT phase. In some animals, tumor volume in the ultrasound-only group slightly exceeded that of controls, yielding a negative TGI%, possibly due to a biomodulatory effect in which low ROS levels stimulate tumor growth. This effect was inconsistent and not statistically significant.

H&E-stained histological images revealed isolated areas of autonecrosis in the control groups; superficial necrosis likely caused by ultrasound-induced mechanical effects in the US group; superficial necrosis in the PDT group; and extensive necrotic regions across the irradiated cross-sectional area in both the SDT and SPDT groups (Figure 1).

The percentage of Ki67-positive cells was 34$\pm$17 % in the control group, 56$\pm$29% (n.s. *vs* control; **p vs SDT; *p *vs* SPDT; n.s. vs PDT) in the ultrasound group, 7$\pm$3% (**p *vs* control; *p *vs* PDT, SPDT) in the SDT group, 35$\pm$20% (n.s. *vs* control and SPDT) in the PDT group, and 25$\pm$16% (n.s. *vs* control) in the SPDT group. These results indicate that the SDT group exhibited a significantly stronger antiproliferative effect compared to all other groups. While the PDT and SPDT groups showed moderate reductions in tumor volume at the endpoint, Ki67 expression did not differ significantly from the control group.

IV. Conclusions

Under the applied light and ultrasound conditions, ALA-mediated SDT showed promising potential for the treatment of cutaneous melanoma. Since SPDT did not significantly enhance therapeutic efficacy, further optimization of parameters

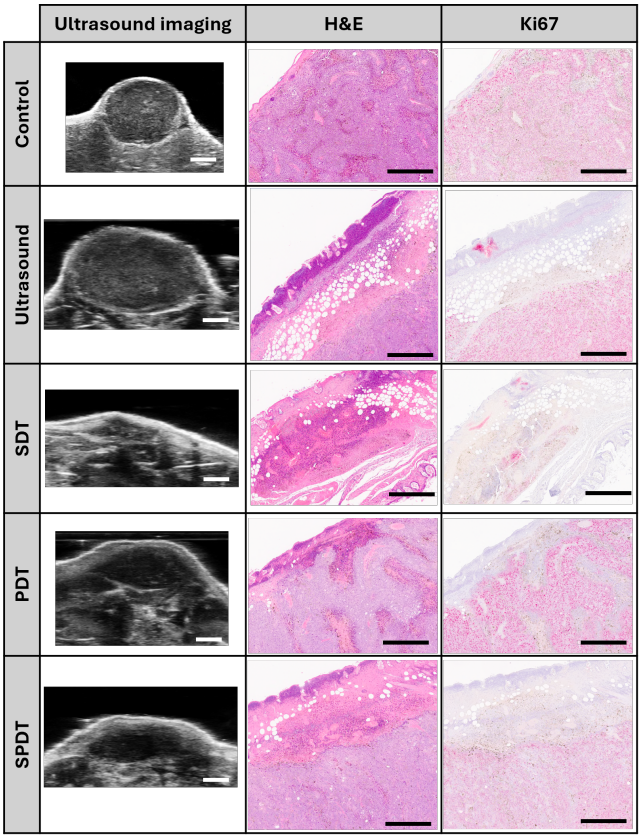

Fig. 1. Representative images of ultrasound imaging, H&E staining, and Ki67 immunofluorescence staining of tumors at the experimental endpoint for all the treatment groups. White scale bars=1.5 mm; black scale bars=0.5 mm.

such as irradiation sequence, oxygen availability, and energy dose is needed to clarify its synergistic effects. In addition, our results demonstrate that the conical aluminum waveguide is an effective tool for targeting small tumors, enabling precise delivery of ultrasound energy to localized regions that are difficult to reach with conventional irradiation.

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